THE IMPACT OF FABP1/SCP2/SCPX ABLATION IN MICE CHALLENGED WITH HIGH PHYTANIC ACID AND HIGH FAT PAIR-FED DIETS

A Dissertation

by

SHERRELLE MARION MILLIGAN

Submitted to the Office of Graduate and Professional Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Chair of Committee,	Ann Kier
Co-Chair of Committee,	Friedhelm Schroeder
Committee Members,	Ian Tizard, DVM
	Albert Mulenga
Head of Department,	Ramesh Vemulapalli

December 2018

Major Subject: Veterinary Pathobiology

ABSTRACT

In vitro studies suggest that fatty acid bind protein 1 (*Fabp1*) and sterol carrier protein 2/sterol carrier protein x (*Scp2/Scpx*) gene products facilitate the uptake, metabolism and detoxification of dietary phytol in mammals. In fact, individually ablating FABP1 or SCP2/SCPx each impairs hepatic branched-chain fatty acid uptake and/or metabolism. Studies also suggest that singly ablating *Fabp1* or *Scp2/Scpx* genes may exacerbate the impact of a high fat diet on whole body phenotype and non-alcoholic fatty liver disease (NAFLD). However, concomitant upregulation of FABP1 in SCP2/SCPx null mice complicates interpretation of each protein's physiological role in studies where only one of these proteins has been ablated. Therefore, the impact of ablating both *Fabp1* and *Scp2/Scpx* genes was explored in phytol and high fat diet fed mice.

In the phytol study, triple gene ablation (TKO) increased hepatic total lipid accumulation, primarily phospholipid, by mechanisms involved in increasing hepatic levels of proteins in the phospholipid synthesis pathway while reducing expression of proteins in targeting fatty acids towards the triacylglycerol synthesis pathway. Concomitantly, TKO also reduced expression of proteins in targeting fatty acids towards the triacylglycerol synthesis pathway. In the high fat study, wild type (WT) male mice exhibited higher hepatic lipid accumulation than WT females and TKO increased hepatic lipid accumulation in both. The greater hepatic lipid accumulation in WT males was associated with high hepatic expression of enzymes in glyceride synthesis, higher hepatic bile acids and upregulation of transporters involved in hepatic reuptake of serum bile acids. The TKO-induced increases in hepatic glycerides were attributed to sex dependent upregulation of hepatic lipogenic enzymes.

Overall, since individually ablating the *Scp2/Scpx* gene elicits the upregulation of the FABP1 protein, these findings with TKO mice help to resolve the contributions of *Scp2/Scpx* gene ablation on dietary phytol and high fat induced whole body and hepatic lipid phenotype independent of concomitant upregulation of FABP1.

DEDICATION

This dissertation is dedicated to my husband Roderick and my beautiful new son, Asher. Roderick, you have been such a great support throughout the process of me obtaining my PhD. Asher, you are the result of much love and much determination. I love you both.

ACKNOWLEDGEMENTS

I would like to thank my committee chair and co-chair, Dr. Ann Kier and Dr. Friedhelm Schroeder. Since our first encounter in 2012, you have done an exemplary job mentoring and nurturing me. I have accomplished so much under your guidance. I would also like to thank my committee members, Dr. Gregory Martin, Dr. Ian Tizard and Dr. Albert Mulenga for their guidance and support throughout the course of this research. I would also like to thank Dr. Nancy Turner for serving on my committee for most of my graduate study.

Next, I would like to thank my lab members Gregory Martin, Danilo Landrock, Kerstin Landrock, Huan Huang, Avery McIntosh, Sarah Chung and Kimberly Hein, my colleagues and the department faculty and staff for making my experience at Texas A&M as pleasant as possible under the circumstances I faced.

Finally, thanks to my family and friends for their encouragement and to my husband for his patience and love.

CONTRIBUTORS AND FUNDING SOURCES

This work was supported by a dissertation committee consisting of Dr. Ann Kier, Dr. Friedhelm Schroeder, Dr. Gregory Martin, Dr. Ian Tizard and Dr. Albert Mulenga from the Veterinary Pathobiology and Veterinary Physiology and Pharmacology Departments.

The data collection and analysis for Chapter 3 through 6 were made possible by the contributions of Danilo Landrock, Gregory Martin, Kerstin Landrock, Huan Huang, Avery McIntosh, Kimberly Hein and Sarah Chung from the departments of Veterinary Pathobiology and Veterinary Physiology and Pharmacology, College of Veterinary Medicine.

This work was financially supported by US Public Health Service/National Institutes of Health Grants RO1DK41402 (Schroeder and Kier), T35OD010991 (Kier), R25 OD 016574 (Kier), T35OD010991 (Kier), and T32OD011083 (Kier).

NOMENCLATURE

3αHSD	3alpha-hydroxysteroid reductase: An intracellular bile acid binding protein that plays a significant role in bile acid biosynthesis, steroid hormone metabolism, and xenobiotic metabolism, displays a pattern of liver specific expression, and functions to inactivate circulating steroid hormones.
ABCA1	ATP-binding cassette sub-family A member 1: A membrane-associated protein involved in nascent HDL formation. With cholesterol as its substrate, it functions as a cholesterol efflux pump in the cellular lipid removal pathway.
ABCG1, 4, 5 or 8	ATP-binding cassette sub-family G member 1, 4, 5 or 8: Involved in hepatic HDL secretion: critically involved in the regulation of lipid-trafficking mechanisms in macrophages, hepatocytes, and intestinal mucosa cells. ABCG1 participates in cholesterol and phospholipid efflux. ABCG4 plays an important role in cellular cholesterol homeostasis and functions as either a homodimer or as a heterodimer with another ABC subfamily protein such as ABCG1. The ABCG5 and ABCG8 transporters are also targets in the control and influence of total body sterol homeostasis.
ACAT2	Acetyl-CoA acetyltransferase (Soat2 gene): An enzyme in cholesterol esterification. Cholesterol is essential for efficient intestinal cholesterol

	absorption, and may control the amount of hepatic free (unesterified) cholesterol available for secretion into bile or into HDL.
ACC1	Acetyl-CoA carboxylase (*Acaca* gene): An enzyme that catalyzes the irreversible carboxylation of acetyl-CoA to produce malonyl-CoA for the biosynthesis of fatty acids. When the enzyme is active, the product, malonyl-CoA, is produced, which is a building block for new fatty acids and can inhibit the transfer of the fatty acyl group from acyl CoA to carnitine with carnitine acyltransferase, which inhibits the beta-oxidation of fatty acids in the mitochondria.
ACBP	Acyl CoA Binding Protein: A small (10 Kd) protein that binds medium- and long-chain acyl-CoA esters with very high affinity and may function as an intracellular carrier of acyl-CoA esters.
ACOX1	Peroxisomal acyl-CoA oxidase-1 (*Acox1* gene): The first enzyme of the fatty acid beta-oxidation pathway, which catalyzes the desaturation of acyl-CoAs to 2-trans-enoyl-CoAs.
AFABP	Adipocyte Fatty Acid Binding Protein (FABP4): A member of the intracellular fatty acid binding protein (FABP) family, which is known for the ability to bind fatty acids and related compounds. Its gene is critical in the regulation of the biological function of differentiated adipocytes and may be involved in mediating obesity-induced alterations in adipocyte gene expression.
AGPAT	1-acylglycerol-3-phosphate-O-acyltransferase (*Agpat2* gene): An adipokine which acylates lysophosphatidic acid at the sn-2 position to

	produce phosphatidic acid in the phospholipid and triglyceride synthesis pathway.
ALT	Alanine Aminotransferase: An enzyme found mostly in the cells of the liver and kidney that converts alanine into pyruvate, an important intermediate in cellular energy production. When the liver is damaged, ALT is released into the blood, which makes ALT a useful test for detection of liver damage.
APOA1	Apolipoprotein A1: A component of high-density lipoprotein (HDL), which is a molecule that transports cholesterol and phospholipids through the bloodstream from the tissues to liver.
APOB	Apolipoprotein B: A component of very low-density lipoproteins (VLDLs), intermediate-density lipoproteins (IDLs), and low-density lipoproteins (LDLs), all of which transport fats, including cholesterol, in the bloodstream. Apolipoprotein B-100 allows these particles to attach to specific receptors on the surface of cells, particularly in the liver. The receptors transport low-density lipoproteins into the cell, where they are broken down to release cholesterol. The cholesterol is then used by the cell, stored, or removed from the body.
AST	Aspartate Transaminase: Catalyzes the reversible transfer of an α-amino group between aspartate and glutamate so is therefore an important enzyme in amino acid metabolism. AST is found in the liver, heart, skeletal muscle, kidneys, brain, and red blood cells. Serum AST levels,

	along with serum ALT levels, are commonly measured clinically as biomarkers for liver health.
BA	β-actin: A western blotting normalization protein/housekeeper.
BFABP	Brain fatty acid binding protein (FABP7). A member to the family of intracellular lipid-binding proteins involved in fatty acid uptake and intracellular transport in the brain. FABP7 is also important in brain development.
β-HOB	β-hydroxybutyrate: A ketone body and a physiological measure of fatty acid oxidation.
BSEP	Bile salt export pump (*Abcb11* gene): A unidirectional, ATP-dependent efflux transporter that plays an important role in the elimination of bile salts from liver cells into bile canaliculi for export into the gastrointestinal tract.
C	Free cholesterol: A measure of the circulating cholesterol that has not been esterified and accounts for about 30% of the total cholesterol in plasma. Cholesterol esterification occurs before or after it is released into the circulation, and esterification with any physiologically relevant fatty acid enhances the lipid-carrying capacity of lipoproteins and prevents intracellular toxicity by free cholesterol.
CE	Cholesteryl ester. The preferred form of cholesterol for transport in plasma and as a biologically inert storage or de-toxification form to buffer an excess. Cholesterol esters do not contribute to membrane structures but are packed into intracellular lipid droplets in the cytoplasm.

COX4	Cytochrome c oxidase subunit IV: A western blotting normalization protein/housekeeper.
CPT1a or 2	Carnitine palmitoyltransferase 1a and 2 (*Cpt1a* or *2* gene): CPT1a, located in the outer mitochondrial membrane, is the rate-limiting enzyme for mitochondrial beta oxidation. CPT2, located in the inner mitochondrial membrane, is also involved in mitochondrial beta oxidation. Long-chain fatty acids cannot enter mitochondria unless they are attached to a substance known as carnitine. CPT1A connects carnitine to long-chain fatty acids so they can cross the inner membrane of mitochondria. Once these fatty acids are inside mitochondria, CPT2 removes the carnitine and adds coenzyme A. Long-chain fatty acids must be joined to coenzyme A before they can be metabolized to produce energy.
CRABP1	Cellular retinoic acid binding protein 1: A specific binding protein for retinoic acid, a metabolite of vitamin A (retinol) that mediates the functions of vitamin A required for growth and development. It is thought to play an important role in retinoic acid-mediated differentiation and proliferation processes and may contribute to vitamin A-directed differentiation in epithelial tissue.
CYP7A1	Cytochrome P7A1 (*Cyp7a1* gene). The rate-limiting enzyme in bile acid biosynthesis from cholesterol. High cholesterol diets induce Cyp7A1 gene expression in mice, and the induction of this gene is mediated via oxysterol metabolites of cholesterol-activating LXR, which directly

	regulates transcription of Cyp7A1. Therefore, LXR upregulates Cyp7a1 when cholesterol is high.
DEXA	Dual energy X-ray absorptiometry: Measures body composition in terms of fat and fat-free mass. DEXA generates X-rays at two different energies and makes use of the differential attenuation of the X-ray beam at these two energies to calculate the bone mineral content and soft tissue composition in the scanned region.
DGAT	Diacylglycerol acyltransferase (*Dgat2* gene): Catalyzes the formation of triacylglycerol from diacylglycerol and acyl-CoA. The reaction catalyzed by DGAT is the terminal and only committed step in triglyceride synthesis, and is essential for adipose tissue formation.
FABP1/LFABP	Liver fatty acid binding protein: A fatty acid binding protein that is primarily expressed in the liver, where it is involved in binding, transport and metabolism of long-chain fatty acids, endocannabinoids, phytocannabinoids and other hydrophobic molecules. Altered expression of this protein has been linked to metabolic disorders such as obesity.
FASN	Fatty acid synthase (*Fasn* gene): A multi-enzyme protein that catalyzes fatty acid synthesis. Its main function is to catalyze the synthesis of palmitate from acetyl-CoA and malonyl-CoA, in the presence of NADPH. Its expression is reduced by PPARα.
FATP2, 4, and 5	Fatty acid transport protein 2, 4 and 5: These proteins facilitate the transport of long-chain fatty acids across plasma and intracellular membranes.

FTM	Fat tissue mass: A loose connective tissue composed primarily of adipocytes.
FXR	Farnesoid x receptor: Reduces bile acid synthesis. When activated by its bile acid ligands, FXR translocates to the cell nucleus, forms a heterodimer with RXR and induces the down-regulation of Cyp7A1, the rate-limiting enzyme in bile acid synthesis from cholesterol.
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase: A western blotting normalization protein/housekeeper.
GOT	Glutamic oxaloacetic transaminase: See AST.
GPAT	Glycerol-3-phosphate acyltransferase (*Gpam* gene): A rate-limiting enzyme of triacylglycerol and phospholipid biosynthesis by catalyzing the synthesis of lysophosphatidic acid from glycerol-3-phosphate and long-chain acyl-CoA.
GST	Glutathione s-transferases: Metabolic isozymes with the ability to catalyze conjugation of the reduced form of glutathione (GSH) to xenobiotic substrates for detoxification and excretion in the bile or urine. GST are also involved in bile acid cytosolic transport, and are inhibited by bile acids.
HDL-C	High density lipoprotein cholesterol: Known as the "good" cholesterol because it helps remove other forms of cholesterol from the bloodstream rather than their accumulation within vessels. Higher levels of HDL cholesterol are associated with a lower risk of heart disease.

HMGCR	3-hydroxy-3-methylglutaryl-CoA reductase: The rate-limiting enzyme of the mevalonate pathway, the metabolic pathway that produces cholesterol. This enzyme is directly inhibited by statins, drugs to treat cardiovascular disease by reducing serum cholesterol.
HMGCS1	Cytosolic 3-hydroxy-3-methylglutaryl-CoA synthase: An enzyme which catalyzes the reaction in which Acetyl-CoA condenses with acetoacetyl-CoA to form HMG-CoA. It is the second reaction in the mevalonate-dependent cholesterol biosynthesis pathway.
HSL/CEH	Hormone sensitive lipase/cholesteryl ester hydrolase: Mediates the degradation of triglycerides and formation of free fatty acids (FFA). To be utilized as an energy source, FFAs must be transported from the peripheral tissues into the mitochondria of hepatocytes. Once inside the mitochondria, FFAs undergo beta oxidation, which converts them into acetyl coenzyme A (acetyl-CoA).
I-FABP	Intestinal fatty acid binding protein (FABP2): Participates in the uptake, intracellular metabolism and/or transport of long-chain fatty acids. I-FABP is present in small intestine epithelial cells and liver.
LDL-R	Low density lipoprotein receptor: Mediates the endocytosis of cholesterol-rich LDL and thus maintains the plasma level of LDL. This occurs in all nucleated cells, but mainly in the liver, which removes ~70% of LDL from the circulation. Synthesis of receptors in the cell is regulated by the level of free intracellular cholesterol; if in excess, transcription of the receptor gene is inhibited.

Lipin 2	Phosphatidate phosphatase (*Lpin2* gene): An intracellular protein in the endoplasmic reticulum (ER) that functions as a phosphatidic acid phosphohydrolase enzyme (PAP-1) to catalyze the penultimate step in triglyceride synthesis.
LTM	Lean tissue mass: A component of body composition calculated by subtracting body fat weight from total body weight
LXR	Liver X receptor: A ligand-activated transcription factor expressed in the liver and functions as physiological receptors for oxidized cholesterol metabolites, oxysterols. LXR plays an important role in the regulation of bile acid biosynthesis by regulating the expression of CYP7A1, the rate-limiting enzyme in bile acid biosynthesis. Because production of bile acids from cholesterol is the metabolic pathway for degradation of cholesterol, LXRs serve as "cholesterol sensors" to provide levels of CYP7A1 expression that maintain appropriate concentrations of cholesterol.
MDR2	Multidrug resistance protein 2 (ABCB4): Localized in the canalicular membranes of hepatocytes, MDR2 is necessary for the secretion of phospholipids into bile. Fibrates induce Mdr2 gene expression and biliary phospholipid secretion in the mouse.
MRP2	Multidrug resistance associated protein: Hepatic proteins in cytosolic phosphatidylcholine transport and canalicular secretion into bile.
MTP	Microsomal triglyceride transfer protein. A protein involved in secretion of cholesterol as VLDL.
NEFA	Non-esterified fatty acid: Free fatty acid, usually the result of hydrolysis.

Non-HDL-C	Non-HDL cholesterol: Total cholesterol minus HDL cholesterol. Total cholesterol is the sum of LDL, HDL, and very-low-density lipoproteins (VLDL). VLDL carry triglycerides to tissues and eventually become LDL. Like LDL, it also causes cholesterol to build up on the inside of arteries, creating artery-clogging plaque. The higher the LDL and VLDL levels, the higher the risk of heart disease.
NTCP	Sodium taurocholate cotransporting polypeptide: A sodium-dependent uptake transporter expressed on the basolateral (blood-side) membrane of hepatocytes. It is primarily responsible for the uptake of bile acids from the sinusoids. NTCP is one of the key transporters in the enterohepatic circulation of bile acids.
OATP1 or 2	Organic anion-transporting polypeptide 1 or 2 (*Slco1a1* or *Slc22a7* genes, respectively): A membrane transport protein that mediates the transport of mainly organic anions across the cell membrane including bile acids as well as bilirubin and numerous hormones such as thyroid and steroid hormones across the basolateral membrane in hepatocytes, for excretion in bile.
PCTP	Phosphatidylcholine transfer protein: A specific intracellular phospholipid binding protein that can transfer phosphatidylcholine between different membranes in the cytosol.
PL	Phospholipid: A class of lipids that are a major component of all cell membranes. They can form lipid bilayers because of their amphiphilic characteristic. The structure of the phospholipid molecule generally

	consists of two hydrophobic fatty acid "tails" and a hydrophilic "head" consisting of a phosphate group. The two components are joined together by a glycerol molecule.
PPARα	Peroxisome proliferator activated receptor alpha: A transcription factor and major regulator of lipid metabolism in the liver. Its activation promotes uptake, utilization, and catabolism of fatty acids by upregulating genes involved in fatty acid transport, fatty acid binding and activation, and mitochondrial and peroxisomal fatty acid β-oxidation. It is primarily activated through ligand binding. Synthetic ligands include fibrates, which are used to treat cardiovascular disease caused by hyperlipidemia.
qRT-PCR	Quantitative real-time polymerase chain reaction: qRT-PCR quantitatively detects gene expression through the creation of complementary DNA (cDNA) transcripts from RNA using fluorescent dyes.
RXRα	Retinoid x receptor α: An important regulator of cell growth and differentiation both in fetal and in adult tissues. It is a ligand-inducible transcription factor that belongs to the superfamily of nuclear hormone receptors and is specifically activated by retinoic acid. RXRα forms heterodimers with PPAR-α, which bind peroxisome proliferator receptor elements (PPREs) in and around genes regulated by PPAR-alpha.
SCP2	Sterol carrier protein 2: An intracellular lipid transfer protein that mediates transfer of all common phospholipids, cholesterol and gangliosides between membranes and may play a role in regulating steroidogenesis. The Scp2 gene encodes both the SCP2 and SCPx proteins. The transcript

	initiated from the proximal promoter encodes the longer SCPx protein, and the transcript initiated from the distal promoter encodes the shorter SCP2 protein, with the 2 proteins sharing a common C-terminus.
SCPx	Sterol carrier protein x: A peroxisome-associated thiolase that is involved in the oxidation of branched chain fatty acids. It is encoded by the Scp2 gene (see SCP2).
SEM	Standard error of the mean: A measure of the statistical accuracy of an estimate, equal to the standard deviation of the theoretical distribution of a large population of such estimates. The standard deviation is divided by the square root of the number of measurements.
SHP	Short heterodimer partner: FXR does not directly bind to the CYP7A1 promoter, but induces expression of small heterodimer partner (SHP), which then inhibits transcription of the CYP7A1 gene. In this way, a negative feedback pathway is established in which synthesis of bile acids is inhibited when cellular levels of bile acid are already high.
SR-B1	Scavenger receptor class B member 1: A receptor for HDL that facilitates uptake of cholesteryl esters from HDL in the liver. This process drives the movement of cholesterol from peripheral tissues towards the liver (reverse cholesterol transport), where cholesterol can either be secreted via the bile or be used to synthesize steroid hormones. Reverse cholesterol transport is a protective mechanism against the development of atherosclerosis, which is the principal cause of heart disease and stroke.

SREBP1 and 2	Sterol regulatory element-binding protein 1 and 2: SREBP-1 is active in driving transcription of genes involved in fatty acid synthesis, whereas SREBP-2 is more active in stimulating transcription of genes involved in cholesterol biosynthesis. For example, SREBP 2 downregulates Cyp7a1 when cholesterol is low, preventing cholesterol metabolism to bile acids.
TC	Total cholesterol: The sum of free cholesterol and cholesterol ester.
TG	Triglyceride/triacylglycerol: The simplest lipids formed by fatty acids. TG is made up of three fatty acid esters linked to a single glycerol. Most triacylglycerols contain two or three different fatty acids. TGs are nonpolar, hydrophobic, and insoluble in water.
TKO	Triple knock-out: The TKO in these studies were fatty acid binding protein1/sterol carrier protein-2/sterol carrier protein-x null mice on a C57BL/6NCr background (Fabp1/Scp2/Scpx null or gene-ablated mice)
WT	Wild-type C57BL/6 mice

TABLE OF CONTENTS

Page

CHAPTER I

INTRODUCTION AND LITERATURE REVIEW[1]

Metabolic syndrome is a constellation of clinical findings associated with a significantly increased risk for cardiovascular disease and type 2 diabetes. The clinical findings associated with metabolic syndrome include abdominal obesity, high serum glucose, high serum triglycerides, low serum high-density lipoprotein cholesterol and hypertension (1). Diseases associated with metabolic syndrome are amongst the leading causes of preventable death in the United States (2). In addition, metabolic syndrome also has an association with other health problems including non-alcoholic fatty liver disease (NAFLD) which is the most common of all liver disorders, occurring in 10–58% of the US population (3-7).

While the biochemical basis for neither metabolic disease nor NAFLD is completely understood, three potential liver lipid binding proteins have been identified as potential contributing candidates: First is the hepatic fatty acid binding protein (FABP1). Genomic studies suggest that a very prevalent SNP in human FABP1 resulting in a T94A amino acid substitution is highly associated with NAFLD and reduced ability of hypolipidemic drugs to lower serum triglycerides to target levels (8, 9). FABP1 is the single most prevalent liver cytosolic protein that binds, transports, and targets bound lipidic ligands such as long chain fatty acids and their CoA thioester (10, 11), endocannabinoids (12), bile acids (13), cholesterol (14), and lipidic

---

[1]1. Reprinted from Milligan S, Martin GG, Landrock D, et al. Ablating both Fabp1 and Scp2/scpx (TKO) induces hepatic phospholipid and cholesterol accumulation in high fat-fed mice. Biochim Biophys Acta. 2018;1863(3):323-338. doi: S1388-1981(18)30004-0 [pii]. 2018

2.Reprinted from Milligan S, Martin GG, Landrock D, et al. Impact of dietary phytol on lipid metabolism in SCP2/SCPX/L-FABP null mice. Biochim Biophys Acta. 2017;1862(3):291-304. doi: S1388-1981(16)30328-6 [pii]. 2017

xenobiotics (8, 15, 16) to intracellular organelles for storage, oxidation, receptor regulation, or gene regulation. FABP1 also has high affinity for branched-chain lipids such as phytol-derived phytanic and pristanic acids (17, 18). In vitro studies with cultured cells show that FABP1 enhances the uptake and peroxisomal oxidation of branched-chain fatty acids (19-21). FABP1 has even been detected within peroxisomes, suggesting that it may not only chaperone bound branched-chain fatty acyl-CoA to peroxisomes but also to oxidative enzymes within peroxisomes (22).

Another candidate is sterol carrier protein-2 (SCP2) which also chaperones lipidic ligands associated with the development of metabolic disease and NAFLD. SCP2 binds, transports, and targets bound lipidic ligands such as long chain fatty acids and their CoA thioesters (23-25), endocannabinoids(26, 27), bile acids (28), cholesterol (29, 30), and phospholipids (24, 31, 32) to intracellular organelles for storage, oxidation, excretion, or receptor regulation. In addition, SCP2 and SCPx, both encoded by the same SCP2/SCPx gene through alternate transcription sites, are important contributors to branched-chain fatty acid metabolism (3). Like FABP1, SCP2 has no enzymatic activity, but binds branched-chain lipids such as phytol-derived phytanic and pristanic acids (23). SCP2 was also shown to enhance branched-chain fatty acid cellular uptake and metabolism (33). Hepatic SCP-2 concentration is about 6–8 fold less than that of FABP1 (29, 34), with half in cytosol and the remainder primarily concentrated in peroxisomes (28, 35, 36). Within the peroxisomal matrix, SCP2 directly interacts with fatty acid oxidative enzymes, suggesting a role in presenting bound branched-chain fatty acyl CoAs to these enzymes to facilitate their oxidation (37). In contrast to SCP2 and FABP1, SCPx is localized exclusively in peroxisomes (28, 38, 39). SCPx functions as a ketothiolase enzyme with substrate specificity for

both straight-chain and branched-chain fatty acids (38, 40-43). SCPx is the only known peroxisomal ketothiolase enzyme for β-oxidation of branched-chain fatty acids (42).

In our nutritional studies, we sought to investigate the impact of both a high phytol and a high fat diet on *Fabp1/Scp2/Scpx* knock-out mice. Phytol, a saturated sixteen-carbon chain-length fatty alcohol with four methyl branches, is released by ruminant bacterial cleavage of chlorophyll's side-chain for further conversion to branched-chain fatty acids (44). While branched-chain fatty acids are often present at significant levels in meat and dairy products, serum levels are normally low due to rapid hepatic uptake and metabolism (45-47). However, in peroxisomal disorders of branched-chain fatty acid oxidation, serum and hepatic branched-chain fatty acid levels reach toxic levels (45, 46). Branched-chain fatty acids such as phytanic acid have functional similarity to fibrates, hypolipidemic drugs used to treat cardiovascular disease, diabetes and metabolic syndrome (48, 49). High fat diets, one of the major contributors to the development of metabolic syndrome, have also been shown to induce NAFLD (3, 4, 6, 7, 50, 51). Studying the effects of both a high phytol and high fat diet in mice lacking FABP1, SCP2 and SCPx will shed significant new insights into the roles of these proteins as they relate to whole body phenotype and hepatic lipid metabolism, two strong areas of intertest in the study of metabolic syndrome associated diseases.

It is also important to study the effects of a high phytol and high fat diet in TKO mice in the context of sexual dimorphism as studies have already shown significant differences in males in females concerning fatty acid metabolism. For example, FABP1, SCP2 and SCPx are all intrinsically lower in female than male mice. In fact, FABP1 levels are not only sex specific, they also increase during pregnancy and lactation (34). *Fabp1* gene ablation decreased hepatic triglyceride accumulation in fasted male, but not in female, mice fed standard commercial or

defined control diets, while increasing hepatic cholesteryl ester accumulation in fasted female, but not in male mice fed standard commercial or defined control diet (34). Along with control diets, sexual dimorphism has been demonstrated in the metabolism of a phytol diet as well. For example, male and female mice with lowered SCPx have different responses to phytol in lipid parameters and hepatotoxicity. Females, but not males demonstrated the following: i) increased liver mass and decreased fat tissue mass; ii) histopathological lesions in the liver; and iii) significant changes in hepatic lipid distributions (47). Lastly, while *Fabp1* silencing ameliorates hepatic steatosis, inflammation, and oxidative stress in high fat diet fed male mice with NAFLD (52), the impact of sex differences in the context of high fat diet has not been addressed and although ablating the Scp2/Scpx gene decreases hepatic lipid accumulation in control rodent chow fed male mice (53), the impact of sex differences especially in the context of HFD are unclear.

Next, it is important to illustrate the significance of using a pair-feeding scheme versus an *ad libitum* feeding scheme for our nutritional studies. Mice strongly prefer a high fat diet and will consume more of it in *ad libitum* conditions. This phenomenon can significantly skew many aspects of the study, including whole body composition and lipid metabolic pathways. Therefore, there is a need for controlled, pair-fed dietary studies to study the impact of TKO independent of increased total intake of food.

The present biochemical and morphological investigations sought to determine the effects of genotype, sex and diet on these mice. Previous work has been published that outlined the effects of a high phytol diet on TKO male mice. Therefore, this work focuses on female mice fed a high phytol diet and male and female mice fed a high fat diet. Since individually ablating the *Scp2/Scpx* gene elicits the upregulation of the FABP1 protein, this study with TKO mice helps to

resolve the contributions of *Scp2/Scpx* gene ablation on dietary phytol and high fat induced whole body and hepatic lipid phenotype independent of concomitant upregulation of FABP1.

References

1. Samson SL, Garber AJ. Metabolic Syndrome. Endocrinology and Metabolism Clinics of North America. 2014;43(1):1-23.

2. Danaei G, Ding E, Mozaffarian D, Taylor B, Rehm C. The Preventable Causes of Death in the United States: Comparative Risk Assessment of Dietary, Lifestyle, and Metabolic Risk Factors. PLOS Medicine. 2009;6(4):: e1000058.

3. Gariani K, Philippe J, Jornayvaz FR. Non-alcoholic fatty liver disease and insulin resistance: From bench to bedside. Diabetes & Metabolism. 2013;39(1):16-26.

4. Kawano Y, Cohen DE. Mechanisms of hepatic triglyceride accumulation in non-alcoholic fatty liver disease. J Gastroenterol. 2013 April 01;48(4):434-41.

5. Paz-Filho G, Mastronardi CA, Parker, Parker BJ, Khan A, Inserra A, et al. Molecular pathways involved in the improvement of non-alcoholic fatty liver disease. J Mol Endocrinol. 2013;51(1):167-79.

6. Tailleux A, Wouters K, Staels B. Roles of PPARs in NAFLD: Potential therapeutic targets. Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids. 2012;1821(5):809-18.

7. Vluggens A, Reddy JK. Nuclear receptors and transcription factors in the development of fatty liver disease. Curr Drug Metab. 2012 December 01;13(10):1422-35.

8. McIntosh AL, Huang H, Storey SM, Landrock KK, Landrock D, Petrescu AD, et al. Human FABP1 T94A variant impacts fatty acid metabolism and PPAR-α activation in cultured human female hepatocytes. American Journal of Physiology-Gastrointestinal and Liver Physiology. 2014;307(2):G176.

9. Peng XE, Wu YL, Lu QQ, Hu ZJ, Lin X. Two genetic variants in FABP1 and susceptibility to non-alcoholic fatty liver disease in a Chinese population. Gene. 2012;500(1):54-8.

10. Huan H, McIntosh AL, Martin GG, Landrock KK, Danilo L, Shipra G, et al. Structural and functional interaction of fatty acids with human liver fatty acid-binding protein (L-FABP) T94A variant. The FEBS Journal. 2014;281(9):2266-83.

11. McArthur MJ, Atshaves BP, Frolov A, Foxworth WD, Kier AB, Schroeder F. Cellular uptake and intracellular trafficking of long chain fatty acids. Journal of Lipid Research. 1999 August 01;40(8):1371-83.

12. Huang H, McIntosh AL, Martin GG, Landrock D, Chung S, Landrock KK, et al. FABP1: a novel hepatic endocannabinoid and cannabinoid binding protein. Biochemistry. 2016;55:5243-55.

13. Martin GG, Landrock D, Landrock KK, Howles PN, Atshaves BP, Kier AB, et al. Relative contributions of L-FABP, SCP-2/SCP-x, or both to hepatic biliary phenotype of female mice. Archives of Biochemistry and Biophysics. 2015;588:25-32.

14. Martin GG, Atshaves BP, Huang H, McIntosh AL, Williams BJ, Pei-Jing Pai, et al. Hepatic phenotype of liver fatty acid binding protein gene-ablated mice. American Journal of Physiology-Gastrointestinal and Liver Physiology. 2009;297(6):G1065.

15. Huang H, McIntosh AL, Martin GG, Petrescu AD, Landrock KK, Kier AB, et al. Inhibitors of fatty acid synthesis induce PPARa-regulated fatty acid b-oxidative enzymes: synergistic roles of L-FABP and glucose. PPAR Research. 2013;2013(Article ID 865604):22 pages.

16. Martin GG, McIntosh AL, Huang H, Gupta S, Atshaves BP, Landrock KK, et al. Human liver fatty acid binding protein (L-FABP) T94A variant alters structure, stability, and interaction with fibrates. . 2013;52(51):9347-57.

17. Andrey F, Kimberly M, Billheimer JT, Cho Tae-Hyeon, Friedhelm S. Lipid specificity and location of the sterol carrier protein-2 fatty acid-binding site: A fluorescence displacement and energy transfer study. Lipids. 1997;32(11):1201-9.

18. Wolfrum C, Ellinghaus P, Fobker M, Seedorf U, Assmann G, Borchers T, et al. Phytanic acid is ligand and transcriptional activator of murine liver fatty acid binding protein. J Lipid Res. 1999 April 01;40(4):708-14.

19. Atshaves BP, Storey SM, Petrescu A, Greenberg CC, Lyuksyutova OI, Smith R, et al. Expression of fatty acid binding proteins inhibits lipid accumulation and alters toxicity in L cell fibroblasts. Am J Physiol Cell Physiol. 2002 September 01;283(3):688.

20. Atshaves BP, Storey SM, Huang H, Schroeder F. Liver fatty acid binding protein expression enhances branched-chain fatty acid metabolism. Mol Cell Biochem. 2004 April 01;259(1-2):115-29.

21. Atshaves BP, McIntosh AM, Lyuksyutova OI, Zipfel W, Webb WW, Schroeder F. Liver fatty acid-binding protein gene ablation inhibits branched-chain fatty acid metabolism in cultured primary hepatocytes. J Biol Chem. 2004 July 23;279(30):30954-65.

22. Antonenkov VD, Sormunen RT, Ohlmeier S, Amery L, Fransen M, Mannaerts GP, et al. Localization of a portion of the liver isoform of fatty-acid-binding protein (L-FABP) to peroxisomes. Biochem J. 2006 March 01;394(Pt 2):475-84.

23. Dansen TB, Westerman J, Wouters FS, Wanders RJ, van Hoek A, Gadella TW, et al. High-affinity binding of very-long-chain fatty acyl-CoA esters to the peroxisomal non-specific lipid-transfer protein (sterol carrier protein-2). Biochem J. 1999;339 (Pt 1):198-9.

24. Demel RA, Kalsbeek R, Wirtz KW, Van Deenen LM. The protein-mediated net transfer of phosphatidylinositol in model systems. Biochim Biophys Acta. 1977 April 01;466(1):10-22.

