Phosphoethanolamine improves non-alcoholic steatohepatitis, and enhances muscle insulin signaling, in Pcyt2 heterozygous mice.

by

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Phosphatidylethanolamine (PE) is primarily synthesized by the Kennedy pathway, where a series of enzymatic reactions incorporate ethanolamine and diacylglycerol (DAG). This pathway is dependent on CTP-ethanolamine cytidylyltransferase (ET/Pcyt2), which is the rate limiting enzyme. Pcyt2 heterozygous mice (Pcyt2^{+/−}) accumulate DAG and triacylglycerol due to the lack of utilization in the Kennedy pathway, leading to the development of obesity, insulin resistance, hyperlipidemia, and steatohepatitis. Here, we demonstrate that supplementation with phosphoethanolamine (PEA), the substrate of Pcyt2, has the ability to improve steatohepatitis caused by the lack of DAG utilization. PEA also enhanced insulin signaling to promote protein synthesis in skeletal muscle. Genes involved in phospholipid biosynthesis and lipid metabolism were also stimulated by PEA to maintain phospholipid content and promote lipolysis. The effects of PCYT2 knockdown on human fibroblast cells were examined to provide insight as to how reductions in PCYT2 could result in lipid-related disorders in a human model.
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**Abbreviations**

- ACC, acetyl-CoA carboxylase
- AD, Alzheimer’s Disease
- ADP, adenosine diphosphate
- Akt, protein kinase B
- ALT, alanine aminotransferase
- AMP, adenosine monophosphate
- AMPKα, AMP-activated protein kinase α
- AST, aspartate aminotransferase
- ATGL, adipose triglyceride lipase
- ATP, adenosine triphosphate
- BHMT, betaine-homocysteine S-methyltransferase
- CDP, cytidine diphosphate
- CTL-1, choline transporter-like protein 1
- CTP, cytidine triphosphate
- DAG, diacylglycerol
- DGAT1/2, diglyceride acyltransferase 1/2
- DMEM, Dulbecco’s Modified Eagle Media
- dSREBP, Drosophila sterol regulatory-element binding protein
- EK, ethanolamine kinase
- EPT, ethanolamine phosphotransferase
- FA, fatty acid
- FAS, fatty acid synthase
FBS, fetal bovine serum
GAPDH, glyceraldehyde 3-phosphate dehydrogenase
H&E, hematoxylin and eosin
HMGR, HMG-CoA reductase
HRP, horse-radish peroxidase
HSL, hormone-sensitive lipase
IRS-1, insulin receptor substrate-1
KD4, Pcyt2 knockdown human fibroblasts
LPL, lipoprotein lipase
MAM, mitochondrial associated membrane
MCH58, human fibroblast cell line
mTOR, mammalian target of rapamycin
NAFLD, non-alcoholic fatty liver disease
NASH, non-alcoholic steatohepatitis
PBS, phosphate buffered saline
PC, phosphatidylcholine
PCR, polymerase chain reaction
Pcyt1, CTP:cholinephosphate cytidylyltransferase
Pcyt2, CTP:ethanolaminephosphate cytidylyltransferase
Pcyt2\(^{+/−}\), Pcyt2 heterozygous
PE, phosphatidylethanolamine
PEA, phosphoethanolamine
PEMT, phosphatidylethanolamine N-methyltransferase
PGC-1α, PPARγ coactivator 1-alpha
PI3K, phosphoinositide 3-kinase
PPAR, peroxisome proliferator-activated receptor
PS, phosphatidylserine
PSD, phosphatidylserine decarboxylase
PSS1/2, phosphatidylserine synthase 1/2
PVDF, polyvinylidene difluoride
SAM, S-adenosylmethionine
SCD-1, stearoyl-CoA desaturase-1
SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel
Sirt1, sirtuin 1
SREBP, sterol regulatory-element binding protein
T1D, type one diabetes
T2D, type two diabetes
TAG, triacylglycerol
TBS, Tris buffered saline
TG, triglyceride
TGH, triacylglycerol hydrolase
UPP, ubiquitin proteasome pathway
VLDL, very low density lipoprotein
WT, wild-type mice
1.0 INTRODUCTION

1.1 Cellular membranes and phospholipids

All cells and their associated organelles are provided their framework through the use of lipids and proteins, which are organized into a membrane [1-2]. In addition to providing structure, membranes create a hydrophobic barrier to protect the cell from the outside environment, provide compartmentalization for function within the cell, allow for cell to cell interaction, and allow for the diffusion of necessary compounds into organelles [2-3]. A cell’s and organelle’s biological properties are determined by the types of lipids and proteins that make them up [3]. Phospholipids make up the majority of all cellular membranes [1, 3-4]. When membrane composition is compared across all cellular membranes, similarities are noted as all contain a large amount of phosphatidylcholine (PC) and phosphatidylethanolamine (PE), as well as other membrane components, such as cholesterol and phosphatidylserine (PS) [5]. Phospholipids provide membranes with stability, fluidity, and permeability, while also being a requirement for the function of internal membrane proteins [6]. PC and PE, the most abundant phospholipids in membranes [7, 4], are asymmetrically distributed in cell membranes due to their opposing structure, and polarity [1]. PC contains a large head group and therefore is found on the outer leaflet of the membrane, while PE contains a smaller head group and is found on the inner leaflet [1, 8]. This distribution of PC and PE allow for correct membrane curvature, and maintains cellular integrity [9].

1.2 Phospholipid Biosynthesis

The following section is depicted in Figure 1.
1.2.1 Kennedy Pathway

The Kennedy pathway is the primary pathway responsible for the de novo synthesis of PC and PE [7]. The Kennedy pathway consists of two branches; the cytidine diphosphate (CDP)-choline branch for PC synthesis, and the CDP-ethanolamine branch for PE synthesis [7]. The basis of the Kennedy pathway is forming and incorporating high-energy intermediates, CDP-choline and CDP-ethanolamine, with a lipid anchor, typically diacylglycerol (DAG) [7]. The synthesis of PE through the CDP-ethanolamine branch occurs through a series of three enzymatic reactions. The first step is the phosphorylation of ethanolamine, catalyzed by the adenosine triphosphate (ATP)-dependent enzyme ethanolamine kinase (EK). This reaction forms phosphoethanolamine and adenosine diphosphate (ADP). The next reaction is the rate limiting step of PE synthesis, where the enzyme cytidine triphosphate (CTP):phosphoethanolamine cytidylyltransferase (Pcyt2) uses phosphoethanolamine and CTP to form the high-energy intermediate, CDP-ethanolamine. 1,2-diacylglycerol ethanolaminephosphotransferase (EPT) catalyzes the final reaction in PE synthesis. EPT uses CDP-ethanolamine and DAG to form PE.

The same series of reactions occurs in the CDP-choline branch of the Kennedy pathway, but unlike the CDP-ethanolamine branch, choline is used as a substrate instead of ethanolamine, and the enzymes responsible for catalyzing the reactions are specific for choline-containing substrates. However, there has been evidence that Kennedy pathway enzymes overlap with substrate usage [7]. Specifically, EK has been shown to interact with choline, and some phosphotransferase isoforms can use either CDP-ethanolamine or CDP-choline to make PE or PC, respectively [7]. Only Pcyt2 and CTP:phosphocholine cytidylyltransferase (Pcyt1), the rate limiting enzyme of PC synthesis, are highly specific for their substrates and will not use others [7].
Figure 1. The Kennedy pathway. The Kennedy pathway consists of two branches; CDP-choline (left) and CDP-ethanolamine (right), each responsible for the biosynthesis of PC and PE, respectively. PC and PE can be used for the synthesis of PS, and PS can be decarboxylated to synthesize PE. PC synthesis can also occur by using PE. The first two reactions of the Kennedy pathway occur in the cytosol. CDP-choline and CDP-ethanolamine are synthesized to PC and PE, respectively in the endoplasmic reticulum (ER). Phospholipid synthesis from existing phospholipids occur in mitochondrial associated membranes (MAMs) and in the mitochondria.

*Choline kinase-CK; CTP:phosphocholine cytidylyltransferase-Pcyt1; 1,2-diacylglycerol choline phosphotransferase-CPT; Ethanolamine kinase-EK; CTP:phosphoethanolamine cytidylyltransferase-Pcyt2; 1,2-diacylglycerol ethanolaminephosphotransferase-EPT; Phosphatidylserine synthase 1-PSS1; Phosphatidylserine synthase 2-PSS2; Phosphatidylserine decarboxylase-PSD; Phosphatidylethanolamine N- methyltransferase-PEMT*
1.2.2 PC synthesis by PE methylation

Aside from the Kennedy pathway, PC can be synthesized through the methylation of PE [10-12]. This reaction is catalyzed by phosphatidylethanolamine N- methyltransferase (PEMT) [11-12]. However, PC made via PEMT is distinct from those made de novo through the Kennedy pathway [11-12]. This was demonstrated in MT-58 cells, which are non-viable due to their inability to produce PC via the Kennedy pathway [13]. Introduction of PEMT is unable to rescue MT-58 cells, supporting that PC made through the Kennedy pathway is important for cell growth [12]. PEMT expression is also reduced during embryonic hepatic cell growth in rats [14]. Phospholipid diversity is dictated by a number of factors including, number of double bonds, linkages of hydrocarbons chains, and combination of chain lengths [11]. The diversity makes these phospholipid species functionally different, explaining the differences in function demonstrated by PC synthesized through the Kennedy pathway, versus those synthesized through methylation of PE.

