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THE EFFECT OF STEROL CARRIER PROTEIN 2 GENE ABLATION AND DIETARY CHOLESTEROL ON BRAIN AND LIVER LIPID COMPOSITION IN MALE AND FEMALE MICE

by

Madison Delane Jochim
Bachelor of Science, University of North Dakota, 2019

A Thesis

Submitted to the Graduate Faculty

of the

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in partial fulfillment of the requirements

for the degree of

Master of Science

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This thesis, submitted by Madison Delane Jochim, in partial fulfillment of the requirements for the Degree of Master of Science from the University of North Dakota, has been read by the Faculty Advisory Committee under whom the work has been done and hereby approved.

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7/25/2023

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Department	Biomedical Sciences
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Madison Delane Jochim
July 2023

TABLE OF CONTENTS

LIST OF TABLES

Table Page

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For my father

ABSTRACT

Previous studies have shown that sterol carrier protein 2 (SCP-2) and sterol carrier protein x (SCP-x) are important for mediating the transfer of phospholipids, cholesterol and other sterols within the cell. However, the differences between males and females regarding the impact *SCP2* has on brain and liver lipid metabolism is unknown. To further understand this, we profiled the effects of *SCP2* gene ablation on brain and liver lipid metabolism in male and female mice fed a high fat diet or control diet and analyzed phospholipid and neutral lipid mass and composition using HPLC and TLC. In brains of male mice, *SCP2* gene ablation led to significant increase in brain phosphatidylcholine (PtdCho), and an overall increase in total phospholipid levels in male mice fed a high cholesterol diet. In brains of female mice, there was a significant reduction in cholesterol compared to male mice. In liver, male mice fed a high cholesterol diet had a significant increase in total phospholipid mass accounted for by an increase in ChoGpl mass compared to mice fed a control diet and was seen in both wild type and gene-ablated mice. In both male and female mice there was between an increase in cholesteryl ester mass in mice fed a high cholesterol diet compared to mice fed a control diet. These results demonstrate that *SCP2* facilitates brain and liver ChoGpl, cholesterol, and cholesteryl ester metabolism. Results also suggest that a high cholesterol diet increases cholesterol ester synthesis in the liver where SCP-2 and SCP-x is important for regulating

lipid metabolism. There may also be sex-specific differences in liver and brain lipid metabolism and steady-state lipids levels.

CHAPTER I

INTRODUCTION

Sterol carrier proteins (SCP) are a family of proteins that are either 13.2 kDa or 58 kDa depending on cellular location (Ohba et al., 1994a). The *SCP2* gene is alternatively spliced and is highly conserved across mammalian species and encodes two sterol carrier proteins(Pfeifer et al., 1993). These proteins are SCP-x (58 kDa) and proSCP-2 (15 kDa) each having independent initiation sites and promoters (Moncecchi et al., 1991). ProSCP-2 undergoes post-translational cleavage to form SCP-2 (13.2 kDa) (Murphy, 2002; Schroeder et al., 2000). ProSCP-2 is targeted to the mitochondria and the cytoplasmic side of the peroxisome via N-terminal twenty amino acid targeting sequence and C-terminal sequence, respectively (Ohba et al., 1994; Schroeder et al., 2000). After cleavage SCP-2 is found in the endoplasmic reticulum, peroxisomes, mitochondria, and the cytosol. SCP-x, however, is exclusively targeted to the peroxisomal matrix and has thioesterase activity and lipid binding activity (Atshaves et al., 1999; Gallegos et al., 2001).

Sterol carrier proteins are lipid chaperone proteins and were initially thought to function as a mechanism for intracellular cholesterol movement between membranes (Frolov et al., 1996). More recent studies have shown that SCP-2 is a multifunctional protein that impacts fatty acid and cholesterol uptake and trafficking, consistent with a broader role as a ubiquitous lipid binding protein (Murphy, 2002; Murphy, Stiles, et al.,

2000). This is accomplished through the conformational changes that SCP-2 undergoes in response to the binding of lipids exposing it to the aqueous environment and allowing it to be transferred to another membrane (Frolov et al., 1996). Early studies indicated that SCP-2 does not bind fatty acids (Scallen et al., 1985). However, other data demonstrates that the dielectric constant of the solvent, usually ethanol, in which the fatty acids are dissolved significantly impacts the interaction between the fatty acid and the binding grove of SCP-2 (Schroeder et al., 1995). The binding of fatty acids to SCP-2 was later confirmed and shown to have a similar binding affinity as fatty acid binding protein (FABP) (Murphy, 2002; Stolowich et al., 1997). Not only was binding of fatty acids confirmed, but SCP-2 was shown to increase fatty acid uptake into cells (Murphy, 2002). Additionally, SCP-2 plays a role in the oxidative protection of fatty acids working together with Acyl-CoA oxidase to protect the 2-enoyl bond that is susceptible to oxidative degradation due to exposure to hydroxyl radicals (Dansen et al., 2004).

The intracellular movement of cholesterol is an essential function to produce steroids and bile acids within the cell. While SCP-2 (13.2 kD) mediates the transfer of lipids, it also plays a key role in steroidogenesis as well as stimulating the conversion of 7-dehydrocholesterol to cholesterol (Noland et al., 1980). Targeting of proSCP-2 to mitochondria and peroxisomes increases cholesterol uptake and esterification within the cell (Moncecchi et al., 1996; Murphy & Schroeder, 1997). The initiation of steroidogenesis requires the movement of cholesterol from the outer mitochondrial membrane to the inner mitochondrial membrane (Gallegos et al., 2001). The regulation of this movement in isolated systems, including the mitochondria, has been linked to a functional role of SCP-2 (Scallen et al., 1985). Previous studies showed that purified

SCP-2 stimulates pregnenolone synthesis from cholesterol in the mitochondria, which is the first step in steroidogenesis (Pfeifer et al., 1993). This aligns with the expression levels of SCP-2 being higher in steroidogenic tissue such as ovaries, which correlates to the functional role of SCP-2 in hormone synthesis (Rennert et al., 1991).

With the presence of a peroxisomal targeting sequence, in addition to the mitochondrial targeting sequence, SCP-2 is known to be involved in cholesterol synthesis (Pfeifer et al., 1993). This could be one of the initial mechanisms for cholesterol import to the mitochondria for steroidogenesis. Other studies suggest that free cholesterol is solubilized by binding to transport proteins and shuttled to the mitochondria via transport vesicles in a Golgi-dependent mechanism (Puglielli et al., 1995). Intracellular trafficking of cholesterol may occur through different mechanisms depending on cell type and required location within the cell. However, SCP-2 seems to be an important factor in the trafficking of cholesterol for hormone synthesis, especially in tissues that require increased steroidogenesis, such as liver, brain, adrenal gland, ovaries, and testes.

The second protein encoded by the *SCP2* gene is SCP-x. This protein is exclusively targeted to the peroxisome with the sequence Ala-Lys-Ala and has a more specific function (Van der Krift et al., 1985). SCP-x has been identified as a 3-oxoacyl-CoA thiolase, which catalyzes the thiolytic cleavage of 3-oxoacyl-CoA of straight chain fatty acids, 2-methyl-branched fatty acids and side chains of cholesterol and bile acids during β-oxidation (Antonenkov et al., 1997; Wanders et al., 1997). This is an important step in fatty acid elongation, cholesterol synthesis, and ketone body synthesis (Antonenkov et al., 1997). In addition to its enzymatic activities, SCP-x also acts as a transport protein for lipids between membranes. It has been shown to bind

phosphatidylcholine (PtdCho) and 7-dehydrocholesterol for transport between membranes (Atshaves et al., 1999). It is clear that SCP-2 and SCP-x has important function in lipid transport and synthesis, however the role these proteins have in brain and liver lipid metabolism is poorly understood.

There have been few diet studies on the effects of *SCP2,* with the majority of studies using TKO (triple-knock-out) of SCP-2, SCP-x and FABP. These studies examined high fat diet responses to endocannabinoids in the brain and found that both FABP and SCP2 regulate arachidonic acid and subsequently endocannabinoid pathways in the brain (Martin et al., 2019). FABP1 appears to impact dietary oxidative stress in liver disease as well as affecting triacylglycerols (TAG) levels, both of which impact overall weight gain in mice (Atshaves et al., 2010a; Martin et al., 2009; Smathers et al., 2013). Other studies have just started looking into the consequences of *SCP2* deficiency and high fat/high cholesterol diet. These studies have reported that expression of SCP-2 and SCP-x are important for preventing hyperlipidemia, hepatic steatosis, and other metabolic changes in mice that have been fed a 'Western diet" (Wang et al., 2019). Although these results don't give much insight into the role of *SCP2* on liver and brain metabolism *in vivo,* they do suggest that these sterol carrier proteins play a large role in dietary liver function for cholesterol turn-over and metabolic lipid levels in the brain.

The majority of the work completed on *SCP2* has been exclusively done in male rats and mice. However, with *SCP2* playing an important role in hormone synthesis it is important to also consider the effects on females. The few studies done that include female animals have focused on basic steroidogenic effects and homeostatic cholesterol differences between sexes (Giatti et al., 2019; Segatto et al., 2013). A more recent study

showed that SCP2 deficiency affects hepatic phytol accumulation, and subsequently branch chain fatty acid accumulation, in females at a greater rate than males (McIntosh et al., 2017). It is clear that more research needs to be done to document the sex differences affected by *SCP2* in lipid metabolism, hormone synthesis, and lipid transport.

While many studies indicate that SCP-2 and SCP-x have an important role in the transportation and biosynthesis of various lipids and steroids, and emerging studies hinting at the role *SCP2* plays in diet and sex differences in lipid composition; the effect of SCP-2 and SCP-x and high cholesterol diet on brain and liver lipid composition *in vivo*, and how this affects lipid composition in male and female mice is poorly understood. Therefore, this thesis aims to address the following hypothesis: *SCP2* gene ablation, sex, and high cholesterol diet impacts phospholipid and sterol steady state mass having a greater effect in the liver than brain.