25. Frolov A, Cho T, Billheimer JT, Schroeder F. Sterol Carrier Protein-2, a New Fatty Acyl Coenzyme A-binding Protein. Journal of Biological Chemistry. 1996 December 13;271(50):31878-84.

26. Martin GG, Sarah C, Danilo L, Landrock KK, Dangott LJ, Xiaoxue P, et al. Female Mice are Resistant to Fabp1 Gene Ablation-Induced Alterations in Brain Endocannabinoid Levels. Lipids. 2016;51(9):1007-20.

27. Martin GG, Sarah C, Danilo L, Landrock KK, Huan H, Dangott LJ, et al. FABP-1 gene ablation impacts brain endocannabinoid system in male mice. J Neurochem. 2016;138(3):407-22.

28. Gallegos AM, Atshaves BP, Storey SM, Starodub O, Petrescu AD, Huang H, et al. Gene structure, intracellular localization, and functional roles of sterol carrier protein-2. Prog Lipid Res. 2001 November 01;40(6):498-563.

29. Friedhelm SchroederAndrey FrolovJonathan K. SchoerAdalberto M. GallegosBarbara P. AtshavesNeal J. StolowichA. Ian ScottAnn B. Kier, Schroeder F, Frolov A, Schoer JK, Gallegos AM, Atshaves BP, et al, editors. Intracellular Sterol Binding Proteins: Cholesterol Transport and Membrane Domains. Boston, MA: Springer; 1998.

30. Stolowich N, Frolov A, Petrescu AD, Scott AI, Billheimer JT, Schroeder F. Holo-sterol carrier protein-2. (13)C NMR investigation of cholesterol and fatty acid binding sites. J Biol Chem. 1999 December 10;274(50):35425-33.

31. Schroeder F, Zhou M, Swaggerty CL, Atshaves BP, Petrescu AD, Storey SM, et al. Sterol carrier protein-2 functions in phosphatidylinositol transfer and signaling. Biochemistry. 2003 March 25;42(11):3189-202.

32. GADELLA TW, WIRTZ KW. Phospholipid binding and transfer by the nonspecific lipid-transfer protein (sterol carrier protein 2). European Journal of Biochemistry. 2005;220(3):1019-28.

33. Atshaves BP, Storey SM, Schroeder F. Sterol carrier protein-2/sterol carrier protein-x expression differentially alters fatty acid metabolism in L cell fibroblasts. Journal of Lipid Research. 2003 September 01;44(9):1751-62.

34. Atshaves BP, Martin GG, Hostetler HA, McIntosh AL, Kier AB, Schroeder F. Liver fatty acid-binding protein and obesity. J Nutr Biochem. 2010 November 01;21(11):1015-32.

35. Martin GG, Hostetler HA, McIntosh AL, Tichy SE, Williams BJ, Russell DH, et al. Structure and function of the sterol carrier protein-2 N-terminal presequence. Biochemistry. 2008 June 03;47(22):5915-34.

36. Keller GA, Scallen TJ, Clarke D, Maher PA, Krisans SK, Singer SJ. Subcellular localization of sterol carrier protein-2 in rat hepatocytes: its primary localization to peroxisomes. J Cell Biol. 1989 April 01;108(4):1353-61.

37. Bastiaens PIH, Wouters FS, Wirtz KWA, Jovin TM. FRET microscopy demonstrates molecular association of non-specific lipid transfer protein (nsL-TP) with fatty acid oxidation enzymes in peroxisomes. The EMBO Journal. 1998 Dec 15,;17(24):7179-89.

38. Wander RJ, Denis S, van Berkel E, Wouters F, Wirtz KWA, Seedorf U. Identification of the newly discovered 58kDa peroxisomal thiolase SCPx as the main thiolase involved in both pristanic acid and trihydroxycholestanoic acid oxidation: Implications for peroxisomal β-oxidation disorders. J. Inherit. Metab. Dis. 1998;21:302-5.

39. Wanders RJA, Denis S, Wouters F, Wirtz KWA, Seedorf U. Sterol Carrier Protein X (SCPx) Is a Peroxisomal Branched-Chain β-Ketothiolase Specifically Reacting with 3-Oxo-pristanoyl-

CoA: A New, Unique Role for SCPx in Branched-Chain Fatty Acid Metabolism in Peroxisomes. Biochemical and Biophysical Research Communications. 1997;236(3):565-9.

40. Wanders RJ, Denis S, Wouters F, Wirtz KW, Seedorf U. Sterol Carrier Protein X (SCPx) Is a Peroxisomal Branched-Chain β-Ketothiolase Specifically Reacting with 3-Oxo-pristanoyl-CoA: A New, Unique Role for SCPx in Branched-Chain Fatty Acid Metabolism in Peroxisomes. Biochem Biophys Res Commun. 1997;236(3):565-9.

41. Seedorf U, Brysch P, Engel T, Schrage K, Assmann G. Sterol carrier protein X is peroxisomal 3-oxoacyl coenzyme A thiolase with intrinsic sterol carrier and lipid transfer activity. J Biol Chem. 1994 August 19;269(33):21277-83.

42. Antonenkov VD, Van Veldhoven PP, Waelkens E, Mannaerts GP. Substrate Specificities of 3-Oxoacyl-CoA Thiolase A and Sterol Carrier Protein 2/3-Oxoacyl-CoA Thiolase Purified from Normal Rat Liver Peroxisomes: STEROL CARRIER PROTEIN 2/3-OXOACYL-CoA THIOLASE IS INVOLVED IN THE METABOLISM OF 2-METHYL-BRANCHED FATTY ACIDS AND BILE ACID INTERMEDIATES. Journal of Biological Chemistry. 1997 October 10;272(41):26023-31.

43. Antonenkov VD, Van Veldhoven PP, Mannaerts GP. Isolation and subunit composition of native sterol carrier protein 2/3-oxoacyl-coenzyme A thiolase from normal rat liver peroxisomes. Protein Expr Purif. 2000 April 01;18(3):249-56.

44. Scriver CR, Beaudet AI, Sly WS, Valle D, editors. The Metabolic Basis of Inherited Disease. New York: McGraw-Hill; 1990.

45. Verhoeven NM, Jakobs C. Human metabolism of phytanic acid and pristanic acid. Prog Lipid Res. 2001 November 01;40(6):453-66.

46. Avigan J. The presence of phytanic acid in normal human and animal plasma. Biochim Biophys Acta. 1966 April 04;116(2):391-4.

47. Atshaves BP, Payne HR, McIntosh AL, Tichy SE, Russell D, Kier AB, et al. Sexually dimorphic metabolism of branched-chain lipids in C57BL/6J mice. J Lipid Res. 2004 May 01;45(5):812-30.

48. Kannenberg F, Ellinghaus P, Assmann G, Seedorf U. Aberrant Oxidation of the Cholesterol Side Chain in Bile Acid Synthesis of Sterol Carrier Protein-2/Sterol Carrier Protein-x Knockout Mice. Journal of Biological Chemistry. 1999 December 10;274(50):35455-60.

49. Mackie JT, Atshaves BP, Payne HR, McIntosh AL, Schroeder F, Kier AB. Phytol-induced hepatotoxicity in mice. Toxicol Pathol. 2009 February 01;37(2):201-8.

50. Kamath S, Chavez AO, Gastaldelli A, Casiraghi F, Halff GA, Abrahamian GA, et al. Coordinated defects in hepatic long chain fatty acid metabolism and triglyceride accumulation contribute to insulin resistance in non-human primates. PLoS One. 2011;6(11):e27617.

51. Yang SY, He XY, Schulz H. Fatty acid oxidation in rat brain is limited by the low activity of 3-ketoacyl-coenzyme A thiolase. J Biol Chem. 1987;262(27):13027-32.

52. Mukai T, Egawa M, Takeuchi T, Yamashita H, Kusudo T. Silencing of FABP1 ameliorates hepatic steatosis, inflammation, and oxidative stress in mice with nonalcoholic fatty liver disease. FEBS Open Bio. 2017 June 05;7(7):1009-16.

53. Klipsic D, Landrock D, Martin GG, McIntosh AL, Landrock KK, Mackie JT, et al. Impact of SCP-2/SCP-x gene ablation and dietary cholesterol on hepatic lipid accumulation. American Journal of Physiology-Gastrointestinal and Liver Physiology. 2015;309(5):G399.

CHAPTER II

MATERIALS AND METHODS[2]

Materials

Triacylglycerol (L-type Triglyceride M, TG), free cholesterol (free cholesterol, C), total cholesterol (cholesterol E, TC), phospholipid (phospholipid, PL) and non-esterified fatty acid (HR Series NEFA-HR, NEFA) diagnostic kits from Wako Chemicals (Richmond, VA) were used to determine levels of the respective lipids. β-Hydroxybutyrate (β-hydroxybutyrate LiquiColor, β-HOB) and high-density lipoprotein cholesterol (Direct HDL-Cholesterol, HDL-C) diagnostic kits from Stanbio Laboratory (Boerne, TX) were used to determine levels of β-HOB and HDL-C. Apolipoprotein B (APOB) and apolipoprotein A-I (APOA1) levels were measured using diagnostic kits from Diazyme Labs (Poway, CA). A Bradford protein micro-assay (Cat # 500-0001, bovine gamma globulin) from Bio-Rad (Hercules, CA) was used to determine protein levels. All reagents and solvents used were of the highest grade available.

Generation of Fabp1 Null Mice

To generate Fabp1 null mice, primers 5′-gacctcatccagaaagggaag and 5′-cttttccccagtcatggtctc were designed to amplify from mouse DNA a 156-bp exon 2 fragment. The amplicon was used as a probe in Southern blotting and to screen a P1–129/Ola genomic library (Genome Systems). Extensive restriction mapping and southern blotting of mouse genomic and P1 DNA demonstrated the identity of the P1 clones with the respective genomic regions and the

---

[2]1. Reprinted from Milligan S, Martin GG, Landrock D, et al. Ablating both Fabp1 and Scp2/scpx (TKO) induces hepatic phospholipid and cholesterol accumulation in high fat-fed mice. Biochim Biophys Acta. 2018;1863(3):323-338. doi: S1388-1981(18)30004-0 [pii]. 2018
2.Reprinted from Milligan S, Martin GG, Landrock D, et al. Impact of dietary phytol on lipid metabolism in SCP2/SCPX/L-FABP null mice. Biochim Biophys Acta. 2017;1862(3):291-304. doi: S1388-1981(16)30328-6 [pii]. 2017

absence of pseudogenes. Mouse *Fabp1* cDNA was used to confirm the identity of the P1 clones by southern hybridization and PCR for exons 1 and 4. An 8-kb EcoRV fragment containing the 5′ gene flank plus exons 1 and 2 was subcloned from a P1 clone and used to isolate a 3.9-kb end-filled EcoRV/EcoRI fragment ("long arm") that in turn was ligated into vector pTVO (cut with XhoI and end-filled), a plasmid carrying the neomycin resistance marker, to create an intermediate long arm construct. A 10-kb SacI fragment overlapping the above EcoRV fragment and containing the whole gene as well as its 3′ flank was subcloned from P1 and used to isolate a 1.3-kb BamHI/SalI fragment from the 3′ flank ("short arm"). The short arm was then ligated into the long arm construct (opened with BamHI and SalI), resulting in the targeting vector. The SalI/EcoRI fragment just 3′ of the short arm was partially sequenced to design primers. The targeting construct was opened with NotI and electroporated into HM1 cells. After selection with G418, colonies were screened by PCR for homologous recombination, using primers 5′-ccttctatcgccttcttgacgag and 5′-agcctccagggattggaatg and an annealing temperature of 63 °C. These primers correspond to the neo resistance marker and the 3′ genomic flank outside of the construct, respectively. A fragment of the expected size of 1.4-kb was amplified in 5 out of 200 clones, and 2 clones were expanded and injected into C57Bl/6 blastocysts to create chimeric mice by standard procedures. Heterozygous mice were obtained by breeding the chimeras with C57Bl/6 wild type mice, and experimental wild type and Fabp1 null mice were created by interbreeding of the first-generation heterozygous mice. Some of these mice were used to verify the gene deletion as follows. Liver DNA was purified by standard procedures and used as a template for long PCR to amplify an 8-kb fragment from wild type and a 2.5-kb fragment from Fabp1 null DNA. Cycling conditions were 96 °C, 30 s; 32× (94 °C, 30 s; 63 °C, 30 s; 68 °C, 8 min). One primer (5′-ttcaagcctccagggattggaatg) corresponded to a sequence located immediately

3′ of the short arm homology region (i.e. outside of the recombination construct), the other primer (5′-cctggactgagacttgcctggattg) to a sequence located at the 3′-end of the long homology arm. The long PCR products were further verified by nested PCR for a 157-bp fragment of exon 2, using primers 5′-ccgaggacctcatccagaaag and 5′-tccccagtcatggtctccag at an annealing temperature of 60 °C. With the same exon 2 primers, absence of exon 2 was also verified directly on genomic DNA. In addition, absence of exons 3 and 4 in knockout DNA was also directly confirmed by PCR with genomic DNA. After verification of the targeted gene deletion, another PCR assay was designed for routine single-tube genotyping of tail biopsies. Primers 5′-caaggggtgtcagaaatcgtgc and 5′-ccagtcatggtctccagttcgca amplify 123 bp from exon 2 of the wild type allele, and 5′-aagagcttggcggcgaatgg and 5′-tggccatttgtggctgtgctc amplify 227 bp from the neomycin resistance marker into the 3′ flank of both alleles. An annealing temperature of 68 °C was used. Total liver RNA was isolated with the TRIzol reagent from Invitrogen (Carlsbad, CA), and reverse transcription was performed with random hexamer primers and Moloney murine leukemia virus reverse transcriptase (Invitrogen) according to a standard procedure. Aliquots of the RT reaction were used for PCR, using Fabp1 primers (5′-ctcattgccaccatgaacttctc and 5′-agccttgtctaaattctcttgctgact, amplifying 404 bp) and hypoxanthine phosphoribosyl transferase (HPRT) primers (5′-gcttgctggtgaaaaggacctct and 5′-ggaaatcgagagcttcagactcgtc, amplifying 584 bp) at an annealing temperature of 62 °C.

Chimeric mice were bred with C57BL/6 mice, and the resulting heterozygous offspring were interbred and backcrossed to produce the L-FABP null (–/–) and wild type (+/+) littermate control mice for 4-7 generations.

Generation of Scp2/Scpx Ablated Mice

Scp2/Scpx ablated mice were generated by targeted disruption of the *Scp2* gene through homologous recombination. The targeting construct was designed to replace exon 16 with the neomycin cassette of the pUnivec-HPRT vector (a generous gift from Dr. Ramiro Ramírez-Solis, Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, TX). Briefly, a genomic 129/sv Lambda FIX® library (Stratagene, La Jolla, CA) was screened with a 150 bp DNA fragment containing exon 16 of the *Scp2* gene. Positive clones were confirmed by extensive restriction mapping and sequence analysis. Two consecutive genomic DNA fragments, a 2.0 kb PstI clone (containing intronic sequences upstream of exon 16) and a 4.5 kb PstI clone (containing intronic sequences immediately upstream of exon 16, exon 16 itself, and intronic sequences downstream of exon 16) formed the backbone of the targeting construct. The 3′ homology arm was generated by ligating a blunt-ended 2.6 kb XbaI fragment from the 4.5 kb PstI clone into Litmus 39 vector (New England Biolabs, Ipswich, MA) predigested with EcoRV. This plasmid was digested with SacI/PstI and ligated with an 875 bp fragment from the 4.5 kb PstI clone digested with SacI/PstI. The resulting vector was digested with BamHI/HindIII to give a 2,824 bp fragment that was ligated into the pGEM-3Zf vector (Promega, Madison, WI) predigested with BamHI/HindIII. Digestion with EcoRI/HindIII released the 3′ homology region (with an incorporated KpnI site later used for Southern blot screening). The 3′ homology fragment was blunt-ended with T4 DNA polymerase and ligated into the pUnivec-HPRT vector (predigested with NotI and blunted with T4 DNA ligase) to yield an intermediate targeting construct named pUni+3′ vector. The 5′ homology region was prepared by digesting the 4.5 kb PstI clone with AccI/PstI to isolate a 2,562 bp fragment that was then ligated to an AccI/PstI fragment isolated from the 2.0 kb PstI clone. Digestion with BamHI/AccI released the 5′

homology fragment. The targeting construct was completed by blunt-end ligation of the 5′ arm into the intermediate construct pUni+3′ vector (predigested with NheI and then blunted with T4 DNA polymerase). Once complete, the targeting construct was opened with ClaI and electroporated into a 129/Ola-derived embryonic stem cell line E14 maintained on feeder layers. After selection with G418 (200 μg/ml) and gancyclovir (2 μM), DNA was isolated from surviving clones, digested with KpnI, and screened by Southern blot analysis according to standard protocols. Using a 200 bp 5′ probe external to the targeting construct, targeted clones were identified by the presence of a 6 kb band indicating that exon 16 was replaced with the neomycin cassette of pUnivec-HPRT vector. Three positive clones were expanded and injected into C57BL/6NCr blastocysts to create chimeric mice by standard procedures. Three male chimeras were identified by coat color and bred to C57BL/6NCr females to determine germline transmission of the targeted allele. Tail DNA from mixed coat-colored (agouti) F1 offspring was screened by PCR to verify the genotype of each animal. Initial characterization of the *Scp2* gene ablation by Western analysis was performed on F2 *Scp2*−/− homozygous mice generated by interbreeding heterozygous F1 animals. The heterozygous F1 animals were backcrossed to a N6 C57BL/6NCr background before interbreeding to produce the Scp2/Scpx null mice.

Generation of TKO Mice

Scp2/Scpx null mice were crossed with Fabp1 null mice to produce Fabp1−/+/Scp2/Scpx−/+ mice, which were then intercrossed to produce Fabp1−/−/Scp2/Scpx−/− triple-null (TKO) mice. Fabp1/Scp2/Scpx null (TKO) mice were backcrossed> 10 generations to the C57BL/6NCr background. The term TKO refers to loss of all three proteins even though they are encoded by only two genes (i.e. *Fabp1* and *Scp2/Scpx*). Mice were housed in controlled conditions (T= 25 °C, H = 60–70% added humidity) and 12:12 h light/dark cycle. Mouse protocols were approved

by the Texas A&M Institutional Animal Care and Use Committee in compliance with the Guide for the Care and Use of Laboratory Animals. Mice were monitored daily for injury or disease, sentinel monitored quarterly, and were shown free of all known rodent pathogens.

Dietary Phytol Study

One week before the study, all mice were moved from a standard pelleted rodent chow to a modified AIN-76A phytol-free, phytoestrogen-free pelleted control diet (5% calories from fat, diet no. D11243, Research Diets, New Brunswick, NJ). The control diet was used to minimize potential complications due to phytoestrogens (which exert estrogenic effects on the central nervous system, induce estrus, and stimulate growth of the genital tract of female animals) (1) or phytol metabolites (e.g. phytanic acid), known to be the most potent naturally-occurring fatty acid ligand inducers of PPARα (2-4). After the one week on control chow, test mice were transferred to a modified AIN-76A pelleted rodent diet supplemented with 0.5% phytol (5% calories from fat, diet no. D01020601, Research Diets, New Brunswick, NJ), which is approximately ten times the total amount of free phytol and phytanic acid on average available in commercial laboratory rodent diets (5). Humans consume 50-100 mg of dietary phytol and phytanic acid per day (6). The mice fed the 0.5% phytol diet ingested ~250 mg of phytol per day. Although dairy products such as butter, margarine, and cheeses can contain up to 500 mg phytanic acid/100 g wet weight (6), substantial amounts would need to be ingested to reach levels equivalent to those consumed in the 0.5% phytol feeding study (700 g/70 kg/day for human consumption). Phytol toxicity is not normally a problem with healthy individuals. However, it becomes an issue with peroxisomal disorders, such as Refsum's disease, which result in an inability to metabolize phytol (6).

Two feeding groups contained 8 animals each which were fed *ad libitum*: (a) WT mice fed the 0.5% phytol diet, and (b) TKO mice fed the 0.5% phytol diet. The study was stopped after seven days because TKO mice exhibited a low tolerance for the 0.5% phytol diet, which manifested as a 20% percent loss in body weight. Body weight and food intake were measured every other day.

Dietary High Fat Study

Two groups of 16 each WT male, WT female, TKO male, and TKO female mice aged 7 weeks were individually housed in Tecniplast Sealsafe IVC cages with external water bottles and wire lid holders for food pellets. Mice were acclimated for 1 week on a defined, 10 kcal% fat control chow (#D12450B, Research Diets, New Brunswick, NJ) known to be free of phytol and phytoestrogen for reasons described above. In each group of 16 individually- housed mice, 8 mice were continued an additional 12 weeks on this same defined diet while the other 8 mice were pair-fed based upon food weight an isocaloric high fat diet (HFD, # D12451, Research Diets, New Brunswick, NJ). The high fat diet was formulated by modifying the defined control diet by increasing fat from 10 kcal% to 45 kcal% while decreasing carbohydrate from 70 kcal% to 35 kcal% while keeping protein constant as described (7).

Whole Body Phenotype by Dual-Energy X-ray Absorptiometry (DEXA)

To determine fat tissue mass (FTM) and lean tissue mass (LTM), mice were anesthetized at the beginning (day 0) and end (day 7) for the phytol study and end day 84 for the high fat study) using a ketamine/xylazine mixture (0.01 mL/g body weight; 10 mg ketamine/mL and 1 mg xylazine/mL in 0.9% saline solution). Dual-energy X-ray absorptiometry (DEXA) images of each mouse were obtained using a Lunar PIXImus densitometer (Lunar Corp., Madison, WI) after calibration using a phantom mouse with known bone mineral density and fat tissue mass.

Whole body fat tissue mass (FTM) and bone-free lean tissue mass (LTM) were obtained by exposing the entire mouse, minus the head region, to sequential beams of high- and low-energy X-rays and taking X-ray images on a luminescent panel. Soft tissue mass was differentiated from bone mass by measuring the ratios of attenuation at different energies followed by separating soft tissue mass into FTM and LTM.

Animal Euthanasia

At the end of the study after overnight fast, blood was collected from the anesthetized mice via cardiac puncture followed by cervical dislocation as the secondary form of euthanasia according to the AVMA Guidelines for the Euthanasia of Animals. Blood was coagulated overnight at 4 °C, followed by centrifugation at 14,000 rpm for 20 min at 4 °C to process to serum and stored at−80 °C for subsequent lipid and protein analysis, and final DEXA images were taken.

Tissue Collection

Likewise, livers were collected, flash frozen in liquid nitrogen, and stored at −80 °C for subsequent histopathology and analysis of lipids, western blotting, and/or qRT-PCR as described in the following sections.

Liver Histopathology

At the time of liver collection, liver slices were taken near the portal hepatis, fixed for 24 h in 10% neutral buffered formalin, put into individual cassettes with 70% alcohol, processed and embedded in paraffin, sectioned (4–6 μm), and stained with hematoxylin and eosin for histological evaluation.

Liver Homogenization and Protein Analysis

Liver samples (~0.1 g) were minced extensively followed by the addition of 0.5 mL PBS (pH 7.4) and homogenization with a motor-driven pestle (Tekmar Co, Cincinnati, OH) at 2000 rpm. A Bradford protein micro-assay (Bio-rad, Hercules, CA) was used to determine protein levels in the liver homogenates according to the manufacturer's instructions. Protein levels were determined on aliquots of homogenates in Costar 96-well assay plates (Corning, Corning, NY) and read using a BioTek Synergy 2 micro-plate reader (BioTek Instruments, Winooski, VT).

Liver and Serum Lipid Analysis

Liver homogenate lipid classes (TG, free C, total C, PL, and NEFA) were measured using Wako diagnostic kits in accordance with the manufacturer's instructions. Liver cholesteryl ester concentration (CE) was determined by subtracting free (non-esterified) cholesterol concentration from the total concentration. Serum lipids (free C, total C), HDL-cholesterol (HDL-C)], APOA1 and APOB levels were determined using commercially available diagnostic kits in accordance with the manufacturer's instructions. The assays were modified to support the use of 96-well plates and micro-plate reader as described above. Serum cholesteryl ester (CE) concentrations were calculated by subtraction of serum free C from serum total C. Serum non-HDL-C was calculated by subtracting serum HDL-C from serum total C.

Western Blotting

The following antibodies were used in Western blotting to determine protein levels in liver homogenates: Rabbit and goat polyclonal antibody to mouse acyl-CoA-binding protein (ACBP) (SC-23474), APOA1 (SC-23606), ATP-binding cassette sub-family G members 1, 4, 5 and 8 (ABCG1, 4, 5 and 8) (SC-11150, SC-33825, SC-25796, SC-30010), brain fatty acid binding protein (B-FABP) (SC-30088), bile salt export pump (BSEP)

(SC-17294), carnitine palmitoyltransferase 1a (Cpt1a) (SC-31128), carnitine palmitoyltransferase 2 (Cpt2) (SC-20671), cellular retinoic acid binding protein 1 (CRABP I) (SC-10062), fatty acid transport protein 4 (FATP4) (SC-5834), farnesoid x receptor (FXR) (SC-13063), glyceraldehyde 3-phosphate dehydrogenase (GADPH) (SC-13063), intestinal fatty acid binding protein (I-FABP) (SC-16063), multidrug resistance protein (MDR2) (SC-8313), LDL-Receptor (LDL-R) (SC-11826), liver x receptor (LXR) (SC-1201), microsomal triglyceride transfer protein (MTP) (SC-33116), retinoid x receptor α (RXRα) (SC-553), sterol regulatory element- binding protein 1 (SREBP1) (SC-367), sterol regulatory element binding protein 2 (SREBP2) (SC-8151), short heterodimer partner and small heterodimer partner (SHP) (SC-15283), 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR) (SC-27578), cytoplasmic 3-hydroxy-3-methylglutaryl-CoA synthase (HMGCS1) (SC-33829), hormone sensitive lipase (HSL) (SC-25843), multidrug resistance associated protein 2 (MRP2) (SC-5770) and phosphatidylcholine transfer protein (PCTP) (SC-23672) were obtained from Santa Cruz Biotechnology (Dallas, TX). Rabbit and mouse polyclonal antibody to mouse acetyl-CoA acetyltransferase (ACAT2) (ab66259), APOB (ab31992), cytochrome c oxidase subunit IV (COX4) (ab16056), fatty acid transport protein 2 (FATP2) (ab83763) and fatty acid transport protein 5 (FATP5) (ab89008) were purchased from Abcam (Cambridge, MA). Sheep polyclonal antibody to bovine catalase (W90080C) was purchased from BioDesign (Dublin, OH). Rabbit polyclonal antibody to mouse glutamic oxaloacetic transaminase (GOT) (12248), FABP1 (11294), SCP2 (12249), and SCPx (11306) were prepared as described (6, 8). Goat polyclonal antibody to mouse glutathione s-transferase (GST) (27457701V) was obtained from GE Life Sciences (Pittsburgh, PA). Bacterial polyclonal antibody to mouse 3alpha-hydroxysteroid reductase (3αHSD) (H9117-01) was from US Biological (Peabody, MA). Rabbit polyclonal

antibody to mouse PPARα (PA1-822A) was obtained from Pierce Antibody (Rockford, IL) and to mouse scavenger receptor class B member 1 (SR-B1) (NB400-104), ATP-binding cassette transporter member 1 (ABCA1) (NB400-105) from Novus Biological (Littleton, CO). Alkaline phosphatase-conjugated goat polyclonal antibody to rabbit IgG (product # A3687) and rabbit polyclonal antibody to goat IgG (product # A4187) were from Sigma-Aldrich (St. Louis, MO). Alkaline phosphatase-conjugated rabbit polyclonal antibody to mouse IgG (product # ab6729) was from Abcam (Cambridge, MA). Antibodies to housekeeper proteins were obtained as follows: Mouse monoclonal antibody to mouse GAPDH (MAB374) from Millipore (Billerica, MA); rabbit polyclonal antibody to mouse cytochrome c oxidase subunit IV (COX4) (ab16056) from Abcam (Cambridge, MA) and mouse polyclonal antibody to mouse β-Actin (BA) (SC-47778) from Santa Cruz Biotechnology (Dallas, TX). Since the proteins of interest were differentiated by size from COX4, BA or GAPDH on the tricene gels, the membrane blots were cut into two, and each was probed with antisera against the protein of choice and against COX4, BA or GADPH housekeeper proteins to ensure uniform protein loading. Alkaline-phosphatase conjugated goat anti-rabbit or mouse IgG were used to visualize protein bands. Blot images were then obtained using an Epson Perfection V700 Photo scanner (Long Beach, CA) and image proteins (mean 8-bit grayscale density) quantitated by densitometric analysis using NIH Image (available by anonymous FTP). Levels of each protein were then normalized to the mean level of the respective housekeeper protein (COX4, BA or GAPDH). Proteins with no source of pure protein were expressed as relative fold differences between samples. Western blots where pure protein was available (SCPx, SCP2, FABP1, and ACBP in the phytol study) linear standard curves were generated for quantitative analysis. Band intensities on the Western blots were analyzed as described above and then plotted against the protein amount to generate a standard

curve within the linear range of each protein. Changes in protein expression between liver homogenate samples were quantitated by comparing the sample with the standard curve on each blot.

Real-time Quantitative RT-PCR

The following probes were obtained from Applied Biosystems (Foster City, CA) for quantitating gene specific mRNA levels in mouse liver homogenates as follows: glycerol-3-phosphate acyltransferase (GPAT) (Gpam; Mm00833328_m1), 1-acylglycerol-3-phosphate-Oacyltransferase (AGPAT) (Agpat2; Mm00458880_m1), Lipin 2 (Lpin2; Mm00522390_m1), diacylglycerol acyltransferase (DGAT) (Dgat2; Mm00499536_m1), acetyl-coA carboxylase (ACC1) (Acaca; Mm01304285_m1), acetyl-coA carboxylase (ACC1) (Acaca; Mm01304285_m1), fatty acid synthase (FASN) (Fasn; Mm00662319_m1), 3-hydroxy-3-methylglutaryl-CoA (HMGCR) (Hmgcr; Mm01282492_m1), 3-hydroxy-3-methylglutaryl-CoA synthase (HMGCS1) (Hmgcs1, Mm01282492_m1), sodium-taurocholate cotransporting polypeptide (NTCP) (Slc10; Mm01302718_m1), organic anion-transporting polypeptide 1 (OATP1) (Slco1a1; Mm01267414_m1), organic anion-transporting polypeptide 2 (OATP2) (Slc22a7; Mm00460672_m1), carnitine palmitoyltransferase 1a (CPT1A) (Cpt1a; Mm00550438_m1), carnitine palmitoyltransferase 2 (CPT2) (Cpt2; Mm00487202_m1), acyl-CoA oxidase-1 (ACOX1) (Acox1; Mm00443579_m1), acetyl-CoA acetyltransferase (ACAT2) (Soat2; Mm00448823_m1), bile salt export pump (BSEP) (Abcb11; Mm00445168_m1), sterol regulatory element-binding protein 2 (SREBP2) (Srebf2; Mm01306289_m1), cytochrome P7A1, CYP7A1 (Cyp7a1, Mm00484152_m1) and ATPbinding cassette sub-family G member 5 (ABCG5) (Abcg5; Mm01226965_m1) and ATP-binding cassette sub-family G member 8

(ABCG8) (Abcg8; Mm00445977_m1). Briefly, total mRNA was isolated from livers and purified using an RNeasy minikit (Qiagen, Valencia, CA) as per the manufacturer's protocol. Concentrations and quality of mRNA were measured using the ND-1000 method (Nanodrop Technologies, Inc., Wilmington, DE) where a 260/280 ratio of 1.9–2.1 was accepted as mRNA of good quality. For qRT-PCR, expression patterns were assessed using TaqMan® One-Step PCR Master Mix reagent kit, gene-specific TaqMan PCR probes, and primers. An ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) and thermal cycling conditions of 48 °C for 30 min, 95 °C for 10 min before the first cycle, 95 °C for 15 s and 60 °C for 1 min, repeated 40 times, were used for quantitating mRNA expression. Prior to amplification, total mRNA was first reverse transcribed in the first step of the thermal cycler protocol (48 °C for 10 min) using TaqMan one step chemistry. mRNA expression was normalized to 18S RNA (a housekeeping gene) and made relative to the control mouse group (male WT mice on high fat diet) set to one for final calculations.

Statistics

Data were expressed as averages ± standard error of the mean (SEM). For the phytol study, statistical analysis was performed using t-test (GraphPad Prism, San Diego, CA, San Jose, CA). Values with $p < 0.05$ were considered statistically significant. For the high fat study, statistical analysis was performed by one-way ANOVA followed by Newman-Keuls post hoc analysis (GraphPad Prism, San Diego, CA, San Jose, CA and Sigma Plot, Systat, San Jose, CA). Statistical differences of $p < 0.05$ were considered significant.

References

1. Thigpen JE, Setchell KD, Ahlmark KB, Locklear J, Spahr T, Caviness GF, et al. Phytoestrogen content of purified, open- and closed-formula laboratory animal diets. Lab Anim Sci. 1999 October 01;49(5):530-6.

2. Wolfrum C, Ellinghaus P, Fobker M, Seedorf U, Assmann G, Borchers T, et al. Phytanic acid is ligand and transcriptional activator of murine liver fatty acid binding protein. J Lipid Res. 1999 April 01;40(4):708-14.

3. Ellinghaus P, Wolfrum C, Assmann G, Spener F, Seedorf U. Phytanic acid activates the peroxisome proliferator-activated receptor alpha (PPARalpha) in sterol carrier protein 2-/ sterol carrier protein x-deficient mice. The Journal of biological chemistry. 1999 Jan 29,;274(5):2766.

4. Hanhoff T, Benjamin S, Börchers T, Spener F. Branched-chain fatty acids as activators of peroxisome proliferator-activated receptors. European Journal of Lipid Science and Technology. 2005 Oct;107(10):716-29.

5. Mackie JT, Atshaves BP, Payne HR, McIntosh AL, Schroeder F, Kier AB. Phytol-induced hepatotoxicity in mice. Toxicol Pathol. 2009 February 01;37(2):201-8.

6. Atshaves BP, Payne HR, McIntosh AL, Tichy SE, Russell D, Kier AB, et al. Sexually dimorphic metabolism of branched-chain lipids in C57BL/6J mice. J Lipid Res. 2004 May 01;45(5):812-30.

7. Atshaves BP, Martin GG, Hostetler HA, McIntosh AL, Kier AB, Schroeder F. Liver fatty acid-binding protein and obesity. J Nutr Biochem. 2010 November 01;21(11):1015-32.

8. Atshaves BP, Petrescu AD, Starodub O, Roths JB, Kier AB, Schroeder F. Expression and intracellular processing of the 58 kDa sterol carrier protein-2/3-oxoacyl-CoA thiolase in transfected mouse L-cell fibroblasts. Journal of Lipid Research. 1999 Apr 1,;40(4):610.