1.2.3 PC and PE as substrates for PS synthesis.

PS composes approximately 10% of biological membrane phospholipids [15]. PS synthesis occurs through a calcium-dependent base-exchange reaction [10, 16]. This reaction involves the head group of a pre-existing phospholipid, usually PC or PE, being transferred and replaced by an L-serine residue [10, 17]. Phosphatidylycerine synthase (PSS) catalyzes this reaction, with different isoforms of the enzyme catalyzing the transfer from specific phospholipids [10]. Specifically, PSS1 catalyzes the head group transfer from PC, while PSS2 transfers the head group from PE [15]; however in vitro, PSS1 has demonstrated the ability to use PE as a substrate for the reaction [17]. Following synthesis, PS can be decarboxylated by
phosphatidylserine decarboxylase (PSD) to synthesize PE. In contrast, PC cannot be synthesized from PS, making the PSS1 reaction irreversible.

1.2.4 Localization of phospholipid synthetic pathways

The Kennedy pathway occurs throughout the cell where multiple cellular compartments are involved [8]. These compartments include the cytosol, ER, and mitochondria, although the mitochondria are only involved in the PS biosynthetic pathways [8]. Choline and ethanolamine are not made de novo within the cell and are therefore produced by the metabolism of lipids acquired through one’s diet. Choline and ethanolamine are transported into the cell via a carrier, choline transporter-like protein 1 (CTL1), that are shared between both substrates [8]. In the cytoplasm, the first two steps of the Kennedy pathway occur by CK/EK and Pcyt1/2. CDP-choline and CDP-ethanolamine are then made into their respective phospholipids in the ER through the action of CPT and EPT [8].

PSS1 and PSS2 reside primarily in mitochondria-associated membranes (MAMs) [18]. MAMs are endoplasmic reticulum (ER)-like membranes that are associated with mitochondria [18]. PSD is localized primarily in the mitochondria [19]. In fact, the bulk of mitochondrial PE is synthesized through PSD rather than the CDP-ethanolamine branch of the Kennedy pathway [20]. This makes PSD highly important for mitochondrial integrity since the majority of mitochondrial membranes are composed of PE [20]. Interestingly, PSD has demonstrated preference for newly synthesized PS, rather than membrane PS [20], suggesting the importance of PSS1/2 localization with MAM, since MAM mediates the import of PS into the mitochondria [20-21]. This is specifically demonstrated in the CHO mutant strain R-41, where PS translocation into the mitochondria was impaired [21]. The R-41 mutant strain was defective in
producing PE through PSD, despite PS biosynthesis being unaffected [21].

Majority of PEMT enzymes are present exclusively in hepatocytes [10]. Interestingly, there are two PEMT isoforms that exist; PEMT1 and PEMT2. Each isoform has a different localization within the cell. PEMT1 is localized primarily on the ER membrane [22], while PEMT2 is present on MAMs [23]. Taken together, the connectivity of all phospholipid biosynthetic enzymes in the cytosol, ER, MAM, and mitochondria, provides an excellent means by which the cell can maintain homeostatic levels of PC, PE, and PS.

1.3 Relationship between phospholipids and disease progression

1.3.1 PC and liver function.

Approximately 70% of ER membrane PC is made through the Kennedy pathway, while the other 30% is made from the PE to PC converting enzyme, PEMT [24-25]. Both these pathways have been of particular interest when analyzing the effects of altered phospholipid content on human health. Specifically, altered PC content through choline deficiency [26], knockout of PEMT [9], or a combination of both [27] has been demonstrated to play a role in the progression liver diseases.

Mice lacking PEMT undergo hepatic ER stress, leading to the development of steatohepatitis [9]. With the addition of a choline-deficient diet, mice lacking PEMT experience liver failure [26]. Choline deficient hepatocytes have decreased PC content and demonstrate a decreased rate of very low-density lipoprotein (VLDL) secretion [27]. VLDLs, which contain a phospholipid layer rich in PC, are responsible for the transport of triglycerides (TG) out of the liver [27]. Active PC synthesis via the Kennedy pathway, which is highly dependent on the
availably of choline, is therefore required for VLDL secretion [27]. Impairments in PC synthesis via the Kennedy pathway may lead to the development of hepatosteatosis.

1.3.2 PE and heart function.

PE and its downstream signaling events play a large role in maintaining heart function [28]. Alterations in PE distribution in sarcolemmal membranes of heart myocytes lead to sarcolemmal disruptions [29]. In diabetic hearts, abnormalities in PE were shown to contribute to membrane dysfunction and defective contractility [30]. In Drosophila easily shocked (eas) mutants, which demonstrate disruptions in PE synthesis, the alterations in PE content lead to heart failure [28]. This was due to the chronic stimulation of Drosophila sterol regulatory element-binding protein (dSREBP) [28]. dSREBP, like mammalian SREBP, is a transcription factor responsible for regulating lipid homeostasis [28]. When stimulated, SREBP induces cholesterol and lipogenic gene expression [28]. In wild-type Drosophila, a decrease in PE membrane content triggers the release of dSREBP, where it induces fatty acid synthesis [28, 31], and this result is reversed following restoration of homeostatic levels of PE in the membrane [28]. The stimulation of dSREBP in Drosophila eas mutants lead to an increase in the expression of lipogenic genes, which in turn stimulate the accumulation of fat within myocytes, ultimately leading to heart failure [28].

1.3.3 PE and Alzheimer’s Disease

Neuronal membranes contain the largest amount of PE compared to all other organs [6, 32]. Neuronal cells are responsible for receiving, processing, and transmitting information, and all these processes are dependent on the lipid composition of the membrane [6]. In the brains of those with Alzheimer’s disease (AD), the total amount of PC and PE was lower [6]. Specifically,
the decrease in PE was due to a decrease in PE-plasmalogens, which is a PE phospholipid that contains an ether bond at the sn-1 position of an alkenyl group. PE-plasmalogens have the tendency to be attacked by free radicals due to their ether bond [6]. In AD, there is increased oxidative stress and in turn, PE-plasmalogens may decrease due to the increase in free radical content [6]. PE-plasmalogens can also be degraded by plasmalogen-selective phospholipases, which may be dysregulated in AD [6].

1.4 Pcyt2

As mentioned, Pcyt2 controls the rate limiting step in the CDP-ethanolamine branch of the Kennedy pathway [24]. Pcyt2 was purified from rat liver in the mid 1970’s [33], and was more recently shown to have three isoforms; Pcyt2α, Pcyt2β, Pcyt2γ [34]. Pcyt2 is unique in that it is encoded by a single gene, while other enzymes in the Kennedy pathway show redundancy with each enzyme being encoded on at least two different genes [34]. Although present on a single gene, the functional redundancy in Pcyt2 through the presence of different isoforms is controlled by alternative splicing [34]. Pcyt2α and Pcyt2β are well characterized due to their higher metabolic activities, while Pcyt2γ is metabolically inactive [34]. Pcyt2α and Pcyt2β differ by the presence of an 18-amino acid linker peptide that is only found within Pcyt2α [34]. This linker peptide is located between two catalytic domains [35], and therefore its absence alters the catalytic activity of the enzyme, as demonstrated through significantly different Km and Vmax of the two isoforms [34].

1.4.1 Role of Pcyt2 in the development of metabolic syndrome

Due to the role of phospholipids in disease progression, it is only fair to assume that Pcyt2 dysfunction may be a contributing factor for certain diseases. Pcyt2 is ubiquitously
expressed in multiple tissues, and many have looked into the effects of Pcyt2 expression on disease progression at a tissue-specific and whole-body level [36-40]. The muscle-specific knockout, and hepatic deletion of Pcyt2 both result in the accumulation of DAG and triacylglycerol (TAG) in their respective knockout tissues [36-37]. In addition, both knockout models demonstrated an up-regulation of SREBP [36-37], and hepatic deletion also lead to the activation of peroxisome proliferator-activated receptor gamma (PPARγ), which regulates lipid storage [37]. The alterations in lipid metabolism, in which lipogenesis is favoured, that are noted within these tissue-specific studies may indicate that Pcyt2 is a contributing factor to the development of lipid-related disorders. To further investigate this theory, our lab has generated a Pcyt2 heterozygous mouse (Pcyt2(+/-)), in which Pcyt2 expression and activity is reduced on a whole-body level [38]. It is important to note that these mice are heterozygous, as the complete deletion of Pcyt2 resulted in embryonic lethality [38], demonstrating that Pcyt2 function, and de novo PE synthesis, is essential for growth and development.

