Eluication Of Lipid Metabolic Pathways In Differentiating Giardia Lamblia Using High Resolution Mass Spectrometry

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ELUICATION OF LIPID METABOLIC PATHWAYS IN DIFFERENTIATING GIARDIA LAMBLIA USING HIGH RESOLUTION MASS SPECTROMETRY

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Dedication

Dedication of this dissertation is to my wife Lillian, parents Wayne and Debbie, brothers Colby and Corbin, grandparents, aunts and uncles, cousins, and canine companion Oliver.

Additionally, to my mentors Siddhartha Das, Ph.D. and Igor Almeida, D.Sc. For whom without their support, and constant push to strive for excellence, I would not be here today.

Thank you.
ELUICATION OF LIPID METABOLIC PATHWAYS IN DIFFERENTIATING *GIARDIA LAMBLIA* USING HIGH RESOLUTION MASS SPECTROMETRY

by

CAMERON CONNER ELLIS, B.Sc.

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Abstract

*Giardia lamblia* is an intestinal protozoan found worldwide, including the U.S. This parasite exists in two morphologic stages—a replicative trophozoite and a relatively dormant yet viable cyst. While exposures of cysts to gastric acid during passage through the human stomach induces excystation, factors in the small intestine, where trophozoites colonize trigger encystation or cyst formation. Transformation into cyst stage is essential for *Giardia* to survive in the environment for months before infecting new hosts. Because of its small genome size (11.7 Mb), metabolic pathways in *Giardia* are highly reduced. As far as lipid metabolism is concerned, only limited number of lipid genes are present in this parasite. Therefore, to compensate, *Giardia* scavenges lipids from its environment, remolds and use them to assembles membranes and vesicles. Earlier, using ESI-LTQ-MS, we have demonstrated that phosphatidylglycerols (PGs), phosphatidylcholines (PCs) and phosphatidylethanolamines (PEs) are major glycerol-based phospholipids in *Giardia* and abundant in non-encysting and encysting trophozoites, as well in cysts. The GC/MS analysis identified palmitate (16:0), palmitoleate (16:1), oleate (18:1), and linoleate (18:2) as major fatty acids in *Giardia*. In the current study, we employed high-resolution liquid chromatography tandem mass spectrometry (LC-MS/MS) for quantitative lipidome and proteome analysis to further elucidate the lipid metabolic pathways that is operative in *Giardia*. Vegetative and encysting trophozoites as well as osmotically resistant cysts were cultured in the laboratory, collected, and subjected to lipid extraction or tryptic digestion. Lipidomic analysis revealed the dynamic changes of overall lipid and fatty acid (mostly C16, C18 moieties) profiles throughout the life stages of *Giardia*. Lipid classes that were found in cysts included lyso-phosphatidylcholine (LPC, P- and O-), lyso-phosphatidylethanolamine (LPE), diacylglycerol (DG), triacylglycerol (TG), cholesterol ester (CE), PC, P-PC (Plasmalogen), and ceramides.
Sphingomyelin (SM), PG, bis (monoacylglycerol) phosphate (BMP) were present mostly in encysting trophozoites rather than cysts. Ether linked PC species revealed to be most abundant in trophozoites. Proteomic analysis identified increased levels of enzymes from lipid elongation pathway—i.e., acyl-CoA ligases, long-chain fatty acid elongase (LCFAELO), oxidoreductase and enoyl-CoA reductase in cysts. To further explore how fatty acid elongase influences the lipid metabolism, giardial fatty acid elongase (gFAELO) was overexpressed, followed by lipidomic analysis. It was observed that the overexpression of gFAELO induced the generation of long chain fatty acids and upregulated Cer, PE, LPC, LPE, and LPA in cysts. These studies suggest that although limited, Giardia maintains an active and robust lipid metabolism in all stages including the water-resistant non-motile cysts. It is possible that active lipid metabolism in cysts is required for the successful excystation to produce infective membranous trophozoites.

Abbreviations: BMP, bis-monoacyl-glycerophosphate; CE, cholesterol ester, DG, diacylglycerol; FA, fatty acid, HBMP, hemi bis(monoacylglycerol)phosphate; LCFA, long-chain fatty acid; LPC, lyso-phosphatidylcholine; LPE, lyso-phosphatidylethanolamine; MUFA, mono-unsaturated fatty acids; PC, Phosphatidylcholine, PE, phosphatidylethanolamine: PG, phosphatidylglycerol; SM, sphingomyelin; VLCFA, very long chain fatty acid.
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Chapter 1: Introduction

1.1 Giardia and Giardiasis

Giardiasis, caused by an intestinal protozoan, *Giardia lamblia*, is a major public health problem worldwide (Einarsson et al., 2016; Levine et al., 2013). *Giardia* infection is spread through the fecal-oral route via contaminated water and food. It is estimated that over 1 billion people are infected with *Giardia* worldwide and of these ~200 million people display some symptoms (Torgerson et al., 2015). Giardiasis is also prevalent in western countries, where both adults and children are infected with asymptomatic syndrome. In contrast, ~30%–40% of people (especially children) in developing nations are infected with *Giardia*, and the infection could be both symptomatic and asymptomatic (Dunn et al., 2020). *Giardia* is a non-invasive parasite and maintains two-staged life cycle—i.e., trophozoites and cysts. Exposure of cysts to gastric acid during passage through the stomach triggers excystation that releases two vibrant/motile trophozoites from cysts. Newly excysted trophozoites (also known as “excyzoite”) colonize and attach on the luminal surface of the small intestine. The attachment of *Giardia* on intestinal epithelial cells is critical for binary fission (replication), causing diarrhea-like symptoms followed by encystation and cyst formation. The formation of water-resistant cysts allows *Giardia* to survive in the environment for months before transmitting to a new host (Einarsson et al., 2016; Barash et al., 2017). As no vaccines are currently available to treat giardiasis, chemotherapeutic agents are the only options to control the infection (Argüello-García et al., 2020). Metronidazole (Mtz) and its derivatives (tinidazole, nitazoxanide, albendazole, and mebendazole) are a commonly prescribed treatments for giardiasis (Lopez-Velez et al., 2010; Nabarro et al., 2015). All of these drugs, however, may cause side effects (Riches et al., 2020). For example, Mtz is a genotoxic agent and a potential carcinogen, and its frequent use gives rise to drug-resistant
giardiasis (Nabarro et al., 2015; Bendesky et al., 2002). Other drugs from the nitrothiazole and benzimidazole groups are also used, but like Mtz, they can produce toxicity and may interfere with pregnancy (Riches et al., 2020). Among the non-metronidazole classes of compounds, several repurposed drugs such as auranofin, fumagillin, orlistat, omeprazole, disulfiram, and NBDHEX have showed promising activity against the Giardia parasite in culture (Riches et al., 2020). Our laboratory has demonstrated that the synthetic analogues of phosphonoxins (Staake et al., 2010) and triazoxins (Pagola et al., 2020) are effective against Giardia trophozoites in low micromolar concentrations. All these compounds (as previously described) either have multiple targets or their mode of action has yet to be identified.

1.2 Lipid Metabolism and Remodeling by Giardia

The focus of my research is to elucidate the lipid metabolic pathways in Giardia by lipidomic and proteomic approaches. Because Giardia has limited lipid synthesis ability and generate new lipids possibly by remodeling reactions (Yichoy et al., 2010; Duarte et al., 2019), I hypothesize that these limited and disjoined lipid pathways could be targeted for drug development. In fact, there are several instances, where lipids, fatty acids and their transport proteins were considered as drug targets. For example, phosphatidylinositol transfer proteins can serve as potential targets in combating fungal infections (Khan et al., 2021). It was proposed that phospholipid and fatty acid synthesis in malaria parasite could be exploited to develop anti-malarial agents (Ben Mamoun et al., 2010). Bioactive lipids such as prostaglandins, leukotriens and hydroxy eicosatetraenoic acids are known targets for developing drugs against cancer, inflammatory diseases, and fibrosis (Evans and Hutchinson, 2010). Likewise, the small molecule inhibitors were designed to target sphingolipid (SL) metabolism in Cryptococcus (Mor et al., 2015).
Because of its small genomic size (Morrison et al., 2007), the lipid pathways in *Giardia* are greatly reduced. The parasite colonizes in the duodenum below the bile duct, where it is exposed to dietary lipids and bile salts that serve as major source of lipids for *Giardia* (Adam, 2001). Bile salts are likely to be involved in carrier-mediated uptake of lipids (Das et al., 1997). Transport of lipids are also dependent on their hydrophobic and hydrophilic properties. For instance, ceramide is internalized by clathrin-coated vesicles (Hernandez et al., 2007), and phospholipids are likely to be taken up by phospholipid-transporting ATPases or flipases (Yichoy et al., 2010). Once internalized, the lipid remodeling can take place either by acyl or head-group exchange reactions (Das et al., 2001; 2002). For example, free and conjugated fatty acids e.g., oleate, myristate, palmitate, and arachidonate can be taken up by *Giardia* directly from the culture medium and incorporated into membrane lipids to produce remodeled PLs (Gibson et al., 1999). Exchange of bases or head-groups of PLs likely to occurs through the activities of various enzymes. For instance, when live *Giardia* is labeled with [³H]-myo-inositol it is incorporated into phosphatidylinositol (PI) with the help of inositol-PI headgroup-exchange enzymes. The newly generated PI then incorporated into downstream glycolipids (Subramanian et al., 2000). Likewise, *Giardia* expresses the genes for other headgroup exchange enzymes such as phosphatiylserine decarboxylase (*psd*) and phosphatidylglycerol phosphate synthase (*pgps*), indicating headgroup remodeling reactions are also carried out by this parasite (Yichoy et al., 2009, 2010).

Because lipids and fatty acids are critical for growth and encystation of *Giardia* (Mendez et al., 2015); we evaluated the stage-specific changes of lipid profiles in this parasite and compared that with the culture medium. Electrospray Ionization-Quadrupole Time-of-Flight Mass Spectrometry (ESI-QTOF-MS) and Gas Chromatography-Mass Spectrometry (GC-MS) were employed to analyze phospholipids (PLs) and fatty acids, respectively. Major phospholipids identified in
*Giardia* were PC (25 species), SM (3 species), PG (17 species), PE (6 species) and PI (2 species). It was noticed that the relative abundance of these lipids remained unchanged in trophozoites, encysting cells and cysts. (*Yichoy et al., 2009*). The analysis of culture medium revealed the presence of only lyso-PC (3 species), of PCs (5 species) and a single species of diacylglycerol (DAG), further supporting the idea that *Giardia* generates new lipids (i.e., PG, PE, and PI) acquired from the host by head group and fatty acid remodeling reactions (*Yichoy et al., 2009*). Fatty acids (C16:0, C18:0, and C18:1) were identified as major species in *Giardia*. Traces of other fatty acids including C10:0, C12:0, C14:0, C15:0, C17:0, C18:3, C19:0, C20:0, C22:0, C24:0, C26:0 and C28:0 were also detected. Like PLs, fatty-acid composition remained unaltered in trophozoites, encysting cells and cysts. In serum and bile, the major fatty acids identified were C16:0, C18:0, C18:1, and C18: 2. Therefore, it is likely that few long chain fatty acids (i.e., C24:0, C24:1, C23:0, etc.), which are present in *Giardia*, could be synthesized de novo by the action of elongase enzyme (*Yichoy et al., 2009*). The sphingolipidomic analysis (Duarte et al., 2019) indicate that more than 100 species of SLs are present in *Giardia* and many of them are obtained from the growth medium. These include sphingoid bases, ceramide 1-phosphate (Cer-1-P), N-acetyl hexosamine trihexosylceramide (HexNAc-Hex3Cer), trihexosylceramide (Hex3Cer), dihexosylceramide (Hex2Cer), monohexosylceramide (HexCer), ceramide (Cer), sphingomyelin (SM), mono-sialodihexosylceramide (SA-Hex2Cer) and di-sialodihexosylceramide (SA2-Hex2Cer). Many of these lipids are also present in the culture medium and scavenged by the parasite before releasing back to the medium. Results indicate that the release SLs are remodeled at the fatty acyl moieties (Duarte et al., 2019). This supports the idea that a bidirectional active SL transport pathway is present and utilized by this parasite for maintaining growth and inducing encystation (Duarte et al., 2019).
1.3 Stories Told by the Genome Database

According to the genome database (GiardiaDB.org) approximately thirty-four lipid/fatty acid metabolic genes are present in *Giardia*. These include genes for PL synthesis, long chain fatty acid elongase, sterol synthesis, phospholipases, SL metabolism and several genes for lipid signaling pathways as shown in *Table-1*. Thus, based on the genome database as well as the biochemical and lipidomic analysis, a comprehensive lipid metabolic pathway in *Giardia* has been proposed and elucidated in *Figure 1*.

