GLOBAL SPHINGOLIPID PROFILE OF *GIARDIA LAMBLIA* DURING STAGE DIFFERENTIATION: THE INFLUENCE OF SPHINGOMYELIN ABUNDANCE ON CYST VIABILITY

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Dedication

To Minerva and Aurelio.

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by

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Abstract

Giardia lamblia is a protozoan parasite and a major cause of the waterborne-illness (known as giardiasis) worldwide. Giardiasis is endemic in developing countries and is a leading cause of non-viral- and non-bacterial-associated intestinal disorders. Acute symptoms of giardiasis include diarrhea, cramps, and malabsorption. The disease is often self-limiting, although the infection can result in long-term disorders such as chronic fatigue, stunted cognitive skills, and failure to thrive even after the parasite has been cleared.

This parasite exists in two morphologic forms—infective trophozoites and transmissible, water-resistant cyst, which is passed by the fecal-oral route and is likely to be spread by contaminated drinking water. Infection can occur upon ingestion of as few as 10 cysts, and an infected individual sheds more than 10^9 cysts in the feces per day. Although giardiasis can effectively be treated with nitroheterocyclic compounds, such drugs can cause unpleasant side effects and ultimately lead to drug-resistant parasites. Therefore, it is necessary to identify novel targets for developing new anti-giardial agents. One of these targets could be sphingolipid (SL) pathways because the results from our laboratory indicate that SL genes and gene products play critical roles in regulating encystation (cyst formation) of *Giardia.* We have shown previously that SL metabolism in *Giardia* is limited to five putative enzymes, which are differentially regulated during the formation of water-resistant cysts. We have also shown that the silencing of the giardial glucosylceramide synthase gene (gGlcT1) using anti-gGlcT1 morpholino oligonucleotide results in inhibition of growth and the production of viable cysts.

The goal of my dissertation is to establish the sphingolipidome of *Giardia* during encystation to provide a global perspective of the SL changes that take place during this critical stage of the parasite's life cycle and to show how *Giardia* can modulate these lipids. In Specific Aim 1, I used mass spectrometry to elucidate the sphingolipid profile of *Giardia* during encystation as well as culture media sources of SLs. In Specific Aim 2, I investigated the

parasite's ability to generate ceramide from the hydrolysis of sphingomyelin (SM) via two putative sphingomyelinase (SMase) enzymes identified by mining the *Giardia* genome project.

Briefly, I found that the levels of ceramide and glycosylceramides increased significantly (~10 fold) during encystation followed by a decline in cysts. The level of sphingomyelin (SM), on the other hand, increased sharply in the cyst population. Analysis indicated that SM-enriched cysts are viable and undergo excystation in culture. It was also observed that *Giardia* expresses two active acid SMase enzymes (gASMLPD3b1 and gASMLPD3b2) with distinct pH requirements, one of which shows the characteristics of a secreted SMase. My results indicate that a dynamic metabolic conversion among various classes of SLs occurs during giardial encystation, and that this conversion could be critical for completing excystation and producing infection in the human gut.

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GalNAc, β(1-3)-N-acetyl-d-galactosamine
CWP1, cyst wall protein 1
CWP2, cyst wall protein 2
CWP3, cyst wall protein 3
ESV, encystation specific vesicle
h.p.i. hours post induction
PV, peripheral vacuole
TVN, tubular vesicle network
ER, endoplasmic reticulum
DNA, deoxyribonucleic acid
NO, nitric oxide
VSP, variable surface protein
SREBP, sterol regulatory element binding protein
HCNCp, high-cysteine non-variant cyst protein
ATP, Adenosine tri-phosphate
TLC, thin layer chromatography
qTOF, quadrupole time of flight
Lyso-PC, lyso-phosphatidylcholine
PC, phosphatidylcholine
PG, phosphatidylglycerol
PE, phosphatidylethanolamine
CDP-DAG, cytidine diphosphate-diacylglycerol
SM, sphingomyelin
Cer, Ceramide
PalmA, palmitic acid
SL, sphingolipid
gSPT1, giardial serine palmitoyltransferase 1
gSPT2, giardial serine palmitoyltransferase 2
gGlcT1, giardial glucosylceramide transferase 1
GCS, glucosylceramide synthase
gASMLPD3b1, giardial acid sphingomyelinase-like phosphodiesterase 3b 1
gASMLPD3b2, giardial acid sphingomyelinase-like phosphodiesterase 3b 2
SMPDL-3b, sphingomyelinase phosphodiesterase like 3b
SAGE, serial analysis of gene expression
qRT-PCR, quantitative reverse transcription polymerase chain reaction
PPMP, D-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propano
GSLs, glycosphingolipids
3KSR, 3-ketosphinganine reductase
Lass, longevity assurance gene (dihydroceramide synthase)
CerS, ceramide synthase
CerK, ceramide kinase
SMase, sphingomyelinase