The whole-body knockdown of Pcyt2 in our Pcyt2(+/-) mice was successful as Pcyt2 mRNA and protein expression, as well as activity was reduced in various tissues [38]. The reductions in Pcyt2 activity correlated with reductions in the rate of PE synthesis, while maintaining overall PE content [38]. Decreasing de novo PE synthesis via the Kennedy pathway generates excess DAG due to the lack of its utilization in the final step of the CDP-ethanolamine branch [39-40] (Figure 2). The accumulation of DAG is toxic to cells; therefore, it must be stored as TAG. For TAG synthesis from DAG to occur, fatty acids (FA) must be available, and for this reason, lipogenesis within this model is increased [39]. Specifically, Pcyt2(+/-) mice demonstrate increases in the expression of Srebp, diglyceride acyltransferase 1/2 (Dgat1/2), and fatty acid synthase (Fas), all of which play a role in providing FAs for TAG synthesis [39]. Pcyt2(+/-)
mouse model is the first to show that DAG not utilized for phospholipid synthesis is channeled into TAG [40]. This was further proven by an in vitro study using Pcyt2-deficient hepatocytes, where reintroduction of Pcyt2 restored DAG and TAG levels to that of controls, and FA synthesis by SREBP, DGAT1/2, and FAS was reduced [40].

The accumulation of DAG and TAG, is associated with the development of obesity and insulin resistance in both hepatic and skeletal muscle [41], which are two characteristics of metabolic syndrome. Metabolic syndrome is a term used to describe a group of conditions that increase one’s risk for cardiovascular disease and type 2 diabetes [42]. These conditions include obesity, insulin resistance, hyperlipidemia, decreased serum high-density lipoproteins (HDL), and increased blood pressure [42]. Classically, the presence of at least three of the five conditions indicates that one has metabolic syndrome [42]. The accumulation of DAG and TAG, and increases in FA synthesis in the Pcyt2(+/−) mouse model, results in the development of metabolic syndrome [39]. Specifically, Pcyt2(+/−) mice experience hypertriglyceridemia, insulin resistance, and obesity [39], and male mice also develop hypertension [48].

The presence of obesity frequently results in the development of other conditions, including non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH) [43-46]. NAFLD and NASH are similar in that they both demonstrate elevated lipid droplets within the liver [43-45]. In NASH, however, there is also evidence of inflammation [45-47], making those with NASH more likely to experience liver failure [43]. During insulin deprivation, lipolysis is suppressed, causing an increased amount of fatty acids to be delivered to the liver [43-45, 47]. The increased delivery of fatty acids, in addition to decreased transport out of the liver, contributes to lipid droplet formation [43, 47]. The insulin resistant state of Pcyt2(+/−) mice, as well as the increases in TAG formation within this model, cause the development of
NASH [39].

**Figure 2.** Pcyt2 knockdown leads to the accumulation of DAG and TAG. Knockdown of Pcyt2 results in impaired PE synthesis through the CDP-ethanolamine branch of the Kennedy pathway. The reaction carried out by EPT, which incorporates DAG into the phospholipid, does not occur as readily, therefore decreasing the utilization of DAG for PE synthesis. DAG therefore accumulates, and is stored as its energy substrate TAG. Accumulation of DAG and TAG is associated with the development of metabolic syndrome.

*Ethanolamine kinase-EK; CTP:phosphoethanolamine cytidylyltransferase-Pcyt2; 1,2-diacylglycerol ethanolaminephosphotransferase-EPT.*
1.4.2 Reversing the development of metabolic syndrome caused by Pcyt2 deficiency

Recently, work has been done in order to provide a therapeutic option to reverse the development of metabolic syndrome in Pcyt2 knockdown mice, as this may provide a means by which metabolic syndrome can be reversed in the general population. Due to the connectivity of the phospholipids within the Kennedy pathway, it was postulated that increasing PC synthesis through choline supplementation would allow for the greater utilization of DAG and ultimately reduce the lipid accumulation that gives rise to metabolic syndrome [49]. Choline supplementation had a significant effect on reducing weight gain that is characteristic of the Pcyt2(+/−) mouse genotype [49]. Choline supplementation was able to stimulate the expression of lipolytic genes, specifically adipose triglyceride lipase (Atgl), lipoprotein lipase (Lpl), and hormone-sensitive lipase (Hsl) [49]. In addition, the expression of Fas, Srebp, and Dgat1/2 were repressed due to choline supplementation [49]. The altered expression of these genes reduced the amount of plasma and tissue TAG content [49]. Choline also increased the expression of mitochondrial regulators Ppara and PPARγ coactivator 1-alpha (Pgc-1α), thus providing more mitochondria for fatty acid oxidation [49]. Taken together, choline provided an excellent means by which metabolic syndrome can be reversed from stimulating phospholipid metabolism.

Reversing the phenotype of our model by enhancing DAG utilization through the CDP-ethanolamine branch of the Kennedy pathway is less understood. Phosphoethanololamine (PEA), the substrate of Pcyt2, is an existing supplement that is sold to consumers by a wide variety of online retailers. PEA supplements are suggested for an improvement in immune function, for stress relief, or improvements in overall well-being. Due to its availability, the implications of PEA supplementation on the treatments of certain diseases has been examined. PEA has been shown to have anti-tumor activity when supplemented to cancer cells due it its ability to induce
apoptosis [50] and inhibit angiogenesis [51]. In addition, PEA supplementation demonstrated the ability to inhibit metastasis in mice with renal carcinoma, and its efficacy was much higher than the common anti-cancer drug, Sunitinib [51]. There may also be implications of PEA on diabetic treatments, as those with type 2 diabetes demonstrate reduced PEA concentrations, therefore supplementing with PEA may improve insulin signaling [52]. PEA’s promising results as a treatment for other diseases, in addition to being the direct substrate of Pcyt2, makes it an excellent candidate as a treatment option for our $Pcyt2^{(+/-)}$ mice.

1.5 Rationale

$Pcyt2$ knockdown has shown to play an important role in the development of metabolic syndrome, and associated lipid disorders [39-40]. Stimulating the CDP-choline branch of the Kennedy pathway through choline supplementation has shown that aspects of metabolic syndrome, including obesity and hypertriglyceridemia, can indeed be reversed [49]. However, little is known about the effects of stimulating the CDP-ethanolamine branch. This thesis will focus on the effect of PEA, the substrate of Pcyt2, on reversing the development of metabolic syndrome in $Pcyt2^{(+/-)}$ mice. Providing PEA will allow for greater substrate availability for the remaining Pcyt2 to drive the synthesis of PE, therefore allowing for greater DAG utilization. In this thesis, the effects of PEA supplementation on all aspects of metabolic syndrome, including obesity, hepatosteatosis, and insulin sensitivity will be examined, as well as any changes PEA may induce on the Kennedy pathway. It is hypothesized that PEA will stimulate the Kennedy pathway to utilize DAG, and improve characteristics of metabolic syndrome, and the associated disorders, within this model. The effects of $Pcyt2$ knockdown in human fibroblast cells will also be examined to provide a better understanding of how this deficiency translates to a human model. Specifically, how the Kennedy pathway is altered due to $Pcyt2$ knockdown in these cells.
will be investigated, as well as how lipid metabolism is affected. *It is hypothesized that Pcyt2 knockdown cells will demonstrate similar alterations in phospholipid and lipid metabolism that is demonstrated in Pcyt2<sup>+/−</sup> mice, where lipid synthesis is favoured and DAG accumulates.*

### 2.0 EXPERIMENTAL PROCEDURES

#### 2.1 Animal Treatments

*Pcyt2<sup>+/−</sup> (HET) mice were generated as previously described [38], and knockdown was confirmed through mouse ear notch genotyping. All procedures were approved by the University of Guelph’s Animal Care Committee and were in accordance with guidelines by the Canadian Council on Animal Care. Mice were exposed to a 12-h light / 12-h dark cycle beginning with light at 7am. Eight-month and ten-month old mice were fed a standardized died (Harlan Teklad S-2335) *ad libitum* and had free access to water. Female mice were housed based on group (WT, HET, HET+PEA), while male mice were individually housed. For supplemented groups, phosphoethanolamine (PEA) (Sigma-Aldrich 268674) was provided through water for eight weeks. PEA is available as a supplement for general consumers, therefore the concentration of PEA in water was calculated to mimic the daily dosage of PEA recommended for humans per kilogram body weight. When calculated, the daily dosage of PEA for our mice was 3 mg. Since mice typically consume 3 mL of water per day, the concentration of PEA in the water provided was 1 mg/mL. Daily water consumption was measured for the first two weeks of supplementation to ensure that 3 mL was being consumed. Body weight was measured every 7 days during the supplementation period.*

#### 2.2 Blood Biochemistry
Blood was collected immediately after euthanization. Serum was separated by centrifugation and sent to the Animal Health Laboratory (University of Guelph) for biochemical analysis on metabolites characteristic in metabolic syndrome development, as well as markers for liver and kidney function. These metabolites include; cholesterol, glucose, triglyceride, urea, ALT, and AST. Values are presented as mmol/L for cholesterol, glucose, triglyceride, and urea. ALT and AST values are presented as U/L. Mice were not fasted prior to sample collection.

