Insights into the Role of Phosphatidylethanolamine *N*-methyltransferase and the PC:PE Ratio in Liver Function

by

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Abstract

PEMT is quantitatively important in the liver and is responsible for approximately 30% of hepatic PC synthesis, with the remaining 70% synthesized by the CDP-choline pathway. HFD-fed *Pemt*$^{-/-}$ mice and L*Pcyt1a*$^{-/-}$ mice both develop NAFLD associated with reduced VLDL secretion and a reduced hepatic PC:PE molar ratio. Interestingly, the severity of NAFLD was greater in HFD-fed *Pemt*$^{-/-}$ mice, which was associated with a greater decrease in the hepatic PC:PE molar ratio, compared to L*Pcyt1a*$^{-/-}$. *Pemt*$^{-/-}$ mice are also protected from DIO and IR but L*Pcyt1a*$^{-/-}$ mice are not suggesting a role for PEMT, beyond PC deficiency, in mediating these effects. However, dietary supplementation of choline into the HFD ameliorates the protection against DIO and IR and improves NAFLD. Aside from the role of PC in VLDL secretion, a large proportion of PC is also secreted into bile. This thesis endeavors to delineate the effects of hepatic PEMT deficiency and PC:PE imbalance in biliary secretion, resistance to DIO and IR, and NAFLD development during over nutrition.

Interestingly, portions of the ER located close to the canaliculus are enriched in PEMT. Phospholipid balance and asymmetrical distribution by ATPase Phospholipid Transporting 8B1 (ATP8B1) on the canalicular membrane is required for membrane integrity and biliary processes. We hypothesized that PEMT is an important supplier of PC to the canaliculus and that PEMT activity is critical for the maintenance of canalicular membrane integrity and bile formation upon HFD feeding, when there is an increase in overall hepatic PC demand. *Pemt*$^{+/+}$ and *Pemt*$^{-/-}$ mice were fed a chow diet, an HFD, or a choline-supplemented HFD (CSHFD). *Pemt*$^{-/-}$ mice developed cholestasis, i.e., elevated plasma bile acid (BA) concentrations and decreased biliary secretion rates

of BAs and PC, during HFD feeding. Maximal BA secretory rate was reduced more than 70% in HFD-fed *Pemt*$^{-/-}$ mice. Hepatic ABCB11/bile salt export protein (BSEP), responsible for BA secretion, was decreased in *Pemt*$^{-/-}$ mice and appeared to be retained intracellularly. Canalicular membranes of HFD-fed *Pemt*$^{-/-}$ mice contained fewer invaginations and displayed a smaller surface area than *Pemt*$^{+/+}$ mice. Choline supplementation prevented and reversed the development of HFD-induced cholestasis. Thus, we propose that hepatic PC availability is critical for bile formation. Dietary choline supplementation might be a potential noninvasive therapy for specific subset of patients with cholestasis.

We have previously suggested that PC insufficiency and the severely reduced hepatic PC:PE molar ratio, which reduces membrane integrity, contributes to the development of cholestasis in HFD-fed *Pemt*$^{-/-}$ mice. Since the majority of hepatic PC is synthesized through the CDP:choline pathway, we developed an inducible L*Pcyt1a*$^{-/-}$ (iL*Pcyt1a*$^{-/-}$) mouse to investigate the effects of PC insufficiency on biliary secretion. We report that the biliary secretion of bile acids (BA) is not impaired, and that the biliary secretion of phospholipids is surprisingly increased in iL*Pcyt1a*$^{-/-}$ *mice*. This suggests that CTα is not required for biliary secretion under HFD conditions and may play a role in regulating biliary phospholipid secretion.

Small amounts of PEMT protein have been detected in extra-hepatic tissues, such as white adipose, testis, and kidneys. We investigated if the lack of *hepatic* PEMT is responsible for the metabolic phenotype, or if the low expression of PEMT in other tissues contributes to the protection against diet induced obesity and insulin resistance in *Pemt*$^{-/-}$ mice. Furthermore, we investigated if decreasing PEMT expression with

antisense oligonucleotides (ASO) would result in metabolic benefits in both lean and obese mice, without negatively impacting liver health. We both restored hepatic PEMT in *Pemt*$^{-/-}$ mice, via adeno associated virus delivery, and decreased hepatic PEMT with ASOs targeted against PEMT. Weight gain, insulin sensitivity, and indices of liver function were determined.

We report that the protection against diet induced weight gain and insulin resistance, as well as the development of NAFLD is dependent on hepatic PEMT activity. NAFLD, associated with a significant decrease in the hepatic PC:PE ratio, was exacerbated by PEMT deficiency in obese mice, suggesting that phospholipid insufficiency promotes NAFLD progression during obesity/over-nutrition. Hepatic PEMT is critical for maintaining phospholipid balance, which is crucial for a healthy liver.

Preface

This thesis is original work by Sereana Wan. All procedures regarding animal handling, feeding and surgeries were approved by the University of Alberta's Institutional Animal Care Committee in accordance with guidelines of the Canadian Council on Animal Care. The contributions made by the candidate, Sereana Wan, and the co-authors of these studies are described below.

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List of Abbreviations

AAV	Adeno-associated virus
ALT	Alanine amino transferase
ASBT	Apical sodium dependent bile acid transporter
ASO	Antisense oligonucleotide
ATP8B1	Atpase Phospholipid Transporting 8B1
BA	Bile acids
BRIC	Benign recurrent intrahepatic cholestasis
BSEP/ABCB11	Bile salt export protein
CA	Cholic acid
Cd68	Cluster of differentiation 68
CDCA	Chenodeoxycholic acid
CEPT	CDP-choline:1,2-diacylglycerol choline/ethanolamine phosphotransferase
CK	Choline kinase
Col1a1	Collagen type I alpha 1 chain
CPT	CDP-choline:1,2-diacylglycerol cholinephosphotransferase
CS	Choline supplementation
CT	CTP:phosphocholine cytidylyltransferase
CYP27A1	Sterol 27-hydroxylase
CYP7A1	Cholesterol 7α-hydroxylase
CYP8B1	Cytochrome P450 enzyme sterol 12α-hydroxylase
DAG	Diacylglycerol

DIO	Diet induced obesity
ER	Endoplasmic reticulum
ET	CTP:phosphoethanolamine cytidylyltransferase
FGF19	Fibroblast growth factor 19
FXR	Farnesoid X receptor
GFP	Green fluorescence protein
GNMT	Glycine *N*-methyltransferase
HFD	High-fat diet
IR	Insulin resistance
KD	Knock down
MAT1A	Methionine adenosyltransferase 1A
MCA	Muricholic acid
MDR2	Multiple drug-resistant protein 2
MTP	Microsomal triacylglycerol transfer protein
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
Nox2	NADPH oxidase
NTCP	Sodium-taurocholate co-transporting polypeptide
OATP	Organic anion transporting polypeptide
OST	Organic solute transporter
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PEMT	Phosphatidylethanolamine *N*-methyltransferase

PFIC	Progressive familial intrahepatic cholestasis
PL	Phospholipid
PS	Phosphatidylserine
PSD	Phosphatidylserine decarboxylase
Shp	Small heterodimer partner
TG	Triacylglycerols
TUDCA	Tauroursodeoxycholic acid
VLDL	Very low density lipoprotein
Zfp36l1	Zinc finger protein 36 like 1

Chapter 1

Introduction

1.1 Overview of phosphatidylcholine (PC) and Phosphatidylethanolamine (PE) synthesis

Phosphatidylcholine (PC) is the major phospholipid of all mammalian cell types and subcellular organelles. In general, PC comprises 40-50% of total cellular phospholipids, although different cell types, individual organelles and even the two leaflets of organelle membranes contain distinct phospholipid compositions. The second most abundant phospholipid in mammalian membranes is phosphatidylethanolamine (PE), which is enriched in mitochondrial inner membranes (~40% of total phospholipids) compared to other organelles (15-25% of total phospholipids).

1.1.1 PC Biosynthesis

In all nucleated mammalian cells PC is synthesized by the CDP-choline pathway, also called the Kennedy pathway (Kennedy and Weiss 1956, Kennedy 1957) (Fig. 1.1). Choline enters the cell via three classes of choline transporters - the high affinity transporter (CHT1), the intermediate affinity transporters (CTL family) and the low affinity organic cation transporters (OCT family) [reviewed in (Traiffort, O'Regan et al. 2013)]. Upon entering the cell, choline is rapidly phosphorylated by ATP to phosphocholine via the cytosolic enzyme choline kinase (CK) encoded by two distinct genes, *Chka* and *Chkb* from which CKα and CKβ, respectively (Fig. 1.1), are derived [reviewed in (Aoyama, Liao et al. 2004, Fagone and Jackowski 2013)]. The second reaction of the choline pathway for PC synthesis is the conversion of CTP and phosphocholine to CDP-choline via the enzyme CTP:phosphocholine cytidylyltransferase (CT) (Fig. 1.1). Two isoforms of CT have been identified in mice - CTα, which is encoded by the *Pcyt1a* gene, and CTβ, which is the product of the *Pcyt1b* gene. CTα is ubiquitously expressed in mammalian tissues

whereas CTβ is most highly expressed in the brain (Lykidis, Baburina et al. 1999, Karim, Jackson et al. 2003). CTα is a soluble protein that contains a nuclear localization signal and resides primarily in the nucleus (Wang 1993, Wang, MacDonald et al. 1993, Northwood, Tong et al. 1999, Gehrig and Ridgway 2011, Aitchison, Arsenault et al. 2015). In contrast, CTβ lacks a nuclear localization signal and, consequently, is exclusively extra-nuclear. Both CT isoforms are amphipathic proteins that can associate reversibly with membranes, particularly those enriched in diacylglycerol (DAG) and fatty acids [(Pelech, Pritchard et al. 1983, Cornell and Vance 1987, Johnson 2003); reviewed in (Cornell and Northwood 2000, Cornell and Ridgway 2015)]. In addition, the association of CTα with membranes results in dephosphorylation of the protein thereby increasing its enzymatic activity (Houweling, Jamil et al. 1994, Chong, Taneva et al. 2014). Thus, upon translocation to membranes, CTα becomes activated [reviewed in (Cornell and Northwood 2000, Cornell and Ridgway 2015); (Chong, Taneva et al. 2014)]. The reaction catalyzed by CT is, under most metabolic conditions, the rate-limiting reaction for PC synthesis via the CDP-choline pathway (Choy, Farren et al. 1979). The final reaction in the CDP-choline pathway is catalyzed by the integral membrane protein CDP-choline:1,2-diacylglycerol cholinephosphotransferase (CPT) and the dual-specificity protein, CDP-choline:1,2-diacylglycerol choline/ethanolamine phosphotransferase (CEPT) (Henneberry and McMaster 1999, Henneberry, Wistow et al. 2000) (Fig. 1.1). These proteins are tightly embedded in the endoplasmic reticulum (ER) membrane (van Golde, Fleischer et al. 1971, Vance and Vance 1988, Henneberry, Wright et al. 2002) and transfer phosphocholine from CDP-choline to DAG thereby generating PC.

Figure 1.1

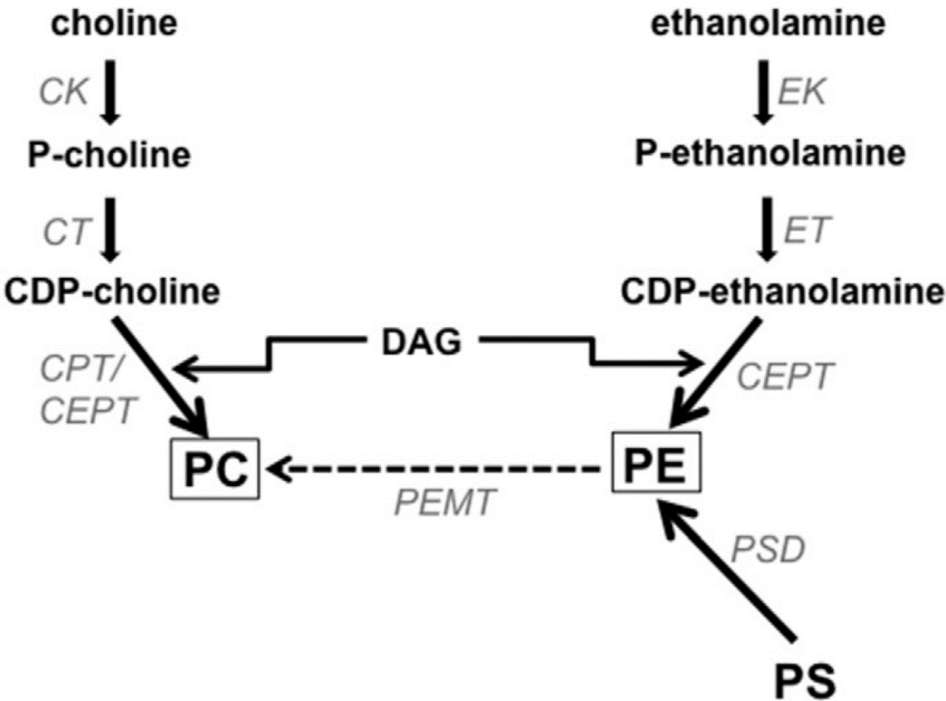

Figure 1.1 Biosynthetic pathways for phosphatidylcholine (PC) and phosphatidylethanolamine (PE) in mammalian cells

Abbreviations for enzymes are shown in gray. PC can be made in mammalian cells by two biosynthetic pathways. In the choline pathway, which operates in all nucleated mammalian cells, choline enters the cell and is phosphorylated to phosphocholine by the cytosolic enzyme choline kinase (CK). The phosphocholine is converted to CDP-choline by CTP:phosphocholine cytidylyltransferase (CT), an amphitropic protein that is mainly located in the nucleus. In the final step of this pathway for PC synthesis, phosphocholine is transferred from CDP-choline to diacylglycerol (DAG) by the integral ER membrane proteins, CDP-choline:1,2-diacylglycerol cholinephosphotransferase (CPT), and to a lesser extent by the dual-specificity protein CDP-choline:1,2-diacylglycerol choline/ethanolamine phosphotransferase (CEPT), resulting in production of PC. In the other PC biosynthetic pathway, PE is converted to PC by three successive methylation reactions catalyzed by phosphatidylethanolamine *N*-methyltransferase (PEMT) using *S*-adenosylmethionine as the methyl-group donor. PEMT activity has been localized to the ER and mitochondria-associated membranes (MAM). The dotted line indicates that in mammals, the reaction catalyzed by PEMT occurs essentially only in hepatocytes.

PE is made by two spatially-separated biosynthetic pathways. In the ethanolamine pathway, which parallels the choline pathway for PC synthesis, ethanolamine is phosphorylated to phosphoethanolamine by the cytosolic enzyme ethanolamine kinase (EK). Another cytosolic protein, CTP:phosphoethanolaminecytidylyltransferase (ET) converts phosphoethanolamine and CTP to CDP-ethanolamine. The final step in the pathway is catalyzed by the ER integral membrane dual-specificity protein, CDP-ethanolamine:1,2-diacylglycerol choline/ethanolamine phosphotransferase (CEPT) which transfers phosphoethanolamine to DAG to generate PE in the ER. The alternative pathway for PE synthesis, the phosphatidylserine (PS) decarboxylase (PSD) pathway occurs only in mitochondrial inner membranes. PS is imported from its site of synthesis in the ER/MAM to mitochondrial inner membranes where PSD converts PS to PE.

In addition to the choline pathway for PC synthesis, which is ubiquitously expressed in all nucleated mammalian cells, the liver also utilizes an alternative pathway for PC synthesis [(Bremer, Figard et al. 1960, Sundler and Akesson 1975); reviewed in (Vance 2014)]. In this pathway, PE is methylated to PC via three sequential methylation reactions (Fig. 1.1) in which *S*-adenosylmethionine (AdoMet) is the methyl group donor. The enzyme phosphatidylethanolamine *N*-methyltransferase (PEMT) catalyzes all three methylation reactions [reviewed in (Vance 2013, Vance 2014)]. Although the liver is the only mammalian tissue that contains significant PEMT protein or activity (Vance and Ridgway 1988, Ridgway and Vance 1989, Tasseva, van der Veen et al. 2016), a small amount of PEMT activity (less than 1% of that in liver) has been reported to increase in adipocytes during differentiation (Cole and Vance 2010, Horl, Wagner et al. 2011). In rodents, approximately 30% of PC biosynthesized in the liver is derived from the PEMT pathway with the remaining 70% of PC being generated by the CDP-choline pathway (DeLong, Shen et al. 1999). PEMT is a small (~20 kDa) integral membrane protein that is located in the ER (Ridgway and Vance 1987) and is highly enriched in portions of the ER which lie in close proximity to the canaliculus – the site of biliary secretion (Sehayek, Wang et al. 2003).

1.1.2 PE Biosynthesis

The two major pathways utilized by mammalian cells for the biosynthesis of PE are the ethanolamine pathway (Kennedy and Weiss 1956) and the phosphatidylserine decarboxylase (PSD) pathway (Borkenhagen, Kennedy et al. 1961, Percy, Moore et al. 1983, Zborowski, Dygas et al. 1983) (Fig. 1.1). The PE derived from these two pathways is synthesized in two spatially separated organelles: the ER and mitochondria,

respectively. Small amounts of PE can also be synthesized by a base-exchange reaction in the ER catalyzed by phosphatidylserine synthase-2 in which the serine residue of phosphatidylserine (PS) is exchanged for ethanolamine (Sundler, Akesson et al. 1974, Bjerve 1984). The reactions of the CDP-ethanolamine pathway for PE synthesis parallel those of the choline pathway for PC synthesis (Kennedy and Weiss 1956). Ethanolamine is imported into the cell via transporters that are incompletely defined. The ethanolamine is subsequently phosphorylated by two cytosolic, ethanolamine-specific kinases that are particularly abundant in the liver and reproductive tissues (Lykidis, Wang et al. 2001, Tian, Jackson et al. 2006, Gustin, Western et al. 2008) (Fig. 1.1). The second reaction of the CDP-ethanolamine pathway is catalyzed by the cytosolic enzyme CTP:phosphoethanolamine cytidylyltransferase (ET), which is encoded by the *Pcyt2* gene (Nakashima, Hosaka et al. 1997, Poloumienko, Cote et al. 2004) (Fig. 1.1). This enzyme converts CTP and phosphoethanolamine to CDP-ethanolamine and pyrophosphate, and is normally the rate-limiting enzyme of the pathway (Sundler 1975, Sundler and Akesson 1975, Tijburg, Houweling et al. 1987). The ET protein contains two similar catalytic motifs, both of which are required for activity (Nakashima, Hosaka et al. 1997, Tie and Bakovic 2007); however, unlike CT, ET activity is not regulated by reversible binding to membranes. The final step of this pathway for PE synthesis is mediated by CEPT, as well as by CDP-ethanolamine:1,2-ethanolaminephosphotransferase (Henneberry and McMaster 1999, Horibata and Hirabayashi 2007) (Fig. 1.1). These enzymes are integral membrane proteins of the ER and convert CDP-ethanolamine and DAG to PE.

The other major pathway for PE synthesis in mammalian cells is the PSD pathway that operates exclusively on the outer aspect of mitochondrial inner membranes (Borkenhagen, Kennedy et al. 1961, Percy, Moore et al. 1983, Zborowski, Dygas et al. 1983) (Fig. 1.1). The substrate for the decarboxylase is PS that is made by two PS synthases, PSS1 and PSS2, which are restricted to ER membranes (Hübscher, Dils et al. 1959, Suzuki and Kanfer 1985, Kuge, Saito et al. 1997, Bergo, Gavino et al. 2002, Arikketh, Nelson et al. 2008). These two PS synthases are highly enriched in the MAM (Stone and Vance 2000). The translocation of PS from the ER/MAM to the site of PSD in mitochondrial inner membranes is ATP-dependent and is the rate-limiting step for PE synthesis via this route (Voelker 1989, Shiao, Lupo et al. 1995). PS-derived PE can be rapidly exported, by unknown mechanisms, from mitochondria to other cellular organelles, such as the ER and plasma membrane (Vance, Aasman et al. 1991, Shiao, Lupo et al. 1995, Kainu, Hermansson et al. 2012). However, the PE that is present in mitochondrial membranes is preferentially derived *in situ* from PSD rather than from the ethanolamine pathway in the ER (Shiao, Lupo et al. 1995). Thus, the pools of PE made by the two spatially separated pathways (in the ER from CDP-ethanolamine and in mitochondria from PSD) are, at least to some extent, compartmentalized. This conclusion is supported by experiments in knockout mice in which genetic elimination of either PE biosynthetic pathway is embryonically lethal despite normal, or enhanced, operation of the other pathway (Steenbergen, Nanowski et al. 2005, Fullerton, Hakimuddin et al. 2007, Leonardi, Frank et al. 2009).

1.2 The subcellular roles of PC and PE synthesis

1.2.1 Phospholipid metabolism and lipoprotein secretion

Phospholipids are required for the formation and stability of lipoproteins. Together with cholesterol, phospholipids (particularly PC) form a monolayer surrounding a neutral lipid core consisting of triacylglycerols (TG) and cholesteryl esters. Besides the major lipids – TG, phospholipids and (esterified) cholesterol – other lipids, such as DAG and sphingolipids – are also present in low amounts on lipoproteins. In humans, the assembly of the TG-rich lipoproteins (i.e. very low density lipoproteins, VLDLs), in the liver requires apolipoprotein (apo) apoB100, whereas assembly of TG-rich chylomicrons in the intestine is dependent on apoB48 (Davidson and Shelness 2000). In addition to apoB, PC is required for assembly and secretion of VLDLs and chylomicrons (Skipski, Barclay et al. 1967). It has been estimated that PC comprises 60-80% of the phospholipids on the surface of apoB-containing lipoproteins (Skipski, Barclay et al. 1967, Agren, Kurvinen et al. 2005). Therefore, it is not surprising that reduced PC synthesis impairs the secretion of TG-rich lipoproteins.

In the liver, assembly of VLDL particles starts with the translation of apoB on ER-bound ribosomes. ApoB is co-translationally translocated into the ER lumen where phospholipids, cholesterol, cholesteryl esters and TG are recruited onto apoB from the ER membrane (Manchekar, Richardson et al. 2004). The lipidation of apoB is dependent on the microsomal triacylglycerol transfer protein (MTP) (Ingram and Shelness 1997, Kulinski, Rustaeus et al. 2002, Manchekar, Richardson et al. 2004), which is thought to interact directly with apoB thereby facilitating the translocation of apoB into the ER lumen [(Liang and Ginsberg 2001); reviewed by (Hussain, Shi et al. 2003)]. Although MTP has

a preference for binding TG and cholesteryl esters, it also transfers phospholipids to apoB (Wetterau, Aggerbeck et al. 1992). Another ER luminal protein, the phospholipid transfer protein, is also involved in transferring phospholipids to apoB in the early stages of lipoprotein assembly (Manchekar, Liu et al. 2015). After formation of a nascent TG-poor, apoB-containing particle, the bulk of lipids is added to apoB in the ER lumen (Rusiñol, Verkade et al. 1993, Yamaguchi, Gamble et al. 2003), or possibly in the Golgi apparatus (Gusarova, Brodsky et al. 2003), in a process that seems to be independent of MTP (Kulinski, Rustaeus et al. 2002). Insufficient availability of lipids for the initial lipidation of apoB leads to diminished translocation or mis-folding of apoB, resulting in degradation of apoB via ER-associated co-translational degradation and the ubiquitin-proteasome pathway (Fisher, Zhou et al. 1997, Liao, Yeung et al. 1998, Zhou, Fisher et al. 1998). Low PC levels, or a low PC/PE ratio, in the ER membrane causes degradation of the particle (Verkade, Fast et al. 1993). Thus, the availability and transfer of lipids to apoB is crucial for successful secretion of VLDL.

1.2.2 Requirement of hepatic PC synthesis for VLDL secretion

In the 1930s, the importance of PC for VLDL secretion was first established when Best and Huntsman (Best and Huntsman 1932) showed that dietary choline could prevent lipid (TG) deposition in the liver. Subsequently, choline deficiency was shown to induce hepatic steatosis in rodents (Yao and Vance 1990, Rinella and Green 2004, Rizki, Arnaboldi et al. 2006) as well as humans (Zeisel, Da Costa et al. 1991, Buchman, Dubin et al. 1995), at least in part due to reduced VLDL secretion (Yao and Vance 1990). Furthermore, methionine adenosyltransferase 1A (MAT1A) knockout mice have low levels of AdoMet, a substrate for PC synthesis via the PEMT biosynthetic pathway, and

decreased VLDL secretion (Cano, Buque et al. 2011). Mechanistic studies in rat hepatocytes incubated with methionine- and/or choline-deficient medium, which limited substrate availability for both PC biosynthetic pathways, impaired the secretion of apoB and lipids associated with VLDL (Yao and Vance 1988, Verkade, Fast et al. 1993, Fast and Vance 1995). The choline-deficient medium caused the production of abnormal, PC-deficient VLDL particles that were degraded intracellularly (Verkade, Fast et al. 1993). More recently, the consequence of reduced PC biosynthesis on VLDL secretion was directly investigated in mouse models that lacked either PEMT or CTα specifically in the liver. The fasting concentrations of TG and apoB100 in plasma of liver-specific *Pcyt1α* $^{-/-}$ (L*Pcyt1α* $^{-/-}$) were 50% lower than in control mice because of diminished VLDL secretion (Jacobs, Devlin et al. 2004, Jacobs, Lingrell et al. 2008). Similarly, hepatocytes from mice lacking PEMT secreted 50% and 70% less TG and apoB100, respectively, than did wild-type hepatocytes (Noga, Zhao et al. 2002). In addition, plasma from fasted *Pemt* $^{-/-}$ mice fed a high fat/high cholesterol diet for 3 weeks contained less TAG and apoB100 compared to control mice, due to reduced VLDL secretion (Noga and Vance 2003). Moreover, the rate of secretion of VLDL-TG was 70% lower in *Pemt* $^{-/-}$ mice fed a high-fat diet than in *Pemt* $^{+/+}$ mice fed the same diet (van der Veen, Lingrell et al. 2016). Together, these studies clearly show that hepatic PC biosynthesis via each pathway for PC biosynthesis is independently required for normal VLDL secretion during HF-feeding.

It has recently been proposed that increased hepatic PC biosynthesis stimulates production of VLDL particles. Mice lacking glycine *N*-methyltransferase (GNMT) have elevated (50-fold) hepatic AdoMet levels (Martinez-Una, Varela-Rey et al. 2013), leading to increased flux through the PEMT pathway and increased VLDL secretion (Martinez-

Una, Varela-Rey et al. 2013, Martinez-Una, Varela-Rey et al. 2015). Most of the excess PC synthesized in these mice is subsequently catabolized to DAG and re-esterified to TG (van der Veen, Lingrell et al. 2012, Martinez-Una, Varela-Rey et al. 2013). It is therefore unclear whether the increase in VLDL-TG secretion in *Gnmt*$^{-/-}$ mice is due to increased availability of PC or TG or both.

1.2.3 Importance of phospholipids in lipid droplet formation

As a mechanism for preventing lipotoxicity, excess intracellular fatty acids are incorporated into TGs that are stored in cytosolic lipid droplets (Listenberger, Han et al. 2003). The neutral lipids in the lipid droplet core are surrounded by a monolayer of phospholipids [reviewed in (Pol, Gross et al. 2014)]. The relative abundance of PC and PE on the surface of lipid droplets is important for their dynamics: inhibition of PC biosynthesis during conditions that promote TG storage increases the size of the lipid droplets since the ratio of surface area (phospholipid content) to volume (TG) of larger droplets is less than that of smaller droplets (Guo, Walther et al. 2008, Krahmer, Guo et al. 2011, Aitchison, Arsenault et al. 2015). Additionally, an increase in the relative amount of PE on lipid droplets can promote fusion of the droplets (Hafez and Cullis 2001). Thus, addition of PC to the surface of expanding lipid droplets reduces the relative abundance of PE and thereby prevents coalescence of lipid droplets (Guo, Walther et al. 2008).

Adipocytes are specialized cells for storing excess energy as TAG in lipid droplets. During differentiation of 3T3-L1 cells into adipocytes, the requirement for PC is increased to facilitate expansion of lipid droplets, and CTα expression is strongly stimulated (Aitchison, Arsenault et al. 2015). Interestingly, during differentiation of 3T3-L1 fibroblasts into adipocytes, a process that involves storage of TG in lipid droplets, PEMT expression

is strongly induced (Cole and Vance 2010). In concert with increased PEMT expression during this differentiation, mRNAs encoding PS synthase-1 and PSD were induced, suggesting a tight regulation of phospholipid biosynthetic pathways during adipocyte differentiation and lipid droplet expansion (Horl, Wagner et al. 2011). Knock-down of PEMT in 3T3-L1 adipocytes increased basal hydrolysis of TAG (Horl, Wagner et al. 2011). Moreover, adipocytes from *Pemt*$^{-/-}$ mice appear to be resistant to diet-induced hypertrophy: when *Pemt*$^{-/-}$ mice were fed a high-fat diet, their adipocytes were smaller in diameter than those of *Pemt*$^{+/+}$ mice (Jacobs, Zhao et al. 2010). However, we recently reported that PEMT deficiency in mice did not impair adipocyte differentiation or lipolysis, but marginally reduced *de novo* lipogenesis in white adipose tissue (Gao, van der Veen et al. 2015). Intriguingly, a recent study showed that single nucleotide polymorphisms in the *PEMT* gene correlate with the percentage of body fat mass in humans (Sharma, Langberg et al. 2013). Increased levels of *PEMT and PCYT1A* mRNA in adipose tissue was positively correlated with fat mass and waist-hip ratio, underscoring the role of PC biosynthesis in lipid droplet formation and/or adipocyte differentiation.

1.3 Role of PC and PE in liver health and disease

The two major phospholipids in plasma membranes of all mammalian cells are PC and PE. PC and PE predominantly reside on the outer and inner leaflets of the plasma membrane bilayer, respectively (Verkleij, Zwaal et al. 1973, Devaux 1991). In several mouse models and in human studies, a change in not only the absolute concentrations of these phospholipids, but more critically in the overall hepatic ratio between PC and PE, is a key determinant of liver health. Changes in the hepatic PC/PE molar ratio have been linked to development of non-alcoholic fatty liver disease (NAFLD) in humans (Li, Agellon

et al. 2006), as well as liver failure (Walkey, Yu et al. 1998), impairment in liver regeneration (Ling, Chaba et al. 2012), and the severity of alcoholic fatty liver disease (Kharbanda, Mailliard et al. 2007). Thus, in the following sections we shall discuss the critical role of the hepatic PC/PE molar ratio in liver health.

1.3.1 Physiological effects of a decreased hepatic PC:PE molar ratio

CTα, encoded by the gene *Pcyt1a*, is the rate-limiting enzyme of the major pathway for hepatic PC synthesis (Fig. 1.1). When L*Pcyt1a*$^{-/-}$ mice were fed a chow diet, sex-dependent effects on hepatic lipid metabolism were noted (Jacobs, Devlin et al. 2004). In female L*Pcyt1a*$^{-/-}$ mice, hepatic PC levels were 20% lower than in control mice, without a concomitant decrease in PE. In response to this decrease in the amount of hepatic PC, VLDL secretion was impaired and TAG accumulated in the liver. In female, but not male, L*Pcyt1a*$^{-/-}$ mice hepatic TAG levels were 20% higher than in floxed-control mice (Jacobs, Devlin et al. 2004). When L*Pcyt1a* $^{-/-}$ mice were challenged with a high-fat diet, the sex disparity was eliminated and the impact of a decreased level of hepatic PC became much more evident. The hepatic PC/PE ratio in L*Pcyt1a*$^{-/-}$ mice (1.4) was significantly lower than in control mice (1.7), and the L*Pcyt1a*$^{-/-}$ mice developed non-alcoholic steatohepatitis (NASH). Within 1 week of feeding the L*Pcyt1a*$^{-/-}$ mice a high-fat diet, plasma levels of alanine aminotransferase (a marker for liver damage) and hepatic TAG levels were 7-fold and 3-fold higher, respectively, than those in control mice (Niebergall, Jacobs et al. 2011, Ling, Chaba et al. 2012).