CHAPTER III

PHYTOL STUDY RESULTS[3]

Body Weight, Liver Weight, Liver Histopathology and Serum AST and ALT

Dietary phytol significantly decreased weight gain in WT mice, an effect exacerbated by TKO (Chapter 3 Fig. 1A). The phytol-induced weight loss was attributable to both reduced food consumption and reduced energy intake. Phytol decreased food consumption by 16±3% in WT and nearly twice as much by 29±2% in TKO mice (not shown). When body weight change was expressed on the basis of energy intake, i.e. body weight gain per kcals of food consumed, phytol decreased energy intake in WT mice, an effect markedly exacerbated nearly 3-fold in by TKO (Chapter 3 Fig. 1B). This suggested that TKO exacerbated the effect of dietary phytol on weight loss, not only by decreasing food intake but also by altering metabolism.

Dietary phytol was associated with mild hepatocellular necrosis in both WT and TKO (not shown). These observations were supported by measurement of serum values of ALT and AST, other indicators of hepatocyte damage. Although dietary phytol increase ALT and AST values similarly in both WT and TKO mice, all values were within the normal range of mouse serum ALT and AST values (less than 150 and 330 units/l, respectively) shown for blood chemistry and hematology in 8 inbred strains of mice, MPD: Eumorphia. Mouse Phenome Database web site, The Jackson Laboratory, Bar Harbor, Maine USA. http://phenome.jax.org [Cited 29 Oct, 2014]). Taken together, these measures indicated that the major changes in whole body and liver lipid

[3] Reprinted from Milligan S, Martin GG, Landrock D, et al. Impact of dietary phytol on lipid metabolism in SCP2/SCPX/L-FABP null mice. Biochim Biophys Acta. 2017;1862(3):291-304. doi: S1388-1981(16)30328-6 [pii]. 2017

phenotype elicited by TKO in phytol fed mice detailed in the following sections were not due to marked hepatotoxicity.

Body Composition: Fat Tissue Mass and Lean Tissue Mass

PIXImus dual-energy x-ray absorptiometry (DEXA) scans were done to determine if any decline in weight gain, was due to selective loss of fat or protein. Representative PIXImus images of mice from the beginning (Chapter 3 Fig. 2A,B) and end (Chapter 3 Fig. 2C,D) of the dietary study suggested gross differences in body composition. Phytol-fed WT mice appeared visually smaller at the end of the study (Chapter 3 Fig. 2A,C). This difference appeared even more marked in phytol-fed TKO mice (Chapter 3 Fig. 2B,C). DEXA analysis of 8 mice to resolve FTM (Chapter 3 Fig. 2E) and LTM (Chapter 3 Fig. 2F) showed that dietary phytol increased FTM while concomitantly decreasing LTM (Chapter 3 Fig. 2E,F). In contrast, TKO decreased both FTM and LTM (Chapter 3 Fig. 2E,F).

Hepatic Lipid and Glyceride Accumulation

Phytol-fed TKO mice had markedly more total hepatic lipid than phytol-fed WT mice (Chapter 3 Fig. 3A). This change was attributed primarily to a nearly 3-fold increase in phospholipid (Chapter 3 Fig. 3C) while triacylglycerol actually decreased about 25% (Chapter 3 Fig. 3D). Hepatic phospholipid accumulation was associated with a more than two-fold increase in expression of the key enzymes in synthesis of phosphatidic acid (precursor of both phospholipids and triacylglycerols), i.e. *Gpam* (Chapter 3 Fig. 3E) and *Agpat* (Chapter 3 Fig. 3F) and increased expression of *Acc1*, the rate limiting enzyme in *de novo* fatty acid synthesis (Chapter 3 Fig. 4C). Though *Dgat* is a key enzyme in the synthesis of triacylglycerol and was significantly higher in TKO mice than WT mice (Chapter 3 Fig. 3H), this increase was not reflected in hepatic triacylglycerol levels. On the contrary, triacylglycerol levels were significantly less in TKO mice.

This decrease is attributed to decreased expression of *Fasn*, a key enzyme in fatty acid synthesis (Chapter 3 Fig. 4C) and increased expression of *Lipe* a key enzyme in triacyglycerol hydrolysis.

Taken together, these findings suggested that the TKO induced hepatic lipid accumulation by favoring fatty acyl targeting towards phospholipids at the expense of triacylglycerol in phytol-fed TKO phytol-fed mice.

Hepatic Fatty Acid Oxidation

Hepatic glyceride accumulation in phytol-fed TKO mice may arise not only from increased *de novo* lipogenesis, but also from increased uptake and/or decreased oxidation of fatty acids.

While loss of FABP1 and SCP-2, both facilitators of fatty acid uptake, was expected to increase serum non-esterified fatty acid (NEFA) level, on the contrary TKO did not significantly changes serum NEFA (Chapter 3 Fig. 5A). This lack of increase in serum NEFA was attributable in part to concomitant downregulation of two hepatic membrane fatty acid transporters, FATP5 (Chapter 3 Fig. 5B) and GOT (Chapter 3 Fig. 5E). FATP2 and FATP4, however, remained unchanged (Chapter 3 Fig. 5C,D).

TKO did not decrease hepatic (Chapter 3 Fig. 6B) or serum (Chapter 3 Fig. 6A) levels of β-hydroxybutyrate (β-OHB), a measure of hepatic fatty acid oxidation. This was reflected in no change in the levels of hepatic expression of key enzymes in mitochondrial (CPT1A, CPT2) or peroxisomal (p-thiolase) fatty acid β-oxidation (Chapter 3 Fig. 6C,D,F). As expected, the other major enzymes in peroxisomal fatty acid β-oxidation, i.e. SCPx (58kDa) and SCPx (43kDa), were not detectable in these TKO mice. In addition, there was no significant change in the expression of nuclear receptors regulating transcription of fatty acid β-oxidative enzymes (Chapter 3 Fig. 6I,J).

Thus, the TKO-induced increase in hepatic glycerides was not associated with either increased expression of membrane transport proteins involved in fatty acid uptake or a decrease in fatty acid β-oxidation. Instead, the TKO-induced increase in hepatic glyceride was associated with increased fatty acid synthesis, represented by an increase in key enzymes, particularly an increase in *Acc1*, the rate limiting enzyme in fatty acid synthesis (Chapter 3 Fig. 4C).

Hepatic Cholesterol Accumulation

The finding that TKO increased total neutral lipid, despite decreasing triacylglycerol, suggested upregulation of the other major neutral lipid species, cholesterol and/or cholesteryl ester perhaps as a result of the upregulated expression of target genes in cholesterol synthesis (*Hmgcs1*, *Hmgcr*) (Chapter 3 Fig. 4E,F). However, there was no effect on hepatic total cholesterol, free cholesterol or cholesteryl ester levels (Chapter 3 Fig. 7A-C) nor was there a change in the expression of the cholesteryl ester synthetic enzyme (acyl CoA cholesterol acyltransferase, ACAT 2) (Chapter 3 Fig. 7D). In contrast, there was marked upregulation of hepatic expression of the *Lipe* gene, which codes for the degradative enzyme cholesteryl ester hydrolase (CEH) (Chapter 3 Fig. 7E). Thus, upregulation of *Lipe* did not actively decrease hepatic cholesterol levels in phytol-fed TKO.

Serum Levels of Cholesterol and Lipoproteins in Serum Cholesterol Transport

TKO significantly increased both serum cholesterol and cholesterol ester in phytol fed mice (Chapter 3 Fig. A,B). This increase is attributed to increased lipoprotein rich in apoB as well as increased non-HDL-C (Chapter 3 Fig. F, G) in the serum. These parameters, along with increased expression of *Lipe*, however, did not contribute to decreased hepatic accumulation of cholesterol. These findings suggest that TKO, in combination with phytol, has a "protective" effect against hepatic cholesterol accumulation.

Hepatic Expression of Proteins Involved in Cholesterol Basolateral Uptake/Efflux and Canalicular Secretion

TKO and dietary phytol differentially impacted hepatic expression of proteins involved in hepatic cholesterol uptake and loss.

TKO did not alter hepatic levels of scavenger receptor B1 (SR-B1) or low-density lipoprotein receptor (LDL-R), the key receptors in bidirectional uptake/efflux of high density lipoprotein (HDL) cholesterol and low-density lipoprotein (LDL) cholesterol (Chapter 3 Fig. 9C, D), or apoA1, involved in hepatic cholesterol secretion to nascent-HDL (Chapter 3 Fig. 9A) at the basolateral membrane. In contrast, TKO markedly decreased hepatic accumulation of apoB, the key apoprotein in LDL (Chapter 3 Fig. 9B). Concomitantly, TKO did not affect expression of ATP binding cassette proteins localized in the canalicular membrane for transporting hepatic cholesterol into bile, i.e. *Abcg5*, *Abcg8* (Chapter 3 Fig. 9E,F). A low level of apoB in the liver was reflected by high levels of apoB in the serum in TKO mice.

Bile Acid and Transport

Since bile acid level in large part drives biliary cholesterol secretion (1-3), hepatic expression of proteins involved in bile acid reuptake from serum (*Oatp1, Oatp2, Ntcp*), bile acid cytosolic transport (FABP1, SCP2, 3αHSD, GST), and biliary secretion of bile acid (BSEP) and phospholipid (MDR) at the canalicular membrane was examined.

With regards to basolateral proteins in bile acid uptake from serum, TKO significantly decreased hepatic expression of *Oatp1* (Chapter 3 Fig. 10A), but increased that of *Oatp2* (Chapter 3 Fig. 10B) and *Ntcp* (Chapter 3 Fig. 10A,C). TKO decreased expression of the nuclear regulatory protein FxR, but did not affect the nuclear regulatory protein LxR (Chapter 3 Fig. 10H,I). These

proteins are responsible for regulating transcription of target genes in bile acid metabolism and uptake.

Regarding cytosolic bile acid binding/transport proteins, TKO abolished expression of the major murine cytosolic bile acid binding/transport protein FABP1 (Chapter 3 Fig. 11A), and this null effect was not compensated for by upregulation of the other known cytosolic bile acid binding/transport proteins (3αHSD, GST, Chapter 3 Fig. 10D-E).

With regards to canalicular membrane proteins involved in hepatic bile acid export into bile, TKO did not affect expression of the bile salt export protein BSEP (Chapter 3 Fig. 10H) or the canalicular membrane protein for phospholipid transport into bile (MDR, Chapter 3 Fig. 10G).

Other Fatty Acid Binding and Transport Proteins

The possibility of potential compensatory upregulation of other fatty acid and cholesterol binding proteins occurred in response to loss of FABP1 and SCP2/SCPx (TKO) was examined. As expected, TKO alone resulted in complete loss of FABP1 (Chapter 3 Fig. 11A) as well as all SCP2/SCPx gene products, SCP2 (Chapter 3 Fig. 11B), SCPx (58kDa) (Chapter 3 Fig. 6G), and SCPx (43kDa) (Chapter 3 Fig. 6H). These ablations resulted in compensatory upregulation of other known FABP family members including I-FABP (Chapter 3 Fig. 11D) and A-FABP (Chapter 3 Fig. 11F) with no compensatory upregulation of B-FABP (Chapter 3 Fig. 11E) or CRABP1 (Chapter 3 Fig. 11G).

References

1. Tiangang Li, John Y. L. Chiang. Regulation of Bile Acid and Cholesterol Metabolism by PPARs. PPAR Research. 2009;2009:1-15.

2. Michael Trauner, James L. Boyer. Bile Salt Transporters: Molecular Characterization, Function, and Regulation. Physiological Reviews. 2003 Apr 1;83(2):633-71.

3. Repa JJ, Mangelsdorf DJ. The Role of Orphan Nuclear Receptors in the Regulation of Cholesterol Homeostasis. Annual Review of Cell and Developmental Biology. 2000 Nov;16(1):459-81.

CHAPTER IV

HIGH FAT STUDY RESULTS[4]

Whole Body Phenotype: Impact of Sex and TKO in High Fat Fed Mice

Singly ablating *Fabp1* increases weight gain (1-3) while singly ablating *Scp2/Scpx* decreases weight gain (4) in a sexually dimorphic manner. Since singly ablating these genes concomitantly upregulates expression of the non-ablated gene in a sex-dependent manner, it was important to determine the impact of ablating both genes on whole body phenotype in both male and female mice on a high fat diet (HFD).

Although wild-type (WT) male mice ate more food than WT females, TKO did not significantly impact total food consumed by either males or females (Chapter 4 Table 1). Consistent with greater food intake, WT male mice gained more total body weight than WT female mice, but TKO had no effect on either males or females. Furthermore, when total body weight was calculated on the basis of total high fat food consumed, WT males converted more of this increased high fat food intake to weight gain than WT females. But again, TKO did not alter total weight gain/total high fat food consumed (Chapter 4 Table 1). Male WT mice gained approximately 2-fold more than female WT mice on both HFD and control diet when measuring total body weight gain per high fat diet consumed in kcal. While the male TKO mice body weight gain/total HFD consumed was much higher than the female TKO, both the male and female TKO mice body weight gain/total HFD consumed was comparable to that of their WT mice counterparts. Also, the male and female TKO mice on control diet had similar increases in

[4]Reprinted from Milligan S, Martin GG, Landrock D, et al. Ablating both Fabp1 and Scp2/scpx (TKO) induces hepatic phospholipid and cholesterol accumulation in high fat-fed mice. Biochim Biophys Acta. 2018;1863(3):323-338. doi: S1388-1981(18)30004-0 [pii]. 2018

total body weight gain/total control diet consumed. Unlike the male TKO mice, the female TKO mice exhibited no increase resulting from HFD as compared to control diet.

PIXImus dual-energy x-ray absorptiometry (DEXA) scans were performed to determine if any difference in weight gain was due to selective loss of lean tissue mass (LTM) or fat tissue mass (FTM) as described in Methods. Both male and female WT mice on HFD gained significantly more FTM as compared to their counterparts on control diet. The male TKO mice also gained significantly more on HFD vs control diet but not so for the female TKO mice. WT males had greater weight gain as FTM than their female WT counterparts (Chapter 4 Table 1). TKO on HFD did not alter the relative proportion of LTM vs FTM in either male or female mice.

Final liver weight (grams wet weight) of WT male mice was significantly greater than that of WT females, but was not associated with greater protein content of the liver (Chapter 4 Table 2). TKO did not significantly alter total liver weight or liver protein content of either males or females. Finally, when data were expressed as liver weight/body weight, the parameter was not significantly altered by either sex or TKO (Chapter 4 Table 2).

Effect of Sex and TKO on Clinical and Morphological Hepatic Pathology

Both WT and TKO male mice had higher levels of lipid accumulation in the liver both grossly and histologically where females did not show evidence of lipid accumulation (Chapter 4 Fig. 1 and 2). TKO did not affect Kupffer cell density or size and there was no other histologic evidence of diffuse inflammation in the liver of WT or TKO mice. In both WT and KO mice there were a few very small scattered foci of inflammation which are a common incidental background finding in the liver of mice of all ages (5). These foci were slightly more frequent in TKO mice which may explain the slightly higher ALT and AST in TKO mice (Chapter 4 Table 3). Although AST and ALT were slightly increased in TKO mice the levels were still in the

normal range for mice (less than 150 and 400 units/l, respectively (blood chemistry and hematology in 8 inbred strains of mice, MPD:Eumorphia. Mouse Phenome Database web site, The Jackson Laboratory, Bar Harbor, Maine USA. http://phenome.jax.org [Cited 29 Oct, 2014]) (Chapter 4 Table 3).

Effect of Sex and TKO on Hepatic Accumulation of Glyceride Lipids in High Fat Fed Mice

Total liver lipid levels were higher in HFD-fed WT male than WT female mice (Chapter 4 Fig. 3A) which was associated with increased hepatic lipid accumulation both grossly and histologically and higher levels of phospholipid in WT males than WT females, albeit not significantly (Chapter 4 Fig. 3C). WT males also showed a two-fold increase in expression of the key enzymes in the synthesis of phosphatidic acid (precursor of both phospholipids and triacylglycerols), i.e. GPAT (Chapter 4 Fig 3E) and AGPAT (Chapter 4 Fig 3F). Expression of Lipin 2, a key enzyme in converting phosphatidic acid to diacylglycerol for the synthesis of phospholipids (phosphatidylcholine, phosphatidylethanolamine) was higher in WT males than WT females (Chapter 4 Fig. 3H) also. However, the lack of difference in expression of DGAT (which transacylates diglyceride to form triglyceride) between the WT male and WT female groups (Chapter 4 Fig. 3G) was consistent with no significant difference in liver triacylglycerol levels between WT male and WT female mice (Chapter 4 Fig. 3D). Finally, it is important to note that despite the fact that SREBP1 was actually lower in WT male than WT female livers (Chapter 4 Fig. 4A), liver expression of SREBP1 target enzymes FASN (Chapter 4 Fig. 4C) and ACC1, the rate limiting enzyme of *de novo* fatty acid synthesis, did not differ between the two sexes in WT mice (Chapter 4 Fig. 4B).

TKO significantly increased hepatic total lipid levels in males and females (Chapter 4 Fig. 3A), due primarily to higher levels of phospholipid (Chapter 4 Fig. 3C) while levels of

neutral lipids (Chapter 4 Fig. 3B), especially triacylglycerol (Chapter 4 Fig. 3D) were not altered in either sex. TKO induced increases in hepatic phospholipid in male mice was associated with increased expression of SREBP1 (Chapter 4 Fig. 4A) despite no change in SREBP1 target enzymes ACC1 (Chapter 4 Fig. 4B) and FASN (Chapter 4 Fig. 4C). However, higher liver level of phospholipid in female TKO mice was attributed to increases in expression of enzymes in phospholipid synthesis [GPAT (Chapter 4 Fig 3E), AGPAT (Chapter 4 Fig 3F), Lipin *2* (Chapter 4 Fig. 3H)] as well as increased SREBP1 (Chapter 4 Fig. 4A) and its target enzymes in *de novo* fatty acid synthesis, i.e., ACC1 (Chapter 4 Fig. 4B) and FASN (Chapter 4 Fig. 4C).

Taken together, these findings indicate the presence of significant sexual dimorphism in hepatic accumulation of lipids in HFD fed mice. Nevertheless, TKO induced hepatic accumulation of phospholipid in both sexes with increases in phospholipid synthetic enzymes in female high fat fed mice.

Effect of Sex and TKO on Hepatic Fatty Acid Oxidation in High Fat Fed Mice

Decreased hepatic fatty acid oxidation may also contribute to hepatic accumulation of lipids. The gene products of both the *Fabp1* (2, 6) and *Scp2/Scpx* (4, 7, 8) genes facilitate fatty acid uptake/oxidation. Therefore, it was important to consider potential contributions from loss of both FABP1 as well as SCP2/SCPx that may have reduced hepatic fatty acid oxidation to thereby contribute to hepatic accumulation of lipids. This possibility was tested by examining serum levels of non-esterified fatty acids and β-hydroxybutyrate (β-HOB), a physiological measure of fatty acid oxidation, as well as determining hepatic expression of key receptors and enzymes involved in fatty acid oxidation in HFD fed mice.

Although serum levels of NEFA (Chapter 4 Fig. 5A) were higher in WT males than females, serum levels of β-HOB (Chapter 4 Fig. 5B) did not differ between the WT male and

WT female mice. Similarly, serum glucose (Chapter 4 Fig. 5C), another oxidative substrate was higher in males than females. This change was despite the fact that WT males had higher hepatic expression of fatty acid oxidative enzymes, i.e. CPT1A (Chapter 4 Fig. 5D), CPT2 (Chapter 4 Fig. 5E), and ACOX1 (Chapter 4 Fig. 5F). Nuclear receptors involved in fatty acid oxidation were also measured where PPARα (Chapter 4 Fig. 5G) was higher in WT males than females but RXRα was lower in WT males than WT females (Chapter 4 Fig. 5H).

TKO significantly reduced serum β-HOB (Chapter 4 Fig. 5B) in males while TKO had no effect on serum glucose (Chapter 4 Fig. 5C) in neither males nor females. Decreases in serum β-HOB in TKO males were associated with loss of FABP1 and SCP2/SCPx and reduced expression of the FABP1 target nuclear receptor PPARα (Chapter 4 Fig. 5G) with no effect on the FABP1 target proteins CPT1A (Chapter 4 Fig. 5D), CPT2 (Chapter 4 Fig. 5E) or ACOX1 (Chapter 4 Fig. 5F). For TKO females the unaltered NEFA (Chapter 4 Fig. 5A) and β-HOB (Chapter 4 Fig. 5B) was associated with potentially offsetting increases in expression of several FABP1 target proteins [CPT1A (Chapter 4 Fig. 5D) and CPT2 (Chapter 4 Fig. 5E)], while RXRα (Chapter 4 Fig. 5H) was decreased compared to WT females.

Taken together, these data suggested that the greater lipid accumulation in HFD fed WT males than WT females was not associated with reduced fatty acid oxidation. However, TKO motivated hepatic lipid accumulation through downregulation of the FABP1 targeted nuclear receptors involved in the regulation of peroxisomal beta-oxidation, PPARα and RXRα, in males and females, respectively.

Impact of Sex and TKO on Hepatic Cholesterol Accumulation in High Fat Fed Mice

Both *Fabp1* (9-11) and *Scp2/Scpx* (12-15) gene products bind, enhance uptake, facilitate metabolism, and function in removal of cholesterol from the liver into bile. Therefore, the

impact of sex and TKO on the hepatic accumulation of cholesterol was determined in HFD fed mice.

Hepatic cholesterol levels were markedly sex-dependent and impacted by TKO. The hepatic level of total cholesterol was significantly higher in WT male than WT female mice (Chapter 4 Fig. 6A), due primarily to increased free cholesterol (Chapter 4 Fig. 6B) at the expense of cholesteryl ester (Chapter 4 Fig. 6C). The higher liver cholesterol level in WT males was associated with higher levels of SREBP2 (Chapter 4 Fig. 6D) despite WT females expressing higher levels of SREBP2 target proteins in *de novo* cholesterol synthesis, i.e. HMGCS1 (Chapter 4 Fig. 6E) and HMGCR (Chapter 4 Fig. 6F). Hepatic expression of enzymes in cholesterol esterification, i.e. ACAT2 (Chapter 4 Fig. 6G) and hydrolysis, i.e. HSL (Chapter 4 Fig. 6H) did not account for the hepatic cholesterol accumulation in WT males.

TKO markedly increased hepatic total cholesterol accumulation in females but not males (Chapter 4 Fig. 6A). Increased total cholesterol in TKO females was attributed to increases in both hepatic free cholesterol (Chapter 4 Fig. 6B) and cholesteryl ester (Chapter 4 Fig. 6C). Though males did not experience an increase in total cholesterol, TKO significantly increased cholesteryl ester accumulation (Chapter 4 Fig. 6C). Despite increases in hepatic cholesterol in females, there was no significant change in hepatic level of SREBP2 (Chapter 4 Fig. 6D) as well as no change in expression of HMGCS1 (Chapter 4 Fig. 6E) and HMGCR (Chapter 4 Fig. 6F). Again, altered hepatic expression of enzymes in cholesterol esterification, i.e. ACAT2 (Chapter 4 Fig. 6G) and hydrolysis, i.e. HSL (Chapter 4 Fig. 6H) in TKO mice did not account for the hepatic cholesterol accumulation. In contrast, expression of HSL was higher in TKO females than WT females.

In general, these data indicate that loss of *Fabp1* and *Scp2/Scpx* gene products, rather than major upregulation of *de novo* cholesterol synthesis and esterification, contributed to increased hepatic cholesterol levels in HFD fed TKO mice.

Impact of Sex and TKO on Hepatic Expression of Proteins Involved in Hepatic Uptake and Efflux/Secretion of Cholesterol in High Fat Fed Mice

FABP1 enhances the uptake of HDL-cholesterol into the cytosol (14), while SCP2 facilitates both uptake and efflux of lipoprotein-derived cholesterol into/from the cytosol (13, 14, 16). Therefore, it was essential to determine potential contributions of compensatory alterations in other proteins mediating lipoprotein cholesterol uptake/secretion/excretion in male and female *Fabp1/Scp2/Scpx* gene ablated mice fed HFD.

Hepatic expression of SR-B1, the receptor for uptake of HDL-cholesterol, was significantly lower in WT male than WT female mice (Chapter 4 Fig. 7A). This decrease was offset in part by WT males expressing higher hepatic levels of proteins in hepatic HDL secretion, i.e. APOA1 (Chapter 4 Fig. 7B) and ABCG4 (Chapter 4 Fig. 7E). Concomitantly, ABCG1 (Chapter 4 Fig. 7D), another protein involved in nascent cholesterol secretion, did not differ between the sexes. Liver levels of LDL-R, the receptor for the uptake of LDL-cholesterol, were also markedly higher in WT male than WT female mice (Chapter 4 Fig. 7F). However, again, this increase was offset in part by higher levels of a key hepatic protein involved in secretion of cholesterol as VLDL (precursor of LDL), i.e. APOB (Chapter 4 Fig. 7G). MTP, another protein involved in secretion of cholesterol as VLDL, was unaffected by sex. (Chapter 4 Fig. 7H). Despite sexual dimorphism in hepatic cholesterol accumulation, there was no difference in the major liver canalicular proteins involved in translocating cholesterol into bile, i.e. ABCG5 (Chapter 4 Fig. 7I) and ABCG8 (Chapter 4 Fig. 7J), between males and females.

TKO did not affect hepatic level of SR-B1 in either sex (Chapter 4 Fig. 7A) but did increase hepatic level of several proteins involved in nascent HDL secretion including ABCG1 (Chapter 4 Fig. 7D) and ABCG4 (Chapter 4 Fig. 7E) while significantly decreasing APOA1 (Chapter 4 Fig. 7B) in males. Although TKO had no effect on SR-B1 in females (Chapter 4 Fig. 7A), it differentially impacted proteins involved in nascent HDL formation by increasing ABCA1 (Chapter 4 Fig. 7C), and ABCG1 (Chapter 4 Fig. 7D) and decreasing ABCG4 (Chapter 4 Fig. 7E). TKO increased hepatic expression of ABCG5 (Chapter 4 Fig. 7I) in both males in females while having no impact on ABCG8 (Chapter 4 Fig. 7J).

These data indicated that the higher hepatic cholesterol level in WT males was associated with higher levels of proteins in the non-HDL-cholesterol uptake pathway (LDL-R) despite the higher level of the key protein in nascent LDL secretion, APOB. The TKO-induced increase in hepatic cholesterol in males was associated at least in part with concomitant downregulation of the key protein in nascent HDL secretion (APOA1). In contrast, the TKO-induced increase in hepatic cholesterol in females did not consistently correlate with concomitant upregulation or downregulation of liver proteins in HDL-cholesterol uptake/efflux, LDL-cholesterol uptake, or VLDL-cholesterol secretion.

Effect of Sex and TKO on Hepatic Expression of Intracellular Proteins Involved in Cytosolic Transport of Cholesterol in High Fat Fed Mice

FABP1 facilitates cytosolic transport of cholesterol to the plasma membrane for secretion of cholesterol as lipoprotein (1, 17), to the endoplasmic reticulum for esterification by ACAT2 (18), or to bile canaliculi for biliary cholesterol excretion (10, 15, 19). Similarly, SCP2 also facilitates cholesterol transfer from the plasma membrane to the endoplasmic reticulum (20, 21) for esterification by ACAT2 (22, 23), to mitochondria and peroxisomes for oxidation to steroids

(24, 25) or bile acids (26-28), and to bile canaliculi for biliary excretion (15, 19, 27). Thus, it was important to determine the effect of sex and TKO on hepatic expression of cytosolic cholesterol binding/transfer proteins in HFD fed mice.

The higher hepatic cholesterol level in WT male mice was associated with WT males having higher levels of both FABP1, the most prevalent intracellular cholesterol binding/transport protein (Chapter 4 Fig. 8A) as well as SCP2 (Chapter 4 Fig 6B). However, hepatic levels of other less prevalent intracellular cholesterol binding proteins, GST (Chapter 4 Fig. 8C) and 3αHSD (Chapter 4 Fig. 8D) were lower in WT males than WT females.

TKO resulted in complete loss of FABP1 (Chapter 4 Fig. 8A) and SCP2 (Chapter 4 Fig. 8B) in both males and females. This loss resulted in potential partial compensatory upregulation of 3αHSD in males (Chapter 4 Fig. 8D) while neither GST (Chapter 4 Fig. 8C) nor 3αHSD (Chapter 4 Fig. 8D) were upregulated in females.

Taken together, the finding of higher hepatic cholesterol levels in WT males than WT females was associated with higher levels of FABP1 and SCP2. In TKO mice, the absence of FABP1 and SCP2 was compensated for only in part by increased level of one less common cholesterol binding protein, i.e. 3αHSD, and not GST (Chapter 4 Fig. 8C, D), in males, while both 3αHSD (Chapter 4 Fig. 8D) and GST (Chapter 4 Fig. 8C) were decreased in females.

Effect of Sex and TKO on Serum Lipoproteins and Apoproteins in High Fat Fed Mice.

The findings in the preceding sections suggest potential contributions of sex and TKO to altering serum apoprotein and lipoprotein profile in HFD fed mice. This possibility was examined as described in Methods. In addition, serum apolipoprotein bound cholesterol has been shown to be affected by FABP1 expression, where FABP1 ablation significantly reduces serum

HDL cholesterol levels in mice fed a standard chow. Therefore, it was essential to examine the effects of ablation in mice fed a HFD diet.

FABP1 upregulation in male WT mice contributed to higher serum levels of HDL-cholesterol (Chapter 4 Fig. 9A) and APOA1 (Chapter 4 Fig. 9B), although the ratio of APOA1/HDL-cholesterol did not significantly differ between the sexes (Chapter 4 Fig. 9C). Higher levels of HDL-C in WT males also were associated with lower SR-B1, the hepatic receptor for HDL-C. Despite increased expression of LDL-R, the hepatic receptor for LDL-C, the serum level of non-HDL-cholesterol (Chapter 4 Fig. 9D) and APOB (Chapter 4 Fig. 9E) were higher in WT males, but the ratio of non-HDL-cholesterol/APOB was not significantly different (Chapter 4 Fig. 9F). In addition, TKO males had higher levels of serum non-HDL-C (Chapter 4 Fig. 9A), APOB (Chapter 4 Fig. 9 E) and APOB/non-HDL-C ratio (Chapter 4 Fig. 9F) than TKO females.

In male mice, TKO decreased serum levels of HDL-cholesterol (Chapter 4 Fig. 9A) and APOA1 (Chapter 4 Fig. 9B) without altering the ratio of APOA1/HDL-cholesterol (Chapter 4 Fig. 9C). While TKO decreased APOB levels in both males and females (Chapter 4 Fig. 9E), the level of non-HDL-cholesterol was not significantly altered (Chapter 4 Fig. 9D). Though the ratio of APOB/non-HDL-C was increased in TKO males, decreased APOB levels in TKO females, albeit not significantly, contributed to a decreased APOB/non-HDL-C ratio (Chapter 4 Fig. 9F).

Overall, sex and TKO affected serum lipoprotein and apoprotein levels in males and females. Further, higher serum HDL-C in WT males was associated with higher hepatic FABP1 and lower hepatic SR-B1. Though LDL-R levels were increased in both WT and TKO male groups compared to both female groups, non-HDL-C levels only differed between TKO males

and females. These data confirm that *Fabp1/Scp2/Scpx* gene ablation yields a sexually dimorphic response in serum apolipoproteins involved in serum cholesterol transport.

Impact of Sex and TKO on Hepatic Proteins involved in Biliary Bile Formation in High Fat Fed Mice

Bile acid production and canalicular excretion drives co-secretion of cholesterol into bile to regulate hepatic cholesterol level. FABP1 binds bile acids (9, 10, 29, 30) and enhances transfer/targeting of HDL-derived cholesterol (and cholesteryl ester after hydrolysis) to the bile canaliculus for biliary excretion (10, 15, 19). SCP2 enhances cholesterol transfer to mitochondria and peroxisomes for oxidation to bile acids [30,54,81] and to the bile canaliculus for cholesterol biliary excretion (15, 19, 27). Therefore, the impact of sex and TKO on hepatic expression of proteins involved in bile acid production and dynamics was determined in HFD fed mice.

Hepatic levels of bile acids were significantly higher in WT males than WT females (Chapter 4 Fig. 10A) which was associated with decreased levels in the expression of the rate limiting enzyme in hepatic bile acid synthesis, i.e. CYP7A1 (Chapter 4 Fig. 10B), as bile acids repress the transcription of CYP7A1. Higher hepatic bile acids were also associated with higher levels of hepatic proteins involved in bile acid re-uptake from the blood, i.e. OATP1 (Chapter 4 Fig. 10C) or OATP2 (Chapter 4 Fig. 10D). However, BSEP (Chapter 4 Fig. 10F), which codes for a protein involved in canalicular bile acid secretion, was also higher in WT males than females. Another hepatic protein in canalicular bile acid secretion into bile, MDR2 (Chapter 4 Fig. 10G) along with hepatic proteins in cytosolic phosphatidylcholine transport and canalicular secretion into bile, i.e. MRP2 (Chapter 4 Fig. 10E) and PCTP (Chapter 4 Fig. 10H) remained unchanged between WT males and females.

Though expression of CYP7A1 in WT males was less than females, levels of the nuclear regulatory protein that contributes to hepatic cholesterol homeostasis by upregulating CYP7A1 in the presence of high cholesterol, i.e. LXR (Chapter 4 Fig. 11A), was higher in WT males than females. This also held true for TKO males compared to TKO females. WT males also expressed higher levels of a nuclear regulatory protein involved in downregulating the import and synthesis of bile acids, i.e. SHP (Chapter 4 Fig. 11C). FXR, which induces SHP (31) was not affected by sex in WT mice (Chapter 4 Fig 9. B).

TKO had little effect on hepatic bile acid levels in either male or female mice (Chapter 4 Fig. 10A). While complete loss of key proteins involved in bile acid formation, binding, and cytosolic transport (FABP1, SCP2, SCPx), was expected to lower liver bile acid levels, concomitant upregulation of the key synthetic enzyme (CYP7A1, Chapter 4 Fig. 10B) in males, and apical proteins in bile acid reuptake from serum, i.e. OATP1 (Chapter 4 Fig. 10C), OATP2 (Chapter 4 Fig. 10D) and MRP2 (Chapter 4 Fig. 10F) in both sexes, apparently compensated to maintain liver bile acid levels. This was in spite of a TKO induced upregulation of a major canalicular bile acid export pump (BSEP, Chapter 4 Fig. 10E) while another canalicular bile acid pump was not affected (MDR, Chapter 4 Fig. 10H). TKO induced upregulation of SHP in both males and females compared to their WT counterparts (Chapter 4 Fig. 11C) but did not impact hepatic expression of other nuclear receptors regulating transcription of proteins in bile acid synthesis in either males or females (Chapter 4 Fig. 11A-B).

Taken together, these findings suggested that HFD fed WT males' higher liver bile acid level was associated with greater reuptake despite increased BSEP, involved in canalicular bile acid secretion. TKO did not alter hepatic bile acid levels largely due to compensatory

upregulation of the rate limiting bile acid synthetic enzyme in males and of apical transporters for bile acid reuptake in both sexes.