To address this hypothesis male and female *SCP2* gene-ablated mice were fed a high cholesterol diet over the course of twelve weeks and compared to wild-type male and female mice fed a control diet. Levels of phospholipids, cholesterol, and cholesteryl esters were measured, and the impact of SCP-2/SCP-x, diet, and sex was assessed. In chapter 3, we investigated the effect of *SCP2* gene-ablation and high cholesterol diet on phospholipid composition in brain, cholesterol and cholesteryl ester mass in brain, and sex differences in brain. In chapter 4, we investigate the effect of SCP2 gene-ablation and high cholesterol diet on phospholipid composition in liver, cholesterol and cholesteryl ester composition in liver, and sex differences in liver. Our hypothesis is consistent with previous studies where *SCP2* impacts lipid metabolism and peroxidation, however this study aims to profile the effects of S*CP2* gene ablation in the mouse model when treated

with high cholesterol diet and determine sex differences in lipid composition; an important step in elucidating functions of SCP-2 and SCP-x in brain and liver lipid metabolism.

In brain, the following statistically significant changes were observed between groups. We observed a 17% increase in total phospholipid mass accounted for by a 21% increase in ChoGpl mass in male gene-ablated mice fed a high cholesterol diet compared to the other groups. In female mice, brain cholesterol mass was reduced in all groups by about 50% as compared to male mice, although a similar change was not observed in total phospholipid mass. There were no significant changes in cholesterol between groups in each sex, supporting previous studies that the brain is a cholesterol privileged organ. Cholesteryl ester mass was decreased by 61% in *SCP2* gene-ablated female mice fed a high cholesterol diet, and by 50% in male gene-ablated mice fed a high cholesterol diet compared to the other groups, although a similar change was not observed in gene-ablated mice fed a control diet. This suggest that in the absence of *SCP2* paired with a high cholesterol diet there is likely a complex signaling mechanism that results in reduced brain cholesteryl ester formation in both male and female mice.

In liver, the following statistically significant changes were observed between groups. We observed a 7% increase in total phospholipid mass accounted for by a 18% increase in ChoGpl mass in male *SCP2* gene-ablated mice fed a high cholesterol diet compared to male gene-ablated mice fed a control diet. A similar effect was observed in male wild-type mice fed a high cholesterol diet where a 28% increase in total phospholipid mass was accounted for by a 29% increase in ChoGpl mass compared to wild-type mice fed a control diet. In both male and female mice there was between a

70%-90% increase in cholesteryl ester mass in mice fed a high cholesterol diet compared to mice fed a control diet. This increase was greater in the wild-type mice than the SCP2 gene-ablated mice, suggesting that SCP-2/SCP-x is important for cholesteryl ester formation in the liver. Female mice had a 48%-69% increase in cholesteryl ester mass compared to male mice, likely due to the effect of estrogen on VLDL formation. This suggests that a high cholesterol diet increases liver secretion of VLDL, where phosphatidylcholine (ChoGpl) is found on the exterior of the lipoprotein whereas cholesteryl esters are a key component of the lipoprotein core.

Sterol carrier protein 2 (SCP-2) is a ubiquitous and evolutionarily conserved protein that plays a crucial role in lipid metabolism. SCP-2 is involved in the transfer of lipids such as cholesterol, fatty acids, and phospholipids between cellular membranes and is necessary for the proper functioning of these lipids in cells. This protein is expressed in various tissues including liver, brain, gonads, and heart and has been implicated in several physiological processes such as lipid transport, steroidogenesis, endocannabinoid metabolism, inflammation, fatty acid metabolism, and cancer progression. Defects in *SCP2* have been linked to several diseases including hypercholesterolemia, liver disease, vascular inflammation, atherosclerosis, and cancer (McIntosh et al., 2017; Wang et al., 2019; Xu et al., 2023). Understanding the role of SCP-2 and SCP-x in lipid metabolism and its interactions with other cellular components is crucial for the development of new treatments for these diseases.

CHAPTER II

METHODS

Animals

Male and female wild-type C57/6N and *SCP2* gene-ablated mice were used by Dr. Friedhelm Schroeder at Texas A&M University. The animal protocols were performed at Texas A&M University in the Schroeder Lab and reported as follows. Mice were 8 weeks of age at study initiation with seven animals per group. The mouse groups were: male wild-type on control diet (CO), male *SCP2* gene-ablated on CO, male wild-type on high cholesterol diet (HCD), male *SCP2* gene-ablated on HCH, female wild-type on CO, female *SCP2* gene-ablated on CO, female wild-type on HCH, and female *SCP2* gene-ablated on HCD. Each genotype consisted of 14 animals and were randomly assigned to their feeding group. Mice were maintained on their respective diet and water for twelve weeks. The mice were fasted overnight then anesthetized and euthanized as per the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

Mouse Surgery

Mouse surgery was performed at Texas A&M University in the Schroeder Lab where the following protocol was followed. Mice were randomly assigned for euthanasia and the isolated brain and liver from each mouse was flash-frozen using liquid nitrogen and stored at -80°C for Western blots and high-performance liquid chromatography

(HPLC) for lipid analysis. Each mouse was processed separately to minimize metabolic changes from the procedure.

Tissue Lipid Extraction

Frozen brain and liver tissue were homogenized in 1 mL ice-cold buffer (10 mM potassium phosphate, pH 8.0/1 mM dithiothreitol) and stored in -80°C until lipid extraction. Lipids were extracted by adding the previous homogenate to a 7 mL Tenbroeck homogenizer and homogenized by using a 5 mL rinse of hexane:isopropanol (3:2 v/v) (HIP), transferring to a new glass test tube and repeating the rinse a second time until a fine consistency was established and transferring to a glass test tube. A final rinse of 2.5 mL HIP was added to the homogenizer then transferred to the respective test tube. The sample was then centrifuged at 3,250 x g for 10 minutes at -10°C to pellet the debris and the liquid phase was transferred to a new glass test tube and dried under a stream of nitrogen and dissolved in *n*-hexane:2-propanol:water (56.7:37.5:5.5 v/v/v) (HIP/H2O) and stored at -80°C. The pellet was left to dry overnight at room temperature to be used for protein analysis. Samples were processed by group and kept on ice throughout the extraction.

Silicic Acid Columns

The separation of phospholipids and neutral lipids were done using silicic acid column chromatography. Long glass Pasteur pipettes were inserted into a stand and filled with glass wool. Approximately 2 cm of chloroform activated Unisil silicic acid was added on top of the glass wool and rinsed with 1 mL of chloroform. Previously extracted lipids were dried under nitrogen, rinsed with HIP, and then dissolved in 200 μL of chloroform. Lipids were transferred to the column and the test tube was rinsed three more

times with 200 μL of chloroform. Neutral lipids were eluted using 1 mL rinses of chloroform/methanol (58:1 v/v) until approximately 10 mL were collected in new glass test tubes. Phospholipids were eluted using 1 mL rinses of 100% methanol until approximately 10 mL were collected in separate glass test tubes. All lipids were dried under nitrogen, rinsed with HIP, and stored in 1 mL of HIP/H2O at -80°C for further chromatography.

Filtration of Phospholipids

Phospholipid samples were filtered prior to HPLC using a 0.2 μm Nylon filter and collected in a new glass test tube. Ultrafiltered phospholipids were dried under nitrogen and stored in HIP/H2O at -80°C for HPLC. This step is important for removing residual proteins left in the sample.

High Performance Liquid Chromatography

Using the filtered brain phospholipid samples, high performance liquid chromatography (HPLC) was used to separate these samples further into individual phospholipid classes. These classes include ethanolamine glycerphospholipid (EtnGpl), lysophosphatidylethanolamine (lysoPtdEtn), phosphatidylinositol (PtdIns), phosphatidylserine (PtdSer), choline glycerophospholipid (ChoGpl), sphingomyelin (CerPCho), and lysophosphatidylcholine (lysoPtdCho) which were used for further analysis. The HPLC configuration used was a Beckman Coulter Gold 127 Solvent Module, Beckman Coulter Gold 166 Detector, Supelco Zorbax silica column (25 cm x 4.6 mm, 5 μm), and Kipp & Zonen BD-41 Dual Channel Electrical Pen Lift Chart Recorder. The solvents used were (A) n-hexane:2-propanol (3:2 v/v) and (B) n-hexane:2-propanol:water (56.7:37.8:5.5 v/v/v). The program used was proportioned using 70% A

and 30% B with a flow rate on 1.5 mL/minute, then stepped increases the proportion of B until 100% B at approximately 100 minutes from sample injection. The detector absorbance was set at 205 nm. Fractions of phospholipid classes were manually collected in acid washed glass test tubes and dried in an oven. Phospholipid mass was assessed by measuring lipid phosphorous.

Thin-Layer Chromatography

The neutral lipid fraction collected from the silicic acid columns were further separated using thin-layer chromatography (TLC) into cholesterol and cholesteryl esters for continued analysis. Other major classes also separated are free fatty acids, triacylglycerol, monoacylglycerol, 1,2-diacylglycerol, and 1,3-diacylglycerol. TLC was performed on heat-activated Silica Gel G glass plates and ran in an enclosed glass container. 50 μL of each neutral lipid sample was spotted on the plate in its respective lane using n-hexane:2-propanol:water (56.7:37.8:5.5 v/v/v) and dried for one minute in 110°C oven. The mobile phase consisted of petroleum ether:diethyl-ether:acetic acid (75:25:1.3 v/v/v) and plates were developed for approximately 50 minutes. (Moncelli et al., 1994) Plates were removed from the container and then placed in iodine vapors to visualize the lipids. Cholesterol and cholesteryl ester fractions were marked and scraped into glass test tubes for further analysis.

The filtered liver phospholipid samples were further separated using TLC into ethanolamine glycerphospholipid (EtnGpl), phosphatidylinositol (PtdIns), phosphatidylserine (PtdSer) running with lysophosphatidylethanolamine (lysoPtdEtn), choline glycerophospholipid (ChoGpl), sphingomyelin (CerPCho), and lysophosphatidylcholine (lysoPtdCho). TLC was performed on heat-activated Silica Gel

H glass plates and ran in an enclosed glass container. Phospholipid samples (50 µL) were spotted on the plate using n-hexane:2-propanol:water (56.7:37.8:5.5 v/v/v) and dried for one minute in 110°C oven. The mobile phase consisted of chloroform:methanol:acetic acid:water (50:37.5:3:2 v/v/v) and plates were developed for approximately 90 minutes. Plates were removed from the container and placed in iodine vapors to visualize the lipids. Separated phospholipid classes were marked and scraped into acid washed glass test tubes and quantified by analysis of lipid phosphorous.

