INTEGRATING EPIDEMIOLOGICAL AND MOLECULAR APPROACHES TO STUDY THE TRANSMISSION AND TREATMENT OF *GIARDIA LAMBLIA*

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ABSTRACT

Giardia lamblia, a eukaryotic intestinal parasite, is one of the major causes of diarrheal disease, giardiasis, worldwide. The pathophysiologic complications of Giardia infections include small intestinal barrier dysfunction and decreased intestinal surface area. These factors can in turn result in small intestinal malabsorption and contribute to undernutrition of the infected host.

Undernutrition is an especially important concern for children under five as this is a period of critical childhood growth and development. However, our understanding of the global childhood morbidity associated with Giardia infections is limited as observational studies addressing the association between G. lamblia infection and undernutrition in children, measured either as anthropometric indicators of growth [weight-for-age (WAZ), weight-for-height (WHZ), height-for-age (HAZ)], or by decreases in serum micronutrient (vitamins and minerals) levels, have been inconclusive.

Thus, in Part I of the research presented here, we performed two different systematic reviews and meta-analyses pooling individual epidemiological studies to obtain summary estimates of the overall qualitative and quantitative effects of G. lamblia infection and growth (Chapter 3) and micronutrient malnutrition (Chapter 4).

We found that Giardia infection, with or without diarrhea, was statistically significantly associated with mild and moderate growth deficits (WAZ WHZ, and HAZ > -2 SD). Infection
was also significantly negatively associated with decreased levels of serum zinc and iron. However, infection did not increase the odds of either severe growth deficits (WAZ, WHZ, or HAZ < -2 SD) or severe micronutrient deficiencies (anemia). With a growing awareness of the long-term negative consequences of childhood undernutrition, this research provides evidence supporting treatment of all infected children in order to minimize the impacts to childhood growth and development.

Current anti-
*Giardia* treatments are recommended to treat symptomatic giardiasis given limited tolerance and the potential for harsh side effects. Additional concerns such as high rates of clinical resistance and treatment failures raise the development of new therapeutics against *Giardia* to a high priority. In Part II of the research presented here (Chapter 6), we describe a bottom up approach to rational drug development using Surface Plasmon Resonance (SPR) as a tool to identify small molecule compounds targeting putative methyltransferase enzymes in *Giardia*. 
The writing of this dissertation is dedicated to my parents who have encouraged and supported me to achieve my goals every step of the way.

Many thanks,
Sweta R. Batni
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Chapter 1: *Giardia* and Giardiasis

1.1: Introduction

*Giardia lamblia* (syn. *Giardia intestinalis*, *Giardia duodenalis*), an enteric, protozoan parasite, is one of the major causes of diarrheal disease, giardiasis, worldwide. *Giardia* is estimated to contribute to approximately 280 million cases of symptomatic disease in humans annually (2) with around 500,000 new cases reported each year (3). Long-standing estimates of prevalence report ~33% of all people in developing countries have had giardiasis (4). However, more recent studies have reported infection rates ranging from 90% to 100% in some developing countries (5, 6). *Giardia* is also a public health concern in developed countries. The pathogen has been identified as the most common human intestinal parasite in the U.S. (7, 8). Worldwide, approximately 2% of adults and between 6% -8% of children in developed countries are infected annually (4). In addition to humans, *G. lamblia* is capable of causing disease in pets (e.g. dogs, cats, ferrets), livestock (e.g. cattle, sheep, goats, pigs), and wildlife (e.g. beavers).

*Giardia* is also a significant waterborne pathogen. In fact, *Giardia* was identified as the most common cause of waterborne outbreaks in the United States (9) (7). Ingestion of water that has been fecally contaminated with *Giardia* cysts is the most common mode of parasite transmission. As such, individuals living in resource limited settings with poor water quality and a lack of sanitation are at greatest risk of infection.

Giardiasis may also impose substantial economic costs at the individual and societal levels. For example, individuals with giardiasis can incur significant medical costs and losses to productivity due to persisting giardial symptoms. *Giardia* is also the most
persistent pathogen diagnosed in cases of travelers’ diarrhea (10); travel-related giardiasis can result in substantial economic costs by interfering with travel, trade, and/or tourist industry revenues (11). Lastly, giardiasis in young farm animals may result in substantial economic losses due to lower animal productivity, impaired feed efficiency, and severe weight loss (12, 13).

1.2: Biology of *Giardia lamblia*

1.2.1 Structure

*Giardia* exists in two forms: the trophozoite and the cyst. Trophozoites are the disease causing form of the parasite, while *Giardia* cysts are the infective stage of the parasite. Trophozoites are approximately 12 µm - 15 µm long, 5 µm - 9 µm wide, and have a characteristic pear, or teardrop, shape with bilateral symmetry (14). Each trophozoite contains two similar nuclei each of which is transcriptionally active and diploid resulting in a tetraploid (4N) organism. Additionally, each nucleus contains an approximately equal amount of DNA (14-16). *In situ* hybridization with single copy genes supports the generally held assumption that each nucleus has the same complement of genes and chromosomes (14).

The trophozoite cytoskeleton includes a median body, eight flagella organized in four pairs--anterior, ventral, posterior/lateral, and caudal--and a ventral disk which each play important roles in mediating parasite motility, attachment, and survival in the small intestine of infected hosts (reviewed in Adam, 2001)(17).

Cysts are non-motile, oval shaped, approximately 5 µm by 7 µm - 10 µm in diameter, and are covered by a cyst wall that is between 0.3 µm to 0.5 µm thick.
Additionally, cysts are metabolically dormant having a metabolic rate of only 10%-20% of that found in trophozoites (18). The cyst wall has an outer filamentous layer composed of N-acetylgalactosamine and three different cyst wall proteins (CW1, CW2, CW3) and an inner membranous layer composed of two membranes. These cyst wall components help to protect the parasite and contribute to its prolonged survival in the environment under a variety of harsh environmental conditions.

1.2.2 Parasite Life Cycle

The parasite has a relatively simple two-stage life cycle: the quiescent cyst and the vegetative trophozoite. Infection is most commonly initiated following the ingestion of *Giardia* cysts from fecally contaminated water or food; however, direct person-to-person, fecal-oral transmission of *Giardia* may also occur (19). Upon ingestion, the dormant cyst becomes metabolically active and undergoes excystation. Excystation is initially triggered when a cyst comes in contact with the stomach acids and digestive enzymes of an infected host; the excystation process is completed when the cyst passes from the stomach into the upper host small intestine and differentiates into its trophozoite form.

*Giardia* is a non-invasive parasite; thus attachment of *Giardia* trophozoites to the enterocytes in the upper small intestine of an infected host is necessary for the parasite to initiate and maintain disease. Eventually, trophozoites are pushed through the small intestine, by peristalsis of the gut, to the colon where the process of encystation, marked by differentiation of the motile, disease causing trophozoite back into the non-motile, infectious cyst, is induced in response to host factors including higher levels of bile salts, lower levels of cholesterol, and a basic pH (14).
1.2.3 Genotypes of *G. lamblia*

*Giardia* is capable of infecting a diverse range of vertebrate hosts. The *Giardia* genus is composed of six species in total; however, *G. lamblia* is the only species known to infect mammals (20). Eight genetically distinct assemblages, A-H (21), make up the *G. lamblia* species complex. Of these, only two assemblages, A (WB) and B (GS), and their respective subtypes, have been demonstrated to infect humans (20). The remaining assemblages (C-H) have been found in dogs (assemblages C and D), hoofed livestock (E), cats (assemblage F), rats (assemblage G), and marine mammals (assemblage H) (22).

Whole genome sequencing has found inter-genetic differences in the two assemblages. For example, comparative genomics of the two *G. lamblia* genomes (WB and GS,) show 77% nucleotide and 78% amino-acid identity in orthologous proteins (23). Intra-genetic differences have also been documented in the two assemblages. For example, assemblage B isolates are highly heterogeneous with only two isolates exhibiting identical enzyme profiles. On the other hand, assemblage A isolates display little genetic variation (24).

The prevalence of assemblage A as compared to assemblage B infections varies geographically, though assemblage B infections appear to be more common worldwide, especially in *Giardia* endemic regions (20). There is also evidence of differences in virulence and pathogenicity between the two assemblages. For example, a recent study of 207 human *Giardia* isolates in Sweden found a significant association between assemblage B infections and flatulence (25). Likewise, an earlier study of human volunteers experimentally infected with different human *Giardia* isolates found that the GS isolate (assemblage B) was statistically significantly more frequently associated with the presence
of diarrhea or loose stools than the WB isolate (assemblage A) (26). These examples suggest that parasite strain may be an important determinant for the severity of infections; however, the evidence demonstrating a definitive correlation between genotype and phenotype has been inconclusive to date (27-31). Additionally, the occurrence of mixed *Giardia* infections at both the inter- (e.g. A + B) and intra- (e.g. A1 + A2) assemblage levels have been documented in several studies (32) (20, 33). The presence of mixed infections further complicates efforts aimed at determining whether different assemblages are associated with differences in virulence and pathogenicity.

1.3: Disease Transmission

In general, a limited availability of clean water and/or poor sanitation and hygiene practices influence the overall risk of *Giardia* infection in a population. Though anyone can be infected with the parasite, the populations at greatest risk of infection include: travelers to countries with a high prevalence of *Giardia*; individuals in close contact with feces, or fecally contaminated surfaces, from infected individuals such as in day care settings or nursing homes; individuals who ingest *Giardia* contaminated water, either through routine, or recreational, consumption, from untreated sources (e.g. lakes, rivers, streams, ponds, wells); backpackers, campers, or hikers who consume unsafe water from untreated sources; and individuals who come into contact with *Giardia* contaminated feces during oral-anal sexual contact (4).

*Giardia* is readily transmitted between hosts; infected individuals can shed anywhere between 1-10 billion cysts per gram of stool per day back into the environment for either up to a week, as typically found in acute infections, or for longer periods (e.g.
several weeks to months) as may occur in chronic infections. Shed cysts are immediately infectious and environmentally stable able to persist in a variety of different external conditions, such as in cold water or highly acidic environments, for up to several months. Thus, cysts can rapidly accumulate in the environment for prolonged periods of time where they remain capable of infecting a susceptible host(s). The infectious dose of *Giardia* is low; consumption of water contaminated with as few as 10 infectious cysts is sufficient to initiate a novel infection and establish disease in a newly infected host (4).

About half (~50%) of the infections during epidemics are clinically asymptomatic (14, 34). Asymptomatic individuals play an important role in parasite transmission and in maintaining high levels of disease spread during *Giardia* outbreaks, as these individuals can shed cysts at rates equivalent to symptomatic individuals.

1.4: Clinical Disease and Pathophysiology

1.4.1 Symptomatology

The clinical presentation of giardiasis is highly variable; symptoms may be either acute, resolving within two to four days, or chronic, with symptoms lasting for several weeks to months. Although diarrhea is the major symptom of giardiasis, infected individuals may present with either clinically asymptomatic disease, or with a broad range of mild, moderate, or severe disease symptoms such as fatigue, abdominal bloating or cramping, flatulence, nausea, weight loss and a failure to thrive. If diarrhea is present, it may occur with or without malabsorption syndrome (35). Symptomatic human infections comprise only a fraction of all *Giardia* infected cases (36) with the vast majority of infections resulting in clinically asymptomatic disease.
Children have been documented to be at increased risk of developing more serious consequences from *Giardia* infections (reviewed in Halliez, 2013) (37). For example, infections in early childhood have been associated with having negative impacts on childhood growth and development such as lower cognitive function (38); impairments to height and weight (39); nutrient deficiencies (40); and a failure to thrive (41). Additionally, a recent study found that approximately 1/3 of *Giardia* infected patients can express long-term extra-intestinal symptoms (42). Extra-intestinal manifestations of *Giardia* include ocular pathologies, arthritis, allergy, or muscular complications (reviewed in Halliez et al., 2013) (37).

1.4.2 Pathophysiology

Multiple different pathophysiological consequences have been observed as a result of *Giardia* infections. It has been well established that *Giardia* initiates enterocyte apoptosis (43, 44) and small intestinal barrier dysfunctions (43, 45) upon infection. Infections have also been demonstrated to result in a diffuse shortening of the epithelial brush border microvilli (46, 47), a reduced total intestinal absorptive surface area (44, 48), and reduced disaccharidase activities (49).

Cotton et al. suggest that *Giardia* induced pathophysiology helps contribute to the development of diarrheal disease via intestinal malabsorption and maldigestion (50). Specifically, it has been suggested that the diffuse shortening of brush border microvilli and reduced total absorptive surface area as a result of infection cause the malabsorption of nutrients and electrolytes. This small intestinal malabsorption then creates an osmotic gradient that draws water into the intestinal lumen resulting in rapid peristalsis and subsequent diarrheal disease. Additionally, mast cell degranulation and adaptive immune
responses (51) have been demonstrated to lead to increased intestinal transit rates that may result in *Giardia* induced diarrheal disease (reviewed in Cotton et al., 2011) (50). Persistent *Giardia* infection has also demonstrated to cause growth impairment and crypt hyperplasia in a murine malnutrition model (52).

### 1.5: Factors Contributing to Disease Symptomatology and Pathophysiology

Additional evidence suggests that *Giardia* infections are capable of modulating host immune responses through a multifactorial process that involves complex, yet poorly understood, host-parasite interactions (reviewed in Cotton et al., 2015) (53).

#### 1.5.1 Host Factors

The role of the host’s immune status in influencing not only host susceptibility to *Giardia* infection, but also the variable clinical presentation observed in giardiasis has been well established. Experimental evidence has demonstrated that intestinal pathology during *Giardia* infection is driven in part by the host immune system (54). For example, immunocompromised individuals have been found to have an increased susceptibility to *Giardia* infection (55) and are at increased risk of developing severe and chronic giardiasis (reviewed in (36, 50)). On the other hand, even some immunocompetent *Giardia* infected individuals may present with persistent giardiasis symptoms that can last for years (35). Certain aspects of the host immune responses that have been suggested to play a role in contributing to *Giardia* induced pathophysiology are outlined below.
Inflammation

Our understanding of the host inflammatory responses to infection remains unclear as experimental studies have produced conflicting results. While it is plausible that high parasite burdens as a result of infection can contribute to subsequent increases in intestinal permeability which in turn could induce pro-inflammatory intestinal responses (reviewed in Cotton et al., 2015) (53), neither histological analyses of human assemblage A *Giardia* infections (48, 56), nor data from *in vivo* mouse models of *G. muris* infections (47), show any signs of overt mucosal inflammation.

Conversely, findings from more recent studies suggest that assemblage B *Giardia* infections may in fact induce pro-inflammatory intestinal responses. For example, a study conducted in Norway found a positive association between a small subset of human patients chronically infected with assemblage B *Giardia* infections and duodenal inflammation (57). Additionally, animal studies have noted robust intestinal inflammatory responses (58) including the activation of pro-inflammatory signaling pathways (e.g. MAPK and NF-κB) that result in the production of pro-inflammatory cytokines and chemokines (e.g. TNF-α and CXCL8) in response to assemblage B infections (59).

There is also a growing body of evidence (reviewed in (37)) suggesting that *Giardia* infections are associated with an increased risk of developing post-infectious chronic gastrointestinal inflammatory disorders such as irritable bowel syndrome (IBS) (60-62). However, understanding the mechanisms by which *Giardia* infection causes inflammation, as well as studying its role in either initiating, or exacerbating, post-infectious chronic gastrointestinal inflammatory disorders requires further investigation.
Intestinal Mucus Layer

The intestinal tract is lined with a layer of mucus that serves as part of the host’s innate immune response against colonization by pathogenic microorganisms. For example, intestinal goblet cells secrete mucus as a nonspecific immunological host defense to prevent giardial colonization of the intestinal epithelia. However, instances of parasite immunomodulation of host mucus have also been demonstrated. For example, Cotton et al., 2015 (unpublished), observed disruption of the intestinal mucus by infection with assemblage B *Giardia* parasites in mice. Additionally, it has also been reported that mucus protects *Giardia* trophozoites and facilitates parasite survival by inhibiting host lipase activity and by decreasing the toxicity of host lipolysis products present in the intestinal fluid (63). These findings suggest that perhaps immunomodulation of the intestinal mucus layer may be one strategy employed by *Giardia* to facilitate parasite survival in, and colonization of, infected hosts (53). However, the exact mechanisms by which the parasite interacts with the intestinal mucus layer to facilitate disease remain unknown.

Giardia, L-Arginine, Nitric Oxide (NO)

The complex host parasite interactions between *Giardia*, L-Arginine, and nitric oxide (NO) may also contribute to the severity and pathophysiology of disease. Both arginine and NO play important roles in host innate immune responses against *Giardia* infections. L-arginine acts as the sole amino acid substrate for NO production by Nitric Oxide Synthase 2 (NOS2); NO is a broad-spectrum host cell-signaling molecule that is involved in many physiological and pathological processes. One of its key roles is in host immune responses as an antimicrobial defense given its toxicity to many pathogens.
Several lines of evidence have confirmed the importance of NO as a host defense against *Giardia* (64, 65).

However, L-arginine also acts as the primary source of energy for *Giardia* trophozoites. In fact, L-arginine consumption by *Giardia* is one of the multiple means the parasite employs to limit the host production of NO. Limiting the amount of arginine available for host utilization in turn decreases the amount of NO produced by the host. As a result of arginine depletion, the parasite is able to minimize its exposure to NO, thus evading an important host immune response (reviewed in Cotton et al., 2015) (53). However, the extent to which host NO and L-arginine impact the parasite and how these interactions may influence disease severity and pathogenesis remains to be determined.

*Acquired Immunity*

Lastly, a mixed adaptive immune response, mediated by both B- and T-cells, has been reported to play an important role in facilitating parasite clearance. Both animal and human studies have confirmed the central role that B cells, or antibody-producing cells, play in host anti-giardial defenses. For example, an animal study found that immunoglobulin (Ig) A deficient mice were unable to eradicate either *G. muris* or *G. lamblia* infections demonstrating that the specific host production of IgA antibodies are required for parasite clearance (66).

T cells have also been demonstrated to play a fundamental role in *Giardia* clearance not only by activating and stimulating the differentiation of B cells to generate anti-*Giardia* specific antibodies, but also by acting through B-cell-independent mechanisms (67). For example, CD4⁺ T cells have been found to be required for the control of acute *G. lamblia* infections in mice; a requirement that persists in the absence of B cells (67). Further
elucidating the roles of the different components of host adaptive immune responses in contributing to the pathophysiology and severity of disease is an important topic for further consideration.

**Microbiome**

Differences in host microbiota compositions between individuals may contribute to variability in disease pathology, symptomatology, and susceptibility. For example, the use of probiotics in controlling bacterial and viral diseases has long been demonstrated, however, research into whether probiotics can also be beneficial in controlling intestinal parasitic infections has only emerged during the last decade (68). A recent animal study found that mice fed daily with a probiotic seven days prior to being infected with *Giardia* efficiently reduced the excretion of both cysts and trophozoites as compared to infected mice that were not administered probiotic prior to infection (69). Recent evidence also suggests that host nutritional and microbial statuses are other factors that may be associated with disease severity and pathogenesis; however, the exact mechanisms by which these factors play a role in contributing to variable disease outcomes in the host needs to be further elucidated (70).

### 1.5.2 Parasite Factors

*Giardia* does not invade the mucosa, yet is able to attach and detach to the host intestinal epithelium to cause disease, survive, and replicate in the hostile environment of the small intestine. The parasite is also able to avoid parasite clearance either from host peristalsis, or from the continual renewal of host intestinal epithelial cells every three to five days (71, 72).
Antigenic Variation

Surface antigenic variation has been shown to play an important role in host immune evasion in *Giardia* and giardiasis. Specific *Giardia* antigens involved in surface antigenic variation belong to a family of variant specific-surface proteins (VSPs). VSPs are a family of related cysteine-rich proteins that contain a frequent number of CXXC motifs and range in size from 22.3 kDa to over 200kDa. VSPs coat the entire trophozoite surface including the flagella (73). Parasites express only one VSP on their surface at a time except during VSP switching and differentiation when multiple VSPs are expressed simultaneously. Clonal VSPs switch to a new VSP every 6.5-13.5 generations *in vivo* (73). This antigenic variation ensures that even small populations of recently cloned parasites express different VSPs on their trophozoite surfaces at any given time. It has been estimated that there are approximately 150 different VSPs present in one *Giardia* genome. Thus, an infected host can be exposed to far greater than 150 antigenically unique VSPs (73).

Presenting a wide assortment of VSPs to an individual host not only increases the parasite’s chance of successfully evading host immune responses, but also increases the likelihood that *Giardia* can either initiate a new infection or cause repeated, concurrent, or recurrent infections in individuals as commonly occurs in *Giardia* endemic areas (73). Thus, parasite antigenic variation has important implications not only for parasite survival and disease outcomes, but also for parasite transmission and disease control efforts.
1.6: Disease Detection, Treatment, and Control

1.6.1 Detection

The microscopic ova and parasite examination (O&P) is the traditional method used for stool parasite testing in *Giardia* infections (74). However, the sensitivity of the O&P test is greatly impacted by the number of stool samples collected and submitted for testing. For example, the microscopic examination of one stool sample allows for the detection of around 60% - 72% of *Giardia* infections (75), whereas the examination of three stool samples increases the sensitivity of detection by approximately 11% for *Giardia* (75, 76).

However, because of intermittent, or low-levels of cyst shedding, (77), it is often recommended to examine more than 3 stool samples collected over multiple days in order to ensure the accurate detection of all infections (78). Yet, ensuring compliance of study participants in the proper collection of multiple stool samples over prolonged periods of time may be challenging. Additionally, microscopic identification requires trained personnel to prepare, stain, and examine stool samples for *Giardia* cysts further adding to the time and labor required in the microscopic detection and diagnosis of infections.

Given these limitations, direct fluorescent antibody (DFA), immunochromatographic (IC), and enzyme immunoassays (EIAs), which each has a higher test sensitivity (> 90%) than microscopy for detecting *Giardia* infections (75), are commercially available and have replaced microscopy as the routine diagnostic method of choice in many hospitals and public health laboratories (79). Additionally, molecular methods, such as PCR, are more sensitive and specific than microscopy for the detection of pathogenic protozoa. However, it may not be feasible to implement either
immunoassays or molecular tests routinely in the field due to considerations such as potential limited laboratory access and higher costs (75).

1.6.2 Treatment and Control

Giardiasis is self-limiting in the majority (>85%) of all infected cases (36) suggesting the presence of effective host defenses against infection. However, because of the observed association of *Giardia* infections not only with negative long-term impacts to childhood growth and development, but also with post infectious extra-intestinal disease symptoms, anti-*Giardia* treatment is recommended for both children and adults with symptomatic giardiasis.

First line therapeutics against giardiasis include members of the 5-nitroimidazole (5-NI) compound class (metronidazole, tinidazole, ornidazole, and secnidazole); of these metronidazole and tinidazole have consistently demonstrated the greatest *in vitro* activity against giardiasis (reviewed in Gardner and Hall, 2001) (74). Metronidazole, a broad-spectrum antimicrobial agent with activity against anaerobic and microaerophilic pathogens, is the most commonly used drug to treat giardiasis worldwide (74). In brief, metronidazole acts as prodrug whose antiparasitic activity is dependent on reduction of its nitro group by electron transport protein ferredoxins present in *Giardia*’s anaerobic metabolic pathways. This reduction reaction favors the intracellular transport of metronidazole allowing it to covalently bind to DNA macromolecules which in turn results in DNA damage due to a loss of helical structure, impaired template function, introduction of double strand breaks, and eventual trophozoite death (74). Metronidazole has also been demonstrated to inhibit trophozoite respiration and motility (18). Additionally, the reduction of metronidazole leads to the formation of toxic free radical intermediates that
can react with cellular components to kill the parasite (80). A typical course of metronidazole is administered as three oral doses of 250 mg each daily for 5-10 days with a median efficacy in adult and pediatric populations of 92% (74). Tinidazole is the preferred drug for treating giardiasis in the U.S. given its high efficacy, increased tolerability, and convenient dosing schedule (80).

In recent years, alternative therapeutics have also been included for routine use in the antigiardial armamentarium (reviewed in Gardner and Hill, 2001) (74). For example, the benzimidazole, albendazole, widely used abroad as an antihelmintic agent, is now also being considered as one of the most promising new anti-Giardia treatment options. Albendazole has been demonstrated in a meta-analysis to be as effective as metronidazole in treating giardiasis (81). Additionally, albendazole has a comparatively benign side effect profile and simple dosing regimen (single versus multiple doses per day) (80).

However, these common drug treatment options against giardiasis, are usually, but not always effective. Anti-Giardia therapies fail in up to 20% of cases suggesting that drug resistance is an issue of concern (80). For example, each of the three described pathways required for the activation of 5-NI drugs in Giardia have been implicated in drug resistance (80). Additionally, frequent recurrence of infections, of up to 90% (82) for some individuals living in Giardia endemic areas, also suggests parasite resistance to metronidazole.

Drug resistance is not the only issue of concern. The widespread use of current anti-Giardia treatments is also limited by factors including: the limited safety and efficacy in special populations (e.g. pregnant women); reports of poor drug compliance; the high incidence of undesirable side effects such as headache, vertigo, gastrointestinal
complications (e.g. nausea, vomiting, anorexia, constipation), and bitter, metallic taste; and the reports of significant treatment failures of some drugs in clearing the parasite from the intestine (83). In addition, the potential for *Giardia* infections to produce persistent clinical symptoms that could negatively impact childhood growth and development and quality of life, as well as the growing recognition of associations of *Giardia* infections with post infectious extra-intestinal complications, further strengthen the case for the need to develop novel, more effective anti-*Giardia* treatments. Despite the growing recognition of all these factors however, the number of anti-*Giardia* therapeutics has not significantly increased in the last decades (83).

Control strategies to minimize *Giardia* associated morbidity in humans are limited as there is currently no commercially available human *Giardia* vaccine or other medical prophylaxis to prevent infection in humans, and the commercially available veterinary vaccine is of limited efficacy in treating canine infection. Vaccine candidates against human *Giardia* infections have demonstrated low efficacy in vaccine trials as these potential candidates have been unsuccessful thus far in mounting a fully protective immune response against *Giardia* (reviewed in Faubert, 2000) (84). Also, efforts in the rational development and design of an effective anti-*Giardia* vaccine have been stalled by a limited understanding of the key immunogenic proteins associated with *Giardia* infections and the high variability of infections due to VSP switching by the parasite. Additionally, the fact that there are no identical VSPs between the WB and GS isolates (85) further complicates the development of an effective vaccine that will simultaneously protect against mixed assemblage (e.g. A + B) *Giardia* infections.
1.7: Concluding Thoughts

*Giardia lamblia*, a ubiquitous eukaryotic parasite, is one of the major causes of diarrheal disease, giardiasis, worldwide. *Giardia* is transmitted by ingestion of fecally contaminated food or water, thus individuals living in developing countries without access to clean water or proper sanitation are at increased risk of chronic, multiple, and/or recurring infections. The clinical symptomatology and pathophysiology of giardiasis is highly variable. However, recent evidence suggests that repeated and/or persistent disease in children has been found to be negatively associated with childhood growth and development. Given its significant disease burden, and its links to developmental shortfalls among children, *Giardia* was included as part of the World Health Organization’s (WHO) Neglected Disease Initiative in 2004 (86).

As *Giardia* is an intestinal parasitic infection that is associated both with diarrheal outcomes and is associated with negatively impacting child health and development, there is growing recognition among the global public health community of the importance for further understanding the true impact of *Giardia* infections in order to minimize the impact to human health. Specifically, providing a better understanding of both the epidemiology of infection, as well as gaining insights into the mechanisms by which infection leads to variable disease severity, duration, and pathogenicity in infected hosts will lead to the development of targeted, effective strategies designed to improve the treatment and control of this widespread human pathogen, thus minimizing its morbidity and global disease burden.
Chapter 2: Malnutrition

2.1: Introduction

Malnutrition, or “bad nutrition,” is a significant global public health problem throughout the developing world. Although malnutrition is a broad term that can be used to refer to either over- or undernutrition (87), the specific focus of our research is on undernutrition which broadly refers to a nutritional deficiency that is caused from either a lack of food intake or an inability of the body to convert or absorb the nutrients consumed (i.e. poor bioavailability).

It has been estimated that maternal and childhood malnutrition is the underlying cause of 3.5 million deaths, 35% of the disease burden in children younger than 5 years of age, and 11% of the total global disability adjusted life years (DALYs) (87). Although individuals of any age may be malnourished in the course of their life spans, young children, especially those < 5 years of age (88), are at increased risk for developing severe complications as a result of undernutrition--the effects of which can be both immediate and enduring. For example, in the short term, undernutrition can cause delayed physical growth and motor development (e.g. decreases in weight, height, and neural plasticity); a greater degree of socio-behavioral problems (e.g. deficient social skills, decreased attention, or a decreased ability to communicate); and lower cognitive development (e.g. decreased abilities to learn, think analytically, or form working memory) (89). These short-term deficits in early childhood growth and development can also have long-term devastating consequences at both the individual and societal levels in the forms of lower overall educational attainment and decreased workforce productivity.
2.2: Types of Malnutrition

2.2.1 Protein-Energy (Calorie) Malnutrition/Undernutrition

Causes and Outcomes

The most common form of undernutrition worldwide is known as protein-energy, or protein-calorie, malnutrition (undernutrition), subsequently referred to here as protein-energy malnutrition (PEM). PEM is defined by deficiencies either in the consumption of macronutrients (e.g. proteins, carbohydrates, and fat) due to poor dietary quality, or in poor bioavailability of the macronutrients consumed due to the presence of underlying enteric infections such as *Giardia*. The body relies largely on macronutrients not only in order to perform its key functions such as maintaining proper cellular structure and function, but to also provide the energy needed to perform these daily functions. A body’s metabolic demand for macronutrients is dynamic, but remains especially important during early childhood as this represents a period of active growth and development. Adults and older children can access proportionally larger reserves of energy than young children during periods of reduced macronutrient intake. Thus, young children, especially children < 5 years of age, are most at risk for PEM.