References

1. Atshaves BP, Martin GG, Hostetler HA, McIntosh AL, Kier AB, Schroeder F. Liver fatty acid-binding protein and obesity. J Nutr Biochem. 2010 November 01;21(11):1015-32.

2. Atshaves BP, McIntosh A, Storey S, Landrock K, Kier A, Schroeder F. High Dietary Fat Exacerbates Weight Gain and Obesity in Female Liver Fatty Acid Binding Protein Gene-Ablated Mice. Lipids. 2010 Feb;45(2):97-110.

3. McIntosh A, Atshaves B, Landrock D, Landrock K, Martin G, Storey S, et al. Liver Fatty Acid Binding Protein Gene-Ablation Exacerbates Weight Gain in High-Fat Fed Female Mice. Lipids. 2013 May;48(5):435-48.

4. Klipsic D, Landrock D, Martin GG, McIntosh AL, Landrock KK, Mackie JT, et al. Impact of SCP-2/SCP-x gene ablation and dietary cholesterol on hepatic lipid accumulation. American Journal of Physiology-Gastrointestinal and Liver Physiology. 2015;309(5):G399.

5. McInnes EF. Background lesions in laboratory animals. Repr. ed. Edinburgh [u.a.]: Saunders/Elsevier; 2014.

6. Atshaves BP, McIntosh AM, Lyuksyutova OI, Zipfel W, Webb WW, Schroeder F. Liver fatty acid-binding protein gene ablation inhibits branched-chain fatty acid metabolism in cultured primary hepatocytes. J Biol Chem. 2004 July 23;279(30):30954-65.

7. Murphy EJ. Sterol carrier protein-2 expression increases NBD-stearate uptake and cytoplasmic diffusion in L cells. American Journal of Physiology - Gastrointestinal and Liver Physiology. 1998 Aug 1;275(2):237-43.

8. Seedorf U, Ellinghaus P, Roch Nofer J. Sterol carrier protein-2. Biochim Biophys Acta. 2000 June 26;1486(1):45-54.

9. Martin GG, Atshaves BP, Landrock KK, Landrock D, Storey SM, Howles PN, et al. Ablating L-FABP in SCP-2/SCP-x null mice impairs bile acid metabolism and biliary HDL-cholesterol secretion. American journal of physiology. Gastrointestinal and liver physiology. 2014 Dec 1;307(11):G1143.

10. Martin GG, Landrock D, Landrock KK, Howles PN, Atshaves BP, Kier AB, et al. Relative contributions of L-FABP, SCP-2/SCP-x, or both to hepatic biliary phenotype of female mice. Archives of Biochemistry and Biophysics. 2015;588:25-32.

11. Martin GG, Atshaves BP, Huang H, McIntosh AL, Williams BJ, Pei-Jing Pai, et al. Hepatic phenotype of liver fatty acid binding protein gene-ablated mice. American Journal of Physiology-Gastrointestinal and Liver Physiology. 2009;297(6):G1065.

12. Martin GG, Hostetler HA, McIntosh AL, Tichy SE, Williams BJ, Russell DH, et al. Structure and function of the sterol carrier protein-2 N-terminal presequence. Biochemistry. 2008 June 03;47(22):5915-34.

13. Storey SM, Atshaves BP, McIntosh AL, Landrock KK, Martin GG, Huang H. Effect of sterol carrier protein-2 gene ablation on HDL-mediated cholesterol efflux from cultured primary mouse hepatocytes. American Journal of Physiology - Gastrointestinal and Liver Physiology. 2010 Jul 1;299(1):244-54.

14. Storey SM, McIntosh AL, Huang H, Landrock KK, Martin GG, Landrock D, et al. Intracellular cholesterol-binding proteins enhance HDL-mediated cholesterol uptake in cultured primary mouse hepatocytes. American Journal of Physiology - Gastrointestinal and Liver Physiology. 2012 Apr 15;302(8):824-39.

15. Wang J, Bie J, Ghosh S. Intracellular cholesterol transport proteins enhance hydrolysis of HDL-CEs and facilitate elimination of cholesterol into bile. Journal of Lipid Research. 2016 Sep 1;57(9):1712-9.

16. Moncecchi D, Murphy EJ, Prows DR, Schroeder F. Sterol carrier protein-2 expression in mouse L-cell fibroblasts alters cholesterol uptake. Biochimica et biophysica acta. 1996 Jul 26;1302(2):110.

17. Neeli I, Siddiqi SA, Siddiqi S, Mahan J, Lagakos WS, Binas B, et al. Liver Fatty Acid-binding Protein Initiates Budding of Pre-chylomicron Transport Vesicles from Intestinal Endoplasmic Reticulum. Journal of Biological Chemistry. 2007 Jun 22,;282(25):17974-84.

18. Jefferson JR, Slotte JP, Nemecz G, Pastuszyn A, Scallen TJ, Schroeder F. Intracellular sterol distribution in transfected mouse L-cell fibroblasts expressing rat liver fatty acid-binding protein. Journal of Biological Chemistry. 1991 Mar 25,;266(9):5486.

19. Hafer A, Katzberg N, Muench C, Scheibner J, Stange EF, Seedorf U, et al. Studies with sterol carrier protein 2 (SCP2) gene knockout mice identify liver fatty acid binding protein (FABP1) as intracellular cholesterol transporter contributing to biliary cholesterol hypersecretion and gallstone formation. Gastroenterology. 2000;118(4):A926.

20. Frolov A, Woodford JK, Murphy EJ, Billheimer JT, Schroeder F. Fibroblast membrane sterol kinetic domains: modulation by sterol carrier protein-2 and liver fatty acid binding protein. Journal of Lipid Research. 1996 Sep 1,;37(9):1862.

21. Schroeder F, Gallegos AM, Atshaves BP, Storey SM, Mcintosh AL, Petrescu AD, et al. Recent Advances in Membrane Microdomains: Rafts, Caveolae, and Intracellular Cholesterol Trafficking. Experimental Biology and Medicine. 2001 Oct;226(10):873-90.

22. Gavey KL, Noland BJ, Scallen TJ. The participation of sterol carrier protein2 in the conversion of cholesterol to cholesterol ester by rat liver microsomes. Journal of Biological Chemistry. 1981 Mar 25,;256(6):2993.

23. Murphy EJ, Schroeder F. Sterol carrier protein-2 mediated cholesterol esterification in transfected L-cell fibroblasts. Biochimica et Biophysica Acta (BBA)/Lipids and Lipid Metabolism. 1997;1345(3):283-92.

24. Chanderbhan RF, Kharroubi AT, Noland BJ, Scallen TJ, Vahouny GV. Sterol Carrier Protein2: Further Evidence for Its Role in Adrenal Steroidogenesis. Endocrine Research. 1986;12(4):351-70.

25. Atshaves BP, McIntosh AL, Landrock D, Payne HR, Mackie JT, Maeda N, et al. Effect of SCP-x gene ablation on branched-chain fatty acid metabolism. Am J Physiol Gastrointest Liver Physiol. 2007 March 01;292(3):939.

26. Fuchs M, Hafer A, Christian MA, Kannenberg F, Teichmann S, Scheibner J, et al. Disruption of the Sterol Carrier Protein 2 Gene in Mice Impairs Biliary Lipid and Hepatic Cholesterol Metabolism. Journal of Biological Chemistry. 2001 Dec 21,;276(51):48058.

27. Kannenberg F, Ellinghaus P, Assmann G, Seedorf U. Aberrant Oxidation of the Cholesterol Side Chain in Bile Acid Synthesis of Sterol Carrier Protein-2/Sterol Carrier Protein-x Knockout Mice. Journal of Biological Chemistry. 1999 December 10;274(50):35455-60.

28. Muench C, Hafer A, Katzberg N, Scheibner J, Stange EF, Seedorf U, et al. Relevance of the Sterol Carrier Protein-2 Gene for Bile Acid Synthesis and Gallstone Formation in Genetically Susceptible Mice. . 2000:1167.

29. Martin GG, Atshaves BP, McIntosh AL, Mackie JT, Kier AB, Schroeder F. Liver fatty-acid-binding protein (L-FABP) gene ablation alters liver bile acid metabolism in male mice. The Biochemical journal. 2005 Nov 1,;391(Pt 3):549-60.

30. Martin GG, Atshaves BP, McIntosh AL, Mackie JT, Kier AB, Schroeder F. Liver fatty acid binding protein gene ablation potentiates hepatic cholesterol accumulation in cholesterol-fed female mice. American Journal of Physiology - Gastrointestinal and Liver Physiology. 2006 Jan 1,;290(1):36-48.

31. Denson LA, Sturm E, Echevarria W, Zimmerman TL, Makishima M, Mangelsdorf DJ, et al. The Orphan Nuclear Receptor, shp, Mediates Bile Acid-Induced Inhibition of the Rat Bile Acid Transporter, ntcp. Gastroenterology. 2001;121(1):140-7.

CHAPTER V

PHYTOL STUDY CONCLUSIONS AND DISCUSSION[5]

Since accumulation of branched-chain fatty acids derived from dietary phytol is toxic (1, 2), most research has focused on identification of the individual peroxisomal enzymes in their metabolism and the pathological consequences of genetic mutations therein (3-7). In contrast, much less is known about how these poorly soluble branched-chain lipids traffic through the hepatocyte cytosol for metabolism. *In vitro* ligand binding (8-11) and transfected cell (8-10, 12) studies suggest FABP1 and SCP2/SCPx gene products act as potential 'chaperones'. Further, a highly prevalent human FABP1 SNP (26-38% allele frequency) (13-19) resulting in FABP1 T94A substitution alters uptake/metabolism of another branched-chain lipid, cholesterol (14, 18, 20, 21). Human subjects expressing the FABP1 T94A variant exhibit marked triglyceride accumulation in liver (18, 20), elevated triglyceride and LDL cholesterol in serum (14, 22, 23), and increased incidence of cardiovascular disease (14, 16, 22). Although less prevalent, a mutation in the human SCPx gene completely abolishes SCPx activity, markedly elevates serum levels of branched chain fatty acids (phytanic acid, pristanic acid), and induces neurodegeneration (24, 25). While gene-targeted mice ablated in FABP1 (10, 26-31), SCPx (32), or SCP2/SCPx (33-37) have proven useful in resolving respective physiological roles, concomitant upregulation of the non-ablated chaperone, especially FABP1 in SCP2/SCPx null or SCPx null mice, complicates interpretation of phenotype (32, 35, 36). The current study with

[5]Milligan S, Martin GG, Landrock D, et al. Ablating both Fabp1 and Scp2/scpx (TKO) induces hepatic phospholipid and cholesterol accumulation in high fat-fed mice. Biochim Biophys Acta. 2018;1863(3):323-338. doi: S1388-1981(18)30004-0 [pii].

mice ablated in both SCP2/SCPx and FABP1 genes (SCP2/SCPx/FABP1 triple gene ablation or TKO) provided several important insights:

First, ablating both FABP1 and SCP2/SCPx significantly affected whole body phenotype in mice fed a high phytol diet as compared to ablating each individually. In earlier findings, feeding dietary phytol alone decreased body weight of female wild-type (WT) mice (38), an effect not further exacerbated upon ablating FABP1 (26). In contrast, singly ablating SCPx or SCP2-SCPx markedly exacerbated phytol diet-induced weight loss several-fold (32, 35). Likewise, ablating FABP1 in SCP2/SCPx null mice (i.e. TKO) nearly doubled the dietary phytol-induced weight loss, an effect attributed to both decreased food consumption and energy intake. These data indicated that concomitant upregulation of FABP1 in SCP2/SCPx null or SCPx null mice (32, 35, 36, 39) did not diminished and/or obscure the impact of SCP2/SCPx ablation on dietary phytol-induced weight loss. This outcome suggests that SCP2/SCPx may play a greater role than FABP1 in exacerbating phytol-induced weight loss.

Second, the TKO data presented herein suggested that SCP2/SCPx gene products, much more than the FABP1, contributed to phytol oxidation. In response to dietary phytol, peroxisomal oxidation products of phytanic acid and pristanic acid accumulate concomitant with upregulation of FABP1, SCP2, SCPx and peroxisomal oxidative enzymes in WT mice (38). FABP1 gene ablation did not further exacerbate this dietary phytol-induced increase likely due to concomitant upregulation of SCPx, a key peroxisomal enzyme in phytol oxidation (26). In contrast, singly ablating SCPx or SCP2-SCPx markedly decreased dietary phytol-induced accumulation of phytol metabolites despite concomitant upregulation of FABP1 (32, 35, 36, 39) and some fatty acid oxidative enzymes (32, 35). Ablating FABP1 in SCP2/SCPx null mice (i.e. TKO) did not further exacerbate dietary-phytol induced oxidation and upregulation of fatty acid

oxidative enzymes. These findings suggested that SCP2/SCPx gene products may play a greater role in phytol oxidation than FABP1. Consistent with this possibility, nearly half of SCP2 is peroxisomal, while all of SCPx is peroxisomal and SCPx is the only known peroxisomal ketoacyl-CoA thiolase involved in branched chain fatty acid oxidation (40). Despite these findings, however, FABP1 may serve a lesser role in phytol metabolism, i.e. in re-uptake of excess phytol metabolites from serum (10). FABP1 enhances fatty acid uptake (41-43), accounts for 80-90% of hepatic cytosol binding/transport capacity for fatty acids and fatty acyl-CoAs (44, 45), and is the most prevalent cytosolic transporter for targeting these ligands to mitochondria and peroxisomes for oxidation (37, 46, 47). Loss of FABP1 alone decreases hepatocyte uptake of phytanic acid (10). In contrast, ablation of SCP2/SCPx significantly decreases serum NEFA, attributable in large part to marked upregulation of FABP1 (35, 39). TKO offset the effect of FABP1 upregulation in SCP2/SCPx ablated mice since serum NEFA levels were unaltered. Taken together, these findings suggested that in the context of high dietary phytol, SCP2/SCPx has a greater role in oxidation while FABP1 may have a role in reuptake.

Third, TKO markedly increased hepatic lipid accumulation, primarily due to increased phospholipid, in phytol-fed female mice. Earlier it was shown that dietary phytol alone increased hepatic lipid accumulation, primarily by increasing phospholipid in female wild-type (WT) mice (38), an effect exacerbated upon FABP1 ablation (26). FABP1 is known to stimulate the rate limiting enzyme in phospholipid synthesis (GPAT) *in vitro* (48-50). FABP1 overexpression in transfected cells increases fatty acid uptake and incorporation into triglyceride, albeit quantitatively not as much as into phospholipid (41). In contrast, singly ablating SCPx or SCP2-SCPx decreased hepatic total lipid, primarily by decreasing triglyceride (32, 35) concomitant with upregulation of FABP1 (32, 35, 36, 39). Ablating FABP1 in SCP2/SCPx mice (i.e. TKO)

fed phytol mice reflected a combined phenotype where phospholipid was increased nearly 5-fold at the expense of triglyceride. The increased hepatic phospholipid was associated with increased transcription of the rate limiting gene in *de novo* fatty acid synthesis (*Acc1*) and enzymes in phospholipid synthesis (*Gpam*, *Agpa*t). Taken together, these findings suggested that in the context of phytol-diet, FABP1 and SCP2/SCPx play somewhat different roles in hepatic lipid accumulation, differing in preferentially impacting phospholipid versus triglyceride.

Fourth, phytol diet TKO increased cholesterol in the serum, but not liver. Dietary phytol alone did not alter hepatic cholesterol accumulation in female wild-type (WT) mice (26). However, FABP1 gene ablation significantly increased only hepatic free cholesterol, but not cholesteryl ester or total cholesterol, in phytol-fed females (26). Likewise, singly ablating SCPx did not significantly alter hepatic total, free, or esterified cholesterol in female mice (32) while ablating SCP2/SCPx increased hepatic total and esterified cholesterol level (39)—effects likely attributed to concomitant greater upregulation of FABP1 (32, 35, 36, 39). FABP1 overexpression in cultured cells enhances cholesterol uptake and intracellular targeting for esterification (51, 52). Concomitant FABP1 upregulation in SCP2 overexpression mice also induces hepatic cholesterol accumulation (53-55). Taken together, these data suggested that FABP1 upregulation may counteract the effect of ablating SCP2/SCPx or SCPx on hepatic cholesterol.

In summary, the results presented herein with TKO mice shed significant new insights into the roles of FABP1 and SCP2/SCPx genes in whole body weight gain and hepatic lipid metabolism in response to dietary phytol. TKO exacerbated dietary phytol-induced whole-body weight loss, especially lean tissue mass (LTM). TKO also increased hepatic total lipid accumulation, primarily phospholipid, in response to upregulation of hepatic levels of proteins in

the phospholipid synthesis. Increased hepatic lipid accumulation was not attributable to any concomitant upregulation of membrane fatty acid transport/translocase proteins involved in fatty acid uptake (FATP2, FATP4, FATP5 or GOT) or cytosolic proteins involved in fatty acid intracellular targeting (ACBP). Since individually ablating SCPx or SCP2/SCPx elicited concomitant upregulation of FABP1, these findings with TKO mice significantly contribute to our understanding of the respective roles of these genes in dietary phytol metabolism.

References

1. Verhoeven NM, Jakobs C. Human metabolism of phytanic acid and pristanic acid. Prog Lipid Res. 2001 November 01;40(6):453-66.

2. Avigan J. The presence of phytanic acid in normal human and animal plasma. Biochim Biophys Acta. 1966 April 04;116(2):391-4.

3. Jansen GA, Wanders RJA. Alpha-Oxidation. BBA - Molecular Cell Research. 2006;1763(12):1403-12.

4. Theil AC, Schutgens RB, Wanders RJ, Heymans HS. Clinical recognition of patients affected by a peroxisomal disorder: a retrospective study in 40 patients. European journal of pediatrics. 1992;151(2):117-20.

5. van den Brink D, Wanders R. Phytanic acid: production from phytol, its breakdown and role in human disease. Cell Mol Life Sci. 2006 Aug;63(15):1752-65.

6. Wanders RJ, Vreken P, Ferdinandusse S, Jansen GA, Waterham HR, van Roermund CW, et al. Peroxisomal fatty acid alpha- and beta-oxidation in humans: enzymology, peroxisomal metabolite transporters and peroxisomal diseases. Biochemical Society transactions. 2001;29(Part 2):250-67.

7. Wanders RJA, Komen J, Ferdinandusse S. Phytanic acid metabolism in health and disease. BBA - Molecular and Cell Biology of Lipids. 2011;1811(9):498-507.

8. Atshaves BP, Storey SM, Petrescu A, Greenberg CC, Lyuksyutova OI, Smith R, et al. Expression of fatty acid binding proteins inhibits lipid accumulation and alters toxicity in L cell fibroblasts. Am J Physiol Cell Physiol. 2002 September 01;283(3):688.

9. Atshaves BP, Storey SM, Huang H, Schroeder F. Liver fatty acid binding protein expression enhances branched-chain fatty acid metabolism. Mol Cell Biochem. 2004 April 01;259(1-2):115-29.

10. Atshaves BP, McIntosh AM, Lyuksyutova OI, Zipfel W, Webb WW, Schroeder F. Liver fatty acid-binding protein gene ablation inhibits branched-chain fatty acid metabolism in cultured primary hepatocytes. J Biol Chem. 2004 July 23;279(30):30954-65.

11. Andrey F, Kimberly M, Billheimer JT, Cho Tae-Hyeon, Friedhelm S. Lipid specificity and location of the sterol carrier protein-2 fatty acid-binding site: A fluorescence displacement and energy transfer study. Lipids. 1997;32(11):1201-9.

12. Atshaves BP, Storey SM, Schroeder F. Sterol carrier protein-2/sterol carrier protein-x expression differentially alters fatty acid metabolism in L cell fibroblasts. Journal of Lipid Research. 2003 September 01;44(9):1751-62.

13. Robitaille J, Brouillette C, Lemieux S, Pérusse L, Gaudet D, Vohl MC. Plasma concentrations of apolipoprotein B are modulated by a gene–diet interaction effect between the LFABP T94A polymorphism and dietary fat intake in French-Canadian men. Molecular Genetics and Metabolism. 2004;82(4):296-303.

14. Fisher E, Weikert C, Klapper M, Lindner I, Möhlig M, Spranger J, et al. L-FABP T94A is associated with fasting triglycerides and LDL-cholesterol in women. Molecular Genetics and Metabolism. 2007;91(3):278-84.

15. Martin O. Weickert, Christian v. Loeffelholz, Michael Roden, Visvanathan Chandramouli, Attila Brehm, Peter Nowotny, et al. A Thr94Ala mutation in human liver fatty acid-binding protein contributes to reduced hepatic glycogenolysis and blunted elevation of plasma glucose

levels in lipid-exposed subjects. American Journal of Physiology - Endocrinology And Metabolism. 2007 Oct 1,;293(4):1078-84.

16. Yamada Y, Kato K, Oguri M, Yoshida T, Yokoi K, Watanabe S, et al. Association of genetic variants with atherothrombotic cerebral infarction in Japanese individuals with metabolic syndrome. International Journal of Molecular Medicine. 2008 Jun;21(6):801-8.

17. Bu, Liming|Salto, Lorena M.|De Leon, Kevin J.|De Leon, Marino. Polymorphisms in fatty acid binding protein 5 show association with type 2 diabetes. Diabetes Research and Clinical Practice. 2011;92(1):82-91.

18. Peng XE, Wu YL, Lu QQ, Hu ZJ, Lin X. Two genetic variants in FABP1 and susceptibility to non-alcoholic fatty liver disease in a Chinese population. Gene. 2012;500(1):54-8.

19. Maria Luisa Mansego, Fernando Martínez, Maria Teresa Martínez-Larrad, Carina Zabena, Gemma Rojo, Sonsoles Morcillo, et al. Common Variants of the Liver Fatty Acid Binding Protein Gene Influence the Risk of Type 2 Diabetes and Insulin Resistance in Spanish Population. PLoS One. 2012 Mar 1,;7(3):e31853.

20. McIntosh AL, Huang H, Storey SM, Landrock KK, Landrock D, Petrescu AD, et al. Human FABP1 T94A variant impacts fatty acid metabolism and PPAR-α activation in cultured human female hepatocytes. American journal of physiology. Gastrointestinal and liver physiology. 2014 Jul 15,;307(2):G164.

21. Huang H, McIntosh AL, Landrock KK, Landrock D, Storey SM, Martin GG, et al. Human FABP1 T94A variant enhances cholesterol uptake. BBA - Molecular and Cell Biology of Lipids. 2015 Jul;1851(7):946-55.

22. Brouillette C, Bossé Y, Pérusse L, Gaudet D, Vohl M. Effect of liver fatty acid binding protein (FABP) T94A missense mutation on plasma lipoprotein responsiveness to treatment with fenofibrate. J Hum Genet. 2004 Aug;49(8):424-32.

23. Tian Y, Li H, Wang S, Yan J, Chen Z, Li Z, et al. Association of L-FABP T94A and MTP I128T Polymorphisms with Hyperlipidemia in Chinese Subjects. Lipids. 2015 Mar;50(3):275-82.

24. Ferdinandusse S, Kostopoulos P, Denis S, Rusch H, Overmars H, Dillmann U, et al. Mutations in the Gene Encoding Peroxisomal Sterol Carrier Protein X (SCPx) Cause Leukencephalopathy with Dystonia and Motor Neuropathy. The American Journal of Human Genetics. 2006;78(6):1046-52.

25. Horvath R, Lewis-Smith D, Douroudis K, Duff J, Keogh M, Pyle A, et al. SCP2 mutations and neurodegeneration with brain iron accumulation. Neurology. 2015 Nov 24,;85(21):1909-11.

26. Barbara P. Atshaves, Avery L. McIntosh, H. Ross Payne, John Mackie, Ann B. Kier, Friedhelm Schroeder. Effect of branched-chain fatty acid on lipid dynamics in mice lacking liver fatty acid binding protein gene. American Journal of Physiology - Cell Physiology. 2005 Mar 1,;288(3):543-58.

27. Hostetler HA, McIntosh AL, Atshaves BP, Storey SM, Payne HR, Kier AB, et al. Liver type fatty acid binding protein (L-FABP) directly interacts with peroxisome proliferator-activated receptor-alpha in cultured primary hepatocytes. The Journal of Lipid Research. 2008 Nov 20,.

28. Huang H, Starodub O, McIntosh A, Atshaves BP, Woldegiorgis G, Kier AB, et al. Liver fatty acid-binding protein colocalizes with peroxisome proliferator activated receptor alpha and enhances ligand distribution to nuclei of living cells. Biochemistry. 2004 Mar 9,;43(9):2484-500.

29. Martin GG, Atshaves BP, McIntosh AL, Mackie JT, Kier AB, Schroeder F. Liver fatty-acid-binding protein (L-FABP) gene ablation alters liver bile acid metabolism in male mice. The Biochemical journal. 2005 Nov 1,;391(Pt 3):549-60.

30. Gregory G. Martin, Barbara P. Atshaves, Avery L. McIntosh, John T. Mackie, Ann B. Kier, Friedhelm Schroeder. Liver fatty acid binding protein gene ablation potentiates hepatic cholesterol accumulation in cholesterol-fed female mice. American Journal of Physiology - Gastrointestinal and Liver Physiology. 2006 Jan 1,;290(1):36-48.

31. Anca D. Petrescu, Huan Huang, Gregory G. Martin, Avery L. McIntosh, Stephen M. Storey, Danilo Landrock, et al. Impact of L-FABP and glucose on polyunsaturated fatty acid induction of PPARα-regulated β-oxidative enzymes. American Journal of Physiology - Gastrointestinal and Liver Physiology. 2013 Feb 1,;304(3):241-56.

32. Atshaves BP, McIntosh AL, Landrock D, Payne HR, Mackie JT, Maeda N, et al. Effect of SCP-x gene ablation on branched-chain fatty acid metabolism. Am J Physiol Gastrointest Liver Physiol. 2007 March 01;292(3):939.

33. Kannenberg F, Ellinghaus P, Assmann G, Seedorf U. Aberrant Oxidation of the Cholesterol Side Chain in Bile Acid Synthesis of Sterol Carrier Protein-2/Sterol Carrier Protein-x Knockout Mice. Journal of Biological Chemistry. 1999 December 10;274(50):35455-60.

34. Seedorf U. Functional Analysis of Sterol Carrier Protein-2 (SCP2) in the SCP2 Knockout Mouse. In: Chang TY, Freeman DA, editors. Intracellular Cholesterol Trafficking. Boston, MA: Springer US; 1998. p. 235-52.

35. Seedorf U, Raabe M, Ellinghaus P, Kannenberg F, Fobker M, Engel T, et al. Defective peroxisomal catabolism of branched fatty acyl coenzyme A in mice lacking the sterol carrier protein-2/sterol carrier protein-x gene function. Genes & development. 1998;12(8):1189-201.

36. Michael Fuchs, Andrea Hafer, Christian MÃ¼nch, Frank Kannenberg, Sandra Teichmann, JÃ¼rgen Scheibner, et al. Disruption of the Sterol Carrier Protein 2 Gene in Mice Impairs Biliary Lipid and Hepatic Cholesterol Metabolism. Journal of Biological Chemistry. 2001 Dec 21,;276(51):48058.

37. Atshaves BP, McIntosh AL, Payne HR, Gallegos AM, Landrock K, Maeda N, et al. SCP-2/SCP-x gene ablation alters lipid raft domains in primary cultured mouse hepatocytes. Journal of Lipid Research. 2007 Oct 1,;48(10):2193-211.

38. Mackie JT, Atshaves BP, Payne HR, McIntosh AL, Schroeder F, Kier AB. Phytol-induced hepatotoxicity in mice. Toxicol Pathol. 2009 February 01;37(2):201-8.

39. Klipsic D, Landrock D, Martin GG, McIntosh AL, Landrock KK, Mackie JT, et al. Impact of SCP-2/SCP-x gene ablation and dietary cholesterol on hepatic lipid accumulation. American Journal of Physiology-Gastrointestinal and Liver Physiology. 2015;309(5):G399.

40. Gallegos AM, Atshaves BP, Storey SM, Starodub O, Petrescu AD, Huang H, et al. Gene structure, intracellular localization, and functional roles of sterol carrier protein-2. Prog Lipid Res. 2001 November 01;40(6):498-563.

41. Murphy EJ, Prows DR, Jefferson JR, Schroeder F. Liver fatty acid-binding protein expression in transfected fibroblasts stimulates fatty acid uptake and metabolism. Biochimica et Biophysica Acta (BBA)/Lipids and Lipid Metabolism. 1996;1301(3):191-8.

42. Murphy EJ. L-FABP and I-FABP expression increase NBD-stearate uptake and cytoplasmic diffusion in L cells. American Journal of Physiology. 1998 Aug 1,;38(2):G244.

43. Prows DR, Murphy EJ, Schroeder F. Intestinal and liver fatty acid binding proteins differentially affect fatty acid uptake and esterification in L-cells. Lipids. 1995 Oct;30(10):907-10.

44. Gregory G. Martin, Heike Danneberg, Leena S. Kumar, Barbara P. Atshaves, Erdal Erol, Michael Bader, et al. Decreased Liver Fatty Acid Binding Capacity and Altered Liver Lipid Distribution in Mice Lacking the Liver Fatty Acid-binding Protein Gene. Journal of Biological Chemistry. 2003 Jun 13,;278(24):21429-38.

45. Martin GG, Huang H, Atshaves BP, Binas B, Schroeder F. Ablation of the liver fatty acid binding protein gene decreases fatty acyl CoA binding capacity and alters fatty acyl CoA pool distribution in mouse liver. Biochemistry. 2003 Oct 7,;42(39):11520-32.

46. Hostetler H, Lupas D, Tan Y, Dai J, Kelzer M, Martin G, et al. Acyl-CoA binding proteins interact with the acyl-CoA binding domain of mitochondrial carnitine palmitoyl transferase I. Mol Cell Biochem. 2011 Sep;355(1):135-48.

47. Woldegiorgis G, Bremer J, Shrago E. Substrate inhibition of carnitine palmitoyltransferase by palmitoyl-CoA and activation by phospholipids and proteins. Biochimica et biophysica acta. 1985 Nov 14,;837(2):135.

48. Jolly CA, Hubbell T, Behnke WD, Schroeder F. Fatty Acid Binding Protein: Stimulation of Microsomal Phosphatidic Acid Formation. Archives of Biochemistry and Biophysics. 1997 May 1,;341(1):112-21.

49. Schroeder F, Jolly CA, Cho T, Frolov A. Fatty acid binding protein isoforms: structure and function. Chemistry and Physics of Lipids. 1998;92(1):1-25.

50. Bordewick U, Heese M, Börchers T, Robenek H, Spener F. Compartmentation of hepatic fatty-acid-binding protein in liver cells and its effect on microsomal phosphatidic acid biosynthesis. Biological chemistry Hoppe-Seyler. 1989 Mar;370(3):229.

51. J R Jefferson, J P Slotte, G Nemecz, A Pastuszyn, T J Scallen, F Schroeder. Intracellular sterol distribution in transfected mouse L-cell fibroblasts expressing rat liver fatty acid-binding protein. Journal of Biological Chemistry. 1991 Mar 25,;266(9):5486.

52. Murphy EJ, Schroeder F. Sterol carrier protein-2 mediated cholesterol esterification in transfected L-cell fibroblasts. Biochimica et Biophysica Acta (BBA)/Lipids and Lipid Metabolism. 1997;1345(3):283-92.

53. Atshaves BP, McIntosh AL, Martin GG, Landrock D, Payne HR, Bhuvanendran S, et al. Overexpression of sterol carrier protein-2 differentially alters hepatic cholesterol accumulation in cholesterol-fed mice. Journal of Lipid Research. 2009 Jul 1,;50(7):1429.

54. Zanlungo S, Amigo L, Mendoza H, Glick J, Rodriguez A, Kozarsky K, et al. Overexpression of sterol carrier protein-2 in mice leads to increased hepatic cholesterol content and enterohepatic circulation of bile acids. Gastroenterology. 2000;118(4):A997.

55. Zanlungo S, Amigo L, Mendoza H, Miquel JF, Vío C, Glick JM, et al. Sterol carrier protein 2 gene transfer changes lipid metabolism and enterohepatic sterol circulation in mice. Gastroenterology. 2000 Dec;119(6):1708-19.

CHAPTER VI

HIGH FAT STUDY CONCLUSIONS AND DISCUSSION[6]

Although singly ablating *Fabp1* or *Scp2/Scpx* genes may exacerbate the impact of HFD on whole body phenotype and hepatic lipid metabolism, dietary regimen (mice prefer and consume more *ad libitum* fed HFD), sex-differences, and/or concomitant upregulation of the non-ablated gene complicate interpretation of such findings. This is especially evident in mice singly ablated in *Scp2/Scpx* or *Scpx* wherein hepatic levels of FABP1 are concomitantly upregulated (1,2,3). To further resolve the impact of *Scp2/Scpx* gene ablation on whole body and hepatic phenotype independent of FABP1 upregulation, the current study was undertaken by examining the impact of also ablating *Fabp1* in *Scp2/Scpx* null mice (i.e. *Fabp1/Scp2/Scpx* ablation or TKO). In order to preclude potential complications of data interpretation, these studies were performed by: i) pair-feeding high fat diet (HFD) independent of increased preference and intake of HFD (rev. in (4) and ii) phytoestrogen-free and phytol-free HFD independent of potential estrogenic (5,6) or PPARα (7,8,9) induction effects of these lipophilic molecules. The data provided the following new insights on the impact of these genes on whole body and liver lipid phenotype in both male and female mice pair-fed HFD.

First, female WT mice consumed more total high fat diet as percent of body weight than their male WT counterparts, but the WT male mice gained significantly more body weight than female WT mice. The study herein continued from 8 weeks to 20 weeks. Studies involving C57BL/6 mice examining various high fat diets, demonstrate various weight gains for male vs

---

[6]Milligan S, Martin GG, Landrock D, et al. Impact of dietary phytol on lipid metabolism in SCP2/SCPX/L-FABP null mice. Biochim Biophys Acta. 2017;1862(3):291-304. doi: S1388-1981(16)30328-6 [pii].

female mice. It is difficult to compare each study due to the different starting ages, substrains, duration, diet compositions, and microbiota. In one particular study wherein C57BL/6N mice were put on a HFD at 4 weeks of age, females gained similar weight to males until about after 16 weeks duration and overtook the males in body weight after about 30 week duration (10). However another HFD study involving C56BL/6J mice beginning at 3 weeks (after weaning) and lasting until the mice were 9 months, showed the male WT on HFD continuing to gain weight at a similar or slightly higher rate (11). Further, while the male mice outdistanced their counterparts on normal diet throughout, the female mice on HFD were similar to their counterparts on normal diet up to about 4 months of age (11). A different study involving C57BL/6J mice revealed that the fatty acid profile of HFD was linked to sex differences in weight gain (12). Further complicating direct comparisons, the C57BL/6J substrain is more sensitive to high fat diet-induced obesity than the C57BL/6N substrain (13).