2.3 Tissue Homogenization and Protein Preparation

Hepatic tissue and skeletal muscle from each mouse, weighing approximately 35 mg each, were homogenized in 300 µL of lysis buffer (1 M Tris pH 7.4, 0.5 M EDTA, 0.1 M NaF) with protease and phosphatase inhibitors. Following homogenization, samples were centrifuged for 10 minutes at 10,000 g at 4ºC. Supernatant was collected and protein concentration was determined using BCA protein assay (Thermo Scientific 23225). Samples were prepared for immunoblotting by diluting with lysis buffer with inhibitors (described above) and loading dye. Samples were then heated at 95ºC for 10 minutes. All samples were prepared to contain 25 µg of protein.

2.4 Western Blotting

5 µg of protein was loaded on a 6% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) and run at 120V for 1 h. Gels were transferred to a polyvinylidene difluoride (PVDF) membrane for 1 h at 22V. To ensure equal loading and successful protein transfer, membranes were stained with Ponceau S. Membranes were blocked with 5% milk in (TBS) containing 0.5% Tween 20 for 1 h at room temperature. Membranes were then probed for the protein of interest with a rabbit polyclonal antibody diluted 1:1000 in 5% Bovine Serum Albumin (BSA) in TBS-T.
Antibody incubation was done overnight at 4°C. Following incubation, membranes were washed in TBS-T for 15 minutes then incubated with goat-anti-rabbit IgG linked to horse-radish peroxidase (HRP) at a dilution of 1:5000 for 2 h at room temperature. A chemiluminescent peroxidase substrate assay (Sigma-Aldrich CPS160-1KT) was applied to the membrane, and bands were visualized on X-ray film. Densitometry analyses was done using ImageJ 1.46 software. Bands were normalized to Ponceau S stained membranes.

2.5 Hepatic Gene Expression

Hepatic tissue, weighing between 50-100mg was homogenized in 1 mL of TRIZol Reagent (Thermo Scientific 15596-018) to isolate mRNA. cDNA was synthesized using 2.5 µg of mRNA using Superscript III reverse transcriptase (Invitrogen 18080044). Expression of various genes involved in phospholipid and lipid metabolism were determined by polymerase chain reaction (PCR) using poly A primers. Primer sequences and conditions listed in Table 1. Reactions were standardized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH).
2.6 Hepatic Triglyceride Content

Hepatic tissue, weighing approximately 200 mg was homogenized in 500 µL of phosphate buffered saline (PBS) in 5% Tween 20. Samples were heated for 5 minutes at 95ºC then cooled to room temperature. The heating/cooling process was repeated, then insoluble material was removed through centrifugation at room temperature. A triglyceride assay kit (Wako Diagnostics 994-02891 and 998-02992) was used to determine the quantity of triglycerides in samples. Samples were prepared for the assay using manufacturer’s instructions. A600 absorbance values were measured and normalized to tissue weight.
2.7 Histological Analysis

Fresh livers were collected and fixed in 10% formalin in PBS and embedded in parafilm until histopathologic examination. Sections were de waxed in xylene and rehydrated in ethanol. Sections were stained with hematoxylin and eosin (H&E) to examine quantity of lipid droplets, as well as 0.1% Picrosirius red for collagen content. All staining was performed at the Ontario Veterinary College, Department of Pathobiology, University of Guelph.

2.8 PCYT2 Knockdown Cells and Cell Culture

PCYT2 knockdown was performed by a lentivirus as previously described [53]. PCYT2 knockdown (KD4) and MCH58 human fibroblast cells were grown in Dulbecco’s Modified Eagle Media (DMEM, high glucose) (Fisher Scientific SH3002201) supplemented with 10% fetal bovine serum (FBS) and 2 µg/mL puromycin. Cells were cultured at 37ºC with 5% CO2. PCYT2 protein expression and activity has been previously demonstrated to be successful in KD cells [50-51], therefore was not measured in this thesis.

2.9 Cellular Gene Expression

KD4 and MCH58 were grown to 90% confluence and harvested in 1 mL of TRIzol Reagent (Thermo Scientific 15596-018) to isolate mRNA. cDNA preparation and PCR was done using same procedures as described for hepatic tissue. Primer sequences and PCR conditions are listed in Table 2. Reactions were standardized to GAPDH. All measurements were performed in triplicates.
Table 2: Primer Sequences and melting temperatures (Tm) for polymerase chain reaction (PCR) using human fibroblast RNA.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Forward 5'-3'</th>
<th>Primer Reverse 5'-3'</th>
<th>Tm (°C)</th>
</tr>
</thead>
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<tr>
<td>CTL-1</td>
<td>CTCTTCTGCATTGGGATGGGA</td>
<td>CTGGGACACGCTGCTACACA</td>
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<tr>
<td>PCYT1</td>
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<td>TGTTTGGGTTCCCCCAGTCC</td>
<td>53</td>
</tr>
<tr>
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<td>GATTTGCAGCAGAGCCGTT</td>
<td>56</td>
</tr>
<tr>
<td>PSS2</td>
<td>GCACCGACACCCCTTTACT</td>
<td>GTTTAGGGCGGTTCCTGAG</td>
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</tr>
<tr>
<td>PSD</td>
<td>CATCTGGACGGTGGGGTGA</td>
<td>CGACTCCAGGGAGTAGGTGA</td>
<td>58</td>
</tr>
<tr>
<td>PEMT</td>
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<td>GGGTCTTGCTGTTCCCATCGT</td>
<td>56</td>
</tr>
<tr>
<td>TGH</td>
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</tr>
<tr>
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</tr>
<tr>
<td>GAPDH</td>
<td>ACCACAGTCATGCCATCAC</td>
<td>TCCACACCCCTGTTGCTGTA</td>
<td>58</td>
</tr>
</tbody>
</table>

2.10 Statistical Analysis

PEA trials were conducted on male and female mice with 3-5 mice per group. Male mice (n=3 per group) were used for Western Blot, gene expression, and histology analysis. Statistical significance between groups is determined by one-tailed Student’s t-test or one-way ANOVA with Tukey’s post hoc test. A p of ≤ 0.05 at 95% confidence interval was considered significant. Data is presented as mean ± S.E.M. All statistical tests were performed with Graphpad Prism 6 software (NIH).
3.0 RESULTS

3.1 The effects of PEA supplementation on Pcyt2 heterozygous mice.

3.1.1 PEA supplementation does not prevent weight gain in Pcyt2\(^{(+/-)}\) mice, but affects tissue mass.

Adult-onset obesity is one of the characteristics of Pcyt2\(^{(+/-)}\) mice [39]. The weight gain demonstrated by this model was previously prevented through choline supplementation [49]. PEA was not able to reduce body mass throughout the eight-week supplementation period in both male and female mice (Figure 3 A, B). Following euthanization, liver, adipose, and quadriceps muscle tissue were excised and weighed. Pcyt2 knockdown in both male and female mice demonstrated little change in tissue weight, when compared to wild-type (WT) litter mates. However, when normalized to total body weight, PEA treated male Pcyt2\(^{(+/-)}\) mice showed a significant increase in skeletal muscle mass, while showing no effect on the other tissues (Figure 4 A). Female Pcyt2\(^{(+/-)}\) mice treated with PEA showed no changes in tissue mass when compared to WT and untreated Pcyt2\(^{(+/-)}\) litter mates (Figure 4 B).

3.1.2 PEA improves liver damage.

Biochemical analysis was performed on mouse serums for metabolites that are markers of metabolic syndrome. Cholesterol, triglyceride, and glucose, some of the many markers for obesity-related disorders, did not change with the knockdown of Pcyt2, and is not improved by PEA supplementation (Figure 5 A). Similarly, urea, a marker for kidney function, remained the same across all groups (Figure 5 A). ALT and AST are liver-specific enzymes commonly used as markers for liver function. High serum concentrations of ALT and AST indicate that there is
liver damage, and is commonly noted in those with NAFLD and NASH. $P_{cyt2}^{(+/-)}$ mice, which develop hepatosteatosis [39], have elevated serum concentrations of ALT when compared to WT (Figure 5 B). The addition of PEA to $P_{cyt2}^{(+/-)}$ mice restored ALT concentrations to that seen in the WT group, and reduced concentrations of AST, indicating that PEA has the ability to restore liver function that is lost by $P_{cyt2}$ knockdown.