The body adapts to a reduction in macronutrient intake by an increased use of its energy reserves (i.e. muscle and fat), which in turn corresponds to a decrease in growth (i.e. weight and/or height). As a result, undernourished individuals tend to be thinner and/or shorter than well-nourished individuals (90). The effects of PEM on growth may be acute, chronic, or both acute and chronic. Deficits in weight correspond to acute malnutrition, as an individual’s weight can respond rapidly and reversibly to dietary changes resulting in
weight losses, whereas deficits in height are indicative of chronic malnutrition, or non-reversible linear growth faltering over a long period of time (90).

The most serious, and more lethal, forms of PEM refer to a group of severe disorders, marasmus, kwashiorkor, or the intermediate states of marasmus and kwashiorkor, seen almost exclusively in children in developing countries (91). Marasmus is caused by an inadequate dietary intake of protein and calories (energy), whereas kwashiorkor is caused when an individual consumes a low-protein, high carbohydrate diet. Edema, or swelling, is a characteristic feature of kwashiorkor, but is absent in marasmic states. Both marasmus and kwashiorkor first develop in early childhood during weaning and persist throughout the lifespan of the child. Children that develop either of these severe forms of PEM in early childhood will never recover their full weight or height potentials. Marasmus is a significant public health problem affecting over 50 million children worldwide, whereas kwashiorkor is more common in areas of the world with high poverty and limited food supplies (e.g. sub-Saharan Africa, Southeast Asia, and Central America) (91). Kwashiorkor is rarely found in countries where people have access to sufficient quantities of food. Thus, the majority of our studies did not assess the relationship of *Giardia* infection with these extreme forms of undernourishment. The small number that we identified in our systematic review were abstracted in our systematic analysis, but were too few in number (n<5) to include in a meta-analysis (See Chapter 3).

**Measures of PEM**

Anthropometric indicators, height-for-age (HA), weight-for-age (WA), and weight-for-height (WH), are the standard measures used for monitoring the physical growth, and estimating the nutritional status, of children < 5 years of age. Mid-upper arm
circumference (MUAC) is also an anthropometric indicator of nutritional status in children < 5 years of age, though it is not commonly used in observational studies; and therefore, was not included in our meta-analyses as there were too few studies (n<5) to pool in order to determine an overall impact.

The basic measurements that constitute anthropometric information in children include: age, sex, height, and weight. Once this information is collected, an individual child’s measurement(s) are then compared to the median height-for-age, weight-for-age, or weight-for-height of healthy children of the same age and sex in an internationally accepted reference population (e.g. child growth standards jointly developed by the National Center for Health Statistics US Centers for Disease Control and Prevention (CDC) and the World Health Organization (WHO) (92).

Anthropometric indicators can be expressed in relationship to a select reference population of healthy children by using one of two statistical methods: Z scores or percentage of median (90). Of these statistical methods however, expressing anthropometric measurements in terms of Z scores is the most frequently used method for assessing growth, and was the measure reported for all the observational studies used in our meta-analyses (See Chapters 3 and 4). Z scores are a measure of the difference in the value of height or weight for an individual and the median value of height or weight for the reference population divided by the standard deviation (SD) of the reference population. Thus, Z scores provide a description of how many SDs away an individual’s height or weight measurement lies from the median reference population.

The physical growth of children < 5 is an accepted indicator of the nutritional wellbeing of the population they represent. Thus, in order to assess population nutritional
status from anthropometric indicators and further understand the relationship between *Giardia* infection and its impact on anthropometric indicators of growth, we used the same classifications of cut off values to delineate mild/moderate and severe malnutrition that were used by individual study authors when interpreting the results of our systematic review and meta-analyses. These cutoffs were based on guidance outlined by the WHO (90). For example, HAZ, WAZ, and WHZ scores that lie between -2 and the median (e.g. \(-2 < Z < 0\)) defined mild/moderate impacts on malnutrition. The terms stunted, wasted, or underweight were used to define more severe measures of malnutrition and are based on the following WHO child growth standard cutoffs for height and weight: HAZ < -2 (stunted), WAZ < -2 (wasted), and WHZ < -2 (underweight) (See Chapter 3). HAZ, WAZ, or WHZ scores of less than -3 SD from the median as compared to the child growth standard cutoffs for height and weight (e.g. \(Z < -3\)) define the most severe forms of undernutrition (i.e. marasmus, kwashiorkor, or an intermediate marasmus/kwashiorkor); however, these were not included in our meta-analysis as there were too few studies (n<5) to pool.

2.2.2 Micronutrient Malnutrition (MM)

*Introduction*

The second type of undernutrition is known as micronutrient malnutrition (MM). MM is caused by deficiencies in one or more micronutrients (i.e. essential vitamins and minerals). Micronutrients must be obtained from the diet, as the body does not produce them. Thus, individuals with poor dietary quality, who consume too few of one, or many, micronutrients may suffer from MM. MM may also result from poor bioavailability of the micronutrients consumed, due to repeated or persistent infections. Although
micronutrients are only required in small amounts in the body, they serve vital functions in ensuring proper development, maintaining immune function (especially seen with vitamin A and zinc), and promoting overall health and wellbeing. Over 2 billion people are at risk for MM worldwide (93), with the highest prevalence rates found in Southeast Asia and Sub-Saharan Africa. Micronutrient malnutrition (MM) is an especially important concern in maternal or child health as nutritional deficits in the mother can negatively affect a child’s survival and development (87).

Measuring MM

While many severe forms of micronutrient deficiencies are associated with distinct clinical symptoms, most people are not severely micronutrient deficient, and thus do not show any overt clinical symptoms. Therefore, solely relying on the use of clinical methods of detection to assess for micronutrient deficiency in a given population is limited. Clinical assessment can also prove problematic in resource poor settings where individuals may not have access to a trained medical professional capable of accurately performing a clinical assessment. Thus, clinical assessments may underestimate the true risk of subclinical MM in a population.

Instead, a better measure of assessing the micronutrient status of a population is through the use of biochemical methods, which involve the determination of micronutrient levels in body fluids. The use of biochemical markers to detect milder micronutrient deficiencies in which there are no apparent clinical signs or symptoms provides a more comprehensive picture not only of the prevalence of mild or moderate micronutrient deficiencies in a population, but also helps identify target groups at increased risk for developing severe micronutrient malnutrition.
Biochemical micronutrient assessment first involves the collection of serum samples from study volunteers. Next, samples must be analyzed in a laboratory to identify the serum level of the marker of interest. Lastly, individual values are compared to predetermined age-specific cutoffs, unique for each micronutrient, that have been jointly determined by the WHO and CDC. See Principles of Nutritional Assessment by Rosalind Gibson for more detailed information on interpretive criteria for each micronutrient (94).

2.3: Enteric Infections and Malnutrition

2.3.1 Overview

Diarrheal disease is a leading cause of childhood mortality and morbidity worldwide. It is estimated that there are nearly 1.7 billion cases of diarrhea annually (95). Children under 5 years of age (children < 5) bear a large burden of diarrheal disease. This is most likely due to impaired absorptive function due to repeated and persistent enteric infections and diarrheal disease. Maintaining a healthy intestinal tract is critical during this period of infancy and early childhood, because unlike many other species, the predominant brain and synapse development occurs in humans in the first two years after birth (96). Hence, the absorption of key nutrients during this time is critical to ensuring the optimal growth and development of the body, brain, and neuronal synapse affecting cognitive development (96).

Each year, diarrhea kills approximately 760,000 children < 5, making it the second leading cause of death in this population (95). On average, children < 5 in resource poor settings with a lack of access to clean water and sanitation, experience at least three diarrheal episodes a year as a result of repeated exposure to diarrheal causing pathogens in
contaminated water sources (95). Diarrhea impairs the absorption of key nutrients needed for child growth and development, and thus, diarrhea is also a leading annual cause of malnutrition (95). Further, it has been well demonstrated that repeated bouts of diarrhea, rather than a single acute episode, have the greatest impacts on childhood growth likely due to the compounding effects of impaired nutrient absorption with each bout of diarrhea (reviewed in Guerrant et al., 2008) (97). In fact, it has also been demonstrated that malnourished children have a greater incidence, longer duration, and increased severity of diarrheal disease (98).

Leonardo Mata, in his published studies observing children in rural population in Guatemala in the 1960s, was among one of the first researchers to have documented an association of diarrhea and enteric infections on childhood growth and development (99). Since this time, numerous studies have made similar observations on the impact of enteric infections and diarrheal diseases on growth. The adoption of the Sustainable Development Goals at the end of 2015 (100) has brought about renewed focus and efforts by the global health community to better define the role of enteric infections on childhood growth and development. While the evidence linking malnutrition and enteric infections is largely based on studies examining the association of diarrheal disease and malnutrition, there is limited evidence that even asymptomatic enteric infections, without overt dehydrating diarrhea, can result in deficits in growth (reviewed in Guerrant et al., 2008) (97).

2.3.2 Enteric Infections

The etiologic agent(s) of enteric infections include a number of different viral (e.g. rotavirus), bacterial (e.g. V. cholera O1, E. coli 0157, Shigella sp., Salmonella spp.), protozoan (e.g. Giardia lamblia, Entamoeba histolytica), and helminthes (e.g. Ascaris,
*Trichuris* species (96). Thus, co-infections are common given that enteric pathogens share a common mode of disease transmission--through ingestion of contaminated water and/or food. However, the focus of our analysis is on examining the associations of *Giardia* infections and malnutrition.

### 2.3.3 Giardia and Malnutrition

A recent meta-analysis found a negative association of *Giardia* infections with persistent diarrhea in young children (101). Both experimental and observational studies suggest that diarrhea in giardiasis is a result of small intestinal malabsorption. Persistent *Giardia* infection has also been demonstrated to cause growth impairment and crypt hyperplasia in a murine malnutrition model (52).

Many of the pathophysiological consequences associated with malnutrition, such as intestinal epithelial cell apoptosis (102) and increased intestinal permeability (103), have also been found in both human and animal studies to occur in giardiasis (43-45, 104) (See Pathophysiology in Chapter 1) suggesting a synergistic relationship in which the presence of one factor, either malnutrition, or *Giardia* infection, may increase the susceptibility to, or severity of, the other.

Numerous independent epidemiologic studies have documented varying impacts of *Giardia* infection on childhood growth; however, the results of these individual studies have been inconclusive. For example, while a number of these studies have found a negative association between *Giardia* infection and growth deficits (41, 105-110) other similarly conducted independent epidemiologic analysis (111, 112) found no association. Many of these same individual epidemiologic studies (41, 106, 107) also analyzed the
effects of _Giardia_ infection with micronutrient deficiencies again with similarly conflicting results.

2.4: **Using Epidemiologic Approaches to Examine the Association between _Giardia_ Infection and Malnutrition**

Historically, systematic reviews and meta-analyses have been used in the field of clinical medicine to summarize the overall efficacy of treatment interventions and guide the practice of evidenced based clinical medicine based on qualitative (systematic reviews) and quantitative (meta-analyses) reviews of the literature. In brief, the goal of a systematic review of the scientific literature is to identify, apprise, select, and synthesize all available evidence that specifically addresses a research question (113). Systematic reviews rely on the careful identification and evaluation of all available evidence on a particular topic of interest by using explicit, reproducible, and systematic methods. Thus, systematic reviews offer an advantage over narrative reviews in that they minimize selection bias in the process of data gathering.

Often systematic reviews include a meta-analysis component that involves using statistical techniques to synthesize the data from several independent studies that address the same research question into a single quantitative estimate or summary effect size (113). Meta-analyses can help identify patterns, find sources of disagreement, or ascertain interesting relationships among various different study results. The qualitative and quantitative results found through performing a systematic review and meta-analysis respectively can guide public health decision-making. Additionally, systematic reviews
and meta-analyses can also be used to generate new hypotheses in the field that can then be tested in experimental studies.

The growing recognition of the broader utility of systematic reviews and meta-analyses for uses outside of clinical medicine and towards synthesizing the often-conflicting findings from independent observational studies in *Giardia* has only recently been demonstrated. For example, Muhsen and Levine, 2012 performed a systematic review and meta-analysis to examine the role of *G. lamblia* as an enteric pathogen (exposure) capable of causing diarrheal illness in young children (outcome) in developing countries. The authors chose this epidemiological approach not only as a way to synthesize the number of conflicting reports found in observational studies of the association of *Giardia* infection on pediatric diarrhea, but also to quantify a summary effect reflecting the overall burden of diarrheal disease that can be attributed to *Giardia* infection (101).

In Part I of my research, I extended the analysis performed by Muhsen and Levine and performed two unique systematic reviews and meta-analyses in order to synthesize all published literature and provide an overall estimate of the association of both asymptomatic and symptomatic *Giardia* infections (exposure), with or without overt diarrhea and two types of undernutrition (outcome).

### 2.5: Micronutrients

As described in the parasite life cycle in Chapter 1, attachment of *Giardia* trophozoites to the host upper small intestine, duodenum, is necessary to cause disease. The duodenum is also the site of the body where a number of micronutrients are absorbed. Thus, numerous studies examining the impact of *Giardia* infection on micronutrient levels
have looked for relationships between infection and micronutrients absorbed in the duodenum. However, these studies have largely focused on the associations of *Giardia* infection (exposure) and iron, vitamin A, and zinc (outcome) as these three micronutrients have long been recognized as micronutrient deficiencies of significant public health concern in the malnutrition literature.

However, there are also a number of other micronutrients that are absorbed in the duodenum including: calcium, phosphorus, magnesium, copper, selenium, thiamine, riboflavin, niacin, folate, and the other fat soluble vitamins—vitamin D, E, and K—for example whose levels may be impacted by *Giardia* infection, and thus were included as search terms in our systematic review and meta-analysis.

We were also interested in examining the overall association of *Giardia* infection with vitamin B12 in our systematic review and meta-analysis. Despite the fact that vitamin B12 is absorbed in the ileum and not the duodenum, a number of isolated case reports, using histological analyses, have reported an association between *Giardia* and vitamin B12 absorption (114-121). For this reason, we also included vitamin B12 as a search term in our analysis (See Chapter 4).

Brief descriptions of the roles, clinical outcomes of deficiencies, biochemical markers used to assess status, etc. of each of the micronutrients (vitamins and minerals) of relevance to our systematic review and meta-analysis are provided below. See *Principles of Nutritional Assessment* by Rosalind Gibson for more detailed information on each micronutrient outlined below (94).
2.5.1 Vitamins

Vitamin A, D, E, and K

Vitamins A, D, E, and K are all fat-soluble vitamins with diverse roles in maintaining vital body functions (94). Of these four different fat soluble vitamins, vitamin A has the largest disease burden with vitamin A deficiencies (VAD) estimated to be responsible for 0.6 million deaths annually (87).

Vitamin A has a clearly defined role in maintaining vision; thus, vitamin A deficiency (VAD) can result in a number of ocular manifestations of varying severity. One of the earliest and most common ocular manifestations seen in individuals with moderate to severe vitamin A deficiencies (VAD) is night blindness, defined as a condition in which a person cannot see in dim light. Depending on the extent and duration of VAD, a spectrum of other ocular manifestations including conjunctival xerosis, Bitot’s spots, keratomalacia, and xerophthalmia may also occur. In addition to its essential role in vision, vitamin A is also required for maintaining the integrity of epithelial cells, and has an important role, via the regulatory action of retinoic acid, in innate and adaptive immune functions. As a result, VAD results in impaired immune responses with lowered resistance to infections. For example, studies have found associations with VAD and an increased risk of mortality from diarrheal disease and measles (122, 123).

Serum vitamin A circulates largely in the form of a 1:1 complex of retinol and retinol binding protein; either total serum vitamin A or serum retinol levels may be assayed to measure vitamin A status and detect subclinical deficiencies.
**Vitamins D and E**

Vitamin D is a generic term for a group of related fat-soluble substances, and their metabolites, and includes vitamin D$_2$, which is derived from the ergosterol found in yeast and plants and vitamin D$_3$, which originates from the action of sunlight or UV light on the precursor 7-dehydrocholesterol found in the skin or from dietary sources (e.g. fatty fish and organ meats). The principal role of vitamin D is to ensure the adequate intestinal absorption of calcium and phosphorus and to regulate bone mineralization. Vitamin D also plays a critical role as an immune modulator, and in the regulation of cell differentiation, proliferation, and apoptosis. The body’s requirement for vitamin D may be met either by dietary intake and/or by skin synthesis.

Severe vitamin D deficiency in adults can produce osteomalacia, a condition characterized by a failure in the mineralization of bone resulting in weak bones, diffuse skeletal bone tenderness, proximal muscle weakness, and an increased frequency of fractures. In infants and children severe vitamin D deficiency can result in rickets, a condition marked by the softening and weakening of bones. Rickets is rare in developed countries due to widespread vitamin D supplementation; however, it remains a public health issue of concern for children exposed to conditions associated with extreme poverty such as famine and starvation. Rickets may also arise in children with malabsorption syndromes.

25-hydroxyvitamin D (25(OH)D) is the most abundant metabolite of all the vitamin D derivatives; thus, measuring the serum concentration of 25(OH)D is the most useful way to assess vitamin D status and detect subclinical deficiencies.
Vitamin E functions primarily as a lipid antioxidant. More than 90% of the vitamin E found in human serum is in the form of α-tocopherol; thus, concentration of serum α-tocopherol is the most commonly used biochemical marker to assess vitamin E status. Although vitamin E deficiency as a result of inadequate dietary intake has never been described in humans, it may occur in disease states where severe and chronic malabsorption of fat exists (e.g. celiac disease, PEM). Alternatively, secondary vitamin E nutritional deficiency may occur in fat-malabsorption syndromes.

Vitamin K

Vitamin K functions as a coenzyme in the synthesis of the active forms of several proteins involved in blood coagulation and bone metabolism. There are two main types of vitamin K. Phylloquinone, the major form of dietary vitamin K1, is widely distributed in plant and animal tissues; whereas vitamin K2 is synthesized by the microbiological flora of the normal gut. Primary nutritional deficiency of vitamin K in healthy individuals is very rare, however, vitamin K deficiency may be commonly seen in people at risk for malnutrition. Additionally, it may be a secondary outcome of some disease states (e.g. biliary obstruction, liver disease, and malabsorption syndromes). Additionally, broad-spectrum antibiotics, which destroy the intestinal flora, may also produce secondary vitamin K deficiency. Newborn infants may be at increased risk of vitamin K deficiencies as it is poorly transported across the placenta; however, vitamin K deficiency is generally not a risk to infants living in most developed countries as these infants routinely receive a dose of vitamin K either intramuscularly or orally within 6 hours of birth. If present, however, vitamin K deficiency in newborns is associated with hemorrhagic disease. Serum vitamin K levels are rarely used to determine whether a vitamin K deficiency exists; rather
initial suspicion of vitamin K deficiency is made based on clinical assessments in cases where unexpected or excessive bleeding occurs.

*The B vitamins and Folate*

Vitamin B12 and folate are two related vitamins; severe deficiencies in both vitamins cause megaloblastic anemia, or an anemia characterized by abnormally large red-cell precursors in the bone marrow (megaloblasts) and larger than normal red cells in the peripheral blood (macrocytic cells).

More specifically, vitamin B12 is the general term used to describe all the corrinoids that exhibit the biological activity of cyanocobalamin. Cobalamin-containing coenzymes function in the body in the degradation of certain amino acids and odd-chain fatty acids, as well as having a link with nucleic acid metabolism. The clinical presentation of vitamin B12 deficiency is indistinguishable from folate deficiencies; symptoms first occur in the hematopoietic system and include megaloblastic anemia. Other clinical manifestations of vitamin B12 are also known to occur both in the gastrointestinal and neurological systems. Of note, patients suffering from tropical sprue (a small intestinal malabsorption disease commonly found in the tropics marked by abnormal flattening of the villi and inflammation of the lining of the small intestine) may also develop vitamin B12 deficiency. Total serum vitamin B12 levels are measured to detect subclinical vitamin B12 deficiency.

Folate is the general term used to describe folic acid and its related compounds which each function as coenzymes involved in the transfer of single-carbon atom groups [e.g. methyl (CH₃), methylene (CH₂), methenyl (CH), formimino (CH=N=H), and formyl (CHO)] and perform steps involved in intermediary metabolism. Interestingly, the
malabsorption of folate has been documented secondary to infection with *Giardia* (115), but whether this finding is seen in other studies has not been determined. However, the main clinical manifestations of folate deficiency first appear in the hematopoietic system, due to its rapid proliferation of cells; one of the earliest signs of folate deficiency in humans is the hypersegmentation of neutrophils first in the bone marrow and then in the peripheral blood eventually leading to macrocytosis of the erythrocytes. Folate deficiencies have also been associated with Fragile X syndrome and 5,10 methylene-tetrahydrofolate reductase—two genetic disorders. Lastly, it has been established that folate also plays an essential role in the prevention of neural tube defects and as such is of particular importance during pregnancy. As is seen with vitamin B12 deficiencies, symptoms affecting the gastrointestinal and neurological systems may also occur. Biochemical tests that measure serum folate levels or folate levels in the erythrocytes are generally used in the nutritional assessment of folate status. Vitamin B12 deficiency blocks the uptake of folate by the tissues. Given this association, tests for folate deficiencies should be conducted in conjunction with an assessment of serum vitamin B12 concentrations.

*Thiamine, riboflavin, niacin*

The B vitamins—thiamin, riboflavin, and niacin—act as coenzymes or prosthetic groups in a variety of reactions involved in the catabolism of carbohydrates, fats, and proteins. Specifically, thiamin has a major role in carbohydrate metabolism; riboflavin is an essential component of two important coenzymes involved in a number of oxidative enzyme systems involved in electron transport; and niacin is the term used to describe a group of compounds involved in the synthesis of a number of reactions involved in intracellular respiration, oxidation, and nonredox reactions. All three vitamins are stored
for short periods of time, have no major tissue storage reservoir, are water soluble, rapidly excreted in the urine, and have a fast rate of catabolism. As a result, deficiencies in any of these vitamins develop more quickly than for either the fat-soluble vitamins, or vitamin B12.

Clinical symptoms associated with deficiencies in any of these three B vitamins are not highly specific (e.g. insomnia, irritability, loss of appetite, and weight loss). The classical syndrome of thiamine deficiency is beriberi, which produces symptoms affecting the cardiovascular, muscular, nervous, and gastrointestinal systems. The classical signs of riboflavin deficiency, termed ariboflavinosis, include: angular stomatitis (inflammation of the mucosa of the mouth); cheilosis (dry scaling of the lips and mouth); and glossitis (inflammation of the tongue). Pellagra is the characteristic syndrome of niacin deficiency and is characterized by skin and neurological complications (e.g. dermatitis and depressive psychosis). Although clinical deficiencies associated with any of these vitamins are rarely seen in developed countries as a result of food fortification programs, these clinical conditions are still found in parts of the world with low-protein, high carbohydrate diets (e.g. Southern Africa, Egypt, India, and China). Additionally, poor bioavailability of each of these micronutrients continues to be an issue in undernourished populations, despite the implementation of food fortification or vitamin supplementation programs, likely as a result of enteric infections.

Functional biochemical tests are most frequently used to confirm a clinical diagnosis of any of these vitamin deficiencies and to detect subclinical states. The biochemical markers generally used to assess deficiencies in any of these vitamins vary based on micronutrient and are as follows: transketolase levels are used to detect
erythrocyte thymine levels and the measurement of the functional activity coefficient of erythrocyte glutathione reductase (EGR) for riboflavin status. However, no biochemical or physiological function tests currently exist that reflect body stores of niacin.

2.5.2 Minerals

Iron

Iron is an essential micronutrient that plays an essential role in many metabolic processes. Aerobic metabolism is critically dependent on maintaining normal concentrations of several iron containing proteins that mediate oxygen transport, storage, and utilization. About 70% of the iron found in the body is present in hemoglobin, the oxygen-carrying pigment of the red blood cells. The remaining 30% is found as either storage iron in the liver (~25%), or in the form of myoglobin (~4%), the oxygen-binding storage protein found in muscle.

Iron deficiency is the primary cause of nutritional anemia, and is the most frequently occurring micronutrient deficiency in low-income and industrialized countries. Iron deficiency anemia is of particular concern in infancy and early childhood due to its associations with cognitive development. Iron deficiencies may arise from inadequate dietary intakes, poor iron absorption, or a combination of these factors. The most widely recognized physical manifestation of iron deficiency is nutritional anemia which can be further categorized in three stages based on increasing severity: iron depletion, marked by a progressive reduction in the amount of iron liver storage; iron-deficient erythropoiesis (iron deficiency without anemia), which is characterized by the exhaustion of iron stores; and lastly, iron-deficiency anemia (IDA), which is characterized by exhaustion of liver
stores, declining levels of circulating iron, and the presence of microcytic anemia, or a reduction in the concentration of hemoglobin in the red blood cells.

Venous blood hemoglobin levels are the biomarker used in the diagnosis of anemia; however, three interrelated variables--serum iron, total iron binding capacity, and transferrin saturation are useful for differentiating between milder forms of nutritional iron deficiency and anemia. Inflammation, infection, and chronic disease states such as PEM typically produce low hemoglobin and serum iron, and transferrin values.

Zinc

Zinc has a number of functions that are vital to body functioning. Examples include: cellular metabolism; function or regulation of numerous enzymes; maintenance of protein structure; synthesis and degradation of DNA, RNA, and ribosomes; stabilization of the structure of certain DNA binding proteins (zinc-fingers). Zinc also plays a role in the absorption, mobilization, transport, and metabolism of other micronutrients, such as vitamin A, most likely through its involvement in protein synthesis and cellular enzyme functions (reviewed in Christian and West, 1998) (124). The clinical manifestations of even marginal zinc deficiencies include slow physical growth; secondary zinc deficiency has been documented in cases of malabsorption syndromes (e.g. Crohn’s disease).

Zinc also plays an important role in modulating the host’s immune response to infectious diseases, which has been extensively reviewed(125). The association of zinc deficiency with diarrhea-associated morbidity has been well established. In fact, a recent meta-analysis of randomized controlled trials (RCT) of zinc supplementation found that zinc supplementation reduced both the severity and duration of both acute and persistent diarrhea in children (126). Zinc supplementation trials have also found an association of
lower zinc status and an increased susceptibility and higher rates of acute respiratory infections (127, 128). Serum and plasma zinc concentrations are the most widely used measures to assess zinc status at the population level.

*Copper and selenium*

Copper is an essential component of many enzyme systems especially those that catalyze oxidation-reduction reactions. One of the earliest clinical manifestations of copper deficiency in humans is persistent neutropenia (low neutrophil count) usually followed by the development of microcytic anemia [small red blood cells and low mean corpuscular volume (MCV)]. Less frequently, copper deficiency has been associated with impaired growth. Copper deficiency in humans is rare, though it has been described in cases associated with prolonged diarrhea as this prevents copper reabsorption from the bile. Therefore, individuals with malabsorption syndromes (e.g. tropical sprue, celiac disease) are at risk for copper deficiency. Conversely, infection (129) and inflammation (130) are associated with elevated serum copper levels; however the overall impact to an individual experiencing both infection and malabsorption simultaneously on serum copper levels has not been clearly elucidated in the literature. Serum copper or serum ceruloplasmin levels are most often used to assess copper status.

Selenium plays an essential role in several important metabolic pathways including antioxidant defense systems, thyroid hormone metabolism, and redox control of enzymes and proteins. In humans, two diseases are linked to severe endemic selenium deficiency: Keshan’s disease (cardiomyopathy) and Kashin-Beck’s disease (a chronic bone disorder), which both have been shown to occur predominantly in young children and women of child-bearing age living in areas of Northeast China, where the soil is low in selenium.
Additionally, children with severe PEM (marasmus or kwashiorkor) tend to have a low biochemical selenium status likely due to an inadequate intake of proteins. A combination of biomarkers is recommended for assessing selenium status, however, measuring urine selenium concentration is the most commonly used indicator for assessing nutritional status.

*Calcium, Phosphorus, and Magnesium*

Calcium, magnesium, and phosphorus are the major mineral components of the body. Of these, calcium is the most abundant mineral in the body and plays an essential role in making up the structural component in bone and soft tissues. Calcium also plays a regulatory role in several metabolic processes including enzyme activation, vascular contraction and vasodilation, muscle contractability, nerve transmission, hormone function, and membrane transport. Several clinical conditions are associated with impaired calcium absorption such as Crohn’s and celiac disease. Chronic calcium deficiency occurs from a habitually inadequate intake or poor calcium absorption and is an important factor associated with reduced bone mass. An individual’s vitamin D status affects the efficiency of calcium absorption in the body. There are no satisfactory tests used to routinely assess an individual’s calcium status; serum calcium concentrations should not be used as a measure of calcium status because they are strongly homeostatically controlled and remain constant under most conditions. Biochemical markers of bone remodeling may be used to assess the bone mineral content of calcium either directly or indirectly; however, many of these methods are invasive, costly, labor intensive, and require specialized personnel, thus are of limited value in epidemiological field studies.
Phosphorus is the second most abundant mineral in the body and plays an essential role in all the energy-producing reactions of the cells. It may also play a functional role in many physiological buffer systems and in the activation of many catalytic proteins by phosphorylation. Dietary deficiency of phosphorus is rare; however, malabsorption syndromes (e.g. Crohn’s and celiac disease) may reduce serum phosphorus concentrations. Rickets is also associated with low serum phosphorus levels because a vitamin D metabolite plays an essential role in the efficacy of phosphorus absorption. Serum phosphorus is the most commonly used as the biochemical marker of nutritional phosphorus levels.

Magnesium acts as a metal activator or cofactor in over 300 enzyme systems and may be required for the transport of other ions such as potassium and calcium. It is also involved in protein synthesis and energy metabolism. Magnesium also acts in the neuromuscular system by stabilizing nerve axons and influencing the release of neurotransmitters. Hence, a magnesium deficiency in humans likely results in neuromuscular dysfunctions. Serum magnesium levels have also been found to be low in malnourished children (131). Serum magnesium concentration is the most frequently used measure to assess magnesium status.