Phospholipid Mass

Both total phospholipid mass and major phospholipid class mass were quantified via lipid phosphorus assay. All samples were processed in chromic acid washed glass test tubes to prevent contamination. To assess total lipid phosphorous total phospholipids were dried after filtration and dissolved in 1 mL of n-hexane:2-propanol (3:2 v/v). 20 µL were placed in the acid washed test tube for the phosphorus assay. Phospholipid classes collected from HPLC were dried overnight at 85°C in an oven. All phospholipid samples were digested with 500 µL of water and 650 µL of perchloric acid and placed in a heat block at 185°C for one hour (Rouser, G., Siakotos, A., and Fleischer, 1969). Samples were allowed to cool at room temperature before 500 µL of aqueous ascorbic acid (10% w/v), 500 µL of aqueous ammonium molybdate (2.5% w/v), and 3.3 mL of deionized water was added and vortexed well. Samples were then placed in 110°C heat block for five minutes, then removed and allowed to cool to room temperature. This colormetric assay was quantified via absorbance at 797 nm on Beckman Coulter DU Series 600 spectrophotometer and compared to a standard curve of known concentration of

phosphorus. Phospholipids were normalized to nmol of phosphorus per milligrams of protein as samples were previously prepared as homogenates.

Cholesterol Mass

Cholesterol and cholesteryl esters were quantified using an iron binding colormetric assay in which ethanol is used as a solvent for the neutral lipid samples previously separated via TLC (Bowman & Wolf, 1962). The previous TLC scrapings of cholesterol and cholesteryl esters of each sample were placed in a screw top glass test tube in which 3 mL of ethanol was added along with 3 mL of 'working iron reagent'. The working iron reagent consisted of ferrous choride hexahydrate:concentrated phosphoric acid (2.5 g/100 mL) were 8 mL was q.s to 100 mL with sulfuric acid. Tubes were capped tightly and vortexed for one minute. Because this is an exothermic reaction tubes were left to cool at room temperature. Samples were then centrifuged at 2,500 x g for twenty minutes to pellet the silica gel scrapings from TLC. This assay was quantified via absorbance at 550 nm on Beckman Coulter DU Series 600 spectrophotometer and compared to a standard curve of known concentrations of cholesterol. Cholesterol and cholesteryl esters were normalized to nmol of each per milligram of protein.

Protein Mass

Protein mass was determined via the Bradford method, which utilizes protein-dye binding of Coomassie Brilliant Blue G-250 to create a colormetric assay quantified by the absorbance at 595 nm (Bradford, 1976). The dried pellet previously separated via lipid extraction was incubated in 4 mL (brain samples) and 8 mL (liver samples) of freshly made 0.2 M KOH overnight at 65°C. The stock Bradford reagent was made fresh by dissolving 200 mg of Coomassie Blue G-250 in 100 mL of ethanol then adding 200 mL

of phosphoric acid. The working Bradford reagent was made fresh by adding 150 mL of the stock reagent and q.s to 1000 mL using water. The working reagent was then double filtered and stored in an airtight container. 20 µL of each sample was pipetted into new glass test tubes then 5 mL of 'ready-to-use' Bradford reagent was added, tubes were vortexed and incubated at room temperature for ten minutes before quantified on Beckman Coulter DU Series 600 spectrophotometer and compared to known concentrations of BSA standards. Total protein per sample was calculated and necessary to normalize phospholipid and neutral lipid mass within each sample.

Calculations

Standards for phosphorus mass, cholesterol mass, and protein mass were plotted in Microsoft Excel against absorbance were a linear trendline and equation were generated and used to calculate sample mass. Samples were then normalized by dividing phosphorus mass and cholesterol mass by protein mass for each sample.

Statistics

Statistical analysis was completed using GraphPad Prism 9.5.0 by Dotmatics Scientific Software (San Diego, CA). Wild-type and SCP-2 gene ablated groups without diet and sex variables were compared and statistical significance was measured by using two-tailed paired Student's t-test where significance was defined as $p < 0.05$. Male and female wild-type and gene ablated groups without the diet variable were compared and statistical significance was measured by using a two-way ANOVA with Tukey's multiple comparisons test where significance was defined as $p < 0.05$. All groups, including diet, sex, and gene-ablation variables were compared and statistical significance was measured

by using multi-way ANOVA with Tukey's multiple comparisons test where significance was defined as $p < 0.05$.

CHAPTER III

RESULTS

Study 1: Sterol Carrier Protein 2 Gene Ablation and Dietary Cholesterol Increase Choline Glycerophospholipid, Total Phospholipid Content, and Cholesterol Mass in Male and Female Mouse Brain

In this study, brain phospholipid and neutral lipid mass was assessed in *SCP2* gene-ablated male and female mice fed a high cholesterol or control diet. The major classes of phospholipids (EtnGpl, lysoPtdEtn, PtdIns, PtdSer, ChoGpl, lysoPtdCho, CerPCho), and neutral lipid mass (cholesterol and cholesteryl esters) were measured. Phospholipid mass was normalized to protein in each sample and values expressed as nmol/mg protein. Phospholipid composition was calculated because it is a useful tool to assess if one phospholipid is increased relative to another, thereby giving some indication of alterations in biosynthetic pathways. In addition, because the molar composition is independent of sample volume or losses during sample handling, the total phospholipid mass (nmol/mg protein) for each sample was used to calculate individual phospholipid mass for the respective samples using the molar composition. Cholesterol and cholesteryl ester mass was quantified and normalized to sample protein with values expressed as nmol/mg of protein.

3.1 Effect of Sterol Carrier Protein 2 Gene Ablation on Steady State Phospholipid and Neutral Lipid Mass in Brain

Phospholipid Mass

There were no significant differences in phospholipid mass or molar composition between wild-type and *SCP2* gene-ablated mice (Table 1).

Table 1. Effect of *SCP2* gene-ablation on phospholipid mass and molar composition in brain.

	WT		**KO**		**WT**		**KO**	
Class	Mean	SD	Mean	SD	Mean	SD	Mean	SD
	nmol/mg protein				mol%			
EtnGpl	497	106	566	70	44	5	46	6
PtdIns	32	11	23	9	3	1	2	1
lysoPtdEtn	13	8	8	4	1	1	1	1
PtdSer	92	27	71	22	7	2	7	2
ChoGpl	447	93	426	55	40	5	39	4
CerPCho	46	17	35	9	4	1	4	2
lysoPtdCho	16	14	13	6	1	1	1	1
TOTAL	1142	226	1141	119				
	n=14		n=14		n=14		n=14	

Values are expressed as nmol/mg protein and represent mean ± standard deviation, n = 14. The '*' represents significance between wild type and *SCP2* gene-ablated mice. Wild-type is represented by WT, and *SCP2* gene-ablation is represented by KO. Statistical analysis is two-tailed Students t-test. Statistical significance is defined as $p<0.05$.

Cholesterol Mass

There were no significant differences in cholesterol or cholesteryl ester mass between wild-type and *SCP2* gene-ablated mice (Table 2).

Table 2. Effect of *SCP2* gene-ablation on neutral lipid mass in brain.

	WT		**KO**	
Class	Mean	SD	Mean	SD
	nmol/mg protein			
Cholesteryl Esters	66	28	65	13
Cholesterol	308	145	365	234
	n=14		n=14	

Values are expressed as nmol/mg protein and represent mean ± standard deviation, n = 14. The '*' represents significance between wild type and *SCP2* gene-ablated mice. Wild-type is represented by WT, and *SCP2* gene-ablation is represented by KO. Statistical analysis is two-tailed Students t-test. Statistical significance is defined as $p<0.05$.

3.2 Effect of *SCP2* Gene Ablation on Brain Steady State Phospholipid and Neutral Lipid Mass and Differences between Male and Female Mice

Phospholipid Mass

When analyzing the difference between male and female phospholipid mass in brain without the effect of diet only control treatment groups were used. Interestingly, there was a base-line significant difference between wild-type male mice and wild-type female mice, with females having 13% more total phospholipid mass than males in brain (Table 3). There were no other significant differences between groups in brain phospholipid mass.

Table 3. *SCP2* gene-ablation increases total phospholipid mass in male mice and difference in steady state total phospholipid mass between sex in brain.

	MWT		**MKO**		**FWT**		**FKO**	
Class	Mean	SD	Mean	SD	Mean	SD	Mean	SD
				nmol/mg protein				
EtnGpl	461	38	584	47	532	141	549	87
PtdIns	27	7	16	7	37	12	29	5
lysoPtdEtn	6	3	5	2	20	4	10	3
PtdSer	95	21	59	19	89	32	82	19
ChoGpl	425	61	416	56	470	117	435	58
CerPCho	41	9	33	10	50	23	37	8
lysoPtdCho	6	5	15	8	25	14	12	2
TOTALS	1061	132^	1129	85	1224	279^	1154	152
	n=7		n=7		n=7		n=7	

Values are expressed as nmol/mg protein and represent mean ± standard deviation, n = 7. The '*' represents significance between wild type and *SCP2* gene-ablated mice within each sex. The '^' represents significance between corresponding male and female mice. Male wild-type mice are represented by MWT, male gene-ablated mice are represented by MKO, female wild-type mice are represented by FWT, and female gene-ablated mice are represented by FKO. Statistical analysis is two-way ANOVA with Tukey's multiple comparisons test. Statistical significance is defined as $p<0.05$.

Phospholipid Composition

There was a significant difference in ethanolamine glycerophospholipid (EtnGpl) between female *SCP2* gene-ablated mice and female wild-type mice with a 9% increase in molar composition in the absence of *SCP2* in brain (Table 4). Male knockout mice had 9% more EtnGpl than female knockout mice. There were no other significant differences between groups in brain phospholipid molar composition.

Table 4. *SCP2* gene-ablation increases the proportion of EtnGpl in female mice.