**Figure 3.** PEA supplementation has no effect on overall body weight in $P_{cyt2}^{(+/-)}$ mice. The weight (in grams) of male (A) and female (B) mice during eight weeks of treatment demonstrated that PEA does not reduce body weight. Results are expressed as mean ± SEM (n=3-5 per group).
Figure 4. PEA increases muscle mass in male Pcyt2\(^{+/−}\) mice. Male Pcyt2\(^{+/−}\) mice (A) showed a significant increase in muscle mass when normalized to overall body weight. Female Pcyt2\(^{+/−}\) HET mice (B) showed no differences in tissue weight. Results are expressed as mean ± SEM (n=3-5 per group). Statistical significance determined by one-way ANOVA with Tukey’s post hoc test. *p<0.05.
Figure 5. Serum parameters show that PEA improves liver damage. PEA supplementation does not change serum amounts of cholesterol, glucose, triglyceride, and urea (A). However, in Pcyt2\(^{+/−}\) mice, PEA decreases ALT and AST (B), which are biochemical markers of liver damage. Male and female values were combined. Results are expressed as mean ± SEM (n=6-8 per group). Statistical significance determined by one-way ANOVA with Tukey’s *post hoc* test. *p<0.05.
3.1.3 PEA stimulates the insulin signaling cascade and mTOR activation in muscle tissue.

The insulin signaling cascade includes a variety of proteins, whose activation or inactivation can result in different cellular processes, including cell growth and proliferation, and protein synthesis. Insulin receptor substrate 1 (IRS-1), phosphoinositide 3-kinase (PI3K), and protein kinase B (PKB/Akt) are important regulators of this signaling cascade. Due to the increase in muscle mass that was seen in male Pcyt2\(^{(+/)}\) mice, the proteins from this cascade were investigated. These proteins were isolated from the collected skeletal muscle tissue of WT, HET, and HET+PEA mice and their content was examined by immunoblotting (Figure 6 A-C). Pcyt2 knockdown had no effect on IRS-1 protein content; and the addition of PEA did not play a role in increasing its expression (Figure 6 A). The same trend was noted for PI3K expression (Figure 6 B). PEA induced Akt activation by phosphorylation (Figure 6 C). Akt activation results in multiple signaling events, including the phosphorylation and activation of mammalian target of rapamycin (mTOR). The activation of Akt by PEA is also demonstrated by the activation of mTOR, seen only in the HET+PEA group (Figure 6 D).
Figure 6. Expression of proteins involved in insulin signalling and protein synthesis are upregulated by PEA in muscle tissue. IRS-1 (A) and PI3K (B) protein expression are unchanged in Pcyt2<sup>−/−</sup> mice following the addition of PEA. Increases in the phosphorylation, and activation, of Akt (C) and mTOR (D) occur as a result of PEA supplementation. Results are expressed as mean ± SEM (n=3 per group). Statistical significance determined by one-way ANOVA with Tukey’s post hoc test. *p<0.05.
3.1.4 PEA inhibits fatty acid synthesis by inducing mitochondrial biogenesis in muscle.

Sirtuin 1 (Sirt1) and AMP-activated protein kinase α (AMPKα) are both mediators of mitochondrial biogenesis, and in turn beta-oxidation and ATP production in cells. In Pcyt2(+/-) mouse skeletal muscle, Sirt1 protein content is decreased (Figure 7 A). PEA supplementation restored some of Sirt1 protein content in the Pcyt2(+/-) mice, although not to that of WT (Figure 7 A). As a kinase, AMPKα, which is active when phosphorylated, has the ability to phosphorylate other proteins. Acetyl-CoA carboxylase (ACC), who plays a role in fatty acid synthesis, is one of the many proteins whose activity is in part controlled by AMPKα phosphorylation. ACC phosphorylation, and inactivation, was not affected by Pcyt2 knockdown (Figure 7 C). However, PEA supplementation demonstrated greater ACC inactivation when compared to both WT and Pcyt2(+/-) mice. This effect was seen without changes in AMPKα activation (Figure 7 B).
Figure 7. Mitochondrial biogenesis is stimulated, while FA synthesis is impaired by PEA in muscle. Sirt1 protein expression (A) is downregulated as a result of Pcyt2 knockdown. PEA greatly increased the phosphorylation of ACC (C). PEA had no effect on the phosphorylation of AMPKα (B). Results are expressed as mean ± SEM (n=3 per group). Statistical significance determined by one-way ANOVA with Tukey’s post hoc test. *p<0.05.
3.1.5 PEA does not impact insulin signaling and mitochondrial biogenesis in hepatic tissue.

The above data demonstrated that PEA supplementation resulted in changes in the insulin-signaling cascade, and fatty acid synthesis in skeletal muscle, therefore it was important to see if the same changes occurred in the liver. The same insulin signaling and mitochondrial biogenesis proteins examined in skeletal muscle were also examined by immunoblotting in hepatic tissue collected from the WT, HET, and HET+PEA groups (Figure 8 A-D; Figure 9 A-C). Unlike muscle tissue, PEA increased PI3K protein content (Figure 8 B), while having no effect on protein content of IRS-1 (Figure 8 A), or the activation of Akt and mTOR (Figure 8 C, D). In hepatic tissue, PEA played no role in increasing Sirt1 protein content (Figure 9 A), but did lower the quantity of phosphorylated and activated AMPKα (Figure 9 B). Lack of AMPKα activation seen in Pcyt2\(^{(+/-)}\) mice supplemented with PEA had no effect on ACC inactivation (Figure 9 C).
Figure 8. PEA increases the expression of PI3K in the liver. IRS-1 protein expression (A), and activation of Akt (C) and mTOR (D), in the liver is unaffected by PEA. PI3K protein expression (B) is stimulated in Pcyt2^{+/-} mice following PEA supplementation. Results are expressed as mean ± SEM (n=3 per group). Statistical significance determined by one-way ANOVA with Tukey’s post hoc test. *p<0.05
Figure 9. **PEA has no effect on mitochondrial biogenesis and does not inhibit FA synthesis within the liver.** PEA had no effect on the expression of Sirt1 (A). AMPKα phosphorylation, and activation, (B) decreases as a result of Pcryt2 knockdown, and is not improved with the addition of PEA. Phosphorylation and inactivation of ACC is unaffected by PEA (C). Results are expressed as mean ± SEM (n=3 per group). Statistical significance determined by one-way ANOVA with Tukey’s *post hoc* test. **p<0.01.
3.1.6 PEA supplementation alleviates hepatosteatosis.

Liver steatosis occurs as a result of Pcyt2 knockdown in mice [39]; however, it is unknown if PEA has the ability to alleviate it. Liver sections were collected and stained with H&E to view the quantity and size of lipid droplets present. In Pcyt2\(^{(+/-)}\) mice, there were a large number of lipid droplets that accumulated within the liver (Figure 10 A, middle panels), when compared to WT (Figure 10 A, left panels). PEA supplementation reduced the accumulation of lipid droplets (Figure 10 A, right panels). This result is further shown through a triglyceride assay performed on liver samples (Figure 10 B). Similar to the H&E data, the triglyceride assay showed that Pcyt2\(^{(+/-)}\) mice have an increased quantity of triglycerides in the liver, and it is reduced by PEA supplementation (Figure 10 B).

3.1.7 PEA stimulates the expression of lipid metabolism genes.

In order to explain the decrease in the accumulation of lipid droplets in the liver of Pcyt2\(^{(+/-)}\) mice following PEA supplementation, it was important to examine the effects of PEA on the mRNA expression of genes involved in lipid metabolism. PEA had no effect on the hepatic mRNA expression of triacylglycerol hydrolase (Tgh) and Lpl (Figure 11 A, B), however it increased the expression of Atgl (Figure 11 C). PEA had no effect on Ppara or Ppar\(\gamma\) expression (Figure 11 D, E).
Figure 10. Hepatosteatosis is improved by PEA in Pcyt2<sup>−/−</sup> mice. H&E stained liver sections demonstrate that Pcyt2 knockdown results in hepatosteatosis, the accumulation of lipids in the liver (A, middle panels). PEA reduces the accumulation of these lipid droplets within the liver (A, right panels). A triglyceride assay (B) further showed this result, as liver triglycerides are increased as a result of Pcyt2 knockdown, and reduces following PEA supplementation. Results are expressed as mean ± SEM (n=3 per group). Statistical significance determined by one-way ANOVA with Tukey’s post hoc test. *p<0.05, **p<0.01.
Figure 11. Hepatic mRNA expression of lipolytic genes are increased by PEA. The mRNA expression of Tgh (A), Lpl (B), Ppara (D), and Pparγ (E) are unchanged following the addition of PEA in Pcyt2(+/−) mice. However, PEA did increase the expression of Atgl (C). Results are expressed as mean ± SEM (n=3 per group). Statistical significance determined by one-way ANOVA with Tukey’s post hoc test. *p<0.05.
3.1.8 PEA alleviates liver fibrosis.