2.6: Concluding Thoughts

Global efforts to reduce the global burden of disease from malnutrition, especially in children < 5 have placed emphasis on implementing improved treatment and control strategies of diarrheal disease. As Giardia is a major cause of diarrheal disease, identifying whether Giardia infections initiate or exacerbate childhood malnutrition has major
implications for the field. Numerous independent observational studies have drawn associations between *Giardia* infection and two types of undernutrition, growth and micronutrient malnutrition, however, the results from these individual studies have been inconclusive. Thus, in Part I of the research presented in chapters 3 and 4, I provided a more complete understanding of the association of *Giardia* infection with malnutrition outcomes. Specifically, in Chapters 3 and 4, I performed two unique systematic reviews and meta-analyses in order to determine the extent of the impact of *Giardia* infections on anthropometric indicators of growth (Chapter 3) and micronutrient malnutrition (Chapter 4). Lastly, in Chapter 5, I discuss the public health implications of these findings.
Chapter 3: Anthropometric Impact of *Giardia* Infection in Children: A Systematic Review and Meta-Analysis

3.1: Introduction

*Giardia lamblia* (referenced simply as *Giardia* subsequently) is one of the most prevalent intestinal protozoan parasites in the world, with the highest rates of infection occurring in children in regions without regular access to improved water and sanitation. Long-standing estimates of infection rates (~33% of all adults in developing regions of the world)(19) appear to have actually been overly conservative, with a more recent study finding that almost 90% of children in an urban slum tested positive for *Giardia* by one year of age (5). *Giardia* continues to be a health concern in industrialized regions as well (19, 132). *Giardia* is readily transmitted between hosts: even asymptomatic individuals shed cysts at a high rate of $10^8$-$10^9$ cysts per day for weeks to months following infection, and as few as ten cysts have been demonstrated to be sufficient to initiate a new infection. Although *Giardia* was included in 2004 as part of the WHO Neglected Disease Initiative (86), attention to the parasite has remained low compared to other intestinal pathogens, for which there is clearer evidence of morbidity and mortality from infection. Giardiasis is correlated with malabsorptive diarrhea: even in the U.S., *Giardia* is responsible for an estimated 15% of clinical pediatric diarrheal visits (133). Yet, a majority of infections are self-limiting without apparent clinical consequences, and some have proposed that *Giardia* could also be considered a commensal microbe in many people (134). In a recent WHO Neglected Tropical Disease report and follow-up analysis, *Giardia* was no longer mentioned as a Neglected Tropical Disease (135, 136).
Likewise, aspects of *Giardia*-associated malnutrition and its consequences are particularly problematic in the literature, especially for children. We focus here on undernutrition, which, in turn, is divided into protein-energy malnutrition (PEM) and micronutrient malnutrition. While there are multiple direct indicators for micronutrient deprivation (e.g., hemoglobin level and anemia for iron deficiency), PEM is typically assessed through anthropometric measures. The World Health Organization (WHO) defines three anthropometric measures of malnutrition using Z-scores: weight-for-age (WAZ), height-for-age (HAZ), and weight-for-height (WHZ). The most severe anthropometric consequences of malnutrition are in turn respectively defined as being “underweight” (with a weight-for-age Z-score less than -2 standard deviations from the mean), “stunting” (height-for-age), and “wasting” (weight-for-height). Recent efforts to reduce the rates of childhood undernutrition have made significant progress; however, with worldwide estimates of childhood malnutrition at 15% underweight, 25% stunted, and 8% wasted, and with hundreds of millions of children still at risk, we will miss the projected goals set in the Millennium Development Goals (100). In aggregate, it is estimated that 45% of all children’s deaths under the age of five are attributable directly or indirectly to malnutrition (137).

In response to these statistics, there has been increased recognition among public health officials of the need to gather the evidence to make a stronger case between neglected enteric pathogens (NEP) and malnutrition (138, 139). Therefore, in this study we conduct the first systematic review and meta-analyses to examine the impact of *Giardia* infection and anthropometric growth indicators as signs of undernutrition and find that
even sub-clinical infections in children have a deleterious impact on childhood growth and development.

3.2: Methods

This systematic review was developed in line with the PRISMA guidelines (140).

3.2.1 Search Strategy

We conducted a systematic review to identify all relevant studies addressing the association between *Giardia* infection and anthropometric indicators of growth. Two independent authors (SRB and HGE) each searched the MEDLINE, EMBASE, and Cochrane electronic databases, without either language or date restrictions, using the following search terms: “*Giardia*”, “anthropo*”, “anthropometric”, “malnutrition”, “malnourished”, “BMI”, “height”, “stunt*”, “weight”, “wasting”, “wasted”. We also conducted an additional electronic search of the Google Scholar database. Searches in Google Scholar were limited to English-only studies published from January 1, 1980–June 30, 2014 and included the search terms “child” or “children”. Google searches were also subject to the following exclusions: NOT “gerbil”, “jird”, “mouse”, “mice” OR “rat”; NOT “celiae” OR “coeliac”; NOT “immunodeficient”, “immunodeficiency”, “immunosuppressed”, “immunocompromised”, “HIV” OR “AIDS”; NOT “irritable bowel syndrome” OR “Crohn’s disease”; and NOT “refugee” OR “refugees”. Hand-searches of the reference and citation lists of all relevant articles found through the different web database searches were performed to identify any additional published studies meeting study inclusion criteria.
3.2.2 Selection Criteria

Two independent reviewers (SRB and HGE) initially screened the title and abstracts of each identified article to select potential full text articles for further data extraction according to study inclusion and exclusion criteria. All longitudinal cohort, case-control, and cross-sectional epidemiological study designs, as well as any randomized control trials and longitudinal studies with treatment intervention that addressed the association between *Giardia* infection as the exposure and growth measured by weight-for-age, height-for-age, or a combination of height and weight-for-age as study outcomes in children were included in this review. Additional inclusion criteria were as follows: the study performed a microscopic diagnosis of *Giardia* infection; included children ≤ 15 years old; and published in English from January 1, 1980-June 30, 2014. No geographical restrictions were applied.

3.2.3 Data Extraction

Two authors (SRB and HGE) developed a data extraction form in Excel. The following data were extracted from each study: bibliographic information (e.g. author name, year of publication, full reference citation); geographic/location of study (e.g. country of study, urban/rural); epidemiological study design (e.g. longitudinal cohort, case-control, cross-sectional, randomized control trial, other); study demographics (e.g. age range and gender distribution of study population); total study size; anthropometric indicators [e.g. height, height-for-age Z score (HAZ), weight, weight-for-height Z score (WHZ), weight-for-age Z score (WAZ), body mass index (BMI), mid-upper arm circumference (MUAC), or head circumference (HC)]; statistical outcome measured (e.g. mean Z scores, odds ratios (OR), number and percent, p-value, other non-Z score
measurements); and other potentially relevant information [e.g. data analysis type (univariate, bivariate, or multivariate) and comparison groups (e.g. infected versus uninfected)].

3.2.4 Quality Assessment of Studies

Two authors (SRB, HGE) independently performed a quality assessment of each study prior to its inclusion in the final systematic review and meta-analyses. The quality assessment score system was internally developed and adapted based on guidance outlined in the Strengthening the Reporting of Observational Studies in Epidemiology checklist (STROBE Statement) (141).

Briefly, each study was first broken down into 4 main sections (introduction, methods, results, discussion/conclusions). Each of these main sections was further parsed into a total of 18 metrics (Table 3.6.2.1) that pertained to a core, general component common to different epidemiological study designs. Metrics were assigned a score of either zero or one based on the presence (1) or absence (0) of relevant study information. Any studies that received a score of 0 on any of the 18 metrics were excluded from the final systematic review and further meta-analyses. Disagreements were resolved by discussion and consensus.

3.2.5 Meta-Analyses

Random effects meta-analyses of studies comparing infection with *Giardia* (exposure) and Z score measurements (outcome) were performed using R statistical software (142). Papers published by the same research group, addressing the same research question, were checked for potential duplicate data. When duplicate data was detected, we used the largest published data set in our meta-analyses. Additionally, in longitudinal
studies, we only used baseline data in our meta-analyses. Statistical significance for each pooled estimate was defined as $p < 0.05$.

Three separate random effects meta-analyses using the inverse variance method were performed on each of the following continuous variables: height-for-age (HAZ), weight-for-height (WHZ), and weight-for-age (WAZ). The sample sizes, means, and standard deviations for both *Giardia* infected and *Giardia* uninfected children were abstracted from each study and used to compute pooled standardized mean differences, corresponding 95% confidence intervals (95% CI), and $p$ values for each indicator respectively. We converted any studies reporting a standard error of the mean (SEM) to a standard deviation (SD) using the conversion formula found in the Cochrane Handbook for Systematic Reviews of Interventions (113). For studies that reported only subgroup estimates, we calculated an overall effect by combining these estimates using the formula for combining means and standard deviations also provided in the Cochrane Handbook for Systematic Reviews of Interventions (113).

Three additional random effects meta-analyses using an inverse variance method were performed on a distinct set of studies that analyzed associations between *Giardia* infection and either stunting (HAZ $< -2$ SD), wasting (WHZ $< -2$ SD), or underweight (WAZ $< -2$ SD). The author, year, total numbers of children infected and uninfected with *Giardia* respectively, and the outcome(s) of interest (stunting, wasting, or underweight) were abstracted from each study in order to compute pooled odds ratios (OR), 95% CI, and $p$ values for each anthropometric indicator. Only unadjusted risk estimates were used in meta-analyses of studies that reported an odds ratio (OR).
Heterogeneity among studies was evaluated using Cochran’s Q statistic (143). Heterogeneity was considered statistically significant if \( p < 0.10 \) (113). An \( I^2 \) statistic was also calculated to quantify the percentage of total variation across studies that is due to heterogeneity rather than chance; it was interpreted following the guidelines outlined in the Cochrane Handbook for Systematic Reviews of Interventions (113). When heterogeneity was detected, sensitivity analyses were performed in order to test the effects of sample size on the Q statistic and its associated p value (113). We created Begg’s funnel plots (144) and performed Egger’s linear regression test (145) to evaluate the presence of publication bias for each meta-analysis.

### 3.2.6 Sign Test

We conducted a sign test of all studies in the qualitative analysis that addressed an association between *Giardia* and anthropometric indicators of malnutrition (143). This technique allowed the impact of every study included in the systematic review to be quantified regardless of whether the numerical data presented in each separate study could be combined statistically in meta-analyses. We calculated p values comparing the total number of included studies that found a negative association, regardless of statistical significance, between *Giardia* infection and at least one anthropometric indicator (WAZ, WHZ, or HAZ) to the number of included studies that found no association. We also performed subgroup analyses using the sign test to compute p values for studies that measured anthropometric indicators as either continuous (means) or dichotomous (OR) variables. All sign test analyses were completed according to the protocol in the R statistical software manual (142).
3.3: Results

3.3.1 Systematic Review

Search results are presented in Figure 3.5.2.1. Initial database and hand searches yielded a total of 3525 studies; 1363 of these were identified as unique articles and underwent a title and abstract review. We excluded 95 studies that were conducted in highly selected subpopulations (e.g., celiac patients, people with HIV/AIDS, immunocompromised individuals, individuals with irritable bowel syndrome or Crohn’s disease, or refugee(s) populations) in this step due to the possible introduction of confounding variables; we additionally excluded 540 other studies for failure to meet full inclusion criteria (e.g., not in English, not in children, not an original study). We assessed the remaining 728 articles in a full text review. We excluded 621 studies at this stage for failing to meet full inclusion criteria: 392 studies did not focus on *Giardia* as a variable in the analyses, 196 studies did not examine anthropometric outcomes of infection, 29 studies looked more generally at enteric pathogens other than *Giardia*, 2 studies assessed malnutrition as exposure and *Giardia* infection as outcome, and 2 studies were unavailable as full text. The remaining 107 articles met the inclusion criteria and were included in the systematic review.

We excluded 81 of the 107 articles used in the systematic review from the meta-analysis for the following five reasons: (1) 38 studies provided quantitative estimates examining the association of *Giardia* infection and malnutrition that were too variable to combine (39, 40, 56, 108, 146-157) (111, 158-163) (164-175) (176-179) (180-185); (2) 29 studies provided insufficient quantitative information of the association between *Giardia* infection and malnutrition to be pooled (104, 109, 186-202) (110, 203-206) (207-211); (3)
Four studies calculated a risk ratio (instead of an odds ratio) for the association of *Giardia* infection on malnutrition (112, 212-214); (4) Three studies contained duplicate data sets that we had included in other studies (186, 215, 216); (5) We performed meta-analyses only when five or more studies measured the same anthropometric indicator; thereby excluding seven studies that only collected data on an alternative anthropometric malnutrition indicator [e.g., body mass index (BMI), mid-upper arm circumference (MUAC), or head circumference (HC)]. See Table 3.6.2.2 for baseline characteristics of the studies included in the systematic review only.

To capture the full breadth of evidence from the articles in the systematic review, we conducted a sign test on all studies that reported findings for an association between *Giardia* infection and anthropometric measures of malnutrition. 91 of the 107 studies included these findings and were evaluated using the sign test: the 26 studies from the meta-analyses, plus 65 studies that were in the systematic review only. Using the broadest criteria – identification of at least one anthropometric measure negatively impacted by *Giardia* infection – 60 of the 91 studies reported this finding (p = 0.003). Interestingly, when the studies were divided into two groups, those that measured anthropometric indicators as a continuous variable (means) showed that *Giardia* infection was significantly associated with impaired growth (39 of 56 studies; p=0.004), while the studies that measured anthropometric indicators as a dichotomous variable (OR) did not show a significant association. (Details of these analyses can be found in Table 3.6.2.3.)

### 3.3.2 Baseline Characteristics of Studies Included in Meta-Analyses

Tables 3.5.1.1 and 3.5.1.2 present the baseline characteristics of studies included in the meta-analyses of continuous and dichotomous variables respectively. The 26 studies
in the meta-analyses were grouped into two main categories based on their characterization of the association between infection and the anthropometric indices of malnutrition as either a dichotomous (n=13) or as a continuous (n=13) variable. Studies that measured the indices as a dichotomous variable recorded the odds ratios for one or more of the anthropometric indicators (WAZ, HAZ, and WHZ); these studies classified growth impairment as severe (<-2 SD), i.e., capturing only children who were underweight (WAZ), stunted (HAZ), and wasted (WHZ).

The majority of studies that reported continuous outcome variables (n=9) and those that analyzed dichotomous variables (n=12) were cross-sectional epidemiological study designs, allowing for comparison between infected children with uninfected children from the same location. The studies represent broad geographic distribution: Latin or South America (41, 106, 217-219) (105, 107, 207, 220-223); Africa (224-227) (228); the Middle East (229-233); and Asia (234, 235) (236, 237)

3.3.3 Meta-Analyses

*Giardia infection and Weight-for-Age (WAZ)*

Weight-for-age (WAZ) anthropometric analyses provide information about acute malnutrition because weight can be affected by short-term nutritional deficiencies (90). Random effects meta-analysis of nine studies (223, 233) (105, 207, 217, 218, 220, 222, 231) comparing the mean WAZ scores for children infected with *Giardia* as compared to uninfected children found a statistically significant association (pooled mean difference = -0.26, 95% CI: -0.41, -0.12; p = 0.0005) with $I^2 = 22.2\%$ indicating there may be low heterogeneity (Fig. 3.5.2.2). When we performed a random effects meta-analysis of twelve studies (41, 107, 219, 221, 224, 225, 227-230, 232, 234) comparing the association between
*Giardia* infection and severe weight-for-age status (underweight: WAZ < -2 SD) we found no statistically significant association between *Giardia* infection and underweight status (pooled OR = 1.18, 95% CI: 0.89, 1.56; p = 0.26) and a moderate level of heterogeneity ($I^2 = 47.5\%$) (Figure 3.5.2.2). There was no evidence of publication bias for either continuous (Egger’s test, p=0.15) or dichotomous (Egger’s test p=0.14) variables, respectively (Fig. 3.6.1.1).

*Giardia infection and Height-for-Age (HAZ)*

Height-for-age (HAZ) anthropometric analyses provide information about chronic malnutrition because height is typically only affected by long-term nutritional deficiencies (90). Random effects meta-analysis of twelve studies (106, 207, 217, 218, 226) (105, 220, 222, 223, 231, 233, 235) comparing the mean HAZ scores for children infected with *Giardia* as compared to uninfected children found a statistically significant negative association between infection and HAZ (pooled mean difference = -0.29, 95%CI: -0.41,-0.16; p < 0.0001) with $I^2$: 43.5% indicating there may be moderate heterogeneity (Fig. 3.5.2.3). In the meta-analysis of ten studies (107, 221, 232, 234, 237) (41, 219, 227, 228, 230) comparing the association between *Giardia* infected and uninfected children with severe height-for-age status (stunting: HAZ scores < -2 SD), we found no statistically significant association (pooled OR = 1.26, 95%CI: 0.81, 1.92; p = 0.33) with $I^2$: 81.3% indicating there may be moderate or substantial heterogeneity (Fig. 3.5.2.3). There was no evidence of publication bias for either the meta-analysis of continuous (Egger’s test, p=0.04) or dichotomous variables (Egger’s test, p=0.90) (Fig. 3.6.1.1).
**Giardia infection and Weight-for-Height (WHZ)**

Weight-for-height (WHZ) anthropometric analyses can provide information about both acute and chronic malnutrition (90). In the random effects meta-analysis of twelve studies (223, 233, 235, 236) (105, 207, 220, 222, 231) (217, 218, 226) that compared WHZ scores between children infected with *Giardia* or uninfected, we found a statistically significant negative association (pooled mean difference $= -0.14$, 95% CI: -0.25, -0.03; $p = 0.01$) with $I^2$: 20% indicating there may be low heterogeneity (Fig. 3.5.2.4). When we performed a meta-analysis of the six studies (41, 107, 228, 230, 232, 234) comparing the association between *Giardia* infected and uninfected children with severe weight-for-height status (wasting: WHZ $< -2$ SD), we found no statistically significant association (pooled OR $= 0.89$, 95% CI: 0.62, 1.29; $p = 0.55$) with $I^2$: 6.9% indicating there may be low heterogeneity (Fig. 3.5.2.4). There was no evidence of publication bias for either the continuous (Egger’s test, $p = 0.71$) or dichotomous (Egger’s test, $p=0.71$), respectively (Fig. 3.6.1.1).

### 3.4: Discussion

Our overall meta-analysis results revealed statistically significant support for the impact of *Giardia* infection on childhood nutritional status and growth. More specifically, we found that *Giardia* infection negatively affected children’s anthropometric measures of weight and height, indicating that the infection may have been associated with both acute and chronic undernutrition outcomes. We did not detect a significant association between *Giardia* infection and the more extreme categories of underweight, stunting and wasting. Because the studies we evaluated included both symptomatic and asymptomatic children,
our findings extend an earlier report that focused exclusively on the negative impact of *Giardia*-associated diarrheal disease (101), providing evidence for long-term negative health consequences of clinically asymptomatic *Giardia* infections. This work thus supports the recent call to the global health community to “restructure our theoretical framework to broaden our concept of ‘diarrheal’ disease to include ‘asymptomatic’ enteric infections” (138).

In this study we examined studies that assessed the impact of *Giardia* infection in terms of anthropometric indicators. Undernutrition can be caused by both caloric deprivation (often also termed protein-energy malnutrition because of the vital role of proteins in providing nutritional resources) and/or micronutrient (e.g., vitamin A, iron, zinc) deprivation. Because most of the studies included in our meta-analysis did not define the micronutrient status of the subjects, we assume that caloric deprivation was the primary cause of the growth impairment, but we cannot rule out either a role for micronutrients in growth or for secondary health consequences from micronutrient deficiencies in infected children.

Our analyses cannot define directionality in the association between *Giardia* infection and undernutrition. Because *Giardia* infection is associated with malabsorption, it is difficult to ascertain whether the observed nutrient deprivation is a consequence of this malabsorption or whether infected individuals consume fewer calories. A recent study in mice demonstrated that infection with *G. lamblia* parasites negatively impacted weight gain and that undernourished mice (on low protein diets) were in turn more susceptible to the negative impact of parasite infection (52). The authors suggest that both alter the architecture of the small intestine mucosal surfaces as a possible explanation for their
mutually deleterious and negatively synergistic effects. This is sometimes described as “environmental enteropathy” (138) in individuals from developing countries. Indeed, both infected and uninfected individuals included in this meta-analysis were often undernourished compared to WHO growth standards, raising the possibility that the children were more susceptible to infection because of their nutrient-deprived status. Yet, the majority of the cross-sectional studies available for analysis (39 out of 41) reported Giardia infection as the exposure and malnutrition as the outcome, with only two published reports looking at the inverse relationship. Thus, while we can reasonably conclude that Giardia infection results in mild malnutrition among children, we do not have the data to argue for a causal effect in the opposite direction.

Generally, there is strong evidence for a correlation between undernutrition and diarrheal disease. In regions of the world with inadequate sanitation and access to clean drinking water, children experience multiple bouts of diarrhea each year and diarrheal disease remains the second leading cause of mortality in children under five with an estimate of slightly under 2 million deaths annually (95, 238, 239). Historically, attention has focused on bouts of acute diarrhea and the associated mortality from dehydration; however, there is also a profound impact of chronic (persistent) diarrhea on nutrient absorption and morbidity. Diarrhea is now recognized as the leading cause of malnutrition in children under five (95, 240). Most research has focused on the anthropometric growth consequences from this intersection, but a growing body of evidence also argues for long-term impacts on cognition and human development (97, 241). Researchers have identified a “vicious cycle” between malnutrition and childhood infections in which the incidence of
each exacerbates the likelihood and impact of the other through a yet poorly understood interaction between the host and host defenses and the parasite (238, 242-246).

Delineating a clear connection specifically between giardiasis and malnutrition has proven more difficult, marked by a lack of consensus on the direction of Giardia’s association with diarrhea (101, 139, 247, 248). Indeed, the overlap in geography and chronology between the two has made it harder – not easier – to prove a direct correlation because of the confounding nature of coincident occurrence. Multiple studies have demonstrated altered intestinal morphology and function in humans and other animals as a consequence of infection with Giardia (reviewed in (37, 50, 54)), but a causal link to malnutrition has only recently been documented. Bartelt and colleagues (2013) found that infection with Giardia impaired growth in a mouse model and further exacerbated the effects of a malnourished diet (52). The clearest evidence thus far comes from a recent systematic review and meta-analysis that examined the association between Giardia infection and diarrheal disease (101). The authors found that while Giardia was not associated with acute diarrhea, a limited subset of their analysis demonstrated a significant correlation with persistent diarrhea (defined as having a duration >14 days). Yet because only a small percentage of giardiasis cases result in diarrhea, the consequence of infection remains unclear for the estimated billion or more asymptomatic carriers of the parasite each year.

Therefore, one of our most striking findings is that even subclinical infection with Giardia can result in long-term growth impairment in children. Giardia infections have long been associated with malabsorptive persistent diarrhea, long considered less consequential than the pathology of bloody diarrhea or the dehydration risks from acute
watery diarrhea (249). The absence of overt symptoms in the majority of individuals infected with *Giardia* is perhaps the reason that the global health impact of such a ubiquitous human pathogen has been historically underestimated. The majority of studies included in our analysis were cross-sectional, with subjects randomly recruited from local populations and not drawn from patients with documented disease. Yet even in these largely asymptomatic children, the consequences of *Giardia* infection on growth were both acute (as reflected in weight-for-height, and weight-for-age measures) and chronic (as reflected in height-for-age measures).

We also believe that the negative association between *Giardia* infection and childhood growth and development may have been overlooked because the infection has a rather diffuse impact on anthropometric indices. Thus, the effect is only apparent when growth is measured as a continuous variable (represented as means, medians, and other measures of central tendency and overall distribution) and not as discrete binary outcomes (e.g. underweight, stunting, and wasting). Another limitation of our review is that the majority studies did not diagnose *Giardia* infection (or its indirect measure: cyst shedding), preventing us from drawing any conclusions about the possible effects of parasite burden.

There is an emerging literature on the negative association between enteric infections and diarrhea in general (97, 241, 250) and *Giardia* infections in particular (38, 109, 149, 232, 251-253) on cognitive function.

Our meta-analysis had a number of limitations. Accurate detection of infection status is perhaps the most significant. First, infection rates are likely to be underestimated because of the low sensitivity of microscopic detection methods. In the studies that specifically identified children with only *Giardia* infections, the most commonly used
methods of detecting *Giardia* infection in field studies are formalin-ether sedimentation microscopy to visually identify parasites or commercial ELISA to detect parasite antigen. While false positives are rare, both methods of detection can miss low-grade infections, and because cyst shedding is intermittent (26), analysis of single stool samples (as is common) can result in false-negatives. False negatives would result in a bias against our findings, as truly infected children with lower anthropometric scores could potentially have been incorrectly classified as controls in the studies we abstracted. Finally, as few if any studies reported the specific assemblages of *Giardia lamblia* (23), we were not able to estimate potential associations of these parasitic subpopulations on child growth and development.

A second limitation deals with polyparasitism. In regions of the world without access to safe drinking water and basic sanitation, children can be infected simultaneously with multiple enteric pathogens (e.g., *Giardia* and *Ascaris*). It is unclear what effect polyparasitism might have on anthropometric outcomes and to what extent we can ascribe negative consequences on anthropometric indicators to each respective pathogen.

Finally, we chose not to include studies that focused on subjects with known inflammatory or immune disorders (e.g., HIV/AIDS, irritable bowel syndrome, celiac disease, Crohn’s disease). The enhanced susceptibility of immunosuppressed individuals to *Giardia* infections is well-documented (55), but it presented a potentially confounding influence that we chose to exclude in our analyses. *Giardia*’s intersection with gastrointestinal inflammation is even more complicated. Numerous studies have shown that *Giardia* infection reduces inflammation (reviewed in (50)), yet other studies have
shown that some infections can be associated with inflammatory bowel diseases either as cause or effect (62, 254).

The meta-analysis we present here provides supporting evidence for the deleterious impacts of *Giardia* infection on children’s growth and development. Our findings also have implications for policy: others have recently proposed that correctly diagnosing, treating, and preventing childhood intestinal infections are essential to reduce the incidence of stunting (139), a conclusion supported by our results. If true, the results can further predict a potentially life-long toll on health and quality of life – a cost in DALYs that far exceeds current estimates – and point to the need to renew attention toward the “Neglected Enteric Protozoa” if global goals for disease reduction and positive human development are to be reached (138).