In comparing total body weight gain per high fat diet consumed in kcal, the male WT mice gained approximately 2-fold more than female WT mice. The 2-fold increase was similar to the WT male vs female gain on control diet. In contrast, the TKO male vs female mice on control diet did not differ in total body weight gain per total food consumed on control diet. While the female TKO mice exhibited no increase as compared to control diet, the male TKO mice significantly gained more than the male WT mice on the control diet—comparable to that of the WT male mice on HFD.

The body weight gain exhibited by the male WT vs female WT mice was associated with increased hepatic levels of the two major cytosolic long chain fatty acid (LCFA) and LCFA-CoA binding proteins, i.e. FABP1 and SCP2. Even in the context of control-chow fed diets, hepatic

protein levels of FABP1, SCP2 and SCPx are also higher in livers of male than female mice (14, 15, 16, 17).

Second, male WT mice exhibited greater hepatic lipid accumulation than their female counterparts fed the same HFD. While FABP1 stimulates both anabolic and catabolic aspects of LCFA metabolism, in the context of HFD the net effect of the anabolic processes appeared greater in WT male than WT female mice. The WT male mice's greater hepatic lipid accumulation was associated directly with: i) Higher hepatic levels of FABP1 and SCP2. FABP1 is known to enhance uptake (18-22) and cytosolic transport of exogenous LCFA (18, 23, 20) for anabolic processes such as esterification in the endoplasmic reticulum (ER) (24-26), and lipoprotein budding from ER for secretion (27, 28). Likewise, SCP2 also enhances uptake of LCFA and cholesterol (29, 30), LCFA cytosolic transport (30), and esterification in the endoplasmic reticulum (31-34) ; ii) Higher hepatic expression of several key enzymes in the synthesis of phospholipids (GPAT, AGPAT, and Lipin *2*). In contrast, over all, LCFA catabolism was not higher in HFD-fed WT males than females. Consistent with the higher hepatic expression of FABP1 and SCP2 in HFD-fed WT males than females, hepatic expression of LCFA oxidative enzymes was higher in HFD-fed WT males than females (CPT1A, CPT2, and ACOX1). FABP1 facilitates LCFA/LCFA-CoA targeting for oxidation in mitochondria and peroxisomes (18, 35) as well as targeting to nuclei for LCFA and LCFA-CoA activating PPARα transcription of oxidative enzymes (36, 37). SCP2 also enhances LCFA oxidation (38). Nevertheless, the HFD-fed WT male and female mice did not differ in LCFA oxidation as indicated by unaltered serum β-HOB levels. This suggested that in the context of HFD, the higher levels of FABP1 and SCP2 in WT males than females resulted in preferential targeting of LCFA towards anabolic metabolism (i.e. lipid synthesis and storage). Consistent with this

possibility, the greater body weight gain of HFD-fed WT males was associated with 5-fold greater gain as FTM as well as LTM as compared to females.

Third, while TKO male mice had less weight gain than WT mice on control diet, the TKO had little effect on whole body phenotype in either male or female HFD-fed mice as compared to their WT counterparts. Singly ablating the *Fabp1* gene increases whole body weight gain in females and less so in males (39-42). Conversely, singly ablating the *Scp2/Scpx* gene decreases body weight, especially in females (3, 17).Taken together, these findings suggested that the reduced whole body weight of *Scp2/Scpx* gene ablated mice was not attributable to concomitant upregulation of FABP1. The two genes appeared to impact oppositely the whole body weight gain in HFD fed mice.

Fourth, TKO significantly increased hepatic accumulation of lipid (phospholipid, cholesterol, cholesteryl ester) in both male and female HFD-fed mice. Despite the loss of both FABP1 and SCP2, three factors contributed to increased hepatic lipid accumulation. They include the following: i) Upregulation of other cytosolic lipid ligand (LCFA, cholesterol) 'chaperone' proteins (3αHSD) in TKO males but not females; ii) Upregulation of key proteins in phospholipid synthesis (GPAT, AGPAT, and/or Lipin 2) in females and *de novo* LCFA synthesis (SREBP1 in males and females; ACC1 and FASN in females*).* Singly ablating *Fabp1* decreased hepatic triacylglycerol in males while increasing phospholipid, cholesterol and cholesteryl ester in females (43). Despite concomitant upregulation of FABP1, singly ablating *Scp2/Scpx* increased hepatic accumulation of lipid (phospholipid, triacylglycerol, cholesteryl ester) in both male and female mice (17). Taken together, these data indicated that FABP1 and SCP2/SCPx had a greater impact on hepatic triacylglycerol and cholesterol, respectively, but in a sex dependent manner; iii) Decreased LCFA oxidation as indicated by decreased serum β-HOB in

male mice. Despite downregulation of hepatic expression of nuclear receptors regulating expression of LCFA oxidative enzymes in both males and females (PPARα and RXRα, respectively), expression of several LCFA oxidative enzymes (CPT1A, CPT2) was upregulated in TKO females but not males. Singly ablating *Fabp1* reduced LCFA oxidation as well as ligand-induced PPARα transcription of LCFA oxidative enzymes (18, 19, 39, 44-46). Despite concomitant upregulation of FABP1, singly ablating *Scp2/Scpx* also decreased LCFA oxidation (3). Again, these findings suggested that SCP2/SCPx may have a greater impact on LCFA oxidation than FABP1.

Fifth, sex, more than *Fabp1/Scp2/Scpx* gene ablation, played a significant role in hepatic bile acid synthesis, reuptake and secretion. WT male mice exhibited higher levels of hepatic bile acid with concomitant downregulation of CYP7A1 expression compared to WT females. WT males also exhibited increased proteins involved in the reuptake of bile acid from serum (OATP1 and OATP2) despite increases in BSEP and SHP which are involved in the secretion and decreased synthesis of bile acids, respectively. FABP1 and SCP2/SCPx have been shown to play distinct, but complimentary roles in hepatic bile acid homeostasis where FABP1 is involved in the retention of bile acids and SCP2/SCPx functions in the formation and biliary secretion of bile acids. These findings suggest that the increase in hepatic bile acid concentration in WT mice was impacted by upregulation of FABP1 and SCP2 and specifically, their bile acid retention and formation functions. Again, TKO had no effect on hepatic bile acid levels. This was due to the concomitant upregulation of bile acid synthetic and reuptake proteins (CYP7A1, OATP1, OATP2) and contrasting upregulation of proteins involved in inhibiting the synthesis and promoting the canalicular secretion of bile acids (SHP, MRP2 and BSEP).

Sixth and finally, ablation of *Scp2/Scpx* in *Fabp1* null mice (TKO) impacted hepatic phospholipid mass to a greater extent than ablating *Fabp1* alone—especially in the context of HFD. For example, in HFD-fed mice TKO significantly increased liver phospholipid mass in both males and females (shown herein)—in marked contrast to LKO alone which did not alter hepatic phospholipid level in either sex (47). However, in control-fed mice, TKO selectively increased hepatic phospholipid in females but not males (not shown)—similarly as shown for LKO in control-fed females vs males (47-49, 19). In contrast, ablating only the *Scp2/Scpx* gene (DKO) alone had no effect on hepatic phospholipid content in either male or female control-fed mice (17). Taken together, these findings suggest that the impact of *Scp2/Scpx* and *Fabp1* gene products on hepatic phospholipid phenotype is complex, highly dependent on the expression of one or both these genes, and is significantly dependent on the context of sex and dietary fat. Both direct and indirect mechanism(s) may be involved. For example, both FABP1 and SCP2 are known to bind fatty acyl-CoA and directly stimulate microsomal glycerol-3-phosphate acyltransferase (GPAT)—the rate limiting step in synthesis of phosphatidic acid from which other phospholipid subclasses are derived (24, 26, 34, 50). Whether FABP1 and/or SCP2 may also and possibly differentially impact other fatty acyl-CoA transacylation enzymes in the formation of phospholipid classes vs triacylglycerols is not known. Alternately, FABP1 (but not SCP2) is known to transfer bound ligands (fatty acids, fatty acyl-CoAs, xenobiotics) through the cytosol, into nuclei wherein it binds to nuclear receptors (e.g. PPARα) to facilitate ligand transfer and activate transcription of numerous genes in fatty acid metabolism (rev. in (39, 37, 51, 52, 53). While the absence of FABP1 and SCP2 (i.e. TKO) would thereby normally be expected to reduce PPARα transcriptional activity, inhibition of phytol metabolism elicits the opposite response since in phytol-fed mice (54). TKO markedly inhibits phytol metabolism in phytol-fed

mice and induces accumulation of high levels of phytol metabolites (phytanic acid and pristanic acid) that are potent PPARα activators (54). These metabolites in turn induce transcription of key enzymes regulating the synthesis of phosphatidic acid (*Gpam*, *Agpat*) as well as synthesis/hydrolysis of triacylglycerol (*Lipin*, *Dgat*) (54). Resolving whether FABP1 also either directly impacts enzyme activities and/or transcription of mRNAs encoding enzymes in phospholipid synthetic steps downstream from *Gpat* (e.g. *Pcyt1a*, *Pemt*, *Pcyt2*, *Ptdss1* and *2*) is beyond the timeframe and scope of this manuscript.

In summary, both *Fabp1* and *Scp2/Scpx* gene products play important roles in regulating whole body phenotype and hepatic lipid accumulation in mice. Hepatic FABP1 protein level is greatly upregulated in human NAFLD (55-57) and in animal models of NAFLD (58, 59). Furthermore, a highly prevalent SNP in the coding region of the human *Fabp1* gene results in a T94A amino acid substitution (57, 60-62) that is associated with NAFLD (57, 63). Three additional promising fields of research on potential roles of FABP1 (and potentially SCP2) in the development of NAFLD are: i) The role of microbiome alteration in NAFLD pathogenesis. Studies have shown that gut dysbiosis not only leads to human obesity, but also that many microbial cell components resulting from gut dysbiosis contribute to hepatic inflammation and steatosis (64). It has also been shown that fecal transplantation from mice with NAFLD into healthy wild-type mice results in NAFLD in those healthy mice (65). Environmental modifiers such as differences in gut microbiome have been suggested to contribute to the phenotype of *Fabp1* gene ablated mice (66). ii) The impact of FABP1 on the hepatic endocannabinoid system in the development of NAFLD. Recent discoveries with *Fabp1* gene ablated mice have resolved a link between FABP1, the endocannabinoid (EC) system and NAFLD. FABP1 is the most prevalent endocannabinoid and cannabinoid binding/'chaperone' protein present in hepatic

cytosol of mice (16, 62). Ablation of the *Fabp1* gene increased hepatic levels of AEA and 2-AG, endogenous agonists of the cannabinoid receptor CB1, which have been associated with NAFLD (16). Although SCP2 also binds AEA and 2-AG with high affinity (16, 67, 68), *Scp2/Scpx* gene ablation decreases brain levels of endocannabinoids but concomitantly increases CB1 levels (69). However, nothing is known regarding the impact of ablating the *Scp2/Scpx* gene or SNPs therein on the hepatic endocannabinoid system and/or NAFLD. iii) TKO results in different metabolic patterns between male and female mice due to sex differences in FABP1 and SCP2/Scpx expression as well as to *Fabp1* and *Scp2/Scpx* having different metabolic functions in health and disease. For example, phytol (a normal dietary constituent derived from the side chain of chlorophyll) markedly decreases body weight gain and increases weight loss more so in female than male WT mice (70-72). This difference has been attributed to the lower expression of the protein products of the *Fabp1* and/or *Scp2/Scpx* genes, both of which are involved in phytol metabolism (18, 70, 72, 3, 113). With regards to human disease, increased FABP1 level and human genetic variation in the *Fabp1* gene have been associated with development of NAFLD as indicated above. While *Scp2/Scpx* gene ablation in mice is associated with altered phytol, lipid metabolism, and neurotoxicity (3, 18, 70, 73) human genetic variation in *Scp2/Scpx* also results in neurotoxity exhibited as neurodegeneration, brain iron accumulation, leukencephalopathy, dystonia, and motor neuropathy (74, 75).

Overall in the setting of health as compared to excessive adiposity, our study supports that endogenous (sex) and phenotypic (WT vs TKO) factors impact the metabolic functions of the genes, *Fabp1* and *Scp2/Scpx* wherein lipid accumulation in mice fed a high fat diet highlights

the role of *Fabp1* and/or *Scp2/Scpx* genes as potential targets for therapeutic intervention in the treatment of NAFLD.

References

1. B P Atshaves, A.L. McIntosh, D. Landrock, H.R. Payne, J. Mackie, N. Maeda, J.M. Ball, F. Schroeder, A.B. Kier, Effect of SCP-x gene ablation on branched-chain fatty acid metabolism., Am. J. Physiol. 292 (2007) pp. 939-951.

2. M Fuchs, A. Hafer, C. Muench, F. Kannenberg, S. Teichmann, J. Scheibner, E.F. Stange, U. Seedorf, Disruption of the sterol carrier protein 2 gene in mice impairs biliary lipid and hepatic cholesterol metabolism., J. Biol. Chem. 276 (2001) pp. 48058-48065.

3. U Seedorf, M. Raabe, P. Ellinghaus, F. Kannenberg, M. Fobker, T. Engel, S. Denis, F. Wouters, K.W.A. Wirtz, R.J.A. Wanders, N. Maeda, G. Assmann, Defective peroxisomal catabolism of branched fatty acyl coenzyme A in mice lacking the sterol carrier protein-2/sterol carrier protein-x gene function, Genes and Development 12 (1998) pp. 1189-1201.

4. G G Martin, Landrock D., Chung S, Dangott LJ, D.R. Seeger, Murphy EJ, M.Y. Golovko, A.B. Kier, F. Schroeder, Fabp1 gene ablation inhibits high fat diet-induced increase in brain endocannabinoids., J Neurochem. 140 (2017) pp. 294-306.

5. J E Thigpen, K.D. Setchell, K.B. Ahlmark, J. Kocklear, T. Spahr, G.F. Caviness, M.F. Goelz, J.K. Haseman, R.R. Newbold, D.B. Forsythe, Phytoestrogen content of purified, open- and closed-formula laboratory animal diets., Lab. An. Science 49 (1999) pp. 530-536.

6. J E Thigpen, K.D. Setchell, M.F. Goelz, D.B. Forsythe, The phytoestrogen content of rodent diets., Envron. Health Persp. 107 (1999) p.A182-A183.

7. P Ellinghaus, C. Wolfrum, G. Assmann, F. Spener, U. Seedorf, Phytanic acid activates the peroxisome proliferator-activated receptor alpha (PPARalpha) in sterol carrier protein-2-/sterol carrier protein x-deficient mice., J. Biol. Chem. 274 (1999) pp. 2766-2772.

8. T Hanhoff, S. Benjamin, T. Borchers, F. Spener, Branched-chain fatty acids as activators of peroxisome proliferators., Eur. J. Lip. Sci. Technol. 107 (2005) pp. 716-729.

9. C Wolfrum, P. Ellinghaus, M. Fobker, U. Seedorf, G. Assmann, T. Borchers, F. Spener, Phytanic acid is ligand and transcriptional activator of murine liver fatty acid binding protein., J. Lipid Res. 40 (1999) pp. 708-714.

10. D Medrikova, Z.M. Jilkova, K. Bardova, P. Janovska, M. Rossmeisl, J. Kopecky, Sex differences during the course of diet-induced obesity in mice: adipose tissue expandability and glycemic control., Int. J. Obesity 36 (2012) pp. 262-272.

11. LL Hwang, CH Wang, TL Li, SD Chang, LC Lin, CP Chen, CT Chen, KC Liang, IK Ho, WS Yang, LC Chiou, Sex differences in high fat diet induced obesity, metabolic alterations and learning, and synaptic plasticity deficits in mice., Obesity 18 (2010) pp. 463-469.

12. S E Akoum, V. Lamontagne, I. Cloutier, J.-F. Tanguay, Nature of fatty acids in high fat diets differentially delineates obesity-linked metabolic syndrome components in male and female C57BL/6J mice., Diabetology and Metabolic Syndrome 3 (2011) pp. http:/www.dmsjournal.com/content3-1/34.

13. A Nicholson, P.C. Reifsnyder, R.D. Malcolm, C.A. Lucas, G.R. MacGregor, W. Zhang, E.H. Leiter, Diet-induced obesity in two C57BL/6 substrains with intact or mutant nicotinamide nucleotide transhydrogenase (Nnt) gene., Obesity 18 (2010) pp. 1902-1905.

14. B P Atshaves, A.L. McIntosh, H.R. Payne, J. Mackie, A.B. Kier, F. Schroeder, Effect of branched-chain fatty acid on lipid dynamics in mice lacking liver fatty acid binding protein gene., Am. J. Physiol. 288 (2005) p.C543-C558.

15. B P Atshaves, H.R. Payne, A.L. McIntosh, S.E. Tichy, D. Russell, A.B. Kier, F. Schroeder, Sexually dimorphic metabolism of branched chain lipids in C57BL/6J mice., J. Lipid Res. 45 (2004) pp. 812-830.

16. H Huang, A.L. McIntosh, G.G. Martin, Landrock D., S. Chung, K.K. Landrock, L.J. Dangott, S. Li, A.B. Kier, F. Schroeder, FABP1: a novel hepatic endocannabinoid and cannabinoid binding protein., Biochemistry 55 (2016) pp. 5243-5255.

17. D Klipsic, D. Landrock, G.G. Martin, A.L. McIntosh, K.K. Landrock, J.T. Mackie, F. Schroeder, A.B. Kier, Impact of SCP-2/SCP-x gene ablation and dietary cholesterol on hepatic lipid accumulation., Am. J. Physiol. Gastrointest. and Liver Phys. 309 (2015) p.G387-G399.

18. B P Atshaves, A.L. McIntosh, O.I. Lyuksyutova, W.R. Zipfel, W.W. Webb, F. Schroeder, Liver fatty acid binding protein gene ablation inhibits branched-chain fatty acid metabolism in cultured primary hepatocytes., J. Biol. Chem. 279 (2004) pp. 30954-30965.

19. G G Martin, H. Danneberg, L.S. Kumar, B.P. Atshaves, E. Erol, M. Bader, F. Schroeder, B. Binas, Decreased liver fatty acid binding capacity and altered liver lipid distribution in mice lacking the liver fatty acid binding protein (L-FABP) gene., J. Biol. Chem. 278 (2003) pp. 21429-21438.

20. E J Murphy, L-FABP and I-FABP expression increase NBD-stearate uptake and cytoplasmic diffusion in L-cells., Am. J. Physiol. 275 (1998) p.G244-G249.

21. E J Murphy, D.R. Prows, J.R. Jefferson, F. Schroeder, Liver fatty acid binding protein expression in transfected fibroblasts stimulates fatty acid uptake and metabolism, Biochim. Biophys. Acta 1301 (1996) pp. 191-198.

22. D R Prows, E.J. Murphy, F. Schroeder, Intestinal and liver fatty acid binding proteins differentially affect fatty acid uptake and esterification in L-Cells, Lipids 30 (1995) pp. 907-910.

23. M J McArthur, B.P. Atshaves, A. Frolov, W.D. Foxworth, A.B. Kier, F. Schroeder, Cellular uptake and intracellular trafficking of long chain fatty acids, J. Lipid Res. 40 (1999) pp. 1371-1383.

24. C A Jolly, T. Hubbell, W.D. Behnke, F. Schroeder, Fatty acid binding protein: Stimulation of microsomal phosphatidic acid formation., Arch. Biochem. Biophys. 341 (1997) pp. 112-121.

25. C A Jolly, T. Hubbell, W.D. Behnke, F. Schroeder, Fatty acid binding protein: stimulation of microsomal phosphatidic acid formation., Arch. Biochem. Biophys. 341 (1997) pp. 112-121.

26. C A Jolly, D.A. Wilton, F. Schroeder, Microsomal fatty acyl CoA transacylation and hydrolysis: fatty acyl CoA species dependent modulation by liver fatty acyl CoA binding proteins, Biochim. Biophys. Acta 1483 (2000) pp. 185-197.

27. I Neeli, S.A. Siddiqi, S. Siddiqi, J. Mahan, W.S. Lagakos, B. Binas, T. Gheyi, J. Storch, C.M. Mansbach, Liver fatty acid binding protein initiates budding of pre-chylomicron transport vesicles from intestinal endoplasmic reticulum., J. Biol. Chem. 282 (2007) pp. 17974-17984.

28. S A Siddiqi and C.M. Mansbach, Phosphorylation of Sar1b releases the liver fatty acid binding protein from a multiprotein complex in the intestinal cytosol enabling it to bind to ER and bud the pre-chylomicron transport vesicle., J. Biol. Chem. 287 (2012) pp. 10178-10188.

29. D M Moncecchi, E.J. Murphy, D.R. Prows, F. Schroeder, Sterol carrier protein-2 expression in mouse L-cell fibroblasts alters cholesterol uptake, Biochim. Biophys. Acta 1302 (1996) pp. 110-116.

30. E J Murphy, Sterol carrier protein-2 expression increases fatty acid uptake and cytoplasmic diffusion in L-cell fibroblasts., Am. J. Physiol. 275 (1998) p.G237-G243.

31. H Chao, J.T. Billheimer, A.B. Kier, F. Schroeder, Microsomal long chain fatty acyl CoA transacylation: differential effect of SCP-2, Biochim. Biophys. Acta 1439 (1999) pp. 371-383.

32. H Chao, M. Zhou, A. McIntosh, F. Schroeder, A.B. Kier, Acyl CoA binding protein and cholesterol differentially alter fatty acyl CoA utilization by microsomal acyl CoA: cholesterol transferase., J. Lipid Res. 44 (2003) pp. 72-83.

33. E J Murphy and F. Schroeder, Sterol carrier protein-2 mediated cholesterol esterification in transfected L-cell fibroblasts., Biochim. Biophys. Acta 1345 (1997) pp. 283-292.

34. O Starodub, C.A. Jolly, B.P. Atshaves, J.B. Roths, E.J. Murphy, A.B. Kier, F. Schroeder, Sterol carrier protein-2 immunolocalization in endoplasmic reticulum and stimulation of phospholipid formation., Am. J. Physiol. 279 (2000) p.C1259-C1269.

35. H A Hostetler, D. Lupas, Y. Tan, J. Dai, M.S. Kelzer, G.G. Martin, G. Woldegiorgis, A.B. Kier, F. Schroeder, Acyl-CoA binding proteins interact with the acyl-CoA binding domain of mitochondrial carnitine palmitoyltransferase I., Mol. Cell. Biochem. 355 (2011) pp. 135-148.

36. H A Hostetler, M. Balanarasimha, H. Huang, M.S. Kelzer, A. Kaliappan, A.B. Kier, F. Schroeder, Glucose regulates fatty acid binding protein interaction with lipids and PPARa, J. Lipid Res. 51 (2010) pp. 3103-3116.

37. H A Hostetler, A.L. McIntosh, B.P. Atshaves, S.M. Storey, H.R. Payne, A.B. Kier, F. Schroeder, Liver type Fatty Acid Binding Protein (L-FABP) interacts with peroxisome proliferator activated receptor-a in cultured primary hepatocytes., J. Lipid Res. 50 (2009) pp. 1663-1675.

38. B P Atshaves, A.L. McIntosh, H.R. Payne, A.M. Gallegos, K. Landrock, N. Maeda, A.B. Kier, F. Schroeder, Sterol carrier protein-2/sterol carrier protein-x gene ablation alters lipid raft domains in primary cultured mouse hepatocytes., J. Lipid Res. 48 (2007) pp. 2193-2211.

39. B P Atshaves, G.G. Martin, H.A. Hostetler, A.L. McIntosh, A.B. Kier, F. Schroeder, Liver fatty acid binding protein (L-FABP) and Dietary Obesity., Journal of Nutritional Biochemisty 21 (2010) pp. 1015-1032.

40. B P Atshaves, A.L. McIntosh, A.B. Kier, F. Schroeder, High dietary fat exacerbates weight gain and obesity in female liver fatty acid binding protein gene ablated mice., Lipids 45 (2010) pp. 97-110.

41. G G Martin, B.P. Atshaves, A.L. McIntosh, J.T. Mackie, A.B. Kier, F. Schroeder, Liver Fatty Acid Binding Protein GeneAblated Female Mice Exhibit Increased AgeDependent Obesity, J. Nutr. 138 (2008) pp. 1859-1865.

42. G G Martin, B.P. Atshaves, A.L. McIntosh, J.T. Mackie, A.B. Kier, F. Schroeder, Liver fatty acid binding protein gene ablation enhances age-dependent weight gain in male mice., Mol. Cell. Biochem. 324 (2009) pp. 101-115..

43. G G Martin, B.P. Atshaves, H. Huang, A.L. McIntosh, B.W. Williams, P.-J. Pai, D.H. Russell, A.B. Kier, F. Schroeder, Hepatic phenotype of liver fatty acid binding protein (L-FABP) gene ablated mice., Am. J. Physiol. 297 (2009) p.G1053-G1065.

44. H Huang, A.L. McIntosh, G.G. Martin, A.D. Petrescu, K. Landrock, D. Landrock, A.B. Kier, F. Schroeder, Inhibitors of fatty acid synthesis induce PPARa-regulated fatty acid b-oxidative enzymes: synergistic roles of L-FABP and glucose., PPAR Research 2013 (2013) pp. 1-22.

45. A D Petrescu, H. Huang, G.G. Martin, A.L. McIntosh, S.M. Storey, D. Landrock, A.B. Kier, F. Schroeder, Impact of L-FABP and glucose on polyunsaturated fatty acid induction of PPARa regulated b-oxidative enzymes., Am. J. Physiol. Gastrointest. and Liver Phys. 304 (2013) p.G241-G256.

46. A D Petrescu, A.L. McIntosh, S.M. Storey, H. Huang, G.G. Martin, D. Landrock, A.B. Kier, F. Schroeder, High glucose potentiates liver fatty acid binding protein (L-FABP) mediated fibrate induction of PPARa in mouse hepatocytes., Biochim. Biophys. Acta 1831 (2013) pp. 1412-1425.

47. G G Martin, D. Landrock, S. Chung, L.J. Dangott, A.L. McIntosh, J.T. Mackie, A.B. Kier, F. Schroeder, Loss of fatty acid binding protein-1 alters the hepatic endocannabinoid system response to a high fat diet., J. Lip. Res. 58 (2017) pp. In press 10-2-17-JLR/2017/077891.

48. G G Martin, B.P. Atshaves, A.L. McIntosh, J.T. Mackie, A.B. Kier, F. Schroeder, Liver fatty acid binding protein (L-FABP) gene ablation alters liver bile acid metabolism in male mice., Biochem. J. 391 (2005) pp. 549-560.

49. G G Martin, B.P. Atshaves, A.L. McIntosh, J.T. Mackie, A.B. Kier, F. Schroeder, Liver fatty acid binding protein (L-FABP) gene ablation potentiates hepatic cholesterol accumulation in cholesterol-fed female mice., Am. J. Physiol. 290 (2006) p.G36-G48.

50. U Bordewick, M. Heese, T. Borchers, H. Robenek, F. Spener, Compartmentation of hepatic fatty-acid-binding protein in liver cells and its effect on microsomal phosphatidic acid biosynthesis, Biol. Chem. Hoppe-Seyler 370 (1989) pp. 229-238.

51. F Schroeder, A.D. Petrescu, H. Huang, B.P. Atshaves, A.L. McIntosh, G.G. Martin, H.A. Hostetler, A. Vespa, K. Landrock, D. Landrock, H.R. Payne, A.B. Kier, Role of fatty acid binding proteins and long chain fatty acids in modulating nuclear receptors and gene transcription., Lipids 43 (2008) pp. 1-17.

52. R L Smathers, J.J. Galligan, C.T. Shearn, K.S. Fritz, K. Mercer, M. Ronis, D.J. Orlicky, N.O. Davidson, D.R. Petersen, Susceptibility of L-FABP -/- mice to oxidative stress in early-stage alcoholic liver., J. Lipid Res. 54 (2013) pp. 1335-1345.

53. T Velkov, Interactions between human liver fatty acid binding protein and peroxisome proliferator activated receptor drugs., PPAR Research 2013 (2013) pp. 1-14.

54. S Milligan, G.G. Martin, D. Landrock, A.L. McIntosh, J.T. Mackie, F. Schroeder, A.B. Kier, Impact of dietary phytol on lipid metabolism in SCP2/SCPx/L-FABP null mice., Biochim. Biophys. Acta - Mol. Cell Biol. Lip. 1862 (2017) pp. 291-304.

55. M Charlton, K. Viker, A. Krishnan, S. Sanderson, B. Veldt, A.J. Kaalsbeek, M. Kendrick, G. Thompson, F. Que, J. Swain, M. Sarr, Differential expression of lumican and fatty acid binding protein-1: new insights into the histologic spectrum of nonalcoholic fatty liver disease., Hepatology 49 (2009) pp. 1375-1384.

56. C Guzman, M. Benet, S. Pisonero-Vaquero, M. Moya, M.V. Garcia-Mediavilla, M.L. Martinez-Chantar, J. Gonzalez-Gallego, J.V. Castell, S. Sanchez-Campos, R. Jover, The human liver fatty acid binding protein (FABP1) gene is activated by FOXA1 and PPARa; and repressed by C/EBPa: implicaiton in FABP1 down-regulation in nonalcoholic liver disease., Biochim. Biophys. Acta 1831 (2013) pp. 803-818.

57. A L McIntosh, H. Huang, S.M. Storey, K. Landrock, Landrock D., A.D. Petrescu, S. Gupta, B.P. Atshaves, A.B. Kier, F. Schroeder, Human FABP1 T94A variant impacts fatty acid metabolism and PPARa activation in cultured human female hepatocytes., Am. J. Physiol. Gastrointest. and Liver Phys. 307 (2014) p.G164-G176.

58. J N Baumgardner, K. Shankar, L. Hennings, T.M. Badger, M.J.J. Ronis, A new model for nonalcoholic steatohepatitis in the rat utilizing total enteral nutrition to overfeed a high-polyunsaturated fat diet., Am. J. Physiol. Gastrointest. and Liver Phys. 294 (2007) p.G27-G38.

59. S Y Yang, X.Y. He, H. Schulz, Fatty acid oxidation in rat brain is limited by the low activity of 3-ketoacyl-coenzyme A thiolase, J. Biol. Chem. 262 (1987) pp. 13027-13032.

60. C Brouillette, Y. Bose, L. Perusse, D. Gaudet, M.-C. Vohl, Effect of liver fatty acid binding protein (FABP) T94A missense mutation on plasma lipoprotein responsiveness to treatment with fenofibrate., J. Hum. Gen. 49 (2004) pp. 424-432.

61. E Fisher, C. Weikert, M. Klapper, I. Lindner, M. Mohlig, J. Spranger, H. Boeing, J. Schrezenmeir, F. Doring, L-FABP T94A is associated with fasting triglycerides and LDL-cholesterol in women., Mol. Gen. and Metab. 91 (2007) pp. 278-284.

62. F Schroeder, A.L. McIntosh, G.G. Martin, H. Huang, Landrock D., S. Chung, K.K. Landrock, L.J. Dangott, S. Li, M. Kaczocha, E.J. Murphy, B.P. Atshaves, A.B. Kier, Fatty acid binding protein-1 (FABP1) and the human FABP1 T94A variant: Roles in the endocannabinoid system and dyslipidemias., Lipids 51 (2016) pp. 655-676.

63. X-E Peng, Y.L. Wu, Q.-Q. Lu, Z.-J. Ju, X. Lin, Two genetic variants in FABP1 and susceptibility to non-alcoholic fatty liver disease in a Chinese population., Gene 500 (2012) pp. 54-58.

64. L Zhu, R.D. Baker, S.S. Baker, Gut microbiome and nonalcoholic fatty liver diseases., Pediat. Res. 77 (2014) pp. 245-251.

65. T L Roy, M. Llopis, P. Lepage, A. Bruneau, S. Rabot, C. Bevilacqua, P. Gerard, Intestinal microbiota determines development of non-alcoholic fatty liver disease in mice., Gut 62 (2012) pp. 1787-1794.

66. E P Newberry, S. Kennedy, Y. Xie, J. Luo, H. Jiang, D.S. Ory, N.O. Davidson, Phenotypic divergence in two lines of L-FABP(-/-) mice reflects substrain differences and environmental modifiers., Am. J. Physiol. Gastrointest. and Liver Phys. 309 (2015) p.G648-G661.

67. C J Hillard, H. Huang, C.D. Vogt, B.E. Rodrigues, T.S. Neumann, D.S. Sem, F. Schroeder, C.W. Cunningham, Endocannabinoid Transport Proteins: Discovery of Tools to Study Sterol

Carrier Protein-2., in: V.E.Patricia H.Reggio (Ed.), Methods In Enzymology: Endocannabinoid Transport Proteins, Methods in Enzymology, Academic Press imprint of Elsevier, Cambridge, MA, USA, 2017, pp. 99-122.

68. E S Liedhegner, C.D. Vogt, D.S. Sem, C.W. Cunninham, C.J. Hillard, Sterol carrier protein-2: binding protein for endocannabinoids., Mol. Neurobiol. 50 (2014) pp. 149-158.

69. C J Hillard, E. Liedhegner, J. Tinklenberg, K. Stuhr, E. Doncheck, L. Du, C. Cunningham, Genetic deletion of SCP-2 selectively enhances amygdalar endocannabinoid signaling., FASEB J. 29 (2015) p.Supplement 770.9.

70. B P Atshaves, H.R. Payne, A.L. McIntosh, S.E. Tichy, D. Russell, A.B. Kier, F. Schroeder, Sexually dimorphic metabolism of branched chain lipids in C57BL/6J mice., J. Lipid Res. 45 (2004) pp. 812-830.

71. Landrock D., S. Milligan, G.G. Martin, A.L. McIntosh, K. Landrock, F. Schroeder, A.B. Kier, Effect of Fabp1/Scp-2/Scp-x ablation on whole body and hepatic phenotype of phytol-fed male mice., Lipids 52 (2017) pp. 385-397.

72. A L McIntosh, S.M. Storey, H. Huang, A.B. Kier, F. Schroeder, Sex-dependent impact of Scp-2/Scp-x gene ablation on hepatic phytol metabolism., Arch. Biochem. Biophys. 635 (2017) pp. 17-26.

73. S M Storey, H. Huang, A.L. McIntosh, G.G. Martin, A.B. Kier, F. Schroeder, Impact of Fabp1/SCP-2/SCP-x gene ablation (TKO) on hepatic phytol metabolism., J. Lipid Res. 58 (2017) pp. 1153-1165.