As depicted in *Figure 1*, a vibrant and metabolically active trophozoite expresses putative phospholipid transfer ATPase/flippase (gPLT/gFLIP) that allows the parasite to import PC and lyso-PC from the growth medium. *Giardia* has the genes that encode secreted and intracellular phospholipase B (PLB) enzymes hydrolyzes *Sn*1 and *Sn*2 FAs from PC to produce lyso-PC and soluble glycerophosphorylcholine (*Table-1*)

*Table 1. Lipid Metabolic Genes in Giardia lamblia.*

<table>
<thead>
<tr>
<th>Gene Annotation</th>
<th>NCBI Accession Number</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Phospholipid</strong></td>
<td></td>
</tr>
<tr>
<td>PI Synthase/ CDP-DAG-inositol 3-phosphatidyltransferase</td>
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</tr>
<tr>
<td>PI transfer protein alpha isoform</td>
<td>XP_001705528</td>
</tr>
<tr>
<td>PGP synthase/ CDP-DAG-glycerol-3-phosphate-3-phosphatidyltransferase</td>
<td>XP_769290</td>
</tr>
<tr>
<td>PS decarboxylase</td>
<td>XP_779868</td>
</tr>
<tr>
<td>PS synthase</td>
<td>XP_001707737</td>
</tr>
<tr>
<td><strong>Fatty Acid</strong></td>
<td><strong>Phospholipid-transporting ATPase IA, putative</strong></td>
</tr>
<tr>
<td>---------------</td>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td><strong>Phospholipid-transporting ATPase IIB, putative</strong></td>
<td>XP_001704293, XP_001707954</td>
</tr>
<tr>
<td><strong>Fatty Acid</strong></td>
<td>1-acyl-sn-glycerol-3-phosphate acyl transferase (SLC) 4</td>
</tr>
<tr>
<td><strong>Acyltransferase (SLC 3)</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Lysophosphatidylcholine acyltransferase/Lyso-PAF acyltransferase (SLC 2)</strong></td>
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<td><strong>Lysophosphatidic acid acyltransferase, putative</strong></td>
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</tr>
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<td><strong>Fatty acid elongase 1</strong></td>
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<td><strong>Long chain fatty acid CoA ligase 5</strong></td>
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<td><strong>Long chain fatty acid CoA ligase, putative</strong></td>
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<tr>
<td>Sphingolipid</td>
<td>Glycosyltransferase-1</td>
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<td>-----------------------</td>
</tr>
<tr>
<td>Sphingomyelinase B</td>
<td></td>
</tr>
<tr>
<td>Sphingomyelinase 3b</td>
<td></td>
</tr>
<tr>
<td>Serine palmitoyltransferase-1</td>
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</tr>
<tr>
<td>Serine palmitoyltransferase-2</td>
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</tr>
<tr>
<td><strong>Signaling</strong></td>
<td>Target of rapamycin (TOR)</td>
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<tr>
<td>Phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase</td>
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<tr>
<td>Type II inositol-1,4,5-trisphosphate 5-phosphatase precursor</td>
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<td>Inositol 5-phosphatase 4</td>
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<td>Phosphatase</td>
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<td>PI-3-kinase, catalytic, alpha polypeptide</td>
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<tr>
<td>Phosphatidylinositol-4-phosphate 5-kinase, putative</td>
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<td>PI-4-kinase</td>
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</tbody>
</table>
Both PC and lyso-PC are taken up by gPLT/gFLIP-mediated facilitated diffusion and converted to various downstream lipid molecules (indicated by red fonts). For example, PC can be converted to PE by adding methyl group; however, no methyltransferase (MTase) gene was annotated in the database. Table I also indicates thatgpsd and gpss genes are present in *Giardia* is capable of synthesizing PS from PE and PE from PS by base-exchange reactions. Phosphatidylglycerol (PG) is synthesized de novo as proposed earlier (*Yichoy et al.*, 2009) and PC may serve as a major precursor. The gene (*gpgps*) encoding PGPs was identified and shown to express in non-encysting and encysting cells (*Yichoy et al.*, 2009). It is not unlikely that a novel PC-to-PG remodeling enzyme may be present and used by the parasite to produce PG from PC directly. Nevertheless, such a kind of enzyme has yet to be identified in eukaryotic cells. Cellular lyso-PC can be hydrolyzed to soluble glycerophosphorylcholine with the help of giardial lysophospholipase (LPL) enzyme encoded by *glpl* gene. Conversely, lyso-PC can be converted to PC by the action of lysophosphopholipase acyltransferase (LAAT, encoded by *glaat*) that helps *Giardia* to upsurge its PC pool (*Figure 1*). Regarding FA (green) and neutral lipids (black) metabolism, our model proposes that most FAs originate from the environment and likely to be taken up by facilitated diffusion (*Gibson et al.*, 1999). Once internalized, FAs undergo elongation and desaturation processes. The presence of giardial desaturase enzyme was reported earlier by Ellis et al. (1996), and the gene (*gelo*) that is likely to encode elongase was annotated in the database (*Table 1*) and characterized by us (unpublished). Diacylglycerol (DAG) or other neutral
lipids present in the medium (Yichoy et al., 2009) can also be obtained by membrane diffusion and/or via transport proteins. Intracellular DAG can form triacylglycerol (TAG) by 1-acyl-sn-glycerol-3-phosphate acyl transferase (SLC) enzymes encoded by gslcs genes. Diacylglycerol (DAG) can be activated by cytidine diphosphate (CDP) to produce CDP-DAG, which then can be used as a precursor to synthesize PI. Newly synthesized PI can be utilized to generate PIP, PIP$_2$, and PIP$_3$ by Giardia PIKs for cellular signaling (Cox et al., 2006; Hernandez et al., 2007).

Like PLs and FAs, SL pathways (shown by pink fonts) in Giardia are highly reduced. It expresses only 5 genes i.e., giardial serine-palmitoyltransferase-1 and -2 (gspt-1 and -2), giardial glucosylceramide transferase (gglct), and giardial acid sphingomyelinase-like phosphodiesterase 3b1 and 3b2 (gasmlpd 3b1 and 3b2). It is possible that PalmA obtained from the growth medium is converted to Palm-CoA by acyl-CoA ligase and then is used by the parasite to synthesize 3-ketosphinganine by the action of serine-palmitoyltransferase enzymes encoded by gspts. We have proposed earlier (Hernandez et al., 2008) that 3-ketosphinganine regulates ceramide uptake in Giardia by controlling its endocytic machinery, and this is important because ceramide is not synthesized by this parasite de novo. The newly acquired ceramide is then used by Giardia as precursors to synthesize GlcCer by glucosylerceramide synthase (encoded by gglct1) which may serve as a key regulator of encystation and cyst production (Hernandez et al., 2008). Taken together, it has been proposed that ceramide uptake and GlcCer synthesis is important for the encystation of Giardia.
1.4 Rationale, Hypothesis, Chapters 2 & 3 Overview, and Approach

Because of its limited lipid synthesis ability, *Giardia* imports majority of lipids and fatty acids from the small intestinal milieu, remolds them accordingly and utilizes for growth, encystation and excystation (Das et al. 2001, 2002; Yichoy et al. 2010). Inhibitors of lipid pathways block growth and encystation (Mendez et al. 2015; Hahn et al. 2013), supporting the idea that lipid metabolism is important for *Giardia*.

![Figure 1. Proposed lipid metabolic pathways in Giardia.](image)

**Step 1:** The entry of PC and lyso-PC in *Giardia* with the help of gPLT or gFLIP. **Step 2 and 3:** Diacylglycerol (DAG) and FA are internalized by specific transporter(s) from the growth medium. **Step 4:** Internalized PC can be converted to PS with the help of gPSS1-like enzyme encoded by putative *gpps* gene. **Step 5:** *Giardia* expresses *psd* gene (Yichoy et al., 2009), and its possible encoded product (gPSD) may facilitate PE synthesis. **Step 6:** The putative *gpps* can also encode gPSS2-like enzyme for the synthesis of PS from PE. **Step 7 and 8:** *Giardia* expresses phospholipase B gene (*gplb*) that helps to produce lyso-PC and glycerophosphocholine. **Step 9:** Because PG is the major phospholipid in *Giardia* and is not present in the growth medium (Yichoy et al., 2009), it is likely that *Giardia* synthesizes PG not only by CDP-DAG pathway (step 16) but also by the headgroup remodeling reaction. **Step 10:**
Similarly, PI is synthesized from PC by base or headgroup exchange reactions from PG. However, the presence of these 2 pathways – i.e., PC->PG and PG->PI – is yet to be elucidated in Giardia or other eukaryotic cells. **Step-11:** PI can be converted to various phosphinositides facilitated by gpiks as mentioned before (Cox et al., 2006; Hernandez et al., 2007b). **Step-12:** Diacylglycerol (DAG) obtained from the growth medium can be converted to CDP-DAG (step 13) and serves as a precursor for TAG (step 14), PG (step 15), and PI (step 16). **Step-17:** Exogenous FAs can produce unsaturated FAs (MUFA and PUFA) and elongated FAs as depicted in steps 18 and 19. Exogenous PalmA can be used as a precursor to synthesize 3-ketosphinganine with the help of gspts (steps 20 and 21). Similarly, both ceramide and SM can be acquired from the growth medium (step 22) by endocytic and non-endocytic pathways. Exogenously obtained SM can be hydrolyzed by gsmaseps to produce ceramide (step 23), and ceramide can be used to synthesize GlcCer (step 24). PC, phosphatidylcholine; lyso-PC, lyso-phosphatidylcholine; PS, phosphatidylserine; PE, phosphatidylethanolamine; PIP, phosphatidylinositol phosphate; PIP2, phosphatidylinositol bisphosphate; PIP3, phosphatidylinositol triphosphate; DAG, diacylglycerol; TAG, triacylglycerol; CTP, cytidine triphosphate; FA, fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; SM, sphingomyelin; GlcCer, glucosylceramide. For better understanding, phospholipid pathways are shown in red, neutral lipids (DAG and TAG) in black, FAs in green, and SLs in pink.

Since Giardia cysts transmit the infection (giardiasis) and participate in excystation process, it is likely that active lipid metabolic pathways are operative in cysts and involved in generating membranes and vesicles in excysting trophozoites (Chavez-Mungia et al., 2004). Recently, using deep proteomic analysis, Balan et al. (2021) reported that encystation triggers the upregulation of lipid pathways, especially in cysts, indicating lipid molecules are important for the cyst viability and excystation. Therefore, the major objectives of my proposal are: (1) to investigate changes of global lipid pattern and elucidate the remodeling reactions during encystation and cyst production and (2) characterize the FA elongase pathway that generate long chain FAs and fatty acyl-conjugated lipids during encystation.
1.4.1 Hypothesis

**Hypothesis:** Although morphologically dormant, *Giardia* cysts are metabolically active and uses lipid remodeling pathways to generate new lipids (by passing the synthesis of entirely new molecule) to support the energy production and membrane/organelle biogenesis of excysting trophozoites (excyste).

1.4.2 Chapter 2 Overview: Elucidation of Global Lipid Changes/Remodeling during Encystation and Cyst Production

Preliminary results using the high-resolution mass spectrometry suggest that the quantities of long chain fatty acyl moieties and degree of unsaturation are increased during encystation and remain high in cysts. The data also indicate that cyst utilizes long chain (unsaturated) fatty acyl conjugated lipid molecules for energy storage and to generate new membranes in excysting trophozoites. It is anticipated that the proposed Aim, will establish that *Giardia* has required machineries to carry out the fatty acid remodeling of membrane lipids (commonly known as the Land’s cycle), where an old fatty acid is replaced by a new one most likely at the sn-2 position ([Das et al., 2001](#)). This will also provide information if a robust lipid metabolic pathway is present in cysts as presented by my results in Aim-2 (Please see the results section).

1.4.3 Chapter 3 Overview: Investigate the Influence of Fatty acid Elongase (gFAELO) on Lipid Metabolism in *Giardia* cysts

As mentioned in Aim-1, increased in fatty acyl chain length and degree of unsaturation are observed in cysts rather than trophozoites. Proteomic analysis (Aim-1) supports the idea that limited but active fatty acid elongation pathway is present in *Giardia*. We have shown earlier that gFAELO expression is increased during encystation. Furthermore, the overexpression of elongase enzyme inhibits cyst production and reduces cyst wall thickness drastically; implicating that
gFAELO activity could be linked to excystation. Therefore, the goal of Aim-2 is to evaluate lipid metabolic/remodeling pathway in gFAELO overexpressing cysts. To accomplish this goal, gFAELO will be overexpressed and subjected to encystation followed by cyst lipidome analysis. It is expected that the proposed Aim should provide information regarding the fatty acid elongation that is operative in cysts and should provide information about how the gFAELO influences membrane/organelle biogenesis in excysting trophozoites.

1.4.4 Approach

Previous studies in Giardia global shotgun lipidomic analysis revealed 53 species, from manual lipid annotation, thin layer chromatography, and offline silica fraction (Yichoy et al., 2009). Since then, improvements in separation, ionization, and analyte detection by mass spectrometry have been made (Yang et al., 2011). For example, targeted sphingolipid (SL) analysis in Giardia using a TSQ Endura Triple Quadrupole Mass Spectrometer (Thermo Scientific), revealed the presence of 97 neutral and 37 acidic SLs in Giardia including sphingoid bases, ceramide (Cer), hexosylceramides, ceramide-1-phosphate, sphingomyelin (SM) and sialyl-glycosylceramides (Duarte et al., 2019). In my proposal, I have taken the approach of incorporating new techniques, such as the inclusion of reverse phase ultra-performance liquid chromatography orbitrap high resolution data dependent mass spectrometry (UPLC-HR-MS/MS) along with the newly available open-source software for peak deconvolution, MS/MS lipid identification (LipidMatch flow and MS-DIAL) and statistical analysis (Metaboanalyst v5.0) (Koelmel et al., 2017; Tsugawa et al., 2020; Pang et al., 2021). Proteomic analysis was performed on a licensed version of Proteome Discoverer and exported to a licensed version of Scaffold for analysis. Wild type and fatty acid elongase expressing trophozoites and cysts were cultured in the laboratory,
collected, and subjected to lipid extraction or tryptic digestion followed by the high-resolution mass spectrometric analysis as described above.
Chapter 2: Elucidation of Global Lipid Changes/Remodeling During Encystation and Cyst Production

2.1 MATERIALS AND METHODS

2.1.1 Growth and Encystation of *Giardia*

*Giardia* trophozoites (strain WB, ATCC No. 30957) were cultured in TYI-S-33 medium supplemented with adult bovine serum (ABS, 5%) and bovine bile (0.5%) at 37° C. The antibiotic piperacillin (100 μg/ml) was added during routine culturing of the parasite (Gillin et al., 1989). Trophozoites were harvested by centrifugation (1,500 x g) for 5 minutes at 4°C, followed by three phosphate-buffered saline (PBS) washes. In vitro encystation was carried out following the method described earlier (Gillin et al., 1987). Briefly, trophozoites were cultured in TYI-S-33 medium with (pH 7.8) containing adult bovine serum (10%), lactic acid (10%), and porcine bile (5%) (Gillin et al., 1987). Cells were allowed to encyst for 72 hours (h) and harvested by centrifugation at 1,000 x g for 5 minutes at 4°C, followed by subsequent sterile water washes to isolate water-resistant cysts.