3.4.1 Acknowledgement

The authors would like to thank Theodore J. Picou for technical assistance with R Statistical Software.
### 3.5: Tables and Figures

#### 3.5.1 Tables

**Table 3.5.1.1** Baseline characteristics of studies included in random effects meta-analyses of continuous anthropometric variables

<table>
<thead>
<tr>
<th>Authors, year</th>
<th>Study Design</th>
<th>Age</th>
<th>Location</th>
<th>Study Size</th>
<th>Diagnosis of <em>G. lamblia</em></th>
<th>Anthropometric Variable(s) Measured</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alvey et al., 2013</td>
<td>CS</td>
<td>3-7</td>
<td>Guatemala</td>
<td>87</td>
<td>ELISA</td>
<td>WAZ, HAZ, WHZ</td>
</tr>
<tr>
<td>Veenemans et al., 2011</td>
<td>LCT</td>
<td>0.5-5</td>
<td>Tanzania</td>
<td>612</td>
<td>ELISA</td>
<td>HAZ</td>
</tr>
<tr>
<td>Boeke et al., 2010</td>
<td>CS</td>
<td>5-12</td>
<td>Colombia</td>
<td>386</td>
<td>Sed &amp; Micro</td>
<td>WAZ, HAZ, WHZ</td>
</tr>
<tr>
<td>Quihui et al., 2010</td>
<td>CS</td>
<td>6-10</td>
<td>Mexico</td>
<td>98</td>
<td>Sed &amp; Micro</td>
<td>WAZ, HAZ, WHZ</td>
</tr>
<tr>
<td>Matos et al., 2008</td>
<td>CS</td>
<td>0-4</td>
<td>Brazil</td>
<td>629</td>
<td>Sed &amp; Micro</td>
<td>WAZ, HAZ, WHZ</td>
</tr>
<tr>
<td>Quihui-Cota et al., 2008</td>
<td>CS</td>
<td>6-10</td>
<td>Mexico</td>
<td>400</td>
<td>Sed &amp; Micro</td>
<td>WAZ, HAZ, WHZ</td>
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<td>Sadjiadi and Tanideh, 2005</td>
<td>CS</td>
<td>3-6</td>
<td>Iran</td>
<td>300</td>
<td>Sed &amp; Micro</td>
<td>WAZ, HAZ, WHZ</td>
</tr>
<tr>
<td>Prado et al., 2005</td>
<td>LCT</td>
<td>0-4</td>
<td>Brazil</td>
<td>597</td>
<td>Sed &amp; Micro</td>
<td>WAZ, HAZ, WHZ</td>
</tr>
<tr>
<td>Moya-Camarena et al., 2002</td>
<td>LCT</td>
<td>3-6</td>
<td>Mexico</td>
<td>13</td>
<td>Zn Sulfate</td>
<td>WAZ, HAZ, WHZ</td>
</tr>
<tr>
<td>Fraser et al., 2000</td>
<td>LC</td>
<td>0-1.5</td>
<td>Israel</td>
<td>251</td>
<td>Sed &amp; Micro</td>
<td>WAZ, HAZ, WHZ</td>
</tr>
<tr>
<td>Tsuyuoka et al., 1999</td>
<td>CS</td>
<td>4-24</td>
<td>Brazil</td>
<td>360</td>
<td>Sed &amp; Micro</td>
<td>WAZ, HAZ, WHZ</td>
</tr>
<tr>
<td>Egger et al., 1990</td>
<td>CS</td>
<td>3-8</td>
<td>Thailand</td>
<td>343</td>
<td>Sed &amp; Micro</td>
<td>HAZ, WHZ</td>
</tr>
<tr>
<td>Janoff et al., 1990</td>
<td>CS</td>
<td>0-5</td>
<td>Thailand</td>
<td>202</td>
<td>Sed &amp; Micro</td>
<td>WHZ</td>
</tr>
</tbody>
</table>

---

1. LC: Longitudinal Cohort, CC: Case Control, CS: Cross Sectional, RCT: Randomized Clinical Trial, LCT: LC+Treatment
2. Age range of study participants in years
3. Total study sample size
4. Method of diagnosing *G. lamblia* infection: Sedimentation (Sed), Microscopy (Micro), ELISA, Zinc Sulfate Concentration (Zn Sulfate)
5. WAZ: Weight-for-Age, HAZ: Height-for-Age, WHZ: Weight-for-Height
Table 3.5.1.2 Baseline characteristics of studies included in random-effects meta-analysis of dichotomous anthropometric variables

<table>
<thead>
<tr>
<th>Authors, year</th>
<th>Study Design</th>
<th>Age</th>
<th>Location</th>
<th>Study Size</th>
<th>Diagnosis of <em>G. lamblia</em></th>
<th>Anthropometric Variable(s) Measured</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ignatius et al., 2014</td>
<td>CS</td>
<td>0-4</td>
<td>Rwanda</td>
<td>558</td>
<td>Sed&amp;Micro; ICA; PCR</td>
<td>WAZ</td>
</tr>
<tr>
<td>Daneshvar and Rahimkhani, 2013</td>
<td>CC</td>
<td>0-4</td>
<td>Iran</td>
<td>142</td>
<td>Sed&amp;Micro</td>
<td>WAZ</td>
</tr>
<tr>
<td>Ignatius et al., 2012</td>
<td>CS</td>
<td>0-4</td>
<td>Rwanda</td>
<td>492</td>
<td>Sed&amp;Micro; PCR</td>
<td>WAZ</td>
</tr>
<tr>
<td>Inabo et al., 2011</td>
<td>CS</td>
<td>0.5-12</td>
<td>Nigeria</td>
<td>374</td>
<td>ELISA</td>
<td>WAZ, HAZ</td>
</tr>
<tr>
<td>Botero-Garcés et al., 2009</td>
<td>CS</td>
<td>0.5-6</td>
<td>Colombia</td>
<td>2035</td>
<td>Sed&amp;Micro</td>
<td>WAZ, HAZ, WHZ</td>
</tr>
<tr>
<td>Silva et al., 2009</td>
<td>CS</td>
<td>0.5-6</td>
<td>Brazil</td>
<td>405</td>
<td>Sed&amp;Micro</td>
<td>WAZ, HAZ</td>
</tr>
<tr>
<td>Worku, 2009</td>
<td>CS</td>
<td>6-14</td>
<td>Ethiopia</td>
<td>322</td>
<td>Sed&amp;Micro</td>
<td>WAZ, HAZ, WHZ</td>
</tr>
<tr>
<td>Sereebutra et al., 2006</td>
<td>CS</td>
<td>2.5-7</td>
<td>Guatemala</td>
<td>131</td>
<td>ELISA</td>
<td>HAZ</td>
</tr>
<tr>
<td>Almerie et al., 2008</td>
<td>CS</td>
<td>6-12</td>
<td>Syria</td>
<td>1469</td>
<td>Sed&amp;Micro</td>
<td>WAZ, HAZ, WHZ</td>
</tr>
<tr>
<td>Simsek et al., 2004</td>
<td>CS</td>
<td>0.5-5</td>
<td>Turkey</td>
<td>160</td>
<td>Sed&amp;Micro</td>
<td>WAZ, HAZ, WHZ</td>
</tr>
<tr>
<td>Sackey et al., 2003</td>
<td>CS</td>
<td>0-14</td>
<td>Ecuador</td>
<td>244</td>
<td>Sed&amp;Micro</td>
<td>WAZ, HAZ, WHZ</td>
</tr>
<tr>
<td>Muniz-Junqueira et al., 2002</td>
<td>CS</td>
<td>0-5</td>
<td>Brazil</td>
<td>124</td>
<td>Sed&amp;Micro</td>
<td>WAZ, HAZ</td>
</tr>
<tr>
<td>Awasthi and Pande, 1997</td>
<td>CS</td>
<td>1.5-3.5</td>
<td>India</td>
<td>1061</td>
<td>Sed&amp;Micro</td>
<td>WAZ, HAZ, WHZ</td>
</tr>
</tbody>
</table>

6 LC: Longitudinal Cohort, CC: Case Control, CS: Cross Sectional, RCT: Randomized Clinical Trial, LCT: LC+Treatment
7 Age range of study participants in years
8 Total study sample size
9 Method of diagnosing *G. lamblia* infection: Sedimentation (Sed), Microscopy (Micro), Immunochromatographic Assay (ICA), ELISA, PCR
10 Underweight: (WAZ < -2 SD), Stunting: (HAZ < -2 SD), Wasting: (WHZ < -2 SD)
3.5.2 Figures

Figure 3.5.2.1 Flow chart of systematic review

Figure 3.5.2.1 is a flow chart outlining the systematic review following the PRISMA guidelines.
Figure 3.5.2.2: *Giardia* infection and Weight-for-Age (WAZ). There is a significant negative association between *Giardia* infection and WAZ > -2 (top panel). However, as shown in the bottom panel *Giardia* infection does not significantly increase the odds of being underweight (WAZ < -2). In the top panel, blue boxes designate positive standardized mean differences and the red boxes are negative standardized mean differences; in the bottom panel blue boxes are odds ratios (OR) > 1, red boxes OR < 1.
Figure 3.5.2.3 *Giardia* infection and Height-for-Age (HAZ)

### Table: Giardia infection and Height-for-Age (HAZ)

<table>
<thead>
<tr>
<th>Author, Year</th>
<th>Country</th>
<th>Total Infiltrated</th>
<th>Mean SD</th>
<th>Mean SD</th>
<th>W(random) MD</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Avey et al., 2013</td>
<td>Guatemala</td>
<td>48</td>
<td>-3.400 1.100</td>
<td>39</td>
<td>-2.200 1.000</td>
<td>6.2%</td>
</tr>
<tr>
<td>Veeremans et al., 2011</td>
<td>Tanzania</td>
<td>192</td>
<td>-2.440 0.750</td>
<td>366</td>
<td>-2.400 0.670</td>
<td>17.9%</td>
</tr>
<tr>
<td>Boldo et al., 2010</td>
<td>Colombia</td>
<td>26</td>
<td>-1.160 0.980</td>
<td>360</td>
<td>-0.720 0.880</td>
<td>7.3%</td>
</tr>
<tr>
<td>Quihui et al., 2010</td>
<td>Mexico</td>
<td>49</td>
<td>-1.200 1.100</td>
<td>85</td>
<td>-0.700 1.000</td>
<td>7.1%</td>
</tr>
<tr>
<td>Matos et al., 2008</td>
<td>Brazil</td>
<td>32</td>
<td>-0.771 1.056</td>
<td>137</td>
<td>-0.340 1.085</td>
<td>6.8%</td>
</tr>
<tr>
<td>Quihui-Cota et al., 2008</td>
<td>Mexico</td>
<td>40</td>
<td>-0.460 0.900</td>
<td>70</td>
<td>-0.380 0.800</td>
<td>8.7%</td>
</tr>
<tr>
<td>Sadjadi and Tarkhish, 2005</td>
<td>Iran</td>
<td>71</td>
<td>-0.570 1.120</td>
<td>229</td>
<td>-0.220 1.150</td>
<td>9.9%</td>
</tr>
<tr>
<td>Prado et al., 2004</td>
<td>Brazil</td>
<td>85</td>
<td>-0.630 1.090</td>
<td>512</td>
<td>-0.190 1.040</td>
<td>11.9%</td>
</tr>
<tr>
<td>Moya-Camarena et al., 2002</td>
<td>Mexico</td>
<td>7</td>
<td>0.080 0.700</td>
<td>6</td>
<td>-0.020 0.600</td>
<td>2.9%</td>
</tr>
<tr>
<td>Fraser et al., 2000</td>
<td>Israel</td>
<td>185</td>
<td>-1.150 0.870</td>
<td>24</td>
<td>-0.840 0.980</td>
<td>6.7%</td>
</tr>
<tr>
<td>Tsuyukoa et al., 1999</td>
<td>Brazil</td>
<td>38</td>
<td>-0.514 1.139</td>
<td>313</td>
<td>-0.226 1.120</td>
<td>7.4%</td>
</tr>
<tr>
<td>Egger et al., 1990</td>
<td>Thailand</td>
<td>31</td>
<td>-2.170 0.980</td>
<td>147</td>
<td>-1.670 1.020</td>
<td>7.6%</td>
</tr>
</tbody>
</table>

Random effects model

### Table: Odds Ratio of Giardia infection and Height-for-Age (HAZ)

<table>
<thead>
<tr>
<th>Author, Year</th>
<th>Country</th>
<th>Events Infiltrated</th>
<th>Odds Total</th>
<th>Events Uninfected</th>
<th>Odds Total</th>
<th>W(random) OR</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inabo et al., 2011</td>
<td>Nigeria</td>
<td>77</td>
<td>155</td>
<td>118</td>
<td>219</td>
<td>12.7%</td>
<td>0.84 [0.56; 1.28]</td>
</tr>
<tr>
<td>Botero-Garcés et al., 2009</td>
<td>Colombia</td>
<td>106</td>
<td>554</td>
<td>186</td>
<td>1461</td>
<td>13.7%</td>
<td>1.92 [1.25; 2.11]</td>
</tr>
<tr>
<td>Silva et al., 2009</td>
<td>Brazil</td>
<td>10</td>
<td>94</td>
<td>17</td>
<td>270</td>
<td>9.4%</td>
<td>1.77 [0.78; 4.02]</td>
</tr>
<tr>
<td>Worku, 2009</td>
<td>Ethiopia</td>
<td>5</td>
<td>29</td>
<td>44</td>
<td>145</td>
<td>7.9%</td>
<td>0.47 [0.17; 1.31]</td>
</tr>
<tr>
<td>Almune et al., 2008</td>
<td>Syria</td>
<td>10</td>
<td>206</td>
<td>79</td>
<td>920</td>
<td>10.6%</td>
<td>0.54 [0.28; 1.07]</td>
</tr>
<tr>
<td>Seebehra et al., 2006</td>
<td>Guatemala</td>
<td>12</td>
<td>33</td>
<td>33</td>
<td>96</td>
<td>9.4%</td>
<td>1.13 [0.49; 2.57]</td>
</tr>
<tr>
<td>Simsek et al., 2004</td>
<td>Turkey</td>
<td>26</td>
<td>45</td>
<td>11</td>
<td>80</td>
<td>9.1%</td>
<td>8.58 [3.60; 20.46]</td>
</tr>
<tr>
<td>Sackey et al., 2003</td>
<td>Ecuador</td>
<td>30</td>
<td>58</td>
<td>56</td>
<td>169</td>
<td>11.2%</td>
<td>2.16 [1.16; 3.97]</td>
</tr>
<tr>
<td>Muniz-Junqueira et al., 2002</td>
<td>Brazil</td>
<td>2</td>
<td>12</td>
<td>6</td>
<td>27</td>
<td>4.2%</td>
<td>0.70 [0.12; 4.10]</td>
</tr>
<tr>
<td>Awasthi and Pande, 1997</td>
<td>India</td>
<td>34</td>
<td>60</td>
<td>617</td>
<td>980</td>
<td>11.8%</td>
<td>0.77 [0.45; 1.30]</td>
</tr>
</tbody>
</table>

Random effects model

---

Figure 3.5.2.3: *Giardia* infection and Height-for-Age (HAZ). There is a significant negative association between *Giardia* infection and HAZ > -2 (top panel). However, as shown in the bottom panel *Giardia* infection does not significantly increase the odds of stunting (HAZ < -2). In the top panel, blue boxes designate positive standardized mean differences and the red boxes are negative standardized mean differences; in the bottom panel blue boxes are odds ratios (OR) > 1, red boxes OR < 1.
Figure 3.5.2.4 Giardia infection and Weight-for-Height (WHZ). There is a significant negative association between Giardia infection and WHZ > -2 (top panel). However, as shown in the bottom panel Giardia infection does not significantly increase the odds of wasting (WHZ < -2). In the top panel, blue boxes designate positive standardized mean differences and the red boxes are negative standardized mean differences; in the bottom panel blue boxes are odds ratios (OR) > 1, red boxes OR < 1.
3.6: Supplemental Figures and Tables

3.6.1 Figures

Figure 3.6.1.1 Funnel Plots

Figure 3.6.1.1: Funnel plots of studies examining the association of Giardia infection and WAZ, HAZ, and WHZ. Each circle represents a unique study analyzed in the meta-analysis. Effect estimates are plotted on the X-axis; the measure of study size is on the Y-axis. Effect sizes from smaller studies scatter more widely at the bottom of the graph, with the spread narrowing among larger studies. The dotted vertical line is the overall summary estimate from the meta-analysis of studies. The left panel is studies looking at milder/moderate malnutrition (WAZ, HAZ, WHZ > -2) using means. The right panel is studies examining the association between infection and more severe measures of malnutrition (WAZ, HAZ, WHZ < -2) assessing these indicators in terms of dichotomous variables or odds ratios (OR).
### 3.6.2 Tables

Table 3.6.2.1 Summary of scoring system to assess study quality.

<table>
<thead>
<tr>
<th>Section</th>
<th>0 points</th>
<th>1 point</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Introduction</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Background/Rationale</td>
<td>No explanation of scientific background and rationale for study</td>
<td>Explanation of scientific background and rationale for study</td>
</tr>
<tr>
<td>Research Question</td>
<td>No statement of research question</td>
<td>Stated research question, including any prespecified hypotheses</td>
</tr>
<tr>
<td>Study Objective(s)</td>
<td>No statement of study objective(s)</td>
<td>Stated study objective(s)</td>
</tr>
<tr>
<td><strong>Methods</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Study Design</td>
<td>No description of study design</td>
<td>Presented description of study design including type of study conducted:</td>
</tr>
<tr>
<td></td>
<td>Descriptive Case study</td>
<td>Longitudinal Cohort (with or without treatment intervention)</td>
</tr>
<tr>
<td></td>
<td>Not epidemiological study</td>
<td>Cross-Sectional</td>
</tr>
<tr>
<td></td>
<td>(example: Review article)</td>
<td>Case-Control</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Randomized Control Trial</td>
</tr>
<tr>
<td>Setting</td>
<td>Did not describe setting, location, or relevant dates</td>
<td>Described setting, location, and relevant dates for: periods of recruitment, exposure, data collection, and follow-up</td>
</tr>
<tr>
<td>Participants</td>
<td>Did not describe study population</td>
<td>Included description of study population such as eligibility criteria, inclusion and exclusion criteria, sources and methods of participant and control selection, and case and control definitions (if applicable)</td>
</tr>
<tr>
<td>Diagnostic Criteria</td>
<td>Did not describe</td>
<td>Described diagnostic methods used for diagnosis of <em>G. lamblia</em> infection</td>
</tr>
<tr>
<td>Variables</td>
<td>Did not describe</td>
<td>Clearly defined all variables collected including outcomes, exposures, predictors, and potential confounders</td>
</tr>
<tr>
<td>Quantitative Variables</td>
<td>Did not describe</td>
<td>Described how quantitative variables were handled in data analysis</td>
</tr>
<tr>
<td>Data sources/measurement</td>
<td>Did not describe</td>
<td>Described sources of data and details of methods of data assessment (measurement) and analysis</td>
</tr>
<tr>
<td><strong>Results</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Participants</td>
<td>Did not report numbers of study participants at each stage of study</td>
<td>Reported numbers of participants in each stage of study (total number of potential participants, examined for eligibility, included in study, lost to follow up, analyzed)</td>
</tr>
<tr>
<td>Descriptive data</td>
<td>Did not provide demographics of study participants</td>
<td>Provided characteristics of study participants including information on potential confounders</td>
</tr>
</tbody>
</table>
**Outcome data**

| Did not report numbers of outcome events or summary measures | Reported numbers of outcome events or summary measures analyzed |

**Main results**

| Did not report summary of main results | Reported estimates and their precision. Noted whether estimates are unadjusted, or (if applicable) confounder adjusted estimates |

**Discussion/Conclusions**

**Key results**

| Did not summarize key study results | Summarized key results with reference to study objectives |

**Limitations**

| Did not discuss study limitations | Discussed study limitations including discussion on potential sources of bias or imprecision |

**Interpretation of Study Results**

| Did not describe | Provided some interpretation of study results considering objectives, limitations, multiplicity of analyses, results from similar published studies, and other relevant evidence |

**Generalizability**

| Did not discuss | Discussed the generalizability (external validity) of the study results |

---

**Table 3.6.2.2 Baseline characteristics of studies included in systematic review only (Sample)**

<table>
<thead>
<tr>
<th>Author, year</th>
<th>Country</th>
<th>Study Design</th>
<th>Age&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Study Size</th>
<th>Micronutrient(s) Examined&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Authors&lt;sup&gt;c&lt;/sup&gt; Findings&lt;sup&gt;d&lt;/sup&gt;</th>
<th>Reason for Exclusion&lt;sup&gt;e&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ahmed et al., 1991</td>
<td>Saudi Arabia</td>
<td>CS</td>
<td>4 - 19</td>
<td>1426</td>
<td>Fe</td>
<td>Giardia lamblia was the most common pathogenic parasite and its high infection rate seemed to be associated with lower hemoglobin (Hb) level</td>
<td>2a</td>
</tr>
<tr>
<td>Ahmed et al., 1990</td>
<td>Saudi Arabia</td>
<td>CS</td>
<td>6 - 12</td>
<td>717</td>
<td>Fe</td>
<td>G. lamblia was found to be the most common intestinal parasite among children of all socio-economic classes. Anemia was more prevalent among parasitically infected children</td>
<td>2a</td>
</tr>
<tr>
<td>Aini et al., 2007</td>
<td>Malaysia</td>
<td>CS</td>
<td>2 - 15</td>
<td>281</td>
<td>Fe</td>
<td>None of the infections studied (including giardiasis) showed significant</td>
<td>2b</td>
</tr>
</tbody>
</table>

<sup>a</sup> CS: Cross sectional; CC: Case Control; LC: Longitudinal Cohort; T: Treatment intervention; S: Supplementation

<sup>b</sup> Age range of study population in years

<sup>c</sup> Total study size

<sup>d</sup> Copper (Cu), Folate, Iron (Fe), Zinc (Zn), Vitamin A, Vitamin B12

<sup>e</sup> Author’s reported findings specific to *Giardia* infection and mean serum micronutrient levels

<sup>f</sup> 1: Heterogeneous outcome; 2: Insufficient data--2a: *Giardia* not isolated as a variable in analysis; 2b: No raw data; 3: Duplicate data
Table 3.6.2.3 Sign Test (Sample)

Table 1: Sign Test for All Studies Included in Systematic Review (N=91) (107-16)

<table>
<thead>
<tr>
<th>Association Found?</th>
<th>How Measured?</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Means (M)</td>
<td>Odds Ratio (OR)</td>
<td></td>
</tr>
<tr>
<td>Yes (n=60)</td>
<td>39</td>
<td>21</td>
<td></td>
</tr>
<tr>
<td>No (n=31)</td>
<td>17</td>
<td>14</td>
<td></td>
</tr>
</tbody>
</table>

p values

|          | p = 0.003 | p = 0.005 | p = 0.31 |

Table 2: Sign Tests for All Studies Included in Systematic Review by Malnutrition Indicator (N=91)

Table 2a: Chronic Malnutrition (Height, Height-for-Age (HA), Height-for-Age Z (HAZ), Head Circumference (HC))

<table>
<thead>
<tr>
<th>Association Found?</th>
<th>How Measured?</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Means (M)</td>
<td>Odds Ratio (OR)</td>
<td></td>
</tr>
<tr>
<td>Yes (n=35)</td>
<td>24</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>No (n=24)</td>
<td>11</td>
<td>13</td>
<td></td>
</tr>
</tbody>
</table>

p values

|          | p = 0.19 | p = 0.04 | p = 0.84 |

Table 2b: Acute Malnutrition (Weight, Height-for-Age (WA), Weight-for-Age Z (WAZ), Mid Upper Arm Circumference (MUAC))

<table>
<thead>
<tr>
<th>Association Found?</th>
<th>How Measured?</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Means (M)</td>
<td>Odds Ratio (OR)</td>
<td></td>
</tr>
<tr>
<td>Yes (n=38)</td>
<td>27</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>No (n=18)</td>
<td>8</td>
<td>10</td>
<td></td>
</tr>
</tbody>
</table>

p values

|          | p = 0.01 | p = 0.002 | p = 1.00 |
Chapter 4: *Giardia* Infection and Micronutrient Deficiencies: A Systematic Review and Meta-Analysis

4.1: Introduction

*Giardia lamblia* (syn. *Giardia intestinalis, Giardia duodenalis*), a eukaryotic intestinal parasite, is one of the major causes of diarrheal disease (*giardiasis*) worldwide. Long-standing estimates of infection rates report that ~33% of people in developing countries have had giardiasis (4). However, a more recent study conducted in children between birth up to one year of age, found that the cumulative probability of infection was ~90% in an urban slum in Bangladesh (5) suggesting that the prevalence of *Giardia* in developing countries may be higher than previously estimated. *Giardia* continues to pose a public health threat in developed countries as well. It has been identified as the most common human intestinal parasite (7, 8), and the most common cause of waterborne outbreaks (7, 9), in the United States. *Giardia* is also the most persistent pathogen diagnosed in cases of travelers’ diarrhea (10). The pathogen is transmitted through the ingestion of cysts found in contaminated food or water and/or can be transmitted person-to-person through fecal-oral transmission. Cyst shedding is intermittent, and infected individuals can shed as many as $10^9$ cysts per gram of stool per day for weeks to months following infection. However, *Giardia* has a low infectious dose; ingestion of as few as 10 cysts may cause disease (4). The clinical presentation of *Giardia* is highly variable. Infected individuals can present as clinically asymptomatic carriers or may present with a varying range of mild, moderate, or severe disease symptoms (e.g. fatigue, abdominal bloating, weight loss, diarrhea, or failure to thrive). The pathophysiology of *Giardia* infections is equally variable with complications believed to include: heightened rates of enterocyte apoptosis; small intestinal barrier dysfunction; and a diffuse shortening of epithelial microvilli among others (reviewed in (50)). These factors are thought to
contribute to *Giardia* associated-nutrient malabsorption and micronutrient malnutrition. In fact, *Giardia* has been identified as one of the most important intestinal parasites related to nutritional status (255).

Undernutrition, a nutritional deficiency resulting from either a lack of food intake or an inability of the body to convert or absorb the nutrients consumed, is a significant global public health problem accounting for over ⅓ of child deaths worldwide and 11% of the total global disability adjusted life years (DALYs) annually (87). Though undernutrition encompasses deficits both in protein or caloric intake or absorption, known as protein-energy malnutrition (PEM), and in the intake or absorption of essential vitamins and minerals, known as micronutrient malnutrition (MM), we specifically focus our analysis here on MM, acknowledging that PEM is also a grave public health concern. Micronutrient deficiencies, defined as deficiencies in essential vitamins or minerals needed for proper growth and development, are one of the causes of micronutrient malnutrition. Estimates report that over two billion people are at risk for MM globally (93, 255). While multiple different micronutrient deficiencies may exist within a population, the most common include: Vitamin A Deficiency (VAD), iodine deficiency disorders (IDD), and iron deficiency anemia (IDA). VAD has been identified as the leading cause of preventable blindness in children (256) and iron deficiency affects approximately 20% to 50% of the world’s population (255). Other micronutrient deficiencies of growing public health concern include zinc, folate, and the B vitamins; however there is limited data on the worldwide prevalence of each (93). Although MM is more frequently associated with the developing world, there is growing recognition that this form of undernutrition represents an important public health problem in some industrialized countries as well. It has been estimated that countries may lose anywhere between 2%-3% of their Gross Domestic Product (GDP) as a result of iron, iodine, and zinc deficiencies (257).
Intestinal parasitic infections, such as with *G. lamblia* or hookworms, are both considered risk factors for micronutrient malnutrition (258). In addition to contributing to more short term, acute impacts of undernutrition, either of these conditions, intestinal parasitic infection or micronutrient malnutrition, may also produce long-term impacts on growth and development. For example, studies have documented the negative effects of *Giardia* infections on childhood physical growth and cognitive development (38, 109, 149, 232). Similarly, micronutrient deficiencies have also been found to impair childhood growth (reviewed in (259)) and cognitive development (reviewed in (260)). However, the literature examining the association between *Giardia* infections and micronutrient malnutrition has been inconclusive to date. Although *Giardia* was included in the World Health Organization’s (WHO) “Neglected Disease Initiative” in 2004 (86), attention to the parasite has remained low compared to other enteric pathogens, for which there is clearer evidence of pathogen-associated morbidity and mortality. In fact, in a recent WHO Report assessing the global impact of neglected tropical diseases, *Giardia* was not included (135).

Recent calls to gather evidence to support the interactions between micronutrient deficiencies and intestinal parasitic infections have emerged in the literature (258, 261). While a previous systematic review and meta-analysis assessed the impacts of helminth infections and micronutrients (262), the effect of *Giardia* infection on different micronutrient deficiencies was not addressed. Therefore, in this study, we conduct the first systematic review and meta-analyses to examine the association of *Giardia* infection and serum micronutrient levels to contribute to the evidence base examining the associations of intestinal parasitic infections on micronutrient malnutrition.
4.2: Methods

This systematic review was developed in line with the PRISMA guidelines (140).

4.2.1 Search Strategy

We conducted a systematic review to identify all relevant studies examining the relationship between *Giardia* infection (exposure) and serum micronutrient levels (outcome). Two independent authors (SRB and RM) each searched the MEDLINE, EMBASE, and Cochrane electronic databases, without either language or date restrictions, using the following search terms: “*Giardia*”, “micronutrient”, “Vitamin A”, “retinol”, “xerophthalmia”, “night blindness”, “Vitamin B”, “Vitamin B12”, “folate”, “Vitamin D”, “bone density”, “osteoporosis”, “calcium”, “Vitamin E”, “iron”, “iron deficiency anemia”, “hemoglobin”, “zine”, “copper”, “phosphorus”, “magnesium”, “selenium”, “thiamin”, “riboflavin”, and “niacin”. We also conducted an electronic search of the Google Scholar database. Searches in Google Scholar were limited to the terms “*Giardia*” and “micronutrient” and included English-only studies published from January 1, 1970–December 1, 2014. Google Scholar searches were also subject to the following exclusions: NOT “gerbil”, “jird”, “mouse”, “mice” OR “rat”; NOT “biochemistry”, NOT “molecular biology”, NOT “mitochondrial evolution”, NOT “genetics”, NOT “celiac” OR “coeliac”; NOT “immunodeficient”, “immunodeficiency”, “immunosuppressed”, “immunocompromised”, NOT “irritable bowel syndrome (IBS)”, and NOT “HIV” OR “AIDS”. Hand-searches of the reference and citation lists of all relevant articles found through electronic database searches were performed to identify any additional published studies meeting study inclusion criteria.

4.2.2 Selection Criteria

Two independent reviewers (SRB and RM) initially screened the title and abstracts of each identified article to select potential full text articles for further data extraction according to study
inclusion and exclusion criteria. All longitudinal cohort, case-control, and cross-sectional epidemiological study designs, as well as any randomized control trials, longitudinal studies with treatment intervention, and supplementation studies that addressed the association between *Giardia* infection and serum micronutrient levels in humans were included in this review. Additional inclusion criteria were defined as follows: the study defined *Giardia* infection as the exposure and serum micronutrient levels as the outcome; the study performed at least a microscopic diagnosis of *Giardia* infection; and the study was published in English between January 1, 1970-December 1, 2014. No further geographical restrictions were applied.

4.2.3 Data Extraction

Two authors (SRB and HGE) developed a data extraction form in Excel. The following data were extracted from each study: bibliographic information (e.g. author name, year of publication, full reference citation); geographic/location of study (e.g. country of study, urban/rural); epidemiological study design [e.g. observational studies (longitudinal cohort, case-control, cross-sectional), treatment studies (randomized control trials or longitudinal cohort with treatment intervention) or supplementation studies]; study demographics (e.g. age range and gender distribution of study population); total study size; micronutrient indicators measured (e.g. minerals or vitamin) statistical outcome measured (e.g. means, odds ratios (OR), numbers and/or percent, p-value); whether the authors reported a statistically significant association between *Giardia* infection and serum micronutrient levels (Y or N), whether the study could also be used in a quantitative meta-analysis (Y or N, if N then reason for exclusion); and other potentially relevant information [e.g. analysis type (univariate, bivariate, or multivariate) and comparison groups (e.g. infected versus uninfected, pre and post treatment, or pre and post supplementation)].
4.2.4 Quality Assessment of Studies

Two authors (SRB, HGE) independently performed a quality assessment of each study in order to assess the generalizability, representativeness, and validity of study results included in the final systematic review and meta-analyses. The quality assessment score system was internally developed and adapted based on guidance outlined in the Strengthening the Reporting of Observational Studies in Epidemiology checklist (STROBE) and as described by others (141, 263, 264).

Briefly, each study was first broken down into 4 main sections (introduction, methods, results, discussion/conclusions). Each of these main sections was further parsed into a total of 18 metrics that pertained to a core, general component common to different epidemiological study designs. Metrics were assigned a score of either zero or one based on the presence (1) or absence (0) of relevant study information. Any studies that received a score of 0 on any one of the 18 metrics were excluded from the final systematic review and further meta-analyses. The score system is available in Table 4.6.1.1. Disagreements were resolved by discussion and consensus.

4.2.5 Meta-Analyses

Random effects meta-analyses were conducted using R statistical software (142) to obtain summary pooled estimates comparing Giardia infection (exposure) and serum micronutrient levels (outcome). Studies identified in the systematic review were first divided according to the following variables: micronutrient(s) studied; statistical outcome variable measured (e.g. means or odds ratio (OR)); and epidemiological study type (e.g. observational or treatment intervention). Next, studies were grouped together if they shared variables in common. Studies falling into each grouping were not mutually exclusive; a single study could be grouped into multiple categories based on the different combinations of indicators reported in the study. For example, the same study could be...
included in both the group of zinc observational studies that measured outcome as mean variables and the group of zinc treatment studies that measured outcomes as mean variables simultaneously. This strategy was replicated for each micronutrient examining the association between *Giardia* infection and serum micronutrient levels that was identified in the systematic review. We further divided studies measuring serum iron levels by the specific marker of iron status used to measure iron levels (e.g. serum iron or ferritin, hemoglobin, or anemia). However, we combined studies that measured mean levels of either serum retinol or serum vitamin A into one meta-analysis to provide an overall estimate of *Giardia* infection and mean serum vitamin A levels (94).