NASH also developed in *Pemt*$^{-/-}$ mice challenged with a high-fat diet. In these mice, the hepatic PC/PE ratio was significantly reduced from ~1.6 to ~ 1.2, and the livers of the *Pemt*$^{-/-}$ mice contained 4-fold more TG than did livers of wild-type mice. The steatosis

eventually progressed to NASH (Jacobs, Zhao et al. 2010, Ling, Chaba et al. 2012, van der Veen, Lingrell et al. 2016). In both the L*Pcyt1a*$^{-/-}$ and *Pemt*$^{-/-}$ mouse models, the severity of NAFLD negatively correlated with the hepatic PC/PE ratio (Ling, Chaba et al. 2012). Importantly, the decrease in the hepatic PC/PE ratio in *Pemt*$^{-/-}$ mice was greater than in L*Pcyt1a*$^{-/-}$ mice, which was surprising considering that the CDP-choline pathway is the predominant pathway for hepatic PC production. However, because PEMT converts PE to PC, PEMT might play a more important role in balancing the hepatic PC/PE ratio.

When *Pemt*$^{-/-}$ mice were fed a choline-deficient diet (which reduced PC production via the CDP-choline pathway) (Li, Agellon et al. 2005, Li, Agellon et al. 2006), hepatic PC concentrations were dramatically reduced by 50% and liver failure occurred within 3 days (Walkey, Yu et al. 1998). The induction of liver failure was due to a large decrease in hepatic PC leading to a drop in the PC/PE ratio falling below a threshold of ~1 (Li, Agellon et al. 2006). Concomitant with the decrease in the hepatic PC/PE ratio, the amount of PE on the outer leaflet of the plasma membrane of primary hepatocytes increased (Li, Agellon et al. 2006), thereby impairing membrane integrity and inducing liver failure. Thus, relatively small alterations in the PC/PE ratio contributed to the development of NAFLD, whereas a more extreme reduction in the PC/PE ratio results in liver failure (Fig. 1.2).

A large proportion of hepatic PC is utilized for biliary secretion by the multiple drug-resistant protein 2 (MDR2 in mice; MDR3 in humans). Therefore, a reduction of bile PC secretion would prevent liver failure in *Pemt*$^{-/-}$ mice fed the choline-deficient diet. In contrast to *Pemt*$^{-/-}$, *Pemt*$^{-/-}$*/Mdr2*$^{-/-}$ mice fed a choline-deficient diet did not develop liver failure and survived beyond 3 months (Li, Agellon et al. 2006). Within 21 days of initiation of the diet, the levels of hepatic PC in *Pemt*$^{-/-}$*/Mdr2*$^{-/-}$ mice decreased by ~50%.

Interestingly, the amount of PE was reduced by a similar amount so that the PC/PE ratio of 1.4 decreased only slightly compared the decreased ratio (to 0.9) in *Pemt*$^{-/-}$ mice fed the choline-deficient diet (Li, Agellon et al. 2006). In other experiments, cellular levels of PE in primary hepatocytes derived from *Pemt*$^{-/-}$ mice fed a choline-deficient diet for 2 days decreased significantly upon treatment of the cells with RNA interference targeted to *Pcyt2* so that PE synthesis via the CDP-ethanolamine pathway was inhibited. The reduction in ET increased the PC/PE ratio from 0.7 to 1.2 (Li, Agellon et al. 2006). Consequently, levels of alanine aminotransferase in the medium were significantly lower than in hepatocytes treated with control, scrambled RNA (Li, Agellon et al. 2006). Conversely, when primary hepatocytes derived from *Pemt*$^{-/-}$/*Mdr2*$^{-/-}$ mice were incubated with arachidonyltrifluoromethyl ketone, a non-specific inhibitor of PE catabolism, the PC/PE ratio decreased from 1.6 to 0.8 and alanine aminotransferase levels in the culture medium rose significantly (Li, Agellon et al. 2006). In combination, these studies demonstrate that the ratio of PC/PE in hepatocytes can regulate membrane integrity and might be a better predictor of liver failure than reduced PC concentrations alone.

The importance of maintaining an appropriate hepatic PC/PE ratio for liver health has clinical relevance, as a significant proportion of patients with NAFLD and NASH have a lower hepatic PC/PE ratio compared to healthy subjects (Li, Agellon et al. 2006, Puri, Baillie et al. 2007). Since elimination of either PEMT or hepatic CTα reduced the hepatic PC/PE ratio and increased liver TAG concentrations in mice, it is not surprising that loss-of-function mutations or deletions in genes encoding either PEMT or CTα are associated with NAFLD in humans. For example, two patients with bi-allelic loss-of-function mutations in *PCYT1A*, the gene encoding CTα, have recently been identified (Payne, Lim

et al. 2014). In these patients, *PCYT1a* gene expression and PC synthesis by the choline pathway were reduced resulting in a reduced PC/PE ratio in EBV-transformed lymphocytes and fibroblasts (Payne, Lim et al. 2014). This reduced PC/PE ratio in cellular membranes was associated with severe fatty liver in both patients (Payne, Lim et al. 2014), supporting the findings from L*Pcyt1a*$^{-/-}$ mice.

A V175M loss-of-function mutation in the human *PEMT* gene has also been discovered (Song, da Costa et al. 2005). However, the association between the V175M mutation and NAFLD in humans appears to be dependent on the ethnicity of the population. Although the mutation was initially found to be associated with NAFLD patients in a study of 87 subjects (Song, da Costa et al. 2005), no correlation was found in 2349 subjects of the Dallas Heart Study (Romeo, Cohen et al. 2006). A large proportion of subjects in both studies were of Caucasian ethnicity. It has also been reported that the V175M mutation is associated with NAFLD and NASH in Chinese and Japanese populations, respectively (Dong, Wang et al. 2007, Zhou, Li et al. 2010). A comprehensive compilation of population studies of the V175M mutation has been analyzed (Tan, Mohamed et al. 2016). A link between NAFLD and the V175M mutation was established and determined to have a stronger correlation in East Asian populations than in Caucasian populations (Tan, Mohamed et al. 2016).

1.3.2 Physiological effects of an increased hepatic PC:PE molar ratio

Up to this point, the importance of maintaining a threshold hepatic ratio of PC:PE has been emphasized and the negative impacts of a decreased ratio have been highlighted. In some instances, however, the PC/PE ratio can be increased above the normal range and this perturbation in the ratio is also associated with NAFLD (Fig. 1.2).

Figure 1.2

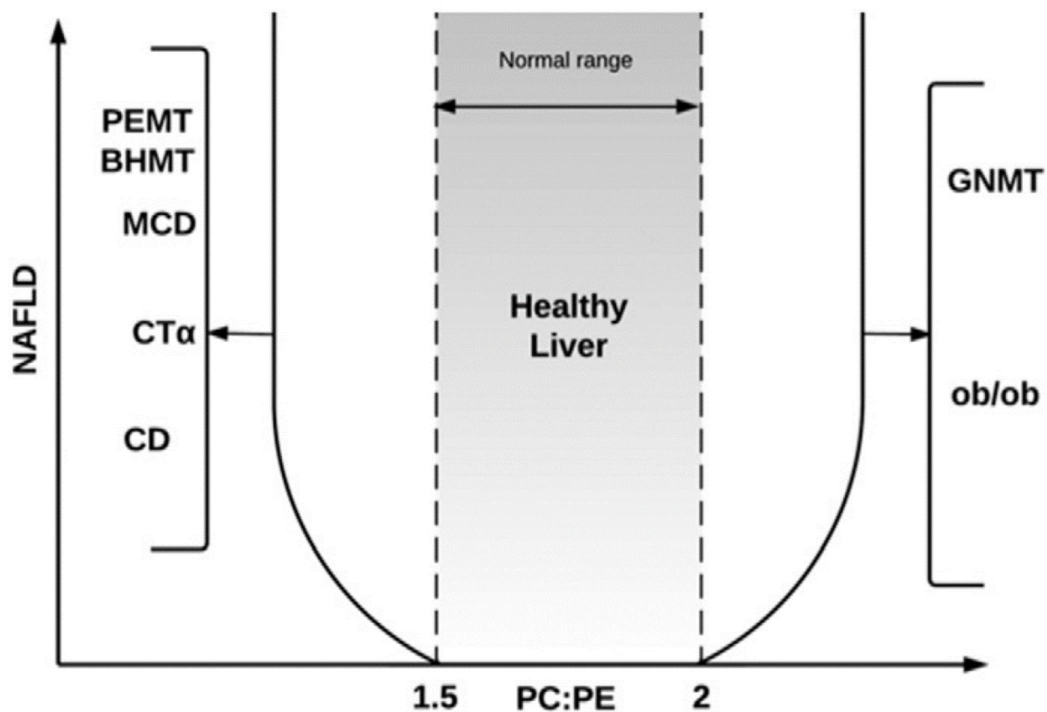

Figure 1.2 The relationship between hepatic PC/PE ratio and NAFLD

NAFLD is a liver disease the severity of which is defined by a pathological score of steatosis, cellular ballooning, portal inflammation and lobular inflammation. Both low and high phosphatidylcholine/phosphatidylethanolamine (PC/PE) ratios are associated with increased NAFLD scores. The normal range for hepatic PC/PE is generally between 1.5-2.0; NAFLD >2.0 and between 1.5-1.0; Liver failure <1.0. A deficiency in PEMT, BNMT, or CTa lead to lower hepatic PC/PE ratio and increased NAFLD severity. In contrast, *Gnmt*$^{-/-}$ and ob/ob mice have increased hepatic PC/PE ratio and develop NAFLD. PEMT: phosphatidylethanolamine *N*-methyltransferase; BHMT: betaine:homocysteine methyltransferase; MCD: methionine- and choline-deficient diet; CTα: CTP:phosphocholine cytidylyltransferase-α; CD: choline-deficient diet; HFD: high-fat diet; GNMT: glycine *N*-methyltransferase; *ob/ob*: leptin-deficient mice.

For example, glycine *N*-methyltransferase (GNMT), which catalyzes a reaction between AdoMet and glycine to form sarcosine and *S*-adenosylhomocysteine, prevents intracellular accumulation of AdoMet, the methyl group donor for the PEMT pathway. In *Gnmt*$^{-/-}$ mice, hepatic AdoMet levels were 50-fold higher than in wild-type mice, leading to a 2.5-fold increase in the rate of PC synthesis through the PEMT pathway (Martinez-Una, Varela-Rey et al. 2013). As a result, the amount of hepatic PE was 50% lower in *Gnmt*$^{-/-}$ mice than in *Gnmt*$^{+/+}$ mice, yet PC was only slightly (albeit significantly) increased (Martinez-Una, Varela-Rey et al. 2013). These data indicate that the liver attempts to maintain phospholipid homeostasis, despite a drastically increased rate of PC synthesis, by stimulating catabolism of PC to DAG, a substrate for TG synthesis through the diacylglycerol acyltransferase pathway (Martinez-Una, Varela-Rey et al. 2013). Hepatic diacylglycerol acyltransferase activity and DAG concentrations in *Gnmt*$^{-/-}$ mice were significantly higher than in their *Gnmt*$^{+/+}$ counterparts (Martinez-Una, Varela-Rey et al. 2013, Martinez-Una, Varela-Rey et al. 2015). Consequently, the hepatic PC/PE ratio rose from ~2 in wild-type mice to ~5 in *Gnmt*$^{-/-}$ mice and the latter mice developed hepatic steatosis by 3 months of age (Martinez-Una, Varela-Rey et al. 2013). In this model, the onset of steatosis was not caused by impaired secretion of TG into VLDL, which was significantly increased in *Gnmt*$^{-/-}$ mice, but by increased hepatic TG synthesis (Martinez-Una, Varela-Rey et al. 2013). When *Gnmt*$^{-/-}$ mice were fed a methionine-deficient diet, the hepatic AdoMet content was lower than that in *Gnmt*$^{+/+}$ mice (Martinez-Una, Varela-Rey et al. 2013) and the reduction in AdoMet normalized the rate of PC synthesis via PEMT (Martinez-Una, Varela-Rey et al. 2013). Consequently, concentrations of PC, PE,

DAG, TG and the PC:PE ratio were also restored to levels in *Gnmt*$^{+/+}$ livers (Martinez-Una, Varela-Rey et al. 2013).

Another murine model that has an increased hepatic PC/PE ratio is the leptin-deficient, *ob/ob* mice (Fu, Yang et al. 2011). In order to accommodate the storage of excess dietary lipids in these mice, the expression of genes involved in hepatic PC synthesis (i.e. *Pcyt1a* and *Pemt*) were up-regulated, thereby increasing the hepatic PC:PE ratio in the ER by ~50% (Fu, Yang et al. 2011). Microsomes isolated from *ob/ob* livers had significantly lower sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCA) activity and disrupted calcium homeostasis when compared to wild-type mice as a result of the altered membrane phospholipid environment (Fu, Yang et al. 2011). Moreover, livers of *ob/ob* mice expressed higher levels of C/EBP homologous protein, a marker of ER stress (Fu, Yang et al. 2011). In addition, levels of SREBP-1c mRNA, and its nuclear, active, form were increased in *ob/ob* mice. Since SREBP-1c increases the expression of several genes involved in lipogenesis, such as fatty acid synthase and diacylglycerol acyltransferase, the levels of mRNAs encoded by these genes were significantly up-regulated in the livers of *ob/ob* mice (Fu, Yang et al. 2011). Consequently, the enhanced hepatic lipogenesis in *ob/ob* mice significantly raised levels of hepatic TAG and induced hepatic steatosis (Perfield, Ortinau et al. 2013). When *ob/ob* mice were treated with shRNA targeted to *Pemt*, the hepatic ER PC:PE ratio was restored to wild-type levels (Fu, Yang et al. 2011). Silencing of *Pemt* with shRNA increased SERCA activity and normalized C/EBP homologous protein expression (Fu, Yang et al. 2011). Reduction of the PC:PE ratio also ameliorated the SREBP-1c-induced activation of fatty

acid synthase and diacylglyerol acyltransferase, thereby significantly decreasing hepatic steatosis (Fu, Yang et al. 2011).

1.4 Metabolic benefits of PEMT deficiency

Aside from the consequences of PEMT deficiency described above (i.e. impaired VLDL secretion and subsequent development of NAFLD), there are also several metabolic benefits associated as well. Our lab has previously shown *Pemt*$^{-/-}$ mice to be resistant to diet induced weight gain and insulin resistance (Jacobs, Zhao et al. 2010). When *Pemt*$^{+/+}$ and *Pemt*$^{-/-}$ mice were fed a HFD for 10 weeks, *Pemt*$^{-/-}$ mice maintained the same weight throughout the 10 weeks whereas *Pemt*$^{+/+}$ mice gained a significant amount of weight (Jacobs, Zhao et al. 2010). Additionally, hematoxylin and eosin (H&E) staining demonstrated HFD-fed *Pemt*$^{-/-}$ mice to be resistant to adipocyte hypertrophy (Jacobs, Zhao et al. 2010). Glucose and insulin tolerance tests in HFD-fed *Pemt*$^{+/+}$ and *Pemt*$^{-/-}$ mice demonstrated improved glucose and insulin tolerance in *Pemt*$^{-/-}$ mice (Jacobs, Zhao et al. 2010, Veen, Lingrell et al. 2019). When hyperinsulinemic euglycemic clamp experiments were conducted, rates of glucose infusion and disposal, as well as suppression of endogenous glucose production, were significantly higher in HFD-fed *Pemt*$^{-/-}$ mice compared to *Pemt*$^{+/+}$ mice (Veen, Lingrell et al. 2019). This improvement in insulin sensitivity was, in part, due to improved hepatic insulin signalling (Veen, Lingrell et al. 2019). Interestingly, decreased hepatic PC:PE molar ratio was found to improve hepatic insulin signalling in vivo and in cell culture (Veen, Lingrell et al. 2019).

1.5 Bile acids

1.5.1 Bile acid synthesis

Bile acids (BAs) are synthesized from cholesterol in the liver in a multistep manner involving the ER, mitochondria, cytosol, and peroxisome (Dawson 2016). BA biosynthesis is briefly outlined in Figure 1.3. There are 2 pathways by which BAs are synthesized in the liver – the "classic" or neutral pathway and the "alternative" or acidic pathway, which synthesize cholic acid or chenodeoxycholic acid, respectively (Dawson 2016). The rate limiting step in the neutral pathway is the conversion of cholesterol to 7α-hydroxycholesterol by cholesterol 7α-hydroxylase (CYP7A1) in the ER (Dawson 2016). In the acidic pathway, the sidechain of cholesterol is modified first by sterol 27-hydroxylase (CYP27A1) which, in the rate limiting step, converts cholesterol to 27-hydroxycholesterol (Dawson 2016). In adult humans and mice, the neutral pathway is the dominant pathway for BA synthesis. The two pathways convene where intermediates of both pathways can be acted upon by cytochrome P450 enzyme sterol 12α-hydroxylase (CYP8B1) (Dawson 2016). CYP8B1 is required for the synthesis of cholic acid (CA) and thereby controls the ratio of CA versus chenodeoxycholic acid (CDCA) produced (Dawson 2016). Intermediates of the "classic" pathway are hydroxylated by CYP8B1 and ultimately result in synthesis of cholic acid (CA) (Dawson 2016). Intermediates of the "alternative" pathway often escape CYP8B1 hydroxylation and result in the synthesis of CDCA (Dawson 2016). The last step in both pathways is the conjugation of the BA to either glycine (dominant in humans) or taurine (dominant in mice) (Dawson 2016). Additionally, BAs are further hydroxylated and converted to α or β muricholic acid (MCA) species in mice. The signalling properties of BAs will be discussed in section 1.5.3.

Figure 1.3

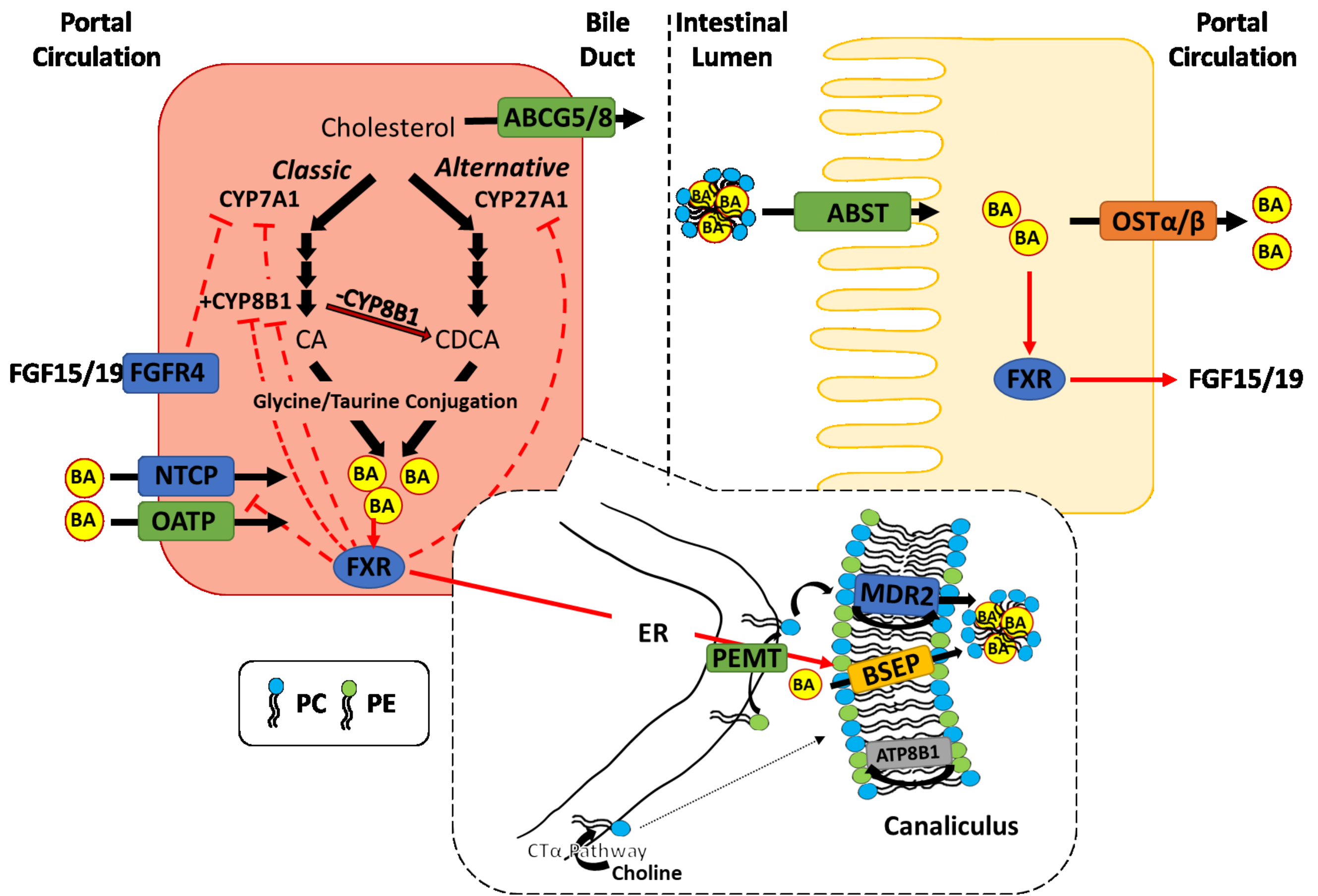

Figure 1.3 Synthesis and enterohepatic circulation of bile acids

Bile acids are synthesized from cholesterol in the liver. There are 2 pathways by which BAs are synthesized in the liver – the "classic" or neutral pathway and the "alternative" or acidic pathway, which synthesize cholic acid or chenodeoxycholic acid, respectively. The rate limiting enzymes in the classic and alternative pathways are cholesterol 7α-hydroxylase (CYP7A1) and sterol 27-hydroxylase (CYP27A1), respectively. CYP8B1 is required for the synthesis of cholic acid (CA) and thereby controls the ratio of CA versus chenodeoxycholic acid (CDCA) produced. The last step in both pathways is the conjugation of the BA to either glycine (dominant in humans) or taurine (dominant in mice).

BAs are secreted into bile by the bile salt export protein (BSEP/ABCB11), PC by multi-drug resistance 3 (MDR3) (Mdr2 in mice), and cholesterol by the ABCG5/ABCG8 dimer. On the canalicular membrane, ATPase Phospholipid Transporting 8B1 (ATP8B1) flips PE from the outer to the inner canalicular membrane. BAs are then stored in the gallbladder and eventually secreted to the intestine to aid in absorption of dietary lipids. The ileum is the major site of BA uptake and the absorption of BAs into enterocytes is mediated by apical sodium dependent bile acid transporter (ASBT). BAs are re-secreted into portal circulation from enterocytes by organic solute transporter (OST) α or β. On the basolateral membrane of hepatocytes, sodium-taurocholate co-transporting polypeptide (NTCP) and organic anion transporting polypeptide (OATP) facilitate the uptake of BAs from portal circulation.

BAs can activate hepatic farnesoid X receptor (FXR) which leads to a suppression in genes involved in BA synthesis (*CYP7A1, CYP8B1, CYP27A1*) and hepatic BA import (*NTCP, OATP1*). Hepatic FXR activation also leads to increased BSEP expression to increase BA export. In enterocytes, high BA concentrations can also induce intestinal FXR activation which then results in the release of fibroblast growth factor 19 (FGF19) (FGF15 in mice) into portal circulation. FGF15/19 activates FGF receptor 4 (FGFR4) on the basolateral membrane of hepatocytes and also leads to the suppression of BA synthesis.

1.5.2 Enterohepatic circulation

The major constituents of bile are BAs and PC which account for 50 and 25% of the dry weight of bile, respectively (Nicolaou, Andress et al. 2012). Biliary PC is required to protect cells against the cytotoxic action of BAs (Smit, Schinkel et al. 1993). Enterohepatic circulation is briefly outlined in Figure 1.3. The majority of BAs are secreted into bile by the bile salt export protein (BSEP/ABCB11), PC by multi-drug resistance 3 (MDR3) (Mdr2 in mice), and cholesterol by the ABCG5/ABCG8 dimer (Smit, Schinkel et al. 1993, Wang, Salem et al. 2001). These proteins, which are critical for proper bile secretion, reside on the canalicular membrane – the site of bile secretion. On the canalicular membrane, ATPase Phospholipid Transporting 8B1 (ATP8B1) flips PE from the outer to the inner canalicular membrane in order to maintain membrane phospholipid asymmetry. BAs are then stored in the gallbladder and eventually secreted to the intestine to aid in absorption of dietary lipids. The ileum is the major site of BA uptake and the absorption of BAs into enterocytes is mediated by apical sodium dependent bile acid transporter (ASBT) (Wong, Oelkers et al. 1995). BAs are re-secreted into portal circulation from enterocytes by organic solute transporter (OST) α or β (Rao, Haywood et al. 2008). The absorption of BAs in the intestine is highly efficient with only 5% of BAs lost and eliminated per cycle through feces, which is the major pathway for cholesterol elimination (Dawson 2016) (Hagenbuch and Meier 1994, Cattori, Eckhardt et al. 1999, Hagenbuch, Adler et al. 2000).

1.5.3 BAs as signalling molecules – BA metabolism

BAs act as signalling molecules which modulate its own metabolism in the liver and intestine, outlined in Figure 1.3. Since high concentrations of BAs are cytotoxic, the

synthesis, import, and export of BAs in the liver are tightly controlled. BAs can activate hepatic farnesoid X receptor (FXR) which leads to an increase in the small heterodimer protein (Shp) (Wang, Chen et al. 1999, Goodwin, Jones et al. 2000, Fiorucci and Distrutti 2015). This activation leads to a suppression in genes involved in BA synthesis (*CYP7A1, CYP8B1, CYP27A1*) and hepatic BA import (*NTCP, OATP1*) (Goodwin, Jones et al. 2000, Sinal, Tohkin et al. 2000). Hepatic FXR activation also leads to increased BSEP expression to increase BA export (Fiorucci and Distrutti 2015). Independent of Shp mediated effects, hepatic FXR activation also induces the expression of the protein ZFP36L1 (Tarling, Clifford et al. 2017). ZFP36L1 increases degradation of *CPY7A1* mRNA and thereby inhibits BA synthesis.

In enterocytes, high BA concentrations can also induce intestinal FXR activation which then results in the release of fibroblast growth factor 19 (FGF19) (FGF15 in mice) into portal circulation (Inagaki, Choi et al. 2005). FGF15/19 activates FGF receptor 4 (FGFR4) on the basolateral membrane of hepatocytes and also leads to the suppression of BA synthesis (Inagaki, Choi et al. 2005).

1.6 Cholestasis

Cholestasis is the impairment or absence of bile flow (Nicolaou, Andress et al. 2012). In the broadest sense cholestasis can be divided into extra-hepatic or intra-hepatic. Extra-hepatic cholestasis refers to disorders in the portion of bile ducts external to the liver, such as gallstones. This thesis will focus on intra-hepatic cholestasis, which refers to disorders influencing bile flow that occur within the liver. Intra-hepatic cholestasis can be drug induced, caused by auto-immune diseases or by either dominant or recessive gene mutations (Nicolaou, Andress et al. 2012). Mutations in genes encoding for proteins

involved in biliary BA and phospholipid secretion lead to 3 types of progressive familial intrahepatic cholestasis (PFIC), described in greater detail in 1.6.1, 1.6.2, and 1.6.3. All PFIC types are due to homozygous gene disruption with loss of protein function and are associated with development of fibrosis, cirrhosis, and end stage liver disease (Nicolaou, Andress et al. 2012, Srivastava 2014). PFIC 2 has the most rapid progression and will progress to end stage liver disease within the first few years of onset (Srivastava 2014). PFIC1 and PFIC3 patients will also develop end stage liver disease within the first decade (Srivastava 2014). Homozygous gene disruption with partial loss of protein function and heterozygous gene mutation can also lead to brief recurrent intrahepatic cholestasis (BRIC). BRIC patients will suffer from intermittent periods of cholestasis but are completely normal and healthy in between occurrences (Srivastava 2014).

Currently, there are limited therapies and surgical methods for management of cholestasis. Not all patients respond to the available treatments and often progress to end stage liver disease and require liver transplantation.

1.6.1 PFIC1

PFIC1 results from mutations in the gene encoding for ATP8B1 (Bull, van Eijk et al. 1998). ATP8B1 is a flippase located on the canalicular membrane, which translocates PE and phosphatidylserine from the outer to the inner leaflet of the membrane bilayer (Srivastava 2014). ATP8B1 is required to maintain membrane asymmetry of PC and PE as large quantities of PC are flipped to the outer leaflet by ABCB4/MDR3. Cells and mice which lack both ATP8B1 and ABCB4/MDR3 maintain canalicular integrity and have improved liver health, respectively, demonstrating a critical role for phospholipid asymmetry (Groen, Romero et al. 2011). ATP8B1 is also necessary for microvillus

development as lack of ATP8B1 leads to a widening of the canalicular lumen, loss of microvillus, and granular bile (Paulusma, Groen et al. 2006)

1.6.2 PFIC2

PFIC2 results from mutations in the gene *ABCB11* encoding for BSEP, which is the major transporter of BAs into bile (Strautnieks, Bull et al. 1998, Wang, Liu et al. 2013). *Abcb11*$^{-/-}$ mice have impaired secretion of BAs, which results in hepatocellular BA accumulation (Zhang, Li et al. 2012, Wang, Liu et al. 2013). BA are cytotoxic and can induces apoptosis and necrosis. PFIC2 patients suffer from high plasma BA concentrations due to hepatocyte injury (Nicolaou, Andress et al. 2012). Interestingly, *Abcb11*$^{-/-}$ mice do not develop cholestasis as severely as humans. This may be due to an increase in tetrahydroxylated BAs species, which are normally not present in bile, which can be secreted into bile by an alternate BA transporter (Wang, Salem et al. 2001, Lam, Wang et al. 2005).

1.6.3 PFIC3

PFIC3 results from mutations in the gene *ABCB4* encoding for the PC floppase ABCB4/MDR3 (Smit, Schinkel et al. 1993). This leads to a complete absence of PC in bile and biliary toxicity. PC is hydrophilic and is necessary to prevent cells from BA toxicity. In addition, biliary PC secretion is tightly associated with, and necessary for, biliary cholesterol secretion (Oude Elferink, Ottenhoff et al. 1996).

1.7 Thesis objectives

PEMT is quantitatively important in the liver and is responsible for approximately 30% of hepatic PC synthesis, with the remaining 70% synthesized by the CDP-choline

pathway (DeLong, Shen et al. 1999). HFD-fed *Pemt*$^{-/-}$ mice and L*Pcyt1a*$^{-/-}$ mice both develop NAFLD associated with reduced VLDL secretion and a reduced hepatic PC:PE molar ratio (Noga, Zhao et al. 2002, Ling, Chaba et al. 2012). Interestingly, the severity of NAFLD was greater in HFD-fed *Pemt*$^{-/-}$ mice, which was associated with a greater decrease in the hepatic PC:PE molar ratio, compared to L*Pcyt1a*$^{-/-}$ mice (Ling, Chaba et al. 2012). *Pemt*$^{-/-}$ mice are also protected from DIO and IR but L*Pcyt1a*$^{-/-}$ mice are not suggesting a role for PEMT, beyond PC deficiency, in mediating these effects. However, dietary supplementation of choline into the HFD ameliorates the protection against DIO and IR and improves NAFLD (Jacobs, Zhao et al. 2010). Aside from the role of PC in VLDL secretion, a large proportion of PC is also secreted into bile (Walkey, Yu et al. 1998). We studied the effects of hepatic PEMT deficiency and PC:PE imbalance in biliary secretion, resistance to DIO and IR, and NAFLD development during over nutrition. The outcomes of these studies will aid delineating the physiological roles of hepatic PEMT in cholestasis and NAFLD, and contribute to potential therapeutic strategies.