2.1.2 Lipidomic Extraction and High-Resolution Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS)

*G. lamblia* samples were subjected to cold CHCl₃:MeOH:H₂O (1:2:0.8, v/v/v) containing 160pmol/µL of EquiSPLASH (AVANTI POLAR LIPIDS, cat no. 330731) deuterated internal standards, vortexed at high speed for 5 minutes, and centrifuged for 10 minutes at 1800 g at room temperature. Supernatant was transferred to a new PTFE lined glass vial (Supelco) and the remaining pellet was extracted two additional times with CHCl₃:MeOH (1:2 v/v), pooled and dried under N₂ gas (Gazos-Lopes et al., 2017).
Dionex Ultimate 3000 (Thermo Scientific) with the autosampler refrigerated at 4°C Kinetex EVO C18 column (Phenomenex, 2.6µm, 100Å, 150 x 2.1mm). Solvent A (60% acetonitrile, 10 mM ammonium formate, 0.1% formic acid), Solvent B (89% isopropanol, 10% acetonitrile, 10 mM ammonium formate) in tandem to a Q-Exactive Plus Hybrid Quadrupole Orbitrap Mass Spectrometer (ThermoScientific) set with an exclusion list ON – Positive mode top 50 ions and Negative mode top 100 ions from a previously run blank injection. Full MS resolution set to 70,000, AGC 1e6, Max IT 75ms, scan range 250 to 1800 m/z (Ruzicka et al., 2014). Data-dependent MS2 resolution 17,500, AGC 1e5, Max IT 75ms, isolation window 1.2 m/z, N(CE) 20, 30, 40, minimum AGC target 5e3, dynamic exclusion 8.0s (ref). The mass spectrometer was equipped with a high flow heated electrospray ionization source (H-ESI II) set to: Sheath gas 40, Aux gas 10, Sweep gas 1, Voltage 3200v, Capillary temperature 300°C, S-RF level 60, Aux gas heater temperature 325°C (Ruzicka et al., 2014).

2.1.3 Proteomic 1D-Liquid Chromatography Mass Spectrometry (LC-MS/MS) Proteomic and Bioinformatic Analysis

Peptides were separated on a New Objective PicoTip Emitter (15 ± 1µm), custom packed with a Phenomenex C18 AQUA (5µm, 125Å porous silica) to 20 cm in length. Data acquisition of eluted peptides were analyzed on a Q-Exactive Plus Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Fisher). Data-dependant acquisition (DDA) was set to top 10 and analyzed by MS1 at 70k and MS2 at 17.5k resolution. Automatic Gain Control (AGC) MS1 at 3e6 and MS2 at 1e5, Max Ion Transmission (IT) 50ms, with a full scan range from 350 to 1600 m/z. Data-dependant MS (dd-MS2) max IT at 100ms and an isolation window at 3.0.
2.1.4 Lipid Identification and Statistical Analysis

Raw data files from the mass spectrometer were loaded into LipidMatch flow for deconvolution and lipid identification, according to the LipidMatch flow instructions (Koelmel et al., 2017). MS-DIAL v4.70 was also utilized for lipid confirmation and identification, according to recommended parameters for the Q-Exactive Plus Orbitrap mass spectrometer (Tsugawa et al., 2020). Lipids identified with ≥85% confidence were only utilized for the analysis. LipidMatch flow identified lipids were referenced against those with a high confidence in MD-DIAL v4.70. Peak area intensities from those identified in LipidMatch flow were normalized with their respective 160pmol/µL internal standard from EquiSplash (AVANTI POLAR LIPIDS). Lipid species that did not have a deuterated internal standard, were normalized with those closest in structure, or Lyso PC-18:1(d7). Normalized lipids were imported to Metaboanalyst v5.0, data filtered by interquartile range (IQR), further normalized by median, and log transformed for multivariate statistics and figure generation (Klatt et al., 2018, Pang et al., 2021).

2.1.5 STRING Generation

Identified proteins to be upregulated in cyst were filtered in excel by statistical significance and their respective accession numbers were imported to STRING (Szklarczyk et al., 2021). Unknowns were excluded from the network. The network was then exported to Cytoscape v3.8.2. Bubbles with no interactions were removed.

2.2 RESULTS

2.2.1 Changes of Global Lipid Profiles During Encystation and Cyst Formation by *Giardia*

*Giardia* trophozoites colonize in the small intestine, where it is expose to bile and dietary lipids. This anerobic lipid environment in the intestine allows trophozoites to survive, replicate and encyst (Das et al., 2002). Genomic and biochemical analysis indicate that lipid synthesis and
metabolic pathways in this parasite is highly reduced, and majority of lipids are acquired from the environment. Studies also suggest that *Giardia* can remodel its lipid and fatty acid molecules and use them to assemble membranes and organelles (*Yichoy et al., 2010; Mendez et al., 2015*). To elucidate the possible lipid metabolic pathways that are operative in *Giardia* and evaluate the changes in lipid profile that takes place during encystation, I utilized HR-LC-MS/MS open-source software LipidMatch flow and MS-DIAL for lipid identification (Figure. 3) (*Koelmel et al., 2017; Tsugawa et al., 2020*). Lipid species identified in both software were confirmed as a positive identification. Interestingly, ~64% of the lipid species identified in LipidMatch flow were glycerophospholipids (PC, PG, PE) and neutral lipids (TG, DG) (Figure 3A). Approximately 70% of the lipids identified in MS-DIAL positive ion mode were neutral lipids (DG, TG), one phospholipid (PC), ceramides (Cer) and sphingomyelin (SM) (Figure 3B). Additionally, ~74% identified in MS-DIAL negative ion mode were glycerophospholipids (PE, PG, PG), free fatty acids (FA), and ether linked PC (Figure 3B). Most of the major lipids found throughout LipidMatch flow, were also identified in MS-DIAL, therefore the LipidMatch flow peak areas were utilized throughout the remainder of the study.

Lipids were normalized and analyzed on Metaboanalyst (Figure 4). The Principal Component Analysis (PCA) highlights the variance in the lipids identified in trophozoites, 24h encystation and cyst, noting differences in overall expression throughout the transition from trophozoites to cysts. Variance throughout each stage were dramatically different, with 10.9% variance in PC2 and large variance in PC1 at 76.9% (Figure 4A). Lipids were separated by species and represented as a global snapshot (Figure 4B). In this high-resolution analysis, two unique phospholipids were identified, they are bis(monoacylglycerol)phosphate (BMP) and hemi bis(monoacylglycerol)phosphate HBMP, with very few oxidized, and lyso species, in addition to
PS (Figure 4B). The heatmap identified a change in lipid profile in encysting cells (24h-post induction of encystation or 24h PIE) compared to non-encysting trophozoites). More drastic changes of upregulated lipids in cysts were observed (Figure 4C). Among the top 50 lipids, the species most drastically effected were PC, PE, Cer-NS, Cer-NDS, PG, DG, SM, and ether linked PC (PC O- or P-) (Figure 4C). Interestingly, more dramatic changes were found in the water-resistant cysts, which are thought to be “metabolically dormant.”

Figures 4D-F compare lipid profiles in various stages by volcano plots. PG and PC species were found to be upregulated in cysts relative to trophozoites. Viewing comparisons between cyst and trophozoites, lipids found to be most significant in terms of upregulation were a mixture of PG and PE (Figure 4D). Species those were downregulated significantly included PC, PS and BMP (Figure 4D). Similarly, comparing trophozoites vs encysting cells, the most upregulated lipid species were found to be an increase in select DG, PG, and PE with carbon chain lengths greater than sixteen (Figure 4E). Downregulated lipids found during encystation were Cer, DG, and PG with a range of carbon chains lengths from C14 to C21 (Figure 4E). We continued comparing differences in lipid species in cyst vs 24h encystation (Figure 4F). In this comparison, we identified an upregulation of PG, and select Cer, that contained carbon chain lengths above C16 with at least one degree of unsaturation in the fatty acyl chain (Figure 4F). Downregulation was noted in three lipids, two of which were PG and one was PC with above carbon chain lengths of C16 (Figure 4F).

2.2.2 Changes of phospholipid profiles during the stage differentiation from trophozoites to cysts

Earlier reports suggest that phospholipids and fatty acids are obtained by *Giardia* from the intestinal milieu or from the growth medium (*Yichoy et al.*, 2010). Bile salts and serum that
constitutes the growth medium are the major source of lipids for *Giardia* (Farthing et al., 1985; Gillin et al., 1987, 1988; Lujan et al., 1996; Kaneda and Goutsu, 1988; Mohareb et al., 1991). Electro-spray ionization quadrupole time-of-flight mass spectrometry (ESI-QTOF-MS) analysis revealed that PCs and PGs are the major phospholipids in this parasite and at least 17 different species of PGs were identified in *Giardia* with various combinations of odd-and even-numbered fatty acids (Yichoy et al., 2009). In addition to PGs, 25 species of PCs, 6 species of PEs, 3 species of SMs and 2 species of PIs were also identified in *Giardia* (Yichoy et al., 2009). Interestingly, except for lyso-PCs and PCs, no other phospholipids were detected in bile and serum, indicating that some of the phospholipids could be synthesized by *Giardia*, especially the PG and PE. The based on this observation, we proposed that *Giardia* has the capacity to synthesize few selective phospholipids most likely by lipid remodeling reactions (Das et al., 2001) by passing the synthesis of entirely new lipids via de novo CDP-DAG mechanism (Figure 4). However, the de novo synthesis of limited phospholipids cannot be ignored at this point. To further understand this and to identify new lipids, we conducted lipidomic analysis using a high-resolution Q-Exactive Plus Hyrbid Quadrupole Orbitrap mass spectrometer (Thermo Scientific). Due to the overall lipid profile in cysts, which are different than trophozoites and encysting cells (Figure 4), we further examined the levels of phospholipids and their acyl chain lengths in water-resistant cysts

2.2.3 While Phosphatidylcholines (PCs) are Abundant in Cysts, Sphingomyelin (SM) is Enriched in Encysting *Giardia*

Phosphatidylcholines (PCs) are major lipids in *Giardia* and some of the PC species are obtained from the growth medium (Yichoy et al., 2009). In this experiment, an attempt was made to identify new PCs and their differential contents in trophozoites, encysting cells and cysts. In the current analysis, it was noted that medium chain fatty acids to long chain fatty acids were
highlighted in a radar plot shown in Figure 5A, while long chain to very long chain fatty acids (LCFAs to VLCFAs) were shown in Figure 5B. Some selective PC moieties i.e., PC 16:0/16:1, PC 16:0/18:3, PC 16:1/18:1, PC 18:0/18:1 was found to be present in cysts (Figure 5A). Those that appeared increased during encystation were PC 14:0/18:1, PC 14:0/18:2, PC 16:0/18:0, PC 17:0/18:0 (Figure 5A). Additionally, there was an increase in LCFA to VLCFA PC, that increased in abundance during encystation and cyst overall, but most notably with PC 18:1/18:2, PC 18:2/18:2 moiety (Figure 5B).

Plasmanyl PC ether (O-PC) and plasmenyl PC ether (P-PC) species were observed to increase globally with the lowest abundances found in trophozoites, increasing in abundance in encyst and finally with greatest abundance to be found in cyst (Figure 6 & 7). Ether linked PC-O was most abundant in cyst PC O-16:0/16:1, PC O-18:1/15:0, PC O-18:0/18:3 (Figure 6). Two species were found to be more abundant during encystation PC O-16:1/18:0, and PC O-16:1/18:2 (Figure 6). Ether linked PC P- plasmalogen species were found to be greatly increased largely throughout encystation and fully formed cyst. With the most abundant species in cyst acyl chain moieties include PC P-16:0/18:1, PC P-16:0/16:1, PC P-18:0/18:1, and PC P-20:1/15:0 during encystation (Figure 7).

Sphingomyelins (SM), however, were identified to be most abundant during encystation, with a dramatic decrease in cysts with the most abundant species being d18:1/16:0 SM (Figure 11). The trend followed an increase of SM species from trophozoites to encysting cells, with a in cyst, except for one species, which was identified as most abundant in all life stages, SM d18:1/18:1 (Figure 11).
2.2.4 Phosphatidylethanolamine Abundant in Water-Resistant Cysts

As previously identified by Yichoy et al. (2009), PE was an abundant species in *Giardia* and proposed to be a newly synthesized phospholipid in this organism. In the current study, several species of PEs were identified and among them 5 species were highly abundant and that included PE 16:0/18:1, PE 16:0/18:2, PE 16:0/20:3, PE 16:1/18:2, PE 18:1/18:1 (Figure 8). The lowest abundant species was PE 15:0/16:1 (Figure 8). Most of the PE species had a notable trend, where the lowest abundant species contained shorter chain fatty acids, highest abundant lipids consisted of long chain fatty acids (LCF) with increased unsaturation (Figure 8). Although trophozoites and encysting cells maintained similar levels of PEs, with an opposing trend of increased abundance with shorter chain lengths, and more saturated fatty acids (Figure 8). The least abundant species found in trophozoites were PE 18:2/20:4, PE 15:0/18:3, with the least abundant found during encystation to be PE 15:0/18:2, PE 17:0/18:2 (Figure 8). Oxidized PEs (OxPE) were also highly abundant in cyst, with a slight increase from trophozoites to encysting cells (Figure 18).

2.2.5 Phosphatidylglycerol (PG) is Increased During Encystation and Abundant in Cysts

Like PE, the PGs are newly identified synthesized lipids in *Giardia* (Yichoy et al., 2009). Our early analysis identified 17 species of PGs in *Giardia*. In the current analysis, I identified 50-55 PG species with the help of LipidMatch Flow and RIKEN MS-DIAL software (Figure 3). It was observed that number of PG species increased during encystation and cyst stage of the parasite displayed the maximum number species. While PG 18:1/18:2, and PG 16:0/18:0 was abundant in encysting cells and cysts (Figure 9), PG 16:0/18:0 the highest abundances of PG in trophozoites (Figure 9).
2.2.6 *Giardia* Cysts are Enriched in Neutral Lipids

The amount of diacylglycerol (DG) increased during the transition to cysts with the highest abundant species was DG 16:0/18:1 and DG 15:0/18:1 (MCFA-> LCFA) (Figure 11A). The most abundant species in encystation was DG 16:1/18:1 (Figure 10A). DG species plotted as LCFA to VLCFA, two DGs were comparable in abundance, DG 18:1/18:2 in cysts, and DG 18:0/18:0 during encystation (Figure 10B). Comparable levels of DG 18:2/18:2 was found in both cysts and encysting cells (Figure 10B). Variable levels of DGs were identified in trophozoites, most of the species were found to be at low abundance, or similar levels compared to encysting and cyst cells (Figure 10).

Triacylglycerol (TG) levels maintained a similar trend in MCFA to LCFA encysting cells and cyst, with the highest abundant species to be found is TG 16:0/16:0/18:1 (Figure 12 Left). Those graphed as LCFA to VLCFA revealed four TG lipids to be in comparable abundance in cyst and during encystation, TG 16:1/16:1/20:0, TG 16:1/17:0/18:0, TG 18:0/18:1/18:2, and TG 18:1/18:1/18:2 (Figure 12 Right). TG levels appeared to be similar in both cysts and during encystation, with the lowest abundance levels found in trophozoites (Figure 12).