The sample sizes, means, and standard deviations for both *Giardia* infected and *Giardia* uninfected individuals were abstracted from each study and used to compute pooled standardized mean differences (SMD) or pooled odds ratios (OR), corresponding 95% confidence intervals (95% CI), and p values for each micronutrient respectively. We converted any studies reporting a Standard Error of the Mean (SEM) to a Standard Deviation (SD) using the conversion formula found in the Cochrane Handbook for Systematic Reviews of Interventions (113). When papers only provided subgroup estimates (e.g. by geographic location, age groups, or genders) we combined individual subgroup estimates in order to obtain an overall effect of *Giardia* infection on serum micronutrient levels in the study population using the formula for combining means and standard deviations provided in the Cochrane Handbook for Systematic Reviews of Interventions (113).

For longitudinal observational studies, we only used data from baseline assessments in order to calculate standardized mean differences (SMD) or odds ratios (OR) for each observational study comparing mean serum micronutrient levels in *Giardia* infected and uninfected individuals pooled in the meta-analyses. Similarly, we used baseline data for our pre-treatment serum
micronutrient levels in *Giardia* infected individuals and compared this to post treatment mean serum micronutrient levels in the same individual in order to calculate SMD for each treatment study. We also performed hand calculations on raw study data in order to obtain means and their associated standard deviations (SD) if the study authors did not report these values. Only unadjusted, estimates were used in meta-analysis of studies calculating an OR. We only chose to perform a meta-analysis if there were a minimum of 5 studies within a given grouping. Papers published by the same research group, addressing the same research question, were checked for potential duplicate data. When duplicate data was detected, we used the largest published data set in our meta-analyses. Statistical significance for each pooled estimate was defined as p value < 0.05.

Heterogeneity between studies was evaluated using the Q and $\tau^2$ statistics (143). Heterogeneity was considered statistically significant if p < 0.10 in order to account for the low power of the test for heterogeneity (113). An $I^2$ statistic was also calculated; it was interpreted following the guidelines outlined in the Cochrane Handbook for Systematic Reviews of Interventions (113). When heterogeneity was detected, sensitivity analyses were performed in order to test the effects of sample size on the Q statistic and its associated p value.

We created Begg’s Funnel Plots (144) and performed Egger’s linear regression tests (145) to evaluate the presence of publication bias for each meta-analysis. Publication bias was considered significant if p < 0.05.

4.2.6 Sign Test

We conducted a sign test on all observational and treatment studies that reported an association between *Giardia* infection (exposure) and serum micronutrient levels (outcome). This technique allowed the impact of every study included in the systematic review to be quantified.
regardless of whether the numerical data presented in each separate study could be combined statistically in meta-analyses. We compared the total number of included studies that found a negative association, regardless of statistical significance, between *Giardia* infection and serum micronutrient levels for each micronutrient identified to the number of included studies that found no association. All sign test analyses were completed according to the protocol found in the R statistical software manual (142).

4.3: Results

4.3.1 Systematic Review

Initial database and hand searches yielded a total of 2104 studies; 1161 of these were identified as unique articles and underwent a title and abstract review. While the vast majority of the 1161 articles identified through a comprehensive search of the different web databases were found either in the MEDLINE or EMBASE databases; Google Scholar was useful in identifying a small number of relevant epidemiological studies that were either published in an international journal that was not currently part of the MEDLINE or EMBASE database collections, or was published as a research dissertation or thesis rather than as a paper that had been published in a scientific journal. We excluded 766 articles at this stage that failed to meet study inclusion criteria: 487 were either not published in English, not about *Giardia*, not conducted in humans (e.g. animals or plants), not an original population study (e.g. reviews, case studies, fictional works), or published prior to 1970; 124 were basic science laboratory studies (e.g. biochemistry, cell or molecular biology, genetics); and 39 studies related to water quality or examining the efficacy of clean water interventions. Additionally, we excluded 90 studies, conducted in highly selected subpopulations (e.g. celiac patients, people diagnosed with HIV/AIDS, immunocompromised
individuals, or patients with irritable bowel syndrome), in this step due to the possible introduction of confounding variables in the analyses. We also excluded an additional 26 studies that measured outcome according to levels of different sugars absorptions (e.g. D-xylose, fructose, or lactose), rather than serum vitamin or mineral levels. We assessed the remaining 395 articles in a full text review. Search results are presented in Figure 4.5.2.1.

We excluded an additional 309 articles at this stage for failing to fully meet the inclusion criteria for this analysis: 85 studies looked at enteric pathogens other than *Giardia*; 129 studies broadly described the clinical management and epidemiology of *Giardia* infection (e.g. detection, diagnosis, surveillance, treatment, and response); 46 studies focused on host immunology and pathology of infection; 47 studies measured another outcome of *Giardia* infection (e.g. growth, cognitive development); and 2 studies were unavailable as full text. The remaining 86 studies met the inclusion criteria and were included in the systematic review.

We excluded 40 of the 86 studies included in the systematic review from the meta-analysis for the following reasons: (1) Studies provided quantitative estimates measuring the association between *Giardia* and serum micronutrient levels that were either too heterogeneous to pool, such as β coefficients, or did not define exposure and outcome per study inclusion criteria (e.g. measured effect of micronutrient supplementation on improving micronutrient levels in *Giardia* infected individuals) (n = 11) (265-275); (2) Studies had insufficient data such that either *Giardia* was not isolated as a variable in the quantitative analysis (n = 14) (189, 276-281) (282-285) (181, 286, 287) or the authors did not provide sufficient raw data to either hand calculate means and standard deviations or determine the total numbers of *Giardia* infected and uninfected individuals with outcome of interest if not provided (n = 13) (115, 186, 196, 288-291) (111, 292, 293) (221, 294, 295); and (3) Studies with duplicate data (n = 2) (296) (297). Table 4.6.1.2 provides the baseline
characteristics of the studies included in the systematic review only (Table 4.6.1.2). Additionally, to capture the full breadth of evidence from each of the studies included in the systematic review, all studies except ones that either did not isolate *Giardia* as a variable in their analyses (n = 14) (189, 276-284) (285, 286) (181, 287), or had duplicate data (n = 2) (296) (297) were included in sign tests of the association of *Giardia* infection and serum micronutrient levels.

**4.3.2 Baseline Characteristics of Studies Included in Meta-Analyses**

Tables 4.5.1.1-4.5.1.3 present the baseline characteristics of studies that were included in different meta-analyses of observational (See 4.5.1.1), treatment (See 4.5.1.2), and studies measuring continuous or dichotomous variables [(e.g. anemia) (4.5.1.3)]. The studies included in our meta-analyses either reported continuous (means) outcome variables (n = 29 observational studies; n = 5 treatment studies) or analyzed the dichotomous (OR) variables (n = 11 observational studies). The majority of studies that reported either continuous outcome variables or analyzed dichotomous variables were cross-sectional epidemiological study designs allowing for comparison between *Giardia* infected and uninfected persons from the same population living in the same location. The studies represent broad geographic distribution: Africa (147, 298-303) (178, 211, 226, 304); the Middle East (305-313); Latin or South America (41, 106, 107, 207, 218, 219, 314, 315); South-East Asia (234, 235, 316-319); and Europe and North America (320-322). The systematic review identified a total of 46 unique articles eligible for meta-analyses; however, we only conducted meta-analyses on groups of studies that contained a minimum of 5 unique articles in them. See Table 4.6.1.2 for baseline characteristics of the studies we excluded from our meta-analyses (323-327).
4.3.3 Meta-Analyses of Observational Studies

There is a negative association between *Giardia* infection and iron (Fe).

We identified a total of 15 unique observational studies (Table 4.5.1.1) that presented data eligible to pool in one of two separate random effects meta-analyses measuring the associations between *Giardia* infection and mean serum iron levels either as studies that measured serum Fe or serum ferritin levels as markers of iron status (n= 10) (106, 147, 298, 302, 307-309, 316, 319, 320) or as studies that measured serum Hb levels (n = 9) (216, 226, 234, 302, 314, 316, 319, 320, 328). We found a statistically significant negative association between *Giardia* infection and mean serum Fe/ferritin levels: [pooled mean difference = -40.1292, 95% CI: (-60.1387, -20.1197), p <0.0001] (Figure 4.5.2.2A). Similarly, we found a statistically significant negative association between infection and mean serum Hb levels [pooled mean difference = -0.4442, (-0.8781, -0.0103), p = 0.04] (Figure 4.5.2.2B). There was a large and significant heterogeneity between the estimates for analyses of serum Fe/ferritin [I²: 98.5%, p < 0.0001] and serum Hb [I²: 87%, p < 0.0001] respectively. We did not find evidence of publication bias for either set of meta-analyses: Serum Fe/Ferritin [Egger’s test, p = 0.89]; Serum Hb [Egger’s test, p=0.95]. (Figure 4.6.2.1) We also examined whether removing one study, Culha and Sangun, 2007 (307), from the meta-analysis of *Giardia* infection and mean serum Fe/ferritin levels would significantly change estimates or heterogeneity between observational studies; however we found that the estimates were not significantly changed [pooled mean difference = -26.19, 95% CI: (-45.52, -6.86), p = 0.008] [I²: 98.4%, p < 0.0001].

We also performed a meta-analysis of 11 studies that reported the association between *Giardia* infection and anemia (serum iron status as a dichotomous variable (OR)) (41, 107, 211, 219, 226, 300, 305, 315, 320, 321, 329) (Table 4.5.1.3). We found no statistically significant
difference in OR between infection and either the presence or absence of anemia [pooled OR: 1.004, 95% CI: (0.78 - 1.29), p = 0.98] (Figure 4.5.2.2C). We also found moderate heterogeneity between the estimates [I$^2$: 58.5%, p = 0.007]. However, there was no evidence of publication bias [Egger’s test, p = 0.36] (Figure 4.6.2.1).

Two separate sign tests of observational studies that reported micronutrient findings as a continuous variable (means) were conducted for each of the different markers used to measure iron status--hemoglobin (Hb) and serum Fe or ferritin. Ten of 19 studies and 7 out of 14 studies eligible for sign tests reported an association between *Giardia* infection and lower serum Hb or serum Fe levels. However, based on the p values of the sign tests for serum Hb (p = 1.0) and serum Fe/ferritin (p = 1.0), we could not reject the null hypothesis of no difference between infection and serum iron. Additionally, we found that 2/12 studies eligible for the sign test reported an association between infection and the presence of anemia. The p value for this set of studies (p = 0.04) allowed us to reject the null hypothesis that there is no difference between infection and anemia. (Table 4.6.1.4)

*There is a negative association between *Giardia* infection and zinc (Zn).*

We identified 12 observational studies that presented data eligible to pool in a random effects meta-analysis of the association between *Giardia* infection and mean serum Zn levels (106, 147, 218, 226, 304, 306-311, 313). The meta-analysis, which included data on 668 *Giardia* infected and 1189 *Giardia* uninfected individuals, found a statistically significant negative association between *Giardia* infection and mean serum zinc levels [pooled mean difference = -19.54, (-24.2287, -14.8481), p < 0.0001] (Figure 4.5.2.3). There was a large and significant heterogeneity between these estimates [I$^2$: 99.6%, p < 0.0001]. We rejected the null hypothesis of no asymmetry in the funnel plot based on the results from Egger’s linear regression test [p = 0.052].
Ten out of 16 studies eligible for sign test found an association between *Giardia* infection and decreased mean serum zinc levels. However, the p value does not allow us to reject the null hypothesis of no difference (p = 0.45). (Table 4.6.1.4)

*There is a lack of a significant association between *Giardia* infection and other micronutrients.*

We also identified other observational studies in our systematic review that examined the associations between infection and serum micronutrient levels of copper (Cu), folate, magnesium, vitamin A, and vitamin B12. Of these, we determined which studies were eligible for meta-analysis and pooled these separately in order to examine the overall associations between *Giardia* infection and mean serum levels of three of the five micronutrients: Cu (n=8) (147, 306-310) (304, 313); vitamin A (n = 8) (106, 178, 207, 299, 301, 303, 317, 318); and vitamin B12 (n=5) (106, 306, 313, 319, 322). Random effects meta-analyses of these studies found no statistically significant associations between any of the three micronutrients (Cu, vitamin A and vitamin B12) and *Giardia* infection pooled mean differences, 95% CI, and p values for Cu: [6.964, (-1.79, 15.71), p = 0.12] (Figure 4.5.2.4A); Vitamin A: [ -0.059, (-0.60, 0.49), p=0.83] (Figure 4.5.2.4B); Vitamin B12: [-29.36, (-101.76, 43.04), p = 0.43] (Figure 4.5.2.4C). There were large and significant heterogeneities between estimates for Cu [I²: 96.3%, p < 0.0001]; vitamin A [I²: 87.6%, p<0.0001]; and vitamin B12 [I²: 93.1%, p < 0.0001]. However, there was no evidence of publication bias for any of the meta-analyses based on the results from Egger’s linear regression test [p = 0.33 (Cu), p = 0.44 (vitamin A), and p = 0.74 (vitamin B12)] (Figure 4.6.2.2). We did not perform meta-analyses to determine the overall effect of infection on mean serum folate levels or mean serum magnesium levels as there were too few studies (n < 5) to pool (Table 4.6.1.3). One out of ten (1/10), 2/5, 3/3, 5/13, and 2/9 observational studies that reported findings as continuous variables (means) were evaluated in separate sign test analyses for Cu, Folate, Magnesium (Mg), Vitamin
A/retinol, and Vitamin B12 observational studies respectively. Of these, we rejected the null hypothesis of no difference in serum micronutrient levels for copper in *Giardia* infected as compared to uninfected individuals (p = 0.02) (Table 4.6.1.4).

4.3.4 Meta-Analyses of Treatment Studies

*Anti-Giardia treatment increases mean serum iron levels.*

We identified 6 studies that measured both pre and post mean serum Hb levels in order to determine the effect of anti-*Giardia* treatment on *Giardia* infected children (302, 312, 314, 319, 330, 331). (Table 4.5.1.2) A random effects meta-analysis on a total of 217 *Giardia* infected, treated children found a statistically significant increase in mean serum Hb levels following treatment for *Giardia* infection pooled mean difference pre versus post treatment: [-0.78 (-1.29, -0.28), p = 0.0023] (Figure 4.5.2.5). There was a large and significant heterogeneity between the estimates [I²: 71.3%, p = 0.004]; however, we did not find evidence of publication bias [Egger’s test, p = 0.67]. (Figure 4.6.2.3). We did find evidence of an overall effect of anti-*Giardia* treatment on mean serum Vitamin A levels (*data not shown*); we did not perform meta-analyses examining the association of anti-*Giardia* treatment for any of the other micronutrients identified in the systematic review as there were too few studies to pool (Table 4.6.1.3). We could not reject the null hypothesis that there is no difference in mean serum values of micronutrients following treatment for *Giardia* infection from the sign test (*data not shown*) (Table 4.6.1.4).

4.4: Discussion

Our overall meta-analyses revealed statistically significant support for the impact of *G. lamblia* infection on serum micronutrient levels. More specifically, through our meta-analyses of observational epidemiologic studies, we found that infection negatively impacted the mean serum
micronutrient levels of two essential trace elements: iron and zinc. (Figures 4.5.2.2A-4.5.2.2C and Figure 4.5.2.3)

Nutrient deficiencies can occur either by malabsorption, or through the inadequate consumption of, macronutrients (e.g. carbohydrates, proteins, or fats) and/or micronutrients (e.g. vitamins and minerals). For this analysis, we chose to focus only on studies that measured the association between infection and serum micronutrient levels. Because *G. lamblia* infection is associated with malabsorption (reviewed in (37, 97, 255)), we assumed any effects we found on serum micronutrient levels was a result of malabsorption due to infection with *G. lamblia*. However, none of the included studies defined the macronutrient status of the study population. Therefore, we cannot rule out the potential confounding roles of macronutrient deficiencies on serum micronutrient levels in infected individuals. Additionally, inadequate dietary intakes of macro- or micronutrients may impact a host’s ability to counter, and/or an individual’s susceptibility to, infection and disease. However, none of the included studies reported the dietary intakes of their study populations. Thus, it is difficult to determine whether the observed decrease in serum micronutrient levels is a cause, or consequence, of *G. lamblia* infection.

One of the most interesting findings from our meta-analyses is that even subclinical infection with *G. lamblia* can result in decreased serum micronutrient levels. However, the effect of asymptomatic infection on serum micronutrient levels was not found for all the micronutrients we assessed. For example, while we found a statistically significant negative association between infection and mean serum zinc and iron levels; we found no significant associations between infection and mean serum levels for vitamin A.

Cross-sectional epidemiologic studies conducted among Orang Asli schoolchildren in rural Peninsular Malaysia found that giardiasis was significantly associated with low serum retinol
levels (186, 289, 323). In contrast, the results from our meta-analysis of eight observational studies examining the impact of *G. lamblia* infection and mean serum vitamin A levels on individuals across different geographic locations globally found no association between *Giardia* and vitamin A.

These inconsistencies could possibly be explained by a number of factors such as: differences in geography, genetics, diet, or environment of the Orang Asli population as compared to the other populations pooled in our analysis. For example, the Orang Asli are the indigenous people and oldest inhabitants of peninsular Malaysia who remain geographically isolated from the rest of the Malaysian population and may have differences in genetic composition, diet, or environmental risk factors that place them at increased risk for vitamin A deficiencies (VAD) as compared to the rest of the Malaysian population. Thus, these factors could confound the association observed in this population. Additionally, differences in study design; differing levels of vitamin A deficiency; or differing burdens of *G. lamblia* infection among the Orang Asli population may also contribute to the negative association found in this population as compared to a lack of association found in the studies pooled in our analysis.

Vitamin A is required for maintaining the integrity of epithelial cells throughout the body; VAD is associated with impaired intestinal immune response and lowered resistance to infection (94). *G. lamblia* infection is also known to impair the integrity of the intestinal epithelium by inducing enterocyte apoptosis (reviewed in (50)). A different study conducted in the same Orang Asli population reported that 82.2% of individuals had ocular manifestations associated with vitamin A deficiency (332). Indeed, though VAD is a major public health problem in over 60 countries worldwide (93), the highest proportion of children impacted by VAD live in Southeast Asia (93). Thus, given the reported high prevalence of vitamin A deficiency observed in this
population and region of the world, these individuals may be more at risk for *Giardia* infection than the other 187 infected individuals, from geographically distinct regions of the world, pooled in our meta-analysis. The high prevalence of VAD could in turn provide a possible contributing factor for the observed association between giardiasis and Vitamin A deficiencies observed in the Orang Asli studies. Additionally, since the majority of our pooled studies did not report the dietary intakes of Vitamin A (Figure 4.5.2.4B), we cannot draw any reasonable conclusions comparing the Orang Asli population with those populations assessed in our meta-analyses. Lastly, the vast majority of studies did not quantify *G. lamblia* infection (or its indirect measure of cyst shedding) preventing us from drawing any conclusions about the possible effects or differences of parasite burden contributing to VAD.

The immune system has been demonstrated to negatively impact both infection (exposure) and micronutrient status (outcome) (333). Host immune status affects both the susceptibility and severity of *G. lamblia* infection. Immunosuppressed individuals have been found to have an enhanced susceptibility to *Giardia* infection (55) and are at increased risk of developing chronic giardiasis [reviewed in (36, 50)]. Additionally, the ability of the host immune system to counter infection and disease is influenced by its nutritional status; nutritional deficiencies have been shown to alter both innate and acquired immune responses to infectious agents. For example, the role of zinc in modulating different aspects of the innate and acquired immune systems has been well characterized (reviewed in (334-336)). Both animal and human studies have demonstrated that zinc deficiency contributes to an increased susceptibility to a number of different infectious diseases (reviewed in (337)). Studies have also suggested complex roles for vitamin A and iron in altering host immune responses; deficiencies in these micronutrients are associated with lowered susceptibilities to infections (reviewed in (333)). Therefore, we eliminated studies conducted in
immunocompromised individuals with known inflammatory or immune disorders (e.g. HIV/AIDS, IBS, or celiac disease), as they would present potentially confounding factors in our analysis.

The significant effects for zinc and iron were only observed when assessing continuous variables using standardized mean differences; however, we found no significant effect when assessing dichotomous variables, or the odds ratio, as was done in studies assessing the association between *G. lamblia* infection and anemia. Categorization of variables into dichotomous outcomes introduces both a conceptual limitation, as well as a statistical loss of power, making it harder to detect effects that may be present. Thus, while our results demonstrate that there is a statistically significant negative association between *G. lamblia* infection and mean serum iron levels, we cannot accurately determine the extent of the association between infection and iron deficiency.

Our meta-analysis had some notable limitations. First, *G. lamblia* is a protozoan parasite that establishes infection by attaching and colonizing the upper small intestine, the primary site of micronutrient absorption from food. While we included a comprehensive set of search terms that included each of the most commonly absorbed micronutrients in the duodenum in our systematic review, we could only perform meta-analyses on the small subset of micronutrients found in the published literature. Therefore, we were not able to estimate the pooled effects of *Giardia* infection on other micronutrients that are also absorbed in the upper small intestine such as calcium or Vitamin E. Additionally, in order to ensure sufficient power in our pooled analyses to detect significant associations between infection and different micronutrients, we did not perform meta-analyses if there were fewer than 5 studies in any one group (observational or treatment) for any of the micronutrients we included in our analyses. So, we cannot determine the pooled effects of infection on certain micronutrients (e.g. folate, magnesium) or the effects of treatment of *G.*
*Giardia* lamblia infections on serum micronutrient levels (e.g. zinc, vitamin A). More studies are needed to address these current gaps in the published literature.

Misclassification of exposure in the assessed studies is another potential limitation of our analysis. In regions of the world without access to clean drinking water or adequate sanitation, individuals can be simultaneously infected with multiple enteric pathogens. While we limited studies in our meta-analyses to include only those that identified subjects as being “infected” or “uninfected” with *G. lamblia*; however, a large number of included studies did not report whether the study population of individuals classified as “uninfected” were truly not infected with any enteric pathogens other than *Giardia*, or whether these individuals were simply not infected with *Giardia*, but could be infected with other enteric pathogens. Therefore, we were unable to determine what, if any, effect polyparasitism might have on serum micronutrient level outcomes.

Additionally, the most commonly used method of detecting *Giardia* infection in our studies was formalin-ether sedimentation microscopy to visually identify parasites in a single stool specimen. Because cyst shedding is intermittent in infected individuals, this method of detection can miss low-grade infections resulting in false negatives (77). False negatives could in turn bias our findings towards the null hypothesis, as infected individuals with decreased mean serum micronutrient levels could potentially have been incorrectly classified as uninfected controls in the studies we abstracted and analyzed. Also, since most studies we analyzed did not differentiate between the different parasitic assemblages of *G. lamblia* infection that infect humans, A and B, we were unable to estimate any potential associations of different *Giardia* assemblages on serum micronutrient levels. Lastly, we also observed large and significant heterogeneities between study estimates in our meta-analyses of largely cross-sectional, observational studies on associations between *G. lamblia* infection and serum zinc and iron levels suggesting there is a high degree of
clinical and methodological diversity among the studies. Thus, there is a clear need for additional well-controlled epidemiological studies with larger population sizes in order to support the findings from our meta-analyses.

In conclusion, we present the first systematic review and meta-analyses on *Giardia* infections and micronutrient status. Results from our meta-analyses presented here provide supporting evidence for the negative associations of *G. lamblia* infections and serum zinc and iron levels. Since all of the studies we evaluated included both clinically symptomatic and asymptomatic individuals, our findings extend earlier analyses that focused exclusively on the negative impacts of *Giardia*-associated diarrheal disease (101) and provides evidence for long-term negative health consequences of clinically asymptomatic *Giardia* infections. However, more evidence is needed on the impacts, if any, of infection on micronutrients other than the ones presented in our analyses. Additionally, more studies are necessary in order to evaluate the pooled effects of treatment of *G. lamblia* infections on serum micronutrient levels, other than iron, as these findings can provide support for public health policy aimed at reducing the burden resulting from *Giardia* infections. Lastly, more studies are needed in order to determine the pooled effects of micronutrient supplementation on outcomes of infection in order to provide additional public health evidence supporting the design of targeted public health nutritional interventions that can promote the Sustainable Development Goal of good health and well-being (100).

4.4.1 Acknowledgement

I would like to thank Dr. Camila Coelho for her technical discussions on the role of the host immune system in response to *Giardia* infections.
### 4.5: Tables and Figures

#### 4.5.1 Tables

Table 4.5.1.1: Baseline characteristics of studies included in random effects meta-analyses of observational studies

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<sup>11</sup> CC: Case control, CS: Cross-sectional, L: Longitudinal  
<sup>a</sup>: Measured serum iron as marker of iron levels  
<sup>b</sup>: Measured serum hemoglobin (Hb) as marker of iron levels  
<sup>c</sup>: Measured serum ferritin as marker of iron levels  
<sup>d</sup>: Measured serum retinol as marker of vitamin A levels  
<sup>e</sup>: Measured serum vitamin A as marker of vitamin A levels
Table 4.5.2 Baseline characteristics of studies included in random effects meta-analysis of treatment studies

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Table 4.5.3 Baseline characteristics of studies included in random effects meta-analysis of anemia.

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12 CS: Cross sectional, CC: Case control, L: Longitudinal, R: Retrospective
4.5.2 Figures

Figure 4.5.2.1 Flow chart of systematic review.

Figure 4.5.2.1 illustrates the flow chart for the systematic review following the PRISMA guidelines. The second to last row indicates the total number of unique studies conducted for each micronutrient. Studies were then further separated into groups based on the study type: observational (O), treatment (T), or supplementation (S). The bottom row indicates the total number of unique studies that were pooled in meta analyses by study type O, T, or S. For the meta-analyses, studies that measured serum iron levels using either serum iron (a) or serum ferritin (c) as markers were combined. Studies measuring serum retinol and serum vitamin A were combined in one meta-analysis to measure serum vitamin A levels.
Figures 4.5.2.2-4.5.2.4 Forest plots of observational studies

4.5.2.2A Serum iron/ferritin

Figure 4.5.2.2A is a forest plot of studies measuring the serum mean levels of iron using serum iron and serum ferritin as markers of iron status. Studies to the left of zero, in blue, indicate lower mean values of serum iron/ferritin in *Giardia* infected as compared to uninfected individuals. Studies to the right of zero, in red, indicate higher mean values of serum iron/ferritin in *Giardia* infected as compared to uninfected individuals.
Figure 4.5.2.2B is a forest plot of studies measuring the serum mean levels of iron using serum Hb as a marker of iron status. Studies to the left of zero, in blue, indicate lower mean values of serum Hb in *Giardia* infected as compared to uninfected individuals. Studies to the right of zero, in red, indicate higher mean values of serum Hb in *Giardia* infected as compared to uninfected individuals.
4.5.2.2C Anemia

Figure 4.5.2.2C is a forest plot of studies measuring the odds ratios of anemia in *Giardia* infected as compared to uninfected individuals. Studies to the left of one, in blue, indicate *Giardia* infection lowers the odds of anemia as compared to uninfected individuals. Studies to the right of one, in red, indicate *Giardia* infection increases the odds of anemia as compared to uninfected individuals.