1.7.1 Objective 1: Study the role of PEMT in biliary secretion under HFD conditions

PC is a large constituent of bile with the equivalent of the entire hepatic pool of PC secreted into bile within 24 hours (Nicolaou, Andress et al. 2012). PEMT has been found to be enriched in portions of the ER which lie in close proximity to the canalicular membrane – the site of bile secretion (Sehayek, Wang et al. 2003). Our lab has previously studied bile secretion in chow fed *Pemt*$^{-/-}$ and *Pemt*$^{+/+}$ mice and found no differences in the secretion of BAs or PC (Verkade, Havinga et al. 2007). However, the importance of asymmetrical phospholipid distribution on the canalicular membrane is critical for proper biliary function, as demonstrated in Atp8b1 deficient mice (Groen, Romero et al. 2011).

We hypothesized that HFD feeding, which further alters the hepatic PC:PE molar ratio and impairs membrane integrity, might attenuate biliary secretion processes and lead to the development of cholestasis in *Pemt*$^{-/-}$ mice. **Thus, the first objective was to study the role of PEMT in biliary secretion under HFD conditions.**

1.7.2 Objective 2: Determine if the hepatic PC:PE molar ratio/ PC availability *or* PEMT-derived PC was critical for biliary processes

HFD-fed *Pemt*$^{-/-}$ mice develop cholestasis associated with a decreased hepatic PC:PE molar ratio (Wan, Kuipers et al. 2019). It has been suggested that PEMT, residing in close proximity to the canalicular membrane, may directly provide PC for biliary secretion and is necessary to maintain the membrane integrity of the canaliculus (Wan, Kuipers et al. 2019). Dietary choline supplementation improves, but does not normalize, the hepatic PC:PE molar ratio and is able to both prevent and treat cholestasis in *Pemt*$^{-/-}$ mice (Wan, Kuipers et al. 2019). **The second objective was to determine if the hepatic PC:PE molar ratio/ PC availability *or* PEMT-derived PC was critical for biliary processes.**

1.7.3 Objective 3: Investigate the metabolic effects of *hepatic* PEMT deficiency in lean and obese mice

PEMT is quantitatively important in the liver but small amounts of PEMT protein have been detected in other tissues such as white adipose tissue, testis, or kidneys (Vance 2014, Watanabe, Nakatsuka et al. 2014). *Pemt*$^{-/-}$ mice are protected against DIO and IR but develop NAFLD (Jacobs, Zhao et al. 2010). Additionally, reduction of hepatic PEMT reduces the PC:PE molar ratio and alleviates ER stress, thereby improving liver

health, in *ob/ob* mice (Fu, Yang et al. 2011). **The third objective was to investigate the metabolic effects of *hepatic* PEMT deficiency in lean and obese mice.**

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Chapter 2

Impaired Hepatic Phosphatidylcholine Synthesis Leads to Cholestasis in Mice Challenged with a High-Fat Diet

2.1 Introduction

Phosphatidylethanolamine *N*-methyltransferase (PEMT) converts phosphatidylethanolamine (PE) to phosphatidylcholine (PC) in the liver and accounts for approximately 30% of hepatic PC synthesis (DeLong, Shen et al. 1999). The remaining 70% of hepatic PC is synthesized from choline through the CDP choline pathway(DeLong, Shen et al. 1999). Our laboratory has previously demonstrated that *Pemt*$^{-/-}$ mice fed a high-fat diet (HFD) are protected from diet-induced obesity and insulin resistance(Jacobs, Zhao et al. 2010). However, HFD-fed *Pemt*$^{-/-}$ mice also develop nonalcoholic fatty liver disease (NAFLD), largely due to impairment in very low-density lipoprotein (VLDL) secretion associated with insufficient availability of PC (Noga, Zhao et al. 2002, Jacobs, Zhao et al. 2010). PEMT deficiency decreases the hepatic PC:PE molar ratio, which impairs membrane integrity and results in endoplasmic reticulum (ER) stress and liver disease (Li, Agellon et al. 2006, Jacobs, Zhao et al. 2010, Ling, Chaba et al. 2012, Gao, van der Veen et al. 2015, van der Veen, Kennelly et al. 2017). Long term HFD feeding in *Pemt*$^{-/-}$ mice leads to progression of hepatic steatosis into nonalcoholic steatohepatitis (NASH) and fibrosis (van der Veen, Lingrell et al. 2017). Interestingly, these effects can largely be prevented by dietary choline supplementation (CS) (Jacobs, Zhao et al. 2010).

The equivalent of the entire hepatic pool of PC is secreted into the bile within 24 hours (approximately 23 mg/day/20g mouse) along with bile acids (BAs) and cholesterol (Walkey, Yu et al. 1998, Nicolaou, Andress et al. 2012). Biliary PC is essential for protection of cells lining the biliary tree from the cytotoxic actions of high concentrations of biliary BA (Smit, Schinkel et al. 1993). BAs, exclusively synthesized from cholesterol

in the liver, are secreted into bile by the bile salt export protein (ATP Binding Cassette Subfamily B Member 11[ABCB11]/bile salt export protein [BSEP]), PC by the flippase ATP Binding Cassette Subfamily B Member 4 (ABCB4)/Multi-drug Resistance 2 (MDR2), and cholesterol by the ABCG5/ABCG8 heterodimer (Smit, Schinkel et al. 1993, Wang, Liu et al. 2013). ATPase Phospholipid Transporting 8B1 (ATP8B1) maintains phospholipid asymmetry of the canalicular membrane that is essential for hepatobiliary transport. Mutations or defects in the above proteins can result in the development of cholestasis—an impairment in secretion of bile and biliary constituents. In humans, mutations in genes encoding ATP8B1, BSEP (*ABCB11*), or ABCB4 result in progressive familial intrahepatic cholestasis (PFIC) types 1, 2 and 3, respectively (Nicolaou, Andress et al. 2012). BAs are effectively maintained in the enterohepatic circulation with only 5% of BAs lost per cycle in the feces, which is the major pathway for cholesterol elimination (Ridgway and McLeod 2016). BAs can activate the farnesoid X receptor (FXR) in both the intestine and liver to maintain hepatic BA homeostasis, i.e., decrease hepatic BA synthesis and uptake while increasing BA secretion (Inagaki, Choi et al. 2005, Fiorucci and Distrutti 2015).

Although PEMT is present throughout the ER, this protein is enriched in portions of the ER that are in close proximity to the canalicular membrane (Sehayek, Wang et al. 2003). We previously observed no differences in bile formation between *Pemt*$^{+/+}$ and *Pemt*$^{-/-}$ mice fed a chow diet (Agellon, Walkey et al. 1999). However, we hypothesized that HFD feeding, which decreases hepatic PC content and reduces membrane integrity in *Pemt*$^{-/-}$ mice, might attenuate biliary secretion processes and thereby induce aspects of cholestasis (Li, Agellon et al. 2006, Jacobs, Zhao et al. 2010). We report that, upon

HFD feeding, PEMT deficiency indeed leads to hallmarks of cholestasis. Dietary choline was able to moderately increase hepatic PC availability and to both treat and prevent the development of cholestasis in HFD-fed *Pemt*$^{-/-}$ mice. Hence, maintaining an adequate supply of hepatic PC is critical for proper biliary secretion.

2.2 Materials and Methods

2.2.1 Animals

All procedures were approved by the University of Alberta's Institutional Animal Care Committee in accordance with guidelines of the Canadian Council on Animal Care. All animals were exposed to a 12-hour light/dark cycle and had free access to drinking water. Male *Pemt*$^{+/+}$ and *Pemt*$^{-/-}$ mice (backcrossed into C57Bl/6 for 7 generations, 6 animals per group), were 8 to 10 weeks old at the start of the study. For 2 to 10 weeks, they were fed a standard chow diet (LabDiet, No. 5001) or a semi-synthetic HFD (catalog No. F3282, Bio-Serv, Flemington, NJ) that contained 60 kcal% from lard and 1.3 g/kg choline chloride. Some mice were fed the HFD supplemented with choline chloride (4g/kg diet; Sigma-Aldrich, St Louis, MO) for 10 weeks. A different set of *Pemt*$^{+/+}$ and *Pemt*$^{-/-}$ mice were placed on the HFD for 6 weeks. At this time, half of the animals were either given the CSHFD or continued on the HFD for an additional 6 weeks. Body weight was monitored weekly during the experiments. Gall bladders were cannulated, and bile was collected for 30 minutes as described (Plosch, van der Veen et al. 2006). Tauroursodeoxycholic acid (Calbiochem) infusions were performed on *Pemt*$^{+/+}$ and *Pemt*$^{-/-}$ mice fed the HFD for 10 weeks as described (Plosch, van der Veen et al. 2006). In short, tauroursodeoxycholic acid was infused into the jugular vein with

stepwise increases in infusion rates (0 -600 nmol/min; 30 minutes per step), during which bile was continuously collected. Animals were not fasted prior to collection of blood by cardiac puncture. Tissues were collected, snap-frozen in liquid nitrogen, and stored at −80^{0}C until further analyses. Samples for histological evaluation were fixed in formalin and subjected to anti-BSEP antibody (PB9414, BosterBio, California) staining. For electron microscopy, samples were preserved in a 3% glutaraldehyde, 3% paraformaldehyde in 0.1 M sodium cacodylate buffer, stored overnight at 4 °C, and analyzed the following morning.

2.2.2 Analytical procedures

Hepatic triacyglycerols (TGs) were measured by a commercially available kit from Roche Diagnostics. Hepatic PC and PE were isolated by thin-layer chromatography and quantified by the phosphorous assay. Plasma alanine amino transferase activity (ALT) was measured using a commercially available kit from Biotron Diagnostics.

For immunoblotting, livers were homogenized in buffer (100 mM Tris–HCl, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, and 0.1 mM PMSF, pH 7.4) containing a protease inhibitor cocktail from Sigma Aldrich (P8340). Proteins were transferred to a polyvinylidene difluoride membrane. Membranes were probed with CCAAT/-enhancer-binding protein homologous protein (catalog No. 2895, Cell Signaling, Beverly, MA), Binding immunoglobulin protein (BiP)/ 78 kDa Glucose regulated protein (GRP78) (catalog No. 3183, Cell Signaling, Beverly, MA), BSEP (catalog No. PB9414, Boster), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (catalog No. AM4300, Ambion), and tubulin (catalog No. T6199, Sigma). Proteins were visualized by an enhanced

chemiluminescence system (Amersham Biosciences, Piscataway, NJ) and quantified using G:Box (Syngene, Cambridge, UK) software. RNA isolation, complementary DNA (cDNA) synthesis, and real-time quantitative polymerase chain reaction were performed as described (Jacobs, Zhao et al. 2010). Messenger RNA (mRNA) levels were normalized to cyclophilin. Secretion of biliary components as well as BA pool composition were determined by described methods (Plosch, van der Veen et al. 2006).

2.2.3 Statistical analysis

Data were analyzed with GraphPad Prism software (GraphPad, La Jolla, CA). All values are means ± SEM. For comparison of groups, 2-way analysis of variance (ANOVA) with Fisher's least significant difference *post hoc* test was used. To compare genotypes on the same feeding regimen, a Student *t* test was used. Level of significance of differences was set at $P < 0.05$. All groups were n = 6.

2.3 Results

2.3.1 *Pemt*$^{-/-}$ mice fed the HFD develop NASH

After 10 weeks of HFD feeding, body weight was lower in *Pemt*$^{-/-}$ mice compared to *Pemt*$^{+/+}$ mice (Fig. 2.1). However, liver weight was dramatically elevated in *Pemt*$^{-/-}$ mice compared to *Pemt*$^{+/+}$ mice (liver weight: 2.76 vs. 1.24 g, respectively) due to increase hepatic TG accumulation (Fig. 2.2 A,B). This steatosis in *Pemt*$^{-/-}$ mice occurred concomitantly with a reduction in the hepatic PC:PE molar ratio, arising from decreased PC and increased PE (Fig. 2.2 C,D). Plasma ALT, a marker for liver damage, was significantly elevated in *Pemt*$^{-/-}$ mice compared to *Pemt*$^{+/+}$ mice (Fig. 2.2 E). These changes in hepatic lipids and plasma ALT developed acutely, i.e., within 2 weeks of the *Pemt*$^{-/-}$ mice being fed the HFD (Fig. 2.3 A-E, K).

Figure 2.1

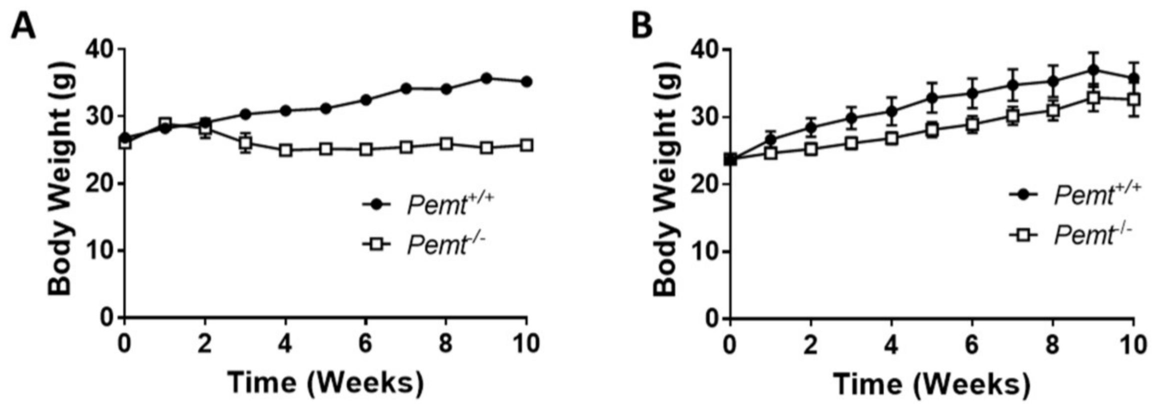

Figure 2.1 *Pemt*$^{-/-}$ mice gain weight on the CSHFD but not the HFD.

Body weight of *Pemt*$^{+/+}$ and *Pemt*$^{-/-}$ mice fed the (A) HFD or (B) CSHFD for 10 weeks.

Values are means ± SEM (n = 6 per group).

Figure 2.2

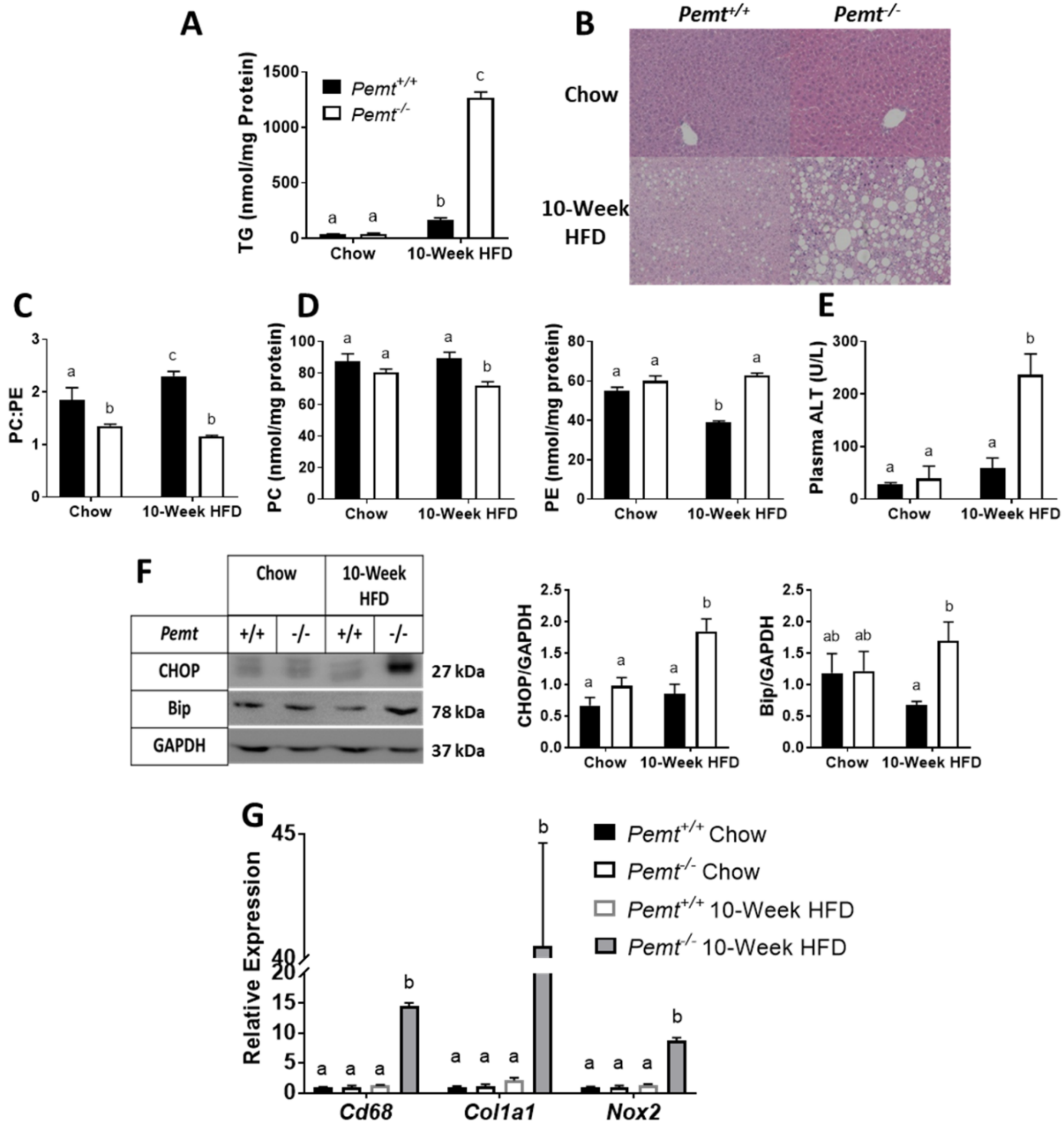

Figure 2.2 *Pemt$^{-/-}$* mice develop NASH when fed the HFD.

Pemt$^{+/+}$ and *Pemt$^{-/-}$* mice were fed the chow diet or the HFD for 10 weeks. Hepatic (A) TG mass. (B) Representative hematoxylin and eosin staining of livers of *Pemt$^{+/+}$* and *Pemt$^{-/-}$* mice fed either the chow diet or HFD for 10 weeks. 20X magnification. (C) PC:PE molar ratio. (D) Mass of PC and PE. (E) Plasma ALT levels. Values are means ± SEM (n = 6 per group). (F) Bip and ER stress responsive C/EBP homologous protein (Schmitt, Kong et al.) and densitometry. Values are means ± SEM (n = 4 per group). (G) mRNA expression of genes involved in inflammation (*Cd68*), fibrosis (*Col1a1*), and oxidative stress (*Nox2*). Values are means ± SEM (n = 5 per group) and are expressed relative to *Pemt$^{+/+}$* mice fed the chow diet. 2-way ANOVA, followed by Fisher's LSD *post hoc* test. Values that do not share a letter are significantly different ($P < 0.05$).

Figure 2.3

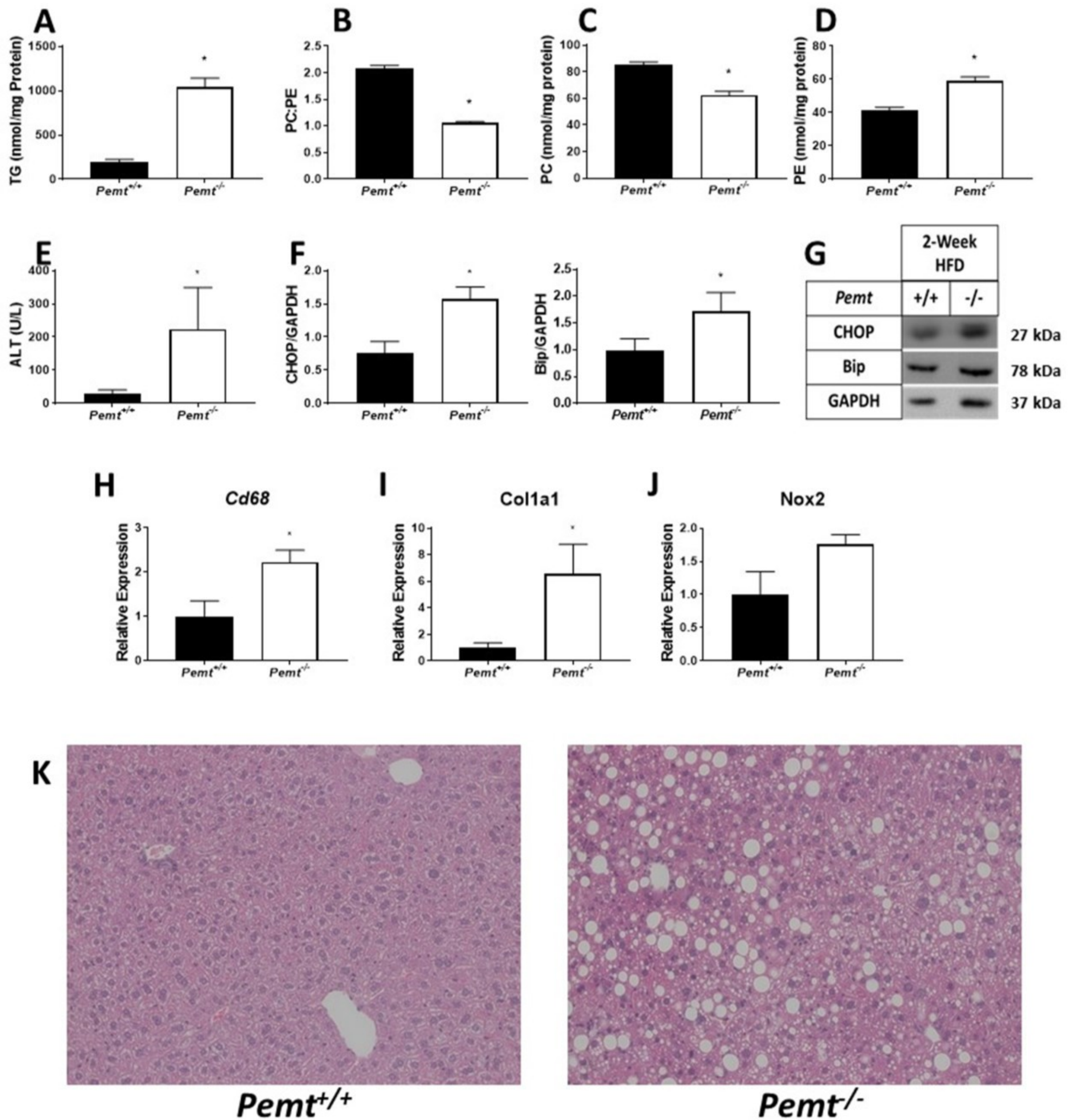

Figure 2.3 *Pemt$^{-/-}$* mice develop NASH on a 2-week HFD.

Pemt$^{+/+}$ and *Pemt$^{-/-}$* mice were fed an HFD for 2 weeks. Hepatic (A) TG, (B) PC:PE ratio, (C) PC, (D) PE, and (E) plasma ALT levels were measured. (G) Representative immunoblots for hepatic CHOP and Bip and (F) protein quantification. Values are means ± SEM (n = 6 per group). Hepatic mRNA levels of genes involved in (H) inflammation, (I) fibrosis, and (J) oxidative stress normalized to cyclophilin mRNA.

The amount of CHOP and BiP, markers for ER stress, were significantly elevated in HFD-fed *Pemt*$^{-/-}$ mice compared to *Pemt*$^{+/+}$ mice (Fig. 2.2 F). Hepatic mRNA levels of cluster of differentiation 68 (*Cd68*), collagen type I alpha 1 chain (*Col1a1*), and NADPH oxidase (*Nox2*)—markers for inflammation, fibrosis, and oxidative stress, respectively—were all significantly elevated in HFD-fed *Pemt*$^{-/-}$ mice compared to *Pemt*$^{+/+}$ mice (Fig. 2.2G). After 2 weeks of the HFD, ER stress, inflammation, and fibrosis were all significantly increased in *Pemt*$^{-/-}$ mice compared to *Pemt*$^{+/+}$ mice (Fig. 2.3 F-J). When the *Pemt*$^{-/-}$ mice were fed the chow diet, despite a lower hepatic PC:PE molar ratio compared to that in *Pemt*$^{+/+}$ mice, there were no differences in hepatic TG content or liver health between genotypes (Fig. 2.2B).

2.3.2 HFD-fed *Pemt*$^{-/-}$ mice develop cholestasis

Previous studies have shown that bile flow and biliary secretion of BAs, PC, and cholesterol are not different between *Pemt*$^{+/+}$ mice and *Pemt*$^{-/-}$ mice fed a chow diet (Verkade, Havinga et al. 2007). Yet, under HFD-fed conditions, *Pemt*$^{-/-}$ mice had a markedly lower rate of bile flow as well as lower biliary secretion rates of BA and PC than *Pemt*$^{+/+}$ mice (Fig. 2.4 A,B). This impairment in biliary secretion in *Pemt*$^{-/-}$ mice resulted in a significant increase in plasma BA concentrations, indicative of cholestasis (Fig. 2.4C). The impairment in biliary BAs and PC secretion was not associated with reduced biliary cholesterol secretion in *Pemt*$^{-/-}$ mice (Fig. 2.4D). Although biliary cholesterol secretion is usually coupled to BA and PC secretion, there are conditions under which it can be uncoupled (Kosters, Frijters et al. 2005, Morgado, Rigotti et al. 2005, Di Ciaula, Wang et al. 2014). Plasma total bilirubin, a marker for the onset of cholestasis, was significantly higher in *Pemt*$^{-/-}$ mice than in *Pemt*$^{+/+}$ mice (Fig. 2.4F)

(Mesa, De Vos et al. 1997). *Pemt*$^{-/-}$ mice fed the chow diet or the HFD for 2 weeks did not exhibit a reduced biliary BA secretion rate or an elevation in plasma BA concentration (Fig. 2.4 A-C; Fig. 2.5 A-E). However, plasma total bilirubin was increased in *Pemt*$^{-/-}$ mice after 2 weeks of HFD feeding (Fig. 2.5F). This indicates an early impairment in biliary elimination of bilirubin, which may suggest early signs of the development of cholestasis.

To determine the maximal secretory rates of BA, we infused increasing amounts of tauroursodeoxycholic acid (TUDCA) into the jugular vein of gallbladder-cannulated *Pemt*$^{+/+}$ and *Pemt*$^{-/-}$ mice fed the HFD for 10 weeks. As expected, the secretion rates of BAs, PC, and cholesterol were increased upon TUDCA infusion in *Pemt*$^{+/+}$ mice. However, these increases were much less pronounced in the *Pemt*$^{-/-}$ mice (Fig. 2.4E). Consequently, *Pemt*$^{-/-}$ mice were not able to tolerate the highest rate of TUDCA infusion and perished (Fig. 2.4E).

Figure 2.4

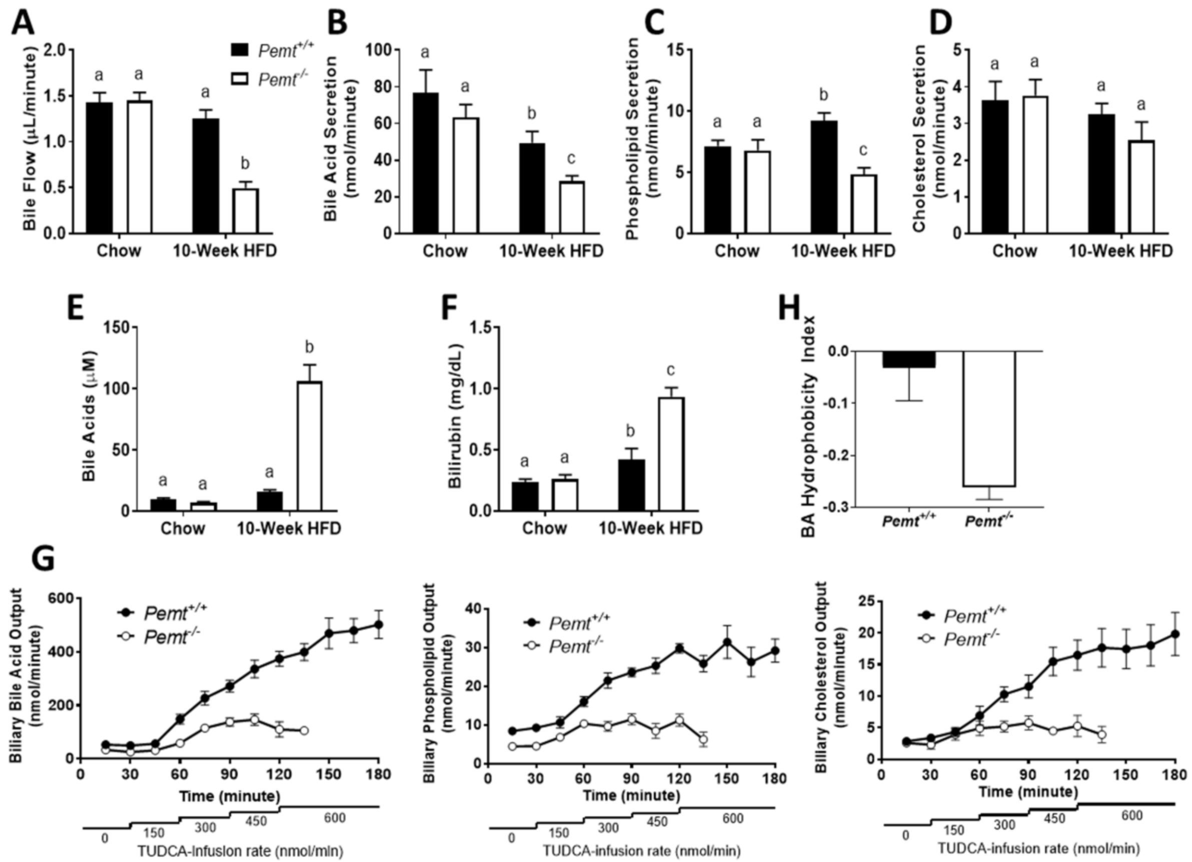

Figure 2.4 *Pemt*$^{-/-}$ mice develop diet-induced cholestasis.

Pemt$^{+/+}$ and *Pemt*$^{-/-}$ mice were fed the chow diet or the HFD for 10 weeks. Basal biliary (A) bile flow, and secretion of (B) bile acids, (C) phospholipids, and (D) cholesterol. Plasma (E) bile acids and (F) total bilirubin concentration. (G) Maximal biliary secretion of bile acids, phospholipids, and cholesterol after taurodeoxycholic acid (TUDCA) infusion in mice fed the HFD for 10 weeks. (H) Hydrophobicity of the bile acid pool after 10 weeks of HFD. Values are means ± SEM (n = 6 per group). 2-way ANOVA, followed by Fisher's LSD *post hoc* test. Values that do not share a letter are significantly different ($P < 0.05$).