Cholesterol esters were found throughout each stage of the *Giardia* lifecycle (Figure 14). CE 18:4 was the most abundant found in encysting cells, with CE 18:2 and CE 18:3 to be most abundant in cyst (Figure 14). The least abundant species found in cyst was CE 18:4 and comparable levels to trophozoites as CE 22:6 (Figure 14). Additionally, a lipid at comparable levels during encystation and trophozoites were CE 17:1 (Figure 14).

2.2.7 Three Ether Linked Phosphatidylinerine Species were Found in Cysts

Phosphatidylserine (PS) was found in lower abundance, compared to other glycerophospholipids, therefore PS and ether linked species of PS were combined onto one radar
plot (Figure 13). Ether linked PS, contained one species with highest abundance PS P-20:0/16:0, with comparable levels of PS P-20:1/18:2 and PS O-18:0/18:3 (Figure 13). There was an unidentified fatty acids PS in cyst labeled PS 34:1, and the PS P-20:1/18:2 with least abundance in trophozoites (Figure 13).

2.2.8 Lyso Species of Lipids were Found to be Abundant in Cyst

Lysophosphatidylcholine (LPC) maintained a similar trend found in other lipid classes, in that, there was a gradual increase in each species during the progression to cysts (Figure 15). However, LPC 20:0 was found to be in lowest abundance, found during encystation (Figure 15). LPC 18:1 was in highest abundance in cyst, followed by LPC 18:0 (Figure 15). Ether linked LPCs were identified, with similar abundances found to be highest overall in cyst, with LPC O-16:1 to be the most abundant (Figure 16). Similar trends were found in oxidized LPC (OxLPC) species, with a OxLPC 24:1(KeOH) (Figure 17).

2.2.9 Novel Lipid Species Bis(monoacylglycerol)phosphate (BMP) and Hemi
bis(monoacylglycerol)phosphate (HBMP) were Identified to be Abundant During
Encystation and Trophozoites.

Bis (monoacylglycerol)phosphate (BMP), a unique lipid which has been shown to be involved in lipid sorting via endosome/lysosome compartments, and a produced from PG with the help of phosphatidylglycerol phosphate synthase (gPGPS) (Grabner et al., 2020; Luquain-Costaz et al., 2020). Although Giardia has limited lipid synthesis ability, it expresses PGPS genes in trophozoites and cysts stages of the parasite (Yichoy et al., 2009). This unique lipid has not been identified previously in Giardia and in other parasites and found to be in higher amounts in trophozoites and 24 h PIE cells (Figure 20). Two of the most abundant BMP species contained saturated fatty acyl moieties identified as BMP 16:0/18:0, and BMP 18:0/18:0 during encystation
(Figure 20). In trophozoites, BMP 16:0/18:3 and BMP 16:0/20:4 was found to be in low abundance (Figure 20). In cysts, BMP appeared to be downregulated with 5 of the species BMP 16:0/16:0, BMP 16:0/18:0, BMP 18:1/18:1, BMP 18:0/20:0, BMP 18:0/18:0 identified to be at significantly lower levels, compared to trophozoites and 24h PIE cells. (Figure 20).

Hemi bis(monoacylglycerol)phosphate (HBMP) is a unique lipid and its function is still not clear. In my analysis, I have identified this unique lipid in *Giardia*, which has been reported previously to be present in *Acinetobacter baumannii* (Tao et al., 2021). Four HBMP species i.e., HBMP 16:0/18:0/18:0, HBMP 16:0/16:0/18:0, HBMP 16:0/18:1/18:1 and HBMP 16:0/18:0/16:0, were found to be abundant in 24h PIE cells (Figure 19). One species that was found to be upregulated in cyst was a LCFA-PUFA, HBMP 16:0/18:1/18:2 (Figure 19).

### 2.2.10 Ceramides are Abundant in Cysts

In our earlier analysis, we discovered several species of ceramides and glycosylceramides in *Giardia* (Duarte et al., 2019). My current lipidomic analysis identified several new species of ceramides including ceramide alpha-hydroxyfatty acid-dihydrosphingosine (Cer-ADS), ceramide alpha-hydroxyfatty acid-phytosphingosine (Cer-AP), ceramide non-hydroxyfatty acid-phytosphingosine (Cer-NP), ceramide non-hydroxyfatty acid-sphingosine (Cer-NS), ceramide non-hydroxyfatty acid-dihydrosphingosine (Cer-NDS) were all found to be upregulated in cyst (Figures 21–23). Cer-NP t18:0/24:0 was identified to be one of the most abundant species found in this group (Figure 21). In the Cer-NS pool, Cer-NS d18:1/17:0, Cer-NS d18:1/18:1, Cer-NS d19:1/16:0, Cer-NS d18:2/22:0, Cer-NS d18:2/24:0 were the highest species found in cysts, with Cer-NS d18:1/16:0 at highest abundance in this species found in 24h PIE (Figure 22). Two species were found in the Cer-NDS pool to be upregulated in cysts, Cer-NDS d28:0/22:0, Cer-NDS d24:0/22:0, with Cer-NDS d18:0/16:0 found in trophozoites at comparable levels to cysts (Figure
A Cer-NDS d18:0/22:0 was shown to be in lowest abundance in this pool of lipids, found during 24 h encystation (Figure 23).

### 2.2.11 Analysis of Free and Conjugated Fatty Acids

Fatty acids play an important role in maintaining growth and encystation of *Giardia*. Using Ellis et al. (1996) reported earlier, those major fatty acids in *Giardia* were C16:0 followed by C18:0, C18:1, and C18:2. Small amounts of C14:0, C15:0, C17:0, C18:3, C19:0, C20:0, C22:0, C24:0, C26:0, and C28:0 were also identified by HPLC analysis. These findings were further confirmed by Yichoy et al. (2009) using GC/MS. Both groups mentioned that the level of fatty acids didn’t change with the encystation. In the current analysis, I carried out FA analysis experiment and observed that the abundance of LCFA and polyunsaturated-LCFA increased in cysts several fold compared to vegetative and encysting trophozoites.

Methylated free fatty acids (FAME) were analyzed utilizing GC/MS. I found that three of the most abundant FFAs were oleic acid (C18:1), palmitic acid (C16:0), and stearic acid (C18:0) (Figure 24A). Additionally, linoleic acid (C18:2) was also identified in slightly increased abundance (Figure 24A). The most abundant FFAs i.e., C16:0, C18:0, C18:1 showed a similar trend: high in trophozoites, low in 24 h PIE, and high again in cysts (Figure 24A). Downregulated FFA during encystation were recovered in cyst at similar abundance levels as trophozoites, except for the mono-unsaturated fatty acids (MUFA) and poly-unsaturated fatty acids (PUFA) (Figure 24A). Mono- and poly-unsaturated fatty acids were present throughout each giardial life stage, however, the overall highest abundance in the unsaturated FFA were found in cysts with carbon chains of C18 (Figure 24A). FA elongation and unsaturation were represented in Figure 24B, highlighting the MUFA and PUFA mostly found in cysts.
Lipids identified throughout the lipidome, conjugated fatty acyl moieties were counted to determine if their conjugations followed a similar trend, compared to the free FAs pool found in Figure 24. Top fatty acyl moieties found to be conjugated to membrane lipids were C16:0, C18:0, C18:1 and C18:2 (Figures 25 & 26). Majority of glycerophospholipids (PC, PE, PG) also contained these top FAs (MUFA and/or PUFA) (Figures 25 & 26). Neutral lipids (DG, TG) maintained a similar trend in containing a MUFA and/or PUFA in DGs and mostly C18:1 in TGs (Figure 10 & 12). Additionally, SM contained nine species with fatty acid carbon tail length of C18:1, in addition to the various ceramides (Figure 23). Oxidized or ether linked lipid species were found to contain at least one degree of unsaturated FA (Figure 25 & 26). Interestingly, BMP and HBMP maintained saturated C16:0 and C18:0 as the most abundant species (Figure 26).

2.2.12 Proteomic Analysis Reveals an Increase in Elongation Pathway

Our analysis (described above) revealed dynamic changes of overall lipid and fatty acid (mostly C16, C18 moieties) profiles throughout the life stages of Giardia with a robust increase in cysts. A recent proteomic analysis by Balan et al. (2021) reported that a changes of lipid metabolism occurs during encystation and many lipids related proteins and enzymes are upregulated in encysting trophozoites and water-resistant cysts. These include putative lipid transporters, steroidogenic acute regulatory protein–related lipid transfer (StARkin), oxysterol binding protein related protein (ORP/Osh) and glycosphingolipid transfer protein (GLTP) families. More recently, it has been reported that the enzymes of CDP-diacylglycerol biosynthesis, phospholipid remodeling and glycerophospho-phosphodiesterase are differentially expressed at the late encystation stage and in cysts (Rojas et al., 2021). These studies encouraged us to analyze lipid metabolic pathways by proteomic analysis to elucidate lipid synthesis, remodeling, and
elongation pathways and whether these pathways are altered during encystation and cyst production.

The proteomic analysis was carried out by high-resolution mass spectrometry analysis as described in Materials and Methods Section. Proteins that were characterized bioinformatically to reveal only statistically significant (p-value ≥ 0.05) proteins upregulated in cysts. Accession numbers from the upregulated proteins were then mapped by the STRING networking online tool and generated KEGG ID annotation (Szklarczyk et al., 2021). STRING networked proteins identified several enzymes involved in metabolic and biosynthesis pathways (Figure 27). Eight proteins were highlighted to be involved in metabolic pathways (gla1100), four enzymes were involved in fatty acid metabolism (gla01212), three were involved in fatty acid elongation (gla00062), two in fatty acid biosynthesis (gla00061), two were also found to be involved in biosynthesis of unsaturated fatty acids (gla01040) (Table 2). Further utilizing the KEGG annotation tool, KEGG Mapper BRITE and KEGG mapper Module, annotations revealed an increase in lipid biosynthesis proteins, and fatty acid elongation in the endoplasmic reticulum (Tables 3 & 4) (Kanehisa et al., 2020; Kanehisa et al., 2022).

Interestingly, the three enzymes that were found to be upregulated in the fatty acid elongation pathway were present in trophozoites and encysting cells, with a sharp increase in abundance in cyst (Figure 28A). One of the mechanisms of fatty acid elongation in Giardia requires a ligation of the free carboxylic acid with the attachment of coenzyme-A (CoA) and malonyl-CoA, or a shuttling of acetyl-CoA into the elongation pathway (Purdy et al., 2015; Guo et al., 2015). Therefore, identified ligases were also added to reveal differential abundances found throughout each Giardia life stage (Figure 28A). Three acyl-CoA synthases/ligases found throughout the proteome, fatty acid elongase 1 (EDO80427), synaptic glycoprotein 2 (identified
by KEGG as enoyl CoA reductase, *EDO78150* and Oxidoreductase short chain dehydrogenase (*EDO 81768*) (Figure 28B). The fourth enzyme would include a dehydratase, not found in our proteomic analysis (Figure 28B). Taking into consideration the lipidomic and free fatty acid analysis, the increase in the fatty acyl elongation pathway, could indicate a link to the increased lipid species found in the lipidomic analysis during encystation and in cysts, along with the increased FFA pool of longer chain lengths (*Figure 4 & Figure 24*).
Chapter 3: Investigating the Influence of Fatty Acid Elongase (gFAELO) on Lipid Metabolism in *Giardia* Cysts

3.1 MATERIALS AND METHODS

3.1.1 Proteomic Analysis Reveals an Increase in Elongation Pathway

Trophozoites were obtained in growth media outlined in section 2.1.1, prior to the incorporation of the oct promoter elongase overexpression plasmid (Figure 2). gFAELO was cloned in pPAC-OCTp-ANX3-3xHA-C provided by Dr. Stefan Svärd ([Jerlström-Hultqvist et al., 2012](#)). pPAC-OCTp-ANX3-3xHA-C contains ornithine-carbamoyl transferase) promoter (Fig. 2). The giardial *ANX3* gene was digested from the plasmid using BamHI and NotI and was replaced with the open reading frame of *gfaelo* (GL50581_2228). The primers 5’ AAAAAGGATCCGCTGGCTGGTCATGGCTTCA 3’ and 5’ AAAAAAGCGGCAGCGCATTAACCATTTCAGTGCAA 3’ were amplified and transformed into chemically competent DH5a *E. coli* cells and plated onto LB plates with 100 ug/ml ampicillin. Colonies were screened by PCR and sequenced at the UTEP DNA Sequencing Core facility. A maxiprep was then performed to ensure enough DNA to be electroporated. Successful endogenous HA tag clones were linearized with FseI prior to electroporation for homologous recombination ([Jerlström-Hultqvist et al., 2012](#)).

3.1.2 Western Blot for Tagged gFAELO

Cell lysates were prepared by incubating in hypotonic buffer for 30 minutes (5 mM HEPES-pH7.4, 0.5 mM EDTA, 0.1% Triton X-100) on ice followed by adding lysis buffer (50
mM HEPES, pH7.4, 250 mM sucrose, 25 mM KCl and 5 mM MnCl2, 10 μM E64, and a protease inhibitor cocktail) as described earlier (Mendez et al., 2013; Robles-Martinez et al., 2017). Samples were sonicated using a 1/8” probe for 3 minutes with a 37% amplitude and 3 sec/9sec pulse-rest and passed through a 27-G X 1 1/4 needle 20 times. Protein concentration of cell lysates were determined using the Bradford assay (BioRad) and 30-40 μg of protein were boiled with 2x Laemmli Buffer and β-mercaptoethanol (BME) at 90°C for 5 minutes. The samples were then applied onto a polyacrylamide gel (10%) for 2 hours at 100V and 300 mA followed by an immunoblot analysis for 35 minutes at 110 V and 300 mA. The PVDF membrane was then incubated overnight in the cold room with 5% milk/TBST (25 mM Tris-pH 7.5, 500 mM NaCl,0.1% Tween 20) with a primary antibody. The mouse HA (hemagglutinin) antibody (SIGMA) was used to detect HA-tagged gGlcT1 (dilution 1:1,000), while tubulin was detected from a mouse tubulin antibody that was a gift from Dr. Giulio Francia (dilution 1:100). The next day, the blots were washed three times with TBST buffer and incubated in the secondary antibody (Thermo) for an hour at room temperature (dilution 1:2,000). After three TBST washes, blots were covered with ECL solution (100 mM Tris-pH 8.8, 2.5 mM luminol, 0.4 mM 4-IPBA, 2.6 mM H2O2) and exposed using the iBright machine (Invitrogen).