74. S Ferdinandusse, P. Kostopoulos, S. Denis, R. Rusch, H. Overmars, U. Dillman, W. Reith, D. Haas, R.J.A. Wanders, M. Duran, M. Marziniak, Mutations in the gene encoding peroxisomal

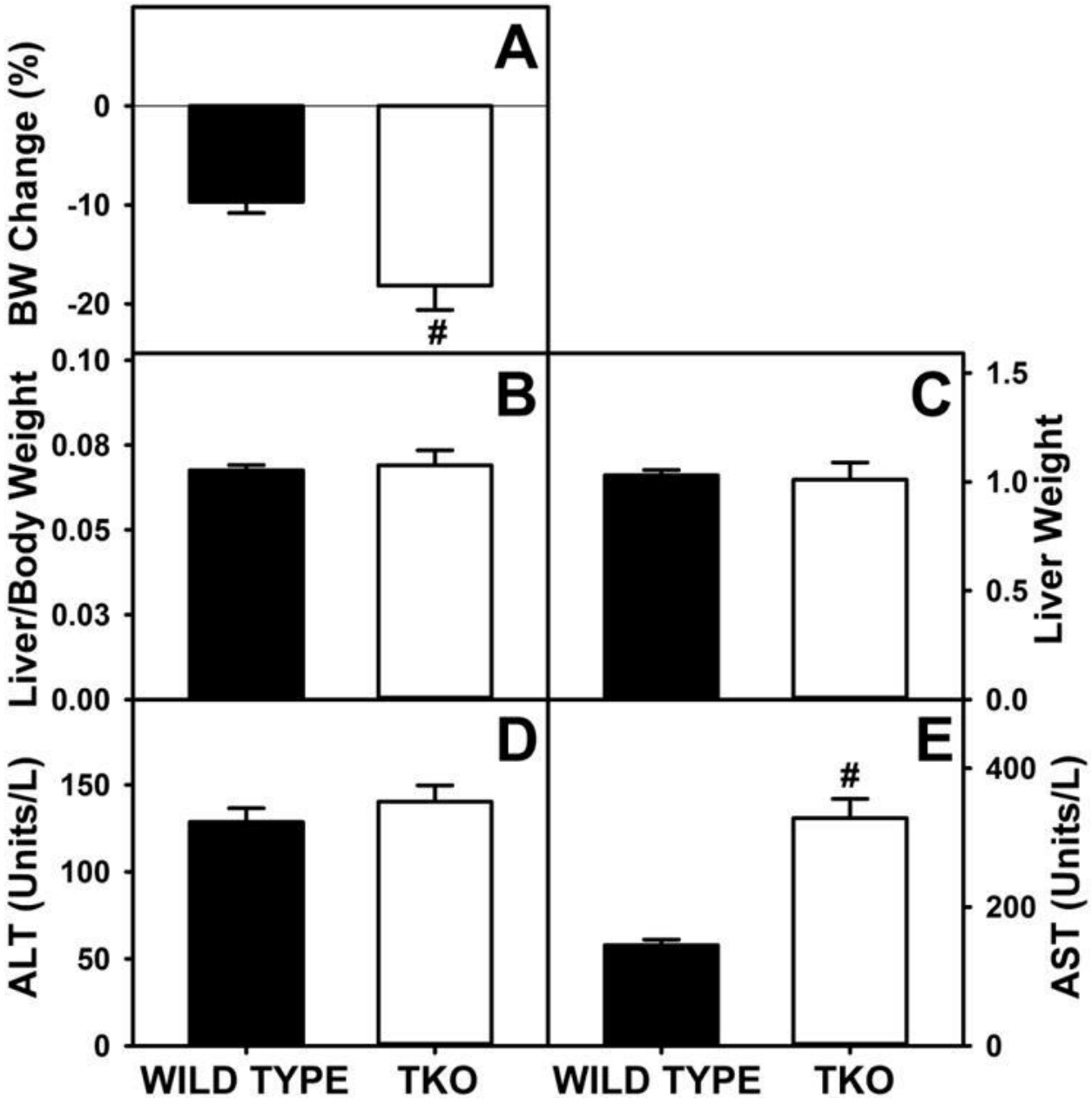
sterol carrier protein X (SCPx) cause leukencephalopathy with dystonia and motor neuropathy., Am. J. Hum. Genet. 78 (2006) pp. 1046-1052.

75. R Horvath, D. Lewis-Smith, K. Douroudis, J. Duff, et al, SCP2 mutations and neurodegeneration with brain iron accumulation., Neurology 85 (20165) pp. 1909-1911.

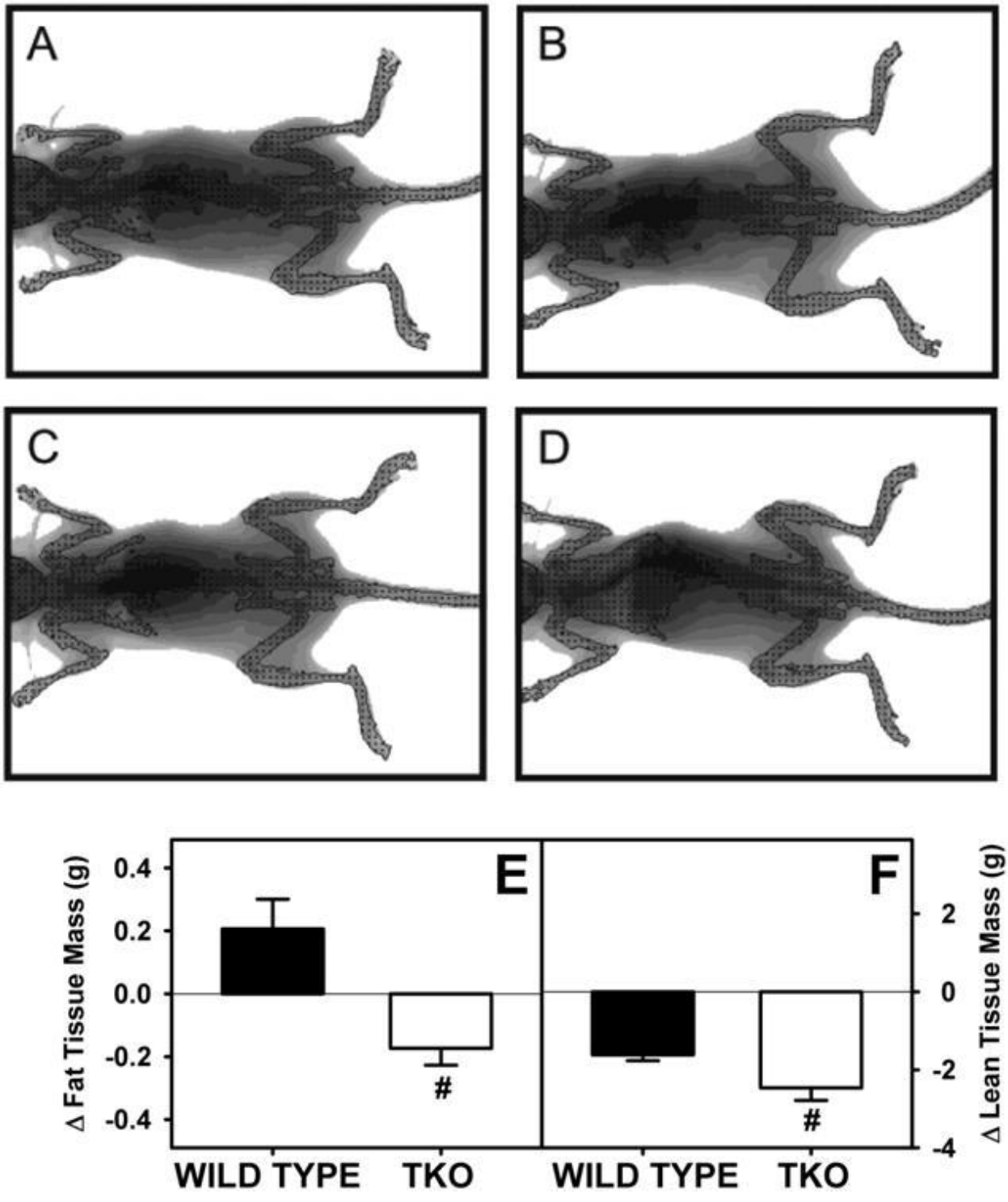
APPENDIX I

CHAPTER III FIGURES

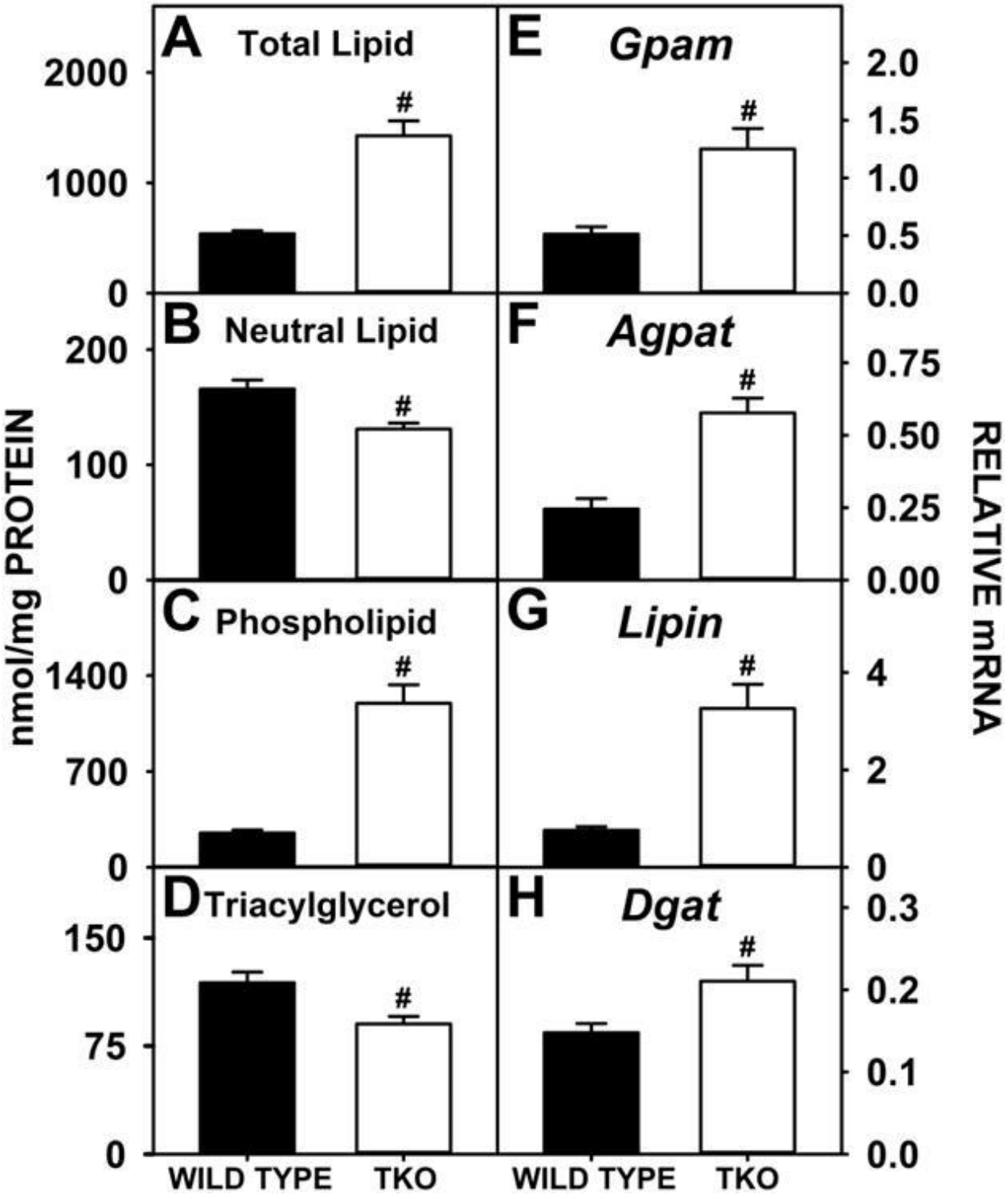
Chapter III Figure 1. Body Weight, Liver Weight and Serum AST and ALT (Milligan, 2017). Percent change in body weight (A), body weight change per energy intake (B), ratio of body weight per gram of liver weight (C), liver weight (D) and serum levels of ALT (E) and AST (F) were determined for wild-type (WT) and L-FABP/SCP2/SCPx gene ablated (TKO) female mice fed a 0.5% phytol diet as described in Experimental Procedures. Means +/- SE; n= 8 animals/group; $^{\#}p < 0.05$ between phytol-fed WT versus phytol-fed TKO mice.


A
BW Change (%)
0
-10
-20
#
B
Liver/Body Weight
0.10
0.08
0.05
0.03
0.00
C
1.5
1.0
0.5
0.0
Liver Weight
D
ALT (Units/L)
150
100
50
0
E
#
400
200
0
AST (Units/L)
WILD TYPE
TKO
WILD TYPE
TKO

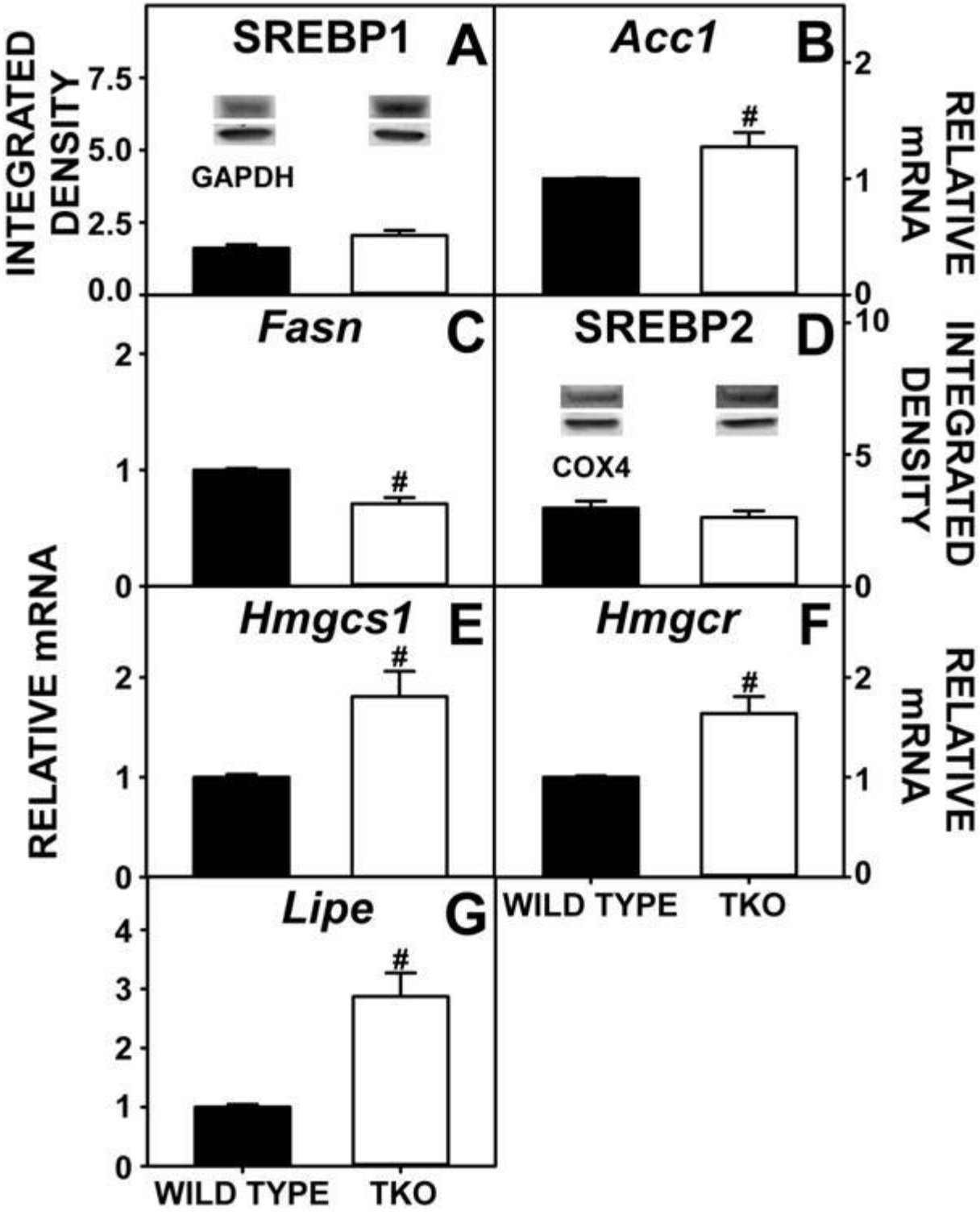
Chapter III Figure 2. Body Composition of Fat Tissue Mass and Lean Tissue Mass (Milligan, 2017). Whole body phenotype of WT and TKO mice fed a 0.5% phytol diet was determined by Lunar PIXImus dual-energy x-ray absorptiometry (DEXA). DEXA resolved whole body fat tissue mass (FTM) and lean tissue mass (LTM). A and B are representative images of control diet fed WT and TKO mice at the end of the study, respectively. C and D are representative images of phytol diet fed WT and TKO mice at the end of the study, respectively. The change in FTM (E) and LTM (F) was determined for 8 mice per group. Means +/- SE; n= 8 animals per group; $^{\#}p < 0.05$ between phytol-fed WT versus phytol-fed TKO mice.


A
B
C
D
E
F
Δ Fat Tissue Mass (g)
0.4
0.2
0.0
-0.2
-0.4
#
WILD TYPE
TKO
2
0
-2
-4
Δ Lean Tissue Mass (g)

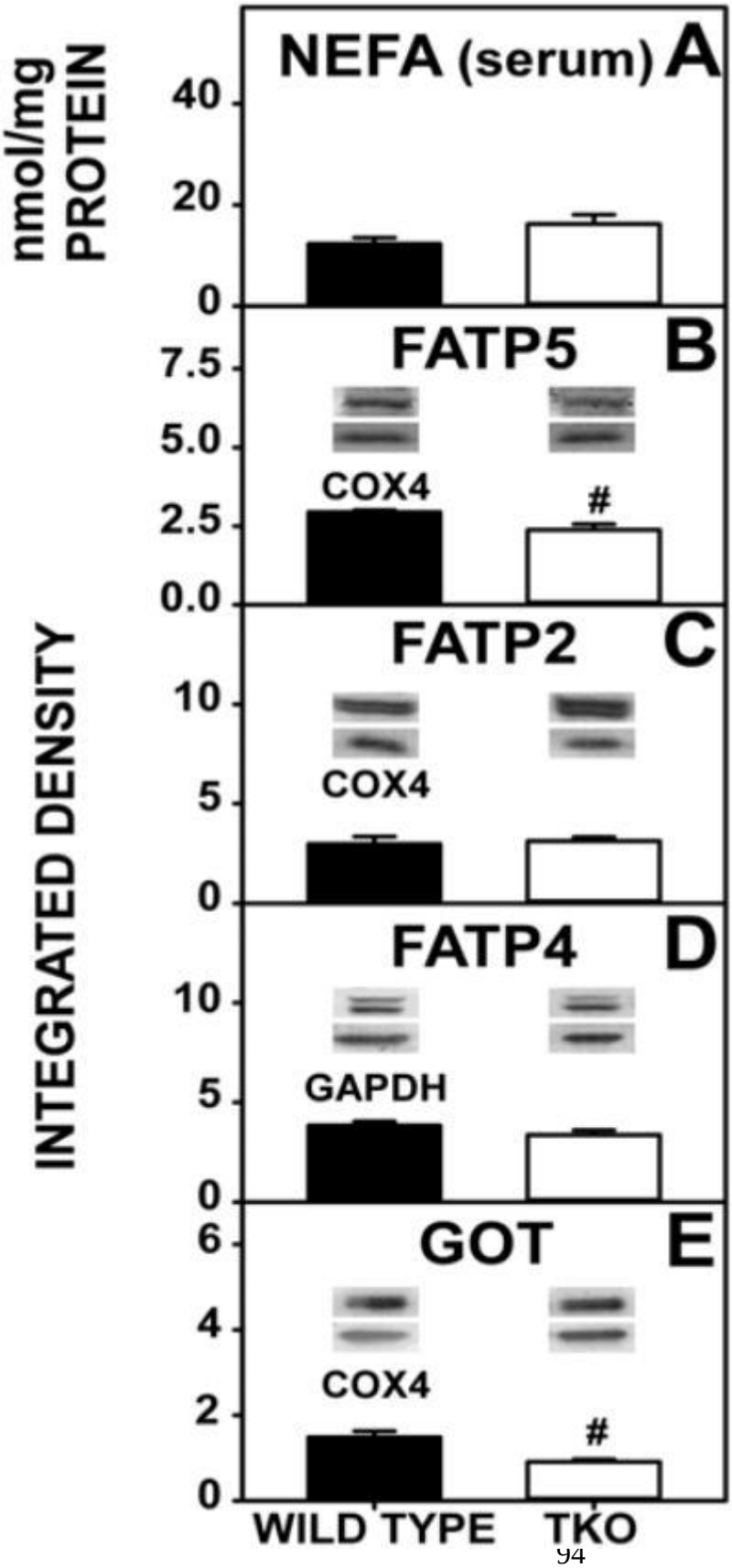
Chapter III Figure 3. Hepatic Lipid Levels and Expression of Key Proteins in Glyceride Synthesis (Milligan, 2017). WT and TKO mice were fed a 0.5% phytol diet as described in Experimental Procedures. Levels of total hepatic lipid (A), hepatic neutral lipid (B), hepatic phospholipid (C) and hepatic triacylglycerol (D) were measured and qRT-PCR was performed to measure the expression of *Gpam* (E), *Agpat2* (F), *Lipin2* (G) and *Dgat2* (H) as described in Methods. Means +/- SE; n= 8 animals per group; $^{\#}p < 0.05$ between phytol-fed WT versus phytol-fed TKO mice.


A Total Lipid
B Neutral Lipid
C Phospholipid
D Triacylglycerol
E Gpam
F Agpat
G Lipin
H Dgat
nmol/mg PROTEIN
RELATIVE mRNA
2000
1000
0
200
100
0
1400
700
0
150
75
0
2.0
1.5
1.0
0.5
0.0
0.75
0.50
0.25
0.00
4
2
0
0.3
0.2
0.1
0.0
#
WILD TYPE
TKO
WILD TYPE
TKO

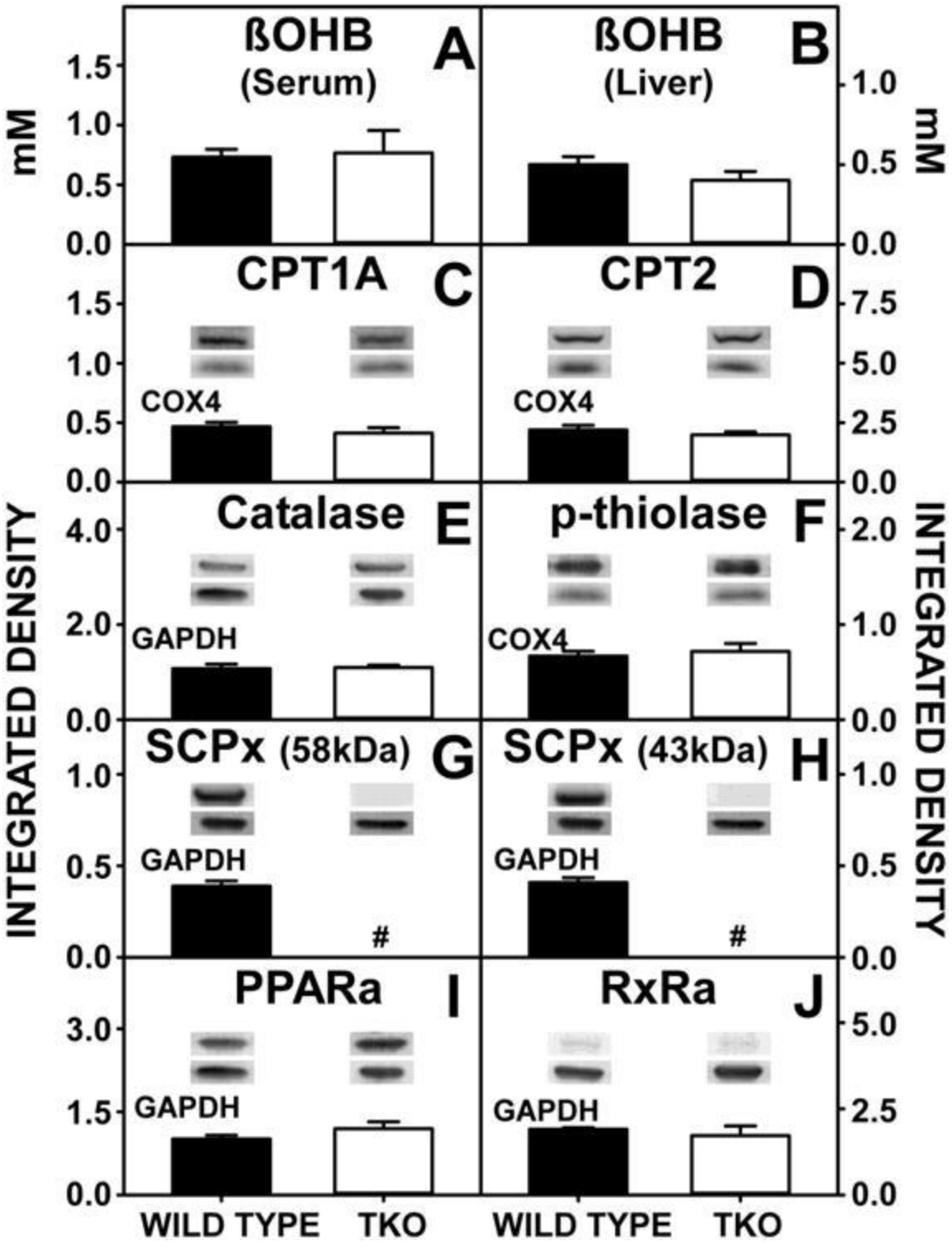
Chapter III Figure 4. Hepatic Expression of SREBPs and Target Genes in *de novo* Fatty Acid and Cholesterol Synthesis (Milligan, 2017). Livers from WT and TKO mice fed a 0.5% diet phytol diet were examined by western blotting as described in Methods to measure relative hepatic protein levels of SREBP1 (A) and SREBP2 (B). The housekeeping gene GADPH or COX4 was used as a loading control to normalize protein expression for SREBP1 and SREBP2, respectively. Inset in Panels A-B show representative western blots of relative protein expression in each mouse group. qRT-PCR was performed to determine relative transcription of SREBP1 target genes Acc1 (C) and Fasn (D) as well as transcription of SREBP2 target genes Hmgcs1 (E) and Hmgcr (F) also as described in Methods. 18S rRNA was used to normalize mRNA expression levels. Means +/- SE; n= 8 animals per group; $^{\#}p < 0.05$ between phytol-fed WT versus phytol-fed TKO mice.


SREBP1
A
INTEGRATED DENSITY
7.5
5.0
2.5
0.0
GAPDH
Acc1
B
RELATIVE mRNA
2
1
0
#
Fasn
C
2
1
0
#
SREBP2
D
INTEGRATED DENSITY
10
5
0
COX4
RELATIVE mRNA
Hmgcs1
E
2
1
0
#
Hmgcr
F
2
1
0
#
RELATIVE mRNA
Lipe
G
4
3
2
1
0
#
WILD TYPE
TKO
WILD TYPE
TKO

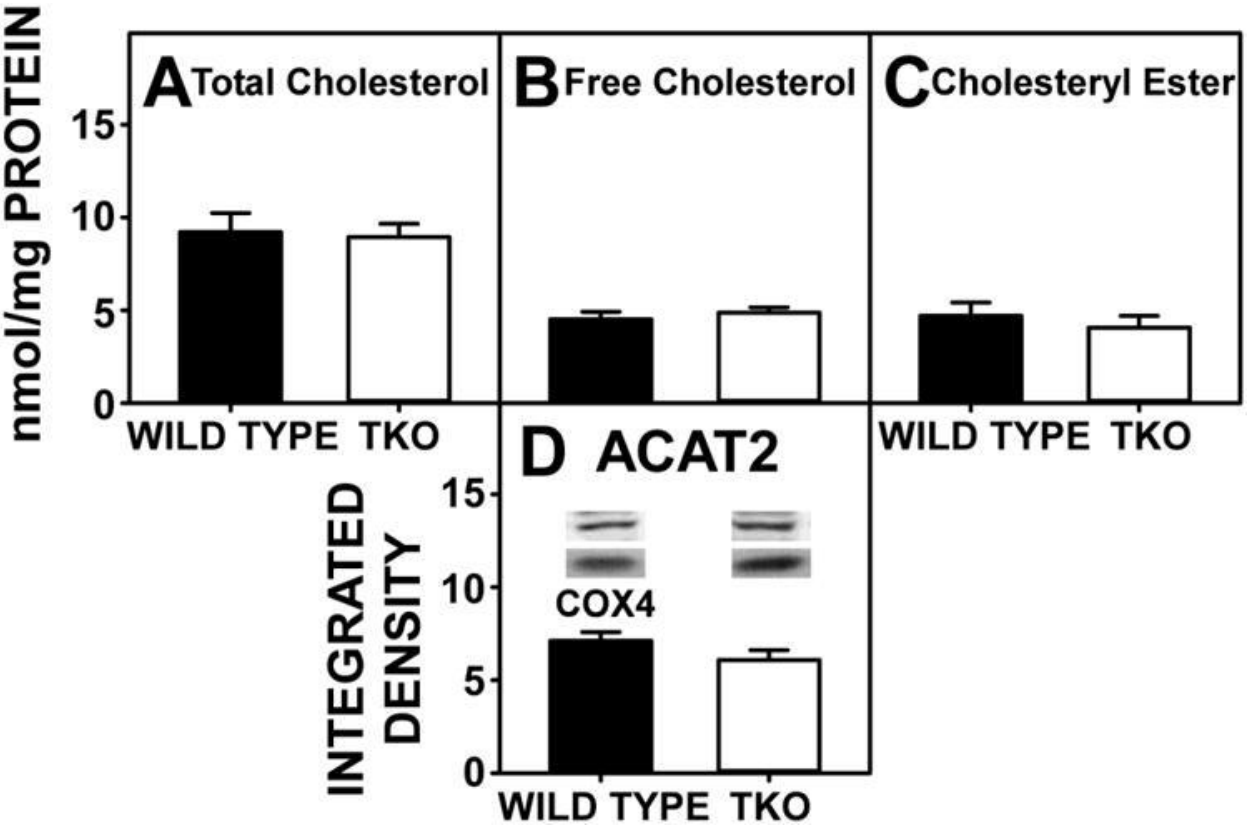
Chapter III Figure 5. Fatty Acid Uptake and Expression of Membrane Fatty Acid Transport Proteins (Milligan, 2017). WT and TKO mice were fed a 0.5% phytol diet as described in Experimental Procedures. Serum NEFA was measured (A) and western blotting was performed to measure the expression of FATP5 (B), FATP2 (C), FATP4 (D), and GOT (E) as described in Methods. The housekeeping gene COX4 was used as a loading control to normalize FATP5, FATP2 and GOT expression. GADPH was used as a loading control to normalize FATP4 expression. Insets in Panels B-E show representative western blots of relative protein expression in each mouse group. Means +/- SE; n= 8 animals per group; $^{\#}p < 0.05$ between phytol-fed WT versus phytol-fed TKO mice.


NEFA (serum)
A
nmol/mg PROTEIN
40
20
0
FATP5
B
7.5
5.0
2.5
0.0
COX4
#
INTEGRATED DENSITY
FATP2
C
10
5
0
COX4
FATP4
D
10
5
0
GAPDH
GOT
E
6
4
2
0
COX4
#
WILD TYPE
TKO

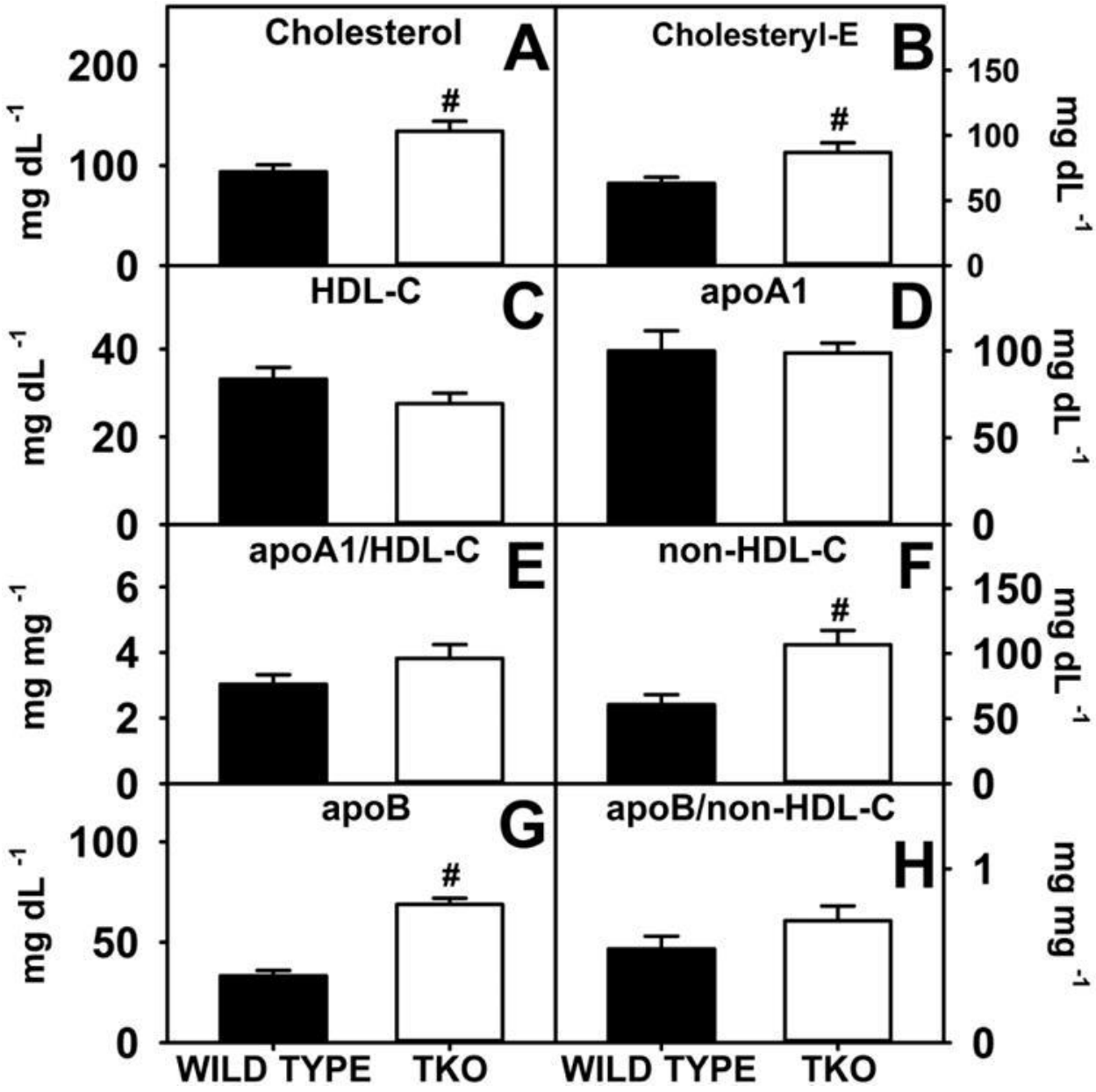
Chapter III Figure 6. Fatty Acid Oxidation (Milligan, 2017). WT and TKO mice were fed a 0.5% phytol diet as described in Experimental Procedures. Serum (A) and liver (B) concentrations of β-hydroxybutyrate (β-OHB) were determined and western blotting was performed to measure the hepatic expression of CPT1A (C), CPT2 (D), catalase (E), p-thiolase (F), SCPx (G and H), PPARα (I) and RxRα (J) as described in Experimental Procedures. The housekeeping gene COX4 was used as a loading control to normalize CPT1A, CPT2 and p-thiolase expression. GADPH was used as a loading control to normalize catalase, SCPx, PPARα and RxRα expression. Inset in Panels C-J show representative western blots of relative protein expression in each mouse group. Means +/- SE; n= 8 animals per group; $^{\#}p < 0.05$ between phytol-fed WT versus phytol-fed TKO mice.