Figure 2.5

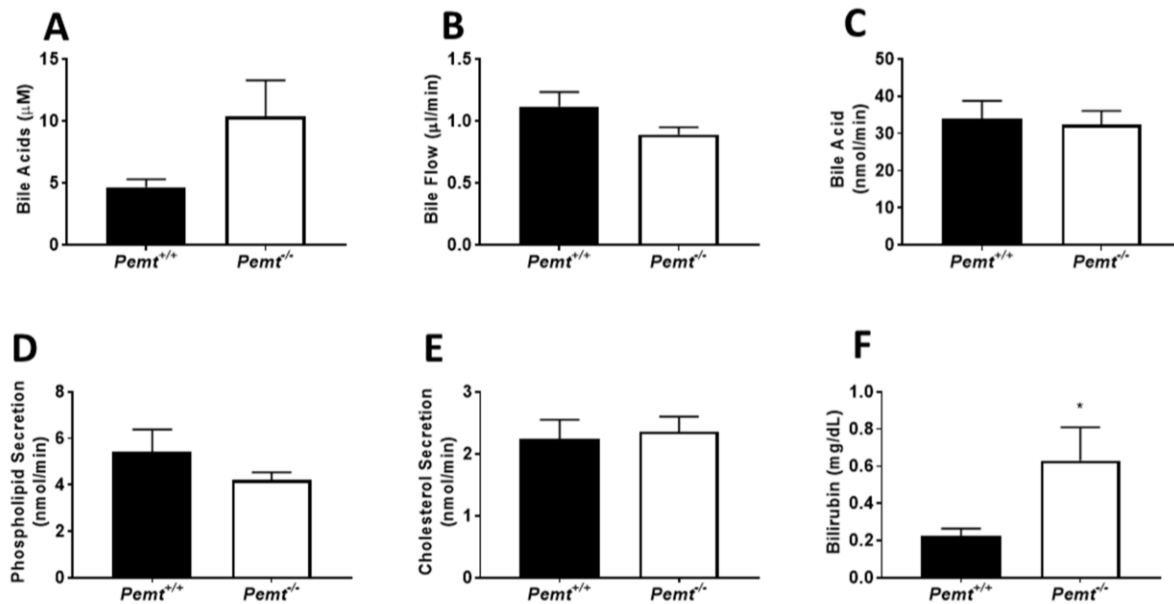

Figure 2.5 Biliary secretion is not impaired after 2 weeks of HFD in *Pemt*$^{-/-}$ mice.

Pemt$^{+/+}$ and *Pemt*$^{-/-}$ mice were fed an HFD for 2 weeks. (A) Plasma bile acid concentration. Basal biliary (B) bile flow, (C) bile acid, (D) phospholipid, and (E) cholesterol secretion. (F) Plasma total bilirubin concentration. Values are means ± SEM (n = 6 per group). Statistics were determined by Student *t* test. *$P < 0.05$.

2.3.3 Decreased hydrophobicity of the BA pool in cholestatic *Pemt$^{-/-}$* mice

In *Pemt$^{+/+}$* and *Pemt$^{-/-}$* mice fed the HFD for 10 weeks, we also determined the composition of the BA pool. The relative plasma concentrations of tauro-β muricholic acid, β-muricholic acid, taurocholic acid, and TUDCA were higher in *Pemt$^{-/-}$* mice than in *Pemt$^{+/+}$* mice, whereas the relative amounts of cholic acid and UDCA were lower (Table 2.1; Fig. 2.6). These changes resulted in a significant decrease in the hydrophobicity of the plasma BA pool in *Pemt$^{-/-}$* mice compared to *Pemt$^{+/+}$* mice (Fig. 2.2F). Consequently, this shifted the BA pool to a less toxic nature by reducing its detergent potency. These changes may represent a compensatory mechanism to reduce BA-induced cytotoxicity in *Pemt$^{-/-}$* mice (Sagawa, Tazuma et al. 1993, Araki, Andoh et al. 2003).

2.3.4 Disruption of genes involved in BA homeostasis in *Pemt$^{-/-}$* mice

When fed a chow diet, the only difference in the expression of genes involved in BA homeostasis was a slightly higher level of multidrug resistance-associated protein (*Mrp2*) mRNA in *Pemt$^{-/-}$* mice compared to *Pemt$^{+/+}$* mice (Fig. 2.7A). After 10 weeks of HFD feeding, mRNA levels of hepatic genes related to BA synthesis (cytochrome P450 family 8 subfamily B member 1[*Cyp8b1*], cytochrome P450 family 27 subfamily A member 1 [*Cyp27a1*]), biliary BA secretion (*Abcb11*), PC secretion (*Abcb4*), and BA import from the circulation (sodium-taurocholate co-transporting polypeptide [*Ntcp*], organic anion co-transporting polypeptide [*Oatp1*]) were all significantly lower in *Pemt$^{-/-}$* mice than in *Pemt$^{+/+}$* mice (Fig. 2.7B). mRNA levels of *Fxr*, the "master regulator" of BA homeostasis, and its effector, small heterodimer partner (*Shp*), were also lower in *Pemt$^{-/-}$* mice than in *Pemt$^{+/+}$* mice (Fig. 2.7B) (Koutsounas, Theocharis et al. 2015).

Table 2.1

Supporting TABLE S1. Plasma concentrations of BA species (umol/L).

Bile Acid Species	*Pemt*$^{+/+}$ Mice	*Pemt*$^{-/-}$ Mice
UDCA	0.050 ± 0.042	0.269 ± 0.262*
CA	0.224 ± 0.017	6.615 ± 7.31*
TUDCA	0.089 ± 0.146	1.204 ± 0.377*
TCA	0.866 ± 1.31	40.4 ± 13.5*
CDCA	0.010 ± 0.023	0.133 ± 0.98*
DCA	0.155 ± 0.143	0.573 ± 0.332*
TCDCA	0.049 ± 0.121	0.666 ± 0.228*
TDCA	0.147 ± 0.090	0.599 ± 0.312*
α-MCA	N.D.	0.272 ± 0.239
β-MCA	0.327 ± 0.167	18.6 ± 15.0*
Tα-MCA	0.292 ± 0.544	3.96 ± 1.16*
Tβ-MCA	0.521 ± 1.04	30.0 ± 0*

Table 2.1 Plasma bile acid composition.

Pemt$^{+/+}$ and *Pemt*$^{-/-}$ mice were fed an HFD for 10 weeks, and plasma bile acid composition was determined. Values are means (µM) ± SD (n = 6 per group). Statistics were determined by Student *t* test. *$P < 0.05$.

α-MCA, alpha-muricholic acid; β-MCA, beta-muricholic acid; CA, cholic acid; CDCA, chenodeoxycholic acid; DCA, deoxycholic acid; TCA, taurocholic acid; TCDCA, taurodeoxycholic acid; TDCA, taurodeoxycholic acid; TUDCA, tauroursodeoxycholic acid; Tα-MCA, tauroalpha-muricholic acid; Tβ-MCA, taurobeta-muricholic acid; UDCA, ursodeoxycholic acid.

Figure 2.6

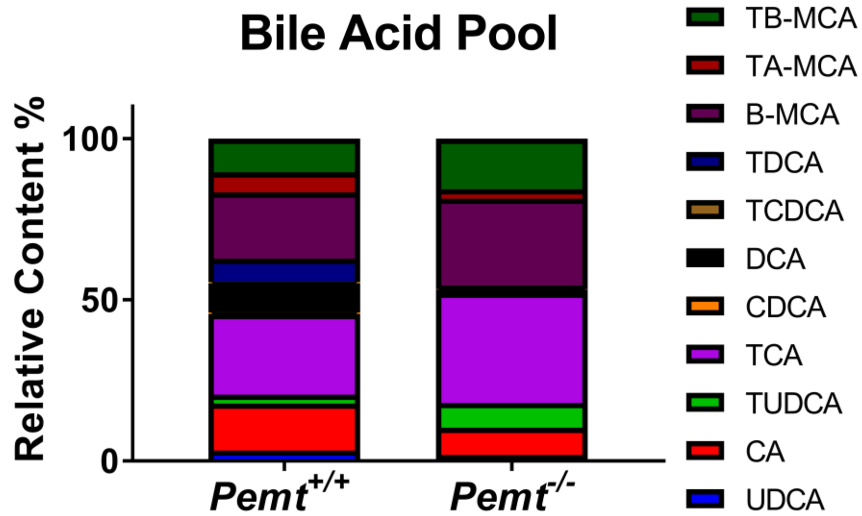

Figure 2.6 Relative composition of bile acid pool.

Relative composition of the plasma bile acid pool.

α-MCA, alpha-muricholic acid; β-MCA, beta-muricholic acid; CA, cholic acid; CDCA, chenodeoxycholic acid; DCA, deoxycholic acid; TCA, taurocholic acid; TCDCA, taurodeoxycholic acid; TDCA, taurodeoxycholic acid; TUDCA, tauroursodeoxycholic acid; Tα-MCA, tauroalpha-muricholic acid; Tβ-MCA, taurobeta-muricholic acid; UDCA, ursodeoxycholic acid.

Figure 2.7

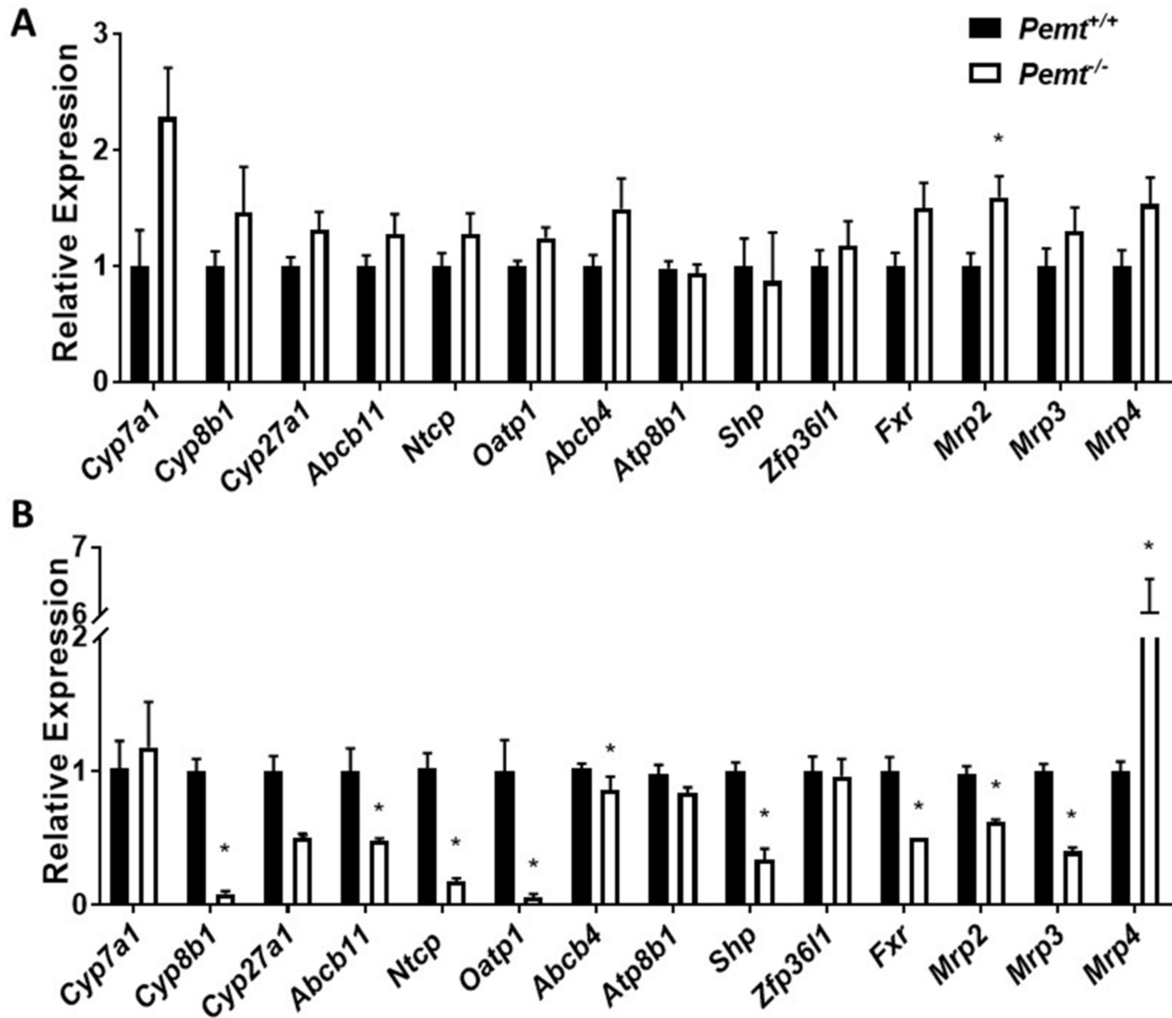

Figure 2.7 Dysregulation of expression of hepatic genes involved in BA homeostasis in *Pemt*$^{-/-}$ mice.

Hepatic mRNA levels of genes involved in bile acid synthesis, biliary secretion and basolateral import of bile acids, biliary secretion of phospholipids, regulators of bile acid synthesis, bile acid homeostasis. and the alternative pathway of bile acid secretion into bile and circulation were determined in *Pemt*$^{+/+}$ and *Pemt*$^{-/-}$ mice fed (A) chow or (B) the HFD and normalized to cyclophilin mRNA levels. Values are expressed relative to *Pemt*$^{+/+}$ mice on the same diet. Values are means ± SEM (n = 5 per group). Student *t* test. *$P < 0.05$.

mRNA levels of zinc finger protein 36 like 1 (*Zfp36l1*), an RNA-binding protein that induces degradation of *Cyp7a1* mRNA, *Cyp7a1,* and *Atp8b1,* were not different between genotypes (Fig. 2.7B) (Tarling, Clifford et al. 2017). *Mrp2, Mrp3,* and *Mrp4* encode proteins that mediate alternative pathways for the secretion of BA metabolites into bile (*Mrp2*) and blood (*Mrp3, Mrp4*). *Mrp2* and *Mrp3* mRNA levels were significantly lower, whereas those of *Mrp4* were massively induced in *Pemt*$^{-/-}$ mice compared to *Pemt*$^{+/+}$ mice (Fig. 2.7B) (Akita, Suzuki et al. 2001). In contrast, the mRNA levels of BA homeostatic genes were not different between *Pemt*$^{-/-}$ and *Pemt*$^{+/+}$ mice fed the HFD for 2 weeks, except for *Oatp1* mRNA, which was lower in *Pemt*$^{-/-}$ mice (Fig. 2.8).

2.3.5 Altered bile canaliculus in *Pemt*$^{-/-}$ mice on HFD

In agreement with *Abcb11* mRNA levels, protein expression of BSEP was significantly lower in *Pemt*$^{-/-}$ mice after 10 (Fig. 2.9A), but not 2, weeks of the HFD (Fig. 2.10). Representative immunohistochemistry slides confirmed that the canaliculus of *Pemt*$^{-/-}$ mice contained smaller amounts of BSEP compared to those of *Pemt*$^{+/+}$ mice (Fig. 2.9B). Interestingly, visualization of the canalicular membrane, through transmission electron microscopy, revealed a marked loss of canalicular structure and surface area and widening of the lumen of the HFD-fed *Pemt*$^{-/-}$ mice compared to the *Pemt*$^{+/+}$ mice (Fig. 2.9C).

Figure 2.8

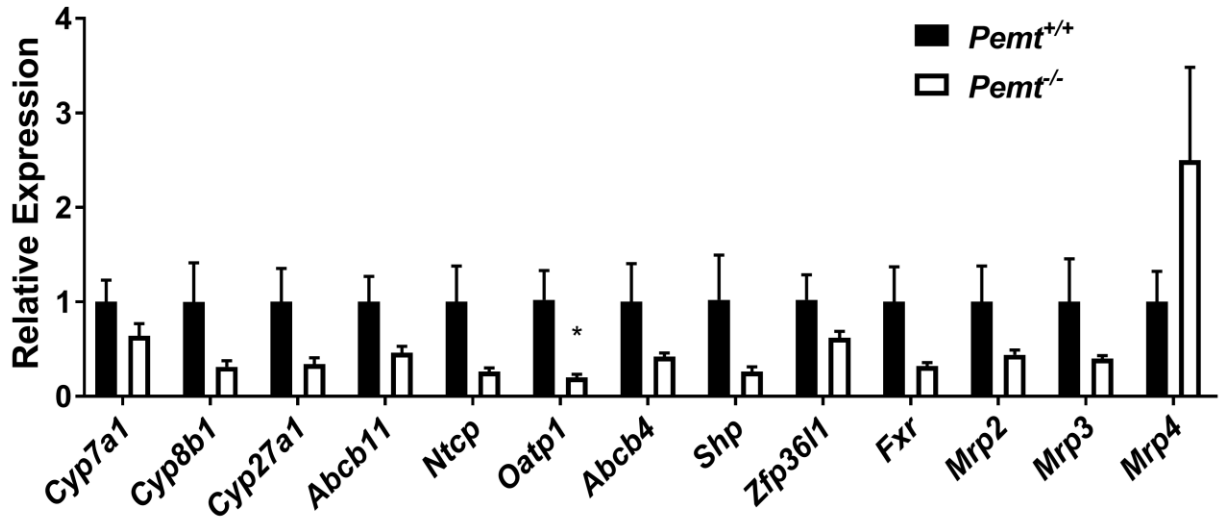

Figure 2.8 Genes related to BA homeostasis are largely unchanged by HFD after 2 weeks.

Pemt$^{+/+}$ and *Pemt*$^{-/-}$ mice were fed an HFD for 2 weeks. Hepatic mRNA levels of genes involved in: bile acid synthesis, biliary secretion of bile acids, basolateral import of bile acids, biliary secretion of phospholipids, and regulators of bile acid synthesis, regulation of bile acid homeostasis, alternative pathway of biliary bile acid secretion, and alternate basolateral BA secretion, normalized to cyclophilin mRNA. Values are expressed relative to *Pemt*$^{+/+}$ mice fed the same diet. Values are means ± SEM (n = 5 per group). To compare groups, a Student *t* test was performed. *$P < 0.05$.

Figure 2.9

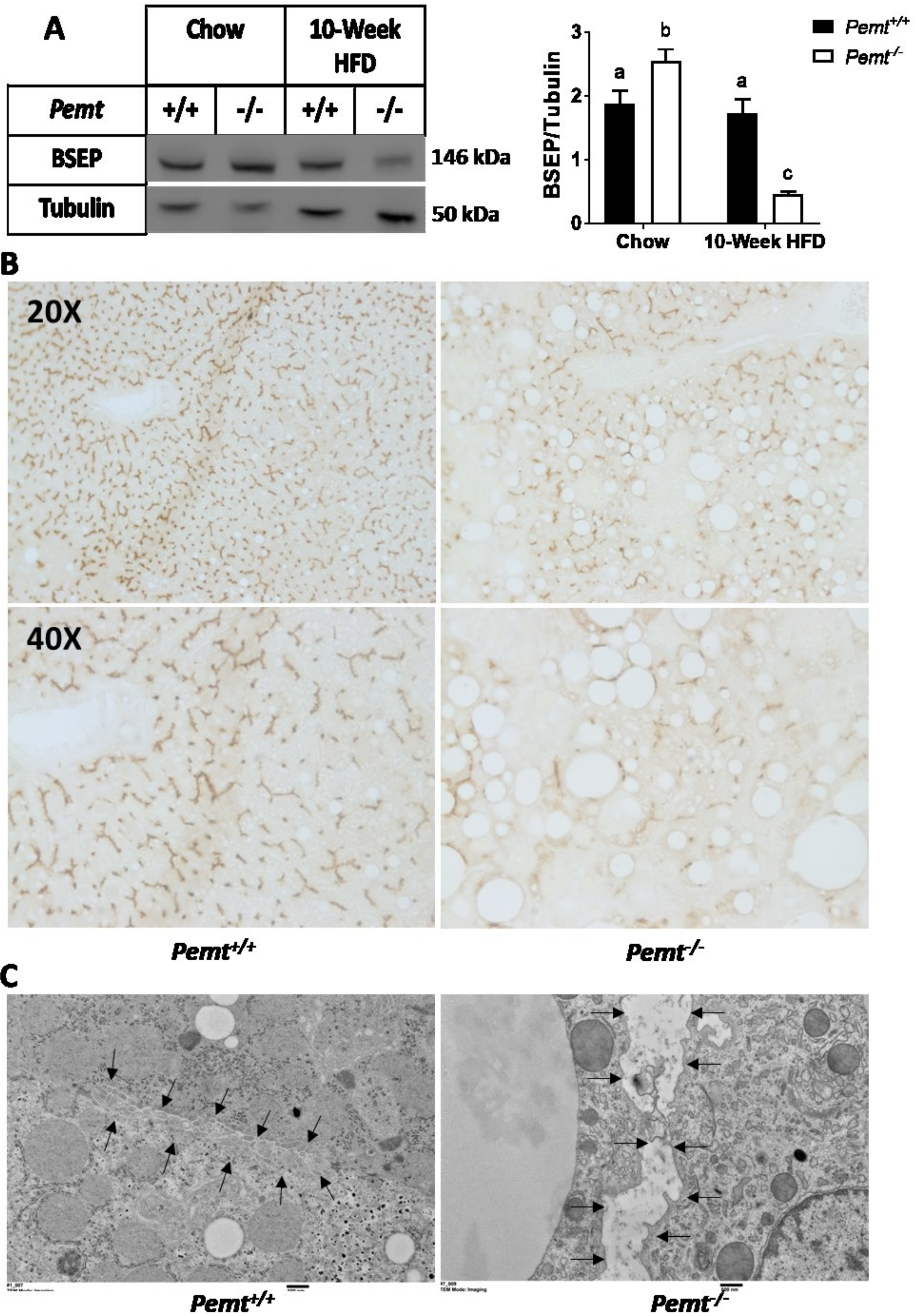

Figure 2.9 Loss of canalicular structure and BSEP deficiency in *Pemt*$^{-/-}$ mice.

(A) Representative immunoblot of hepatic BSEP protein and quantification in *Pemt*$^{+/+}$ and *Pemt*$^{-/-}$ mice fed the chow diet or the HFD for 10 weeks. Values are means ± SEM (n = 6 per group). 2-way ANOVA followed by Fisher's LSD *post hoc* test. Values that do not share a letter are significantly different ($P < 0.05$). Representative (B) immunohistochemistry (above: 20x magnification; below: 40x magnification) for BSEP. (C) Electron microscopy of the canalicular membrane (6,000x magnification) in livers of *Pemt*$^{+/+}$ and *Pemt*$^{-/-}$ mice fed the HFD for 10 weeks. Arrows outline the edge of the canaliculus.

Figure 2.10

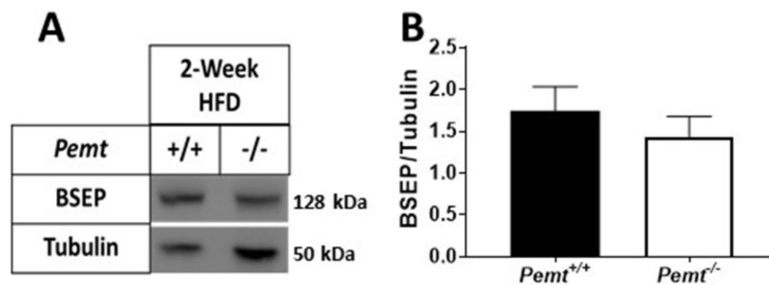

Figure 2.10 . BSEP protein expression not affected after 2 weeks of HFD.

Pemt$^{+/+}$ and *Pemt*$^{-/-}$ mice were fed an HFD for 2 weeks. (A) Representative immunoblot of hepatic BSEP protein and (B) quantification in *Pemt*$^{+/+}$ and *Pemt*$^{-/-}$ mice. Values are expressed relative to *Pemt*$^{+/+}$ mice fed a chow diet. Values are means ± SEM (n = 6 per group). Statistics were determined by Student *t* test. $^{*}P < 0.05$.

2.3.6 CS prevents the development cholestasis in *Pemt*$^{-/-}$ mice

Dietary CS has previously been shown to normalize hepatic PC concentrations in *Pemt*$^{-/-}$ mice to those in wild-type mice (Watkins, Zhu et al. 2003). *Pemt*$^{-/-}$ mice that were fed the CSHFD (3 times normal choline) for 10 weeks did not develop hepatic steatosis, and there was no difference between hepatic PC levels or body weight between genotypes fed the same diet (Fig. 2.1B; Fig. 2.11 A,B,G). However, hepatic PE levels were higher, and thus, the PC:PE ratio was lower, in *Pemt*$^{-/-}$ mice than in *Pemt*$^{+/+}$ mice (Fig. 2.11 C,D). The CSHFD was also unable to normalize plasma ALT levels in *Pemt*$^{-/-}$ mice to those in *Pemt*$^{+/+}$ mice (Fig. 2.11E). However, CHOP and Bip levels as well as mRNA levels of *Cd68, Col1a1,* and *Nox2,* in *Pemt*$^{-/-}$ mice were normalized by the CSHFD (Fig. 2.12 A-C).

Dietary supplementation of *Pemt*$^{-/-}$ mice with choline for 10 weeks normalized plasma BA concentrations, the rate of bile flow, and the amounts of biliary components secreted into bile (Fig. 2.13A-C). Prevention of cholestasis occurred concomitant to a normalization of hepatic Bsep protein levels (Fig. 2.13D). When fed the CSHFD, mRNA levels of BA homeostatic genes were also not different between genotypes (Fig. 2.12D), which is likely related to improved liver health (Figs. 2.11, 2.12).

Figure 2.11

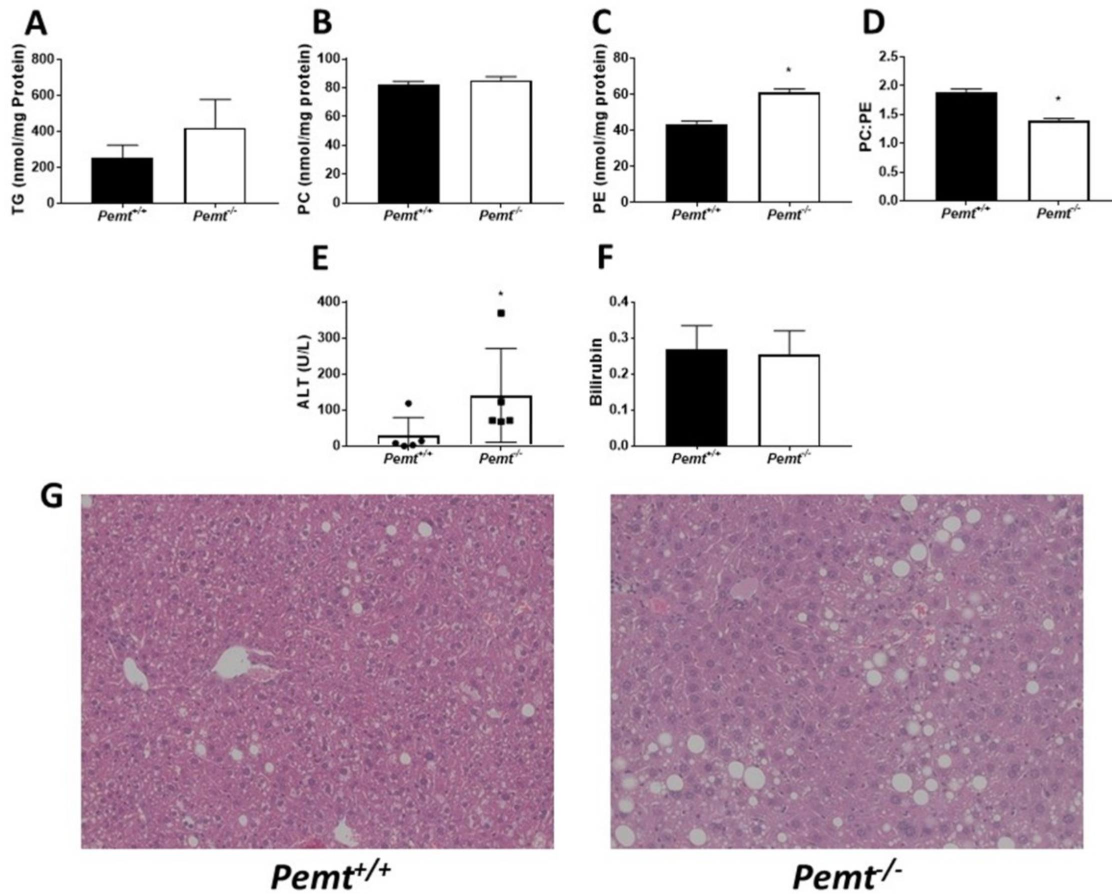

Figure 2.11 Choline-supplemented HFD prevents steatosis and cholestasis in *Pemt*$^{-/-}$ mice.

Pemt$^{+/+}$ and *Pemt*$^{-/-}$ mice were fed a CSHFD for 10 weeks. Hepatic (A) TG, (B) PC, (C) PE, and (D) PC:PE ratio were measured. Plasma (E) ALT and (F) plasma total bilirubin concentration. (G) Representative hematoxylin and eosin staining of livers (20X magnification). Values are means ± SEM (n = 6 per group). Statistics were determined by Student *t* test. Statistics for plasma ALT were determined by a Mann-Whitney *t* test to account for nonparametric distribution of data. *$P < 0.05$

Figure 2.12

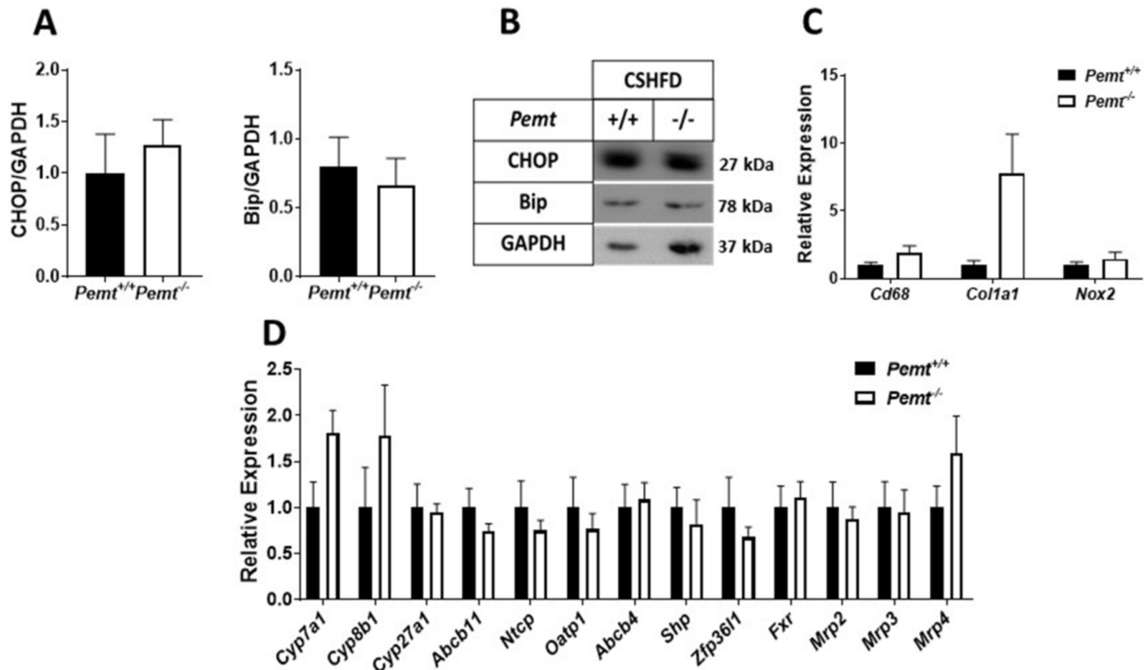

Figure 2.12 Choline-supplemented HFD prevents NASH and alterations in genes and proteins involved in BA homeostasis.

Pemt$^{+/+}$ and *Pemt*$^{-/-}$ mice were fed a CSHFD for 10 weeks. (A) Quantification of (B) representative immunoblots for hepatic CHOP and Bip. (C) Hepatic mRNA levels of genes involved in inflammation, fibrosis, and oxidative stress normalized to cyclophilin mRNA levels from *Pemt*$^{+/+}$ and *Pemt*$^{-/-}$ mice. (D) Hepatic mRNA levels of genes involved in: bile acid synthesis, biliary secretion of bile acids, basolateral import of bile acids, biliary secretion of phospholipids, and regulators of bile acid synthesis regulator of BA homeostasis, alternative biliary BA secretion, and alternative basolateral BA secretion, normalized to cyclophilin mRNA levels from *Pemt*$^{+/+}$ and *Pemt*$^{-/-}$ mice. Values are expressed relative to *Pemt*$^{+/+}$ mice fed a chow diet.