3.1.3 Confocal Microscopy of gFAELO in Trophozoites, Encysting, and Cyst Cells

Giardia trophozoites, encysting cells, and cysts were harvested, washed with 1x PBS, then fixed in 250 ml of 4% paraformaldehyde and incubated for 10 min at room temperature. In the case of Giardia cysts, an overnight treatment in water at 4°C was done prior to fixation, isolating water-resistant cysts. Cells were washed twice with PBS (1x) then blocked with 5% NGS for 20 minutes at room temperature. The samples were labeled with 1:100 of anti-HA tag antibody (Santa Cruz Biotechnology, cat no. sc- 7392) or 1:150 anti-TSA 417 antibody (provided by Dr. Frances
Gillin, UC-San Diego) overnight at 4°C, followed by two washes with 1x PBS and reaction with 1:400 Alexa fluor 568 donkey anti-mouse 568 (cat no. A10037) or Alexa fluor 488 donkey anti-rabbit secondary antibodies (Invitrogen, cat no. A21206) for 1 hour at room temperature (Mendez et al., 2013; De Chatterjee et al., 2015). The samples were washed twice with 1x PBS, followed by incubation at room temperature with 1 mg/mL DAPI solution (cat no. D9542) for 15 minutes and two more 1x PBS washes. 10 mL of sample and 5 mL of Vectashield Antifade Mounting Medium (Vector Laboratories, cat no. H-1000-10) was loaded onto a glass slide followed by placing a coverslip onto the slide. The slides were analyzed with a confocal microscope (Carl Zeiss Laser Scanning Systems LSM 700), using the Zen 2009 software (Carl Zeiss).

3.1.4 Wild Type and gFAELO Overexpressing Cyst Lipidomic Extraction and High-Resolution Liquid Chromatography Mass Spectrometry Data Acquisition

*Giardia* wild type and gFAELO cyst lipids were extracted, and liquid chromatography high resolution mass spectrometry was performed exactly as outlined in the chapter 2 materials and method section 2.1.2.

3.1.5 Wild Type and gFAELO Overexpressing Cyst Lipid Identification and Statistical Analysis

MS-DIAL v4.80 was utilized for lipid identification in both positive and negative ion modes, according to recommended parameters for the Q-Exactive Plus Orbitrap mass spectrometer (Tsugawa et al., 2020). Lipids identified with ≥90% confidence were only included for the analysis. Unknown and Riken labeled metabolites were removed, followed by normalization of EquiSPLASH deuterated standards (AVANTI POLAR LIPIDS) as discussed in section 2.1.2. Normalized peak areas were imported to Metboanalyst v5.0 for multivariate statistics and figure generation as previously mentioned in the materials and method section 2.1.4.
3.2 Results

3.2.1 Confocal Imaging and Western Blot Indicate an Upregulation in the Elongase Pathway During Encystation and Cyst

Since the long-chain fatty acids (LCFAs) participate in the remodeling of glycerol- and sphingosine-based phospholipids and membrane regeneration (Leyssens et al. 2015), and since *Giardia* expresses a single copy of long-chain fatty acid elongase (gFAELO), we cloned and overexpressed giardial fatty acid elongase-1 gene (*gfaelo*-1, GL50581_92729), which was inserted in pPAC-OCTP-Elong-3XHA-C plasmid and expressed in trophozoites under an OCT (ornithine carbamoyltransferase) promoter with hemagglutinin (HA) tag (Jerlström-Hultqvist et al., 2012). For the comparison, a wild-type gFAELO strain was generated by endogenous tagging (homologous recombination) of HA to gFAELO gene using a construct pKS-Elong-3XHA-BSR (Gourguechon & Cande, 2011). Wild-type and overexpressed cell lines were examined by confocal microscopy and western blotting analysis (Figure 29A a-c). Confocal analysis indicated that gFAELO overexpression (+gFAELO) showed extremely low levels of expression (~2.4%) in trophozoites (not visible in the image). However, it increased in 12-hour and 24-hour encysting cells. The maximum expression was observed in cysts (Figure 29 A, B). The localizations of gFAELO in encysting cells were mostly observed in ER/perinuclear membranes (Hernandez et al., 2007; Steaffanic et al., 2010). In cysts, gFAELO was mostly located in vesicle-like structures, which could be ER or novel membrane components that are required for the assembly of membranes of excysting trophozoites.
3.2.2 Western Blot of HA tagged gFAELO Indicates Expression During Encystation and in Cyst.

To further support our results, we performed immunoblot analysis using monoclonal antibody against the HA tag. The immunoblot results support the observation of confocal images demonstrating the gFAELO expression is upregulated during encystation and enriched in cysts (Figure 29A & C). The wild-type cell lines (containing endogenous gFAELO) showed similar pattern of expression, however, encysting cells showed more expression than cysts (Figure 29 C).

3.2.3 Multivariant Statistics Reveals Glycerophospholipids, Neutral lipids, and Ceramides comparing WT vs gFAELO Expressing Cells

In my proteomic analysis (Figure 28), I have demonstrated that a limited long chain FA elongation pathway is present in *Giardia*. We have also provided evidence that a single copy of elongase enzyme is present in this parasite, which is upregulated during encystation (Figure 29). These experiments encouraged us to investigate if elongase enzyme in Giardia actively participate in lipid remodeling reactions. In the current studies, WT and gFAELO overexpressed (+gFAELO) cysts were generated and lipidomic analysis was conducted as mentioned above (in the Method Section). Lipids were extracted and analyzed by positive and negative ion modes. In positive ion mode, the number of lipids identified with MS-DIAL were 662, with 105 (15.8%) species were found to be statistically significant (p-value ≤ 0.05) (Figure 30). From the statistically significant lipids, the top five consisted of PC, DG, PG, PE, and Cer (Figure 30). Negative ion mode lipids were shown to identify 262 lipid species, with 91 of those species were found to be statistically significant, in comparing wild type against gFAELO expressing cells (Figure 30). The 91 (34.7%) statically significant negative ion mode species were found to be PG, Cer, PC, PE, and FA lipids (Figure 30).
3.2.4 PCA Lipidomic Analysis Reveals Changes in +gFAELO Expressing Cells

Principal component analysis (PCA) of cyst wild type and gFAELO positive and negative ion modes revealed differences with no overlap (Figure 31). The positive ion mode PCA x-axis PC 1 revealed a variance of 47.8%, with a PC 2 variance of 27.1% (Figure 31 Left). Negative ion mode PCA x-axis revealed the highest variance of 48.9%, with the y-axis PC 2 at a variance of 19.1% (Figure 31 Right). Both ion modes indicate the total lipid compositions are clustering individually, separated by the highest variance component x-axis.

3.2.5 Heatmaps Reveal Dynamic Changes in the Regulation of +gFAELO Expressing Cyst Lipids

Positive and negative ion mode heatmaps reveal clear changes in cysts overexpressing +gFAELO (Figure 32). Positive ion mode lipids highlight a select group that are upregulated in +gFAELO. Specifically, DG, PE, PC, Cer, Lyso, and NAE, with a down regulation of PG, PE, PC (Figure 32 Left). Negative ion mode top lipids indicate a downregulation in select PG, PC, and PE species (Figure 32 Right). Additionally, the lipid species contained LCFA’s with mostly C16, C18 and above fatty acyl moieties (Figure 32 Right). Upregulated lipids in negative ion mode gFAELO+ cysts were LPE 18:1, LPE 17:0, LPE 16:0, LPC 16:0, LPG O-19:2, very long chain fatty acids 18:0 and FA 20:0, and very long chain ceramide species (Figure 32 Right).

3.2.6 Volcano Plots Further Indicate a Select Group of Lipids with Significance and Upregulation

To further filter statistically significant lipids in cyst, we generated volcano plots in both positive and negative ion modes to determine fold change differences throughout the lipidome (Figure 33). Positive ion mode revealed a select group of upregulated PE’s, namely PE 16:0/18:3 and PE 16:0/14:1 to be the most statistically significant and have the highest fold change value, which was also identified in the positive ion mode heatmap (Figure 32 Left, Figure 33 Left). The
most downregulated lipid, identified by heatmap and volcano plot in WT cyst was DG 18:3/20:4 (Figure 33 Left). Interestingly, when examining the negative ion mode volcano plot, two species were found to be above the fold change cutoff 1.5 and above p-value 0.05, LPA 26:3 and CL 16:0/18:0/22:4/22:6 (Figure 33 Right). However, LPA 26:3 was the most abundantly upregulated lipid in +gFAELO cysts (Figure 33 Right).

3.2.7 Radar Plots Highlight Significant (p-value ≤ 0.05) Lipids Species. Ceramides were Identified to be Upregulated in +gFAELO Expressing Cysts.

Ceramides (Cer) identified in positive ion modes as [M+H]^+, [M+Na]^+, or [M+H-H2O]^+ species were filtered p-value and plotted as a radar graph. Two out of the ten ceramides identified in positive ion mode were found to be downregulated in gFAELO expressing cysts. Eight of the remaining Cer were revealed to be upregulated in gFAELO expressing cysts, with the most abundant identified as Cer 18:2,2O/16:0 (Figure 34 Left). Negative ion mode ceramides were identified as [M-H], or [M+HCOO] and plotted with those with a p-value of 0.05 or less (Figure 34 Right). All the Cer identified in negative ion mode with statistical significance were found to be upregulated in gFAELO expressing cysts, with the most abundant lipid as Cer 18:1;2O/16:0 (Figure 34 Right).

3.2.8 Cholesterol Esters were Shown to be Slightly Downregulated in +gFAELO Cysts.

Cholesterol esters were identified in positive ion mode as [M+NH4]^+ adducts, with all of the species shown to be decreased in abundance compared to the wild type cysts (Figure 35). Two species were found to be in high abundance, CE 18:2, and highest CE 18:3, with a decrease in very long chain – PUFA moieties, CE 20:3, CE 20:4, CE 18:1 (Figure 35).
3.2.9 Fatty Acids were Shown to be an Upregulated Metabolite in +gFAELO Expressing Cells

Eight free fatty acids were found to be upregulated in the gFAELO expressing cysts as the [M-H]- ion (Figure 36). Two species were in highest abundance, FA 20:0, and FA 18:0, two were lowest in abundance in both conditions, FA 18:0:2O and FA 20:0, with four sharing similar abundance levels, FA 20:4:O, FA 20:5:O, FA 22:5:O, FA 28:5 (Figure 36). Interestingly, very long chain fatty acids above 18 carbons were in least abundance throughout the identified free fatty acid pool (Figure 36).

3.2.10 Phosphatidylethanolamine in Positive Ion Mode was again Identified as One of the Most Abundant Species in Giardia and Increased in +gFAELO Cysts

Phosphatidylethanolamines (PEs) were identified in positive ion mode as [M+H]+ with annotated fatty acyl moieties, PE species with [M+Na]+, however, were not labeled with specific fatty acyl moieties (Figure 37). Fourteen PE species were identified as statistically significant (p-value ≤ 0.05) with two in highest abundance in gFAELO expressing cysts, PE 16:0/18:3, and PE 14:0/18:1 (Figure 37). Four were found to be downregulated in gFAELO compared to wild type cysts, PE 16:0/16:1, PE 18:3/18:3, PE 32:1, PE 36:4 (Figure 37).

3.2.11 Phosphatidic Acid and Phosphatidylethanolamine Radar Plots from Negative Ion mode Revealed Similar or Slightly Decreased in +gFAELO Cysts

As two phosphatidic acid (PA) lipids were identified as significant they were simultaniously plotted with negative ion mode phosphatidylethanolamine or ether linked PE significant lipids (Figure 38). The two PA lipids, PA 18:1/18:2, PA 18:1/18:1 were found to be downregulated in gFAELO cysts (Figure 38). Nine PE species and one ether linked PE were identified which were slightly downregulated in +gFAELO cells (Figure 38).
3.2.12 The Neutral Lipid Diacylglycerol was Revealed at Varying Abundance Levels in Giardial Cyst Wild Type and +gFAELO

Eleven out of nineteen diacylglycerol species were found to be upregulated in +gFAELO cysts. The most abundant species of diacylglycerol was identified as DG 18:1/18:2, which was slightly upregulated in WT cells (Figure 39). The second most abundant lipid DG 18:0/18:0 was found to be upregulated in +gFAELO cells (Figure 39). The most downregulated lipid, DG 37:5, was not annotated with fatty acyl moieties (Figure 39).

3.2.13 Significant Lysophosphatidic Acid, Lysophosphatidylcholine, Lysophosphatidylethanolamine, and Lysophosphatidylglycerol Lipids were Upregulated in +gFAELO Cysts.

One lysophosphatidic acid (LPA) species was identified and plotted on the radar plot, in addition to the other significant lyso-lipid species (Figure 40). Four lysophosphatidylcholine’s (LPCs) were found in negative ion mode, with the most abundant containing a fatty acyl moiety of LPC 18:1 (Figure 40). Four lysophosphatidylethanolamines (LPEs) were identified, with one of the species being the most abundant overall, LPE 16:0 (Figure 40). The last abundant LPE was found to be an ether linked species, LPE O-16:1 (Figure 40). A single odd chain PUFA lysophosphatidylglycerol ether linked lipid was found, LPG O19:2 at high abundance compared to the other lyso lipid species (Figure 40). All of the lyso- species lipids that were plotted were highest in abundance in the +gFAELO cysts.

3.2.14 Phosphatidylcholine in Positive Ion Mode

Phosphatidylcholine (PC) in positive ion mode resulted in identifications of 27 PC lipids in both wild type and +gFAELO cysts (Figure 41). The most abundant PC species, slightly increased in WT cells, was a PC 16:0/18:1 followed by a PC 16:1/18:1 and undefined fatty acyl
moieties PC 36:1 (Figure 41). In +gFAELO cells, PC 18:0/18:1 was found to be the most abundant, compared to WT (Figure 41). Additionally, PC 18:2/18:3 and PC 14:0/18:1 were the only PC fatty acyl moieties annotated to be upregulated in +gFAELO cells (Figure 41). Non-annotated lipid species upregulated in +gFAELO were PC 34:2, and PC 36:8 (Figure 41). The remainder of the lipids were downregulated when compared to wild type cysts (Figure 41).