	MWT		**MKO**		**FWT**		**FKO**	
Class	Mean	SD	Mean	SD	Mean	SD	Mean	SD
				mol%				
EtnGpl	44	2	52	4^	43	4	47	3*
PtdIns	3	0	1	1	3	1	3	0
lysoPtdEtn	1	0	0	0	2	0	1	0
PtdSer	9	1	5	1	7	2	7	1
ChoGpl	40	1	37	4	38	2	38	3
CerPCho	4	0	3	1	4	1	3	0
lysoPtdCho	1	0	1	1	2	1	1	0
	n=7		n=7		n=7		n=7	

Values are expressed as nmol/mg protein and represent mean ± standard deviation, n = 7
The '*' represents significance between wild type and *SCP2* gene-ablated mice within each sex. The '^' represents significance between corresponding male and female mice. Male wild-type mice are represented by MWT, male gene-ablated mice are represented by MKO, female wild-type mice are represented by FWT, and female gene-ablated mice are represented by FKO. Statistical analysis is Two-Way ANOVA with Tukey's multiple comparisons test. Statistical significance is defined as $p<0.05$.

Cholesterol Mass

In brain there was significantly less cholesterol in females than males. Regardless of *SCP2* gene-ablation female mice had approximately 50% less cholesterol than male mice (Table 5). There were no other significant differences between groups in brain neutral lipid mass.

Table 5. Female mice have less brain cholesterol than male mice.

	MWT		**MKO**		**FWT**		**FKO**	
Class	Mean	SD	Mean	SD	Mean	SD	Mean	SD
				nmol/mg protein				
Cholesteryl Esters	71	37	60	15	61	15	69	10
Cholesterol	413	117^	504	266^	203	79^	227	50^
	n=7		n=7		n=7		n=7	

Values are expressed as nmol/mg protein and represent mean ± standard deviation, n = 7. The '*' represents significance between wild type and *SCP2* gene-ablated mice within each sex. The '^' represents significance between corresponding male and female mice. Male wild-type mice are represented by MWT, male gene-ablated mice are represented by MKO, female wild-type mice are represented by FWT, and female gene-ablated mice are represented by FKO. Statistical analysis is two-way ANOVA with Tukey's multiple comparisons test. Statistical significance is defined as $p<0.05$.

3.3 Effect of *SCP2* Gene Ablation and High Cholesterol Diet on Brain Phospholipid and Neutral Lipid Mass in Male and Female Mice

Phospholipid Mass

The total mass of phospholipids significantly increased by 17% in *SCP2* gene-ablated male mice that received high cholesterol diet (Table 6). In this same group, there

was also a significant increase in ChoGpl by 21%, which partially contributed to the total phospholipid increase in male mice. *SCP2* gene-ablation significantly and independently increased total phospholipid mass in male mice, however high cholesterol diet increased total phospholipid mass independently (Table 6). The *SCP2* gene-ablated, high cholesterol diet, male mice (Table 6) had significantly higher levels of total phospholipids compered to all female groups (Table 8).

Phospholipid Composition

Male *SCP2* gene-ablated mice fed a high cholesterol diet had a significantly lower EtnGpl composition than wild-type and control diet groups independently (Table 7). This group also was significantly lower by 13% than its female counterpart (Table 9). The male gene-ablated, control diet group had a significantly higher proportion of EtnGpl than all female groups. Male wild-type, high cholesterol diet group had a significantly higher proportion of EtnGpl than it's female counterpart by 11% (Table 7 and Table 9). There was a significantly lower proportion of PtdSer for high cholesterol diet, male wild-type mice compared to control diet, male wild-type mice by 44% (Table 7). *SCP2* gene-ablated male mice fed a high cholesterol diet had a significantly higher proportion of ChoGpl than male control diet gene-ablated mice by 12% (Table 7). There were no other significant differences in phospholipid composition in the brain between groups.

Table 6. Effect of *SCP2* gene-ablation and high cholesterol diet in brain on major phospholipid classes mass in male mice.

	MWC		**MWT**		**MKC**		**MKT**	
Class	Mean	SD	Mean	SD	Mean	SD	Mean	SD
				nmol/mg protein				
EtnGpl	461	38	515	60	584	47	527	51
PtdIns	27	7	16	6	16	7	34	13
lysoPtdEtn	6	3	6	4	5	2	19	11
PtdSer	95	21	54	13	59	19	107	47
ChoGpl	425	61	481	139	416	56	574	118*
CerPCho	41	9	35	10	33	10	74	45
lysoPtdCho	6	5	11	5	15	8	25	21
TOTALS	1061	132^{F}	1118	187	1129	85	1361	211$^{\wedge *F+}$
	n = 7		*n* = 7		*n* = 7		*n* = 7	

Values are expressed as nmol/mg protein and represent mean ± standard deviation, n=7. Male wild-type control diet is represented by MWC, male wild-type high cholesterol diet is represented by MWT, male SCP2 gene-ablated control diet is represented by MKC, and male SCP2 gene-ablated high cholesterol diet is represented by MKT. The '*' represents significance between high cholesterol diet and control diet within gene ablated groups. The '^' represents significance between wild-type and *SCP2* gene-ablated mice within diet treatment groups. Significance between sexes is indicated by 'M or F' and refers to corresponding groups. Significance between sexes where a statistically significant difference is seen between indicated group and all other groups of the opposite sex is indicated by 'M+ or F+'. Statistical analysis is a multi-way ANOVA with Tukey's multiple comparisons test. Statistical significance is defined as $p<0.05$.

Table 7. Effect of *SCP2* gene-ablation and high cholesterol diet in brain on major phospholipid classes molar composition in male mice.

	MWC		**MWT**		**MKC**		**MKT**	
Class	Mean	SD	Mean	SD	Mean	SD	Mean	SD
				mol %				
EtnGpl	44	2	47	6^{F}	52	4^{F+}	39	$7^{\wedge *F}$
PtdIns	3	0	1	0	1	1	2	1
lysoPtdEtn	1	0	1	0	0	0	1	1
PtdSer	9	1	5	1^{*}	5	1	8	2
ChoGpl	40	1	42	7	37	4	42	5^{*}
CerPCho	4	0	3	0	3	1	5	3
lysoPtdCho	1	0	1	1	1	1	2	1
	n = 7		*n* = 7		*n* = 7		*n* = 7	

Values are expressed as nmol/mg protein and represent mean ± standard deviation, n=7. Male wild-type control diet is represented by MWC, male wild-type high cholesterol diet is represented by MWT, male *SCP2* gene-ablated control diet is represented by MKC, and male *SCP2* gene-ablated high cholesterol diet is represented by MKT. The '*' represents significance between high cholesterol diet and control diet within gene ablated groups. The '^' represents significance between wild-type and *SCP2* gene-ablated mice within diet treatment groups. Significance between sexes is indicated by 'M or F' and refers to corresponding groups. Significance between sexes where a statistically significant difference is seen between indicated group and all other groups of the opposite sex is indicated by 'M+ or F+'. Statistical analysis is a multi-way ANOVA with Tukey's multiple comparisons test. Statistical significance is defined as $p<0.05$.

Table 8. Effect of *SCP2* gene-ablation and high cholesterol diet in brain on major phospholipid classes mass in female mice.

	FWC		**FWT**		**FKC**		**FKT**	
Class	Mean	SD	Mean	SD	Mean	SD	Mean	SD
				nmol/mg protein				
EtnGpl	532	141	475	65	549	87	537	127
PtdIns	37	12	34	9	29	5	29	12
lysoPtdEtn	20	4	19	9	10	3	12	2
PtdSer	89	32	82	20	82	19	87	34
ChoGpl	470	117	447	81	435	58	463	102
CerPCho	50	23	42	10	37	8	38	9
lysoPtdCho	25	14	23	10	12	2	12	2
TOTALS	1224	279^{M}	1122	100	1154	152	1179	271
	n = 7		*n* = 7		*n* = 7		*n* = 7	

Values are expressed as nmol/mg protein and represent mean ± standard deviation, n=7. Female wild-type control diet is represented by FWC, female wild-type high cholesterol diet is represented by FWT, female *SCP2* gene-ablated control diet is represented by FKC, and female *SCP2* gene-ablated high cholesterol diet is represented by FKT. The '*' represents significance between high cholesterol diet and control diet within gene ablated groups. The '^' represents significance between wild-type and *SCP2* gene-ablated mice within diet treatment groups. Significance between sexes is indicated by 'M or F' and refers to corresponding groups. Significance between sexes where a statistically significant difference is seen between indicated group and all other groups of the opposite sex is indicated by 'M+ or F+'. Statistical analysis is a multi-way ANOVA with Tukey's multiple comparisons test. Statistical significance is defined as $p<0.05$.

Table 9. Effect of *SCP2* gene-ablation and high cholesterol diet in brain on major phospholipid classes molar composition in female mice.

	FWC		**FWT**		**FKC**		**FKT**	
Class	Mean	SD	Mean	SD	Mean	SD	Mean	SD
				mol %				
EtnGpl	43	4	42	5^{M}	47	$3^{\wedge}$	45	2^{M}
PtdIns	3	1	3	1	3	0	2	1
lysoPtdEtn	2	0	2	1	1	0	1	0
PtdSer	7	2	7	1	7	1	7	1
ChoGpl	38	2	40	6	38	3	39	1
CerPCho	4	1	4	1	3	0	3	0
lysoPtdCho	2	1	2	1	1	0	1	0
	n = 7		*n* = 7		*n* = 7		*n* = 7	

Values are expressed as nmol/mg protein and represent mean ± standard deviation, n=7. Female wild-type control diet is represented by FWC, female wild-type high cholesterol diet is represented by FWT, female *SCP2* gene-ablated control diet is represented by FKC, and female *SCP2* gene-ablated high cholesterol diet is represented by FKT. The '*' represents significance between high cholesterol diet and control diet within gene ablated groups. The '^' represents significance between wild-type and *SCP2* gene-ablated mice within diet treatment groups. Significance between sexes is indicated by 'M or F' and refers to corresponding groups. Significance between sexes where a statistically significant difference is seen between indicated group and all other groups of the opposite sex is indicated by 'M+ or F+'. Statistical analysis is a multi-way ANOVA with Tukey's multiple comparisons test. Statistical significance is defined as $p<0.05$.