2.3.7 CS resolves cholestasis induced by HFD in *Pemt*$^{-/-}$ mice

Because CS was able to *prevent* cholestasis, we hypothesized that it might also *treat* cholestasis induced by HFD in *Pemt*$^{-/-}$ mice. Therefore, we fed *Pemt*$^{+/+}$ and *Pemt*$^{-/-}$ mice the HFD for 6 weeks. After 6 weeks on the HFD, mice of each genotype were split into two groups and either continued on the HFD for another 6 weeks or fed the CSHFD for 6 weeks. As anticipated, after 6 and 12 weeks of the HFD, plasma BA concentrations were dramatically higher in the *Pemt*$^{-/-}$ mice compared to the *Pemt*$^{+/+}$ mice (Fig. 2.14A). However, supplementation of the HFD with choline after 6 weeks of the HFD normalized plasma BA concentrations in *Pemt*$^{-/-}$ mice (Fig. 2.14A). Concomitant to resolution of cholestasis was a marked improvement in liver health in *Pemt*$^{-/-}$ mice. *Pemt*$^{-/-}$ mice fed the HFD for 12 weeks developed hepatic steatosis, which was improved by CS (Fig. 2.14B). mRNA levels of *Cd68* and *Col1a1* were reduced in *Pemt*$^{-/-}$ mice with CS, and levels of *Nox2* were normalized to *Pemt*$^{+/+}$ levels in (Fig. 2.14 F-H). There were no significant changes in hepatic concentrations of PC, PE, or the PC:PE molar ratio between the different dietary regimens, although the PC:PE molar ratio trended to be modestly increased upon CS (Fig. 2.14 C-E). Despite the lack of changes in steady state levels of hepatic phospholipids, CS restored hepatic BSEP and reduced CHOP levels in *Pemt*$^{-/-}$ mice (Fig. 2.14). Surprisingly, Bip levels, another marker of ER stress, were not different between genotypes under any dietary condition in this experiment (Fig. 2.14I).

Figure 2.13

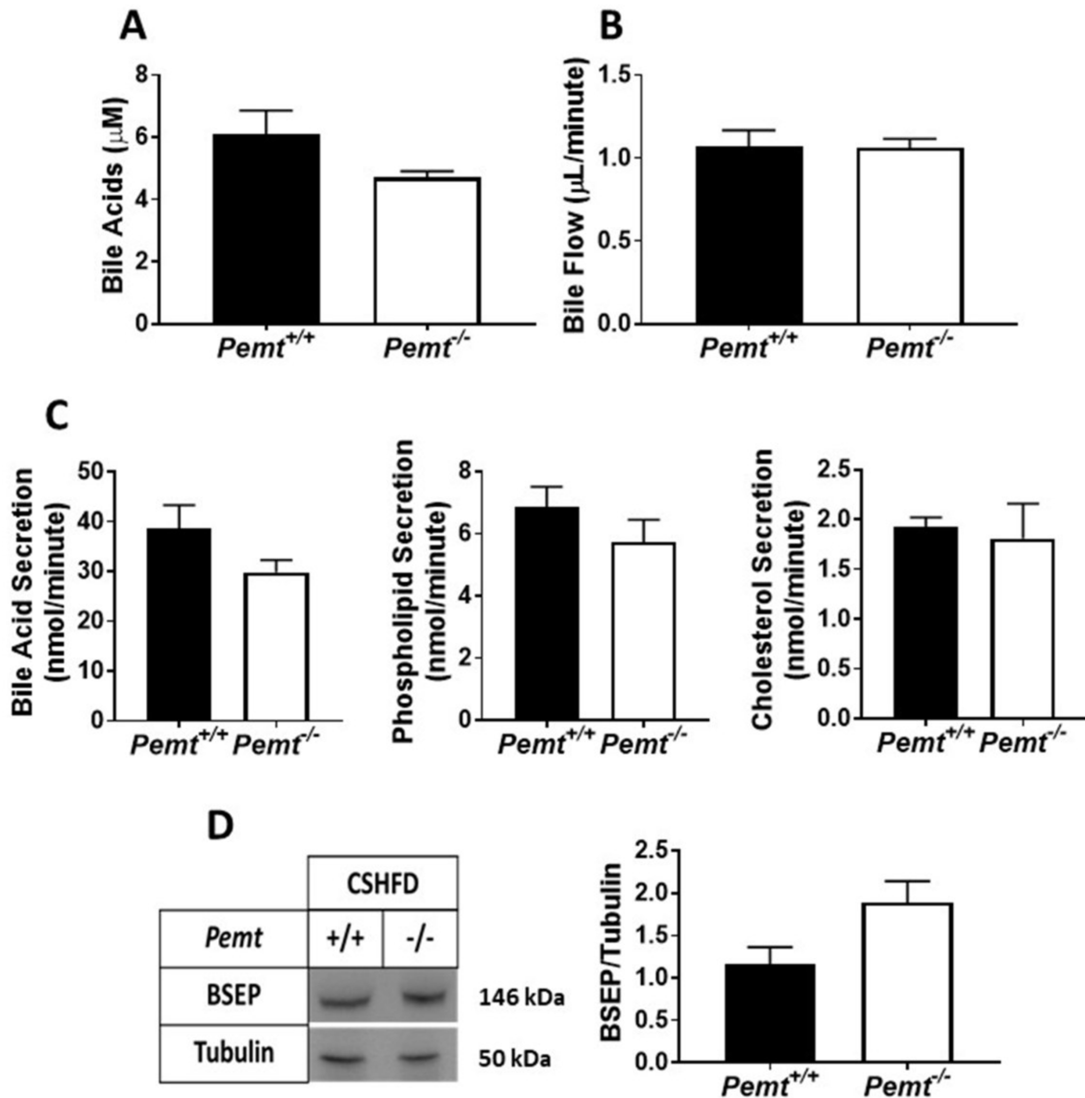

Figure 2.13 Choline supplementation prevents cholestasis in *Pemt*$^{-/-}$ mice.

Pemt$^{+/+}$ and *Pemt*$^{-/-}$ mice were fed the choline-supplemented HFD for 10 weeks. (A) Plasma bile acid concentration. Basal biliary (B) bile flow and (C) bile acid, phospholipid, and cholesterol secretion. (D) Hepatic BSEP protein and quantification, relative to the amount of tubulin. Values are means ± SEM (n = 6 per group). 2-way ANOVA followed by Fisher's LSD *post hoc* test. Values that do not share a letter are significantly different ($P < 0.05$).

Figure 2.14

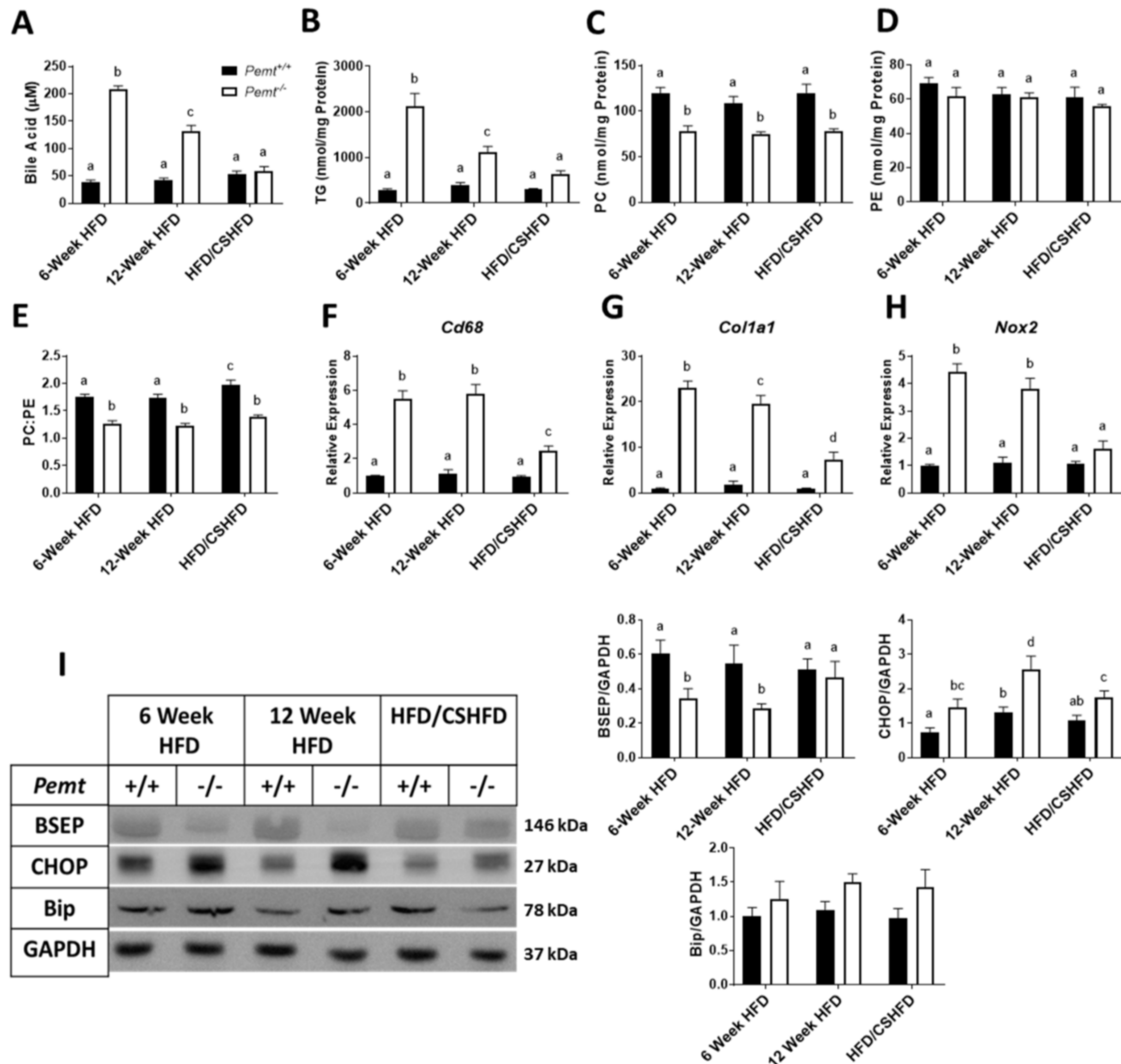

Figure 2.14 Choline supplementation improves liver health and treats cholestasis in *Pemt*$^{-/-}$ mice.

Pemt$^{+/+}$ and *Pemt*$^{-/-}$ mice were fed the HFD for 6 weeks and either continued on the HFD or the choline-supplemented HFD (CSHFD) for an additional 6 weeks. (A) Plasma bile acid concentration. Hepatic (B) TG, (C) PC, (D) PE, and (E) PC:PE ratio. Values are means ± SEM (n = 6 per group). mRNA expression of hepatic genes involved in (F) inflammation, (G) fibrosis, and (H) oxidative stress. Values are means ± SEM (n = 5 per group) (I) Hepatic BSEP, CHOP, and Bip and quantification relative to the amount of GAPDH. Values are means ± SEM (n = 6 per group). 2-way ANOVA followed by Fisher's LSD *post hoc* test. Values that do not share a letter are significantly different ($P < 0.05$).

2.4 Discussion

Pemt$^{-/-}$ mice that are fed the HFD are protected from diet-induced obesity but develop NAFLD, which can progress to NASH (Jacobs, Zhao et al. 2010, Ling, Chaba et al. 2012, van der Veen, Lingrell et al. 2017). We now show that *Pemt$^{-/-}$* mice also develop cholestasis when fed the HFD. This cholestasis can be both prevented and treated by CS. It is interesting to note that the maximal biliary secretory rate is approximately 10-fold higher than physiological BA secretion in mice, demonstrating an impressive mechanism to prevent BA accumulation. However, decreasing PC availability or altering the membrane phospholipid ratio leads to a reduction of the BA secretory capacity and leads to accumulation of BAs in plasma and hepatocytes, which might activate hepatic stellate cells, eventually resulting in fibrosis (Svegliati-Baroni, Ridolfi et al. 2005).

2.4.1 Cholestasis in *Pemt$^{-/-}$* mice mimics PFIC1 and/or PFIC2

In humans, mutations in the genes encoding BSEP or ATP8B1 can result in PFIC2 and PFIC1, respectively (Nicolaou, Andress et al. 2012). Mutations in either gene can also lead to the development of benign recurrent intrahepatic cholestasis (BRIC) types 1 and 2 (Stapelbroek, van Erpecum et al. 2010). Patients with BRIC develop bouts of cholestasis with similar symptoms to their respective PFIC type but are symptomatically normal between recurrences (Stapelbroek, van Erpecum et al. 2010). Clinically, patients with PFIC1/2 present with elevated levels of plasma BA and ALT as well as severely diminished biliary BA secretion and increased portal fibrosis (Nicolaou, Andress et al. 2012). *Atp8b1$^{-/-}$* and *Bsep$^{-/-}$* mice have altered canalicular structure, cholestasis, and elevated plasma BA levels (Wang, Salem et al. 2001, Paulusma,

Groen et al. 2006, Zhang, Li et al. 2012). Similar to the *Bsep*$^{-/-}$ and *Atp8b1*$^{-/-}$ mice, prolonged HFD feeding of *Pemt*$^{-/-}$ mice leads to dramatic elevations in plasma BA concentration and impairment of biliary BA secretion, likely due to the 75% decrease in hepatic BSEP protein as well as the loss of canalicular surface area. ATP8B1 is required for maintaining lipid asymmetry and the PC:PE ratio on the canalicular membrane by flipping PE to the inner leaflet of the canalicular membrane. Although the mechanism by which lack of ATP8B1 induces cholestasis has not been fully elucidated, it has been suggested that loss of lipid asymmetry and PC/PE distribution, similar to HFD-fed *Pemt*$^{-/-}$ mice, reduces BA transport across the canalicular membrane and leads to the loss of canalicular structure. Our results suggest that *Pemt*$^{-/-}$ mice fed the HFD represent a novel model for diet-induced cholestasis that mimics aspects of both PFIC2/BRIC2 and PFIC1/BRIC1.

2.4.2 Dysregulation of genes controlled by FXR

Genes involved in BA homeostasis are tightly regulated by the nuclear receptor FXR in both the liver and the intestine. Under normal conditions, high intrahepatic concentrations of BAs activate FXR, thereby increasing mRNA levels of Shp; decreasing mRNA levels of *Cyp7a1*, *Cyp8b, Ntcp, and Oatp1*; and increasing that of *Abcb11* (Goodwin, Jones et al. 2000, Fiorucci and Distrutti 2015). FXR activation in the intestine also reduces BA synthesis through the secretion of FGF19 (Inagaki, Choi et al. 2005). FXR activation also increases the mRNA levels of *Zfp36l1,* thereby inducing degradation of the *Cyp7a1* transcript (Tarling, Clifford et al. 2017). A working model of hepatic physiology in *Pemt*$^{+/+}$ mice is illustrated in Fig. 2.15A. In contrast, *Pemt*$^{-/-}$ mice fed the HFD for 10 weeks are unable to appropriately regulate the expression of genes

responsible for BA synthesis and export. mRNA levels of *Abcb11*, *Shp,* and *Fxr* are lower, and *Zfp36l1* is unchanged in *Pemt*$^{-/-}$ mice compared to *Pemt*$^{+/+}$ mice, indicating an impairment in FXR signaling (Fig. 2.8B). In agreement, liver-specific FXR-deficient mice have constitutively lower mRNA levels of *Abcb11* than wild-type mice*,* and these levels are not normalized by treatment with an FXR agonist (Schmitt, Kong et al. 2015, Tarling, Clifford et al. 2017). In addition, ER stress and oxidative stress, both of which are evident in *Pemt*$^{-/-}$ mice, can negatively impact hepatic FXR signalling (Kemper, Xiao et al. 2009, Kemper 2011, Balasubramaniyan, Luo et al. 2013, Xiong, Wang et al. 2014). Decreased intestinal BA appearance and elevated levels of muricholic acid species, which are known to be antagonists for FXR, may also repress FXR activation (Sayin, Wahlstrom et al. 2013). Besides the reduction in BA transporters, we also observed a reduction in the phospholipid transporter (*Abcb4*) in *Pemt*$^{-/-}$ mice after 10 weeks of the HFD. This could be a mechanism for preventing a further decrease in the hepatic PC:PE molar ratio to less than 1.0, below which liver failure rapidly occurs (Li, Agellon et al. 2006).

Figure 2.15

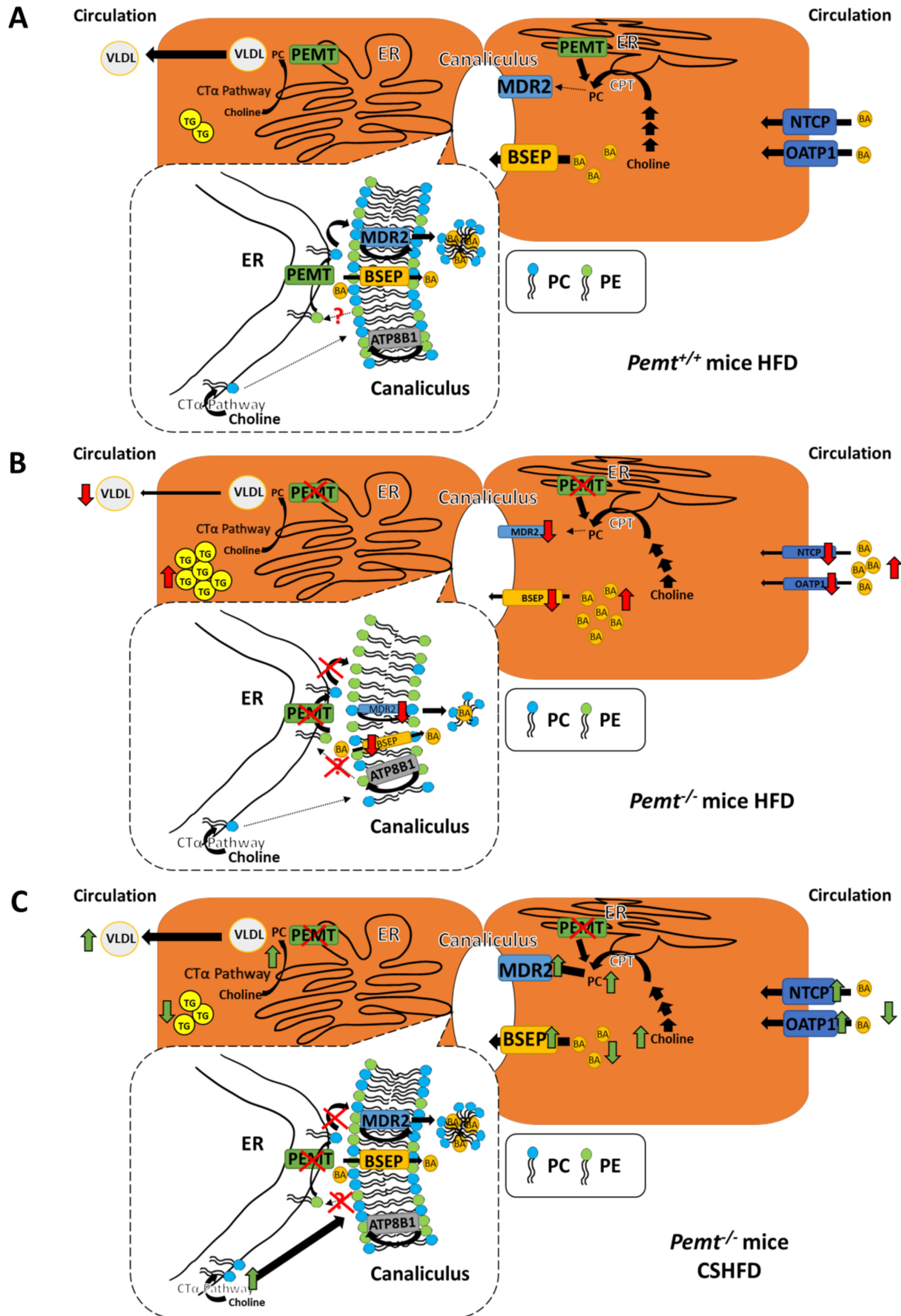

Figure 2.15 Working model of cholestasis in *Pemt*$^{+/+}$ and *Pemt*$^{-/-}$ mice fed the HFD.

(A) When *Pemt*$^{+/+}$ mice are fed the HFD, bile acids (BA) are secreted from the hepatocyte into bile by BSEP and removed from the portal circulation by NTCP and OATP1. Hepatic PC is synthesized either by means of the choline pathway or the PEMT pathway (conversion of PE to PC) in the endoplasmic reticulum (ER). Hepatic PC and TG are secreted into the circulation as VLDL and PC is secreted into bile. MDR2 flips PC from the inner to the outer membrane of the canalicular membrane, from where the PC is extracted into bile by the BAs. ATP8B1 flips PE from the outer to the inner canalicular membrane. Dotted box represents enlargement of area of ER close to the canaliculus. ER domains that are close to the canalicular membrane are enriched with PEMT, thereby providing PC directly to the canalicular membrane for biliary secretion. In addition, PEMT might deplete PE from the canaliculus so that an appropriate PC:PE ratio is maintained. Moreover, PC is made in areas of the ER not close to the canaliculus through the choline pathway and might also provide PC for biliary secretion.

(B) When *Pemt*$^{-/-}$ mice are fed the HFD, the amount of mRNAs encoding NTCP, OATP1, and BSEP is decreased (mechanism not known), leading to the accumulation of BAs in plasma and, likely, in the liver/hepatocyte. Moreover, the amount of MDR2 is decreased in an attempt to conserve hepatic PC. Thus, because PC is synthesized only by the choline pathway in *Pemt*$^{-/-}$ hepatocytes, a reduction in hepatic PC leads to impaired VLDL secretion and, consequently, hepatic steatosis. The lack of PEMT in the hepatocytes reduces the local supply of PC for the canalicular membrane, which cannot be compensated by PC synthesized in the bulk of the ER by the choline pathway. The deficiency of PC in the canalicular membrane would compromise integrity of the canalicular membrane and change in structure of the canaliculus. Arrows in red indicate differences compared to *Pemt*$^{+/+}$ mice.

(C) When *Pemt*$^{-/-}$ mice are fed the choline-supplemented HFD, the increased supply of dietary choline is likely to increase PC synthesis by means of the choline pathway, thereby increasing the availability of PC at the canalicular membrane and restoring membrane integrity. Consequently, the amounts of mRNAs encoding BSEP, MDR2, NTCP, and OATP1 are increased so that hepatocellular and plasma BA concentrations are reduced. The increased amount of PC in the hepatocyte also enhances VLDL secretion and reduces hepatic steatosis. Arrows in green indicate differences compared to *Pemt*$^{+/+}$ mice fed the HFD.

2.4.3 Hepatic changes contributing to cholestasis and BSEP deficiency

One of the features that are well characterized in HFD-fed *Pemt*$^{-/-}$ mice is the lower hepatic PC:PE molar ratio compared to that in *Pemt*$^{+/+}$ mice (Li, Agellon et al. 2006, Jacobs, Zhao et al. 2010, van der Veen, Lingrell et al. 2017). Alterations in the PC:PE ratio are known to negatively impact membrane integrity and liver health (Li, Agellon et al. 2006, van der Veen, Kennelly et al. 2017). *Pemt*$^{-/-}$ mice fed a chow diet have a mild reduction in the hepatic PC:PE molar ratio but do not develop NAFLD or liver damage (Fig. 2.2). However, after *Pemt*$^{-/-}$ mice had been fed the HFD for 2 weeks, the hepatic PC:PE ratio dropped dramatically to approximately 1.05 (approximately 2 in *Pemt*$^{+/+}$ mice), and the plasma concentration of ALT, a marker of liver damage, increased 7-fold compared to that in *Pemt*$^{+/+}$ mice (Fig. 2.3 B,E). This dramatic decrease in the hepatic PC:PE molar ratio results in a loss of membrane integrity and leads to ER stress, which may initiate the onset of cholestasis after 2 weeks of HF feeding. Cholestasis and ER stress can both aggravate inflammation, fibrosis, and oxidative stress, leading to a further dysregulation of FXR-controlled genes. Increased BA levels can activate hepatic stellate cells, thereby aggravating fibrosis (Svegliati-Baroni, Ridolfi et al. 2005). These changes can promote the severe cholestatic phenotype observed when *Pemt*$^{-/-}$ mice were fed the HFD for 10 weeks.

Moreover, we hypothesize that PEMT, which localizes close to the canaliculus, is required to maintain the canalicular PC:PE molar ratio and PC availability (Fig. 2.15B) (Sehayek, Wang et al. 2003). PEMT may remove PE, flipped to the inner canalicular membrane by ATP8B1, to offset the continuous loss of PC into bile, and also produce PC locally for direct delivery to the canalicular membrane. Recent literature reports that

Pemt is a BA-responsive gene and suggests that PEMT plays an important role in maintenance of biliary secretion capacity (Kim, Seok et al. 2018). Thus ultimately, the decreased PC:PE ratio is likely responsible for the loss of canalicular structure and contributes to hepatic BSEP deficiency in $Pemt^{-/-}$ mice.

2.4.4 Dietary CS prevents and treats cholestasis

Interestingly, dietary CS prevented the development of cholestasis in the HFD-fed $Pemt^{-/-}$ mice. Although CS did not normalize the hepatic PC:PE ratio to $Pemt^{+/+}$ levels, the PC:PE ratios were increased to approximately 1.4 after CS compared to approximately 1.1 after HFD, which may have been sufficient to improve hepatic membrane integrity and function (Fig. 2.15C). Strikingly, we were also able to effectively treat $Pemt^{-/-}$ mice that developed cholestasis on an HFD with dietary CS (Fig. 2.14A). Although it was not significant, treatment with dietary choline also increased the PC:PE ratio to approximately 1.4, which was sufficient to restore hepatic BSEP protein levels and improve liver health. This suggests that the canalicular membrane, a major site for phospholipid export, may be sensitive to minute changes in PC:PE balance. Recently, 4-phenyl butyric acid, a drug known to alleviate ER stress, has successfully treated a case of BRIC2 (Hayashi, Naoi et al. 2016). Interestingly, CS reduced ER stress in $Pemt^{-/-}$ mice, which may be an additional mechanism by which cholestasis is alleviated. Phospholipid imbalance is the major underlying cause in patients with PFIC1 and BRIC1. Since there are limited therapies for cholestasis, CS might prove to be effective as a potential addition to therapy for these patients with cholestasis.

In conclusion, we have established that PEMT is a critical modulator of biliary secretion processes and “canalicular health” in mice fed an HFD by maintaining PC availability. Moreover, the experiments revealed that dietary CS might be a novel adjuvant therapy for the subset of patients with cholestasis who have phospholipid imbalance and/or decreased hepatic PC availability.

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Chapter 3

The CDP-choline pathway is not quantitatively important for biliary processes during HFD-feeding

3.1 Introduction

CTP:Phosphocholine cytidylyltransferase (CTα) is the rate limiting enzyme of the CDP choline pathway, which accounts for approximately 70% of hepatic phosphatidylcholine (PC) synthesis (DeLong, Shen et al. 1999). The remaining 30% of hepatic PC is synthesized through the phosphatidylethanolamine *N*-methyltransferase (PEMT) pathway which converts phosphatidylethanolamine (PE) to PC (DeLong, Shen et al. 1999). Our laboratory has previously demonstrated that both liver-specific CTα knock out mice (L*Pcyt1a*$^{-/-}$) and *Pemt*$^{-/-}$ mice fed a high-fat diet (HFD) develop nonalcoholic fatty liver disease (NAFLD), largely due to impairments in very low-density lipoprotein (VLDL) secretion associated with insufficient availability of PC (Noga, Zhao et al. 2002, Jacobs, Lingrell et al. 2008, Jacobs, Zhao et al. 2010). Interestingly, HFD-fed *Pemt*$^{-/-}$ mice develop a more severe form of NAFLD than L*Pcyt1a*$^{-/-}$ mice (Ling, Chaba et al. 2012).

Within 24 hours, the equivalent of the entire hepatic pool of PC is secreted into bile (approximately 23mg/day/20g mouse) along with bile acids (BAs) and cholesterol (Walkey, Yu et al. 1998, Nicolaou, Andress et al. 2012). Cholesterol is converted to BAs exclusively in the liver (Smit, Schinkel et al. 1993). BAs are secreted into bile by the bile salt export protein (ATP Binding Cassette Subfamily B Member 11[ABCB11]/bile salt export protein [BSEP]), and PC by the flippase ATP Binding Cassette Subfamily B Member 4 (ABCB4)/Multi-drug Resistance 2 (MDR2) (Smit, Schinkel et al. 1993, Wang, Liu et al. 2013). ATPase Phospholipid Transporting 8B1 (ATP8B1) is responsible for flopping PE to the inner canalicular membrane, thereby maintaining phospholipid asymmetry which is critical for biliary secretion (Nicolaou, Andress et al. 2012).

Cholestasis – an impairment in the secretion of bile and biliary processes – can result from mutations or defects in the above proteins (Nicolaou, Andress et al. 2012). Improper secretion and circulation of biliary constituents can result in hepatocellular damage, including inflammation and fibrosis, and impaired absorption of dietary lipids (Nicolaou, Andress et al. 2012).

PEMT is an endoplasmic reticulum (ER) protein and is enriched in portions of the ER which are in close proximity to the canalicular membrane (Sehayek, Wang et al. 2003). HFD-fed *Pemt*$^{-/-}$ mice develop cholestasis with severe reductions in bile flow and the secretion of biliary constituents (Wan, Kuipers et al. 2019). These mice also lose regulation of genes associated with BA homeostasis and lose canalicular structure (Wan, Kuipers et al. 2019). We have previously suggested that the severely reduced hepatic PC:PE molar ratio reduces membrane integrity and contributes to the development of cholestasis in HFD-fed *Pemt*$^{-/-}$ mice (Wan, Kuipers et al. 2019). Dietary supplementation with choline was able to both prevent and reverse cholestasis in these mice, suggesting a role for PC insufficiency (Wan, Kuipers et al. 2019). Since the majority of hepatic PC is synthesized through the CDP:choline pathway, we developed an inducible L*Pcyt1a*$^{-/-}$ (iL*Pcyt1a*$^{-/-}$) mouse to investigate the effects of PC insufficiency on biliary secretion. We report that the biliary secretion of bile acids (BA) is not impaired, and that the biliary secretion of phospholipids is surprisingly increased in iL*Pcyt1a*$^{-/-}$ *mice*. This suggests that CTα is not required for biliary secretion under HFD conditions and may play a role in regulating biliary phospholipid secretion.

3.2 Materials and Methods

3.2.1 Animals

All procedures were approved by the University of Alberta's Institutional Animal Care Committee in accordance with guidelines of the Canadian Council on Animal Care. All animals were exposed to a 12-hour light/dark cycle and had free access to drinking water. Male *Pcyt1a*$^{flox/flox}$ mice (backcrossed into C57Bl/6 for 7 generations), were 14 to 16 weeks old at the start of the study and were injected, retro-orbitally, with an adeno-associated virus (AAV) either expressing green fluorescence protein (GFP) (control) or Cre recombinase (iL*Pcyt1a*$^{-/-}$ mice). The AAV was made liver specific with an AAV8 capsid protein and a thyroxine binding globulin promoter at the University of Pennsylvania. For 10 weeks, they were fed a standard chow diet (LabDiet, No. 5001) or a semi-synthetic HFD (catalog No. F3282, Bio-Serv, Flemington, NJ) that contained 60 kcal% from lard and 1.3 g/kg choline chloride. Body weight was monitored weekly during the experiments. Gall bladders were cannulated, and bile was collected for 30 minutes as described (Plosch, van der Veen et al. 2006). Animals were not fasted prior to collection of blood by cardiac puncture. Tissues were collected, snap-frozen in liquid nitrogen, and stored at −80^{0}C until further analyses. For electron microscopy, samples were preserved in a 3% glutaraldehyde, 3% paraformaldehyde in 0.1 M sodium cacodylate buffer, stored overnight at 4 °C, and analyzed the following morning.