3.2.15 Decrease Trend of Phosphatidylcholines (PCs) in Negative Ion Mode

Sixteen phosphatidylcholine (PC) species were identified as statistically in negative ion mode (Figure 42). The abundant PC species was a PC 16:0/18:1 followed by PC 18:0/18:1, PC 18:2/20:4 and an ether linked PC O-18:2/18:1 (PC 16:0>PC1 18:0/18:1>PC 18:2/20:4>PC O-18:2/18:1). Interestingly, the abundance of the remaining PC lipids dropped, with the third most abundant lipid found to be followed by (Figure 42). The amounts of all PC’s identified in negative ion mode were decreased in +gFAELO-expressing cysts (Figure 42). Interestingly, the abundance shorter-chain PC species did not show any remarkable differences between WT and gFAELO cysts (Figure 42).

3.2.16 Phosphatidylglycerol in Positive Ion Mode

Fifteen phosphatidylglycerols (PGs) were identified in WT and +gFAELO cells, in positive ion mode (Figure 43). PG 16:0/18:1 was the most abundant and downregulated in +gFAELO cysts, followed by PG 18:1/18:2 (Figure 43). Interestingly, four of the PG’s identified contained the fatty acyl moiety with 16 carbons, and 10 of the fatty acyl moieties contained 18 carbon chain lengths (Figure 43). Four of the lipids were identified, that were not annotated with a fatty acyl chain lengths (Figure 43). Overall, majority of PG species were found to be downregulated in +gFAELO cells (Figure 43).
3.2.17 Phosphatidylglycerol in Negative Ion Mode

Negative ion mode detected a total of 27 species in negative ion mode, and interestingly 26 of them were downregulated in +gFAELO cysts (Figure 44). Only one shorter chain saturated lipid PG 12:0/16:0, was the only species identified to be upregulated in gFAELO expressing cells (Figure 44). Phosphatidylglycerol 18:1/18:2 was the most abundant lipid identified, followed by PG 18:1/18:1, PG 18:1/22:6, and PG 18:2/18:2 (Figure 43).

3.2.18 Lysophosphatidyl, Phosphatidylinositol, and Monoacylglycerol in Positive Ion Mode

Two lysophosphatidylcholine (LPC) and one lysophosphatidylethanolamine (LPE) were statistically significant, with the LPE 16:0 lipid found to be upregulated in gFAELO (Figure 45). The abundance of a long chain monoacylglycerol (MG 38:2) increased in gFAELO-expressing cysts (Figure 45). The only phosphatidylinositol (PI 32:0) lipid was identified without fatty acyl annotation, downregulated in gFAELO cells, in positive ion mode (Figure 45).

3.2.19 N-acyl Ethanolamine in Positive Ion Mode

N-acyl ethanolamine (NAE) in positive ion mode was identified (Figure 46). Ten NAE species The most abundant NAE was NAE 18:2 in both wild type and gFAELO cysts, with the lipid upregulated in gFAELO (Figure 46). The remaining lipids were all upregulated in gFAELO cells with the second and third NAE species found to be NAE 16:1 and NAE 16:0 (Figure 46).
Chapter 4: Discussion

Generally, lipid molecules play an enigmatic but important role in giardial biology. The lipid-rich environment of the small intestine supports the growth and survival of *Giardia*. It also supports membrane and vesicle biogenesis, raft assembly, encystation, cyst production and excystation (Das et al., 2002; Yichoy et al., 2010; Mendez et al., 2015). Interestingly, *Giardia* has a limited lipid and fatty acid synthesis ability and that majority of its cellular lipids, including phospholipids (PLs), sphingolipids (SLs), neutral lipids, cholesterol, and free fatty acids (FFAs) are obtained from the environment of the small intestines of humans and mammals. Serum and bile, two major constituents of in vitro culture medium, supplies lipids and cholesterol to *Giardia* (Jarroll et al., 1981; Farthing et al., 1985; Gillin et al., 1988) via low-density lipoprotein-like receptors express by trophozoites (Lujan et al., 1996; Rivero et al., 2011). However, evidence suggests that bile (bovine or porcine) facilitates the lipid uptake by parasite and bile acids, especially taurocholic and glycocholic acids are transported to *Giardia* by carrier-mediated uptake process (Kaneda and Goutsu, 1988; Das et al., 1997). Analysis related to lipids and fatty acids content of human *Giardia* isolates revealed that PC and SM are major lipids in this organism, and they were mostly acquired from the culture medium. Reports also support the idea that *Giardia* has limited ability to generate selective membrane lipids that are not present in the culture medium (Gibson et al., 1999; Yichoy et al., 2009). We have demonstrated earlier that PE and PG, which are abundant in *Giardia*, are newly generated lipids and not obtained from the culture medium (Yichoy et al., 2009). However, it is not known if lipid profiles are different in trophozoites, encysting cells and cysts as each stage of the parasite performs different set of biological functions.

I utilized lipidomic, proteomic and bioinformatic methodologies in analyzing the changes of lipid profiles throughout the encystation cycle. I also identified the enzymes of lipid pathways
that are up- or downregulated in trophozoites and cysts. Through global lipidomic analysis, utilizing two open-source software, we were successful in identifying >400 species of lipids in *Giardia* for the first time. Within the lipidome, ~58% of the lipids identified were found to be glycerophospholipids, and 29.2% of the lipids were found to be neutral lipids (Figure 3). Well known glycerophospholipids such as PE and PC were found to be the most abundant in all stages. Lyso-glycerophospholipids (LPC and LPE) were also identified in smaller amount. (Figure 3). Neutral Lipids such as TG and DG were among the highest number of lipids species identified. Another neutral lipid cholesterol ester (CE) was also present but in lower percentage (Figure 3). As far as the difference between trophozoites and cysts are concerned, majority of phosphoglycerides are much more abundant in cysts rather than non-encysting and encysting trophozoites. While the neutral lipids e.g., MCFA-LCFA DGs are slightly higher in encysting trophozoites, LCFA-VLCFA are significantly higher in cysts (Figure 10). Both long chain and very long chain fatty acids containing TGs show equal type of distribution in all three stages (Figure 12). Interestingly, the abundance CE is high in cysts (Figure 14). Long chain FFAs such as C18:0, C18:1 and C18:2 is also abundant in cysts. These data suggest that cysts are more active as far as the abundance of lipids and fatty acids are concerned and, also the cyst lipids contain long chain fatty acyl moieties. Although, the reasons for these unequal distribution of lipids in different stages are not clear, it is possible that cysts are primed for excystation and therefore require a robust and active lipid metabolic machinery.

According to published reports, PG is a major phosphoglyceride in *Giardia* (Gibson et al., 1999; Yichoy et al., 2009). Interestingly, PG is a bacterial lipid and mostly found in bacterial plasma membranes (Yeagle, 1993). In mammalian cells, PG is found in intracellular organelles, especially in mitochondria, where it serves as a precursor for cardiolipin (Schlameet et al., 1993).
PG has been previously identified to be present in photosynthetic organisms, such as, cyanobacteria, and plants (Boudière et al., 2014) and shown to play a key role in thylakoid lipids in Arabidopsis thaliana, with which Giardia shares similarities of many metabolic genes (Xu et al., 2006). As stated above, PG was identified as one of the most abundant lipids in Giardia (Yichoy et al., 2009). We also confirmed throughout our study the presence of PG and the increasing abundance, in general, of the species during encystation and in cysts (Figure 10). The high abundance of PG in Giardia remains as an enigmatic issue as no cardiolipin is synthesized in Giardia (Gibson et al., 1999).

Bis(monoacylglycerol)phosphate (BMP), also referred to as lysobisphosphatidic acid (LBPA), has been shown to be involved in endosomal/lysosomal formation, enriched within internal membranes, and contributes to protein and lipid/cholesterol trafficking from the plasma membrane to the ER (Waite et al., 1987; Delton-Vandenbroucke et al., 2007; Hullin-Matsuda et al., 2007; Casares et al., 2019). Additionally, Hullin-Matsuda et al. (2007) indicated that PG is a precursor to de novo synthesis of BMP. It has been well established that Giardia utilizes environmental lipids to suite the organism’s metabolic needs, while the exact import mechanisms remain unclear (Hernadez et al., 2007; Yichoy et al., 2009). The identification of BMP as a lipid present in Giardia, indicates a potential mechanism for sorting, and the trafficking requirements of trophozoites for Giardia cells transitioning into cyst (Rivero et al., 2013). This is supported by our data, as BMP was a unique lipid found to be upregulated during encystation and trophozoites, while found to be in low abundance in cyst (Figure 20). Thus, it is likely that in Giardia, PG serves as a precursor for BMP.-

Hemi bis(monoacylglycerol)phosphate (HBMP) is a newly identified lipid and its potential function is still not clear. However, one study indicates that HBMP (like BMP, with the addition
of one fatty acyl chain) is involved in endocytic formation and was shown to be cleaved by phospholipase A enzymes (Record et al., 2011). Additionally, BMP and HBMP were also highlighted to be products from PG, with the addition of fatty acids to the opposing glycerol backbone (Hullin-Matsuda et al., 2007; Record et al., 2011). Interestingly, HBMP was found in this study as an additional lipid in Giardia (Figure 19). Abundance levels varied throughout the lifecycle with four HBMP species upregulated during encystation and two of them were abundant in cysts (Figure 19). Three out of the four species of HBMP were highly unsaturated, with combinations of palmitic (C16:0) and stearic (C18:0) acids species, with one species containing two oleic acyl chains (Figure 19). The two identified cyst HBMPs contained at least one acyl chain with a degree of unsaturation (Figure 19).

Recent reports by Balan et al. (2021) and Rojas et al. (2021) demonstrate that various putative lipid pathway enzymes such as lipid transporters, lipid transfer proteins, glycosphingolipid transfer protein (GLTP) families, the enzymes of CDP-diacylglycerol biosynthesis, phospholipid remodeling and glycerophospho-phosphodiesterase are differentially expressed at the late encystation stage and in cysts, suggesting that cysts are metabolically active and more dependent on lipid metabolism than glycolysis (Balan et al., 2021). Our lipid results show that lipid species are more abundant in cysts than trophozoites, further supporting the idea that cysts accumulate various classes of lipids, and these lipids participate in membrane regeneration and energy production during the emergence of new trophozoites (excystoites) by excystation. Our proteomic analysis revealed increased levels of lipid metabolic enzymes and ER proteins that participate lipid/FA biosynthesis pathways (Table 2). Proteins that were shown to be most upregulated in cyst were networked on STRING to reveal interactions (Figure 27). Three key proteins that were found to be involved in multiple pathways were fatty acid elongase,
oxidoreductase, and enoyl-CoA reductase (Figure 27, Table 4). The proteins were also identified in trophozoites and 24-hour encysting cells, which were plotted by weighted spectral count to determine the differential abundance levels throughout the giardial lifecycle (Figure 28A). We identified four enzymes that are abundant in cysts abundance (Figure 28A). For example, enoyl-CoA reductase was shown to have low abundance levels in trophozoites but increased during encystation while drastically increasing in abundance in cyst (Figure 28A). Moreover, this trend was seen throughout each of the four enzymes found in the elongation pathway, whereby the abundance levels increased during cyst transition (Figure 28A). Thus, the elongation pathway, which is upregulated in cysts, could be essential for providing substrates for fatty acid elongation pathway in this parasite. This pathway requires the attachment of coenzyme-A (CoA) to the free carboxylic acid end of a FA by a FA ligase (Balan et al., 2021; Purdy et al., 2015; Guo et al., 2015). I further plotted the ligase enzymes found in our proteomic analysis, which revealed three ligases present and maintained a similar trend in increasing abundance during the transition of trophozoites to cysts (Figure 28A). To get an overall idea about this newly discovered pathway in Giardia, a schematic diagram was generated shown in Figure 28B. One enzyme that was not identified (in the current analysis) but likely to be involved in fatty acid elongation is 3-hydroxy-acyl-dehydratase (Figure 28B). Although there have been previous attempts to identify and elucidate the elongation pathway in Giardia through proteomic analysis (Balan et al., 2021; Purdy et al., 2015; Guo et al., 2015), our current results and the abundance of LCFAs/VLFAs in cysts, motivated us to characterize elongase enzyme (+gFAELO) in this organism. Since only one copy of elongase is present in Giardia, it was easy to overproduce that in trophozoites and follow the reaction profile and its possible role in cysts. Therefore, we then utilized an overexpression plasmid containing HA tag (Figure 29). Results demonstrated that little expression was found in
trophozoites, with increasing expression in encysting trophozoites (24-hour PIE) and cysts (Figure 29). The expression was monitored by confocal and immunoblot analysis, which indicated that the elongation pathway is active and operative in cysts (Figure 29). Interestingly, this highlights our lipid findings, that cyst are more metabolically active than previously thought. Additionally, one reason for such an abundance of lipid accumulation as well as upregulation of elongation pathway enzymes in cysts may indicate a mechanism to primer cyst for excystation, fulfilling the requirements for membrane assembly/generation of excysting trophozoites (excyzoites).

As we have confirmed that the elongation pathway is activated in cysts (Figures 28 & 29), and since Giardia expresses one copy of the elongase gene, my next goal was to carry out lipidomic analysis of elongase expressing cysts. In our lipidomic analysis we identified 662 total lipids in positive ion mode, with which 105 (15.8%) lipids were found to be statistically significant compared to wild type vs +gFAELO cysts (Figure 30 Left). Phosphatidylcholine was found to have the highest number of species, followed by diacylglycerol (DG), phosphatidylglycerol (PG), phosphatidylethanolamine (PE), and ceramides (Cer) with a p-value ≤ 0.05 (Figure 30 Left). In negative ion mode, 262 lipids were identified with 91 (34.7%) species having a p-value ≤ 0.05 (Figure 30 Right). On the other hand, phosphatidylglycerol was found to be the most abundant, followed by ceramides, phsophatidylcholines, phosphatidylethanolamines, and fatty acids in negative ion mode with a p-value ≤ 0.05 (Figure 30 Right). Interestingly, 53.33% of the positive mode lipids were glycerophospholipids, 18.1% were neutral lipids, and 9.52% were ceramides in the top 5 significant lipids pool (Figure 30 Left). Negative mode glycerophospholipids were also the most abundant in cyst at 53.85%, with 18.68% ceramides, and 8.79% fatty acids (Figure 30 Right). In our identification of the most abundant lipids throughout the cyst pool, we further confirm from Chapter 2 that phosphatidylethanolamine and phosphatidylglycerol are two of the
most abundant and significant species found in *Giardia* and now in cyst *(Figure 37 & 38, 43 & 44)* *(Yichoy et al., 2009)*.