ßOHB
(Serum)
A
ßOHB
(Liver)
B
mM
mM
CPT1A
C
CPT2
D
COX4
COX4
Catalase
E
p-thiolase
F
GAPDH
COX4
INTEGRATED DENSITY
INTEGRATED DENSITY
SCPx (58kDa)
G
SCPx (43kDa)
H
GAPDH
GAPDH
#
#
PPARa
I
RxRa
J
GAPDH
GAPDH
WILD TYPE
TKO
WILD TYPE
TKO

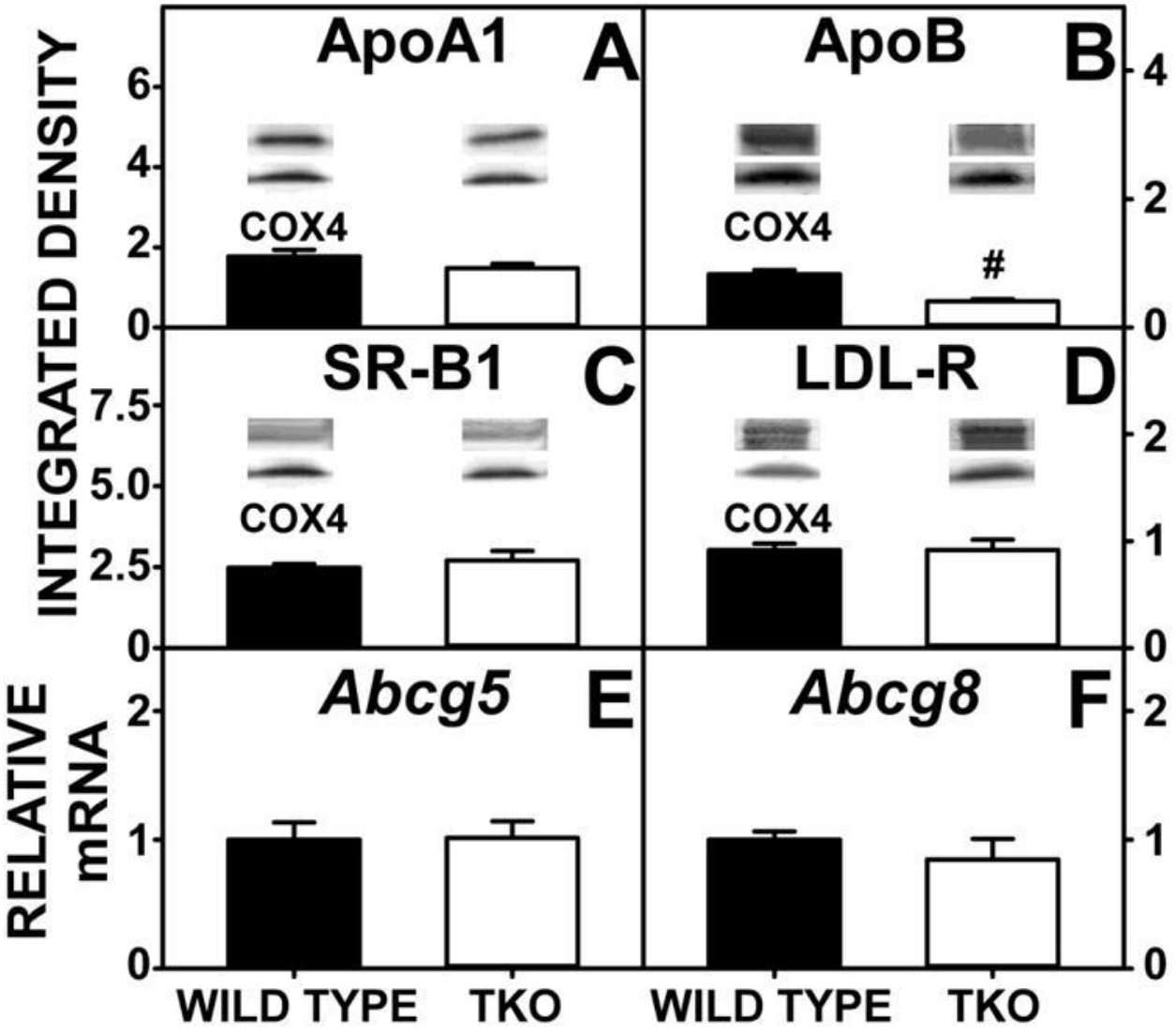
Chapter III Figure 7. Expression of Proteins in the Cholesteryl Ester Synthesis/Hydrolysis Cycle (Milligan, 2017). WT and TKO mice were fed a 0.5% phytol diet as described in Experimental Procedures. Levels of total hepatic cholesterol (A), hepatic free cholesterol (B), and hepatic cholesteryl ester (C) and were assayed; western blots were performed to measure the expression of ACAT2 as described in Experimental Procedures. The housekeeping gene COX4 was used as a loading control to normalize protein expression. Inset in Panel D shows representative western blotting of relative protein expression in each mouse group. qRT-PCR was performed to determine relative transcription of the *Lipe* gene (E) as described in Methods. 18S rRNA was used to normalize mRNA expression levels. Means +/- SE; n= 8 animals per group; $^{\#}p < 0.05$ between phytol-fed WT versus phytol-fed TKO mice.



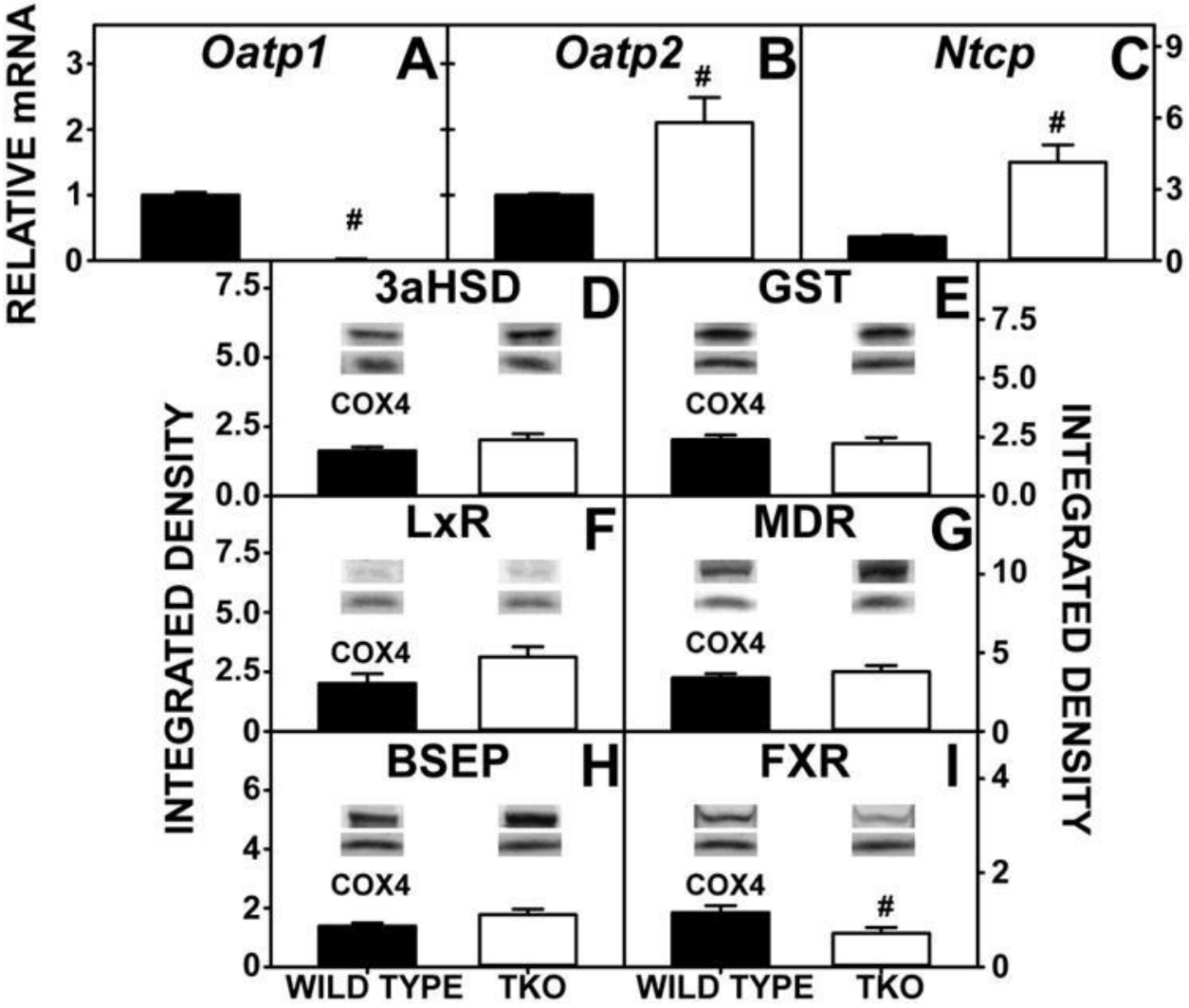
Chapter III Figure 8. Serum Lipid and Lipoprotein Levels (Milligan, 2017). WT and TKO mice were fed a 0.5% phytol diet as described in Experimental Procedures. Serum levels of free cholesterol (A), cholesteryl ester (B), HDL cholesterol (C), apoA1 (D), non-HDL-C (F) and apoB (G) were measured and the apoA1/HDL-C (E) and apoB/non-HDL-C (H) ratios were calculated as described in Experimental Procedures. Means +/- SE; n= 8 animals per group; $^{\#}p < 0.05$ between phytol-fed WT versus phytol-fed TKO mice.


Cholesterol
A
mg dL -1
200
100
0
#
Cholesteryl-E
B
150
100
50
0
mg dL -1
#
HDL-C
C
mg dL -1
40
20
0
apoA1
D
100
50
0
mg dL -1
apoA1/HDL-C
E
mg mg -1
6
4
2
0
non-HDL-C
F
150
100
50
0
mg dL -1
#
apoB
G
mg dL -1
100
50
0
#
apoB/non-HDL-C
H
1
0
mg mg -1
WILD TYPE
TKO
WILD TYPE
TKO

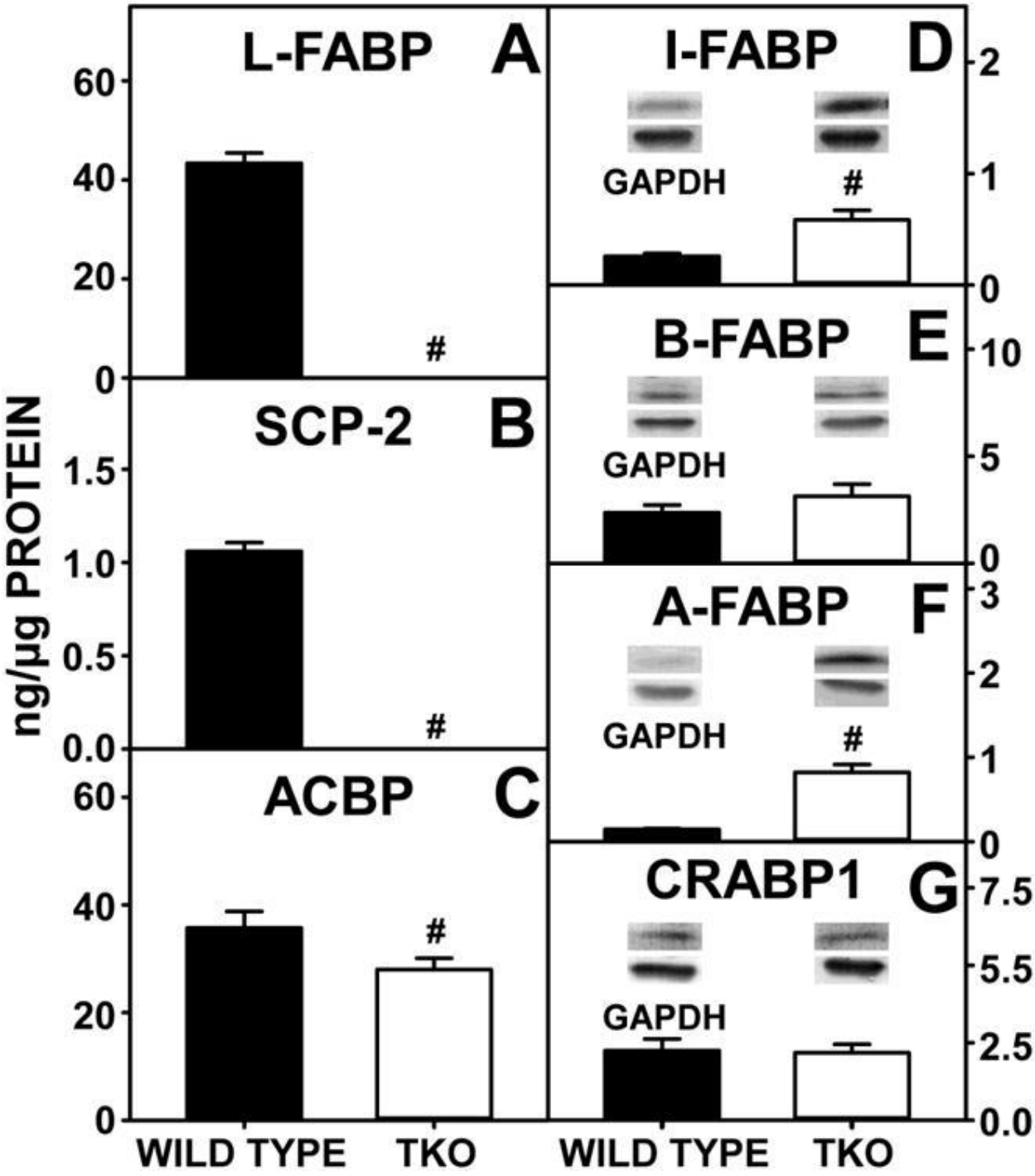
Chapter III Figure 9. Expression of Hepatic Proteins Involved in Cholesterol Uptake, Efflux and Biliary Secretion (Milligan, 2017). WT and TKO mice were fed a 0.5% phytol diet as described in Experimental Procedures. Levels of hepatic apoA1 (A), apoB (B), SR-B1 (C) and LDL-R (D were determined by western blotting as described in Methods. The housekeeping gene COX4 was used as a loading control to normalize protein expression. Inset in Panels A-D show representative western blots of relative protein expression in each mouse group. qRT-PCR was performed to determine relative transcription of *Abcg5* (E) and *Abcg8* (F) as described in Methods. 18S rRNA was used to normalize mRNA expression levels. Means +/- SE; n= 8 animals per group; $^{\#}p < 0.05$ between phytol-fed WT versus phytol-fed TKO.


ApoA1
A
ApoB
B
SR-B1
C
LDL-R
D
Abcg5
E
Abcg8
F
COX4
#
INTEGRATED DENSITY
RELATIVE mRNA
WILD TYPE
TKO
WILD TYPE
TKO

Chapter III Figure 10. Hepatic Expression of Proteins for Bile Acid Transport (Milligan, 2017). WT and TKO mice were fed a 0.5% phytol diet as described inMethods. qRT-PCR was performed to measure the expression of *Oatp1* (A), *Oatp2* (B) and *Ntcp* (C). 18S rRNA was used to normalize mRNA expression levels. Western blotting was performed to measure the expression of 3αHSD (D), GST (E), LxR (F), MDR (G), BSEP (H), and FxR (I). The housekeeping gene COX4 was used as a loading control to normalize protein expression. Inset in Panels D-I show representative western blots of relative protein expression in each mouse group. Means +/- SE; n= 8 animals per group; $^{\#}p < 0.05$ between phytol-fed WT versus phytol-fed TKO mice.



Chapter III Figure 11. Lipid Binding and Transport Proteins (Milligan, 2017). WT and TKO mice were fed a 0.5% phytol diet as described in Methods. Quantitative western blotting was performed to measure the expression of L-FABP (A), SCP2 (B) and ACBP (C) as described in Methods. Additional western blotting was performed to measure the relative expression of I-FABP (D), B-FABP (E), A-FABP (F) and CRABP-1 (G). The housekeeping gene GADPH was used as a loading control to normalize protein expression. Inset in Panels D-G show representative western blots of relative protein expression in each mouse group. Means +/- SE; n= 8 animals per group; $^{\#}p < 0.05$ between phytol-fed WT versus phytol-fed TKO mice.


L-FABP
A
60
40
20
0
#
SCP-2
B
1.5
1.0
0.5
0.0
#
ACBP
C
60
40
20
0
#
ng/µg PROTEIN
WILD TYPE
TKO
I-FABP
D
GAPDH
#
2
1
0
B-FABP
E
GAPDH
10
5
0
A-FABP
F
GAPDH
#
3
2
1
0
CRABP1
G
GAPDH
7.5
5.5
2.5
0.0
WILD TYPE
TKO

References

1. Milligan S, Martin GG, Landrock D, et al. Impact of dietary phytol on lipid metabolism in SCP2/SCPX/L-FABP null mice. *Biochim Biophys Acta*. 2017;1862(3):291-304. doi: S1388-1981(16)30328-6 [pii].

APPENDIX II

CHAPTER IV TABLES AND FIGURES

Chapter IV Table 1. Effect of *Fabp1/Scp2/Scpx* gene ablation and sex on food consumption and body weight in mice fed a high fat diet (Milligan, 2018). Male and female WT and TKO mice on a C57BL/6NCr background were pair-fed a HFD. Total food consumed, average daily food consumption, total body weight (BW) gain, and total body weight (BW) gain/total food consumed were obtained as described in Methods. Whole body fat tissue mass (FTM) and lean tissue mass (LTM) were determined by Lunar PIXImus dual-energy x-ray absorptiometry (DEXA) as described in Methods. WT: wild type; TKO: triple knock out; CO: control diet, HF: high fat diet. Values represent the mean ± SEM, n=6-8. *p <0.05 for TKO vs. WT within the same diet and sex; ^p<0.5 for control diet vs high fat diet within the same genotype and sex; #p <0.05 for male vs. female within the same genotype and diet.

	Male WT		**Male TKO**		**Female WT**		**Female TKO**	
	CO	**HF**	**CO**	**HF**	**CO**	**HF**	**CO**	**HF**
Total Food Consumed (kcal)	1039 ± 27	1143 ± 19^	1021 ± 28	1174 ± 11^	943 ± 15#	1056 ± 17^#	999 ± 26	1085 ± 12^#
Average Daily Food Consumption (kcal/day)	11.8 ± 0.31	13.0 ± 0.22^	11.6 ± 0.32	13.3 ± 0.12^	10.7 ± 0.17#	11.4 ± 0.29^#	12.0 ± 0.19*	12.3 ± 0.13#

Total Food Consumed (%BW)	11.3 ± 0.188	9.3 ± 0.204^	11.6 ± 0.156	9.4 ± 0.147^	14.0 ± 0.197*	12.3 ± 0.353*^	14.1 ± 0.331*	11.8 ± 0.382*^
Total BW Gain (g)	11.0 ± 0.854	15.6 ± 1.351^	8.1 ± 1.407	14.6 ± 1.001^	4.8 ± 0.428#	6.5 ± 0.586#	7.4 ± 0.749	8.3 ± 0.527#
Total BW Gain /Total Food Consumed (g/kcal)	0.0106 ± 0.0006	0.0137 ± 0.001^	0.0078 ± 0.0012*	0.0125 ± 0.0009^	0.0052 ± 0.0005#	0.0063 ± 0.0006#	0.0074 ± 0.0007	0.0077 ± 0.0005#
Total LTM Change (g)	3.5 ± 0.6	3.9 ± 0.4	2.4 ± 0.9	2.9 ± 0.8	3.0 ± 0.3	3.3 ± 0.3	3.0 ± 0.4	3.9 ± 0.4
Total FTM Change (g)	6.3 ± 0.5	11 ± 1^	4 ± 1*	10.3 ± 0.6^	0.6 ± 0.2#	3.3 ± 0.7^#	1.3 ± 0.4#	2.1 ± 0.7#

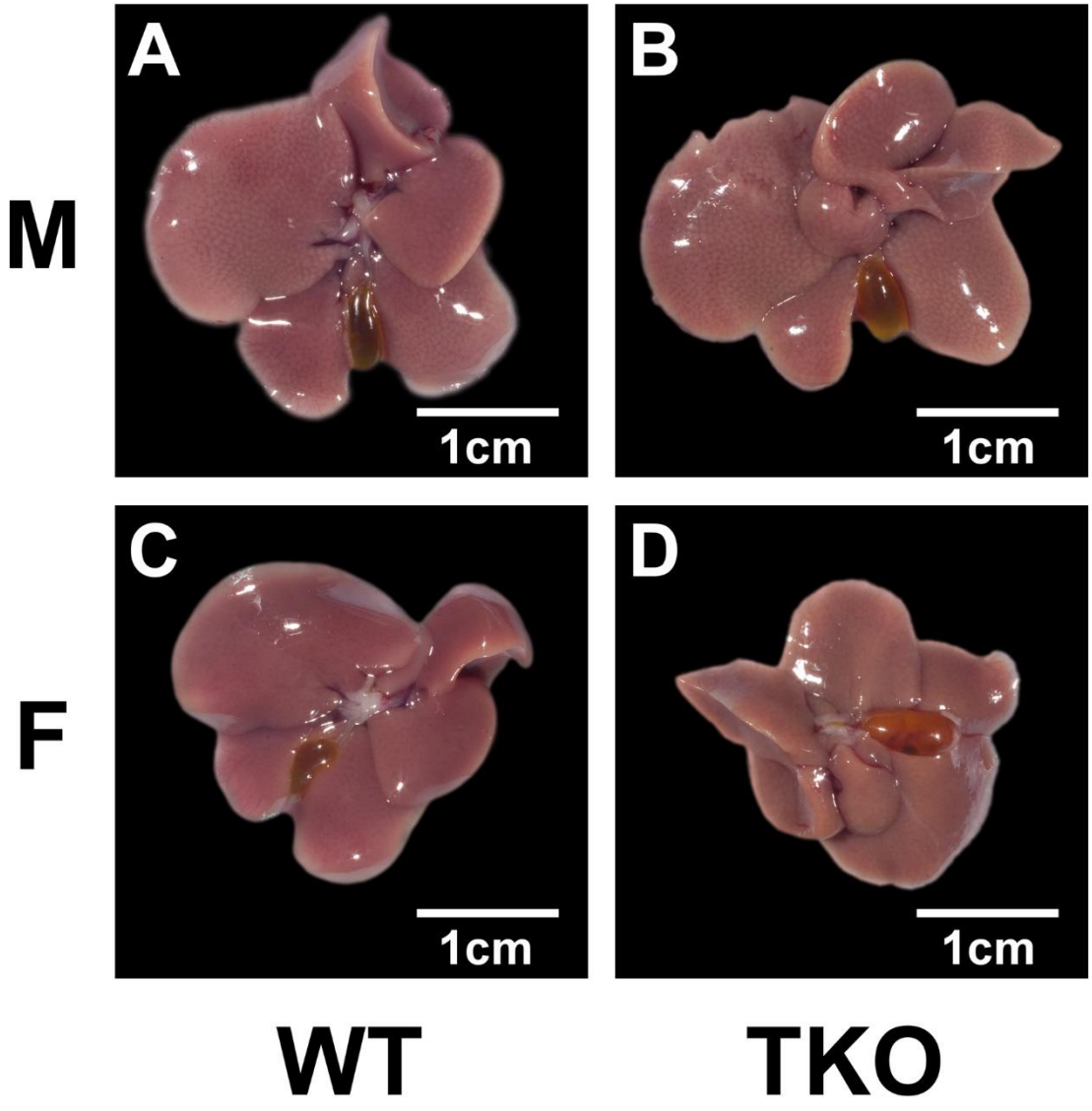
Chapter IV Table 2. Effect of *Fabp1/Scp2/Scpx* gene ablation and sex on liver weight in mice fed a high fat diet (Milligan, 2018). Male and female WT and TKO mice on a C57BL/6NCr background were pair-fed a HFD. Final liver weight, liver weight/body weight (BW) and total liver protein were obtained as described in Methods. WT: wild type; TKO: triple knock out; CO: control diet, HF: high fat diet. Values represent the mean ± SEM, n=6-8. *p <0.05 for TKO vs. WT within the same diet; ^p<0.5 for control diet vs high fat diet within the same genotype; #p <0.05 for male vs. female within the same genotype.

	Male WT		**Male TKO**		**Female WT**		**Female TKO**	
	CO	**HF**	**CO**	**HF**	**CO**	**HF**	**CO**	**HF**
Final Liver Weight (g)	1.30 ± 0.09	1.16 ± 0.08	1.19 ± 0.1	1.24 ± 0.07	0.9 ± 0.02#	0.76 ± 0.02#	1.01 ± 0.06	0.81 ± 0.02#
Final Liver Weight/BW (g/g)	0.041 ± 0.001	0.031 ± 0.001^	0.041 ± 0.002	0.034 ± 0.001^	0.042 ± 0.001^	0.033 ± 0.001	0.044 ± 0.001	0.034 ± 0.002^
Final Total Liver Protein (mg/g)	192 ± 14	165 ± 15	147 ± 12	207 ± 15*^	154 ± 7	151 ± 7	143 ± 8	154 ± 14#

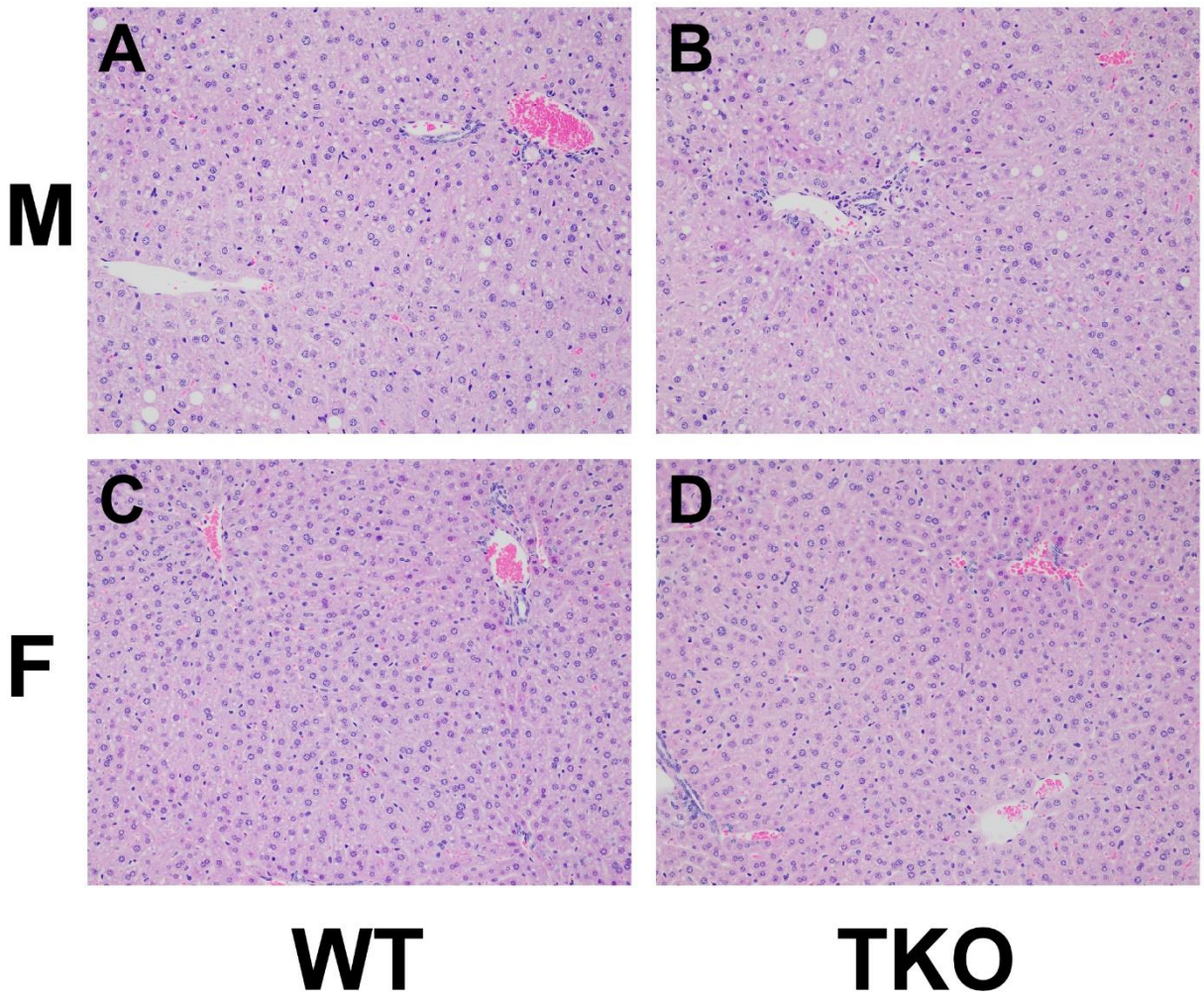
Chapter IV Table 3. Effect of *Fabp1/Scp2/Scpx* gene ablation and sex on AST and ALT in mice fed a high fat diet (Milligan, 2018). Male and female WT and TKO mice on a C57BL/6NCr background were pair-fed a HFD. Final serum AST and ALT were obtained as described in Methods. WT: wild type; TKO: triple knock out. Values represent the mean ± SEM, n=7. *p <0.05 for TKO vs. WT; #p <0.05 for Male vs. Female.

	Male WT	**Male TKO**	**Female WT**	**Female TKO**
AST IU/L	**31 ± 4**	**118 ± 9***	**42 ± 6**	**79 ± 5#***
ALT IU/L	**35 ± 4**	**104 ± 7***	**27 ± 3**	**100 ± 10***

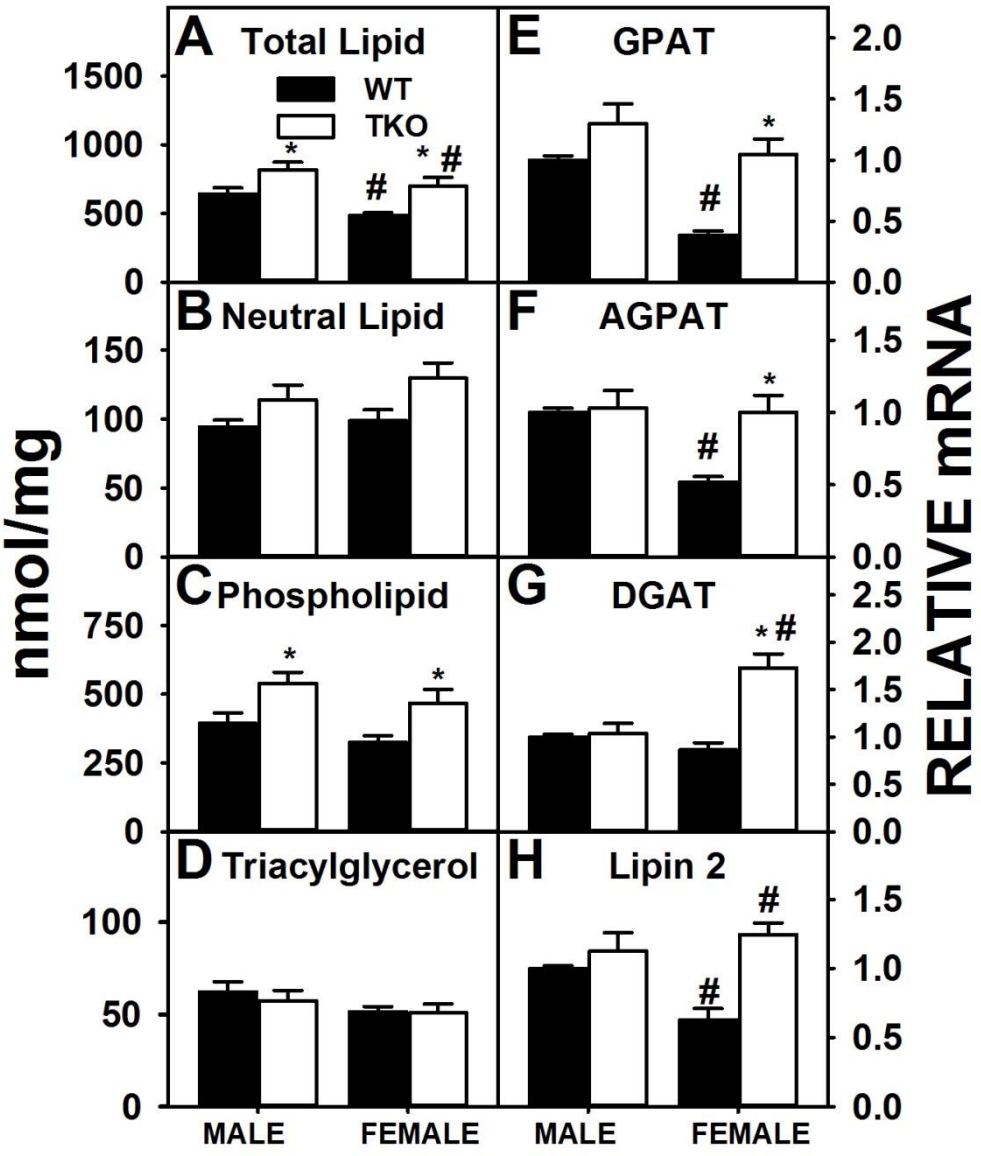
Chapter IV Figure 1. Effect of *Fabp1/Scp2/Scpx* gene ablation and sex on gross liver pathology in high fat fed mice (Milligan, 2018). Male and female WT and TKO mice on a C57BL/6NCr background were pair-fed a HFD. Livers were collected and processed as described in Methods.