3.2.2 Analytical procedures

Hepatic triacyglycerols (TGs) were measured by a commercially available kit from Roche Diagnostics (Ref # 11877771 216). Hepatic PC and PE were isolated by thin-layer chromatography and quantified by the phosphorous assay (Rouser, Fleischer et al. 1970). RNA isolation, complementary DNA (cDNA) synthesis, and real-time quantitative polymerase chain reaction were performed as described (Jacobs, Zhao et

al. 2010). Messenger RNA (mRNA) levels were normalized to cyclophilin. Secretion of biliary components were determined by described methods (Plosch, van der Veen et al. 2006).

3.2.3 Statistical analysis

Data were analyzed with GraphPad Prism software (GraphPad, La Jolla, CA). All values are means ± SEM (n=5-6 per group). For comparison of groups, 2-way analysis of variance (ANOVA) with Fisher's least significant difference *post hoc* test was used. Level of significance of differences was set at $P < 0.05$.

3.3 Results

3.3.1 Generation of inducible L*Pcyt1a*$^{-/-}$ mice

Male *Pcyt1a*$^{flox/flox}$ mice 12-16 weeks of age were injected, retro-orbitally, with an AAV expressing either GFP or Cre. 4 weeks after AAV injection, livers were collected and analyzed for CTα protein content. AAV.Cre treatment significantly reduced hepatic CTα protein levels to approximately 10% of control mice (Fig. 3.1). This is consistent with previous studies in L*Pcyt1a*$^{-/-}$ mice in which CTα protein was less than 30% than that of wild type mice (Niebergall, Jacobs et al. 2011). Hereon, AAV.GFP and AAV.Cre treated mice are referred to as control and iL*Pcyt1a*$^{-/-}$ mice, respectively.

3.3.2 iL*Pcyt1a*$^{-/-}$ mice fed the HFD develop NASH

After 10 weeks of HFD feeding, iL*Pcyt1a*$^{-/-}$ mice gained less weight than control mice (Fig. 3.2A). This results from a lack of weight gain in iL*Pcyt1a*$^{-/-}$ mice during the first 3 weeks of HFD feeding, after which, the rate of weight gain is not different between control and iL*Pcyt1a*$^{-/-}$ mice (Fig. 3.2A). Contrary to literature, both control and iL*Pcyt1a*$^{-}$

$^{/-}$ mice had increased hepatic TG accumulation compared to chow fed mice (Fig. 3.2B) (Niebergall, Jacobs et al. 2011). While there were no significant differences in the amount of PC and PE, the hepatic PC:PE molar ratio was significantly increased in iL*Pcyt1a*$^{-/-}$ mice compared to control mice fed the HFD (Fig. 3.2C). Interestingly, it appears that HFD-feeding reduced hepatic PE and thereby increased the hepatic PC:PE molar ratio in both control and iL*Pcyt1a*$^{-/-}$ mice compared to chow fed animals of the same genotype (Fig. 3.2C). Hepatic mRNA levels of collagen type I alpha 1 chain (*Col1a1*), tumor necrosis factor α (*Tnfα*), and NADPH oxidase (*Nox2*)—markers for fibrosis, inflammation, and oxidative stress, respectively—were all significantly elevated in iL*Pcyt1a*$^{-/-}$ mice compared to controls, independent of diet and hepatic TG accumulation (Fig. 3.2D).

Figure 3.1

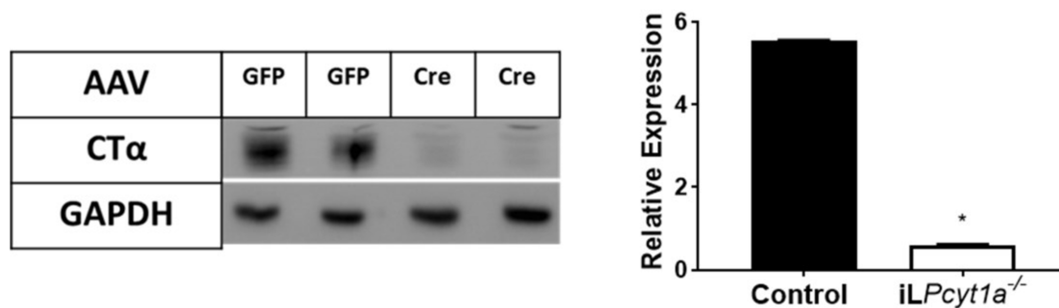

Figure 3.1 Generation of iL*Pcyt1a*$^{-/-}$ mice

Representative immunoblot and densitometry of hepatic CTα. All values are means ± SEM. Student's t-test ($P<0.05$).

Figure 3.2

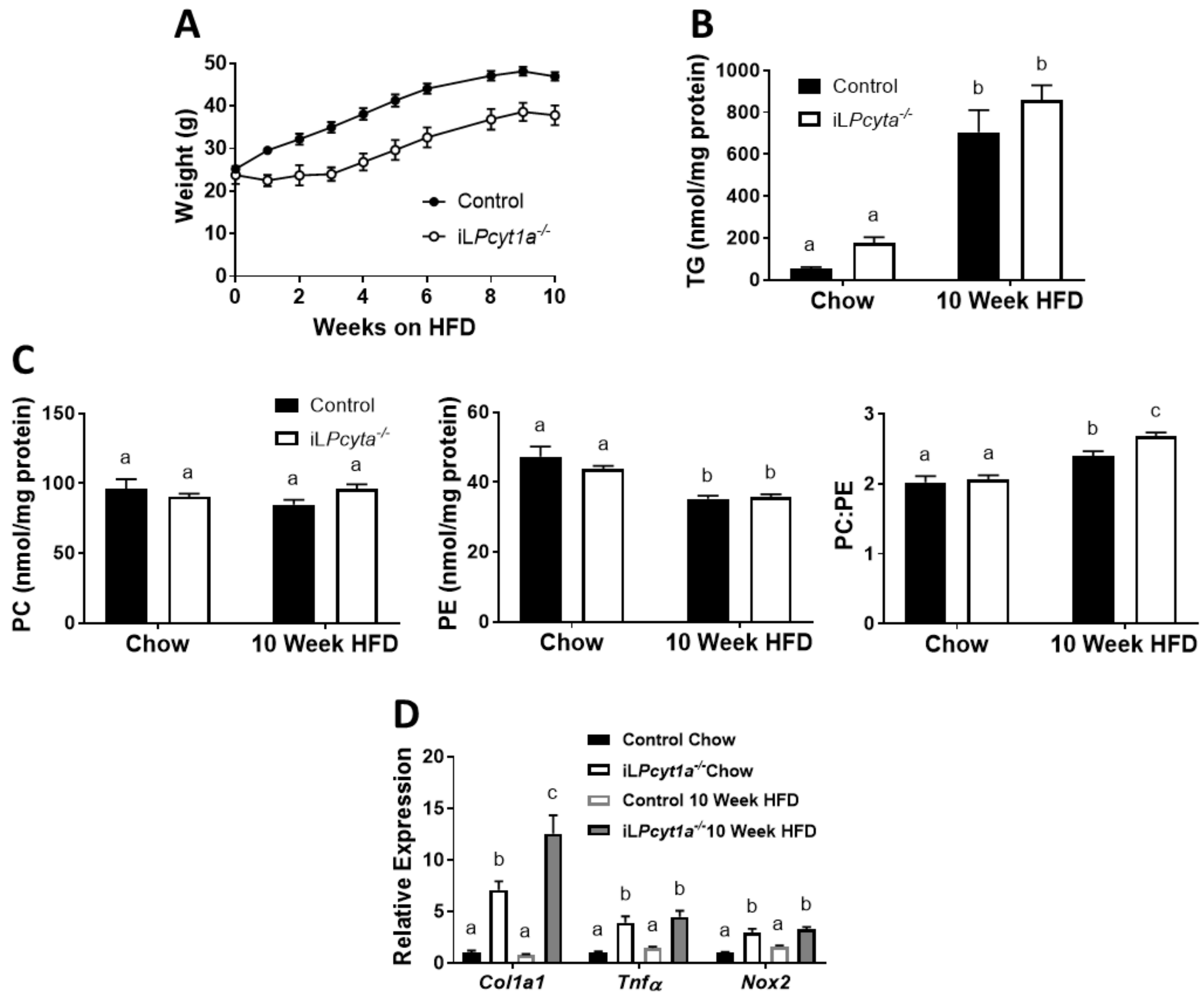

Figure 3.2 iL*Pcyt1a*$^{-/-}$ mice develop NASH when fed the HFD

Control and iL*Pcyt1a*$^{-/-}$ mice were fed the chow diet or the HFD for 10 weeks. (A) Weight gain. (B) Hepatic TG mass. (C) The mass of PC and PE, and the PC:PE molar ratio. (D) mRNA expression of genes involved in fibrosis (*Col1a1*), inflammation (*Tnfα*), and oxidative stress (*Nox2*). Values are means ± SEM and are expressed relative to control mice fed the chow diet. 2-way ANOVA, followed by Fisher's LSD *post hoc* test. Values that do not share a letter are significantly different ($P < 0.05$).

3.3.3 HFD-fed iL*Pcyt1a*$^{-/-}$ mice have altered biliary secretion

Our lab has previously observed that HFD-fed *Pemt*$^{-/-}$ mice develop cholestasis that was associated with a reduced hepatic PC:PE molar ratio and was reversed with choline supplementation (Wan, Kuipers et al. 2019). Interestingly, iL*Pcyt1a*$^{-/-}$ mice fed the HFD had decreased bile flow but the secretion of the major constituents of bile, bile acids and phospholipids, were not decreased compared to control mice (Fig. 3.3 A-C). In fact, the biliary secretion of phospholipids was increased in HFD-fed iL*Pcyt1a*$^{-/-}$ mice. Plasma bile acid concentration, a marker for cholestasis, was not different between HFD-fed control and iL*Pcyt1a*$^{-/-}$ mice (Fig. 3.3D). Secretion of biliary lipids was not different between control and iL*Pcyt1a*$^{-/-}$ mice when fed the chow diet (Fig. 3.3).

Figure 3.3

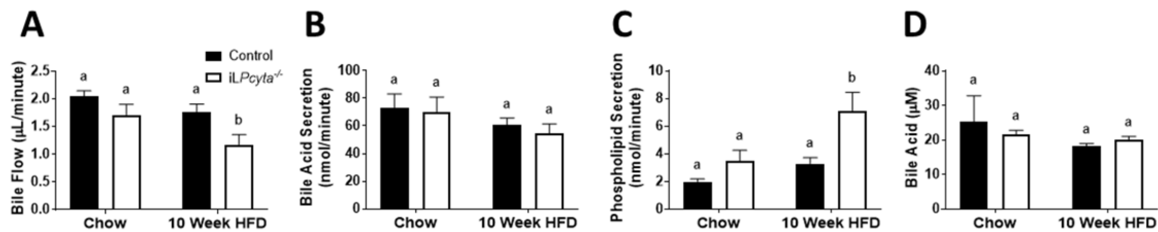

Figure 3.3 Altered biliary secretion in HFD-fed iL*Pcyt1a*$^{-/-}$ mice

Control and iL*Pcyt1a*$^{-/-}$ mice were fed the chow diet or the HFD for 10 weeks. Basal biliary (A) bile flow, and secretion of (B) bile acids and (C) phospholipids. (D) Plasma bile acid concentrations. Values are means ± SEM. 2-way ANOVA, followed by Fisher's LSD *post hoc* test. Values that do not share a letter are significantly different ($P < 0.05$).

3.3.4 Genes involved in BA homeostasis are not altered in HFD-fed iL*Pcyt1a*$^{-/-}$ mice

When fed the chow diet, hepatic mRNA levels of genes responsible for the import of unconjugated BAs from circulation (organic anion co-transporting polypeptide [*Oatp1*]) and biliary secretion of PC (*Mdr2*), were, significantly decreased and increased, respectively, in iL*Pcyt1a*$^{-/-}$ mice compared to control mice (Fig. 3.4A). As described above, these changes in *Oatp1* and *Mdr2* were not associated with any effects in biliary secretion (Fig. 3.3). Interestingly, after 10 weeks of HFD feeding there were no differences in the hepatic mRNA levels of genes associated with BA homeostasis (Fig. 3.4B).

3.3.5 Canalicular membrane is not altered by hepatic CTα deficiency

In previous studies with HFD-fed *Pemt*$^{-/-}$ mice, we observed a marked alteration in the structure of the canalicular membrane (Wan, Kuipers et al. 2019). We observed no loss of structure or widening of the canalicular lumen in iL*Pcyt1a*$^{-/-}$ mice compared to control mice fed the HFD (Fig. 3.5).

Figure 3.4

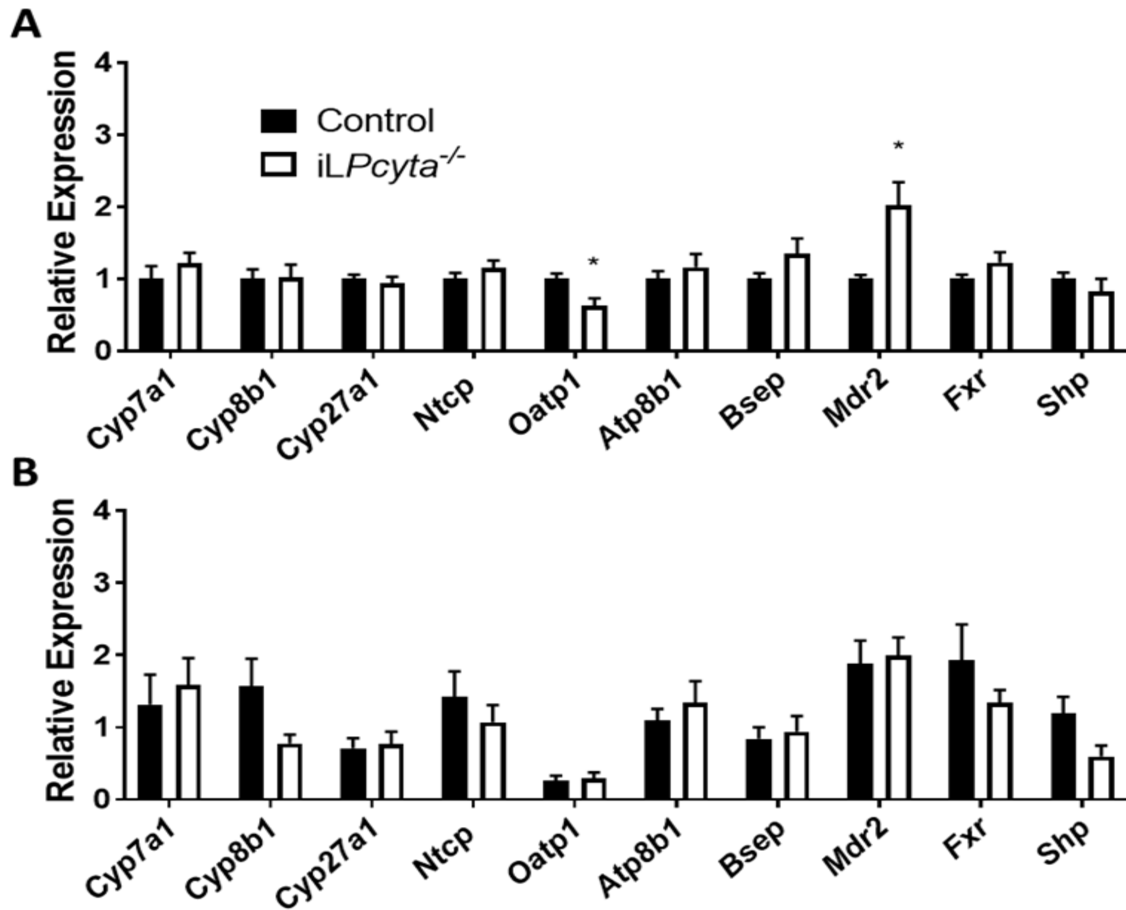

Figure 3.4 Intact regulation of hepatic genes involved in BA homeostasis

Hepatic mRNA levels of genes involved in bile acid synthesis, biliary secretion and basolateral import of bile acids, biliary secretion of phospholipids, regulators of bile acid synthesis, bile acid homeostasis. and the alternative pathway of bile acid secretion into bile and circulation were determined in control and iL*Pcyt1a*$^{-/-}$ mice fed (A) chow or (B) the HFD and normalized to cyclophilin mRNA levels. Values are expressed relative to control mice fed the chow diet. Values are means ± SEM. Student *t* test. *$P < 0.05$.

Figure 3.5

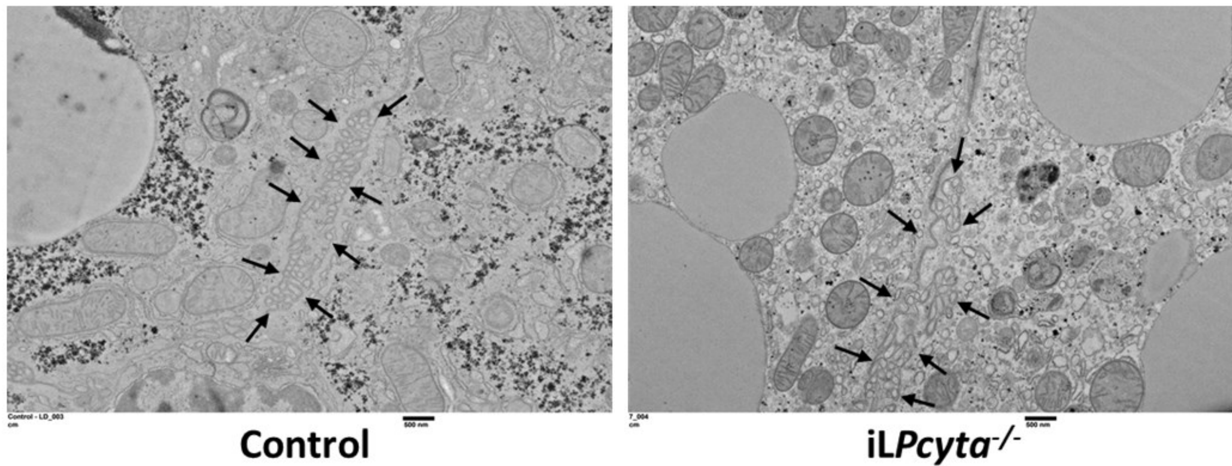

Figure 3.5 Intact canalicular structure in HFD-fed iL*Pcyt1a*$^{-/-}$ mice

Electron microscopy of the canalicular membrane (6,000x magnification) in livers of control and iL*Pcyt1a*$^{-/-}$ mice fed the HFD for 10 weeks. Arrows outline the edge of the canaliculus.

3.4 Discussion

3.4.1 Discrepancies between genetic and inducible L*Pcyt1a*$^{-/-}$ mice

In contrast to the L*Pcyt1a*$^{-/-}$ mice, iL*Pcyt1a*$^{-/-}$ mice gained significantly less weight than control animals when fed the HFD (Fig. 3.1A) (Jacobs, Zhao et al. 2010). Interestingly, iL*Pcyt1a*$^{-/-}$ mice do not gain weight during the first 3 weeks of HFD-feeding. However, from weeks 3-10, the rate of weight gain is no longer different between control and iL*Pcyt1a*$^{-/-}$ mice. This suggests that inducing hepatic deletion of CTα in adult mice acutely alters metabolism and weight gain which can be compensated for in the long

term. Future studies will be conducted to delineate these changes in relation to weight gain.

We have previously observed genetic L*Pcyt1a*$^{-/-}$ mice to have reduced hepatic PC and, consequently, a reduced hepatic PC:PE molar ratio (Niebergall, Jacobs et al. 2011, Ling, Chaba et al. 2012). Additionally, L*Pcyt1a*$^{-/-}$ mice develop NAFLD when fed an HFD (Niebergall, Jacobs et al. 2011, Ling, Chaba et al. 2012). In our current study, where the Pcyt1a gene was ablated in adult mice, the hepatic PC:PE ratio was not different from that of chow fed control mice (Fig. 3.2B). Surprisingly, when fed the HFD, all animals developed NAFLD. It is possible that the accumulation of hepatic TG in control mice may be due issues in the diet batch; long term HFD-feeding with this diet has very recently been observed to induce hepatic TG accumulation in separate animal experiments in our laboratory (data not shown). Also in contrast to L*Pcyt1a*$^{-/-}$ mice, the hepatic PC:PE molar ratio was higher in iL*Pcyt1a*$^{-/-}$ mice compared to control mice. Previous studies have demonstrated hepatic PEMT protein levels to be increased by 2-fold in L*Pcyt1a*$^{-/-}$ mice (Jacobs, Devlin et al. 2004). However, this increase in hepatic PC synthesis through the PEMT pathway was not able to fully compensate for the loss of CTα in L*Pcyt1a*$^{-/-}$ mice (Jacobs, Devlin et al. 2004). Since the amount of hepatic PC was not different between control and iL*Pcyt1a*$^{-/-}$ mice, it suggests that the PEMT pathway was able to compensate for the acute loss of CTα. It is possible that loss of CTα in adult mice, which have established a homeostatic PC:PE molar ratio, further upregulates PEMT to increase PC synthesis. Since PEMT synthesizes PC from PE, it is possible that the increase in PC synthesis proportionally decreased hepatic PE, which leads to the observed increase in the hepatic PC:PE molar ratio in HFD-fed L*Pcyt1a*$^{-/-}$ mice (Fig.

3.2B). Future studies will investigate the role of PEMT in the increased hepatic PC:PE molar ratio observed in iL*Pcyt1a*$^{-/-}$ mice.

Although all animals accumulated hepatic TG and had similar levels of hepatic PC, markers of inflammation, fibrosis, and oxidative stress – hallmarks of NASH – were only increased in iL*Pcyt1a*$^{-/-}$ mice (Fig. 3.4). These data support previous studies which demonstrated that normalized hepatic PC levels was not sufficient to prevent the development of NASH in L*Pcyt1a*$^{-/-}$ mice (Niebergall, Jacobs et al. 2011).

3.4.2 Hepatic phospholipids and cholestasis

Pemt$^{-/-}$ mice fed the HFD develop cholestasis associated with a decreased hepatic PC:PE molar ratio and PC insufficiency (Wan, Kuipers et al. 2019). We believe that PEMT is critical for biliary secretion as it may locally produce PC for biliary secretion. We also suggested that NASH in *Pemt*$^{-/-}$ mice may aggravate cholestatic development. Thus, we hypothesized that, despite the CDP:choline pathway accounting for 70% of hepatic PC synthesis, iL*Pcyt1a*$^{-/-}$ mice would not develop cholestasis when fed the HFD. Indeed, we find no deficiencies in the biliary secretion of BAs and phospholipids, despite a reduction in bile flow, nor any elevations in plasma BAs, a marker of cholestasis, in iL*Pcyt1a*$^{-/-}$ mice fed the HFD (Fig. 3.3). In agreement, we observed no alterations in the hepatic mRNA levels of genes associated with BA homeostasis, which were massively dysregulated in HFD-fed *Pemt*$^{-/-}$ mice (Fig. 3.4B) (Wan, Kuipers et al. 2019). Additionally, HFD-fed iL*Pcyt1a*$^{-/-}$ mice had an intact canalicular structure that was not different from control mice (Fig. 3.5). This demonstrates that the CDP:choline pathway, unlike the PEMT pathway, is not critical for biliary processes during HFD-feeding. It also appears that NASH which develops in both

iL*Pcyt1a*$^{-/-}$ and *Pemt*$^{-/-}$ mice does not contribute to the development of cholestasis. However, since HFD-fed iL*Pcyt1a*$^{-/-}$ mice had an increased hepatic PC:PE molar ratio, it is possible that there is a minimum PC:PE or PC threshold required to maintain biliary processes. Furthermore, the increased biliary secretion of phospholipids from HFD-fed iL*Pcyt1a*$^{-/-}$ mice supports the idea that PEMT may locally synthesize and directly provide PC for the canalicular membrane and, subsequently, biliary secretion. It is also interesting to speculate that, although not significant, *Cyp8b1* tends to be decreased which may increase chenodeoxycholinc acid (CDCA) content in HFD-fed iL*Pcyt1a*$^{-/-}$ mice. CDCA stimulates biliary phospholipid secretion more greatly than cholic acid (CA) and therefore may account for the increased secretion observed in iL*Pcyt1a*$^{-/-}$ mice (Einarsson and Grundy 1980).

In conclusion, we have established that the CDP:choline pathway is not critical for biliary processes, thus demonstrating that the PEMT pathway is sufficient for maintaining canalicular health during HFD-feeding. Our data suggests that the development of cholestasis is independent of NAFLD in *Pemt*$^{-/-}$ mice.

3.5 References

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Chapter 4

Hepatic phosphatidylethanolamine *N*-methyltransferase mediates phospholipid balance and optimal liver health

4.1 Introduction

Phosphatidylethanolamine *N*-methyl transferase (PEMT) converts phosphatidylethanolamine (PE) to phosphatidylcholine (PC), accounting for ~30% of hepatic PC synthesis (DeLong, Shen et al. 1999). The remaining 70% is synthesized through the choline pathway, via the conversion of choline to PC (DeLong, Shen et al. 1999). Although PEMT is almost exclusively expressed in the liver, low levels can be found in various other tissues (Vance 2014). *Pemt*$^{-/-}$ mice fed a high fat diet (HFD) are resistant to diet induced obesity (DIO) and insulin resistance (IR) (Jacobs, Zhao et al. 2010). However, *Pemt*$^{-/-}$ mice rapidly develop non-alcoholic fatty liver disease (NAFLD) when fed a HFD, which can progress to non-alcoholic steatohepatitis (NASH) and fibrosis with prolonged feeding (Jacobs, Zhao et al. 2010, Ling, Chaba et al. 2012). The development of steatosis largely results from impaired secretion of very low density lipoproteins (VLDL) associated with a decreased hepatic PC:PE molar ratio (van der Veen, Lingrell et al. 2016). Lack of PEMT reduces the hepatic PC:PE molar ratio, impairing membrane integrity and leading to endoplasmic reticulum (ER) stress and liver disease (Li, Agellon et al. 2006, Jacobs, Zhao et al. 2010, Ling, Chaba et al. 2012, Gao, van der Veen et al. 2015, van der Veen, Kennelly et al. 2017).

Alterations in the hepatic PC:PE molar ratio have been associated with the development of NAFLD and liver disease, reviewed in (van der Veen, Kennelly et al. 2017). In contrast to *Pemt*$^{-/-}$ mice, knockdown of PEMT in leptin deficient *ob/ob* mice surprisingly reduced steatosis and improved liver health (Fu, Yang et al. 2011). *Ob/ob* mice develop hepatic steatosis and ER stress on a standard chow diet (Bleisch, Mayer et al. 1952, Fu, Yang et al. 2011). The pronounced ER stress has been suggested to be

due to loss of calcium homeostasis (Fu, Yang et al. 2011). This loss of calcium homeostasis was thought to be due to increased PEMT activity which lead to an accumulation of PC and an increase in the PC:PE ratio in the ER (Fu, Yang et al. 2011). Reduction of PEMT activity in *ob/ob* mice reduces the PC:PE molar ratio by reducing PC and thereby alleviates ER stress (Fu, Yang et al. 2011).

While the phenotype of the *Pemt*$^{-/-}$ mouse has been well examined, the importance of *hepatic* PEMT in the phenotype development has not been specifically demonstrated. We hypothesized that PEMT activity, specifically in the liver, is responsible for mediating the protection against diet induced obesity (DIO) and insulin resistance (IR), and for the development of NAFLD. In addition, we hypothesized that decrease of hepatic PEMT can be utilized in *ob/ob* mice to reduce weight gain and improve insulin sensitivity and liver health. We report that the protection against DIO and IR, and the development of NAFLD in *Pemt*$^{-/-}$ mice is dependent upon hepatic PEMT activity. In addition, marked reduction of hepatic PEMT is associated with a reduction in the hepatic PC:PE molar ratio that promotes and aggravates NAFLD in HFD-fed mice and *ob/ob* mice, respectively.

4.2 Materials and methods

4.2.1 Animals

All animal procedures were approved by the University of Alberta's Institutional Animal Care Committee in accordance with guidelines of the Canadian Council on Animal Care. All mice were exposed to a 12-hour light/dark cycle and had free access to food drinking water.

Adeno Associated Virus (AAV) treated mice:

Male *Pemt*$^{+/+}$ and *Pemt*$^{-/-}$ mice (C57BL/6J background), were 8 weeks old at the start of the study and were injected, retro-orbitally, with an AAV either expressing green fluorescence protein (AAV.GFP) or codon optimized human PEMT (AAV.PEMT) (purchased from the University of Pennsylvania Vector Core Lab). The self-complementary AAV was made liver specific with an AAV8 capsid protein and a thyroxine binding globulin promoter at the University of Pennsylvania. For restoration of hepatic PEMT, *Pemt*$^{-/-}$ mice were injected with $1x10^{10}$ genome copies/mouse of AAV.GFP or AAV.PEMT and *Pemt*$^{+/+}$ mice were injected with an equal dose of AAV.GFP. 1 week after AAV administration, mice were fed a semi-synthetic HFD (catalog #F3282, Bio-Serv, Flemington, NJ, USA) that contained 60 kcal% from lard for 10 weeks.

Lean Antisense Oligonucleotide (ASO)-treated mice:

Male C57BL/6J mice were 8-10 weeks old at the start of the study. The mice were intraperitoneally injected weekly with either a scrambled control ASO (GGCCAATACGCCGTCA) or an ASO that inhibits PEMT (αPEMT) (CTTTATTAGTGTGTCG). Both ASOs are modified with a $GalNAc_3$ conjugation for liver specificity (Yu, Graham et al. 2016). Both control and PEMT ASOs were administered at a dose of 25 mg/kg body weight. 1 week after ASO administration, mice were fed the HFD for 10 weeks.

Obese and ob/ob mice treated with ASO:

Male C57BL/6J mice, 8-10 weeks old at the start of the study, were fed the HFD for 6 weeks (obese group) prior to starting ASO treatment for a further 6 weeks. In a

separate trial, 9 week old male C57BL/6J mice and *ob/ob* mice were fed a standard chow diet (LabDiet, #5001) during the 6 weeks of ASO treatment. Both control and anti-PEMT (αPEMT) ASOs were administered at a dose of 25 mg/kg body weight.
All animals were fasted overnight prior to collection of blood by cardiac puncture. Tissues were collected, snap-frozen in liquid nitrogen, and stored at -80ºC until further analyses.

4.2.2 *In vivo* metabolic tests

For the glucose tolerance test, mice were fasted for 12 h, after which they received glucose (2 g/kg body weight) by intraperitoneal injection. For the insulin tolerance test, mice were fasted for 4 h, after which they received insulin (1U/kg body weight) via intraperitoneal injection. Blood glucose levels were measured with a glucometer (Accu-Chek, Indianapolis, IN) immediately before and at indicated times after administration.

4.2.3 Analytical procedures

Hepatic PC, PE, and triacylglycerols (TG), as well as plasma alanine aminotransferase (ALT) and free fatty acids, were quantified as previously described (van der Veen, Lingrell et al. 2016). Immunoblotting was described previously (van der Veen, Lingrell et al. 2017). Membranes were probed with: CCAAT/-enhancer-binding protein homologous protein (Schmitt, Kong et al.) (catalog no. 2895, Cell Signaling, Beverly MA), Bip (catalog no. 3183, Cell Signalling, Beverly MA), and calnexin (catalog no. ADI-SPA-865, Enzo). Proteins were visualized and quantified as described previously (van der Veen, Lingrell et al. 2017). RNA isolation, cDNA synthesis, and real-

time quantitative PCR were performed as described previously (Jacobs, Zhao et al. 2010). mRNA levels were normalized to cyclophilin or 18S ribosomal RNA.

4.2.4 Statistical analysis

Data were analyzed with GraphPad Prism software (GraphPad, La Jolla, CA). All values are means ± SEM. A student's t-test or ANOVA with Fisher's LSD post-hoc test was used as indicated in figure legends. Level of significance of differences was set at $p<0.05$. All groups were n=6-8.