The principal component analysis indicates, when comparing wild type to +gFAELO expressing cysts, that there are differences found throughout the lipidomic pool *(Figure 31)*. These differences are further highlighted in the heatmap, indicating an upregulation of lyso-, ceramides, and select glycerophospholipids, namely PE *(Figure 32)*. Additionally, several glycerophospholipids PG, PC, select PE, PA, and neutral lipids DG were downregulated in +gFAELO cysts *(Figure 32)*. This observation indicates an interplay of metabolic shifts between +gFAELO expression and lipid remodeling. For example, as previously indicated by Yichoy et al. *(2011)*, palmitic acid can be incorporated to be further converted to 3-ketosphinganine, which then in turn can be incorporated in the SM or the ceramide pathway *(Figure 1)*. However, with the overexpression of +gFAELO, we discover a shift in the cyst metabolic pathways, away from the increased encystation CDP-DAG pathway, indicated by the downregulation in PA, PG and DG lipids *(Figure 32, 33, 38, 39, 43)* *(Balan et al., 2021, Rojas et al., 2021)*. Followed by an upregulation of Cer, PE, LPC, LPE, and LPA, suggesting +gFAELO overexpression has an interplay between increased fatty acyl chain length production, and the organism’s ability compensate away from CDP-DAG toward PE and ceramide production *(Figure 32, 33, 34, 37, 38, 40)*.

As shown in the proteomic and pathway analysis, gFAELO is involved by elongating fatty acids *(Figure 28)*. Our +gFAELO expressing cysts, in negative ion mode, we find that the fatty acids are increased overall, with FA 18:0 and FA 20:0 being the most abundant *(Figure 36)*. Cholesterol esters were found to be slightly downregulated or unchanged in comparing wild type to +gFAELO expressing cysts *(Figure 35)*. Lastly, a unique lipid upregulated in +gFAELO was
identified as N-acyl ethanolamine (NAE), which has not been previously identified in *Giardia* (Figure 46). However, Tsuboi et al. (2018) provided a mini review, indicating that NAE is a precursor to an enzymatic catabolic reaction, releasing ethanolamine and free fatty acid. The group indicates this reaction to be carried out by a FA amide hydrolase, not found in *Giardia* (Tsuboi et al., 2018). However, putative hydrolases have been identified in *Giardia* (GiardiaDB, GL50803_12220). Indicating the necessity for further elucidation and characterization of the *Giardia* genome. If a hydrolase capable of cleaving NAE is present in *Giardia*, this could also lend to the increased fatty acid pool and ethanolamine incorporation for further PE, Cer, or lyso- species production (Figure 33, 46). To summarize the body of work, I have updated an illustration from Yichoy et al. (2018) of the lipid pathways found in *Giardia* from our data, a lipidomic and proteomic approach (Illustration 1).
References


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Figure 3. Global Lipidomic Profile of *Giardia* from LipidMatch flow and Riken MS-DIAL

A) Number of lipid species identified from LipidMatch flow. B) Number of lipid species identified from MS-DIAL with ≥85% confidence. Molecules identified through MS-DIAL as Phthalate’s, RIKEN, unknown or without MS2 were removed.
Figure 4. Global Lipidomic profile of Giardia trophozoites, 24-hour encystation, and fully formed cyst from LipidMatch flow and MS-DIAL.

A) Principal component analysis (PCA). B) A Heatmap. C) Number of fatty acid species identified throughout the lipidome, bubbles generated in R-studio (Blighe, Rana, and Lewis (2018)). D-F) Enhanced Volcano Plot generated in R-Studio comparing trophozoites, 24h encysting cells and 72h cyst.
Figure 5. A radar plot depicting the phosphatidylcholine (PC) lipid species. PC species were normalized to 160 pmol/uL of deuterated (PC-d7) spiked internal standard. Trophozoites in blue, encystation in yellow, and cyst in purple were depicted. Normalized peak areas were averaged and plotted on a radar plot, logarithm transformed in reverse order in excel. Species with moieties in highest abundance form toward the center. PC species with post-hoc ANOVA p-value ≥ 0.05 were removed.
Figure 6. A radar plot depicting the ether linked phosphatidylcholine (O-PC) lipid species.

O-PC species were normalized to 160 pmol/μL of deuterated (PC-d7) spiked internal standard. Trophozoites in blue, encystation in yellow, and cyst in purple were depicted. Normalized peak areas were averaged and plotted on a radar plot, logarithm transformed in reverse order in excel. Species with moieties in highest abundance form toward the center. O-PC species with post-hoc ANOVA p-value ≥ 0.05 were removed.
Figure 7. A radar plot depicting the ether linked phosphatidylcholine (P-PC) lipid species.

P-PC species were normalized to 160 pmol/µL of deuterated (PC-d7) spiked internal standard. Trophozoites in blue, encystation in yellow, and cyst in purple were depicted. Normalized peak areas were averaged and plotted on a radar plot, logarithm transformed in reverse order in excel. Species with moieties in highest abundance form toward the center. P-PC species with post-hoc ANOVA p-value ≥ 0.05 were removed.
Figure 8. A radar plot depicting the ether linked phosphatidylethanolamine (PE) lipid species.

PE species were normalized to 160 pmol/uL of deuterated (PE-d7) spiked internal standard. Trophozoites in blue, encystation in yellow, and cyst in purple were depicted. Normalized peak areas were averaged and plotted on a radar plot, logarithm transformed in reverse order in excel. Species with moieties in highest abundance form toward the center.
Figure 9. A radar plot depicting the phosphatidylglycerol (PG) lipid species.

PG species were normalized to 160 pmol/uL of deuterated (PG-d7) spiked internal standard. Trophozoites in blue, encystation in yellow, and cyst in purple were depicted. Normalized peak areas were averaged and plotted on a radar plot, logarithm transformed in reverse order in excel. Species with moieties in highest abundance form toward the center. PG species with post-hoc ANOVA p-value ≥ 0.05 were removed.
Figure 10. A radar plot depicting the diacylglycerol (DG) lipid species.

DG species were normalized to 160 pmol/μL of deuterated (DG-d7) spiked internal standard. Trophozoites in blue, encystation in yellow, and cyst in purple were depicted. Normalized peak areas were averaged and plotted on a radar plot, logarithm transformed in reverse order in excel. Species with moieties in highest abundance form toward the center. Species from poc-hoc ANOVA with p-value ≥ 0.05 were removed.
Figure 11. A radar plot depicting the sphingomyelin (SM) lipid species.

SM species were normalized to 160 pmol/uL of deuterated (SM-d9) spiked internal standard. Trophozoites in blue, encystation in yellow, and cyst in purple were depicted. Normalized peak areas were averaged and plotted on a radar plot, logarithm transformed in reverse order in excel. Species with moieties in highest abundance form toward the center. SM species with p-value ≥ 0.05 were removed.
Figure 12. A radar plot depicting the triacylglycerol (TG) lipid species.

TG species were normalized to 160 pmol/μL of deuterated (TG-d7) spiked internal standard. Trophozoites in blue, encystation in yellow, and cyst in purple were depicted. Normalized peak areas were averaged and plotted on a radar plot, logarithm transformed in reverse order in excel. Species with moieties in highest abundance form toward the center. TG species with post-hoc ANOVA p-value ≥ 0.05 were removed.
**Figure 13.** A radar plot depicting the phosphatidylserine (PS) lipid species.

PS or O-/P- PS species were normalized to 160 pmol/uL of deuterated (PS-d7) spiked internal standard. Trophozoites in blue, encystation in yellow, and cyst in purple were depicted. Normalized peak areas were averaged and plotted on a radar plot, logarithm transformed in reverse order in excel. Species with moieties in highest abundance form toward the center.
Figure 14. A radar plot depicting the cholesterol ester (CE) lipid species.

Cholesterol ester species were normalized to 160 pmol/uL of deuterated (CE-d7) spiked internal standard. Trophozoites in blue, encystation in yellow, and cyst in purple were depicted. Normalized peak areas were averaged and plotted on a radar plot, logarithm transformed in reverse order in excel. CE species with post-hoc ANOVA p-value ≥ 0.05 were removed.
Figure 15. A radar plot depicting the lysophosphatidylcholine (LPC) lipid species.

LPC species were normalized to 160 pmol/uL of deuterated (LPC-d7) spiked internal standard. Trophozoites in blue, encystation in yellow, and cyst in purple were depicted. Normalized peak areas were averaged and plotted on a radar plot, logarithm transformed in reverse order in excel. Species with moieties in highest abundance form toward the center.
Figure 16. A radar plot depicting the ether linked lysophosphatidylcholine (O- or P- LPC) lipid species.

O- or P- LPC species were normalized to 160 pmol/uL of deuterated (LPC-d7) spiked internal standard. Trophozoites in blue, encystation in yellow, and cyst in purple were depicted. Normalized peak areas were averaged and plotted on a radar plot, logarithm transformed in reverse order in excel. Species with moieties in highest abundance form toward the center.
Figure 17. A radar plot depicting the oxidized lysophosphatidylcholine (OxLPC) lipid species.

OxLPC species were normalized to 160 pmol/uL of deuterated (LPC-d7) spiked internal standard. Trophozoites in blue, encystation in yellow, and cyst in purple were depicted. Normalized peak areas were averaged and plotted on a radar plot, logarithm transformed in reverse order in excel. Species with moieties in highest abundance form toward the center.
Figure 18. A radar plot depicting the oxidized phosphatidylethanolamine (OxPE) lipid species.

OxPE species were normalized to 160 pmol/uL of deuterated (PE-d7) spiked internal standard. Trophozoites in blue, encystation in yellow, and cyst in purple were depicted. Normalized peak areas were averaged and plotted on a radar plot, logarithm transformed in reverse order in excel. Species with moieties in highest abundance form toward the center.
Figure 19. A radar plot depicting the hemibismonoacylglycerolphosphate (HBMP) lipid species.

HBMP species were normalized to 160 pmol/uL of deuterated (TG-d7) spiked internal standard. Trophozoites in blue, encystation in yellow, and cyst in purple were depicted. Normalized peak areas were averaged and plotted on a radar plot, logarithm transformed in reverse order in excel. Species with moieties in highest abundance form toward the center.
Figure 20. A radar plot depicting the oxidized bismonoacylglycerolphosphate (BMP) lipid species.

BMP species were normalized to 160 pmol/uL of deuterated (DG-d7) spiked internal standard. Trophozoites in blue, encystation in yellow, and cyst in purple were depicted. Normalized peak areas were averaged and plotted on a radar plot, logarithm transformed in reverse order in excel. Species with moieties in highest abundance form toward the center. BMP species with p-value ≥ 0.05 were removed.
Figure 21. A radar plot depicting the ceramide alpha-hydroxy fatty acid-dihydrosphingosine (Cer-ADS), ceramide non-hydroxyfatty acid-phytosphingosine (Cer-NP), ceramide alpha-hydroxy fatty acid-phytosphingosine (Cer-AP) lipid species.

Species were normalized to 160 pmol/uL of deuterated (LPC-d7) spiked internal standard. Trophozoites in blue, encystation in yellow, and cyst in purple were depicted. Normalized peak areas were averaged and plotted on a radar plot, logarithm transformed in reverse order in excel. Species with moieties in highest abundance form toward the center.
Figure 22. A radar plot depicting the ceramide non-hydroxyfatty acid sphingosine (Cer-NS) lipid species.

The lipid Cer-NS species were normalized to 160 pmol/uL of deuterated (LPC-d7) spiked internal standard. Trophozoites in blue, encystation in yellow, and cyst in purple were depicted. Normalized peak areas were averaged and plotted on a radar plot, logarithm transformed in reverse order in excel. Species with moieties in highest abundance form toward the center.
Figure 23. A radar plot depicting the ceramide non-hydroxyfatty acid-dihydrosphingosine (Cer-NDS) lipid species.

The lipid Cer-NDS species was normalized to 160 pmol/uL of deuterated (LPC-d7) spiked internal standard. Trophozoites in blue, encystation in yellow, and cyst in purple were depicted. Normalized peak areas were averaged and plotted on a radar plot, logarithm transformed in reverse order in excel. Species with moieties in highest abundance form toward the center.
Figure 24. Gas Chromatography Mass Spectrometry (GCMS) of free fatty acids identified in Trophozoites.

A) Identified free fatty acids by GCMS. Compounds were identified by the NIST library. The most abundant fatty acid identified in trophozoites were palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1). B-C) Schematic representation of the fatty acid types being elongated found throughout the FFA pool.
Figure 25. Conjugated fatty acid (CFA) profile of *Giardia* from identified lipid species in LipidMatch flow.

The sum of CFA found throughout key lipid species. Lipid represented were PC, PC ether linked, PE, PG, DG, TG.
Figure 26. Conjugated fatty acid profile of *Giardia* from identified lipid species in LipidMatch flow.

The sum of CFA found throughout additional key lipid species. Lipid species represented are Ceramides, BMP, HBMP, SM, OxPE, PG, PE.
Figure 27. STRING proteomics exported and filtered to cytoscape.

STRING analysis of protein accession numbers only identified to be upregulated in 72h cyst.
Figure 28. Proteomic analysis of specific protein identified in the fatty acid elongation cycle.

A) Proteomic weighted spectral count of identified proteins in trophozoites, during encystation and upregulated in cyst. B) Schematic representation of fatty acid elongation cycle. Proteins labeled in red were found to be upregulated in cyst from Table 1.
Figure 29. Confocal imaging and western blot tracking of +gFAELO of trophozoites, encystation and cyst overexpressing fatty acid elongase.

A) Blue florescence indicates DNA by DAPI, green florescence indicates chlorotoxin, red indicates long chain fatty acid elongase (+gFAELO) with an HA tag. Aa) Trophozoite giardial cells expressing gFAELO+. Ab) Cells 12h after encystation. Ac) Giardia cells 24h encystation. Ad) Fully developed cyst after 72h. B) Calculated percentage of cells overexpressing gFAELO in Trophozoites, 12h encysting, 24h encysting cells and cyst. C) Western Blot analysis of both hemagglutinin tagged gFAELO+ and endogenous gFAELO in Giardia trophozoites, 24h encystation, 72h cyst.
Table 2. KEGG Mapper Pathway annotations. Protein annotations were mapped using the KEGG mapper tool to determine the major pathways upregulated in cyst.