Fibrosis is the presence of scar tissue that can occur due to the accumulation of lipids. In the Pcyt2\(^{+/−}\) mouse model, it was clearly demonstrated that there is a large amount of lipid droplet accumulation. Therefore, it was important to examine if fibrosis develops as a result of lipid accumulation in this model, and if PEA can also alleviate it. Liver sections were stained with picrosirius red, which stains areas of fibrosis a dark red colour. Pcyt2 knockdown does lead to fibrosis, as noted by a thicker deep red colour along the edges of the liver (Figure 12, middle panels). Similarly to what occurred with lipid droplet accumulation, PEA reduces the severity of fibrosis within the liver of Pcyt2\(^{+/−}\) mice (Figure 12, right panels).

Figure 12. PEA reduces fibrosis within the liver. Picrosirius stained liver segments demonstrate that PEA has the ability to reduce fibrosis in Pcyt2\(^{+/−}\) mice, as denoted by the deep red stain.
3.1.9 Most phospholipid metabolism genes are stimulated by PEA.

Pcyt2 is an important component of the CDP-ethanolamine branch of the Kennedy pathway. Since the mouse model is based upon impairments in this pathway, it was important to look at the effects that PEA had on other components of the Kennedy pathway. It was expected that Pcyt2 knockdown would alter phospholipid metabolism so PE content is maintained. Therefore, reductions in the expression of genes involved in reactions that utilize PE, as well as increases in genes involved in synthesizing PE from existing phospholipids, would be noted. However, this was not the case. With the exception of Pss1, Pcyt2 knockdown in mice had no effect on the expression of other phospholipid metabolism genes, when compared to WT mice (Figure 13 A-G). However, the addition of PEA altered the expression of some of phospholipid metabolism genes. The addition of PEA increased the expression of Ctl-1 (Figure 13 A), but did not change the expression of Pcyt1(Figure 13 B). The expression of Pss1 and Pss2 did not change following PEA supplementation (Figure 13 C, D). Like Ctl-1, the expression of Psd increased following PEA supplementation (Figure 13 E). In addition, PEA did not alter the expression of Pemt and betaine-homocysteine S-methyltransferase (Bhmt) (Figure 13 F, G).
Figure 13. PEA stimulates the mRNA expression of phospholipid metabolism genes. The mRNA expression of Ctl-1 (A) and Psd (E), increased in PEA treated Pcyt2+/− mice. The expression of Pss1 (C) decreased due to the knockdown of Pcyt2, however is not restored following PEA supplementation. The expression of Pcyt1 (B), Pss2 (D), Pemt (F), and Bhmt (G) remain unchanged. Results are expressed as mean ± SEM (n=3 per group). Statistical significance determined by one-way ANOVA with Tukey’s post hoc test. *p<0.05, **p<0.01.
3.2 Effect of PCYT2 knockdown on human fibroblast phospholipid and lipid metabolism.

3.2.1 PCYT2 knockdown leads to alterations in phospholipid metabolism gene expression.

Similarly to the Pcyt2 knockdown mouse model, knockdown of this critical gene in human fibroblasts lead to alterations in phospholipid metabolism. Specifically, KD4 cells had reduced mRNA expression of PCYT1, PSS1, PSS2 and PEMT (Figure 14 B-D, F). The mRNA expression of CTL-1 and PSD remained unchanged (Figure 14 A, E).

**Figure 14.** Alterations in the expression of phospholipid metabolism genes in human PCYT2 knockdown cells. Human fibroblast cells with PCYT2 knockdown (KD4) show decreased expression of PCYT1 (B), PSS1 (C), and PSS2 (D). All measurements were performed in triplicates and results are expressed as mean ± SEM (n=3 per group). Statistical significance between groups is determined by one-tailed Student’s t-test. *p<0.05, **p<0.01, ***p<0.001.
3.2.2 *Pcyt2* knockdown induces lipogenesis.

Lipogenesis is increased in *Pcyt2*\(^{(+/-)}\) mice to provide FAs for TAG synthesis from DAG [39]. Therefore, lipid metabolism was examined in KD4 cells to see if *PCYT2* knockdown cause increases in lipogenesis. The lipolytic enzyme, *TGH*, was decreased in KD4 cells, but had no effect on *ATGL* expression (Figure 15 A, B). In addition, mRNA expression of stearoyl-CoA desaturase-1 (*SCD1*), which catalyzes the formation of monounsaturated fatty acids, as well as the expression of *DGAT2*, the enzyme responsible for TAG synthesis from DAG, was increased (Figure 15 C, D). *PPARα* expression remained unchanged, while expression of *PPARγ* was increased due to *PCYT2* knockdown (Figure 15 E, F).

![Figure 15. mRNA expression of lipid metabolism genes following *PCYT2* knockdown in human fibroblasts.](image)

*Figure 15. mRNA expression of lipid metabolism genes following *PCYT2* knockdown in human fibroblasts.* In human fibroblast cells, *PCYT2* knockdown lead to the increased expression of lipogenic genes, *SCD1* (C) and *DGAT2* (D). The expression of *TGH* (A) was decreased as a result of *PCYT2* knockdown. In addition, *PPARγ* (F) expression increased. All measurements were performed in triplicates and results are expressed as mean ± SEM (n=3 per group). Statistical significance between groups is determined by one-tailed Student’s *t*-test. *p*<0.05, **p**<0.01.
There has been continuing interest as to how phospholipid metabolism can contribute to various diseases; however, little has been reported on what can be done in order to prevent or reverse the progression. The main focus of this thesis was the role of Pcyt2. Pcyt2 plays an integral role in the de novo synthesis of PE in the CDP-ethanolamine branch of the Kennedy pathway. As the rate limiting enzyme, changes in Pcyt2 content or function can result in alterations in phospholipid content, which can contribute to the development of multiple diseases. Pcyt2 heterozygous (Pcyt2\(^{+/-}\)) mice specifically develop fatty liver and have impairments in the insulin signaling pathway [39]. In attempts to reverse this phenotype, the effects of stimulating the Pcyt2 reaction in the CDP-ethanolamine branch of the Kennedy pathway was examined. Previous work from our lab demonstrated that choline supplementation has the ability to reverse hepatosteatosis and the impaired muscle insulin signaling in Pcyt2\(^{+/-}\) mice by increasing PC turnover and FA oxidation [49]. The current study examined the effects of Pcyt2 substrate, phosphoethanolamine (PEA), on reversing the liver and muscle phenotype in Pcyt2\(^{+/-}\) mice, as well as how Pcyt2 shRNA knockdown altered phospholipid and lipid metabolism in human fibroblast cells.

4.1 The effects of PEA supplementation on Pcyt2\(^{+/-}\) mice.

4.1.1 PEA effect on weight gain and serum metabolites.

Unlike choline supplementation [49], PEA did not significantly reduce weight gain in both male and female Pcyt2\(^{+/-}\) mice, following an eight-week supplementation period (Figure 3 A-B). In addition, liver weight and adipose tissue weight were not different between mouse groups (Figure 4 A-B). Interestingly, male Pcyt2\(^{+/-}\) demonstrated a significant increase in
muscle mass following supplementation with PEA (Figure 4 A), indicating that PEA played a role in inducing protein synthesis in \( Pcyt2^{(+/-)} \) mice.

Analysis of serum metabolites postprandially and after fasting provides a broad insight into underlying metabolic and organ health concerns [55-57]. Across all mice groups, postprandial serum concentrations of these metabolites did not change significantly with PEA treatments (Figure 5 A). Future analysis on blood samples after fasting may confirm if circulating glucose and triglyceride levels were affected by PEA, as well as if oral glucose and insulin tolerance impairments in this model [39] are improved by PEA.

The status of the liver in \( Pcyt2^{(+/-)} \) mice, with and without PEA, was established based on the serum ALT and AST levels. Serum concentrations of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are important biomarkers of liver damage [58-60]. ALT and AST are intracellular hepatic enzymes, therefore their presence in serum indicates leakage of these enzymes from the hepatocyte, into circulation as a result of hepatocyte damage [58, 60]. The liver plays an important role in maintaining glucose concentrations and distributing lipids to peripheral tissues [59]. Loss of normal hepatic function is correlated with the progression of obesity, diabetes, and non-alcoholic fatty liver disease (NAFLD); therefore, ALT and AST serum concentrations can be used as early detection markers of these diseases [59]. \( Pcyt2^{(+/-)} \) mice demonstrated an increase in serum ALT concentrations, indicating that there was significant damage done to hepatocytes in these mice (Figure 5 B). The addition of PEA reduced ALT and AST serum concentrations suggesting that PEA played a beneficial role in the liver. The reduction in ALT and AST concentrations, as well as the increase in skeletal muscle mass in male \( Pcyt2^{(+/-)} \) mice, that occurred following PEA supplementation, provided initial evidence that
PEA played a beneficial role in $P_{cyt2}^{(r/-)}$ hepatic metabolism and skeletal muscle. Therefore, the specific action of PEA on these tissues was investigated in more detail.