Cholesterol Mass

Male mice had significantly more brain cholesterol than female mice across all groups as seen in Table 10 and Table 11. There was approximately a 50% increase in male cholesterol mass compared to females in the brain. This was a significant enough increase in cholesterol to contribute to a greater cholesterol/phospholipid ratio in males than females (Table 10 and Table 11). *SCP2* gene-ablated, high cholesterol diet male mice had a significantly lower cholesterol/phospholipid ratio than gene-ablated, control diet male mice. It is important to note a decrease in cholesteryl esters in both *SCP2* gene-ablated female and male mice that were fed a high cholesterol diet compared to all other groups. There were no other statistically significant differences for neutral lipids between groups.

Table 10. Effects of *SCP2* gene-ablation and dietary cholesterol on brain cholesterol and cholesteryl ester mass in male mice.

	MWC		**MWT**		**MKC**		**MKT**	
Class	Mean	SD	Mean	SD	Mean	SD	Mean	SD
	nmol/mg protein							
Cholesteryl Esters	71	37	76	14	60	15	30	10
Cholesterol	413	117^{F}	484	154^{F+}	504	266^{F+}	418	67^{F}
C/PL Ratio	0.35	0.10	0.38	0.11	0.51	0.27^{F}	0.28	0.06^{*}
	n=7		n=7		n=7		n=7	

Values are expressed as nmol/mg protein and represent mean ± standard deviation, n=7. Male wild-type control diet is represented by MWC, male wild-type high cholesterol diet is represented by MWT, male *SCP2* gene-ablated control diet is represented by MKC, and male *SCP2* gene-ablated high cholesterol diet is represented by MKT. The '*' represents significance between high cholesterol diet and control diet within gene ablated groups. The '^' represents significance between wild-type and *SCP2* gene-ablated mice within diet treatment groups. Significance between sexes is indicated by 'M or F' and refers to corresponding groups. Significance between sexes where a statistically significant difference is seen between indicated group and all other groups of the opposite sex is indicated by 'M+ or F+'. Statistical analysis is a multi-way ANOVA with Tukey's multiple comparisons test. Statistical significance is defined as $p<0.05$.

Table 11. Effects of *SCP2* gene-ablation and dietary cholesterol on brain cholesterol and cholesteryl ester mass in female mice.

	FWC		**FWT**		**FKC**		**FKT**	
Class	Mean	SD	Mean	SD	Mean	SD	Mean	SD
	nmol/mg protein							
Cholesteryl Esters	61	15	72	8	69	10	22	6
Cholesterol	203	79^{M}	177	88^{M}	227	50^{M}	322	51
C/PL Ratio	0.22	0.11^{M}	0.2	0.11^{M}	0.3	0.1	0.31	0.04
	n=7		n=7		n=7		n=7	

Values are expressed as nmol/mg protein and represent mean ± standard deviation, n=7. Female wild-type control diet is represented by FWC, female wild-type high cholesterol diet is represented by FWT, female *SCP2* gene-ablated control diet is represented by FKC, and female *SCP2* gene-ablated high cholesterol diet is represented by FKT. The '*' represents significance between high cholesterol diet and control diet within gene ablated groups. The '^' represents significance between wild-type and *SCP2* gene-ablated mice within diet treatment groups. Significance between sexes is indicated by 'M or F' and refers to corresponding groups. Significance between sexes where a statistically significant difference is seen between indicated group and all other groups of the opposite sex is indicated by 'M+ or F+'. Statistical analysis is a multi-way ANOVA with Tukey's multiple comparisons test. Statistical significance is defined as $p<0.05$.

CHAPTER IV

RESULTS

Study 2: Sterol Carrier Protein 2 Gene Ablation and Dietary Cholesterol Increase Choline Glycerophospholipid, Total Phospholipid Content, and Cholesteryl Ester Mass in Male and Female Mouse Liver

In this study, liver phospholipid and neutral lipid mass was assessed in *SCP2* gene-ablated male and female mice fed a high cholesterol or control diet. The major classes of phospholipids (EtnGpl, lysoPtdEtn, PtdIns, PtdSer, ChoGpl, lysoPtdCho, CerPCho), and neutral lipid mass (cholesterol and cholesteryl esters) were measured. Phospholipid mass was normalized to protein in each sample and values expressed as nmol/mg protein. Phospholipid composition was calculated because it is a useful tool to assess if one phospholipid is increased relative to another, thereby giving some indication of alterations in biosynthetic pathways. In addition, because the molar composition is independent of sample volume or losses during sample handling, the total phospholipid mass (nmol/mg protein) for each sample was used to calculate individual phospholipid mass for the respective samples using the molar composition. Cholesterol and cholesteryl ester mass was quantified and normalized to sample protein with values expressed as nmol/mg of protein.

4.1 Effect of Sterol Carrier Protein 2 Gene Ablation on Steady State Phospholipid and Neutral Lipid Mass in Liver

Phospholipid Mass

There were no significant differences in phospholipid mass or molar phospholipid composition between wild-type and *SCP2* gene-ablated mice in liver (Table 12).

Table 12. Effect of *SCP2* gene-ablation on phospholipid mass and molar composition in liver.

	WT		**KO**		**WT**		**KO**	
Class	Mean	SD	Mean	SD	Mean	SD	Mean	SD
	nmol/mg protein				mol%			
EtnGpl	61	8	73	16	22	4	23	4
PtdIns	24	5	29	4	8	1	9	1
PtdSer	16	4	16	3	5	1	4	1
ChoGpl	132	28	142	17	47	6	46	4
CerPCho	15	5	15	2	5	1	4	1
lysoPtdCho	42	15	52	12	13	4	14	4
TOTALS	289	47	327	10				
	n=14		n=14		n=14		n=14	

Values are expressed as nmol/mg protein and represent mean ± standard deviation, n=14. The '*' represents significance between wild type and *SCP2* gene-ablated mice. Wild-type is represented by WT, and *SCP2* gene-ablation is represented by KO. Statistical analysis is two-tailed Students t-test. Statistical significance is defined as $p<0.05$.

Cholesterol Mass

There were no significant differences in cholesterol or cholesteryl ester mass between wild-type and *SCP2* gene-ablated mice as seen in Table 13.

Table 13. Effect of *SCP2* gene-ablation on neutral lipid mass in liver.

	WT		**KO**	
Class	Mean	SD	Mean	SD
	nmol/mg protein			
Cholesteryl Esters	42	16	33	12
Cholesterol	37	7	40	11
	n=14		n=14	

Values are expressed as nmol/mg protein and represent mean ± standard deviation, n=14. The '*' represents significance between wild type and *SCP2* gene-ablated mice. Wild-type is represented by WT, and *SCP2* gene-ablation is represented by KO. Statistical analysis is two-tailed Students t-test. Statistical significance is defined as $p<0.05$.

4.2 Effect of SCP2 Gene Ablation on Liver Steady State Phospholipid and Neutral Lipid Mass and Differences between Male and Female Mice

Phospholipid Mass

The total phospholipid mass of male *SCP2* gene-ablated mice significantly increased by 24% compared to male wild-type mice and was accounted for by a 19% increase in ChoGpl (Table 14). Interestingly, there was a base-line significant difference between wild-type male mice and wild-type female mice, with female mice having 25% more total phospholipid mass than males. This was accounted for by a 27% increase in ChoGpl in liver (Table 14). There were no other significant differences between groups in liver phospholipid mass.

Table 14. *SCP2* gene-ablation increases total phospholipid mass in male and female mice and difference in steady state total phospholipid mass between sex in liver.

	MWT		**MKO**		**FWT**		**FKO**	
Class	Mean	SD	Mean	SD	Mean	SD	Mean	SD
				nmol/mg protein				
EtnGpl	57	6	83	18*	64	9	63	5
PtdIns	20	2	27	4	27	3	30	3
PtdSer	14	3	15	3	18	4	17	2
ChoGpl	111	12^	137	19*	153	23	148	14
CerPCho	11	3	14	1	20	2	16	3
lysoPtdCho	37	7	52	16	48	19	53	7
TOTALS	249	20^	326	14*	330	23	328	4
	n=7		n=7		n=7		n=7	

Values are expressed as nmol/mg protein and represent mean ± standard deviation, n=7. The '*' represents significance between wild type and *SCP2* gene-ablated mice within each sex. The '^' represents significance between corresponding male and female mice. Male wild-type mice are represented by MWT, male gene-ablated mice are represented by MKO, female wild-type mice are represented by FWT, and female gene-ablated mice are represented by FKO. Statistical analysis is two-way ANOVA with Tukey's multiple comparisons test. Statistical significance is defined as $p<0.05$.

Phospholipid Composition

There was a significant decrease in the proportion of EtnGpl by 13% in female wild-type mice compared to male wild-type mice in liver, and a 24% decrease in female *SCP2* gene-ablated mice compared to male gene-ablated mice (Table 15). There were no other significant differences between groups in liver phospholipid molar composition.

Table 15. *SCP2* gene-ablation decreases the proportion of ChoGpl in female mice and sex differences in phospholipid molar composition.

	MWT		**MKO**		**FWT**		**FKO**	
Class	Mean	SD	Mean	SD	Mean	SD	Mean	SD
				mol%				
EtnGpl	23	1^	25	5^	20	2^	19	2^
PtdIns	8	1	8	1	8	1	9	1
PtdSer	6	1	5	1	5	1	5	1
ChoGpl	44	3	42	5	46	7	45	4
CerPCho	4	1	4	0	6	1	5	1
lysoPtdCho	15	2	16	5	14	5	16	2
	n=7		n=7		n=7		n=7	

Values are expressed as nmol/mg protein and represent mean ± standard deviation, n=7. The '*' represents significance between wild type and *SCP2* gene-ablated mice within each sex. The '^' represents significance between corresponding male and female mice. Male wild-type mice are represented by MWT, male gene-ablated mice are represented by MKO, female wild-type mice are represented by FWT, and female gene-ablated mice are represented by FKO. Statistical analysis is two-way ANOVA with Tukey's multiple comparison test. Statistical significance is defined as $p<0.05$.