DAG, diacylglycerol
NBD, N-[7-(4-nitrobenzo-2-oxa-1,3-diazole)]
Bodipy, 4,4-difluoro-4-bora-3a,4a-diaza-s-indacene
PBS, phosphate buffered saline
PTFE, polytetrafluoroethylene
DEAE-CL, diethylaminoethyl-chloride
NaOAc, Sodium Acetate
DCE, dichloroethane
LTQ, linear triple quadrupole
TIM, total ion map
HPLC, high pressure liquid chromatography
SIM, select ion monitoring
LC MS/MS, tandem liquid chromatography mass spectrometry
SE, standard error
ESI, electrospray ionization
GM3, mono-sialo dihexosylceramide
GD3, di-sialo dihexosylceramide
GM1, mono-sialo HexNAc trihexosylceramide
LCB, long chain base
NL, neutral loss
BP, base peak
FSGS, focal segmental glomerulosclerosis
ASMase, acid sphingomyelinase
BSA, bovine serum albumin
IVT, *in vitro* translated
GFP, green fluorescent protein
HPTLC, high performance thin layer chromatography
PVDF, polyvinylidene fluoride
SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis
HRP, horse radish peroxidase
ECL, enhanced chemi-luminescence
PFA, paraformaldehyde
DAPI, 4', 6-diamidino-2-phenylindole
FDA, fluorescein diacetate
PI, propidium iodide
MS, mass spectrometry

Introduction

People, especially children, can die from diarrheal diseases. According to the World Health Organization (WHO), diarrhea is the second leading cause of death among children under five, killing an estimated 760,000 children every year (WHO Bulletin 2013). Diarrheal disease is caused by bacteria, viruses, and intestinal parasites, including *Giardia lamblia* (hereinafter *Giardia*), a causative agent of waterborne illness or "giardiasis." Giardiasis is classified under the "Neglected Tropical Diseases" category and is generally self-limiting, but recurrence is common in endemic areas (Watkins and Eckmann 2014). There is no vaccine, and treatment with common anti-parasitic agents is confounded by adverse side effects and decreasing drug susceptibility. Furthermore, intestinal micro flora plays an important role in determining the severity of giardial infection. For example recent evidence suggests that reduction of helminth infection may be associated with the increase of *Giardia* infection (Blackwell *et al.* 2013). Helminthes modulate host lipid levels in order to carry out their own cellular processes (reviewed by Bansal *et al.,* 2005). Similarly, *Giardia* uses the host's lipids for its growth and differentiation because it is unable to synthesize its own lipids *de novo* (Das *et al.* 2001; Yichoy *et al.* 2010). Thus, it is possible that the antagonism observed between these organisms involves competition for intestinal lipids, which they both colonize. Investigation into additional lipids essential for growth and/or differentiation of *Giardia* may provide insight into alternative strategies for combating this disease.