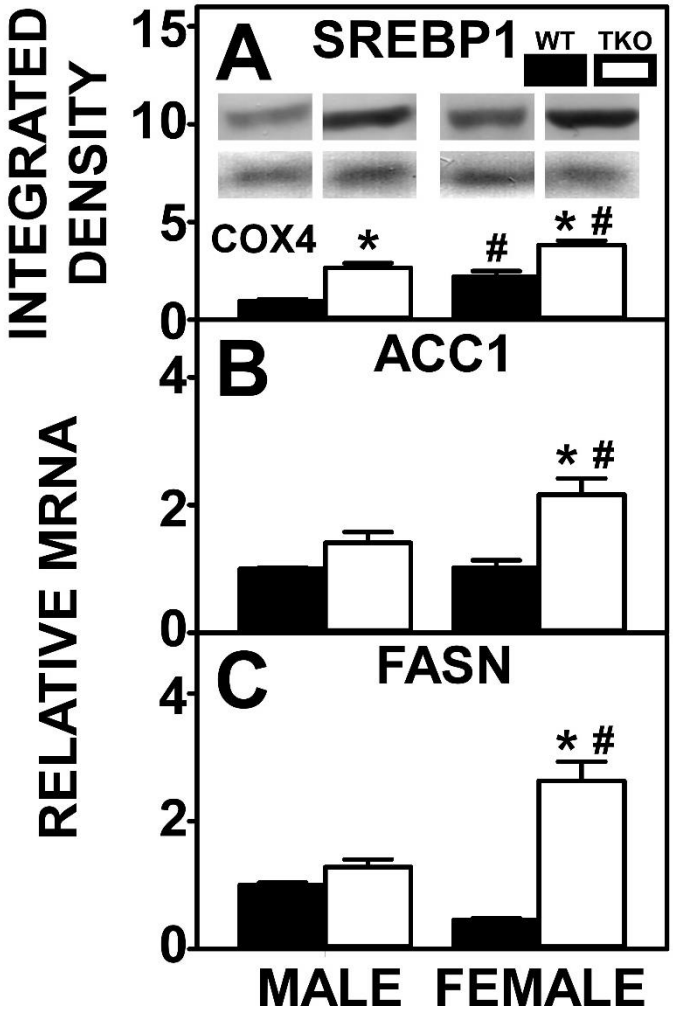
Chapter IV Figure 2. Effects of *Fabp1/Scp2/Scpx* gene ablation and sex on histological liver pathology in high fat fed mice (Milligan, 2018). Male and female WT and TKO mice on a C57BL/6NCr background were pair-fed a HFD. Livers were collected and processed as described in Methods.



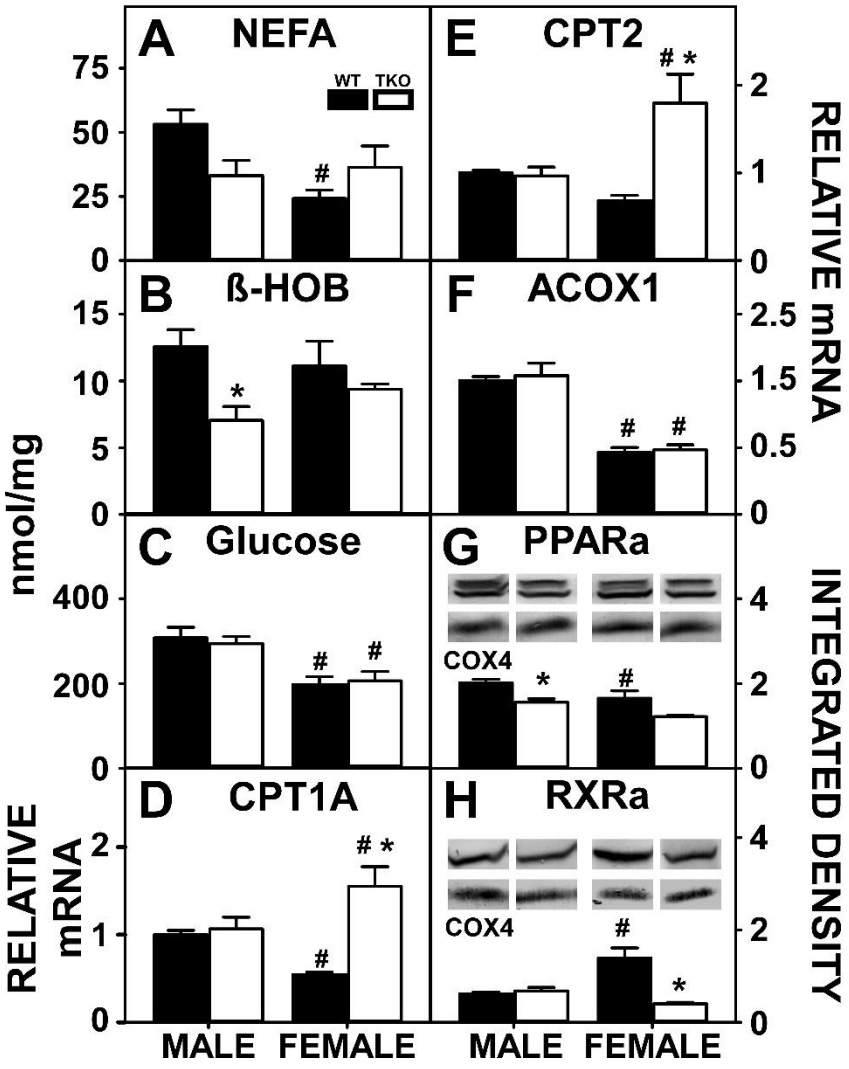
Chapter IV Figure 3. Effects of *Fabp1/Scp2/Scpx* gene ablation and sex on hepatic accumulation of lipids in high fat fed mice (Milligan, 2018). Male and female WT and TKO mice on a C57BL/6NCr background were pair-fed a HFD. Levels of total hepatic lipid (A), hepatic neutral lipid (B), hepatic phospholipid (C) and hepatic triacylglycerol (D) were measured and qRT-PCR was performed to measure the expression of GPAT (Gpam gene) (E), AGPAT (Agpat2 gene) (F), DGAT (Dgat2 gene) (H) and Lipin 2 (Lpin2 gene) (H) as described in Methods. Values represent the mean ± SEM, n=8. *p <0.05 for TKO vs. WT. #p <0.05 for Male vs. Female.


A Total Lipid
WT
TKO
B Neutral Lipid
C Phospholipid
D Triacylglycerol
E GPAT
F AGPAT
G DGAT
H Lipin 2
nmol/mg
RELATIVE mRNA
MALE
FEMALE
MALE
FEMALE

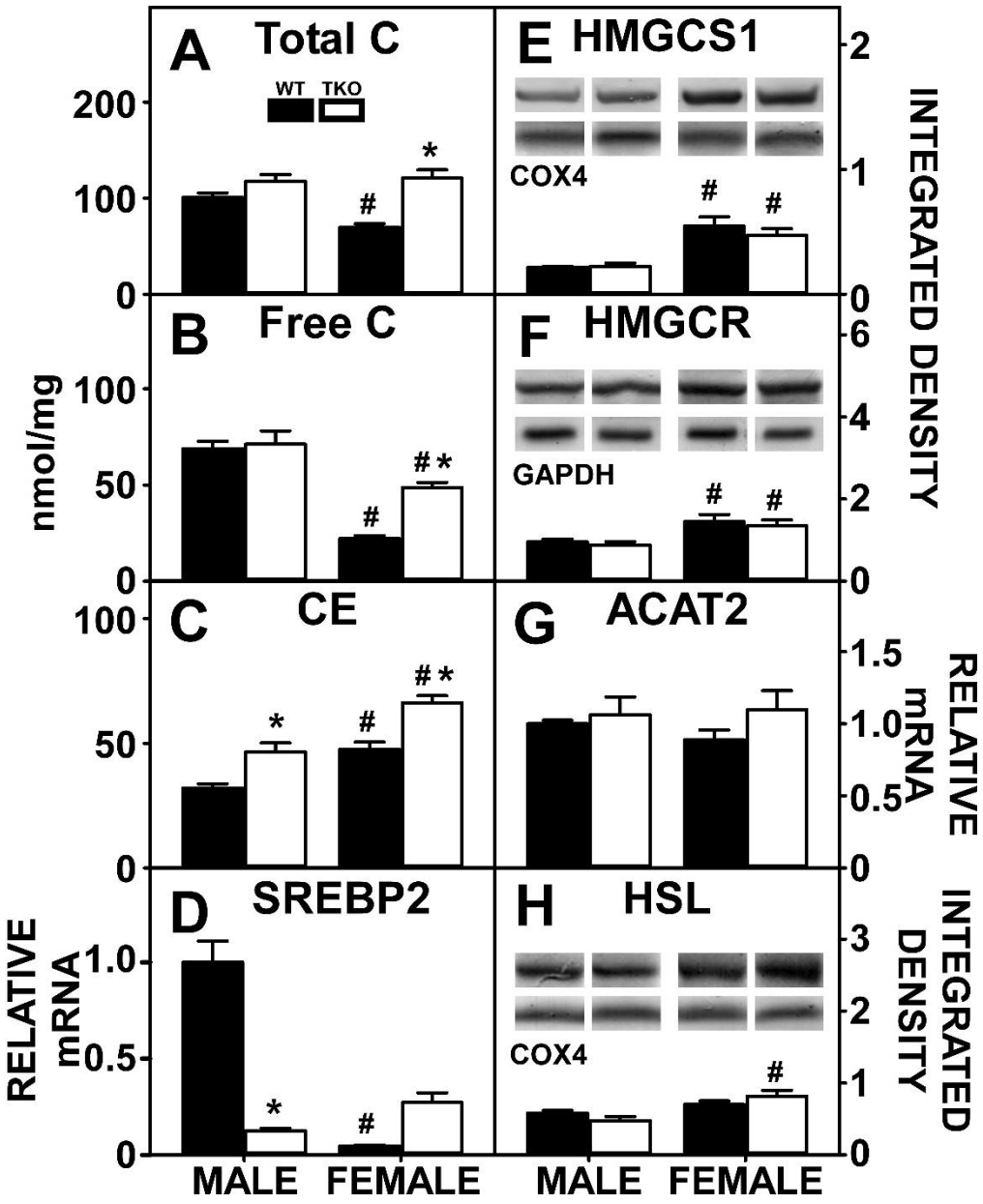
Chapter IV Figure 4. Effects of *Fabp1/Scp2/Scpx* gene ablation and sex on hepatic expression of key proteins in lipid synthesis in high fat fed mice (Milligan, 2018). Livers from male and female WT and TKO mice on a C57BL/6NCr background pair-fed a HFD were examined by western blot to measure relative hepatic protein levels of SREBP1 (A) as described in Methods. The housekeeping gene COX4 was used as a loading control to normalize protein expression of SREBP1. The inset in panel A shows representative western blots of relative protein expression in each mouse group. qRT-PCR was performed to determine relative transcription of the SREBP1 target genes ACC1 (Acaca gene) (B) and FASN (Fasn gene) (C) as described in Methods. Values represent the mean ± SEM, n=8. *p <0.05 for TKO vs. WT. #p <0.05 for Male vs. Female.


INTEGRATED DENSITY
15
10
5
0
A
SREBP1
WT
TKO
COX4
*
#
* #
B
ACC1
RELATIVE MRNA
4
2
0
* #
C
FASN
4
2
0
* #
MALE
FEMALE

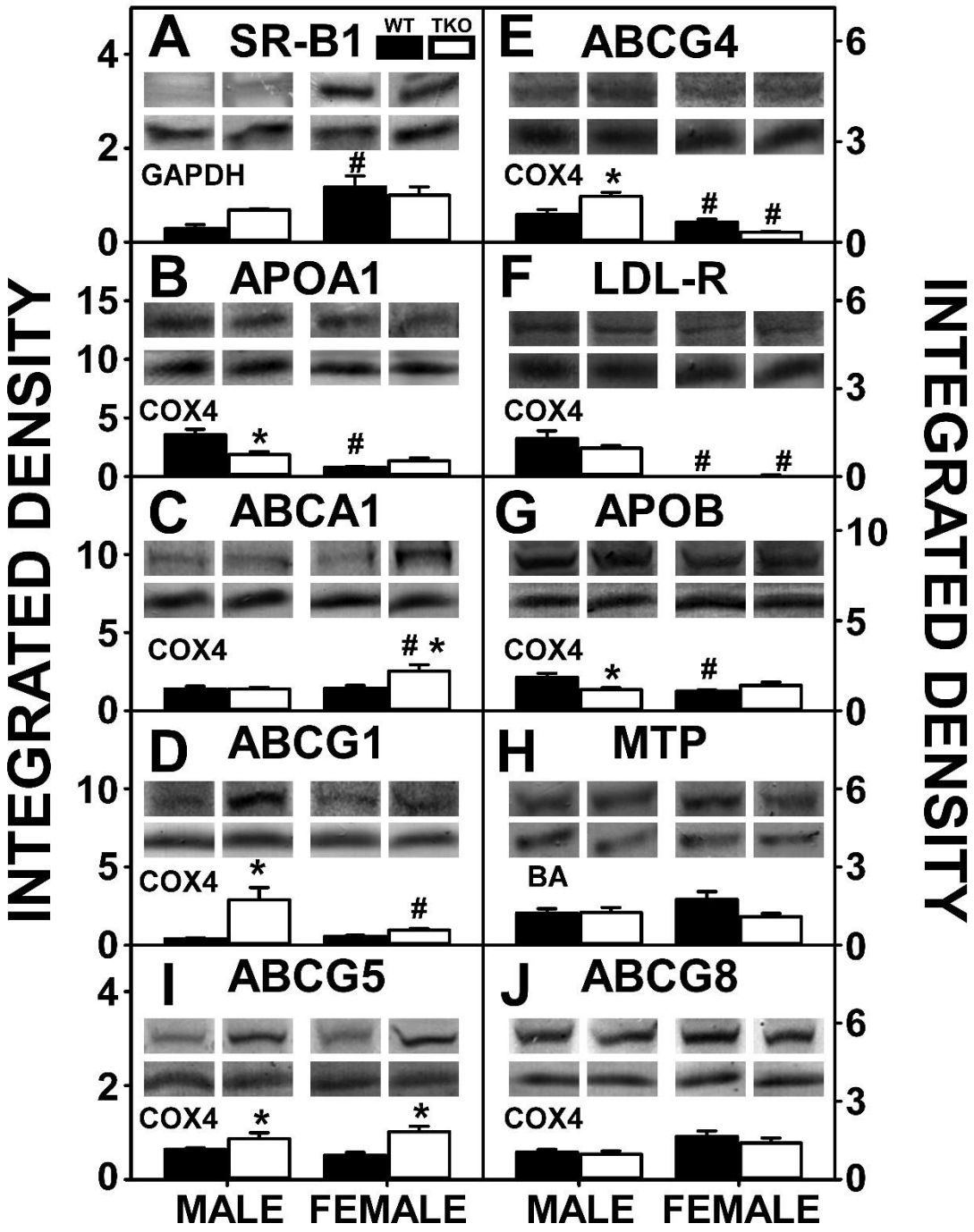
Chapter IV Figure 5. Effects of *Fabp1/Scp2/Scpx* gene ablation and sex on hepatic fatty acid oxidation high fat fed mice (Milligan, 2018). Male and female WT and TKO mice on a C57BL/6NCr background were pair-fed a HFD. NEFA (A), βHOB (B) and glucose (C) were measured as described in Methods. qRT-PCR was performed to determine the relative transcription of the lipid oxidative enzymes *Cpt1a* (D), *Cpt2* (E) and *Acox1* (F) as described in Methods. The nuclear receptors PPARα (G) and RXR (H) were examined by western blot to measure relative hepatic protein levels as described in Methods. The housekeeping gene COX4 was used as a loading control to normalize protein expression of PPARα and RXR. The inset in panels G-H show representative western blots of relative protein expression in each mouse group. Values represent the mean ± SEM, n=8. *p <0.05 for TKO vs. WT. #p <0.05 for Male vs. Female.


A NEFA
WT TKO
E CPT2
B ß-HOB
F ACOX1
C Glucose
G PPARa
COX4
D CPT1A
H RXRa
COX4
nmol/mg
RELATIVE mRNA
RELATIVE mRNA
INTEGRATED DENSITY
MALE FEMALE MALE FEMALE

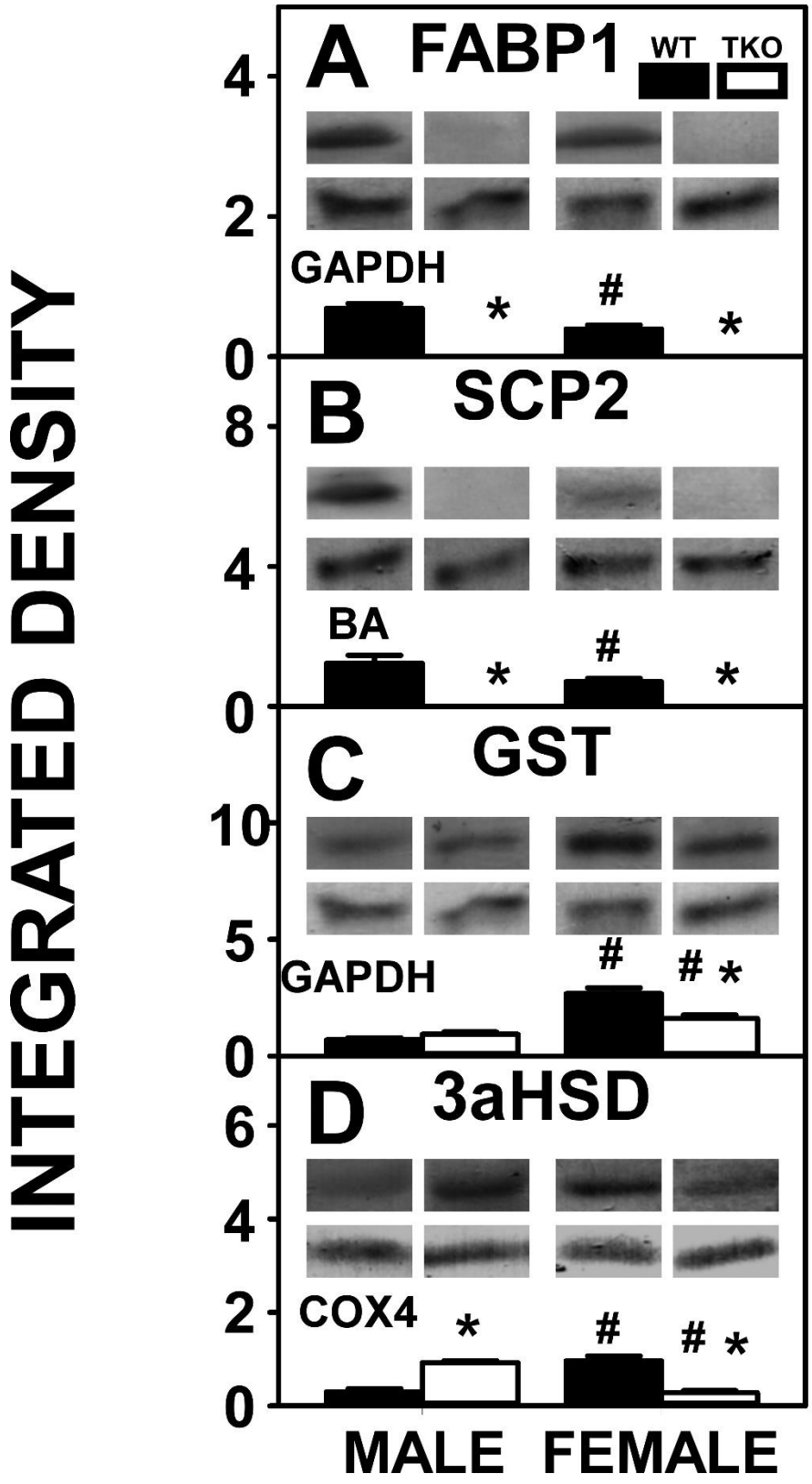
Chapter IV Figure 6. Effects of *Fabp1/Scp2/Scpx* gene ablation and sex on hepatic cholesterol accumulation in high fat fed mice (Milligan, 2018). Male and female WT and TKO mice on a C57BL/6NCr background were pair-fed a HFD. Total C (A), free C (B) and cholesteryl ester (C) were measured as described in Methods. qRT-PCR was performed to determine the relative transcription of SREBP2 *(Srebf2* gene*)* (D) and ACAT2 (*Soat2* gene) (G) as described in Methods. Western blots were performed to measure relative hepatic protein levels of HMGCS1 (E), HMGCR (F) and HSL (H) as described in Methods. The housekeeping gene COX4 was used as a loading control to normalize protein expression of HMGCS1 and HSL and GAPDH was used to normalize HMGCR. The inset in panels E, F and H show representative western blots of relative protein expression in each mouse group. Values represent the mean ± SEM, n=8. *p <0.05 for TKO vs. WT. #p <0.05 for Male vs. Female.


A
Total C
WT
TKO
200
100
0
*
#
E
HMGCS1
COX4
#
#
2
1
0
INTEGRATED DENSITY
B
Free C
nmol/mg
100
50
0
#
#*
F
HMGCR
GAPDH
#
#
6
4
2
0
C
CE
100
50
0
*
#
#*
G
ACAT2
1.5
1.0
0.5
0
RELATIVE mRNA
D
SREBP2
RELATIVE mRNA
1.0
0.5
0
*
#
H
HSL
COX4
#
3
2
1
0
INTEGRATED DENSITY
MALE
FEMALE
MALE
FEMALE

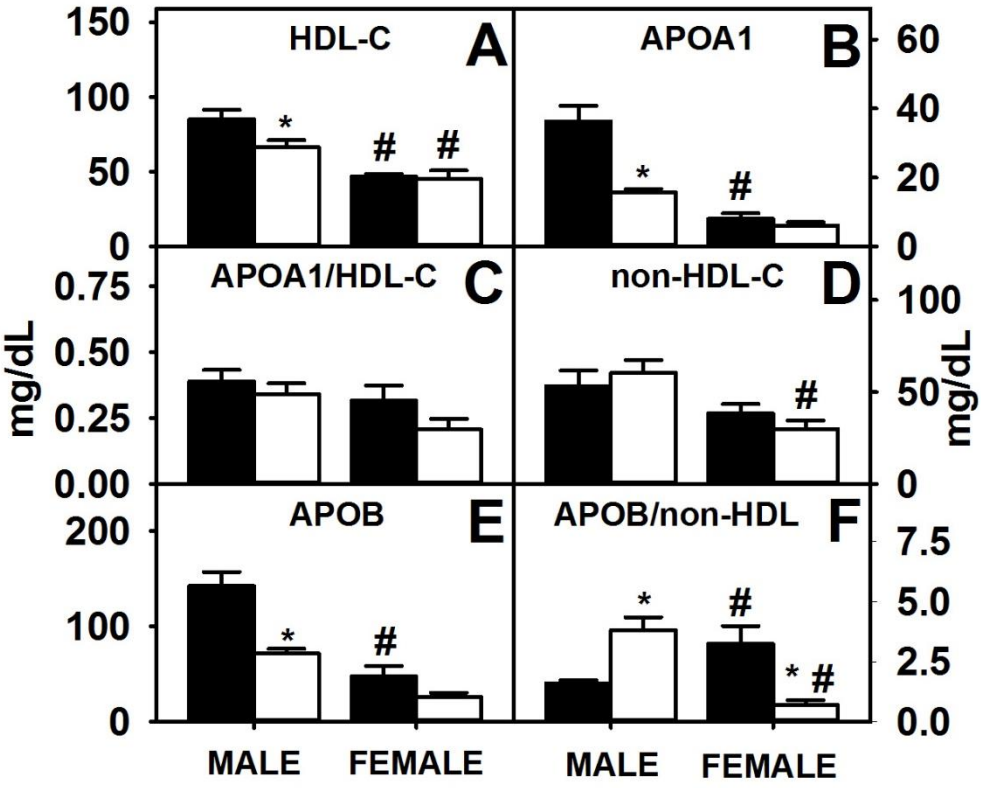
Chapter IV Figure 7. Effects of *Fabp1/Scp2/Scpx* gene ablation and sex on hepatic expression of key proteins in hepatic uptake and efflux/secretion of cholesterol in high fat fed mice (Milligan, 2018). Livers from male and female WT and TKO mice on a C57BL/6NCr background pair-fed a HFD were examined by western blot to measure relative hepatic protein levels of SR-B1 (A), APOA1 (B), ABCA1 (C), ABCG1 (D), ABCG5 (I), ABCG4 (E), LDL-R (F), APOB (G), MTP (H) and ABCG8 (J) as described in Methods. The housekeeping gene GAPDH was used as a loading control to normalize protein expression of SR-B1, COX4 was used to normalize APOA1, ABCA1, ABCG1, ABCG5, ABCG4, LDL-R, APOB and ABCG8 and BA was used to normalize MTP. The insets in panels A-J show representative western blots of relative protein expression in each mouse group. Values represent the mean ± SEM, n=8. *p <0.05 for TKO vs. WT. #p <0.05 for Male vs. Female.


A SR-B1
WT
TKO
GAPDH
B APOA1
COX4
C ABCA1
COX4
D ABCG1
COX4
I ABCG5
COX4
E ABCG4
COX4
F LDL-R
COX4
G APOB
COX4
H MTP
BA
J ABCG8
COX4
INTEGRATED DENSITY
INTEGRATED DENSITY
MALE
FEMALE
MALE
FEMALE

Chapter IV Figure 8. Effects of *Fabp1/Scp2/Scpx* gene ablation and sex on hepatic expression of intracellular proteins involved in cytosolic transport of cholesterol in high fat fed mice (Milligan, 2018). Livers from male and female WT and TKO mice on a C57BL/6NCr background pair-fed a HFD were examined by western blot to measure relative hepatic protein levels of FABP1 (A), SCP2 (B), GST (C) and 3αHSD (D) as described in Methods. The housekeeping gene GAPDH was used as a loading control to normalize protein expression of FABP1 and GST, BA was used to normalize SCP2 and COX4 was used to normalize 3αHSD. The insets in panels A-D show representative western blots of relative protein expression in each mouse group. Values represent the mean ± SEM, n=8. *p <0.05 for TKO vs. WT. #p <0.05 for Male vs. Female.

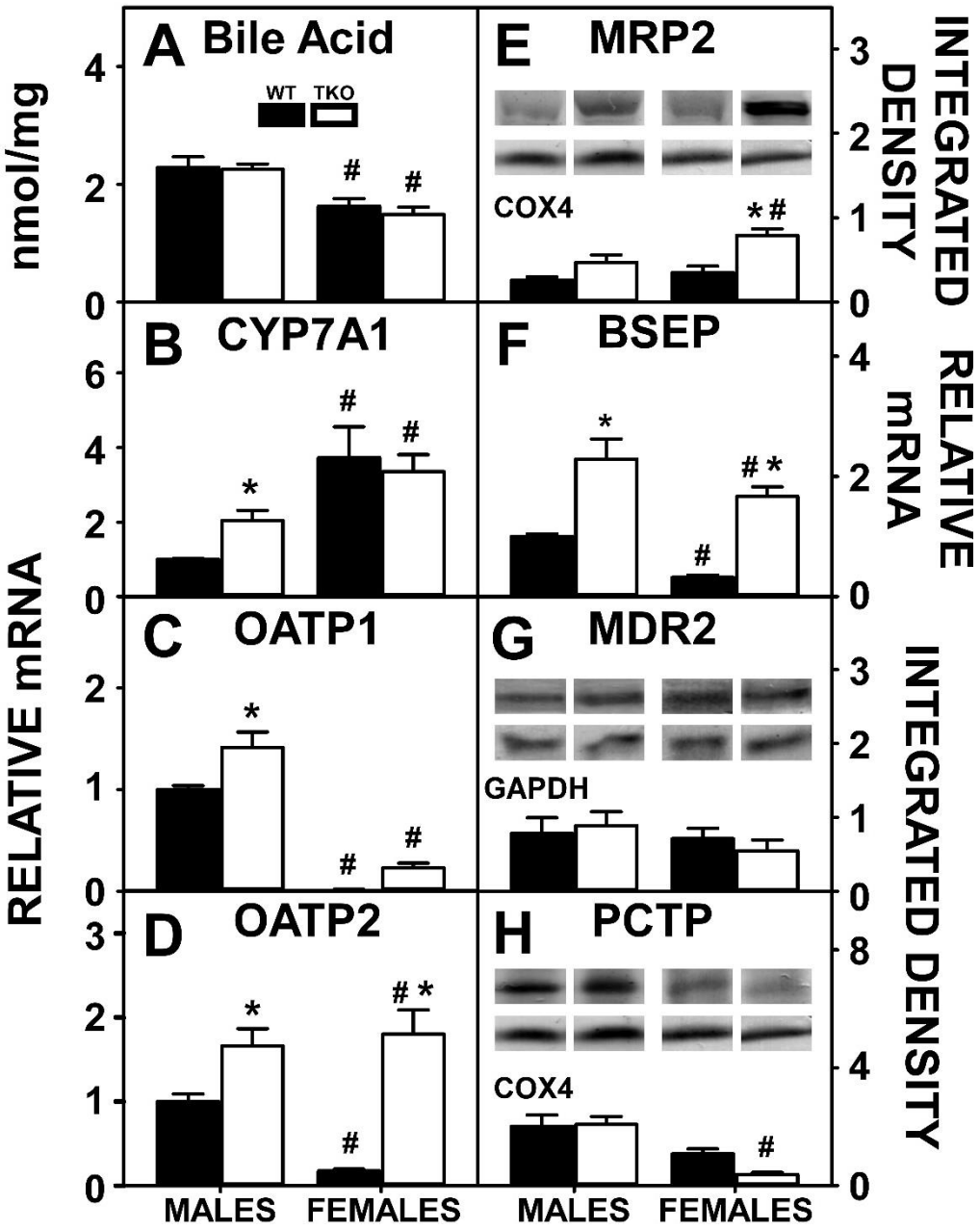

INTEGRATED DENSITY
A FABP1
WT
TKO
4
2
0
GAPDH
*
#
*
B SCP2
8
4
0
BA
*
#
*
C GST
10
5
0
GAPDH
#
*
D 3aHSD
6
4
2
0
COX4
*
#
*
MALE
FEMALE

Chapter IV Figure 9. Effects of *Fabp1/Scp2/Scpx* gene ablation and sex on serum lipoproteins and apolipoproteins in high fat fed mice (Milligan, 2018). Male and female WT and TKO mice on a C57BL/6NCr background were pair-fed a HFD. Levels of serum HDL-C (A), apoA1 (B) and non-HDL-C (D) were measured and ratios of apoA1/HDL-C (C) and apoB/non-HDL-C (F) were calculated as described in Methods. Values represent the mean ± SEM, n=8. *p <0.05 for TKO vs. WT. #p <0.05 for Male vs. Female.

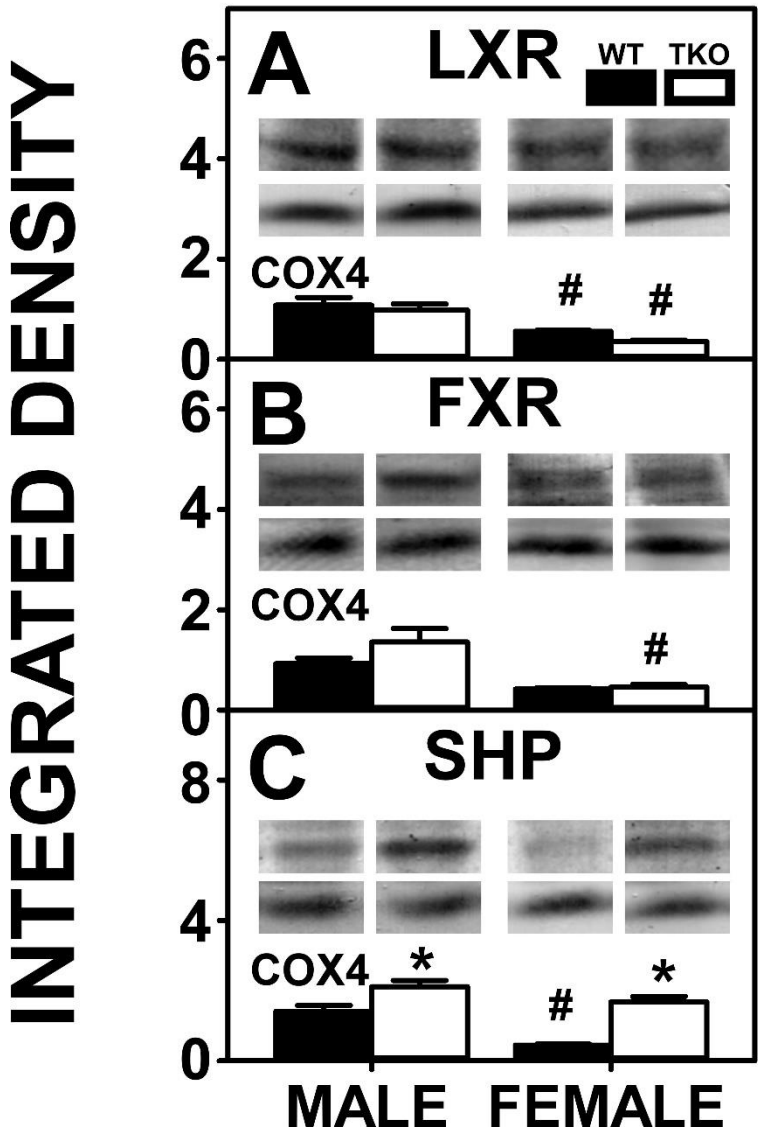


Chapter IV Figure 10. Effects of *Fabp1/Scp2/Scpx* gene ablation and sex on biliary bile formation in high fat fed mice (Milligan, 2018). Male and female WT and TKO mice on a C57BL/6NCr background were pair-fed a HFD. Hepatic Bile acid (A) was measured as described in Methods. qRT-PCR was performed to determine the relative transcription of CYPA1 *(Cyp7a1* gene*)* (B) and *OATP1 (Slco1a1* gene) (C), OATP2 (*Slc227a7* gene) (D) and BSEP (*Abca11* gene) (F) as described in Methods. Western blots were performed to measure relative hepatic protein levels of MRP2 (E) MDR2 (G) and PCTP (H) as described in Methods. The housekeeping gene COX4 was used as a loading control to normalize protein expression of

MRP2 and PCTP and GAPDH was used to normalize MDR2. The insets in panels E, G and H show representative western blots of relative protein expression in each mouse group. Values represent the mean ± SEM, n=8. *p <0.05 for TKO vs. WT. #p <0.05 for Male vs. Female.



Chapter IV Figure 11. Effects of *Fabp1/Scp2/Scpx* gene ablation and sex on hepatic expression of nuclear receptors nuclear receptors regulating transcription of proteins in bile acid synthesis in high fat fed mice (Milligan, 2018). Livers from male and female WT and TKO mice on a C57BL/6NCr background pair-fed a HFD were examined by western blot to measure relative hepatic protein levels of LXR (A), FXR (B) and SHP (C) as described in Methods. The housekeeping gene COX4 was used as a loading control to normalize protein expression of these proteins. The insets in panels A-D show representative western blots of relative protein expression in each mouse group. Values represent the mean ± SEM, n=8. *p <0.05 for TKO vs. WT. #p <0.05 for Male vs. Female.


INTEGRATED DENSITY
A
LXR
WT
TKO
6
4
2
0
COX4
#
#
B
FXR
6
4
2
0
COX4
#
C
SHP
8
4
0
COX4
*
#
*
MALE
FEMALE

References

1. Milligan S, Martin GG, Landrock D, et al. Ablating both Fabp1 and Scp2/scpx (TKO) induces hepatic phospholipid and cholesterol accumulation in high fat-fed mice. Biochim Biophys Acta. 2018;1863(3):323-338. doi: S1388-1981(18)30004-0 [pii].