Cholesterol Mass

In liver there was a significant 49% increase in cholesteryl esters mass in female *SCP2* gene-ablated mice compared to male *SCP2* gene-ablated mice (Table 16). There was a significant 48% increase in cholesteryl ester mass in female wild-type mice compared to male wild-type mice in liver. There were no other significant differences between groups in liver neutral lipid mass.

Table 16. Female mice have more cholesteryl ester mass than male mice.

Class	MWT Mean	MWT SD	MKO Mean	MKO SD	FWT Mean	FWT SD	FKO Mean	FKO SD
	nmol/mg protein							
Cholesteryl Esters	29	4^	22	4^	56	11^	43	8^
Cholesterol	30	2	33	4	43	3	47	11
	n=7		n=7		n=7		n=7	

Values are expressed as nmol/mg protein and represent mean ± standard deviation, n=7. The '*' represents significance between wild type and *SCP2* gene-ablated mice within each sex. The '^' represents significance between corresponding male and female mice. Male wild-type mice are represented by MWT, male gene-ablated mice are represented by MKO, female wild-type mice are represented by FWT, and female gene-ablated mice are represented by FKO. Statistical analysis is two-way ANOVA with Tukey's multiple comparisons test. Statistical significance is defined as $p<0.05$.

4.3 Effect of *SCP2* Gene Ablation and High Cholesterol Diet on Liver Phospholipid and Neutral Lipid Mass in Male and Female Mice

Phospholipid Mass

In liver, male wild-type mice fed a control diet had significantly lower total phospholipid mass by approximately 27% than all other female groups; accounted for by a 32% decrease in ChoGpl within this group. The male wild-type high cholesterol diet mice had a significantly higher total phospholipid mass by 28% than the male control group as well as it's female counterpart (FWT) by 6%. There was a significant independent increase in total phospholipid mass, EtnGpl, and ChoGpl in *SCP2* gene-ablated control diet male mice compared to wild-type control diet male mice. In *SCP2* gene-ablated, high cholesterol diet, male mice the total phospholipid mass was significantly higher by 7% accounted for by a 18% increase in ChoGpl compered to

SCP2 gene-ablated, control diet, male mice. In this same group (MKT), the total phospholipid mass was significantly lower than female, *SCP2* gene-ablated, high cholesterol diet mice (FKT) by 6%. In male mice there was a significant increase in EtnGpl and ChoGpl when treated with high cholesterol diet in wild-type mice. Male wild-type mice also contained 22% significantly higher amounts of EtnGpl when treated with a high cholesterol diet than its female counterpart (FWT) in liver.

In females, there was an independent significant 13% increase in ChoGpl when fed a high cholesterol diet in wild-type mice compared to control diet. There was also an independent significant 13% increase in ChoGpl when fed a high cholesterol diet in *SCP2* gene-ablated female mice compared to control diet. Female, *SCP2* gene-ablated mice, fed a high cholesterol diet had a significantly higher total phospholipid mass than both female *SCP2* gene-ablated control diet by 12%, and female wild-type high cholesterol diet mice by 12%. These phospholipid mass changes significantly contributed to the fluctuation in the phospholipid/cholesterol ratio (Table 21 and Table 22). There were no other significant differences between groups in regard to phospholipid mass in the liver.

Phospholipid Composition

Male wild-type mice that were fed a high cholesterol diet had a significantly higher proportion of EtnGpl by 19% than its female counterpart (FWT), while proportion of ChoGpl, was significantly 17% lower than its female counterpart in liver (Table 18 and Table 20). In male *SCP2* gene-ablated mice fed a high cholesterol diet there was an independent and significant 13% increase in the proportion of ChoGpl compared to male *SCP2* gene-ablated mice fed a control diet (Table 18). In female wild-type mice fed a

high cholesterol diet there was a significant 15% increase in the proportion of ChoGpl than female control diet wild-type mice (Table 20). In female *SCP2* gene-ablated mice fed a high cholesterol diet, there was a significant 11% decrease in the proportion of ChoGpl compared to female wild-type mice fed a high cholesterol diet; and proportion of lysoPtdCho decreased by 31% compared to female *SCP2* gene-ablated mice fed a control diet (Table 20). There were no other significant differences in molar composition of phospholipids in the liver between groups.

Table 17. Effect of *SCP2* gene-ablation and high cholesterol diet in liver on major phospholipid classes mass in male mice.

	MWC		**MWT**		**MKC**		**MKT**	
Class	Mean	SD	Mean	SD	Mean	SD	Mean	SD
				nmol/mg protein				
EtnGpl	57	6	90	20^{*F}	83	$18^{\wedge}$	89	12
PtdIns	20	2	28	7	27	4	26	3
PtdSer	14	3	14	3	15	3	12	2
ChoGpl	111	12^{F+}	156	23^{*}	137	$19^{\wedge}$	168	8^{*}
CerPCho	11	3	16	3	14	1	12	2
lysoPtdCho	37	7	44	15	52	16	41	7
TOTALS	249	20^{F+}	348	29^{*F}	326	$14^{\wedge}$	349	3^{*F}
	n=7		n=7		n=7		n=7	

Values are expressed as nmol/mg protein and represent mean ± standard deviation, n=7. Male wild-type control diet is represented by MWC, male wild-type high cholesterol diet is represented by MWT, male *SCP2* gene-ablated control diet is represented by MKC, and male *SCP2* gene-ablated high cholesterol diet is represented by MKT. The '*' represents significance between high cholesterol diet and control diet within gene ablated groups. The '^' represents significance between wild-type and *SCP2* gene-ablated mice within diet treatment groups. Significance between sexes is indicated by 'M or F' and refers to corresponding groups. Significance between sexes where a statistically significant difference is seen between indicated group and all other groups of the opposite sex is indicated by 'M+ or F+'. Statistical analysis is a multi-way ANOVA with Tukey's multiple comparisons test. Statistical significance is defined as $p<0.05$.

Table 18. Effect of *SCP2* gene-ablation and high cholesterol diet in liver on major phospholipid classes mass in female mice.

	MWC		**MWT**		**MKC**		**MKT**	
Class	Mean	SD	Mean	SD	Mean	SD	Mean	SD
				mol %				
EtnGpl	23	1	26	5^{F}	25	5^{F}	26	4
PtdIns	8	1	8	2	8	1	8	1
PtdSer	6	1	4	1	5	1	3	1
ChoGpl	44	3	45	6^{F}	42	5	48	2^{*}
CerPCho	4	1	5	1	4	0	3	1
lysoPtdCho	15	2	13	4	16	5	12	2
	n=7		n=7		n=7		n=7	

Values are expressed as nmol/mg protein and represent mean ± standard deviation, n=7.Male wild-type control diet is represented by MWC, male wild-type high cholesterol diet is represented by MWT, male *SCP2* gene-ablated control diet is represented by MKC, and male *SCP2* gene-ablated high cholesterol diet is represented by MKT. The '*' represents significance between high cholesterol diet and control diet within gene ablated groups. The '^' represents significance between wild-type and *SCP2* gene-ablated mice within diet treatment groups. Significance between sexes is indicated by 'M or F' and refers to corresponding groups. Significance between sexes where a statistically significant difference is seen between indicated group and all other groups of the opposite sex is indicated by 'M+ or F+'. Statistical analysis is a multi-way ANOVA with Tukey's multiple comparisons test. Statistical significance is defined as $p<0.05$.

Table 19. Effect of *SCP2* gene-ablation and high cholesterol diet in liver on major phospholipid classes molar composition in male mice.

	FWC		**FWT**		**FKC**		**FKT**	
Class	Mean	SD	Mean	SD	Mean	SD	Mean	SD
				nmol/mg protein				
EtnGpl	64	9	70	12	63	5	82	12
PtdIns	27	3	23	3	30	3	38	7
PtdSer	18	4	12	2	17	2	15	2
ChoGpl	153	23	175	14*	148	14	179	18*
CerPCho	20	2	15	2	16	3	18	3
lysoPtdCho	48	19	33	8	53	7	40	7
TOTALS	330	23	328	7^{M}	328	4	371	34$^{*\wedge M}$
	n=7		n=7		n=7		n=7	

Values are expressed as nmol/mg protein and represent mean ± standard deviation, n=7. Female wild-type control diet is represented by FWC, female wild-type high cholesterol diet is represented by FWT, female *SCP2* gene-ablated control diet is represented by FKC, and female *SCP2* gene-ablated high cholesterol diet is represented by FKT. The '*' represents significance between high cholesterol diet and control diet within gene ablated groups. The '^' represents significance between wild-type and *SCP2* gene-ablated mice within diet treatment groups. Significance between sexes is indicated by 'M or F' and refers to corresponding groups. Significance between sexes where a statistically significant difference is seen between indicated group and all other groups of the opposite sex is indicated by 'M+ or F+'. Statistical analysis is a multi-way ANOVA with Tukey's multiple comparisons test. Statistical significance is defined as $p<0.05$.

Table 20. Effect of *SCP2* gene-ablation and high cholesterol diet in liver on major phospholipid classes molar composition in female mice.

	FWC		**FWT**		**FKC**		**FKT**	
Class	Mean	SD	Mean	SD	Mean	SD	Mean	SD
				mol %				
EtnGpl	20	2	21	3^{M}	19	2^{M}	22	3
PtdIns	8	1	7	1	9	1	10	1
PtdSer	5	1	4	1	5	1	4	0
ChoGpl	46	7	54	4^{*M}	45	4	48	$2^{\wedge}$
CerPCho	6	1	4	1	5	1	5	0
lysoPtdCho	14	5	10	3	16	2	11	2^{*}
	n=7		n=7		n=7		n=7	

Values are expressed as nmol/mg protein and represent mean ± standard deviation, n=7. Female wild-type control diet is represented by FWC, female wild-type high cholesterol diet is represented by FWT, female *SCP2* gene-ablated control diet is represented by FKC, and female *SCP2* gene-ablated high cholesterol diet is represented by FKT. The '*' represents significance between high cholesterol diet and control diet within gene ablated groups. The '^' represents significance between wild-type and *SCP2* gene-ablated mice within diet treatment groups. Significance between sexes is indicated by 'M or F' and refers to corresponding groups. Significance between sexes where a statistically significant difference is seen between indicated group and all other groups of the opposite sex is indicated by 'M+ or F+'. Statistical analysis is a multi-way ANOVA with Tukey's multiple comparisons test. Statistical significance is defined as $p<0.05$.