Giardia trophozoites colonize the intestine of mammalian hosts. This is a low-oxygen, nutrient-rich environment, in which the organism must contend not only with the host's immune response but also with the high levels of digestive enzymes constitutively secreted by the intestinal cells. The advantage, however, is that the parasite is able to scavenge these nutrients and thus accumulate metabolic energy that is required to synthesize organelles and macromolecules. A basal eukaryote *Giardia* lack both mitochondria and defined Golgi stack (Regoes *et al.* 2005; Reiner *et al.* 1990) although it is not clear if this is due to secondary loss as

a result of parasitism. What is clear is that the absence of these organelles, or their presence in rudimentary form, does not decrease fitness within the parasite. The same cannot be said for the host, where no benefit is gained from colonization by *Giardia*. In fact, acute giardiasis considerably reduces host fitness, and even after the infection has been cleared the evidence suggests that there are a number of long-term effects of infection, including irritable-bowel syndrome, chronic fatigue, and failure to thrive (Halliez and Buret 2013).

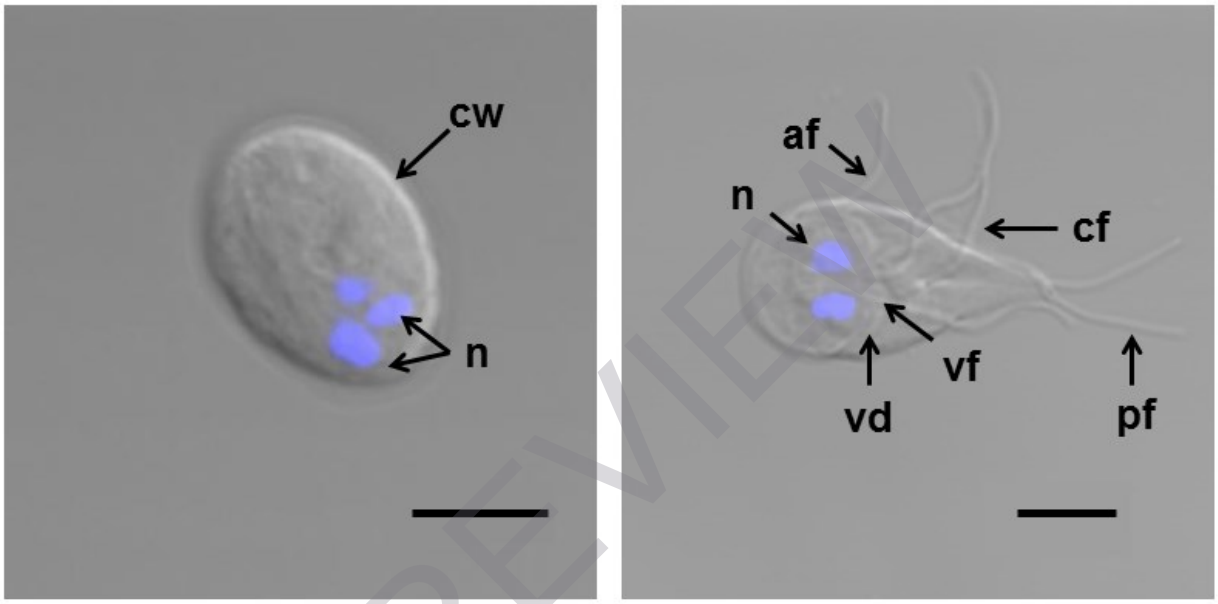

Figure 1.1: *Giardia* morphology

The giardial cyst (infectious stage) and trophozoite (vegetative stage). The active trophozoite stage contains two nuclei (n) and a ventral disc (vd) and ventral flagella (vf) for attachment. For movement the parasite relies on anterior (af), caudal (cf) and posterior flagella (pf). The dormant cyst contains four nuclei (n) enclosed within a cyst wall (cw).

Trophozoites in the intestine swim erratically by beating their flagella and attach to microvilli of intestinal epithelia using a suction-cup-like adhesive disc (House *et al.* 2011). While attached, the flagella continue to beat, and it has been suggested that this is the cause of the major symptoms of giardiasis: diarrhea and malabsorption (Ankarklev *et al.* 2010). It has

also been reported that *Giardia* secretes proteins *in vitro* when co-cultured with human epithelial cells, which can result in decreased membrane function and increased permeability (Ringqvist *et al.* 2008). However, no *Giardia* toxins capable of increasing membrane permeability have yet been identified.