4.3 Results

4.3.1 Hepatic PEMT deficiency protects against diet induced obesity

Our lab has previously shown that whole-body PEMT knockout mice are protected against DIO and IR. Since the PEMT protein has been found in other tissues, such as white adipose tissue and testis, we aimed to delineate the role of PEMT, specifically in the liver, in weight gain and IR (Vance 2014, Watanabe, Nakatsuka et al. 2014). We restored hepatic PEMT in *Pemt*$^{-/-}$ mice by AAV-mediated expression. Hepatic PEMT activity in *Pemt*$^{-/-}$ mice treated with AAV.PEMT was normalized to that of *Pemt*$^{+/+}$ mice (Fig. 4.1A). PEMT or GFP protein was not detected in extra-hepatic tissues (data not shown). Consistent with previous data, AAV.GFP *Pemt*$^{-/-}$ mice did not gain weight after 10 weeks of HFD (Fig. 4.1B). AAV.PEMT *Pemt*$^{-/-}$ mice were not protected against DIO and gained an equal amount of weight to AAV.GFP *Pemt*$^{+/+}$ mice fed the HFD (Fig. 4.1 C).

Figure 4.1

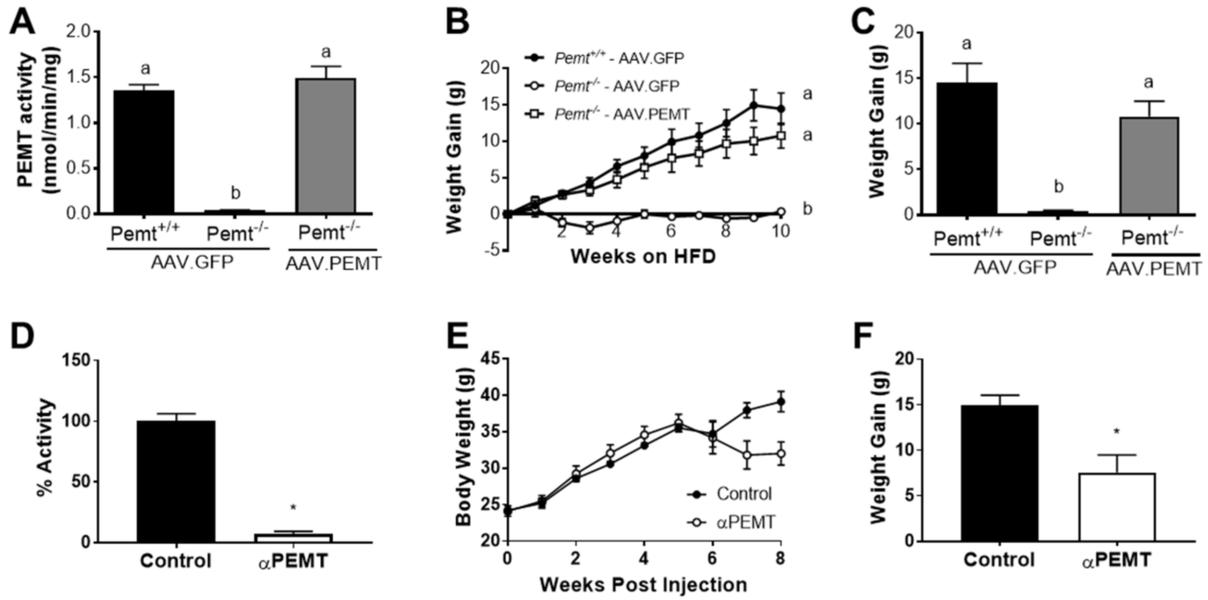

Figure 4.1 Hepatic PEMT activity modulates diet induced weight gain

Pemt$^{+/+}$ mice were injected with control AAV.GFP. *Pemt*$^{-/-}$ mice were injected with either the AAV.GFP or AAV.PEMT, which restores hepatic PEMT. All mice were fed the HFD for 10 weeks. (A) Hepatic PEMT activity. (B) Weekly body weight and (C) weight gain over 10 weeks. Values are means ± SEM. Values that do not share a letter are significantly different ($P < 0.05$).

C57BL/6 mice were fed the HFD and treated with either the control or PEMT ASO for 10 weeks. (D) Hepatic PEMT activity. (E) Weekly body weight and (F) weight gain over 8 weeks. Values are means ± SEM. Student's t-test, * $p<0.05$.

In order to further confirm the role of hepatic PEMT in the physiological effects seen in HFD-fed *Pemt*$^{-/-}$ mice, we also tested the opposite condition. C57BL/6 mice were injected with either a scrambled control ASO or an αPEMT ASO, which specifically reduces hepatic PEMT. Mice were fed the HFD for 10 weeks. αPEMT ASO treatment reduced hepatic PEMT activity by 93% compared to the control group (Fig. 4.1D). Hepatic PEMT knock down (KD) reduced weight gain by 7 grams compared to the control group (Fig. 4.1E). Thus, we demonstrated that the protection against DIO in *Pemt*$^{-/-}$ mice is largely, if not completely, mediated by hepatic PEMT deficiency.

4.3.2 Hepatic PEMT deficiency protects against diet induced insulin resistance

In order to examine the role of hepatic PEMT on insulin sensitivity, GTTs and ITTs were performed at 8 and 9 weeks after AAV treatment, respectively. Consistent with previous data, AAV.GFP *Pemt*$^{-/-}$ mice had a lower fasting blood glucose level and maintained a lower blood glucose level throughout the GTT and ITT, indicating improved glucose tolerance, compared to AAV.GFP *Pemt*$^{+/+}$ mice (Fig. 4.2 A, B). Similar to the effects on weight gain, AAV.PEMT *Pemt*$^{-/-}$ mice are no longer protected against diet induced IR and respond identically to AAV.GFP *Pemt*$^{+/+}$ mice during the GTT and ITT (Fig. 4.2 A, B).

GTTs and ITTs were performed after 5.5 and 6 weeks, respectively, of ASO administration – at which point there were no differences in body weight between groups. The αPEMT group showed improved glucose (Fig. 4.2C) and insulin (Fig. 4.2D) tolerance compared to the control group. Thus, this demonstrates that the protection against diet induced insulin resistance in *Pemt*$^{-/-}$ mice is dependent upon hepatic PEMT deficiency and is independent of body weight.

Figure 4.2

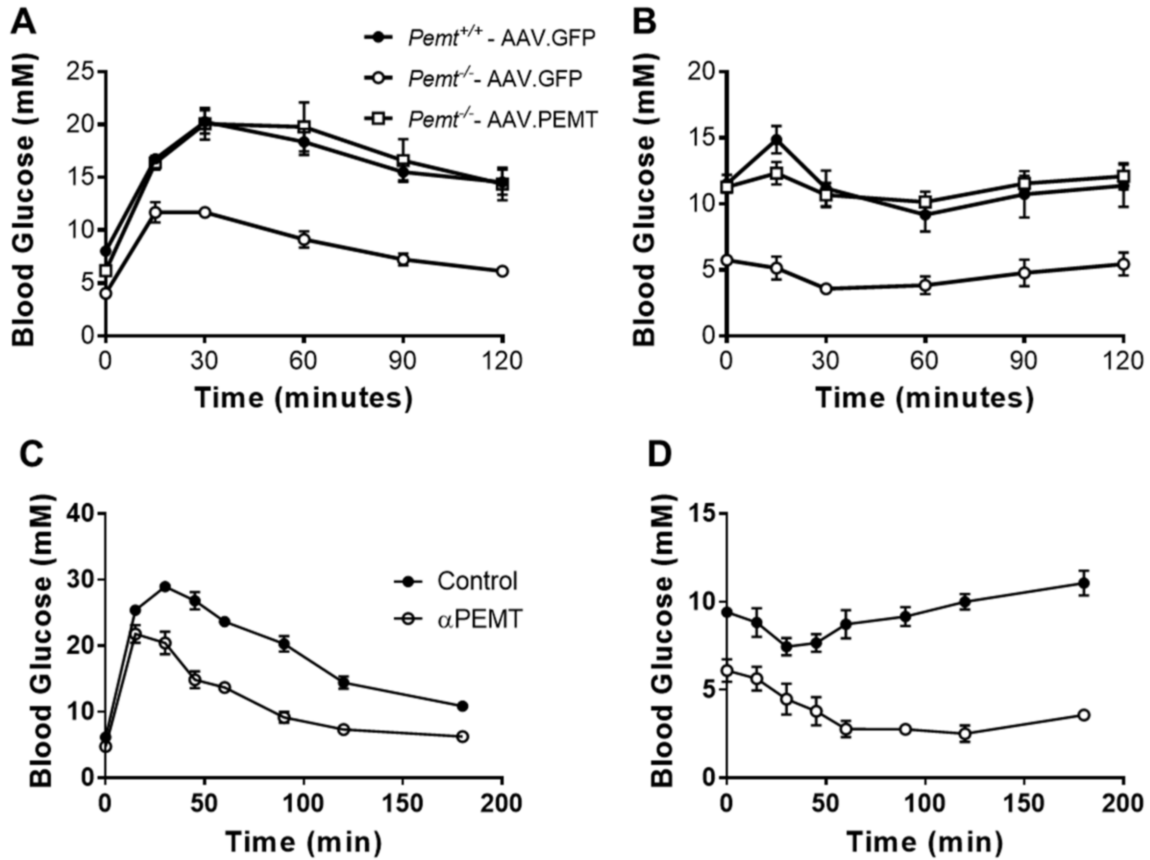

Figure 4.2 Hepatic PEMT activity modulates diet induced insulin resistance

Pemt$^{+/+}$ mice were injected with control AAV.GFP. *Pemt*$^{-/-}$ mice were injected with either the AAV.GFP or AAV.PEMT, which restores hepatic PEMT. All mice were fed the HFD for 10 weeks. (A) GTT. (B). ITT. Values are means ± SEM.

C57BL/6 mice were fed the HFD and treated with either the control or PEMT ASO for 10 weeks. (C) GTT. (D) ITT. Values are means ± SEM.

4.3.3 Knockdown of hepatic PEMT in obese mice reduces weight gain and improves insulin sensitivity

We next investigated whether reducing hepatic PEMT activity would induce weight loss and improve insulin sensitivity in obese mice. PEMT activity was reduced by 84% in the αPEMT group compared to the control group (Fig. 4.3A). Weight gain post-injection was lower in the anti-PEMT group (~1.5g) vs. the control group (8.4g) (Fig. 4.3 B, C). GTTs and ITTs were conducted at 5 and 5.5 weeks after the start of ASO injections. The αPEMT group showed improved glucose (Fig. 4.3D) and insulin (Fig. 4.3E) tolerance compared to the control group.

Figure 4.3

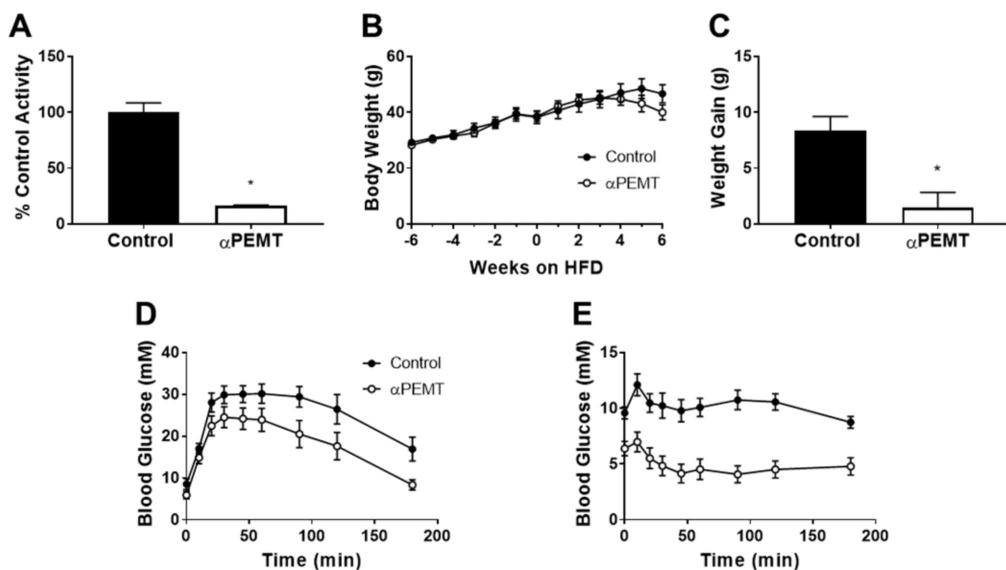

Figure 4.3 Hepatic PEMT knockdown reduces weight gain and insulin resistance in obese mice

C57BL/6 mice were fed the HFD for 6 weeks at which point mice were split into 2 groups and treated with either the control or PEMT ASO and continued on the HFD for a further 6 weeks. (A) Hepatic PEMT activity. (B) Weekly body weight and (C) weight gain over 6 weeks. (D) GTT. (E) ITT. Values are means ± SEM. Student's t-test, * $p<0.05$.

4.3.4 Hepatic PEMT is required for liver health during HFD feeding

Hepatic PC and PE levels appeared to be slightly, but not significantly, decreased and increased, respectively, which led to a significant decrease in the hepatic PC:PE molar ratio in AAV.GFP *Pemt*$^{-/-}$ mice compared to AAV.GFP *Pemt*$^{+/+}$ mice (Fig. 4.4 A-C). There was also a significant increase in hepatic TG and mRNA levels of markers of fibrosis (*Col1a1*), inflammation (*Cd68*), and oxidative stress (*Nox1*) (Fig. 4.4 D, E) in AAV.GFP *Pemt*$^{-/-}$ mice. Restoration of hepatic PEMT in AAV.PEMT *Pemt*$^{-/-}$ mice normalized the hepatic PC:PE molar ratio, TG content, and mRNA levels of markers of fibrosis, inflammation, and oxidative stress to that of AAV.GFP *Pemt*$^{+/+}$ mice (Fig. 4.4 C-E).

Hepatic PEMT knock down did not reduce hepatic PC levels; however, hepatic PE was significantly elevated, resulting in a significant decrease in the hepatic PC:PE molar ratio compared to control mice (Fig. 4.4 F-H). This reduction in the PC:PE ratio was associated with a 4-fold elevation in hepatic TG (Fig. 4.4I). Hepatic mRNA levels of *Col1a1* and *Cd68*, were significantly increased in the αPEMT mice compared to the control mice (Fig. 4.4J). mRNA levels of other markers for fibrosis (*Acta2*), inflammation (*Tnfα*), and oxidative stress (*Hmox1*) were not statistically different between groups (Fig. 4.4J).

Interestingly, KD of hepatic PEMT in obese mice significantly decreased hepatic PC, with no changes in hepatic PE levels (Fig. 4.5 A, B), resulting in a decrease in the hepatic PC:PE molar ratio (Fig. 4.5C). This decrease in the PC:PE ratio was, again, associated with a dramatic elevation in hepatic TG (Fig. 4.5D). mRNA markers of fibrosis (*Acta2*, *Col1a1*), inflammation (*Cd68*, *Tnfα*), and oxidative stress (*Hmox1*, *Nox2*)

were significantly elevated in the αPEMT group compared to the control group (Fig. 4.5E).

Figure 4.4

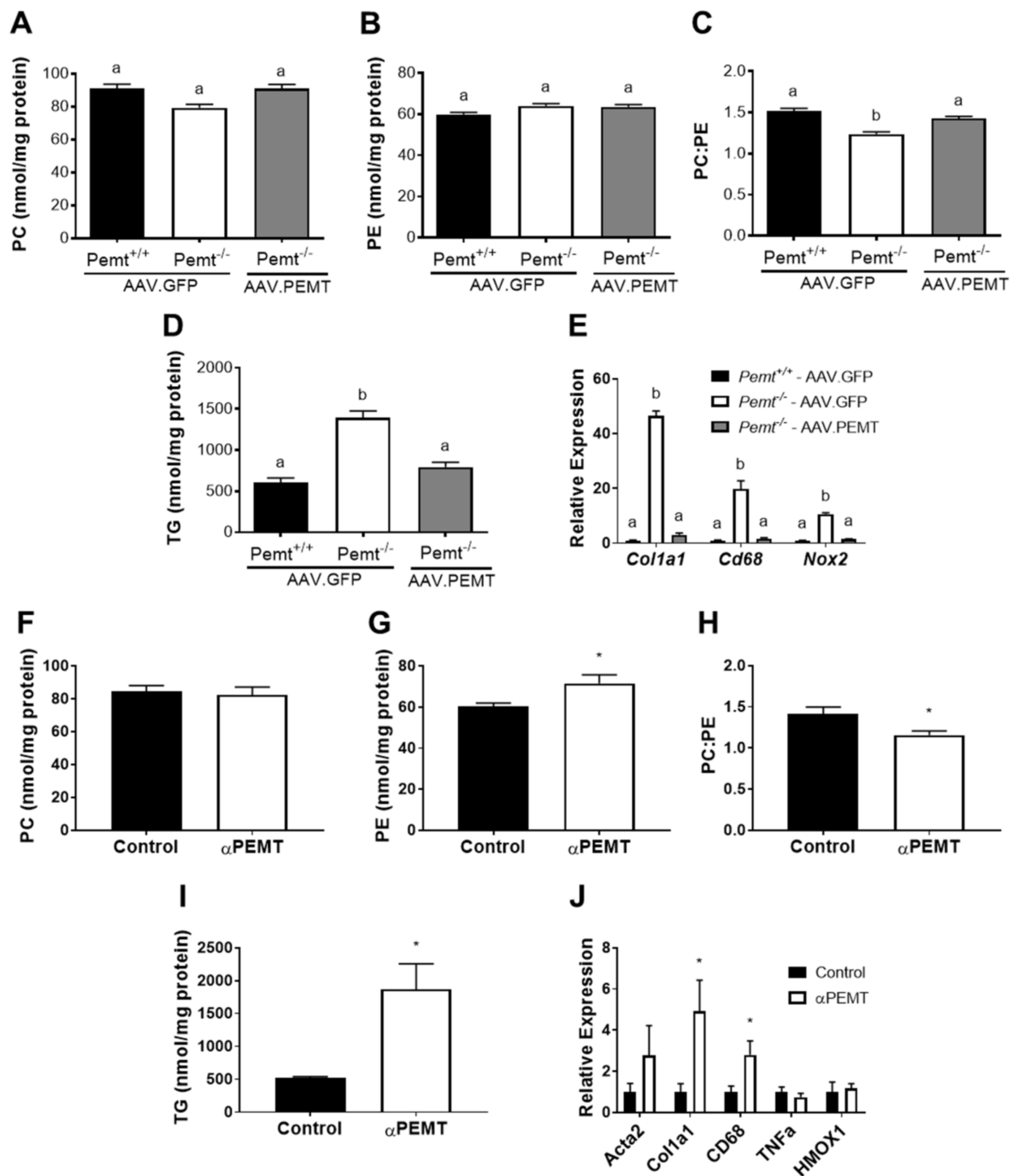

Figure 4.4 Hepatic PEMT activity is critical for liver health during HFD-feeding

Pemt$^{+/+}$ mice were injected with control AAV.GFP. *Pemt*$^{-/-}$ mice were injected with either the AAV.GFP or AAV.PEMT, which restores hepatic PEMT. All mice were fed the HFD for 10 weeks. Hepatic (A) PC and (B) PE mass, (C) PC:PE ratio, and (D) TG content. (E) Hepatic mRNA expression of genes involved in fibrosis (*Col1a1*), inflammation (*Cd68*), and oxidative stress (*Nox2*). Values are means ± SEM and expressed relative to *Pemt*$^{+/+}$ mice. Values that do not share a letter are significantly different ($P < 0.05$).

C57BL/6 mice were fed the HFD and treated with either the control or PEMT ASO for 10 weeks. Hepatic (F) PC and (G) PE mass, (H) PC:PE ratio, and (I) TG content. (J) Hepatic mRNA expression of genes involved in fibrosis (*Acta2, Col1a1*), inflammation (*Cd68, Tnfα*), and oxidative stress (*Hmox1*). Values are means ± SEM and are expressed relative to the control group. Student's t-test, * $p<0.05$.

Figure 4.5

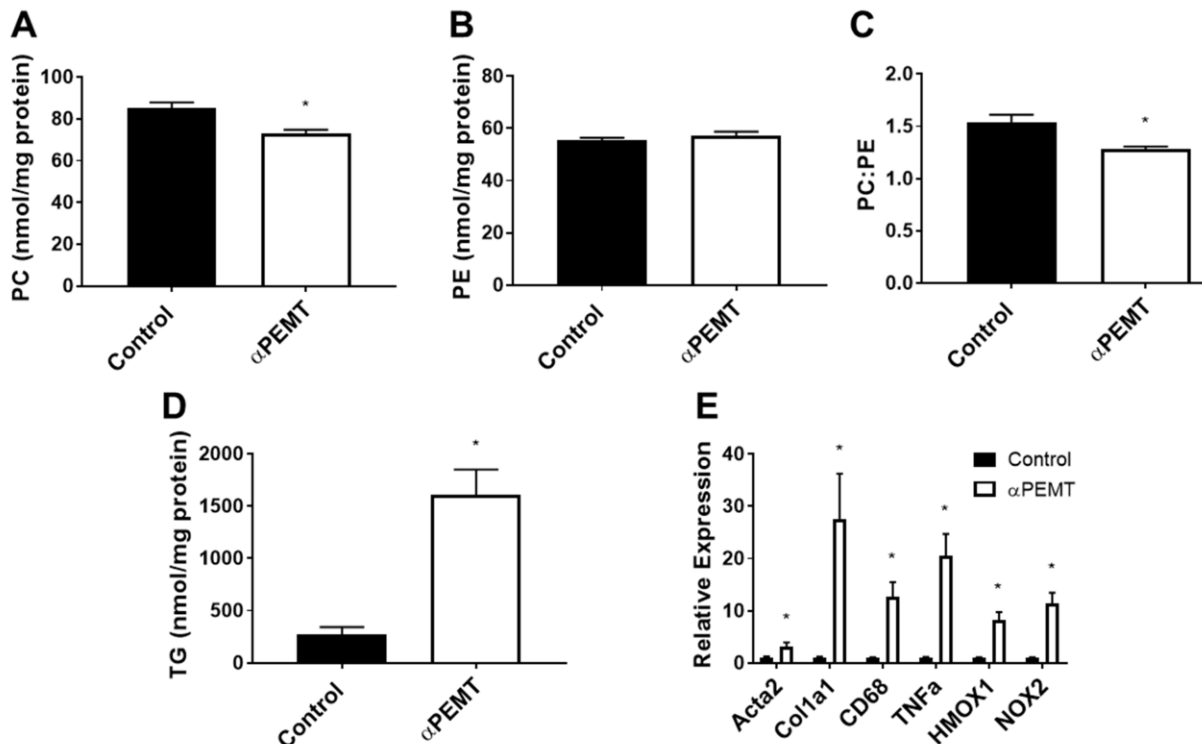

Figure 4.5 Hepatic PEMT knockdown in obese mice results in NASH

C57BL/6 mice were fed the HFD for 6 weeks at which point mice were split into 2 groups and treated with either the control or PEMT ASO and continued on the HFD for a further 6 weeks. Hepatic (A) PC and (B) PE mass, (C) PC:PE ratio, and (D) TG content. (E) Hepatic mRNA expression of genes involved in fibrosis (*Acta2, Col1a1*), inflammation (*Cd68, Tnfα*), and oxidative stress (*Hmox1, Nox2*). Values are means ± SEM and are expressed relative to the control group. Student's t-test, * $p<0.05$.

4.3.5 Knock down of PEMT in *ob/ob* mice reduces weight gain but does not improve insulin sensitivity

We have demonstrated that reduction of PEMT activity reduces diet-induced obesity and improves IR in both lean and obese mice but leads to NASH. We hypothesized that reduction of PEMT in *ob/ob* mice, which has been found to improve liver health and insulin sensitivity, may result in a reduction in weight gain and IR and an improvement of liver health (Fu, Yang et al. 2011). We injected chow-fed *ob/ob* mice with either the scrambled control or αPEMT ASO for 6 weeks. C57BL/6J mice were also fed a chow diet with no ASO injections for the duration of the experiment. PEMT activity was reduced to ~5% in the αPEMT group (Fig. 4.6A). After 6 weeks, hepatic PEMT KD slightly, but significantly, prevented further weight gain compared to the control group (Fig. 4.6 B, C). However, PEMT KD did not improve glucose tolerance (Fig. 4.6D)

4.3.6 Knockdown of PEMT in *ob/ob* mice does not improve liver health

Hepatic PC levels were not different between *ob/ob* mice treated with the control ASO and C57BL/6J wildtype mice (Fig. 4.7A). However, hepatic PE was reduced leading to an increase in the PC:PE molar ratio in the control group compared to the

C57BL/6J mice (Fig. 4.7 B, C). PEMT KD reduced hepatic PC while increasing hepatic PE levels, leading to a significant reduction in the PC:PE molar ratio, below that of both the control group and the C57BL/6J mice (Fig. 4.7 A-C). Hepatic TG was higher in *ob/ob* mice than in C57BL/6J mice but was not different between the control and αPEMT groups (Fig. 4.7D). Plasma alanine-aminotransferase was higher in *ob/ob* mice than in C57BL/6J mice and was further increased by αPEMT-ASO treatment (Fig. 4.7E). mRNA levels of hepatic markers of fibrosis, inflammation, and oxidative stress were not different between the control *ob/ob* group and C57BL/6J mice but were all significantly increased by PEMT KD (Fig. 4.7F). Hepatic protein levels of CHOP and Bip, markers of ER stress, were not different between any groups (Fig. 4.7G).

Figure 4.6

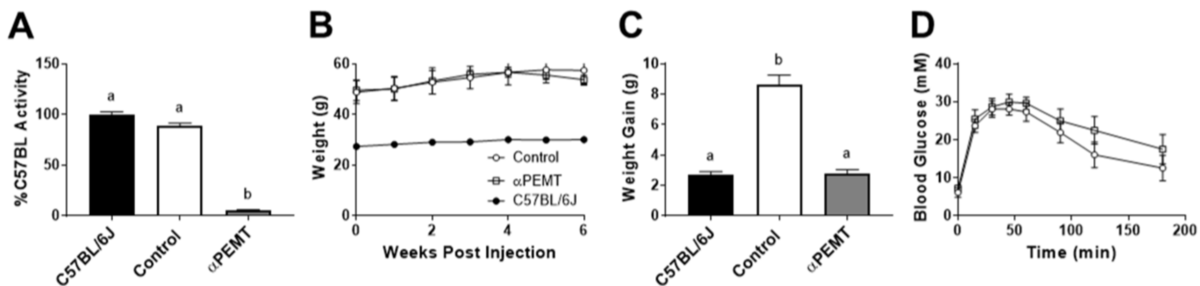

Figure 4.6 Hepatic PEMT knockdown prevents weight gain, but not insulin resistance, in *ob/ob* mice

C57BL/6 mice were maintained on chow without ASO treatment for 6 weeks. *Ob/ob* mice were treated with either the control or PEMT ASO while fed the chow diet for 6 weeks. A) Hepatic PEMT activity. (B) Weekly body weight and (C) weight gain over 6 weeks. (D) GTT. Values are means ± SEM. Two-way ANOVA, followed by Fisher's LSD post hoc test. Values that do not share a letter are significantly different ($P<0.05$).

Figure 4.7

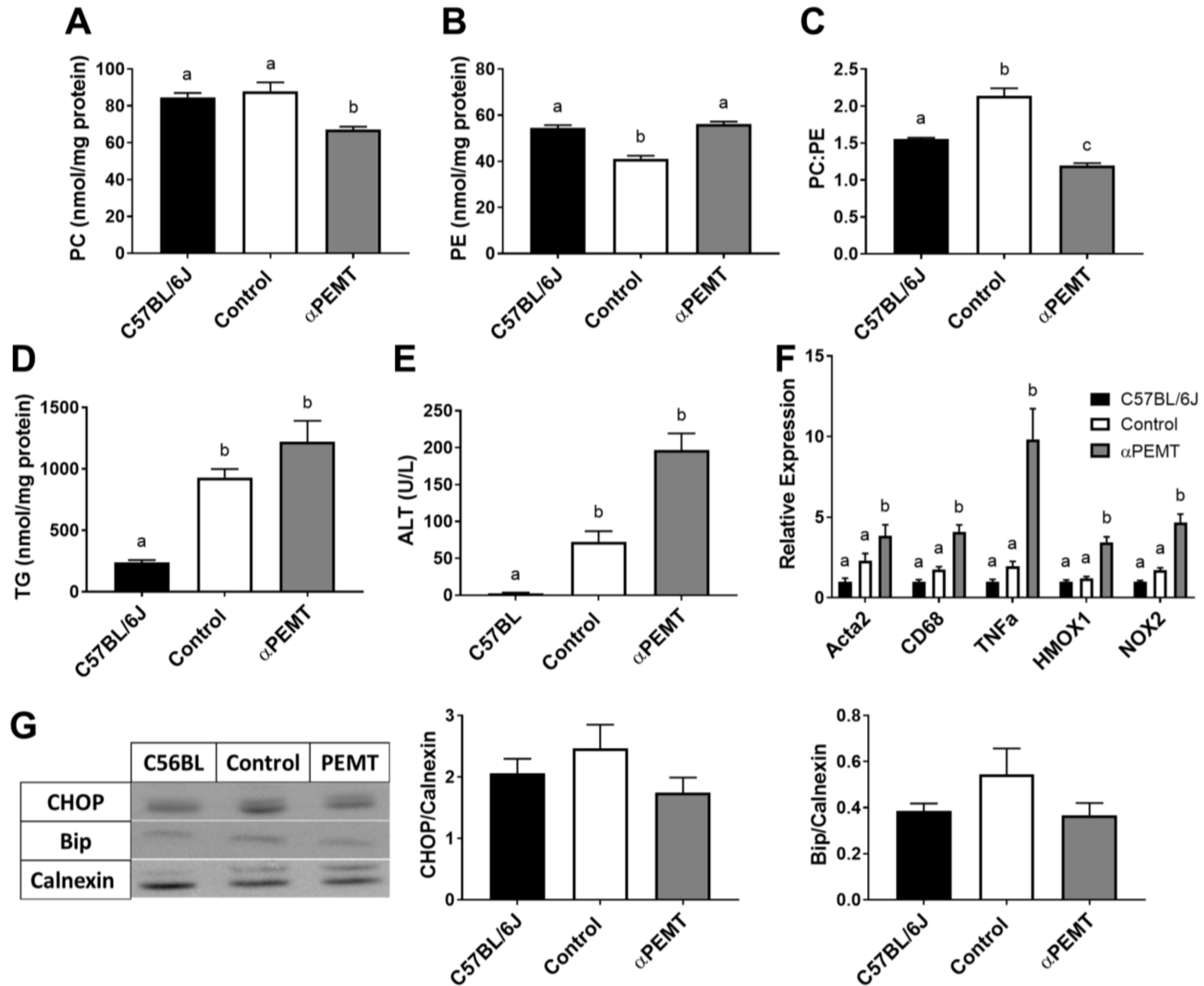

Figure 4.7 Knockdown of hepatic PEMT in *ob/ob* mice is detrimental to liver health and induces NASH

C57BL/6 mice were maintained on chow without ASO treatment for 6 weeks. *Ob/ob* mice were treated with either the control or PEMT ASO while fed the chow diet for 6 weeks. Hepatic (A) PC and (B) PE mass, (C) PC:PE ratio, and (D) TG content. (E) Plasma levels of alanine amino transferase. (F) Hepatic mRNA expression of genes involved in fibrosis (*Acta2*), inflammation (*Cd68, Tnfα*), and oxidative stress (*Hmox1, Nox2*). Values are expressed relative to C57BL/6 mice. (G) Representative immunoblots and densitometry of ER stress responsive C/EBP homologous protein (Schmitt, Kong et al.), and Bip. All values are means ± SEM. Two-way ANOVA, followed by Fisher's LSD post hoc test. Values that do not share a letter are significantly different (P<0.05).