Cholesterol Mass

In contrast to brain, there was no significant difference in cholesterol mass between groups in liver, but there were significant differences in the mass of cholesteryl esters.

In male wild-type mice fed a high cholesterol diet, there was a 90% significant increase in cholesteryl ester mass in liver compared to male wild-type mice fed the

control diet (Table 21), this group (MWT) also significantly had 66% lower cholesteryl ester mass than its female counterpart (FWT) (Table 21 and Table 22). In male *SCP2* gene-ablated mice fed a high cholesterol diet there was significantly 74% less cholesteryl ester mass than wild-type male mice fed a high cholesterol diet (Table 21), this group (MKT) also had significantly 70% less cholesteryl ester mass than its female counterpart (FKT) as seen in Table 21 and Table 22.

In females, wild-type mice fed a high cholesterol diet had significantly 93% more cholesteryl ester mass than female wild-type mice fed a control diet (Table 22). Female *SCP2* gene-ablated mice fed a high cholesterol diet had significantly more cholesteryl ester mass by 82% than female gene-ablated mice fed a control diet; and significantly 71% less cholesteryl ester mass than female wild-type mice fed a high cholesterol diet (Table 22). There were no other significant differences between groups in cholesterol mass in liver.

In both males and females there was a significant decrease in phospholipid/cholesterol ratio in *SCP2* gene-ablated mice fed a high cholesterol diet compared to wild-type mice fed a high cholesterol diet. In female wild-type mice fed a high cholesterol diet there was a significant increase in this ratio compared to female wild-type mice fed a control diet. The most prominent significant difference in phospholipid/cholesterol ratio is the sex difference. Male wild-type mice fed a high cholesterol diet, and both male *SCP2* gene-ablated groups had a significantly lower ratio compared to their female counterparts respectively. However, there was not a significant difference between sexes in wild-type mice fed a control diet.

Table 21. Effects of S*CP2* gene ablation and dietary cholesterol on liver cholesterol and cholesteryl ester mass in male mice.

	MWC		**MWT**		**MKC**		**MKT**	
Class	Mean	SD	Mean	SD	Mean	SD	Mean	SD
	nmol/mg protein							
Cholesteryl Esters	29	4	282	94^{F*}	22	4	73	$17^{F\wedge}$
Cholesterol	30	2	53	4	33	4	35	7
C/PL Ratio	0.12	0.01	0.15	0.01^{F}	0.1	0.01^{F}	0.1	$0.02^{F\wedge}$
	n=7		n=7		n=7		n=7	

Values are expressed as nmol/mg protein and represent mean ± standard deviation, n=7. Male wild-type control diet is represented by MWC, male wild-type high cholesterol diet is represented by MWT, male *SCP2* gene-ablated control diet is represented by MKC, and male *SCP2* gene-ablated high cholesterol diet is represented by MKT. The '*' represents significance between high cholesterol diet and control diet within gene ablated groups. The '^' represents significance between wild-type and *SCP2* gene-ablated mice within diet treatment groups. Significance between sexes is indicated by 'M or F' and refers to corresponding groups. Significance between sexes where a statistically significant difference is seen between indicated group and all other groups of the opposite sex is indicated by 'M+ or F+'. Statistical analysis is a multi-way ANOVA with Tukey's multiple comparisons test. Statistical significance is defined as $p<0.05$.

Table 22. Effects of SCP2 gene ablation and dietary cholesterol on liver cholesterol and cholesteryl ester mass in female mice.

	FWC		FWT		FKC		FKT	
Class	Mean	SD	Mean	SD	Mean	SD	Mean	SD
	nmol/mg protein							
Cholesteryl Esters	56	11	827	223^{M*}	43	8	239	$88^{M*\wedge}$
Cholesterol	43	3	83	14	47	11	53	7
C/PL Ratio	0.13	0.01	0.25	0.04^{M*}	0.14	0.03^{M}	0.14	$0.02^{M\wedge}$
	n=7		n=7		n=7		n=7	

Values are expressed as nmol/mg protein and represent mean ± standard deviation, n=7. Female wild-type control diet is represented by FWC, female wild-type high cholesterol diet is represented by FWT, female *SCP2* gene-ablated control diet is represented by FKC, and female *SCP2* gene-ablated high cholesterol diet is represented by FKT. The '*' represents significance between high cholesterol diet and control diet within gene ablated groups. The '^' represents significance between wild-type and *SCP2* gene-ablated mice within diet treatment groups. Significance between sexes is indicated by 'M or F' and refers to corresponding groups. Significance between sexes where a statistically significant difference is seen between indicated group and all other groups of the opposite sex is indicated by 'M+ or F+'. Statistical analysis is a multi-way ANOVA with Tukey's multiple comparisons test. Statistical significance is defined as $p<0.05$.

CHAPTER V

DISCUSSION

SCP-2 and SCP-x are ubiquitously intracellular proteins highly expressed in the liver, brain, gonads, heart, adrenal glands, and intestine with highest expression being found in the liver (Lin et al., 1995; Moncecchi et al., 1991; Xu et al., 2023). These proteins are localized to the mitochondria, peroxisomes, and nucleoplasm however there is also a general localization within the cytosol (The Human Protein Atlas, 2023). Studies demonstrate that *SCP2* has a broad functional role including: peroxisomal oxidation of branch-chain fatty acids; steroidogenesis; cholesterol esterification; cholesterol trafficking; and more recent studies showing binding of fatty acids and phospholipids (Antonenkov et al., 1997; Pfeifer et al., 1993; Puglielli et al., 1995; Seedorf et al., 2000; Wanders et al., 1997; Wirtz et al., 1998). These studies still leave a gap in knowledge defining differences between males and females in lipid metabolism and the effects of diet on functions of *SCP2.*

Early studies focused on defining the functional roles of *SCP2* were based on data collected from cell culture systems and male mice. The few studies that document roles in female mice or rats focus on *SCP2* knock-out on sex organs and roles in bile acid binding in the liver. These studies suggest that like in male mice, *SCP2* has a role in steroidogenesis in female sex organs (Giatti et al., 2019; Lin et al., 1995). More recent studies demonstrate that SCP-2 and SCP-x are preferentially used in a secondary bile acid

synthesis pathway, however, does not affect hepatic bile acid levels or esterification of cholesterol (Martin et al., 2015). This study also defines the role of liver fatty acid binding protein (L-FABP or FABP1) in hepatic bile acid binding in female mice. L-FABP has been well documented to be a non-specific lipid chaperone that is abundantly expressed in the liver (Bass, 1985). Like SCP-2, FABP facilitates the intracellular transfer of hydrophobic lipids between membranes(Hsu & Storch, 1996; Kim & Storch, 1992; Storch & Bass, 1990; Woodford et al., 1995). The functional difference between males and females has been better documented in FABP and its effects on lipid metabolism. These studies show a baseline difference in L-FABP expression between male and female mice, with an increased level in female liver compared to males (Luxon & Weisiger, 1993), although recent studies suggest males may have higher protein levels than females (Huang et al., 2016). This higher L-FABP expression affects lipid metabolism by increasing phospholipid mass and impacting the esterification of fatty acids and neutral lipids (Murphy, Prows, et al., 2000a; Prows et al., 1995). Increased *Fabp1* expression increases fatty acid and cholesterol uptake and diffusion within the cell (Jefferson et al., 1991; Murphy, Prows, et al., 2000b). With this understanding of sex differences in regard to FABP, it can be predicted that there will also be baseline differences between sexes in regard to SCP-2 and SCP-x, but results are mixed in this regard (Huang et al., 2016; Roff et al., 1992).

Unlike FABP, SCP-2 expression in L-cell fibroblasts decreased phospholipid mass with a decrease in phosphatidylinositol (PtdIns), phosphatidylserine (PtdSer), and choline glycerophospholipid (Murphy, Stiles, et al., 2000). This same study also reported that SCP-2 expression had no effect of free cholesterol mass, causing an increase in the

phospholipid to cholesterol ratio (Murphy, Stiles, et al., 2000). A decrease in total phospholipid mass from SCP-2 expression was also supported in another study (Murphy, 2002), however it also reported that the expression of SCP-2 enhances neutral lipid levels with a specific increase in cholesteryl esters and triacylglycerol (Murphy, 2002). This could suggest that *SCP2* gene ablation would cause a decrease in neutral lipid levels and cause an increase in total phospholipid mass.

More recent studies have investigated the impact of diet, specifically high fat diets, on lipid binding proteins such as FABP and SCP-2/SCP-x. These studies suggest that these binding proteins play a large role in preventing increased lipid mass in liver and brain in mice fed a high fat diet (Atshaves et al., 2010b; Martin et al., 2019). Impaired function of *SCP2* could be a contributing factor in diseases such as fatty liver disease and obesity. Not only that, but recent work reports that *SCP2* is also involved in progressive hypercholesterolemia, vascular inflammation, atherosclerosis, cancer, and it may regulate the brain endocannabinoid system (Martin et al., 2019; Milligan et al., 2018; Myers-Payne et al., 1996; Wang et al., 2019; Xu et al., 2023). There is still a gap in knowledge left from these preliminary studies reporting the impact of *SCP2* on diet. However, it presents an exciting opportunity to study how lipid metabolism and lipid composition are affected by a high cholesterol diet and the presence of *SCP2*.

As previously stated, SCP-2 expression increases neutral lipid levels in L-cell fibroblasts, and it is well known that SCP-2 binds cholesterol to facilitate membrane exchange and intracellular transport (Frolov et al., 1996; Moncecchi et al., 1996; Murphy & Schroeder, 1997; Puglielli et al., 1995). Not only do the proteins encoded by *SCP2* transport cholesterol but it is also involved in cholesterol synthesis and the enzymatic

esterification of cholesterol (Antonenkov et al., 1997; Murphy & Schroeder, 1997). Specifically, SCP-x, a 3-oxoacyl-CoA thiolase, is crucial for β-oxidation during cholesterol synthesis (Antonenkov et al., 1997; Wanders et al., 1997). Whereas SCP-2 stimulates an increase in cholesteryl ester synthesis from the plasma membrane (Murphy & Schroeder, 1997). SCP-2 is also known to be involved in cholesterol synthesis; however, it preferentially stimulates pregnenolone synthesis in the mitochondria (Pfeifer et al., 1993), the first step in steroidogenesis. It is still not known how diet or sex affect these functional roles of *SCP2*, nevertheless impaired function of *SCP2* could be linked to cholesterol buildup and exacerbated when paired with a high fat diet.