Figure 4.5.2.3: Forest plot of *Giardia* infection and serum Zn

Figure 4.5.2.3 is a forest plot of studies measuring the serum mean levels of zinc. Studies to the left of zero, in blue, indicate lower mean values of serum zinc in *Giardia* infected as compared to uninfected individuals. Studies to the right of zero, in red, indicate higher mean values of serum zinc in *Giardia* infected as compared to uninfected individuals.
Figure 4.5.2.4: Forest plots of *Giardia* infections and other micronutrients

4.5.2.4A Copper

Figure 4.5.2.4A is a forest plot of studies measuring the serum mean levels of copper. Studies to the left of zero, in blue, indicate lower mean values of serum copper in *Giardia* infected as compared to uninfected individuals. Studies to the right of zero, in red, indicate higher mean values of serum copper in *Giardia* infected as compared to uninfected individuals.
Figure 4.5.2.4B is a forest plot of studies measuring the serum mean levels of vitamin A. Studies to the left of zero, in blue, indicate lower mean values of serum vitamin A in *Giardia* infected as compared to uninfected individuals. Studies to the right of zero, in red, indicate higher mean values of serum vitamin A in *Giardia* infected as compared to uninfected individuals. Under the author, year column, studies marked with an “a” following the year measured serum retinol as a marker of vitamin A levels, whereas studies marked with a “b” following the year measured serum vitamin A as the marker of vitamin A levels.
Figure 4.5.2.4C is a forest plot of studies measuring the serum mean levels of vitamin B12. Studies to the left of zero, in blue, indicate lower mean values of serum vitamin B12 in *Giardia* infected as compared to uninfected individuals. Studies to the right of zero, in red, indicate higher mean values of serum vitamin B12 in *Giardia* infected as compared to uninfected individuals.
Figure 4.5.2.5: Forest plot of Treatment Studies

*Giardia* infection and serum Hb levels pre- and post-*Giardia* treatment

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Figure 4.5.2.5 is a forest plot of studies measuring the serum mean levels of serum Hb as a marker for iron status pre and post treatment of *Giardia*. Studies to the left of zero, in blue, indicate higher mean values of serum Hb post treatment for *Giardia* as compared to pre-treatment.
### 4.6: Supplementary Tables and Figures

#### 4.6.1 Tables

Table 4.6.1: Scoring system for quality assessment of studies

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<tr>
<td></td>
<td>Not epidemiological study</td>
<td>Longitudinal Cohort (without treatment intervention)</td>
</tr>
<tr>
<td></td>
<td>(example: Narrative review article)</td>
<td>Cross-Sectional</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Case-Control</td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>Treatment:</strong></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Randomized Control Trial</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Longitudinal Cohort with treatment intervention</td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>Supplementation</strong></td>
</tr>
<tr>
<td>Setting</td>
<td>Did not describe setting, location, or relevant dates</td>
<td>Described setting, location, and relevant dates for: periods of recruitment, exposure, data collection, and follow-up</td>
</tr>
<tr>
<td>Participants</td>
<td>Did not describe study population</td>
<td>Included description of study population such as eligibility criteria, inclusion and exclusion criteria, sources and methods of participant and control selection, and case and control definitions (if applicable)</td>
</tr>
<tr>
<td>Diagnostic Criteria</td>
<td>Did not describe</td>
<td>Described diagnostic methods used for diagnosis of <em>G. lamblia</em> infection</td>
</tr>
<tr>
<td>Variables</td>
<td>Did not describe</td>
<td>Clearly defined all variables collected including outcomes (e.g. micronutrient levels), exposures (e.g. <em>Giardia</em> infected versus uninfected), predictors, and potential confounders</td>
</tr>
<tr>
<td>Quantitative Variables</td>
<td>Did not describe</td>
<td>Described how quantitative variables were handled in data analysis</td>
</tr>
<tr>
<td>Data sources/measurement</td>
<td>Did not describe</td>
<td>Described sources of data and details of methods of data assessment (measurement) and analysis</td>
</tr>
<tr>
<td>-------------------------</td>
<td>-----------------</td>
<td>------------------------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td><strong>Results</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Participants</td>
<td>Did not report numbers of study participants at each stage of study</td>
<td>Reported numbers of participants in each stage of study (total number of potential participants, examined for eligibility, included in study, lost to follow up, analyzed)</td>
</tr>
<tr>
<td>Descriptive data</td>
<td>Did not provide demographics of study participants</td>
<td>Provided characteristics of study participants including information on potential confounders</td>
</tr>
<tr>
<td>Outcome data</td>
<td>Did not report numbers of outcome events or summary measures</td>
<td>Reported numbers of outcome events or summary measures analyzed</td>
</tr>
<tr>
<td>Main results</td>
<td>Did not report summary of main results</td>
<td>Reported estimates and their precision. Noted whether estimates are unadjusted, or (if applicable) confounder adjusted estimates</td>
</tr>
<tr>
<td><strong>Discussion/Conclusions</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Key results</td>
<td>Did not summarize key study results</td>
<td>Summarized key results with reference to study objectives</td>
</tr>
<tr>
<td>Limitations</td>
<td>Did not discuss study limitations</td>
<td>Discussed study limitations including discussion on potential sources of bias or imprecision</td>
</tr>
<tr>
<td>Interpretation of Study Results</td>
<td>Did not describe</td>
<td>Provided some interpretation of study results considering objectives, limitations, multiplicity of analyses, results from similar published studies, and other relevant evidence</td>
</tr>
<tr>
<td>Generalizability</td>
<td>Did not discuss</td>
<td>Discussed the generalizability (external validity) of the study results</td>
</tr>
</tbody>
</table>
### Table 4.6.1: Baseline characteristics of studies included in systematic review only (Sample)

<table>
<thead>
<tr>
<th>Author, year (Country)</th>
<th>Study Design</th>
<th>Age(^a)</th>
<th>Study Size(^b)</th>
<th>Micronutrient(s) Examined(^c)</th>
<th>Authors’ Findings(^d)</th>
<th>Reason for Exclusion(^e)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ahmed et al., 1991 (Saudi Arabia)</td>
<td>CS</td>
<td>4 - 19</td>
<td>1426</td>
<td>Fe</td>
<td><em>Giardia lamblia</em> was the most common pathogenic parasite and its high infection rate seemed to be associated with lower hemoglobin (Hb) level.</td>
<td>2a</td>
</tr>
<tr>
<td>Ahmed et al., 1990 (Saudi Arabia)</td>
<td>CS</td>
<td>6 - 12</td>
<td>717</td>
<td>Fe</td>
<td><em>G. lamblia</em> was found to be the most common intestinal parasite among children of all socio-economic classes. Anemia was more prevalent among parasitically infected children.</td>
<td>2a</td>
</tr>
<tr>
<td>Aini et al., 2007 (Malaysia)</td>
<td>CS</td>
<td>2 - 15</td>
<td>281</td>
<td>Fe</td>
<td>None of the infections studied (including <em>giardiasis</em>) showed significant</td>
<td>2b</td>
</tr>
</tbody>
</table>

\(^a\) CS: Cross sectional; CC: Case Control; LC: Longitudinal Cohort; T: Treatment intervention; S: Supplementation

\(^b\) Age range of study population in years

\(^c\) Total study size

\(^d\) Copper (Cu), Folate, Iron (Fe), Zinc (Zn), Vitamin A, Vitamin B12

\(^e\) Author’s reported findings specific to *Giardia* infection and mean serum micronutrient levels

\(^f\) 1: Heterogeneous outcome; 2: Insufficient data--2a: *Giardia* not isolated as a variable in analysis; 2b: No raw data; 3: Duplicate data

### Table 4.6.3: Baseline characteristics of studies excluded from meta-analyses (n<5)

<table>
<thead>
<tr>
<th>Author, year</th>
<th>Country</th>
<th>Study Type(^{13}) (Design)</th>
<th>Age range (years)</th>
<th>Study size</th>
<th>Micronutrient(s) Examined</th>
<th>Statistical Outcome Measured(^{14})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Al-Mekhlafi et al., 2010</td>
<td>Malaysia</td>
<td>Observational: (Cross-sectional)</td>
<td>7 - 12</td>
<td>241</td>
<td>Vitamin A</td>
<td>Odds Ratio (OR)</td>
</tr>
<tr>
<td>Astiazaran-Garcia et al., 2010</td>
<td>Mexico</td>
<td>Treatment</td>
<td>6 - 12</td>
<td>30</td>
<td>Vitamin A</td>
<td>Means</td>
</tr>
<tr>
<td>Olivares et al., 2013</td>
<td>Spain</td>
<td>Treatment</td>
<td>Children</td>
<td>86</td>
<td>Folate, Vitamin B12</td>
<td>Means</td>
</tr>
<tr>
<td>Olivares et al., 2003</td>
<td>Spain</td>
<td>Treatment</td>
<td>0.10 – 15</td>
<td>64</td>
<td>Copper, Magnesium, Zinc</td>
<td>Means</td>
</tr>
<tr>
<td>Yakinci et al., 1998</td>
<td>Turkey</td>
<td>Treatment</td>
<td>7 – 12</td>
<td>34</td>
<td>Vitamin A</td>
<td>Means</td>
</tr>
</tbody>
</table>

\(^{13}\) Observational or Treatment

\(^{14}\) Means or Odds Ratios
Table 4.6.1.4: Sign test analyses

Sign Test of Eligible Studies Measuring Serum Copper Levels

<table>
<thead>
<tr>
<th>Observational Studies</th>
<th>Treatment Studies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative association found (n= 1)</td>
<td>Post-treatment values increased (n= 0)</td>
</tr>
<tr>
<td>Total (n = 10)</td>
<td>Total (n= 2)</td>
</tr>
<tr>
<td>p = 0.02</td>
<td>p = 0.5</td>
</tr>
</tbody>
</table>

Sign Test of Eligible Studies Measuring Serum Folate Levels

<table>
<thead>
<tr>
<th>Observational Studies</th>
<th>Treatment Studies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative association found (n= 2)</td>
<td>Post-treatment values increased (n= 1)</td>
</tr>
<tr>
<td>Total (n= 5)</td>
<td>Total (n= 3)</td>
</tr>
<tr>
<td>p = 1.0</td>
<td>p = 1.0</td>
</tr>
</tbody>
</table>

Sign Test of Studies Using Serum Fe or Serum Ferritin as Marker

<table>
<thead>
<tr>
<th>Observational Studies</th>
<th>Treatment Studies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative association found (n= 7)</td>
<td>Post-treatment values increased (n= 4)</td>
</tr>
<tr>
<td>Total (n= 14)</td>
<td>Total (n= 6)</td>
</tr>
<tr>
<td>p = 1.0</td>
<td>p = 0.69</td>
</tr>
</tbody>
</table>

Sign Test of Studies Using Serum Hemoglobin (Hb) as Marker

<table>
<thead>
<tr>
<th>Observational Studies</th>
<th>Treatment Studies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative association found (n= 10)</td>
<td>Post-treatment values increased (n= 4)</td>
</tr>
<tr>
<td>Total (n= 19)</td>
<td>Total (n= 7)</td>
</tr>
<tr>
<td>p = 1.0</td>
<td>p = 1.0</td>
</tr>
</tbody>
</table>

Sign Test of Observational Studies Measuring Anemia\textsuperscript{15}

<table>
<thead>
<tr>
<th>Observational Studies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative association found (n= 2)</td>
</tr>
<tr>
<td>Total (n= 12)</td>
</tr>
<tr>
<td>p= 0.04</td>
</tr>
</tbody>
</table>

\textsuperscript{15} There were no studies that measured the impact of treatment of \textit{Giardia} infection on anemia.
### Sign Test of Eligible Studies Measuring Serum Magnesium Levels

<table>
<thead>
<tr>
<th>Observational Studies</th>
<th>Treatment Studies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative association found (n= 3)</td>
<td>Post-treatment values increased (n= 1)</td>
</tr>
<tr>
<td>Total (n= 3)</td>
<td>Total (n= 1)</td>
</tr>
<tr>
<td>p= 0.25</td>
<td>p= 1.0</td>
</tr>
</tbody>
</table>

### Sign Test of Eligible Studies Measuring Serum Zinc Levels

<table>
<thead>
<tr>
<th>Observational Studies</th>
<th>Treatment Studies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative association found (n= 10)</td>
<td>Post-treatment values increased (n= 2)</td>
</tr>
<tr>
<td>Total (n= 16)</td>
<td>Total (n= 3)</td>
</tr>
<tr>
<td>p= 0.45</td>
<td>p= 1.0</td>
</tr>
</tbody>
</table>

### Sign Test of Eligible Studies Measuring Serum Vitamin A Levels

<table>
<thead>
<tr>
<th>Observational Studies</th>
<th>Treatment Studies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative association found (n= 5)</td>
<td>Post-treatment values increased (n= 4)</td>
</tr>
<tr>
<td>Total (n= 13)</td>
<td>Total (n= 5)</td>
</tr>
<tr>
<td>p= 0.58</td>
<td>p= 0.38</td>
</tr>
</tbody>
</table>

### Sign Test of Eligible Studies Measuring Serum Vitamin B12 Levels

<table>
<thead>
<tr>
<th>Observational Studies</th>
<th>Treatment Studies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative association found (n= 2)</td>
<td>Post-treatment values increased (n= 2)</td>
</tr>
<tr>
<td>Total (n= 9)</td>
<td>Total (n=3)</td>
</tr>
<tr>
<td>p= 0.18</td>
<td>p= 1.0</td>
</tr>
</tbody>
</table>
4.6.2 Figures

Figures 4.6.2.1-4.6.2.2: Funnel Plots of Observational Studies

Figure 4.6.2.1: Funnel plots of studies examining the association of *Giardia* infection and serum micronutrient levels of Iron (Fe) [Fe/Ferritin, Hemoglobin (Hb), Anemia] and Zinc

Figure 4.6.2.1: Each circle represents a unique study analyzed in the meta-analysis. Effect estimates are plotted on the X-axis; the measure of study size is on the Y-axis. Effect sizes from smaller studies scatter more widely at the bottom of the graph, with the spread narrowing among larger studies. The dotted vertical line is the overall summary estimate from the meta-analysis of studies.
Figure 4.6.2.2: Funnel plots of studies examining the association of *Giardia* infection and serum micronutrient levels of Copper, Vitamin A, Vitamin B12

Figure 4.6.2.2: Each circle represents a unique study analyzed in the meta-analysis. Effect estimates are plotted on the X-axis; the measure of study size is on the Y-axis. Effect sizes from smaller studies scatter more widely at the bottom of the graph, with the spread narrowing among larger studies. The dotted vertical line is the overall summary estimate from the meta-analysis of studies.
Figure 4.6.2.3: Funnel Plot of Treatment Studies Measuring levels of Serum Hb Pre and Post Treatment for *Giardia*.

Figure 4.6.2.3: Each circle represents a unique study analyzed in the meta-analysis. Effect estimates are plotted on the X-axis; the measure of study size is on the Y-axis. Effect sizes from smaller studies scatter more widely at the bottom of the graph, with the spread narrowing among larger studies. The dotted vertical line is the overall summary estimate from the meta-analysis of studies.
Chapter 5: Discussion

5.1: Overview

In Part I of the research presented here, our goal was to examine the association of *Giardia* infection with two indicators of undernutrition: growth and micronutrient malnutrition. By applying two commonly used epidemiologic methods, the systematic review and meta-analysis, we found, both qualitatively and quantitatively, an overall negative association of *Giardia* infection on childhood growth and serum micronutrient levels. Thus, this research contributes to the field by providing evidence in support of the hypothesis that enteric parasitic infections, such as *Giardia*, are risk factors influencing malnutrition (86, 97, 138).

5.2: *Giardia* and Undernutrition

One of the most salient findings from our analyses is that even asymptomatic *Giardia* infections can have a negative, long-term impact on undernutrition outcomes. The role of *Giardia* as a cause of either acute or chronic diarrheal disease has long been established. However, symptomatic *Giardia* infections comprise only a fraction (~20%-80%) of cases worldwide (36, 338). The absence of overt symptoms in the majority of *Giardia* infected individuals is perhaps the primary reason that the global burden of disease for such a widespread human pathogen has historically been underestimated. In fact, the majority of studies included in our analyses were cross-sectional epidemiological study designs composed of study participants who were randomly recruited from the local population where the individual study was conducted. Thus, these cross-sectional studies included both asymptotically and symptomatically infected individuals, rather than only including study participants with documented diarrheal disease. Yet, when pooling...
studies largely composed of asymptomatic individuals, we still found an overall negative impact of *Giardia* infection on undernutrition.

### 5.2.2 *Giardia* and Growth

Our systematic review and meta-analyses of observational epidemiologic studies provide supporting evidence for the deleterious impacts of *Giardia* infection on children’s growth. Specifically, we found that *Giardia* infection does have a significant negative association with both acute [weight-for-age (WAZ) and weight-for-height (WHZ)] and chronic [height-for-age (HAZ)] anthropometric measures of malnutrition. However, we found no association between infection and the more severe anthropometric indicators of malnutrition: underweight (WAZ < -2 SD), wasting (WHZ < -2 SD), or stunting (HAZ < -2 SD). Collectively, these findings suggest that the effect of *Giardia* infection on anthropometric measures is significant, but small.

While mild growth effects may seem inconsequential, anthropometric indicators can reflect more meaningful developmental metrics. Deficits in height for example are negatively associated with brain development and neural plasticity in turn causing delays in motor or cognitive skills (89). For example, Berkman et al., 2002 demonstrated that *Giardia* infections or stunting in infancy was associated with measurable reductions in cognitive assessments performed in children at 9 years of age (38). Thus, overlooking even subtle impacts of *Giardia* infection on growth could have detrimental effects on late childhood development.

### 5.2.3 *Giardia* and Micronutrient Malnutrition (MM)

Our systematic review and meta-analyses of observational epidemiologic studies provide supporting evidence for the negative impact of *Giardia* infection on serum micronutrient levels of two essential trace elements of public health significance: zinc and iron.
The current body of literature examining the associations between *G. lamblia* infection and serum zinc levels has been inconclusive. Animal studies have found that *G. lamblia* infected mice have lower serum zinc levels than uninfected mice regardless of the levels of dietary intake of zinc (339, 340). These findings are consistent with the findings from our meta-analyses of observational studies of the negative association between infection and serum zinc levels. However, the associations between infection and zinc in zinc supplementation or treatment studies are varied. Results from human studies looking at associations between *G. lamblia* and zinc measuring heterogeneous outcomes report varying results on the beneficial impacts of zinc supplementation during infection with *G. lamblia*. For example, a randomized controlled trial (RCT) examining the interaction of vitamin A and zinc supplementation and *G. duodenalis* on the growth velocity of children aged 5 - 24 months found that *Giardia* infected children supplemented with zinc alone had significantly lower growth than did uninfected children (173). Whereas, two different RCTs, one examining the effects of zinc and multinutrient supplementation on *Giardia*-associated diarrhea in children aged 6-60 months (226), the other evaluating the effect of vitamin A and zinc supplementation on the prevalence and duration of *Giardia* infections in children aged 6-15 months of age (271), found beneficial impacts of zinc supplementation on the outcomes of *Giardia* infection (e.g. diarrhea, prevalence, and duration). Observational studies evaluating the impacts of treatment of *G. lamblia* on serum zinc levels have similarly disparate results; some studies report significant increases in the mean serum zinc levels following anti-*Giardia* treatment (218, 310), while another study found no difference in mean serum zinc levels following treatment for *G. lamblia* infection(325). Given both the heterogeneity of study outcomes, as well as the fact that none of the published studies have examined the molecular mechanisms for the observed
interactions between *Giardia* and zinc in animals or humans, additional studies are needed to address this research question.

Similarly, little is known about the giardiasis-iron interaction in animals and humans. Animal studies investigating the effects of *G. lamblia* infection on serum hemoglobin (Hb) levels in both rat (341) and mouse models (342) found that infected animals had statistically significantly lower serum Hb levels than uninfected animals. Our meta-analysis of observational studies also found a negative association between infection and serum Hb levels in humans. Additionally, our pooled analysis of treatment studies found that treatment of *G. lamblia* infection led to an increase in mean serum Hb levels. Even though serum Hb and serum iron concentrations may show distinct responses to infection, we found that the significant negative association between *G. lamblia* and iron held for both observational and treatment studies regardless of whether we pooled studies that measured iron status as serum iron or serum Hb levels. Hence, our meta-analyses provide additional support for the association between *Giardia* and iron; however, we cannot draw conclusions about the biological basis for this interaction. Therefore, more studies are needed to help elucidate this link.

One possible explanation for the reduced serum zinc and iron micronutrient levels found in *Giardia* infected individuals may be due to the pathophysiology of the parasite. *G. lamblia* undergoes surface antigenic variation through the spontaneous expression of a single variant-specific surface protein (VSP) on the trophozoite surface in an infected host. *In vitro* studies have demonstrated that VSPs are metal binding surface proteins capable of binding zinc and iron ions (343-345). Thus, the ability of *G. lamblia* VSPs to bind these ions may play a role in the observed deficiencies of serum zinc and iron in *Giardia* infection; however, studies are needed to elucidate
the exact mechanisms by which micronutrients modulate host-pathogen interactions in *G. lamblia* infections.

### 5.2.4 Other

We excluded studies conducted in immunocompromised individuals with known inflammatory or immune disorders (e.g. HIV/AIDS, IBS, or celiac disease), as they would present potentially confounding factors in our analysis. The host immune system has been demonstrated to negatively impact both susceptibility to infections (exposures) and the resulting variable disease morbidities from infections (333). For example, immunosuppressed individuals have been found to have an enhanced susceptibility to *Giardia* infection (55) and are at increased risk of developing chronic giardiasis (reviewed in (36, 50)). On the contrary, there have also been occasional reports of chronic giardiasis found in the absence of apparent immunodeficiency (77, 338) suggesting that non-immune system mediated host factors may also play a role in influencing either host susceptibility to infection or in the duration and severity of undernutrition outcomes (reviewed in Faubert, 2000) (84). Though the contribution of other these other factors to the varying severity and duration found in host symptomatology and pathophysiology remains unknown, host parasite interactions, for example, may play a role in explaining the finding from our systematic review and meta-analyses that *Giardia* infection significantly impacted mild, but not severe, anthropometric indicators of growth.

Additionally, we did not detect any notable geographic, regional, or local patterns or trends within or between different studies included in our meta-analyses examining the overall associations of *Giardia* infection and either anthropometric indicators of growth or micronutrient malnutrition. While the majority of the study subjects in our included studies were often undernourished when compared to an internationally accepted reference population (e.g. CDC or
WHO), all of the studies included in our pooled analyses compared infected and uninfected individuals within the same population. Thus, we assumed any significant differences found between infected and uninfected individuals within the same study were a result of *Giardia* infection and not a result of potential confounding factors such as diet, nutritional status, climate, or environment as these were considered to be constant between infected and uninfected individuals within the same study. Additionally, we found that the negative association of infection with undernutrition held regardless of geographic location or region where the study was conducted. This suggests that an individual’s infection status (*Giardia*-infected or uninfected) was a greater predictor of undernutrition outcomes than were differences in dietary composition or environmental factors that may be found in different geographic or regional locations. Several studies have documented small intestinal malabsorption as one of the pathophysiological consequences of *Giardia* infection (reviewed in Cotton et al, 2011 (50)). In fact, the reduced intake of macro- (responsible for growth deficits) or micronutrients (responsible for micronutrient malnutrition) (90) could be a result of small intestinal malabsorption of the nutrients consumed. Based on our findings, we can reasonably conclude that the overall negative association we found between infection and either deficiency in growth or in micronutrient malnutrition were likely a result of *Giardia* infection induced small intestinal malabsorption that is independent of any geographic or regional differences.

5.3: Study Limitations

5.3.1 Correlation of Genotype with Phenotype

Most of the individual studies in the field did not differentiate between the two assemblages in their diagnosis of *Giardia* infection, thus, we could not address whether there was an association
of *Giardia* genotype with differences in pathogenicity in either of our systematic reviews and meta-analyses.

Our traditional characterization of *Giardia lamblia* as a single species of protozoan parasite obscures the significant differences between the different assemblages. *Giardia* assemblages A and B both infect humans; however, each assemblage is sufficiently different in its genomic sequence and structure and cellular morphology. Therefore, it has been suggested that these assemblages should be considered different species (346). The body of literature examining the correlation of *Giardia* genotype with phenotype has, with the exception of one study (225) that found an association with assemblage B infections and impaired child growth in Rwanda, been limited to examining the association of assemblage with symptomatic giardiasis, specifically diarrheal outcomes (27-31). Collectively, these studies have pointed to differences in parasite virulence between the A and B assemblages in symptomatic giardiasis, but there is no clear consensus. However, given the variability in these findings it is likely that genotype alone might not explain the observed differences in malnutrition outcomes. Thus, more research is needed in order to help contribute to the evidence base needed to inform appropriate clinical and public health decision-making, policy, and guidance for individuals infected with *Giardia*.

5.3.2 Disease Detection and Diagnosis

The accurate detection of *Giardia* infections is another potential limitation in our analyses. Several different methods exist for the detection of *Giardia*. However, most of the studies included in our systematic reviews and meta-analyses used traditional methods of detection (i.e., sedimentation and microscopy and ELISA) that are notably less sensitive than more modern methods such as PCR (347). Further, given that cyst shedding is intermittent (77), it is generally recommended to examine three or more stool samples prior to diagnosing an individual as *Giardia*.
infected or uninfected. In fact, the sensitivity of using microscopic methods for detection of
*Giardia* infections is further reduced by the collection of fewer than 3 stool samples. For example,
the collection of only one stool sample allows for the detection of only around 60%-80% of
infections, while the sensitivity of detection is 80% - 90% if only two samples are collected on
different days (74, 76). In contrast, the examination of three stool samples will allow for greater
than 90% detection (74, 76). However, the majority of studies included in our analyses did not
explicitly report the number, frequency, or duration of stool sample collection used for the
detection and diagnosis of *Giardia* infection in a given population. Thus, it is possible that some
of these studies missed some cases of low-grade infection in the population resulting in false
negatives. Potential misclassification of exposure resulting in false negatives, could in turn bias
our findings towards the null hypothesis of no difference between infection status and
undernutrition. However, we still found a significant association between exposure and outcome
further strengthening our argument that *Giardia* infection is associated with growth deficits and
decreased mean serum micronutrient levels.

**5.3.3 Association versus Causation**

One potential limitation of our epidemiologic analyses is that since most epidemiologic
study designs are observational, rather than experimental, in nature we can at most determine only
whether a significant association exists between *Giardia* infection and undernutrition rather than
determining a cause-effect relationship. However, Sir Austin Bradford-Hill developed nine
criteria (348) that are widely used in epidemiology as a framework against which we can infer
causality, not just association, when assessing our observational findings. These nine criteria
Plausibility; 7. Coherence; 8. Experiment; and 9. Analogy (348). The findings from our meta-
analyses fulfill 6 of the 9 criteria (strength, consistency, specificity, temporality, biological plausibility, and experiment) as described below providing strong support for the argument that the observed association between *Giardia* infection is causal.

**Strength** of association is the first of the nine criteria that our data satisfies. In both meta-analyses presented in Chapters 3 and 4, we found statistically significant associations (p < 0.05) between infection and WAZ > -2 SD, WHZ > -2 SD, and HAZ > -2 SD (Chapter 3) and infection and serum zinc and serum iron levels (Chapter 4). This statistical significance in turn translates to a large population public health problem; a p value < 0.05 represents a shift in the normal distribution of a population further away from the median of the WAZ, WHZ, or HAZ values of an international healthy reference population. These shifts represent large numbers of children that are impacted by *Giardia* related undernutrition outcomes, and in turn are at risk for developing long term negative impairments to cognitive and developmental deficits.

The second criteria our results demonstrate is a **consistency** of findings. For example, we aggregated individual studies examining the association of *Giardia* infection with undernutrition that were performed across different, heterogeneous populations from discrete geographic locations demonstrating a consistency of findings. We also only included studies in our analysis that defined *Giardia* as exposure and undernutrition as outcome ensuring **specificity** of the association.

There is experimental evidence for **biological plausibility** in support of a causal relationship. In fact, multiple studies have documented altered intestinal morphology and small intestinal malabsorption as a consequence of *Giardia* infection (reviewed in (37, 50, 72)); while Bartelt et al., 2013 demonstrated that persistent *G. lamblia* infection in a mouse model led to impaired growth which was associated with altered intestinal morphology (e.g. crypt hyperplasia)
and epithelial cell apoptosis (52). Thus, we can reasonably infer from our epidemiologic analyses and supporting experimental biology studies that *Giardia* infection is a risk factor causing undernutrition.

We also fulfill the temporality criteria as a reverse cause and effect relationship between *Giardia* infection and undernutrition has been recently documented in animal studies (52). However, the epidemiologic literature examining the inverse relationship defining undernutrition as exposure and *Giardia* infection as outcome is small. Thus, we do not have the data to argue for a causal effect in the opposite direction.

While our systematic reviews and meta-analyses satisfy a number of the Bradford Hill criteria (348) allowing us to infer that there is likely a causal relationship between *Giardia* infection and undernutrition; we acknowledge that the true association between infection and undernutrition is likely both complex and multifactorial requiring more direct experimental evidence to definitively prove that *Giardia* infection causes undernutrition.

5.3.4 Other

In regions of the world without consistent access to safe drinking water or basic measures of sanitation and hygiene, individuals are at a higher risk of exposure to a large number of different environmentally transmitted pathogens (e.g. *Giardia*, helminths). Thus, children are likely to be simultaneously infected with multiple pathogens in addition to *Giardia*. However, none of the studies in our systematic review or meta-analyses examined the potential multiplicative or additive effect modification interactions of multiple infections on undernutrition. Therefore, we were unable to assess what, if any, effect(s) polyparasitism might have on the negative association of *Giardia* infection and malnutrition. Additionally, none of the epidemiological studies included in our analyses quantified the burden of infection, instead classifying *Giardia* infection as a
dichotomous variable (infected or uninfected). Thus, we were unable to evaluate what, if any, effect(s) differing parasite burden may have on undernutrition outcome.

5.4: Public Health Policy Recommendations

The findings from our systematic reviews and meta-analyses demonstrate the role of either asymptomatic or symptomatic *Giardia* infections can play in being a contributing factor to deficits both in childhood growth and micronutrient malnutrition. Thus, implementing effective strategies for the control and treatment of *Giardia* infections can help minimize the global burden of undernutrition.

For example, in the long term, implementing strategies aimed at improving the water quality and sanitation in a community or population can help minimize the risk of waterborne *Giardia* transmission in a population. Similarly, rolling out widespread health behavior and health education campaigns on the importance of maintaining good personal hygiene throughout at risk communities and populations may help limit the fecal-oral transmission of disease in populations. Additionally, investing in biological research and development into effective human vaccines that target the process of preventing encystation in the *Giardia* life cycle may be a promising strategy to control further disease spread in populations.

Perhaps the most significant short-term public health policy recommendation that can have an immediate lasting effect on minimizing the global burden of *Giardia* associated undernutrition, is to implement enhanced surveillance and new treatment guidance that will effectively detect and treat all cases of *Giardia* infection regardless of disease presentation. Current treatment guidelines for asymptomatic patients, and children in particular, is controversial especially in *Giardia* endemic areas where the risk of recurrent infection is high as the perceived costs associated with
treatment have been thought to outweigh the benefits (See Section 1.6.2 Treatment and Control) (74). However, our findings that even asymptomatic infections are significantly associated with deficits in growth and decreased serum micronutrient levels suggest that far larger numbers of children than those with overt diarrhea as a result of infection are actually be at risk for long term negative consequences on cognition and development than previously estimated. Thus, raising the effective treatment of all *Giardia* infected children should be a top priority to minimize the impacts of undernutrition.

5.5: Concluding Thoughts

Our analyses demonstrate that even asymptotically infected individuals can experience both the acute and chronic manifestations of undernutrition. For example, growth deficits in height associated with asymptomatic infections in children, can impact childhood physical and cognitive development. Thus, we argue that treatment should be administered if *Giardia* parasites are detected in the stool even in the cases of asymptomatic infection. However, as described in Chapter 1, Section 1.6.2, current treatment options for *Giardia* are associated with a number of limitations. Therefore, in Part II of the research presented here (Chapter 6), we discuss our progress towards the development of novel therapeutics against *Giardia* using a bottom up approach targeting putative *Giardia* methyltransferase enzymes using Surface Plasmon Resonance (SPR).
Chapter 6: DNA Methylation as a Target for Drug Discovery in Giardia lamblia

6.1: DNA Methylation

The term epigenetics refers to all meiotically and mitotically heritable changes in gene expression not coded for in the DNA sequence. Eukaryotic organisms regulate gene expression through three main epigenetic mechanisms including: covalent histone modifications (e.g. acetylation, methylation, and phosphorylation); silencing of noncoding RNA molecules; and DNA methylation (349). However, our focus here is on exploring DNA methylation, specifically the role of DNA methyltransferase enzymes as drug targets in Giardia.