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Table 3. KEGG Mapper BRITE annotations. Protein annotations were mapped using the KEGG mapper tool to determine the major groups of proteins upregulated in cyst.

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Figure 30. Total number of positive and negative ion modes identified in MS-DIAL v4.80. Identified lipids (>90%) were analyzed separately and normalized.

Post normalization, statistics were calculated with Metaboanalyst v5.0. Total Lipids Found in MS DIAL Positive Ion Mode = 662. Lipids with P-value ≤ 0.05 = 105. Only 15.8% were significant in cysts. Total Lipids Found in MS DIAL Negative Ion Mode = 262. Lipids with P-value ≤ 0.05 = 91. Only 34.7% negative ion mode lipids were significant in cysts.
Cyst identified lipids (>90% confidence) were analyzed separately and normalized by their respective internal standard. Post normalization statistics and PCA were calculated and generated with Metaboanalyst v5.0. (Left) Positive ion mode principal component 1 revealed the highest variance at 47.8%, with principal component 2 at 27.1%. (Right) Negative ion mode principal component 1 was revealed to have the highest variance at 48.9%, with principal component 2 at 19.1%.

**Figure 31. Positive and Negative ion modes principal component analysis (PCA) from MS-DIAL v4.80.**
Figure 32. Positive and Negative ion modes heatmaps from MS-DIAL v4.80.

Cyst identified lipids (>90% confidence) were analyzed separately and normalized by their respective internal standard. Post normalization statistics and heatmaps were calculated and generated with Metaboanalyst v5.0. (Left) Positive ion mode principal component 1 revealed the highest variance at 47.8%, with principal component 2 at 27.1%. (Right) Negative ion mode principal component 1 was revealed to have the highest variance at 48.9%, with principal component 2 at 19.1%.
Cyst identified lipids (>90% confidence) were analyzed separately and normalized by their respective internal standard. Post normalization log 2-fold change and raw p-values were calculated with Metaboanalyst v5.0 two sample t-test. Volcano Plots were generated in R-studio v1.4.1717 with the EnhancedVolcano package (Blighe, Rana, and Lewis (2018)). (Left) Positive ion mode principal component 1 revealed the highest variance at 47.8%, with principal component 2 at 27.1%. (Right) Negative ion mode principal component 1 was revealed to have the highest variance at 48.9%, with principal component 2 at 19.1%.
Figure 34. Positive and Negative ion mode ceramides from MS-DIAL v4.80.

Cyst identified lipids (>90% confidence) were analyzed separately and normalized by an internal standard. Positive ion mode ceramides were normalized 160pmol/µL LPC 18:1(d7) internal standard. Negative ion mode ceramides were normalized by 160pmol/µL LPE 18:1(d7) internal standard. Statistics were calculated using the two-sample t-test in Metaboanalyst v5.0. Positive and Negative normalized ceramide peak intensities with a p-value of ≤ 0.05 were averaged and plotted as a radar plot in Excel. (Left) Ten ceramides in positive ion mode were identified as statistically significant, eight are most abundant in +gFAELO expressing cells, with two to be found most abundant in Wild Type. (Right) Seventeen ceramides in negative ion mode were found all increased in abundance in +gFAELO cyst.
Cyst identified lipids (>90% confidence) were analyzed separately and normalized by 160pmol/µL of CE 18:1 (d7) internal standard. Statistics were calculated using the two-sample t-test in Metaboanalyst v5.0. Positive normalized cholesterol ester peak intensities with a p-value of ≤ 0.05 were averaged and plotted as a radar plot in Excel. Five cholesterol ester species were identified and found to be in increased abundance in wild type.
Figure 36. Negative ion mode identified free fatty acids in MS-DIAL v4.80.

Cyst fatty acid identified species (>90% confidence) were analyzed separately and normalized by 160pmol/µ LPE 18:1(d7) internal standard. Statistics were calculated using the two-sample t-test in Metaboanalyst v5.0. Negative ion mode normalized cholesterol ester peak intensities with a p-value of ≤ 0.05 were averaged and plotted as a radar plot in Excel. Eight fatty acids were identified, all the fatty acids were found to be upregulated in the +gFAELO expressing cells.
Figure 37. Positive ion mode identified in phosphatidylethanolamine in MS-DIAL v4.80.

Cyst fatty acid identified species (>90% confidence) were analyzed separately and normalized by 160pmol/µ PE 18:1(d7) internal standard. Statistics were calculated using the two-sample t-test in Metaboanalyst v5.0. Positive ion mode normalized cholesterol ester peak intensities with a p-value of ≤ 0.05 were averaged and plotted as a radar plot in Excel. Fourteen significant phosphatidylethanolamine species were identified with nine species found to be upregulated in gFAELO expressing cysts. Additionally, one ether linked phosphatidylethanolamine (PE-P) species was found to be upregulated in gFAELO cells.
Cyst PE and PA identified lipid species (>90% confidence) were analyzed separately and normalized by 160 pmol/µL PA 15:0/18:1 (d7). Statistics were calculated using the two-sample t-test in Metaboanalyst v5.0. Negative ion mode normalized cholesterol ester peak intensities with a p-value of ≤ 0.05 were averaged and plotted as a radar plot in Excel. Two PA species were upregulated in +gFAELO expressing cells. Nine PE species were identified to be upregulated in cyst, with one PE 16:0/18:3 was found to be comparable.
Figure 39. Positive ion mode identified diacylglycerol in MS-DIAL v4.80.

The diacylglycerol identified lipid species (>90% confidence) in cyst were analyzed separately and normalized by 160 pmol/µL DG 15:0/18:1 (d7). Statistics were calculated using the two-sample t-test in Metaboanalyst v5.0. Positive ion mode normalized diacylglycerol peak intensities with a p-value of ≤ 0.05 were averaged and plotted as a radar plot in Excel. Eight DG lipids were found to be upregulated in WT cysts. The remaining eleven DG species were identified to be upregulated in gFAELO expressing cells.
Figure 40. Negative ion mode identified lysophosphatidyl lipid species in MS-DIAL v4.80.

Lysophosphatidyl (LPA, LPC, LPE, LPG) identified lipid species (>90% confidence) in cyst were analyzed separately and normalized by 160 pmol/µL LPE 18:1 (d7). Statistics were calculated using the two-sample t-test in Metaboanalyst v5.0. Negative ion mode normalized LPA, LPC, LPE, and LPG peak intensities with a p-value of ≤ 0.05 were averaged and plotted as a radar plot in Excel.
Figure 41. Positive ion mode identified phosphatidylcholine in MS-DIAL v4.80.

Phosphatidylcholine (PC) and ether linked phosphatidylcholine (PC O-) identified lipid species (>90% confidence) in cyst were analyzed separately and normalized by 160 pmol/µL LPC 18:1 (d7). Statistics were calculated using the two-sample t-test in Metaboanalyst v5.0. Negative ion mode normalized PC, PC O- peak intensities with a p-value of ≤ 0.05 were averaged and plotted as a radar plot in Excel.
Figure 42. Negative ion mode identified phosphatidylcholine in MS-DIAL v4.80.

Phosphatidylcholine (PC) and ether linked phosphatidylcholine (PC O-) identified lipid species (>90% confidence) in cyst were analyzed separately and normalized by 160 pmol/µL PA 15:0/18:1 (d7). Statistics were calculated using the two-sample t-test in Metaboanalyst v5.0. Negative ion mode normalized PC, PC O- peak intensities with a p-value of ≤ 0.05 were averaged and plotted as a radar plot in Excel.
Phosphatidylglycerol (PG) identified lipid species (>90% confidence) in cyst were analyzed separately and normalized by 160 pmol/µL PG 15:0/18:1 (d7). Statistics were calculated using the two-sample t-test in Metaboanalyst v5.0. Positive ion mode normalized PG peak intensities with a p-value of ≤ 0.05 were averaged and plotted as a radar plot in Excel.
Figure 44. Negative ion mode identified phosphatidylglycerol in MS-DIAL v4.80.

Phosphatidylglycerol (PG) identified lipid species (>90% confidence) in cyst were analyzed separately and normalized by 160 pmol/µL PA 15:0/18:1 (d7). Statistics were calculated using the two-sample t-test in Metaboanalyst v5.0. Negative ion mode normalized PG peak intensities with a p-value of ≤ 0.05 were averaged and plotted as a radar plot in Excel.
Figure 45. Positive ion mode identified significant lyso species, phosphatidylinositol, and monoacylglycerol in MS-DIAL v4.80.

Lysophosphatidylcholine (LPC), lysophosphatidylethanolamine (LPE), monoacylglycerol (MG), phosphatidylinositol (PI) identified lipid species (>90% confidence) in cyst were analyzed separately and normalized by 160 pmol/µL LPC 18:1 (d7). Statistics were calculated using the two-sample t-test in Metaboanalyst v5.0. Positive ion mode normalized PG peak intensities with a p-value of ≤ 0.05 were averaged and plotted as a radar plot in Excel.
Figure 46. Positive ion mode identified significant N-acyl ethanolamine in MS-DIAL v4.80.

N-acyl ethanolamine (NAE) identified lipid species (>90% confidence) in cyst were analyzed separately and normalized by 160 pmol/µL LPC 18:1 (d7). Statistics were calculated using the two-sample t-test in Metaboanalyst v5.0. Positive ion mode normalized NAE peak intensities with a p-value of $\leq 0.05$ were averaged and plotted as a radar plot in Excel.
Illustration 1. Updated from Yichoy et al. 2001. *Giardia* lipid pathways, highlighting lipid species intake of extracellular milieu and remodeling to BMP / HBMP.

[1 & 2] The transport of lyso-PC and PC from extracellular milieu into *Giardia* ER (Yichoy et al. 2011). [3] PC can be converted to lyso-PC by phospholipase B (gPLB), removing a fatty acid, generating a lyso species (Yichoy et al. 2011). [4] PC would also be converted to PS through one of the potential phosphatidylerine synthases (gPSS)
Conversion from PS to PE would occur by phosphatidylserine decarboxylase (gPSD) (Yichoy et al. 2009 & Yichoy et al. 2011). Additionally, the conversion from PE back to PS would occur by a present phosphatidylserine synthase (gPSS) (Yichoy et al. 2009 & Yichoy et al. 2011). After PC can be converted to PA using a phospholipase D (PLD), unfortunately PLD has not been identified in Giardia; therefore, one possibility a phospholipase B, which many are found in Giardia could maintain a similar function as a PLD (LISOVITCH et al. 2000, McDermott et al. 2020). PA from the extracellular medium would be transported into the cell for conversion to PG or by the conversion of PC to PA (highlighted in [7]). PA to PG remodeling would be converted by the CDP-DAG pathway, in which phosphatidylglycerol phosphate synthase (gPGPS) and phosphatidylglycerol phosphatase (gPGP), present in Giardia would then remodel the PA to PG adding a glycerol to the phosphate group (Yichoy et al. 2009, Yichoy et al. 2011, Bouétique et al. 2014). PG can be converted to PI as a branching pathway of CDP-DAG, with the removal of glycerol and addition of inositol highlighted in Yichoy et al. 2009. The conversion of PG to BMP has been suggested to be remodeled by the removal of one fatty acid via phospholipase (unknown in Giardia, possibly a phospholipase B), leaving an LPG species (Czolkoss et al. 2021). Then the LPG can be converted to BMP by adding a fatty acid to a free hydroxyl on the glycerol head group, perhaps through an acyl transferase, such as a lysophospholipid acyl transferase (gLPAT) (Czolkoss et al. 2021). The mechanism at which the fatty acid is being added to the free phosphate glycerol headgroup has not been clearly identified. However, according to GiardiaDB a lysophospholipid acyl transferase (DHA2_7126) is present in the organism. Little is known about the synthesis of HBMP; however, the mechanisms would require the addition of one fatty acyl moiety in either the PG to HBMP remodel or the BMP to HBMP remodel. In either pathway, three fatty acyl moieties would be present with one hydroxyl group on the a glycerol backbone. Another pathway that could lead to the production of BMP or HBMP is the import of LPA into the cell, possibly by the addition of a fatty acid by gLPAT to PA conversion. The pathway would then be subject to [9, 11 & 12]. DAG can also be a precursor, in that, DAG would be imported into the cell and potentially undergo remodeling through the addition of a phosphate group by a kinase. The kinase would add a phosphate to the free hydroxyl group of the glycerol backbone. However, a giardial DAG-kinase (gDAGK) has yet to be identified. Another, likely mechanism, is the conversion of DAG to PG through the addition of the CDP-DAG pathway previously mentioned, with the presence of gPDPS (Yichoy et al. 2011). Also, a branching pathway from CDP-DAG is the conversion from DAG to PI with the addition of an inositol substituent by a phosphatidylinositol synthase enzyme (Yichoy et al. 2011).
Vita

Cameron Conner Ellis was born on January 4, 1986, and raised in El Paso, Texas. As the oldest of three brothers, he graduated Coronado High School in 2005. After high school, he waited
tables to pay for his education and began attending the University of Texas at El Paso (UTEP). In 2014 he was awarded his Associates of Arts degree from El Paso Community College. In his final years as an undergraduate at UTEP, he joined the lab of Dr. Hugues Ouellet studying cholesterol metabolism in *Mycobacterium tuberculosis* and was admitted into The Campus of Undergraduate Research Initiatives (COURI) his senior year. In April 2016, he was awarded Outstanding Undergraduate Student Presentation at the American Society for Microbiology Rio Grande Branch meeting, and Best Undergraduate Poster Presentation at the 2016 UTEP COURI symposium. He completed Bachelor of Science in Cellular and Molecular Biochemistry in May 2016 from UTEP.

After starting the graduate program, he joined the lab of Dr. Siddhartha Das studying the robust lipid remodeling pathways in differentiation stages of *Giardia lamblia*, by means of proteomic and lipidomic mass spectrometry techniques. During this time, he began working as a part-time Research Associate and Mass Spectrometry specialist in the 5th floor core facility under Dr. Igor Almeida in 2017. During his graduate studies, he has co-authored over a dozen publications across varying collaborative projects. Focused on method development and multi-omic integration, and has had the privilege of working with mentors, colleagues, and students.

This dissertation was typed by Cameron Ellis.