Based upon these previous results, we hypothesized that *SCP2* gene ablation, sex, and high cholesterol diet impacts phospholipid and sterol steady state mass having a greater effect in the liver than brain. This is consistent with previously reported expression studies that show *SCP2* to be highly expressed in the liver where it is used to transport phospholipids and sterols between intracellular membranes (Frolov et al., 1996). To assess this, male and female *SCP2* gene-ablated mice were fed a high cholesterol diet and compared to wild-type male and female mice fed a control diet. Additionally, we measured phospholipid steady state mass, phospholipid molar composition, cholesterol and cholesteryl ester steady state mass in brain and liver to determine the impact of SCP-2/SCP-x and diet in male and female mice.

In this study, *SCP2* gene-ablation paired with high cholesterol diet demonstrated several important differences in brain lipid mass. First, *SCP2* deletion resulted in a 17% increase in total phospholipid mass in the brain of male mice (Table 6), but not in female ablated mice (Table 8). This was accounted for by an expected increase in ChoGpl mass

(21%), which could be due to a decrease in intracellular movement of ChoGpl via SCP-2 (Atshaves et al, 1999). This is consistent with previous data showing that SCP-2 binds ChoGpl for transport between membranes (Atshaves et al., 1999), which may impact its synthesis. This is consistent with the expression of SCP-2 reducing ChoGpl mass in L-cells expressing (Murphy, Stiles, et al., 2000). Second, female mice consistently had approximately 50% less brain cholesterol mass than male mice. This is likely due to the presence of estrogen at a higher level in female mice causing a decrease in cholesterol levels in the brain (ISLAM et al., 1984). This change was not observed in total phospholipid mass suggesting it was not the result of differences in samples between the male and female groups. There were no significant changes in cholesterol between groups within each sex, supporting previous studies that the brain is a cholesterol privileged organ (Björkhem & Meaney, 2004; Pfrieger, 2003). This means that a high cholesterol diet did not affect brain cholesterol levels in male and female mouse groups, however there was a baseline difference between sexes independent of SCP-2 expression.

However, *SCP2* gene-ablation parried with high cholesterol diet had a profound and significant decrease in cholesteryl ester mass in both sexes. Gene ablation under high dietary cholesterol conditions resulted in a 61% decrease in cholesteryl ester mass in female mice (Table 11), and a 50% decrease in male mice compared to the other groups (Table 10). This was not observed in gene-ablated mice fed a control diet, suggesting that in the absence of *SCP2* paired with a high cholesterol diet there is likely reduced trafficking of cholesterol for formation of cholesteryl esters in the brain, which is independent of a change in brain cholesterol mass. We speculate that this is either due to a reduction of acyl-CoA cholesterol acyl transferase activity (ACAT) or a down

regulation by a complex signaling mechanism that affects brain cholesteryl esters based on input from dietary cholesterol in both male and female mice.

Similar to brain, liver demonstrated specific, but limited changes in phospholipid mass. *SCP2* gene-ablated mice paired with a high cholesterol diet resulted in a 7% increase in total phospholipid mass in the liver of male mice, a similar affect also seen in the brain (Table 17). Similar to brain this was accounted for by a 18% increase in ChoGpl mass and could be a result of impaired intracellular movement via SCP-2 consistent with what was reported in a cellular processing study on *SCP2* (Atshaves et al., 1999). A similar effect was observed in male wild-type mice fed a high cholesterol diet where a 28% increase in total phospholipid mass was accounted for by a 29% increase in ChoGpl mass compared to wild-type mice fed a control diet (Table 17), suggesting that this change is independent of SCP-2 expression.

In liver, male and female mice that were fed a high cholesterol diet resulted in a 70%-90% increase in cholesteryl ester mass compared to mice fed a control diet (Tables 21 and 22).. An opposite effect compared to brain where only cholesterol mass changed between male and female mice independent of dietary cholesterol or *SCP2* gene ablation (Tables 10 and 11). This increase in liver cholesteryl esters was greater in the wild-type mice than the *SCP2* gene-ablated mice, suggesting that SCP-2/SCP-x is important for cholesteryl ester formation in the liver. This aligns with previous studies done in L-cell fibroblasts where *SCP2* expression stimulates cholesteryl ester formation (Murphy & Schroeder, 1997). Female mice had a 48%-69% increase in cholesteryl ester mass compared to male mice, likely due to the effect of estrogen on VLDL formation (Palmisano et al., 2017). These results suggest that a high cholesterol diet increases liver

secretion of VLDL. VLDL is a lipoprotein that consists of an outer shell where phosphatidylcholine (ChoGpl) is found and an interior core where cholesteryl esters are a key component (Smith et al., 1978). This helps to explain the increase in ChoGpl and cholesteryl esters in the liver as they are being utilized for VLDL synthesis and secretion. Interestingly, in *SCP2* gene-ablated mice there was approximately a 73% decrease in cholesteryl ester mass compared to wild-type mice (Table 21 and Table 22). This suggests that SCP-2/SCP-x may be involved in VLDL synthesis in the liver, and function as an intracellular membrane transport protein to traffic cholesteryl esters and ChoGpl for the formation of this lipoprotein.

The sterol carrier protein 2 gene (*SCP2*) encodes two ubiquitous and evolutionarily conserved proteins that plays a crucial role in lipid metabolism(Pfeifer et al., 1993). These proteins include proSCP-2 (15 kDa) and SCP-x (58 kDa) where proSCP-2 undergoes post-translational cleavage to form SCP-2 (13.2 kDa) (Moncecchi et al., 1991; Ohba et al., 1994a; Schroeder et al., 2000). SCP-2 is found in the endoplasmic reticulum, peroxisomes, mitochondrial membrane, and the cytosol where it is involved in the transfer of lipids such as cholesterol, fatty acids, and phospholipids between cellular membranes. This protein is expressed in various tissues including liver, brain, gonads, and heart and has been implicated in several physiological processes such as lipid transport, steroidogenesis, endocannabinoid metabolism, inflammation, fatty acid metabolism, and cancer progression. Impaired *SCP2* function has been linked to several diseases including hypercholesterolemia, liver disease, vascular inflammation, atherosclerosis, and cancer (McIntosh et al., 2017; Wang et al., 2019; Xu et al., 2023).

These data validate that *SCP2* facilitates brain and liver ChoGpl, cholesterol, and cholesteryl ester metabolism. Results also suggest that a high cholesterol diet increases cholesterol ester synthesis in the liver where SCP-2/SCP-x is important for the formation of VLDL. These finding help understand the role that SCP-2 and SCP-x have in diseases such as hypercholesterolemia, liver disease, vascular disease, and cardiac disease where VLDL cholesterol is one of the main contributing molecules in the progression of these diseases. It is clear that SCP-2 is important in lipid metabolism and dietary cholesterol has an important role in determining steady-state levels of phospholipids and neutral lipids in the brain and liver. However, when coupled, SCP-2 and increased dietary cholesterol seem to mediate signaling for brain cholesterol levels. Irrespective of *SCP2* gene-ablation and diet these data also suggest a sex difference in steady-state total phospholipid and neutral lipid levels.

While this work supports prior research regarding *SCP2* function in ChoGpl metabolism and cholesteryl ester formation, there are also limitations. This study only measured steady-state lipid levels, not taking into account the dynamic nature of lipid synthesis and catabolism. Phospholipid and neutral lipid movement was not measured via radiotracer studies, limiting our understanding and appreciation of potential complex metabolic interactions in this knockout model. Therefore, these data suggest intracellular movement of cholesterol via SCP-2 in the brain and liver as well as an influence of SCP-2 mainly on ChoGpl levels. During this study male and female brain and liver was extracted and then homogenates were produced. This presents a limitation as homogenates have the potential to facilitate lipid breakdown, although our data does not indicate adverse breakdown in the brain samples. Brain samples are the most susceptible

to these changes and in this case, in the absence of microwave fixation, changes may have occurred despite the careful nature of sample preparation (Murphy, 2010). Although there are limitations present, it is important that future research aims to further define differences in lipid composition and metabolism between males and females. This study also illuminates potential future directions in the mechanistic role that SCP-2 has in VLDL formation and export, especially in light of increased dietary cholesterol uptake. Understanding *SCP2* in both sexes will lead to improved diagnostics and treatment of liver, heart, and vascular disease, hypercholesterolemia, and possibly cancer, while these data herein suggest an equally important role for SCP-2 in the brain as well as the influence of elevated dietary cholesterol on brain-sterol metabolism.

APPENDICES

Abbreviations

ARA: arachidonic acid, 20:4n-6

CerPCho: sphingomyelin

ChoGpl: choline glycerophospholipid

CNS: central nervous system

DAG: diacylglycerol

DGK: diacylglycerol kinase

eCB: endocannabinoids

EtnGpl: ethanolamine glycerophospholipid

FABP: fatty acid binding protein

HIP: hexane:isopropanol

HPLC: high performance liquid chromatography

KCl: potassium chloride

LCFA: long-chain fatty acid

LCFA-CoA: long-chain fatty acyl coenzyme A

lysoPtdCho: lysophosphatidylcholine

lysoPtdEtn: lysophosphatidylethanolamine

MUFA: monounsaturated fatty acid

PtdEtn: phosphatidylethanolamine

PtdIns: phosphatidylinositol

PtdSer: phosphatidylserine

PUFA: polyunsaturated fatty acid

sat: saturated fatty acid

S.D.: standard deviation

SCP: sterol carrier protein

SCP2: sterol carrier protein 2

SCPx: sterol carrier protein x

TAG: triacylglycerol

TLC: thin-layer chromatography

Unsat: unsaturated fatty acid

UV: ultraviolet

VLDL: very low-density lipoprotein

WT: wild-type

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