4.1.2 Role of PEA on skeletal muscle protein metabolism.

Loss of skeletal muscle mass and strength is a normal process that occurs as people age but can be worsened by the presence of certain diseases, including diabetes [61]. This is process is termed myopathy [61]. Myopathy has been suggested to be due to the lack of insulin signaling cascade activation in those with insulin resistance [62-63, 65-67], leading to decreases in overall protein synthesis [64]. Insulin signaling occurs through the IRS-1/PI3K/Akt signaling cascade, where insulin binding causes the phosphorylation of insulin receptor substrate 1 (IRS-1) and downstream activation of phosphoinositide 3-kinase (PI3K) and protein kinase B (Akt/PKB) [66]. In diabetic muscle, this signaling cascade is suppressed, resulting in the activation of ubiquitin proteasome pathway (UPP), as well as caspase-3 [62-63, 66]. PI3K has been deemed the master mediator of these responses [62-63, 66]. Reductions in PI3K activity have been associated with increases in the proteolytic activities of caspase-3 and UPP [62, 66], as well as increases in the production of pro-apoptotic factors [63, 66]. In contrast, activation of PI3K results in the downstream activation of mammalian target of rapamycin (mTOR). mTOR is a multi-domain protein that belongs to the PI3K-related kinase family and is stimulated by a variety of hormones and growth factors, including insulin [67]. When activated, mTOR regulates processes that favour cell and tissue growth, as well as protein synthesis [67].

Due to the increase in muscle mass that occurred in male $P_{cyt2}^{(r/-)}$ mice, which has previously demonstrated to develop insulin resistance [39], it was important to examine how this insulin signaling cascade was affected, and how it could have stimulated protein synthesis. PEA supplementation lead to the increased expression and activation of the insulin signaling cascade
as noted through the increase in Akt phosphorylation (Figure 6 C). The additional notable increase in mTOR activation suggests that there is enhanced protein synthesis in the muscle via mTOR-mediated pathways (Figure 6 D), that may be stimulated by Akt in the insulin signaling cascade. This is a significant result as traditional insulin treatment in diabetics does not have the ability to increase protein synthesis, therefore playing no role in managing the progression of myopathy [68]. PEA proved to be a more successful treatment option for this complication due to its ability to not only improve insulin signaling, but also stimulate protein synthesis. In contrast to skeletal muscle, PEA showed to have a modest effect on the insulin signaling pathway in hepatic tissue. Only the expression of PI3K was enhanced by PEA (Figure 8 B), and there was no effect on mTOR activation.

4.1.3 PEA suppresses fatty acid synthesis in skeletal muscle, but not in the liver.

Increased fatty acid (FA) uptake has been demonstrated in both diabetic and obese models [69-71], which may contribute to muscle insulin resistance. Skeletal muscle FA oxidation occurs predominately in the mitochondria, in order to reduce lipid accumulation, as well as provide energy in the form of ATP [72]. When AMP levels are increased within a cell, AMP-activated protein kinase (AMPK) is activated [72-74]. AMPK is a heterotrimeric protein consisting of a catalytic domain (α) and two regulatory domains (β, γ) [72-74]. Increases in AMP within the cell results in a conformational change in AMPK, allowing for the phosphorylation of the α domain [72-74]. Once activated, AMPK suppresses ATP-consuming processes, such as FA and cholesterol synthesis, and promotes ATP synthesizing processes, such as FA oxidation and glucose uptake [72]. AMPK does this by regulating the actions of acetyl-CoA carboxylase (ACC) and HMG-CoA reductase (HMGR) [72]. When dephosphorylated, ACC is more active and catalyzes the synthesis of malonyl-CoA, which is used to synthesize FAs, as well as inhibit
mitochondrial FA oxidation [72]. PEA has demonstrated the ability to enhance ACC inhibition without impacting the activity AMPK (Figure 7 B, C). By suppressing the activity of ACC through PEA supplementation, FA oxidation can occur more readily within skeletal muscle, which can ultimately improve insulin sensitivity.

AMPKα also has the ability to regulate gene expression [72, 74]. Peroxisome proliferator-activated receptor α (PPARα) and PPAR gamma coactivator 1-alpha (PGC-1α) are two of the many transcription factors and co-activators that are regulated by AMPKα [72, 74]. These transcription factors are also regulated through a deacetylation process mediated by Sirtuin 1 (Sirt1) [72, 74]. When stimulated by either AMPKα or Sirt1, PPARα and PGC-1α promotes mitochondrial biogenesis [74], thereby providing more mitochondria for FA oxidation and ATP production. Sirt1 expression within skeletal muscle was decreased as a result of Pcyt2 knockdown (Figure 7 A), suggesting that there may have been impairments in PPARα and PGC-1α activation and in turn, mitochondrial biogenesis would not have occurred readily to enhance FA oxidation. Although not significant, PEA seemed to restore Sirt1 expression.

Interestingly, these effects are not noted in the liver (Figure 9 A-C). Within the liver, PEA reduced AMPKα expression (Figure 9 B). Sirt1 expression and phosphorylation of ACC remained unchanged across all groups, suggesting that these proteins were not impaired by Pcyt2 knockdown, nor were they enhanced by PEA (Figure 9 A, C).

4.1.4. Steatohepatitis is improved by PEA.

Heptatosteatosis, or NAFLD, is a growing concern among the obese and/or insulin resistant population [43, 47]. The pathogenesis of NAFLD is not clear; however, insulin resistance shows to be the predominant contributing factor [43, 47, 75], with obesity being the most common cause of the insulin resistant state [43]. Lack of insulin signaling in intra- and
extrahepatic tissue contributes to the increased uptake, and decreased export, of FAs from the liver, resulting in NAFLD development. For example, lack of insulin signaling within adipose tissue results in decreased lipolysis and increased release of FAs into circulation [43, 75]. In the liver, uptake of free FAs from circulation occurs readily; however, the lack of insulin signaling results in enhanced TAG and de novo FA synthesis, impaired FA oxidation, and decreased release of TAG into circulation through very low-density lipoprotein (VLDL) secretion [43, 75]. The large accumulation of DAG and TAG within the liver results in an inflammatory response that can lead to the development of non-alcoholic steatohepatitis (NASH), in which fibrosis is present within the liver [45-47].

Treatment options for both NAFLD and NASH are limiting. Metformin, a common medication taken by type 2 diabetics, is used to improve insulin sensitivity in multiple tissues [43]. Due to its actions on insulin signaling, metformin was thought to have the ability to reduce NAFLD progression; however, one review demonstrates that the results are contradictory [43]. There is no known treatment for NASH development. Our Pcyt2(+/−) model has shown to develop hepatosteatosis both in previous work [39], as well as in our current work (Figure 10 A). PEA was successful in reducing the accumulation of FAs and TAG within the liver (Figure 10 A, B), as well as reducing the progression of fibrosis (Figure 12). Although the improvement in NAFLD and NASH may have been due to the increased utilization of DAG in the Kennedy pathway, it is important to note that lipolysis in the liver was also enhanced through the actions of adipose triglyceride lipase (ATGL) (Figure 11 C). Although the name implies that this enzyme is exclusive to adipose tissue, it is also a principal lipase in other tissues, including skeletal muscle and liver [75]. Although not the only hepatic lipase, ATGL is highly active in the liver, as silencing hepatic ATGL results in a 60% decrease in TAG hydrolysis [76]. The ability
of PEA to increase Atgl expression may explain the reduction in TAG accumulation that are noted. Taken together with the previous result showing that PEA improved ALT/AST plasma concentrations, as well as PI3K expression in the liver, PEA proved to be a successful prevention option NAFLD and NASH in diabetic and/or obese individuals.

4.1.5 Alterations in phospholipid metabolism occur following PEA supplementation.

It was previously demonstrated that Pcyt2 knockdown in mice resulted in no changes in phospholipid content [39], suggesting that there are compensatory mechanisms in place to maintain phospholipid homeostasis. To further understand any compensatory changes that may be occurring as a result of Pcyt2 knockdown and PEA supplementation, mRNA expression of key enzymes responsible for PC, PS, and PE synthesis from existing phospholipids were examined. With the exception of Pss1, mRNA expressions of relevant Kennedy pathway enzymes were not changed due to Pcyt2 knockdown in mouse hepatic tissue (Figure 13 A-G). This suggests that there were few compensatory mechanisms in place to maintain phospholipid content within this tissue. However, PEA supplementation was able to enhance the expression of some key regulators of phospholipid metabolism. Firstly, expression of choline transporter-like proteins (CtlI) was increased as a result of PEA (Figure 13 A). CTL1 is highly expressed on the plasma membrane [77] and outer mitochondrial membrane [78] of cells. Choline that enters the cell is subjected to phosphorylation by CK and enters the Kennedy pathway for PC synthesis, which is highly important in the liver due to PC’s abundance in VLDL membranes [26]. Choline not used for PC synthesis may enter mitochondria through CTL1 on the outer mitochondrial membrane [78]. Once transported into the mitochondria, choline is oxidized to betaine by betaine-homocysteine S-methyltransferase (BHMT), which then enters the S-adenosylmethionine (SAM) pathway. SAM is a methyl donor that influences a wide variety of
biological processes including gene expression through DNA methylation, as well as cell growth and differentiation [79]. In the liver, reductions in SAM biosynthesis have been associated with the progression of liver cirrhosis [80-81]. In addition, hepatocyte growth is partly regulated by SAM [79]. Although there were no changes in Bhmt expression in the liver following Pcyt2 knockdown and PEA supplementation (Figure 13 G), the increase that was seen in Ctl1 by PEA may be acting to provide choline for PC synthesis, as well as betaine synthesis for the SAM pathway. This increase in Ctl1 may have provided further evidence that PEA improves hepatosteatosis and liver fibrosis by increasing choline uptake for synthesis of PC for VLDL secretion, and also betaine oxidation to provide methyl groups that aid in hepatocyte growth.