4.4 Discussion

HFD fed *Pemt*$^{-/-}$ mice are protected from DIO but develop NAFLD, which can progress to NASH (Jacobs, Zhao et al. 2010, van der Veen, Lingrell et al. 2016, van der Veen, Lingrell et al. 2017). Here we have demonstrated that these physiological effects are dependent upon hepatic PEMT deficiency. Hepatic PEMT KD, while efficient in reducing weight gain and IR, also results in NAFLD. Likewise, restoration of hepatic PEMT normalizes weight gain and insulin sensitivity, and prevents the development of NAFLD in *Pemt*$^{-/-}$ mice. The development of NAFLD and progression to NASH is associated with a significant decrease in the hepatic PC:PE molar ratio, regardless of changes in the absolute amount hepatic PC and PE.

4.4.1 Metabolic benefits of PEMT deficiency is mediated through hepatic PEMT

The prevalence of NAFLD, obesity, and IR – comorbidities of one another – is rapidly rising in first world nations (Blachier, Leleu et al. 2013, Castro, Kolka et al. 2014, Sung, Lee et al. 2018). Commonly in first world nations, over-nutrition leads to obesity. The increased deposition of lipids in peripheral and hepatic tissues can induce IR (Gaggini, Morelli et al. 2013). NAFLD is often associated with ER stress and dysregulation of lipid metabolism which can also lead to IR (Lebeaupin, Vallee et al. 2018). The development of one disease greatly increases the risk of and susceptibility to another (Sung, Lee et al. 2018). Paradoxically, *Pemt*$^{-/-}$ mice fed a HFD develop NASH and fibrosis but are strikingly protected from obesity and IR. The mechanism behind the protection against obesity has not been fully elucidated, but it has been, in part, attributed to increased energy expenditure and decreased white adipose lipogenesis (Jacobs, Zhao et al. 2010, Gao, van der Veen et al. 2015). Although PEMT

is predominantly found in the liver, we have detected PEMT protein in other tissues, such as adipose and testis (Vance 2014). Others have reported PEMT protein in kidneys; however, we have not reproduced this result using our in-house developed antibody (Vance 2014, Watanabe, Nakatsuka et al. 2014). We hypothesized that the phenotype observed in HFD-fed *Pemt*$^{-/-}$ mice is dependent upon hepatic PEMT activity. Thus, we specifically restored hepatic PEMT activity in *Pemt*$^{-/-}$ mice to that of *Pemt*$^{+/+}$ mice (Fig. 4.1A). We observed that *Pemt*$^{-/-}$ mice with normalized hepatic PEMT activity were no longer protected against DIO (Fig. 4.1 B, C), or IR (Fig. 4.2 A, B). In addition, KD of PEMT, specifically in the liver, was sufficient to partially prevent DIO (Fig. 4.1E, F), and IR (Fig. 4.2 C, D). Hepatic PEMT KD in obese mice also prevented further weight gain and restored insulin sensitivity (Fig. 4.3). In terms of liver health, liver specific restoration of PEMT activity prevented NAFLD development (Fig. 4.4A-G). In contrast, liver specific depletion of PEMT resulted in NAFLD development (Fig. 4.4H-I, Fig. 4.5). This demonstrates that hepatic PEMT activity is indeed critical for the development of the *Pemt*$^{-/-}$ phenotype.

4.4.2 PEMT deficiency promotes insulin sensitivity independent of body weight

Although it is evident that hepatic PEMT deficiency protects against diet induced IR, GTTs and ITTs have, in our laboratory, always been conducted in *Pemt*$^{+/+}$ and *Pemt*$^{-/-}$ mice when there was a significant difference in body weight between groups (Fig. 4.1B) (Jacobs, Zhao et al. 2010, van der Veen, Lingrell et al. 2016, van der Veen, Lingrell et al. 2017). Thus, it was previously difficult to discern the relative role of hepatic PEMT deficiency versus adiposity in IR. However, GTTs and ITTs were performed in our lean and obese mice, when there was not yet a significant difference in body weight,

and showed that hepatic PEMT deficiency improved insulin sensitivity (Fig. 4.2 C, D, Fig 4.3 D, E). These data suggest that independent of weight gain and adiposity, PEMT deficiency leads to an improvement in insulin sensitivity. In agreement, we have recently demonstrated that insulin signaling is improved in primary hepatocytes and hepatoma cells that lack PEMT (Veen, Lingrell et al. 2019). Moreover, this seems to be due to a decrease in the PC:PE molar ratio, or an increase in PE (Veen, Lingrell et al. 2019).

4.4.3 PEMT is crucial for liver health in HFD-fed mice

While hepatic PEMT KD is effective at both preventing and treating obesity and IR in HFD-fed mice, it also results in a decreased hepatic PC:PE molar ratio and NAFLD. In lean mice, the reduction in the hepatic PC:PE molar ratio was associated with an increase in hepatic PE (Fig. 4.4I), yet, in obese mice, it was associated with a decrease in hepatic PC (Fig. 4.5A). In *Pemt*$^{-/-}$ mice, neither hepatic PC nor PE were significantly altered (Fig. 4.4 A, B). However, there was a similar level of steatosis and decrease in the hepatic PC:PE molar ratio in all 3 experiments. In addition, restoration of hepatic PEMT activity in *Pemt*$^{-/-}$ mice normalized the hepatic PC:PE molar ratio and prevented the development of hepatic steatosis. This suggests that the steatosis associated with PEMT deficiency is a result of an altered PC:PE molar ratio, rather than changes in the absolute amounts of individual phospholipids.

Interestingly, it appears that reduction of PEMT activity in adult mice does not lead to the same severity of liver disease as in the genetic *Pemt*$^{-/-}$ mice. *Pemt*$^{-/-}$ mice rapidly develop NASH with inflammation, ER stress, and oxidative stress which can progress to fibrosis when fed the HFD (Fig. 4.4 E-G) (Ling, Chaba et al. 2012, Gao, van der Veen et al. 2015, Lian, Wei et al. 2016, van der Veen, Lingrell et al. 2017). While

knockdown of PEMT in mice fed the HFD for 10 weeks resulted in hepatic steatosis and mildly increased mRNA levels of *Col1a1* and *Cd68*, there were no significant differences in other mRNA markers for inflammation and oxidative stress (Fig 4.4L). However, when obese mice were treated with the αPEMT-ASO, there was a significant increase in hepatic steatosis along with even further increased mRNA levels of markers for inflammation (*Tnfα, Cd68*), fibrosis (*Acta2*, *Col1a1*), and oxidative stress (*Nox2, Hmox1*) (Fig. 5E). In humans the severity of NAFLD and progression to NASH has been positively correlated with obesity (Oh, Kim et al. 2016, Lu, Hu et al. 2018). It is possible that knockdown of PEMT in obese mice, who likely have mild steatosis, could provide another “hit” to the liver that promotes NASH progression.

4.4.4 Disparities in the effects of PEMT deficiency in different murine models

Our laboratory has extensively studied the effects of HFD-feeding and PEMT deficiency in *Pemt*$^{-/-}$ mice. We have previously observed increased hepatic PEMT activity and PC:PE molar ratio in diabetic rats (Hartz, Nieman et al. 2006). Glycine *N*-methyltransferase deficient mice display increased PC synthesis through the PEMT pathway and, consequently, have a dramatically increased hepatic PC:PE molar ratio (Martinez-Una, Varela-Rey et al. 2013). These mice also develop hepatic steatosis and will develop hepatocellular carcinoma when ageing (Martinez-Chantar, Vazquez-Chantada et al. 2008, Martinez-Una, Varela-Rey et al. 2013).

Although the metabolic benefits of hepatic PEMT KD is plagued with NAFLD development in HFD-fed mice, there appear to be several murine models in which PEMT reduction improves liver health. Leptin-deficient *ob/ob* mice develop NAFLD with ER stress on a chow diet (Fu, Yang et al. 2011, Perfield, Ortinau et al. 2013). ER stress

was associated with increased ER PC and an increased PC:PE molar ratio (Fu, Yang et al. 2011). In that model, KD of PEMT has been shown to improve ER stress and liver health (Fu, Yang et al. 2011). Short term (10-14 day) knockdown of PEMT in o*b/ob* mice led to a reduction in the hepatic PC:PE molar ratio and to a reduction in ER stress (Fu, Yang et al. 2011). PEMT KD also reduced hepatic steatosis and improved insulin sensitivity in *ob/ob* mice (Fu, Yang et al. 2011). We therefore hypothesized that αPEMT-ASO treatment in *ob/ob* mice would reduce weight gain and IR without the development of NAFLD. Although PEMT KD in *ob/ob* mice did reduce weight gain, it was ineffective in improving insulin sensitivity (Fig. 4.6 B-D). In agreement with the published ER-specific PC:PE ratio, *ob/ob* mice did indeed have a higher hepatic PC:PE molar ratio than C57BL/6 mice and developed hepatic steatosis (Fig. 4.7C, D) (Fu, Yang et al. 2011). Contrary to literature, despite an elevation in the hepatic PC:PE ratio, we did not observe an increase in ER stress in control *ob/ob* mice compared with C57BL/6 mice (Fig. 7G). Administration of αPEMT-ASO decreased the hepatic PC:PE molar ratio but, also contrary to the literature, did not improve hepatic steatosis (Fig. 4.7C, D) (Fu, Yang et al. 2011). Instead, reduction of PEMT activity resulted in an increase in hepatic mRNA levels of markers associated with inflammation, fibrosis, and oxidative stress as well as plasma ALT levels (Fig. 4.7 E, F). Interestingly, this worsening of liver health and NAFLD progression did not result in a significant increase in markers of ER stress (Fig. 4.7G).

The observed hepatic changes show that long term reduction of PEMT activity results in NASH in *ob/ob* mice as well. In our study, PEMT was dramatically inhibited (5% of normal activity) for 6 weeks which led to a PC:PE ratio (1.2 ± 0.03) that was

below both that of the control *ob/ob* mice (2.1 ± 0.10) as well as the C57BL/6 mice (1.6 ± 0.02) (Fig. 4.7C). Although in the literature, short term PEMT KD in *ob/ob* mice significantly decreased the PC:PE molar ratio compared to control *ob/ob* mice, it is not possible to compare this decrease to values in wild type animals, as they were not included in the KD experiments (Fu, Yang et al. 2011). Since *ob/ob* mice have an increased hepatic PC:PE molar ratio, the large decrease achieved by short term PEMT KD may have *normalized* the ratio to that of wild type mice where as our long term PEMT KD decreased the ratio to *below* that of wild type mice (Fu, Yang et al. 2011). Thus, unlike *ob/ob* mice subjected to a long term PEMT KD, those subjected to a short term PEMT KD may be reaching a healthy PC:PE molar ratio, and therefore, improve liver health. It is also possible that, similar to *Pemt*$^{-/-}$ mice, long term PEMT KD results in the development of cholestasis which causes and exacerbates liver damage (Wan, Kuipers et al. 2019).

In conclusion, we have demonstrated that the phenotype observed in HFD-fed *Pemt*$^{-/-}$ mice is dependent on hepatic PEMT deficiency. In both C57BL/6 and *ob/ob* mice, we observed that decreased PEMT activity had a negative impact on liver health. While it is enticing that diminishing PEMT can reduce diet induced weight gain and IR, it will result in a drop in the hepatic PC:PE molar ratio and cause the development of NAFLD. In addition, significant alterations to the hepatic PC:PE molar ratio during obesity/over-nutrition may promote NASH progression.

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Chapter 5

Conclusion and Future Directions

5.1 Conclusion

PEMT is quantitatively important in the liver and accounts for approximately 30% of hepatic PC synthesis, with the remaining 70% synthesized by the CDP-choline pathway (DeLong, Shen et al. 1999). When fed a high fat diet, both PEMT and liver-specific CTα deficient mice develop NASH associated with reduced VLDL secretion and a reduced hepatic PC:PE molar ratio (Noga, Zhao et al. 2002, Ling, Chaba et al. 2012). Despite the CDP:choline pathway being the major contributor of hepatic PC synthesis, there was a greater decrease in the hepatic PC:PE molar ratio, associated with increased NAFLD severity, in *Pemt*$^{-/-}$ mice compared to L*Pcyt1a*$^{-/-}$ mice (Ling, Chaba et al. 2012). Additionally, *Pemt*$^{-/-}$ mice, but not L*Pcyt1a*$^{-/-}$ mice, are protected against DIO and IR which suggests a role for PEMT, independent of PC deficiency, in mediating these effects (Jacobs, Zhao et al. 2010). However, choline supplementation eliminates the protection against DIO and IR and improves NAFLD in HFD-fed *Pemt*$^{-/-}$ mice (Jacobs, Zhao et al. 2010). Aside from the role of PC in VLDL secretion, a large proportion of PC is also secreted into bile (Walkey, Yu et al. 1998). The experiments outlined in this thesis illuminates the role of PEMT in the development of both cholestasis and NAFLD.

PEMT is enriched in portions of the ER which are in close proximity to the CM and has been thought to cooperate with proteins on the CM for biliary secretion (Sehayek, Wang et al. 2003). In **Chapter 2**, in addition to lowering the hepatic PC:PE molar ratio and inducing NASH, HFD-feeding induced cholestasis in *Pemt*$^{-/-}$ mice; both basal and maximal biliary secretion of BAs, PLs, and cholesterol were decreased and resulted in an accumulation of BAs and bilirubin in plasma. These effects were a result

of a massive dysregulation of hepatic genes involved in BA homeostasis, including a significant decrease in BSEP protein, and a loss of canalicular structure. It has been shown that phospholipid asymmetry on the CM is critical for proper biliary secretion (Groen, Romero et al. 2011). The significantly decreased hepatic PC:PE molar ratio in *Pemt*$^{-/-}$ mice is likely also reflected on the CM and disrupts phospholipid asymmetry. Furthermore, PEMT may locally produce and supply the CM with PC, possibly by removing PE from the CM itself. Thus, PEMT deficiency may dysregulate phospholipid balance even more greatly than what is reflected in the overall hepatic PC:PE molar ratio. Regardless, it is evident that PEMT is required during HFD-feeding for proper biliary secretion.

It has been previously observed that dietary choline supplementation normalizes the hepatic PC:PE molar ratio and, thus, reduces hepatic steatosis and prevents NASH development in HFD-fed *Pemt*$^{-/-}$ mice (Jacobs, Zhao et al. 2010). Dietary choline supplementation was able to both prevent and treat cholestasis in HFD-fed *Pemt*$^{-/-}$ mice. Although CTα has always been believed to be the rate-limiting step for PC synthesis through the CDP:choline pathway, it appears that under HFD conditions the initial substrate, choline, may instead be limiting. Interestingly, choline supplementation was not able to normalize the hepatic PC:PE molar ratio in these experiments. Furthermore, the mechanism by which the hepatic PC:PE molar ratio was decreased was different between the prevention and intervention studies. In the prevention studies, hepatic PC was normalized between genotypes and PE was increased in *Pemt*$^{-/-}$ mice. In the intervention, or treatment, studies, hepatic PC was lower in *Pemt*$^{-/-}$ mice where as PE was not different between genotypes. These data demonstrate that the absolute amount

of hepatic PC is not critical for biliary secretion. It is likely that the CM, a major site for PC loss, is hypersensitive to changes in PC synthesis/availability and thus the cholestatic phenotype can be rescued without necessarily correcting the overall hepatic PC:PE molar ratio. In support, defects in biliary secretion induced by ATP8B1 deficiency can be rescued by ablation of MDR3; there is a reduction in the amount of PC in the CM but this reinstates phospholipid asymmetry and rescues biliary secretion (Groen, Romero et al. 2011). The effects of choline supplementation on biliary secretion clearly demonstrate that insufficient PC synthesis/availability play a role in the development of cholestasis in HFD-fed *Pemt*$^{-/-}$ mice.

HFD-fed L*Pcyt1a*$^{-/-}$ mice also develop NASH and a reduced hepatic PC:PE molar ratio (Niebergall, Jacobs et al. 2011, Ling, Chaba et al. 2012). Following the findings in **Chapter 2**, biliary secretion processes were closely examined in iL*Pcyt1a*$^{-/-}$ mice fed the HFD for 10 weeks (Fig. 5.1). In **Chapter 3**, HFD-fed iL*Pcyt1a*$^{-/-}$ mice develop NASH but have a higher hepatic PC:PE molar compared to control mice. Surprisingly, control mice also developed hepatic steatosis. iL*Pcyt1a*$^{-/-}$ mice did not have impairments in the biliary secretion of BAs and have increased biliary secretion of PLs. It is interesting to speculate that there may be a positive correlation between the hepatic PC:PE molar ratio and biliary phospholipid secretion. In addition, regulation of genes responsible for hepatic BA homeostasis is intact and there are no alterations in canalicular structure in iL*Pcyt1a*$^{-/-}$ mice. The lack of changes in hepatic phospholipid content clearly demonstrates that PEMT is able to fully compensate for the CDP:choline pathway in an inducible model of CTα deficiency (Fig. 5.1).

Figure 5.1

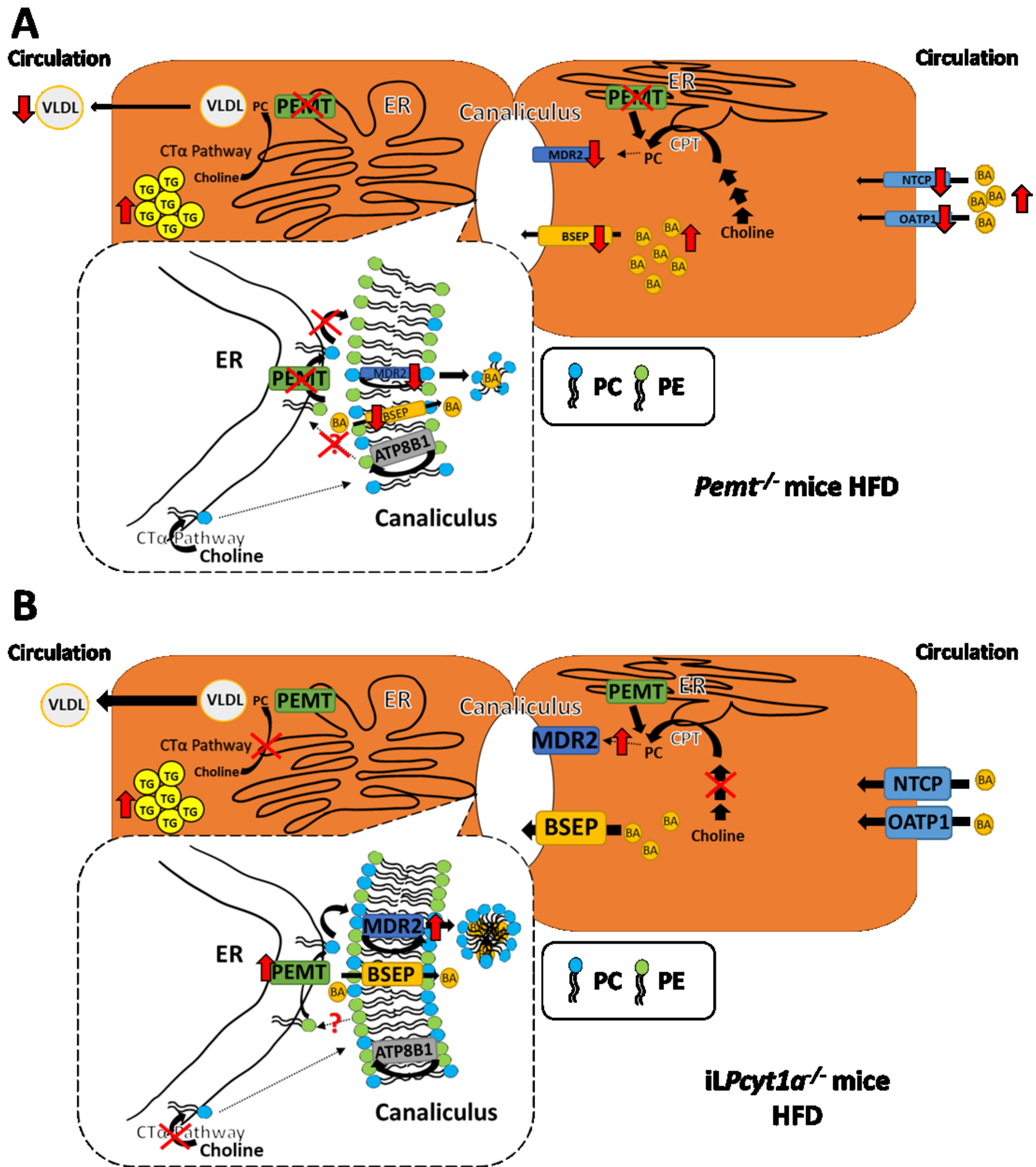

Figure 5.1 NAFLD and biliary processes in *Pemt*$^{-/-}$ and iL*Pcyt1a*$^{-/-}$ mice

(A) When *Pemt*$^{-/-}$ mice are fed the HFD, the amount of mRNAs encoding NTCP, OATP1, and BSEP is decreased (mechanism not known), leading to the accumulation of BAs in plasma and, likely, in the liver/hepatocyte. Moreover, the amount of MDR2 is decreased in an attempt to conserve hepatic PC. Thus, because PC is synthesized only by the choline pathway in *Pemt*$^{-/-}$ hepatocytes, a reduction in hepatic PC leads to impaired VLDL secretion and, consequently, hepatic steatosis. The lack of PEMT in the hepatocytes reduces the local supply of PC for the canalicular membrane, which cannot be compensated by PC synthesized in the bulk of the ER by the choline pathway. The deficiency of PC in the canalicular membrane would compromise integrity of the canalicular membrane and change in structure of the canaliculus. Arrows in red indicate differences compared to *Pemt*$^{+/+}$ mice.

(B) HFD-feeding in iL*Pcyt1a*$^{-/-}$ mice results in an elevated hepatic PC:PE ratio and hepatic steatosis, likely due to impaired VLDL secretion. PEMT derived PC synthesis can compensate for the lack of CTα; the amount of hepatic PC is not different from control mice. PEMT lies in close proximity to the canalicular membrane and the increase in PC synthesis can locally increase PC availability for biliary secretion and, consequently, biliary secretion of PC is increased in iL*Pcyt1a*$^{-/-}$ mice. Canalicular structure is intact, there are no changes in hepatic genes associated with BA homeostasis, and no development of cholestasis. Arrows in red indicate differences compared to control mice.

Figure 5.2

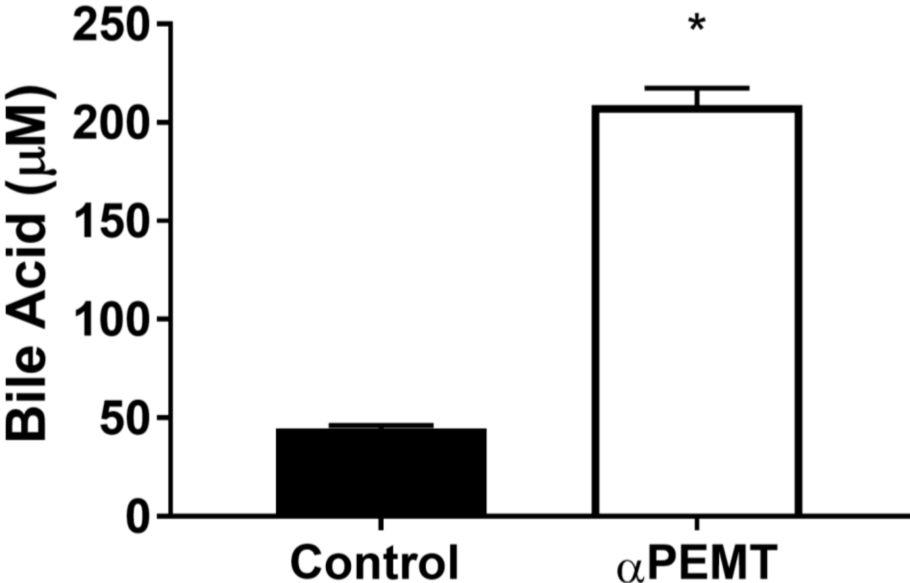

Figure 5.2 Hepatic PEMT deficiency induces cholestasis during HFD-feeding

Plasma bile acid concentration of C57BL/6 mice which were fed the HFD and treated with either the control or PEMT ASO for 10 weeks.

The lack of cholestasis establishes that the CDP:choline pathway is not critical for biliary secretion processes and further support PEMT being required for proper biliary secretion during HFD-feeding (Fig. 5.1).

We have previously suggested that hallmarks of NASH – i.e. ER stress, inflammation, fibrosis, and oxidative stress – and cholestasis may exacerbate one another in HFD-fed *Pemt*$^{-/-}$ mice (Wan, Kuipers et al. 2019). Although NASH develops in both HFD-fed *Pemt*$^{-/-}$ and L*Pcyt1a*$^{-/-}$ mice, the severity of liver disease has been observed to be greater in *Pemt*$^{-/-}$ mice (Ling, Chaba et al. 2012). NAFLD and NASH developed in HFD-fed control and iL*Pcyt1a*$^{-/-}$ mice, respectively, without cholestasis. Thus, it appears that NAFLD, and subsequent progression to NASH, are likely not causal factors in the development of cholestasis. Although NASH may not be driving the development of cholestasis, it is likely that the resulting hepatic accumulation of BAs exacerbates NASH and worsens liver health in *Pemt*$^{-/-}$ mice thus resulting in the severe liver disease previously observed (Ling, Chaba et al. 2012).

PEMT is predominantly found in the liver although small amounts of PEMT protein has previously been observed in other tissues, such as adipose, testis, and kidneys (Vance 2014, Watanabe, Nakatsuka et al. 2014). In addition to developing NAFLD, *Pemt*$^{-/-}$ mice are also protected from DIO and IR (Jacobs, Zhao et al. 2010). In **Chapter 4**, hepatic PEMT was either restored in *Pemt*$^{-/-}$ mice or knocked down in wildtype mice. Hepatic PEMT KD, in both lean and obese mice, reduced weight gain, improved insulin sensitivity, and resulted in NASH associated with a reduced hepatic PC:PE molar ratio. In agreement, restoration of hepatic PEMT in *Pemt*$^{-/-}$ mice reversed the protection against DIO and IR, and normalized the hepatic PC:PE molar ratio,

thereby preventing NAFLD development. Additionally, plasma BA concentrations, a marker of cholestasis, were elevated by hepatic PEMT KD (Fig. 5.2). These data clearly show that the phenotype observed in HFD-fed *Pemt*$^{-/-}$ mice is dependent on hepatic PEMT deficiency.

Both *Pemt*$^{-/-}$ mice and hepatic PEMT KD mice, had reduced hepatic PC:PE molar ratio compared to *Pemt*$^{+/+}$ and control mice, respectively. However, the mechanism by which the decrease in the PC:PE ratio is achieved is different; hepatic PC was decreased and PE was increased in *Pemt*$^{-/-}$ mice and mice which had hepatic PEMT KD, respectively. Interestingly, in all experiments, PEMT deficiency resulted in NASH and a decrease in the hepatic PC:PE molar ratio. Similar to the relationship between the hepatic PC:PE molar ratio and cholestasis, NASH, associated with PEMT deficiency, is also a result of an altered PC:PE molar ratio, rather than changes in the absolute amount of individual phospholipids.

Previous studies in our lab, as well as experiments in this thesis, provide extensive evidence that reductions in the hepatic PC:PE molar ratio results in NAFLD/NASH (Jacobs, Zhao et al. 2010, Ling, Chaba et al. 2012, van der Veen, Lingrell et al. 2017). Additionally, *Gnmt*$^{-/-}$ mice and *ob/ob* mice both have a significantly increased hepatic PC:PE molar ratio and develop NAFLD (Martinez-Chantar, Vazquez-Chantada et al. 2008, Fu, Yang et al. 2011, Martinez-Una, Varela-Rey et al. 2013, Perfield, Ortinau et al. 2013). Short term KD of hepatic PEMT improved ER stress and liver health in *ob/ob* mice (Fu, Yang et al. 2011). In agreement with the literature, we found *ob/ob* mice to have an increased hepatic PC:PE molar ratio compared to controls and to develop NAFLD (Fu, Yang et al. 2011). Hepatic PEMT KD significantly

decreased the hepatic PC:PE molar ratio in *ob/ob* mice to below that of control mice. Consequently, liver health did not improve but rather worsened in *ob/ob* mice when hepatic PEMT was knocked down. It is also possible that long term PEMT KD induced cholestasis, thereby further worsening liver health. These findings further demonstrate that alterations in the hepatic PC:PE molar ratio, regardless of the direction of change, is associated with NAFLD.

In summary, hepatic PEMT is responsible for the development of the HFD-fed *Pemt*$^{-/-}$ phenotype. Hepatic PEMT, but not CTα, is required during HFD-feeding in order to maintain proper biliary secretion processes (Fig. 5.1). The development of neither cholestasis nor NAFLD are dependent upon the absolute amount of hepatic PC or PE, but rather are dependent upon changes in the hepatic PC:PE molar ratio. The CM is hypersensitive to minute changes in phospholipid balance; cholestasis can be rescued in HFD-fed *Pemt*$^{-/-}$ mice by increasing PC synthesis/availability and restore CM integrity without requiring a major increase in the overall hepatic PC:PE molar ratio. Alterations in the hepatic PC:PE molar ratio result in NAFLD which can progress to NASH. While cholestasis may develop independent of NASH in HFD-fed *Pemt*$^{-/-}$ mice, it is likely that cholestasis aggravates NASH progression.

Beyond delineating the role of PEMT and the PC:PE ratio in biliary secretion processes and NAFLD development, this thesis also suggests a potential treatment strategy for cholestasis. Choline supplementation may be a novel adjuvant therapy for a select subset of patients with cholestasis who suffer from phospholipid imbalance and/or hepatic PC insufficiency. Additionally, HFD-fed *Pemt*$^{-/-}$ mice are a novel model

for diet induced cholestasis, specifically mimicking aspects of PFIC2/BRIC2 and PFIC1/BRIC1.

This thesis also demonstrates that PEMT deficiency is likely not a viable therapy for weight gain and insulin resistance in humans as perturbations in the hepatic PC:PE molar ratio lead to the development of NAFLD. However, this does suggest that PC:PE correction may alleviate NAFLD in patients who have an altered hepatic PC:PE ratio.

5.2 Future Directions

While the experiments in this thesis have greatly enhanced our understanding of the functional role of PEMT and the PC:PE ratio in the pathogenesis of cholestasis and NAFLD, there are a few points which may warrant further investigation:

In chapter 2, it is evident that hepatic BA homeostasis is disrupted and that this disruption contributes to the cholestasis present in HFD-fed *Pemt*$^{-/-}$ mice. However, the mechanism by which FXR signalling, and thereby BA homeostasis, is interrupted is still unclear. It is possible that the increase in MCA species, a natural antagonist of FXR, is repressing FXR signalling; however, this does not appear to be the case with all FXR responsive genes (Sayin, Wahlstrom et al. 2013). Additionally, FXR itself is also downregulated. Future studies should aim to delineate the mechanism by which FXR signalling and BA homeostasis is disrupted.

Following the development of cholestasis and rise in plasma BAs, it is possible that peripheral BA signalling may contribute to the resistance to DIO and IR in HFD-fed *Pemt*$^{-/-}$ mice. Peripheral BA signalling has numerous effects which include increased energy expenditure and β-cell protection and proliferation (Watanabe, Houten et al.

2006, Thomas, Gioiello et al. 2009, Kumar, Rajagopal et al. 2012, Kumar, Asgharpour et al. 2016). It would be interesting to determine if the increased energy expenditure and protection against DIO and IR in HFD-fed *Pemt*$^{-/-}$ mice was associated with peripheral BA signalling (Jacobs, Zhao et al. 2010).

Finally, in chapter 3 iL*Pcyt1a*$^{-/-}$ do not gain weight during the first 3 weeks of being fed the HFD. It is unclear what acute changes occur during this time period which prevent weight gain. Future studies should endeavor to delineate the differences between acute and longterm hepatic CTα deletion. Biliary PL secretion is required for secretion of dietary lipids (Voshol, Minich et al. 2000). It is possible that acute CTα deletion rapidly depletes hepatic PC before PEMT is able to compensate. Thus, secretion of dietary lipids may be acutely impaired in iL*Pcyt1a*$^{-/-}$ mice and lead to lack of weight gain.

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