6.1.1 Background

In mammals, as in most (but not all) complex organisms, DNA methylation is essential to gene regulation and is involved in a myriad of biological functions. For example, DNA methylation mediates genomic imprinting and X-chromosome inactivation. Further, DNA methylation has been hypothesized to play roles in protecting the genome from selfish genetic elements and/or may be used as a tool to reduce transcriptional noise in higher eukaryotes (349). In fact, a high degree of methylated DNA sequences were disproportionately found in the DIRS transposable elements in the genome of Dictyostelium discoideum (350). Additionally, a high degree of methylated sequences may also occur in satellite DNA, nonrepetitive intergenic DNA, and exons of genes (351). During mammalian development, DNA methylation and demethylation are orchestrated to achieve major changes in host methylation patterns (352).

DNA methylation is a reversible, epigenetic change that results in the addition of a methyl group at the C5 position of cytosine residues. DNA methylation is a central mechanism for mediating epigenetic gene regulation by modulating the availability of DNA for gene transcription.
More specifically, the pattern of DNA methylation, and as a result the pattern of gene transcription, is set by the actions of a combination of DNA methyltransferase enzymes (DNMT). DNMT enzymes catalyze the transfer of a methyl group from S-adenosylmethionine (SAM) to \( \text{C}^5 \) forming 5-methylcytosine (5mC). Cytosine methylation in the promoter regions of genes results in the repression of gene transcription. Conversely, DNA demethylation is the process in which 5mC is converted back into cytosine, thus restoring transcriptionally active DNA. DNA demethylation occurs either through passive or active mechanisms (349).

DNA methylation can be found in a variety of different fungi (with the exception of \( S. \) cerevisiae); insects (e.g. \( D. \) melanogaster); Coelomates (e.g. zebrafish, claw frog), and mammals (349). DNA methylation primarily occurs in cytosines located in CpG islands, regions of high GC genomic content found near gene promoters. This creates a pattern of modified and unmodified CG sites. CpG islands represent approximately 1%-2% of the total genome and approximately 70%-80% of CpG sites are methylated (351) in mammals; however much lower rates of methylation have been observed in other species.

6.1.2 DNA Methylation in Lower Eukaryotes

Many of the single celled eukaryotes such as the parasitic protists \( T. \) brucei (353) and \( E. \) histolytica (354)possess low levels of DNA methylation (\( \leq 1\% \)). Additionally, studies in \( D. \) melanogaster found weak DNA methylation activities of \( \sim 0.4\% \) in embryos and \( \leq 0.1\% \) in adult flies suggesting a role for DNA methylation in epigenetic regulation in different stages of development (355). Low levels of DNA methylation have also been observed in the organism \( D. \) discoideum (350). Moreover, high concentrations of 5mC sites were found in \( D. \) discoideum transposable elements (350). Together these findings help support the hypothesis that DNA methylation may play a role in contributing to the silencing of
retrotransposons as a way to maintain genome integrity and stability in other eukaryotes with relatively low levels of DNA methylation such as Drosophila (355), T. brucei (353), or E. histolytica (354).

6.2: DNA Methyltransferase Enzymes (DNMT)

6.2.1 Human DNMT Enzymes (hDNMT)

There are three distinct families of eukaryotic DNMT enzymes: DNMT1, DNMT2, and DNMT3; the DNMT3 family of DNMT enzymes can be further broken down into DNMT3A, DNMT3B, and DNMT3L. Mammalian DNMTs 1 and 3 are composed of two domains: the N-terminal regulatory domain and the C-terminal domain that contains the catalytic site. Further, the C-terminal domain contains ten catalytic sequence motifs six of which are conserved in nearly all cytosine methyltransferases from bacteria through plants to mammals (356). Specifically, Motifs I-III form the cofactor binding pocket, motif IV contains the catalytic cysteine, motifs VI, VIII, and X comprise the substrate binding site, and motifs V and VII form the target recognition domain involved in DNA binding (351). Since all known DNMT enzymes use SAM as the methyl donor in the DNA methylation reaction, the active site shows a high structural similarity (357). Additionally, the binding pockets show a high structural conservation of residues interacting with deoxycytidine and the SAM cofactor.

hDNMT 1 and hDNMT 3 Enzymes

Human DNMT1 (hDNMT1) is responsible for duplicating the pattern of DNA methylation during replication (351). hDNMT1 enzymes show a strong preference for hemimethylated DNA, and thus are known as the “maintenance” methyltransferase enzymes. hDNMT1 has also been found to regulate the levels of methylation in differentiated cells (351).
DNMT3A and DNMT3B are known as “de novo methyltransferase enzymes” (351). These enzymes play an essential role in embryonic development as demonstrated by the presence of high levels of de novo methylation of DNA found during gametogenesis and early embryogenesis, whereas only low levels of expression of DNMT3 was observed in differentiated cells (358). The functions of DNMT3a and DNMT3b are at least partially overlapping, though both have been demonstrated to be necessary in ensuring proper development (352). The hDNMT3L protein shows high sequence similarity with DNMT3A; however lacks a conserved segment in its catalytic domain, Motifs IX and X, which is required for methyltransferase activity (351). Therefore, DNMT3L is not an active methyltransferase, but rather has a dual role in binding histone tails and in activating DNMT3A. It was also found to stabilize the conformation of the active-site loop of DNMT3A.

**hDNMT2 Enzymes**

The human DNMT2 family of enzymes (hDNMT2) is relatively small (~ 391 amino acids) in comparison to the other DNA methyltransferases. Further, DNMT2 enzymes are also unique among other eukaryotic methyltransferase enzymes in that while they too possess the conserved catalytic motifs I, IV, VI, IX, and X, in their C terminal domains, hDNMT2s lack the N-terminal regulatory region present in both human DNMT1 and DNMT3s enzymes. Despite these structural differences, DNMT2 enzymes have been confirmed to have very weak DNA methylating activity (359, 360). However, an apparent absence of phenotype was found in dnmt2 knockout cells and no reduction in global DNA methylation levels were observed (361). These findings suggested that human DNMT2 (hDNMT2) enzymes were not necessary for DNA methylation in vivo, rather, perhaps these enzymes actually had a target other than DNA.
In fact, follow up studies have since demonstrated that hDNMT2 enzymes methylate tRNAs with high efficiency. Specifically, mass spectrometry analysis by Goll et al., 2006 revealed that hDNMT2 enzymes methylated cytosine 38 (C38) in the anticodon loop of tRNA\textsuperscript{Asp} in a number of different species (e.g. mouse, Arabidopsis thaliana, and Drosophila melanogaster) (362). Further work investigating the potential functional roles of tRNA methylation, found that a loss of cytosine-C5 methylation caused tRNA degradation and reduced protein synthesis (363) suggesting that RNA cytosine methylation by DNMT2 enzymes promotes tRNA stability and protein synthesis. Additionally, Rai et al., 2007 established catalytic activity of DNMT2 in the cytoplasm of zebrafish embryos and found that a morpholino knockdown of the DNMT2 protein in these embryos conferred differentiation defects in a number of different organs (e.g. retina, liver, and brain) (364). These findings suggest a role for DNMT2 mediated tRNA methylation in zebrafish development.

6.2.2 Dual Specificities for DNMT2 Methyltransferases in Lower Eukaryotes

The role of a single DNMT2 homolog enzyme in mediating low levels of DNA methylation has long been demonstrated in a number of different lower eukaryotic species including Drosophila melanogaster (365), Entamoeba histolytica (354), Dictyostelium discoideum (350), and Trypanosoma brucei (353). In recent years, however, several studies have also found DNMT2 mediated tRNA methylation by a single DNMT2 homolog enzyme in different lower eukaryotic species (366-368). These findings suggest that DNMT2 enzymes have a dual specificity for both DNA and tRNA substrates in lower eukaryotes.
6.3: DNA Methylation and Disease

Altered patterns of DNA methylation are frequently observed in human disease. For example, epigenetic alterations in DNA methylation have been found in different cancers (e.g. colorectal (369); breast (370); and ovarian (371)), as well as in psychiatric disease (e.g. bipolar disorder) (372). In cancer cells, for example, aberrant epigenetic silencing of tumor suppressor genes by promoter DNA hypermethylation is one of the most important epigenetic changes in the pathogenesis of cancers (373). Epigenetic alterations to the DNA that manifest as physical disease states are not immediate, but rather have been found to accumulate over time with increasing age (374).

Inhibition of DNA methylation is a promising strategy for the treatment of a number of diseases. In fact, a number of different “epigenetic therapies” are already in various stages of rational drug development in hopes of being added to the armamentarium of anticancer treatments. The mechanism of action (MOA) for a number of these drugs already in development is to block DNA methylation in the cell. Inhibitors of DNA methylation rapidly reactivate the expression of genes that have undergone epigenetic silencing. Brief descriptions of existing DNMT inhibitors of particular relevance to our work targeting DNMT2 in Giardia follows.

6.3.1 DNMT Inhibitors

DNMT inhibitors can be classified into two broad categories: nucleoside and non-nucleoside inhibitors (375). Nucleoside analogs, after incorporating into DNA, cause covalent trapping and subsequent depletion of DNMT enzymes; while noncovalent DNMT inhibitors directly block the active site of the DNMT enzyme without the necessity for DNA incorporation (351). At present, only two nucleoside analogs, 5-azacytidine (AzaC) and 5-aza-2-deoxycytidine (decitabine) have been clinically developed (376). To date, studies of these two nucleoside analogs
has largely focused on their ability to inhibit methylation by hDNMT1 enzymes, as hDNMT1s have long been proposed as the most interesting target for cancer therapies (375).

5-azacytosine (azacytidine) and 5-aza-2-deoxycytidine (decitabine)

In brief, both azacytidine and decitabine compounds act as prodrugs that must first be incorporated into the DNA in place of cytosine into replicating DNA before they are able to block the activity of DNMT enzymes (328). Thus, these compounds are active only in S-phase cells. While decitabine can be directly transformed into a triphosphate that can then serve as a building block for DNA polymerases, the riboside azacytidine is a ribonucleoside that incorporates directly into RNA rather than DNA (377). Thus, azacytidine first needs to be deoxygenated in its sugar moiety before it can be transformed into a triphosphate that can then be incorporated into the DNA. Hence, the rate of DNA incorporation by azacytidine into DNA is much lower than for decitabine (351). Once incorporated in the DNA, both nucleoside compounds are subject to a covalent addition of a thiol group of the DNMT that serves to trap the enzyme to the DNA resulting in DNA hypomethylation and a reactivation of silenced genes. Aza nucleosides have been currently approved by the Food and Drug Administration (FDA) for the treatment of myelodysplastic syndrome, a group of cancers in which immature blood cells in the bone marrow do not mature or become healthy blood cells (375).

However, because of their ability to incorporate into the DNA, these drugs have relatively low specificity and are characterized by substantial cellular and clinical toxicity as the trapped DNMTs may inhibit RNA and DNA polymerases, which in turn leads to an inhibition of protein biosynthesis and DNA strand breaks (376). An additional disadvantage of these compounds is their instability in aqueous solutions (328). These negative effects may be overcome by use of other nucleoside analogs such as zebularine and 5-fluoro-2’-deoxycytidine (reviewed in Yu and
Wang, 2008 (373)). In preclinical tests, the potency of zebularine was found to be 10-fold lower than for the azacytosines (378). See Sippl and Jung, 2009 for more information (351).

Noncovalent DNMT Inhibitors

Noncovalent DNMT inhibitors act directly on DNMT enzymes without the necessity for incorporation into the DNA (351). Much less is known about these compounds, as few have been validated as directly targeting DNMTs in routine practice (375). Of these, however, RG-108 (NSC401077) has so far proven to be a promising antitumor drug based on a virtual screening for DNMT1 inhibitors, a previously established homology model, and novel docking scoring methodology (357). Another DNMT1 inhibitor, NSC 303530, was also identified using this same process (357). Further characterization of these two compounds showed them to inhibit DNMT activity both in vitro and in vivo (357), thus further investigation into their clinical use is warranted. See Sippl and Jung, 2009 for more information (351).

6.4: Drug Discovery in Giardia

6.4.1 Need for Novel Therapeutics in Giardia

Medical countermeasures against Giardia are currently limited to therapy of established infections. In most cases, treatment regimens with either metronidazole or albendazole alone are effective (379), however, the frequent recurrence of infections, of up to 90%, (82) for some individuals living in Giardia endemic areas suggests parasite resistance is an urgent concern. (See Chapter 1, Section 1.6.2 Treatment and Control)

Additionally, most clinical studies, regardless of the anti-Giardia drug used, have found cure rates of standard therapies to generally fall below 100% (379). Apart from drug resistance, treatment failures can occur when there is a potential for high rates of chronic or recurrent
infections as has been demonstrated among children living in some settings where *Giardia* is hyperendemic (70).

However, the number of anti-*Giardia* therapeutics has not significantly increased in the last decades (83). For these reasons, the development of novel, effective therapeutics against *Giardia* through rational drug design programs has been raised to a high priority. Effectively treating giardiasis is also an important step in disrupting the parasite and interrupting the cycle of disease transmission.

Deciding which method to use in identifying novel small molecule anti-*Giardia* compounds can be challenging. While current high throughput screens of large compound libraries have been a productive strategy for identifying antigiardial drugs already approved for other indications (80), in general, these methods are costly and time and labor intensive due to the extensive training required prior to operation. Thus, large-scale high throughput screenings are often not highly feasible in smaller laboratory settings. Additionally, the application of common genetic and molecular tools to investigate the efficacy of new small molecule compounds are limited in *Giardia* due to the fact that the organism has two transcriptionally active nuclei, is tetraploid, and contains an absence of selectable markers.

Looking to alternative strategies that may be used in the rational drug development of novel anti-*Giardia* compounds is becoming increasingly important. In the research presented here, we describe our work in progress using Surface Plasmon Resonance (SPR) technologies to screen small molecule compounds that inhibit the putative methyltransferase enzyme targets in *Giardia*. 
6.4.2 Surface Plasmon Resonance (SPR)

In brief, SPR uses an optical method to measure a change in the refractive index of the medium in close vicinity of a gold surface. The gold surface is coated with a dextran layer and is typically on a glass support that forms the floor of a small volume flow cell on a sensor chip, through which an aqueous solution, composed of the small molecule compounds, analytes, is continuously passed. Polarized light from a laser source is directed through a prism under the gold film surface where surface plasmons are generated at a critical angle of the incident light. Absorption of light is seen as a decrease in intensity of the reflected light. Thus, this technique can be used to monitor the binding of dissolved molecules, known as analytes in SPR contexts, to receptor, ligand, molecules immobilized on the sensor chip surface. The term “ligand” is applied in SPR in an affinity chromatography context and does not imply that the surface-attached molecule is a ligand for a cellular receptor. Binding of analyte molecules to the ligand changes the surface mass, which in turn changes the refractive index of reflected light. This response is measured in response, or resonance, units (RU), which are used to describe the increase in reflected light. The response is visually displayed as real-time output in the form of a sensorgram (See Appendix A) (1).

6.5: DNA Methylation in Giardia

In recent years, Williams et al. (unpublished) documented, for the first time, the presence of DNA methylation, albeit at extremely low levels (~0.05%), in Giardia (Figure 6.11.1.1). They further showed that DNA methylation is essential for parasite survival (Figure 6.11.1.2) (unpublished). Lastly, bioinformatics analysis performed by Williams et al. (unpublished) have indicated the presence of four putative homologs of DNA methyltransferase 2 (DNMT 2) in
*Giardia* that partially retain important functional domains characteristic of all DNA methyltransferases, but demonstrate high sequence divergence (Table 6.11.2.1). Simple sequence comparisons of these candidates lead them to hypothesize that, *G*/*50803_21512 is the most likely of the four candidates to be a functional methyltransferase enzyme in *Giardia* (Table 6.11.2.1), as it is the only one of the four candidate enzymes found that contains a highly conserved proline-cysteine (PC) dipeptide in the proposed catalytic site of DNMT enzymes (356). The high sequence divergence of the putative gDNMT2 candidates suggests that these may be good targets in drug development.

6.6: Using Surface Plasmon Resonance (SPR) as an Analytic Tool to Identify Novel Putative Methyltransferase Enzyme Inhibitors in *Giardia*

6.6.1 Introduction

SPR will allow me a label-free method to quantitatively, sensitively, specifically, and accurately compare the binding (yes/no) and binding affinities (how strong is the binding) of different small molecule compounds to our putative *Giardia* methyltransferase enzymes. Thus, for the purposes of our research, SPR provides a “bottom up” approach to rational drug development and allow us to test the hypothesis that small molecule compounds must first bind with high affinity to our *Giardia* candidate methyltransferase enzymes in order to subsequently inhibit their enzyme activity and in turn block methylation in the parasite. The information generated from the primary SPR screens described will serve as a critical first step in the drug discovery pipeline of novel chemotherapeutics that may be used in the treatment of giardiasis.
6.6.2 Ligand Selection

For a detailed description of the ligands used in the SPR screens please see Appendix B. In our assays, we chose two putative *Giardia* methyltransferase enzyme candidates (*Gl_21512* and *Gl_9528*) to test in our initial SPR screens. However, for the reasons discussed above, we hypothesize that only one of these enzymes, *Gl_21512*, is the functional DNMT2 homolog in *Giardia* (Table 6.11.2.1). Additionally, we included human DNMT1 and hDNMT2 wild type enzymes as positive controls to compare binding and binding affinity of our putative *Giardia lamblia* methyltransferase enzyme candidates to well characterized methyltransferase enzymes. We also included a mutant hDNMT2 enzyme in which a methylatable cytosine at position 79 in the enzyme active site had been replaced with an alanine (C79A), rendering it a nonfunctional enzyme, to serve as a negative control for our screens.

6.6.3 Analyte Selection

There are a number of small molecule compounds that have been identified as known DNMT inhibitors in the literature; thus, we selected analytes for our SPR screens to be used as either positive (+) or negative (-) controls based on their *in silico* predicted binding energies with hDNMT1 enzyme (357). Positive controls included the two noncovalent DNMT inhibitors RG-108 (NSC 401077) and NSC 303530 (357). We also included two well-characterized nucleoside analogs, azacytidine and 5-aza-2’-deoxycytidine (decitabine), as these have been widely used with demonstrated efficacy to block DNA methylation in human cancer cell lines (reviewed in Yu and Wang, 2008) (373). We also used two negative controls (NSC 19555 and NSC 27929) that were identified from the same study using *in silico* approaches that identified RG-108 and NSC 303530 (357). However, unlike the positive controls, these two compounds were found to have a low predicted binding energies with hDNMT1 (357), and thus, were selected as negative controls for
our SPR assays as we hypothesized that these compounds would also bind with weak affinity to our putative *Giardia* enzymes (Appendix B).

In addition to the six analytes chosen as either positive or negative controls for our screens, we also included S-adenosylmethionine (SAM), cytidine, deoxycytidine, adenosine, and deoxyadenosine as analytes in our first round of SPR. Each of these compounds was predicted to bind to different analytes with varying degrees of binding affinities (See Appendix B, Table B1 for binding predictions of each analyte to ligand). We got all the NSC compounds and both nucleoside analogs (azacytidine and decitabine) from the National Cancer Institute, Developmental Therapeutics Program (NCI/DTP) as they are commercially available, in large quantities, at no cost; however, we purchased the other analytes to be used in our SPR screens from commercially available vendors (See Appendix B). A team of undergraduates, who I helped mentor in the Elmendorf Lab led by Bre Walsh (C’17) and Jess McCann (C’18), also produced four different tRNA\textsuperscript{Asp} in house, following a previously published protocol (380), to use as analytes in our screens (Section 6.7.2, Round 3). These included two wild type tRNAs\textsuperscript{Asp}: human and *Giardia lamblia* (Gl\_tRNA\textsuperscript{Asp}), and two mutant tRNAs\textsuperscript{Asp}: human and Gl where the methylatable cytosine in position 38 of the anticodon loop of tRNA\textsuperscript{Asp} had been replaced with an uracil (C38U) in each mutant.

### 6.7: Materials and Methods

Georgetown University (GU) has a BIACORE\textsuperscript{TM} facility with two BIACORE\textsuperscript{TM} instruments, BIACORE\textsuperscript{TM} T-100 and BIACORE\textsuperscript{TM} 1000. Both BIACORE\textsuperscript{TM} instruments are manufactured by GE Healthcare Life Sciences (1) and can be used to perform SPR screens. All SPR screens for these experiments were performed using the BIACORE\textsuperscript{TM} T-100 instrument.
housed in Georgetown University (GU) Lombardi Cancer Center (LCC). The five general steps used for each SPR screen are outlined below.

6.7.1 Surface Plasmon Resonance (SPR) Assays

Step 1: Activating the CM-5 Chip Surface

CM-5 chips allow for covalent immobilization of ligands to the chip surface via amine coupling. Prior to flowing ligand over the surface of a chip, a chip must be first activated with N-hydroxysuccinimide (NHS). In our assays, activation of the chip surface was achieved by flowing a solution of HEPES and NHS buffer over the CM-5 chip surface for ~ 12 minutes.

Step 2: Immobilizing Ligand to CM-5 Chip Surface

Next, each purified ligand was immobilized on the activated CM-5 Sensor Chip via interaction of exposed lysine residues on the ligand with the carboxymethyl groups on the dextran coated chip surface (1). Each sensor chip has a flow cell (FC) with four separate compartments per chip; thus allowing a continuous flow of aqueous analyte solution over the sensor surface to four different immobilized ligands at once. One FC on each chip was always left blank in each screen; this FC served as a reference in order to detect background levels of binding. Each ligand was diluted (1:100) in 10mM acetate buffer prior to being flowed over its designated the CM-5 chip surface. This process was repeated three times to immobilize three different ligands in three different FC belonging to one chip for every screen. The BIACORE™ software program automatically determined when a “sufficient” quantity of ligand was immobilized to the CM-5 chip surface. All SPR screens were performed at 25° Celsius.
Steps 3 and 4: Analyte Flow

Once each ligand was bound to the chip surface, an ethanolamine solution was first flowed over the chip surface in order to prevent nonspecific binding of the analyte directly to the chip surface (Step 3). Following this, each analyte was then flowed simultaneously over each of the 4 flow cells (Step 4). The BIACORE™ software automatically calculated the binding affinities for each flow cell and each chip separately.

Step 5: Regenerating the CM-5 chip surface

Each chip surface is regenerated between analytes in order to wash away any remaining bound analytes and prevent cross contamination between runs. Surfaces were regenerated using NaOH for ~30 minutes.

6.7.2 Experimental Details

Round One (August, 2015) Binding Only

Four different SPR screens were performed varying conditions in each screen and chip in order to determine the optimum conditions for analyte-ligand binding. Flow cell (FC) 1 was always left blank in each of the four different screens and served as a reference for FCs 2-4. The ligands immobilized and immobilizing conditions of each chip follows. Chip 1: FC2: M. SssI (10 mm acetate, pH 5.0, serial dilutions of 1:100 followed by 1:50); FC3: hDNMT2wt (10 mm acetate, pH 5.0, serial dilutions of 1:200 and 1:100); and FC4: hDNMT1 (10 mm acetate, pH 5.0, serial dilutions of 1:200, 1:100 for 60 seconds, and 1:50); Chip 2: FC2: hDNMT2wt (10 mm acetate pH 5.0, 1:100 dilution); FC3: hDNMT2m (10mM acetate, pH 4.5, serial dilutions of 1:100 followed by 1:50); FC4: MBP (10 mm acetate, pH 5.5, 1:400 dilution); Chip 3: FC2: hDNMT2wt (10 mm acetate, pH 5.0, serial dilutions of 1:200 followed by 1:100); FC3: MBP (10 mm acetate, pH 5.5, 1:200 dilution); FC4: Giardia lamblia (Gl)_9528 (10 mM acetate, pH 4.5, 1:200, serial
dilutions of 1:100, and 1:50); and **Chip 4**: FC2: hDNMT2wt (10 mM acetate, pH 5.0, 1:100 dilution); FC3: MBP (10 mM acetate, pH 5.5, 1:400 dilution); FC4: Gl21512 (10 mM acetate, pH 5.0, serial dilutions of 1:100 and 1:50).

The following eleven different compounds were used as analytes to flow over Chip 1 containing the immobilized ligands noted above. The eleven analytes were: 1. SAM; 2. ribocytidine; 3. deoxycytidine; 4. 5-azacytidine; 5. 5-aza-2-deoxycytidine (decitabine); 6. riboadenosine; 7. deoxyadenosine; 8. NSC303530; 9. NSC401077; 10. NSC21970; and 11. NSC19555. Each analyte was flowed over each FC three times.

All eleven analyte compounds were first diluted either in 10 mM HEPES, 150 mM NaCl (HBS-N) buffer (Compounds 1-7) or 100% DMSO (Compounds 8-11) to a final concentration of 10mM. Next, the 10 mM stock solutions were then further diluted in HBS-N to a final concentration of 40 μM. HBS-N was used as the running buffer for compounds 1-7. The running buffer for compounds 8-11 was HBS-N + 5% DMSO. Based on the optimization results conducted in Run 1, Chip 1 the protocol was further modified by replacing HBS-N with HBS-P to help with non-specific binding and analyte concentrations were lowered from 40 μM to 10 μM in order to minimize any mismatch due to bulk refractive index change for all remaining chips and trials in round 1 of screens. Only 7 of the 11 analytes were used in Chip 3 (Compounds 1-3 and Compounds 8-11). Based on the findings from the optimization run for chip 3, the final DMSO concentration was reduced from 5% to 0.1% DMSO for chip 4.

**Round 2 (November 2015) Binding and Binding Kinetics**

All round two experiments were conducted in order to test the binding and binding affinities between five analytes and three ligands. Experiments were performed at 25°C. The specific chip design was as follows—FC1: Blank, FC2: hDNMT1wt (10 mM acetate, pH 4.5, 1:50
dilution); FC3: hDNMT2wt (10 mM acetate, pH 5.0, 1:50 dilution); FC4: hDNMT2m (10 mM acetate, pH 4.5, 1:50 dilution). The following five analytes were used: 1. NSC19555 (- control); 2. NSC21970 (- control); 3. NSC303530 (+ control); 4. NSC401077 (RG-108, + control); and 5. S-adenosyl methionine (SAM, methyl donor). The running buffer was HBS-P (10 mM HEPES, 150 mM NaCl, 0.02% surfactant P20) maintained at a flow rate of 10 μL/min. Each analyte was tested in triplicate and a range of different compounds: 0 µm, 2.5 µm, 5 µm, 10 µm, 20 µm, and 40 µm were flowed over each FC in one CM-5 chip.

Overnight kinetics experiments for assessing binding kinetics for each analyte compound to each ligand were conducted. Each compound was directly diluted in 100% DMSO (Compounds 1 to 4) and in HBS-P (Compound 5) to a final concentration of 10 mM. The stock solutions were further diluted in HBS-P+0.5% DMSO (Compounds 1 to 4) and in HBS-P (Compound 5). The analyte concentrations used were: 0 µM, 2.5 µM, 5 µM, 10 µM, 20 µM, and 40 µM. The running buffer for compounds 1 to 4 was HBS-P+0.5% DMSO and for compound 5 was HBS-P.

Round 3 (January 2016) Binding and Binding Kinetics

For round 3, we performed SPR screens in order to determine the binding and binding kinetics of four different tRNAs\textsubscript{Asp} (human-tRNA\textsubscript{Asp}, human-tRNA\textsubscript{Asp} C38U, \textit{Giardia lamblia} Gl\textsubscript{tRNA}\textsubscript{Asp}, Gl\textsubscript{tRNA}\textsubscript{Asp} C38U) to three different DNMT ligands. The tRNA compounds were purified in house following protocols adopted from (367). All experiments were performed at 25°C. The specific chip design was as follows-- FC1: Blank; FC2: hDNMT1 (10 mM acetate, pH 4.5, 1:50 dilution); FC3: hDNMT2wt (10 mM acetate pH 5.0, 1:50 dilution); FC4: hDNMT2m (10 mM acetate pH 4.5, 1:50 dilution).

Overnight binding kinetics experiments were performed for each tRNA\textsubscript{Asp} using the following analyte concentrations: 0 nM, 3.75 nM, 7.5 nM, 15 nM, 30 nM, and 60 nM. Each set
of binding kinetics experiments were performed by dissolving the four analytes in two different published tRNA methylation buffers used in in vitro RNA methylation assays Buffer 1 (100 mM Tris-HCl (pH 7.5), 100 mM NaCl, 5 mM MgCl₂, 1 mM DTT, and 5% glycerol) (380) and Buffer 2 (100 mM Tris-HCl (pH 8), 100 mM NH₄OAc, 5 mM MgCl₂, 1 mM DTT, and 0.1 mM EDTA) (381).

6.7.3 Calculating Binding Affinity

The dissociation constant (K_D) was calculated to assess binding affinities of different analyte-ligand binding interactions in Rounds 2 and 3 of SPR screens using the formula

\[ K_D = \frac{k_d}{k_a} \frac{1}{Ms} \]

where \( k_d \) is the rate of dissociation and \( k_a \) is the rate of association. Dissociation constants for each analyte were calculated using the BIACORE™ software.