The reduction in Pssl expression seen following Pcyt2 knockdown is not restored by PEA (Figure 13 C). PSS1 controls the synthesis of PS from PC, suggesting that PS synthesis from PC was not readily occurring in our Pcyt2 knockdown mice. This may have been due to the need of PC for other biological processes. However, PEA did enhance the expression of Psd (Figure 13 E), demonstrating that PE homeostasis was maintained through PS decarboxylation. Although this data provides some evidence of compensatory mechanisms, it would be of interest to further investigate the CDP-choline pathway in order to further understand the compensatory changes that may be occurring. It would also be beneficial to examine how the mRNA expression of these Kennedy pathway enzymes translates to protein expression and activity.

4.2 PCT2 knockdown in human fibroblasts alters phospholipid and lipid metabolism.

The previous [39, 49] and current work on Pcyt2(+/−) mice provided a clear understanding on how Pcyt2 deficiency resulted in altered lipid and phospholipid metabolism. However, how this deficiency translates to humans is not well understood. Using a human fibroblast cell line
(MCH58), a PCYT2 deficient cell line (KD4) was developed in order to investigate the metabolic changes that occur. KD4 cells have shown to be an excellent model to demonstrate cellular changes that result from PCYT2 knockdown [56, 82]. These cells have consistently shown that PCYT2 knockdown is successful, both through mRNA expression [56], as well as protein expression of PCYT2 [54]. In addition, PCYT2 knockdown in these cells show impairments in the CDP-ethanolamine branch, as demonstrated by increases in P-Eth and decreases in CDP-Eth [54, 56], and also shows upregulations in the CDP-choline branch enzymes and intermediates [82]. Consistent with the Pcyt2(+/-) mouse model [39], KD4 cells show no changes in phospholipid content [54, 56, 82], making these cells an excellent model to examine how PCYT2 knockdown may translate to a human model, as they closely resemble our Pcyt2(+/-) mouse model.

Current preliminary data on KD4 cells demonstrated that PCYT2 knockdown impacted phospholipid and lipid metabolism processes. As done with the mouse model, enzymes involved in phospholipid synthesis were examined. The unchanged expression of CTL1 and PSD (Figure 14 A, E) and the decreased expression of PSSI (Figure 14 C) as a result of PCYT2 knockdown was consistent with that of the mouse model. Interestingly, KD4 cells demonstrated a decrease in PCYT1, PSS2, and PEMT expression (Figure 14 B, D, F). Reductions in PCYT1 may result in reduced de novo synthesis of PC to compensate for the lack of PE being synthesized. This is important in order to maintain correct PC/PE ratios within a membrane. Due to the lack of PCYT1, less PC is available for PS synthesis via PSS1, explaining the reduction that was seen in PSSI in this model. Due to the lack of PE being synthesized de novo, expression of PSS2 and PEMT, both of which utilize PE, was reduced in order to preserve the PE being made for other processes. The current findings suggest that synthesis of PC, PE, and PS from existing
phospholipids occurred minimally under the conditions of PCYT2 knockdown. Performing additional experiments with this cell line would provide further insight as to how membrane phospholipids are maintained under conditions of PCYT2 knockdown. This can be done using $[^3\text{H}]$-glycerol, to measure the synthesis, turnover, and pool sizes of individual phospholipids (PE, PC, and PS), in addition to DAG and TAG content. Pulse and pulse-chase radiolabeling with $[^3\text{H}]$-acetate could examine the rates of FA synthesis and remodeling of lipids, thereby providing insight to the compensatory mechanisms in place by the cell to maintain phospholipid homeostasis.

Although the effects of PCYT2 knockdown on phospholipid metabolism in human fibroblast cells has been studied extensively, the role it plays on lipid metabolism has yet to be examined. Our current data shows that PCYT2 knockdown shifted lipid metabolism in order to favour lipogenesis (Figure 15 A-F). Stearoyl-CoA desaturase-1 (SCD1), an enzyme that catalyzes the synthesis of monounsaturated FAs from saturated FAs, has been associated with the development of diet-induced hepatic insulin sensitivity [83]. The increase in SCD-1 expression demonstrated by the KD4 cells (Figure 15 C) suggest that PCYT2 knockdown in this model would result in impaired insulin signaling. TAG synthesis is also increased in this model, as shown through an increased expression of DGAT2 (Figure 15 D). The accumulation of TAG is further exacerbated by the decrease in TGH expression (Figure 15 A).

Although preliminary, the current data involving KD4 cells demonstrated that lipogenesis and elevated TAG may indeed develop as a result of lack of PCYT2 in a human model. Along with the impairments in phospholipid metabolism, which has been discussed to be a contributing factor in metabolic disease development, further validation is provided by the impairments that also occur in lipid metabolism. By favouring lipogenesis, this model is at risk of developing
obesity, insulin resistance, and hepatosteatosis. Further investigation into the effects of PCYT2 knockdown on other human tissues, including skeletal muscle and hepatocytes, should be completed. This will provide insight on insulin resistance and hepatosteatosis development in a human model.

4.3 Future directions

Although the work presented in this thesis shows promising results that PEA improved steatohepatitis and promoted skeletal muscle insulin signaling and growth in Pcyt2(+/-) mice, it would be beneficial to continue with these experiments to gather more information in the areas of reduction in body weight, glucose metabolism, and insulin resistance. In this study, the number of mice, separated by sex, was not sufficient to provide concrete conclusions on weight gain. Furthermore, the plasma metabolites from both sexes needs to be measured separately, with more animals, and at a fasted state. It would also be beneficial to perform an oral glucose tolerance test to ensure that there is a systemic reduction in glucose levels and improvements in insulin sensitivity after PEA treatment.

Additional findings on PEA’s actions on DNA methylation was not presented within this thesis and will also be examined further. PEA treated Pcyt2(+/-) mice demonstrated increases in DNA methylation of four gene targets; Dnajb7, Atg4a, Pitpna, Pnpla2. These genes control a wide variety of cellular processes that may have played a role in improving liver function. How the increases in DNA methylation impacted mRNA and protein expression of these genes are currently being investigated and will hopefully provide further insight into PEA’s beneficial role in the liver.

There is also evidence presented in this thesis that may indicate that PEA may be beneficial for diabetics. In addition to impaired insulin signaling, both type 1 and type 2 diabetics
experience various complications, including diabetic myopathy and NAFLD/NASH. Due to PEA’s success in reducing hepatosteatosis through increased lipolysis, stimulating the insulin signaling pathway, and inducing protein synthesis through mTOR activation in our \( Pcyt2^{(+/-)} \) model, examining the effects of PEA supplementation on a diabetic mouse model would be of interest. By doing so, further insight into a potential new therapeutic treatment would be provided, as traditional insulin therapy does not alleviate the additional complications that arise in diabetics. Furthermore, pancreatic beta cell loss is the primary cause of type one diabetes. Through the activation of mTOR, PEA may prevent pancreatic beta cell loss by stimulating cell growth and proliferation. Therefore, supplementing a pancreatic beta cell line with PEA may provide further validation that PEA is a successful therapeutic treatment for type one diabetes.

### 4.4 Conclusion

The work presented in this thesis demonstrated that stimulating the CDP-ethanolamine pathway by PEA was able to reverse the development of NASH, that is phenotypic to \( Pcyt2^{(+/-)} \) mice, and enhance skeletal muscle insulin signaling and muscle mass within this model. The current study clearly demonstrated that PEA stimulates the flux through the CDP-ethanolamine branch of the Kennedy pathway and improves tissue lipolysis to result in reduced liver lipid accumulation and fibrosis, and also stimulates the insulin signaling pathway and mTOR to induce protein synthesis, while also inhibiting FA synthesis in skeletal muscle. PEA has demonstrated the ability to be a therapeutic treatment for diseases that arise from altered lipid metabolism, impaired insulin signaling, or loss of cellular integrity. Furthermore, this thesis demonstrated that phospholipid metabolism is altered as a result of \( PCYT2 \) knockdown in human fibroblasts, and lipogenesis is favored. This suggests that this knockdown could also result in the development of lipid-related diseases in humans.
5.0 References