For a pathogen to be successful, it must infect as many hosts as possible, and *Giardia* is no exception. However, sensitivity to oxygen and residence in the intestine pose an obstacle for the spread of this non-invasive parasite. To circumvent this, *Giardia* has a biphasic life cycle—the trophozoite is able to form a protective wall made of protein and polysaccharides by encystation. Although it is not known what triggers encystation, the induction of cyst formation *in vitro* described by Boucher and Gillin (1990) has allowed many groups to study this process using varied and sophisticated techniques. The formation of the cyst wall and the subsequent transformation to mature cyst takes place in the lower intestine (Adam 2001). The wall is composed of at least three secreted proteins (CWP1-3), as well as β(1-3)-N-acetyl-d-galactosamine (GalNAc) polymer (Gerwig *et al.* 2002; Gottig *et al.* 2006; Chatterjee *et al.* 2010). This appears to be a highly regulated process, in which the deposition of cyst-wall proteins (CWP1-3) and other materials occurs through concerted trafficking of these proteins in encystation-specific vesicles (ESV) during differentiation (Faso and Hehl 2011). Interestingly, polysaccharides are synthesized on the external surface of plasma membranes of trophozoites, but it is unclear when or how they are incorporated into the cyst wall. Usually, the deposition of the cyst wall is completed by 24-hour post-induction (h.p.i.) of *in vitro* encystation, and it coincides with dramatic morphologic changes as the flagellated trophozoite becomes an aflagellate, oval-shaped cyst, which is water resistant and capable of surviving in the oxidizing environment outside the host.

The cyst is passed by the fecal oral route, and therefore transmission is most often associated with infected water supplies as a result of poor or no treatment practices, poor separation of potable water and waste water lines, or communal bathing (Kramer *et al.* 1996; Karanis *et al.* 2007). As a source of infection and a cause of disease, *Giardia* is an important

organism to study in the hope that giardiasis can be prevented through medical intervention. A vaccine against *Giardia* is confounded by the expression of variable-surface proteins (VSPs), which allow *Giardia* to escape host adaptive immune responses (Prucca and Lujan 2009), but *Giardia* is a parasite and as such maintains only the metabolic pathways that are essential for growth. Thus, an integral part of combating the parasite is an understanding of the pathways that are maintained and those that have been forsaken. This provides a two-fold benefit to the study of this protozoan. First, *Giardia* is an evolutionarily conserved organism, the biology of which provides valuable insight into that of more derived organisms. Second, those pathways and processes maintained by *Giardia* are likely to yield targets for fighting the infection.

1.1 The Simple Life o*f Giardia lamblia*

The eukaryote parasite *Giardia intestinalis* (synonymous with *G. lamblia* and *G. duodenalis*) is a member of the diplomonada group, which also includes free-living organisms. The phylogenetic organization of *Giardia* is not clear, and it is not known whether the protist represents an evolutionarily basal organism that diverged before the mitochondrial fusion of higher eukaryotes or a more conserved organism with secondary loss of some organelles and metabolic pathways due to the parasitic life-style (Hilario and Gogarten 1998; Stechmann and Cavalier-Smith 2002). What is clear is that *Giardia* are minimal in a number of ways, including the lack of common eukaryote organelles, the maintenance and processing of genetic material, and the synthesis of proteins for both structure and metabolism.

Giardia does not possess mitochondria, and therefore it has traditionally been characterized as among the earliest branching eukaryotes. This has been called into question since the discovery of the mitochondria-like organelle, the mitosome (Regoes *et al.* 2005). Interestingly this organelle is not known to perform any of the functions traditionally associated with the mitochondria. No Golgi stack has been identified in *Giardia*. The closest to be found is a transient Golgi network, which appears during encystation and which has been implicated in the secretion of ESVs (Reiner *et al.* 1990; Stefanic *et al.* 2006). Rather than lysosomes, a series