6.8: Preliminary Findings

6.8.1 Round 1 Trial SPR Screens

Round 1 SPR screens were performed using a large set of analytes and ligands in order to determine the optimum binding conditions to be used in subsequent assays and determine the feasibility of using SPR as a tool for discovering small molecule DNMT inhibitors. SAM, which is the methyl donor in C-5 methylation reactions, bound to M. SssI, hDNMT1 and hDNMT2wt and hDNMT2m (wild type and mutant), as well as both putative Giardia lamblia candidate enzymes (Gl_21512 and Gl_9528). Surprisingly, maltose-binding protein (MBP), which was included as a control for the Giardia fusion proteins and thus was not predicted to bind as it lacked catalytic activity, was also observed to bind to every analyte. Additionally, we observed a large amount of noise in the sensorgram outputs indicating a high degree of variability between individual runs (data not shown).
6.8.2 Round 2 SPR Screens

In the second round of SPR screens, we determined both binding (yes/no) and binding affinity by measuring the $K_D$ between ligands and analytes, this time using a much narrower set of compounds in order to better define assay perimeters. Again, similar to what we found in our round 1 screens, SAM bound to all three ligands (hDNMT1 wt, hDNMT2 wt, and hDNMT2m). As illustrated in Figure 6.11.1.3, the sensorgram showed clear association, as indicated by an increase in the binding curves in a dose response fashion (the higher the analyte concentration flowed, the higher the response unit), saturation of binding as demonstrated by a plateau at 15.5 RU, and dissociation upon the end of analyte flow as seen by a drop in the binding curves at 60 seconds (Figure 6.11.1.3). Lastly, the sensorgram shows little variability between trials as evidenced by the high degree of overlap within the three replicates performed for the same run at the same concentration (Figure 6.11.1.3). Further, we were able to calculate the binding affinities of SAM to each of the ligands (Tables 6.11.2.2 and 6.11.2.3) for binding (Table 6.11.2.2) and binding affinities (Table 6.11.2.3) respectively and found consistency in $K_D$ values for SAM between the three ligands. These findings suggest that the SPR assay was optimized for assessing both binding and binding affinities for SAM.

However, despite the fact that we also observed clear binding for two NSC compounds, NSC401077 and NSC 19555, we were unable to calculate the $K_D$ values from the BIACORE™ software as the sensorgram curves were of poor data quality a determination made by the BIACORE™ software program based on the shape of the sensorgram and the fact that the analyte did not stay bound to the ligand for the entire 60 seconds of analyte flow, which prevented the calculation of a $k_d$ value (See Figure 6.11.1.4).
6.8.3 Round 3 SPR Screens

In the third round of SPR screens, we determined both binding (yes/no) and binding affinity by measuring the $K_D$ between wild type (wt) human DNMT1, human DNMT2wt, and human DNMT2 (C79A) ligands and 4 different tRNA$^{\text{Asp}}$ analytes [2 human (wt and C38U) and 2 Giardia (wt and C38U)]. In these screens, both human and Giardia tRNA$^{\text{Asp}}$ isolates bound to all three ligands. As illustrated in Figure 6.11.1.5, the sensorgram showed clear association, as indicated by an increase in the binding curves in a dose response fashion (the higher the analyte concentration flowed, the higher the response unit), saturation of binding as demonstrated by a plateau at ~110 RU, and dissociation upon the end of analyte flow as seen by a drop in the binding curves at 60 seconds (Figure 6.11.1.5). Lastly, the sensorgram shows little variability between trials as evidenced by the high degree of overlap within the three replicates performed for the same run at the same concentration (Figure 6.11.1.5). A similar trend of distinct binding, plateau, and dissociation of analyte to ligand was observed when flowing the $Gl_t$ tRNA$^{\text{Asp}}$ wt analyte over hDNMT1 wt ligand (Figure 6.11.1.6).

Further, we were able to calculate the binding affinities of each tRNA analyte using the two tRNA$^{\text{Asp}}$ methylation buffers 1 and 2 (Tables 6.11.2.4A and 6.11.2.4B) respectively. We found consistency in $K_D$ values for binding affinity when dissolving human tRNA$^{\text{Asp}}$ wt in either of the two different buffers. However, were unable to determine a $K_D$ value when $Gl_t$ tRNA$^{\text{Asp}}$ wt was dissolved in methylation buffer 1 (Tables 6.11.2.4A and 6.11.2.4B). This finding, of being unable to determine the $K_D$ values due to poor binding, was consistent for the Giardia tRNA$^{\text{Asp}}$ isolates (unable to determine binding dissolving analyte in buffer 1 but able to determine binding dissolving analyte in buffer 2). These findings suggest that the SPR assay was optimized for assessing both binding and binding affinities using tRNA methylation buffer 2.
6.9: Discussion

We present preliminary findings from our SPR screens identifying the binding and binding affinities of small molecule analyte compounds to different ligands including hDNMT enzymes and two different putative *Giardia* DNMT enzymes. From these preliminary findings we learned some key take-away messages we can use when going forward with further SPR screens in assessing a tool for the rational drug development DNA methylation inhibitors in *Giardia*.

From our first round of SPR screens, we found that SAM bound to hDNMT enzymes as well as the bacterial M. SssI enzyme. Additionally, we confirmed that it bound consistently across ligands. This was as expected based on our binding predictions (Appendix B, Section 8.2) given that SAM is the methyl donor in reactions catalyzed by cytosine-5 methyltransferase enzymes. Thus, these findings suggest that our SPR assay can be used to reliably detect analyte ligand binding using DNMT enzymes.

Further, from our first round of assay optimization, we also learned the importance of assessing both binding and binding affinities between analyte and ligand. For example, our initial results led us to incorrectly assume that we could detect binding in a large number of analyte compounds, even those we did not predict would bind based on characterization in the literature. However, upon interpretation of the data with our collaborators, we realized that just seeing a robust sensorgram signal is not enough evidence to confirm binding with strong affinity. Rather, the only way to confirm strong binding is by assessing binding affinities. This involves the calculation of a dissociation constant. Determining binding affinity in these screens is important in order to determine the strength of binding, as we hypothesize that compounds that bind with weak affinity would not be promising targets for further drug development. So, we could eliminate a number of ligands and analytes in our second round of SPR testing based on $K_D$ values. Thus,
our second round of testing proved more informative in differentiating the types of binding (e.g. strong from weak).

Another interesting finding from our Round 3 SPR screens is that we could only consistently calculate $K_D$ values when dissolving the analytes in tRNA methylation buffer 2 not when dissolving them in tRNA methylation buffer 1. Although the concentrations for making both buffers were published by the same research group, Jurkowski et al., upon closer evaluation it was determined that the tRNA methylation buffer used in experiments published in the 2012 paper (methylation buffer 2) (381) used a 10 fold greater concentration of MgCl$_2$ and DTT than used for experiments published in their 2008 paper (methylation buffer 1) (380). An explanation for this change was not discussed in the 2012 paper, however, this 10 X increase in concentration may explain why we were able to optimize binding and binding affinities in our SPR screens for Giardia only when dissolving the $Gt$ tRNA$^{A_sp}$ wt and C38U analytes in methylation buffer 2 (381) as opposed to methylation buffer 1 (380).

However, our assay design is not without limitations. The most important limitation may be our choice of sensor chip for our SPR assays. More specifically, we chose to perform our SPR screens using a CM-5 sensor chip that uses amine couple chemistry to covalently immobilize ligands to the sensor surface. While this approach is good in ensuring the capture of a high amount of ligand on the sensor surface, any free lysine exposed on a ligand surface can in theory bind to a CM-5 sensor chip. Thus, we cannot definitely confirm that the ligand is bound in a proper confirmation to ensure analyte binding to the active catalytic site on the enzyme. Going forward, we may switch from the CM-5 sensor chip to the Ni$^{2+}$-NTA chip, as this chip allows for more specific binding due to the affinity of Ni$^{2+}$ with histidine tagged proteins such as our Giardia.
enzymes. However, further time and cost considerations must be taken into account before proceeding with this strategy.

While SPR can help identify binding and binding affinity of small molecule compounds (analytes) to our putative *Giardia* methyltransferase enzyme candidates, it only provides one piece of indirect evidence to support our hypotheses that *Giardia* has one functional DNMT2 enzyme of dual substrate specificity. Thus, the focus of other parallel undergraduate research projects that are currently ongoing, or in development, in the Elmendorf Lab are also aimed at helping characterize our putative enzyme candidates. For example, we can determine whether these enzymes have dual DNA and tRNA substrate specificity using LC/MS or bisulfite sequencing approaches on DNA and tRNA isolated from *Giardia* parasites. Both approaches offer several advantages as they are more sensitive techniques that have been developed for both *in vitro* and *in vivo* detection of tRNA methylation in other single cell eukaryotes (363, 381, 382). Together, these pieces can help contribute to understanding more about DNA methylation in *Giardia* and which of our putative enzyme candidates is a good target to proceed with future SPR screens for rational drug development of anti-*Giardia* therapies.
6.10: Concluding Thoughts

There are currently no publications in the field of *G. lamblia* research examining the role of methyltransferase enzymes as novel drug targets in *Giardia*. However, identifying the binding and binding affinity of our putative methyltransferase enzymes represents a critical first step in contributing to the anti-*Giardia* drug development pipeline as a compound should bind with high affinity to its target in order to be an effective enzyme inhibitor. Additionally, knowledge gained from the characterization of the binding properties of these putative methyltransferase enzyme candidates can help narrow the four putative candidates to the most likely functional methyltransferase enzyme in *Giardia*, thus allowing further insight into the molecular biology of the parasite.

6.10.1 Acknowledgements

I would like to acknowledge the assistance of Dr. Aykut Üren, M.D. for his guidance and expertise in helping us to interpret the SPR data. I would also like to acknowledge Puru Tiwari, Ph.D. for his technical expertise in the operating the BIACORE™ equipment and conducting all the SPR screens for the Elmendorf Lab. I would also like to thank former undergraduates in the Elmendorf Lab, Claire Lang (C’14), Chris Jenkins (C’14), and Darryl Turkahia (C’15), for purifying and preparing the hDNMT C79A, *Gl_21512*, and *Gl_9528* ligands used in the SPR screens. I would also like to thank current undergraduates in the Elmendorf Lab, Bre Walsh (C’17), Jess McCann (C’18), Simon Turkington (C’17), and Grant Rosensteel (C’19), for their assistance in preparing the tRNA<sup>Asp</sup> samples used as analytes in the SPR screens.
6.11: Figures and Tables

6.11.1 Figures
The Elmendorf Lab has demonstrated that DNA methylation is present in *Giardia*, though at very low levels, < 0.1% (See Figure 6.11.1.1), and is essential for parasite survival (See Figure 6.11.2).

**Figure 6.11.1.1. Giardia methylates its DNA at low levels.** Genomic DNA dot blot using anti-5-methyl cytosine (5mC) monoclonal antibodies to detect DNA methylation in *Giardia*. DNA was isolated and collected from both the trophozoite (Lane J) and cyst stages (Lanes K-L) of the *Giardia* life cycle. The presence of DNA methylation was compared to a range of different concentrations of DNA, isolated from mouse DNA (NIH 3T3 DNA, New England Biolabs Inc.), to provide a range (2 to 50 ng) of methylation values for estimation (Lanes A-E). Mouse DNA was selected for comparison of methylation levels, as it is known to possess C5-DNA methylation. DNA isolated from E. coli strains JM109 (dcm+) (Lane G) and ER2925 (dcm-) (Lane H) were used as positive and negative controls respectively; 10x SSC media was also used as a negative control in this assay (Lane F). Total RNA was isolated from trophozoites (Lane I) to ensure that any methylation detected was from DNA, not small contaminating amounts of RNA. (Unpublished, Williams, C. et al.)

**Figure 6.11.1.2. Non-methylatable cytosine analogs 5-Aza-C and 5-Aza-2-dC inhibit Giardia growth in a concentration dependent manner.** *Giardia* trophozoites were grown in the presence of varying concentrations of A) 5-Aza-C, or B) 5-Aza-2-dC and counted by hemacytometer every 12 hours up to 48 (Figure 6.11.1.2A) or 60 hours (Figure 6.11.1.2B) respectively. A one-tailed Student’s t-test, used to determine statistical significance, found that 5-Aza-C treatments of 10, 20, and 50 μM had a p value of <0.001 (Figure 6.11.1.2A), whereas the 5-Aza-2-dC treatments of 1 and 10 μM had a p value of <0.0001 (Figure 6.11.1.2B) when compared to untreated trophozoite cultures. (Unpublished, Williams, C. et al.)
Figure 6.11.1.3: Sensorgram output showing SAM binding to hDNMT1 wt (FC2). Along the X-axis is binding over time over increasing varying enzyme concentrations (2.5 μM, 5 μM, 10 μM, 20 μM, and 40 μM). Each analyte was flowed at a constant rate over the CM-5 chip.

Figure 6.11.1.4: Sensorgram output showing NSC19555 binding to hDNMT1 wt (FC2). Along the X-axis is binding over time over increasing varying enzyme concentrations (2.5 μM, 5 μM, 10 μM, 20 μM, and 40 μM). Each analyte was flowed at a constant rate over the CM-5 chip.
Figure 6.11.1.5: Sensorgram of human-tRNA<sub>Asp</sub> binding to hDNMT1wt (Methylation buffer 2)

Figure 6.11.1.5: Sensorgram output showing binding of human tRNA<sub>Asp</sub> to hDNMT1 wt. Along the X-axis is binding over time over increasing varying enzyme concentrations (3.75 nM, 7.5 nM, 15 nM, 30 nM, and 60 nM). Each analyte was flowed at a constant rate over the CM-5 chip.

Figure 6.11.1.6: Sensorgram of Giardia Gl-tRNA<sub>Asp</sub> binding to hDNMT1wt (Methylation buffer 2)

Figure 6.11.1.6: Sensorgram output showing binding of Giardia, Gl-tRNA<sub>Asp</sub> to hDNMT1 wt. Along the X-axis is binding over time over increasing varying enzyme concentrations (3.75 nM, 7.5 nM, 15 nM, 30 nM, and 60 nM). Each analyte was flowed at a constant rate over the CM-5 chip.
### 6.11.2 Tables

**Table 6.11.2.1:** Three Putative gDNMT candidate genes (Unpublished, Williams, C. et al.)

<table>
<thead>
<tr>
<th>Protein</th>
<th>Length / MW</th>
<th>GiardiaDB Identification</th>
<th>Family/Domains</th>
<th>Expression Profile (SAGE, microarray, MS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GL50803_9528</td>
<td>340 aa / 38.7 kDa</td>
<td>Methyltransferase like-2</td>
<td>SAM-dependent methyltransferase superfamily; methyltransferase 12 domain</td>
<td>SAGE: very low level Microarray: unchanged in stages MS: undetectable</td>
</tr>
<tr>
<td>GL50803_14327</td>
<td>304 aa / 33.8 kDa</td>
<td>Hypothetical protein</td>
<td>SAM-dependent methyltransferase superfamily; methyltransferase 8 domain</td>
<td>SAGE: low level Microarray: upregulated encystation MS: undetectable</td>
</tr>
<tr>
<td>GL50803_21512</td>
<td>291 aa / 32.4 kDa</td>
<td>SAM-dependent methyltransferase</td>
<td>SAM-dependent methyltransferase superfamily; methyltransferase 11 domain</td>
<td>SAGE: undetectable Microarray: upregulated encystation MS: undetectable</td>
</tr>
</tbody>
</table>

**Table 6.11.2.2** Summary of Binding Results from Round 2 SPR screens

<table>
<thead>
<tr>
<th>Protein</th>
<th>NSC 19555</th>
<th>NSC 21970</th>
<th>NSC 303530</th>
<th>NSC 401077</th>
<th>SAM</th>
</tr>
</thead>
<tbody>
<tr>
<td>hDNMT1wt</td>
<td>+</td>
<td>+/-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>hDNMT2 wt</td>
<td>+</td>
<td>+/-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>hDNMT2m</td>
<td>+</td>
<td>+/-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

The top row is the list of analytes flowed over the CM-5 chip. The first column indicates the list of ligands bound to the CM-5 chip. + Indicates presence of binding, - Indicates an absence of binding, and +/- is unable to determine based on sensorgram results.

**Table 6.11.2.3** Summary of K_D Values from Round 2 SPR screens

<table>
<thead>
<tr>
<th>Protein</th>
<th>NSC 19555</th>
<th>NSC 21970</th>
<th>NSC 303530</th>
<th>NSC 401077</th>
<th>SAM</th>
</tr>
</thead>
<tbody>
<tr>
<td>hDNMT1wt</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>270 μM</td>
<td>3.6 μM</td>
</tr>
<tr>
<td>hDNMT2 wt</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>14.7 μM</td>
<td>3.6 μM</td>
</tr>
<tr>
<td>hDNMT2m</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>27.9 μM</td>
<td>13.3 μM</td>
</tr>
</tbody>
</table>

The top row is the list of analytes flowed over the CM-5 chip. The first column indicates the list of ligands bound to the CM-5 chip. NA: K_D values could not be automatically calculated using BIACORE™ software due to poor data quality.
Table 6.11.2.4 Summary of $K_D$ Values from Round 3 SPR screens

### 4A Methylation Buffer 1

<table>
<thead>
<tr>
<th></th>
<th>Human-tRNA$_{\text{Asp}}$ C38U</th>
<th>Human-t-RNA$_{\text{Asp}}$ C38U</th>
<th>$Gl_t$RNA$_{\text{Asp}}$</th>
<th>$Gl_t$RNA$_{\text{Asp}}$ C38U</th>
</tr>
</thead>
<tbody>
<tr>
<td>hDNMT1 wt</td>
<td>52.1 nM</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>hDNMT2 wt</td>
<td>27.4 nM</td>
<td>41.9 nM</td>
<td>36.1 nM</td>
<td>78.8 nM</td>
</tr>
<tr>
<td>hDNMT2 C79A</td>
<td>38.3 nM</td>
<td>54.5 nM</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

The top row is the list of analytes flowed over the CM-5 chip. The first column indicates the list of ligands bound to the CM-5 chip.
Wt: wild type.
NA: $K_D$ values could not be automatically calculated using BIACORE™ software due to poor data quality.

### 4B Methylation Buffer 2

<table>
<thead>
<tr>
<th></th>
<th>Human-tRNA$_{\text{Asp}}$ C38U</th>
<th>Human-t-RNA$_{\text{Asp}}$ C38U</th>
<th>$Gl_t$RNA$_{\text{Asp}}$</th>
<th>$Gl_t$RNA$_{\text{Asp}}$ C38U</th>
</tr>
</thead>
<tbody>
<tr>
<td>hDNMT1 wt</td>
<td>31.9 nM</td>
<td>35.1 nM</td>
<td>29.0 nM</td>
<td>38.6 nM</td>
</tr>
<tr>
<td>hDNMT2 wt</td>
<td>45.2 nM</td>
<td>80.9 nM</td>
<td>20.4 nM</td>
<td>105.3 nM</td>
</tr>
<tr>
<td>hDNMT2 C79A</td>
<td>24.2 nM</td>
<td>70.3 nM</td>
<td>20.0 nM</td>
<td>26.9 nM</td>
</tr>
</tbody>
</table>

The top row is the list of analytes flowed over the CM-5 chip. The first column indicates the list of ligands bound to the CM-5 chip.
Wt: wild type.
Chapter 7: Concluding Thoughts

7.1: Part I

Diarrhea has long been recognized as one of the principal causes of morbidity (disease and illness) and mortality (death) among children under the age of 5 (children < 5) in developing countries. Worldwide, recent estimates have reported that diarrhea causes upwards of 1.6 to 2.1 million deaths annually in children < 5 (383). Maintaining a healthy intestinal tract is of utmost importance in infancy and early childhood (children < 5 years of age). Absorption of nutrients during this time is critical not only in ensuring optimal child growth, but also in developing the neuronal synapses necessary to ensure proper brain development and cognitive functioning (96). However, during the period from birth to weaning, children, especially in developing countries, may lack access to clean water and sanitation and thus, are at an increased risk of exposure and infection caused by enteric pathogens, such as *Giardia lamblia*.

Experimental evidence over recent years has demonstrated that the morbidity caused by *Giardia* is mediated by the pathophysiological changes infection causes in the host (See Chapter 1). In turn, these changes directly impair nutrient absorption resulting in small intestinal malabsorption. Further, recent evidence has suggested that small intestinal malabsorption is present even in asymptomatic enteric infections without overt diarrhea (reviewed in Guerrant et al., 2008) (97). Thus, persistent diarrhea and enteropathy, a term used to refer to disease of the small intestine, resulting from chronic and recurring *Giardia* infections that affect nutrient absorption have the potential to cause long lasting impacts on childhood growth and development. Furthermore, it has been shown in a mouse model of malnutrition, that malnourishment increases the severity of the impact of *Giardia* infection (52).
Collectively, these findings led us to formulate the hypothesis that there is a negative association of *Giardia* infection (exposure) with undernutrition (outcome). However, independent observational studies linking *Giardia* infection and undernutrition in the literature have been inconclusive to date and thus, cannot provide the evidence in support of this hypothesis (See Chapter 2). Thus, we set out to synthesize the evidence and provide a summary quantitative estimate based on all available literature using epidemiologic approaches that will clearly help quantify the burden of *Giardia* infection and undernutrition (See Chapter 2).

Subsequently, in Chapters 3 and 4, we show for the first time that *Giardia* infection, regardless of clinical disease, is significantly negatively associated with mild/moderate growth deficits (See Chapter 3) and is significantly negatively associated with decreased serum iron and zinc levels (See Chapter 4). While these findings do not prove a causal link establishing that *Giardia* causes undernutrition, they do help establish a clear association linking these two variables. Thus, these findings can have significant public health policy implications regarding the treatment of *Giardia* infections (See Chapter 5) which are explored in Part II.

### 7.2: Part II

Included in Chapter 1 is a description of the existing anti-*Giardia* treatments that are in current use for treating giardiasis. Important take away messages from this section are that the efficacy of these treatments is limited due to increasing reports of clinical resistance that can cause treatment failures and thus, high rates of repeated and recurring *Giardia* infections. Additionally, the widespread use of these treatments is not recommended, especially in asymptomatic infections, as the potential for harsh side effects and reports of limited tolerability in special populations (e.g. pregnant women) suggest the costs of these existing treatment options may outweigh the benefits.
However, our findings that even asymptomatic *Giardia* infections are negatively associated with different measures of undernutrition (growth and micronutrient deficiencies), suggest that the rational development and design of novel, effective anti-*Giardia* treatments should be an immediate research priority.

Thus in Part II, we use molecular based approaches to investigate novel treatment targets targeting DNA methylation for *Giardia* infection and giardiasis. Specifically, in Chapter 6, I describe the background research conducted in the Elmendorf Lab that established the presence of DNA methylation in *Giardia*, demonstrated a functional role for methylation in parasite survival, and identified putative DNA methyltransferase 2 (gDNMT2) enzymes in the parasite. This background establishes the rationale for targeting DNA methylation in *Giardia*. Next, in I report on our preliminary findings using Surface Plasmon Resonance (SPR) based technology as a way to determine the binding and binding affinities of novel small molecule compounds that could be further developed as inhibitors of putative gDNMT2 enzymes.

**7.3: Summary**

Together, the research findings presented in Parts I and II of this dissertation contributes a more complete understanding of the true potential impact and burden of *Giardia* infections beyond its association with diarrheal disease. Further, it provides supporting evidence that may be used to calculate the Disability Adjusted Life Years (DALYs), or a measure of the number of years lost to disability (morbidity) as a result of *Giardia* worldwide. Lastly, this research also contributes to our understanding of the molecular biology of the parasite, which in turn can guide future research efforts in rational drug development against this single celled eukaryotic parasite.
Appendices

Appendix A

Figures

Figure A1: Ligand-analyte interactions with sensor surface. The ligand may be immobilized directly as shown on the left or attached through binding to an immobilized capturing molecule (right). For more information see (1) (Figure from: http://www.gelifesciences.com/file_source/GELS/Service%20and%20Support/Downloads/Handbooks/pdfs/Biacore%20Assay%20Handbook.pdf)

Figure A2: Schematic visualization of SPR principles. For more information see (1) (Figure from: http://web.bf.uni-lj.si/bi/sprcenter/2-Senzorgram-completeENG.gif)
Appendix B

Table B1 below shows a summary of the known/predicted binding reactions between the enzymes and analytes that we plan to use to establish SPR as a viable assay for our experiments. Each of the enzymes is described in more detail on the next page. Because the enzymes include a wide array of different fusion (and non-fusion) proteins, we plan on covalently linking the proteins to the chips. (Unpublished, Elmendorf Lab, 2015)

Table 8.2.1. Binding Predictions. Bold font indicates enzymes/analytes currently available.

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>SAM</th>
<th>Cytidine</th>
<th>Deoxyctydine</th>
<th>5-aza-cytidine</th>
<th>5-aza-2-deoxyctydine</th>
<th>Adenosine</th>
<th>Deoxyadenosine</th>
<th>tRNA</th>
<th>tRNA-Asp</th>
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<th>NSC401077 (RG108)</th>
<th>NSC21970</th>
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Figure A3: Sample sensorgram output. For more information please see (1)

(Figure from: http://pubs.acs.org/subscribe/archive/md/v04/i02/figures/arbery-fig2.gif)
List of Ligands

MSssI:
- an E. coli CpG methyltransferase
- not a fusion protein
- purchased from NEB, Cat #M0226S; Sales Order # 50041

hDNMT1:
- the human DNMT1 - specificity for hemi-methylated DNA
- not a fusion protein
- purchased from NEB, Cat #M0230S

hDNMT2
- the human DNMT2 - specificity for tRNA-Asp with weak DNA activity
- fusion protein with GST-tag in baculovirus system
- purchased from BPS Bioscience, Cat #51102
- we are also in the process of purifying this as a Hi-tagged protein in E. coli

hDNMT2m
- a mutant human DNMT2 – C79A mutation; no binding or enzymatic activity
- fusion protein with His-tag in E coli
- generated in-house

Ehmeth
- the Entamoeba histolytica DNMT2 – weak activity for both tRNA-Asp and DNA
- fusion protein with GST-tag in E coli
- we are in the process of purifying this

Gl21512
- one of the candidate Giardia lamblia DNMT2
- fusion protein with MBP-tag in E coli
- purified in-house

Gl9528
- one of the candidate Giardia lamblia DNMT2
- fusion protein with MBP-tag in E coli
- purified in-house

MBP
- the MBP fusion control
- purified in-house
GST
- the GST fusion control
- we are in the process of purifying this

E. coli lysate
- purified in-house

List of analytes
SAM – Sigma Cat# A7007-5mg; lot SLBL1067V

Cytidine – ChemGenes Cat# RP-1184

Deoxycytidine – ChemGenes Cat# DN-1002

5-aza-cytidine – Sigma Cat# A2385-100mg; batch MKBR7212V

5-aza-2deoxycytidine – Sigma Cat# A3656-5mg; batch MKBR6437V

Adenosine – ChemGenes Cat# RP-1183

Deoxyadenosine – ChemGenes Cat# DN-1001

tRNAs – Sigma Cat# R8759-500UN; batch SLBH8277V

tRNA-Asp – producing in-house

NSC303530 – source: NCI DTP; 01/2015

NSC401077 (RG108) – source: NCI DTP; 01/2015

NSC21970 – source: NCI DTP; 01/2015

NSC19555 – source: NCI DTP; 01/2015
Bibliography

16. Yu LZB, C.W. Jr.; Adam, R.D. The two nuclei of *Giardia* each have complete copies of the genome and are partitioned equationally at cytokinesis. Eukaryot Cell. 2002;1:191-9.
49. Solaymani-Mohammadi SS, S.M. Host immunity and pathogen strain contribute to intestinal dissacharidase impairment following gut infection. J of Immunology. 2011;187:3769-75.
65. Matowicka-Karna JK, M.; Kemona, H. Assessment of the levels of nitric oxide (NO) and cytokines (IL-5, IL-6, IL-13, TNF, IFN-gamma) in giardiosis. Folia Histochemica et Cytobiologica. 2011;49(2):280-4.


Heyman MB, G.; Sarrut, S.; Giraud, M.; Evans, L.; Touhami, M; Desjeux, J.F.


Prado MS, Cairncross S, Strina A, Barreto ML, Oliveira-Assis AM, Rego S.


145. Egger MDS, G.; Schneider, M.; Minder, C. Bias in meta-analysis detected by a simple, graphical test. BMJ. 1997;315(7109):629-34.

166


152. Duran CH, Glida; Aguilera, William; Rodriguez-Morales, Alfonso J.; Albano, Carlos; Cortez, Jackeline; Jimenez, Sara; Diaz, Marietta; Incani, Renzo Nino. Giardia lamblia infection is associated with lower body mass index values. The Journal of Infection in Developing Countries. 2010;4(6):417-8.


161. LaBeaud DAM, Maxim; Mungai, Peter; McKibben, Elisabeth; Gildengorin, Ginny; Sutherland, Laura J.; King, Christopher L.; Malhotra, Indu, editor Parasitism in Children Aged Three Years and Under: Effects on Growth and Vaccine Response in Rural Coastal Kenya. 60th Annual Meeting American Society of Tropical Medicine and Hygiene; 2011; Philadelphia, PA.


164. MCCORMICK BJ. FREQUENT SYMPTOMATIC OR ASYMPTOMATIC INFECTIONS MAY HAVE LONG-TERM CONSEQUENCES ON GROWTH AND COGNITIVE DEVELOPMENT.


171. Platts-Mills JM, Benjamin J.; McGrath, Monica; Miller, Mark; Gratz, Jean; Kosek, Margaret; Havit, Alexandre; Amidou, Samie; Zaidi, Anita; Kang, Gagandeep; Haque, Rashidul; Bodhidatta, Ladaporn; Houpt, Eric; on behalf of the MAL-ED Network, editor Association between enteropathogens, diarrhea, and growth in the MAL-ED Cohort: American Society of Tropical Medicine and Hygiene.


169


214. Sivarathinaswamy PK, Deepthi; Sarkar, Rajiv; Ajampur, Sitara S.; Muliyli, Jayaprakash; Ward, Honorine; Kang, Gagandeep, editor Burden and Factors Associated with Giardia infection in infants of South India. 60th Annual Meeting of the American Society for Tropical Medicine and Hygiene; 2011; Philadelphia, PA, USA.
215. Ignatius RG, J.B.; Steininger, C; Shyirambere, C.; Musemakweri, A.; Harms, G.; Mockenhaupt, F.P., editor High Frequency of asymptomatic Giardia intestinalis infections in Rwandan children and association with malnutrition. 7th European Congress on Tropical Medicine and International Health; 2011; Barcelona: Blackwell Publishing Ltd.


306. ARBABI M, ESMAILI N, PARASTOEE K. SERUM LEVELS OF ZINC, COPPER, MAGNESIUM ELEMENTS AND VITAMIN B12 IN CHILDREN WITH GIARDIASIS AND ENTEROBIOSES IN KASHAN, IRAN. 2014.


