Role of Hepatic PEMT and Dietary Choline in the Development of Atherosclerosis

by

Yumna Zia

A thesis submitted in partial fulfillment of the requirements for the degree of

Master of Science

in
Nutrition and Metabolism

Department of Agricultural, Food and Nutritional Science
University of Alberta

Abstract:

Phosphatidylethanolamine *N*-methyltransferase (PEMT) is a hepatic enzyme that converts phosphatidylethanolamine to phosphatidylcholine. *Pemt*$^{-/-}$ mice are protected from obesity and insulin resistance, a phenotype that is reversed with dietary choline supplementation. Additionally, PEMT deficiency reduces plasma lipids and is protective against atherosclerosis when crossed with the low-density lipoprotein receptor (*Ldlr*$^{-/-}$) mice. Recent studies have demonstrated that choline can be metabolized by the gut microbiota into trimethylamine-*N*-oxide (TMAO), which is a novel risk factor for atherosclerosis. The effect of choline supplementation on the development of atherosclerosis in *Pemt*$^{-/-}$/*Ldlr*$^{-/-}$ mice is not known. Therefore, the objective of this thesis was to determine whether reintroducing hepatic PEMT expression or dietary choline supplementation promotes atherosclerosis in *Pemt*$^{-/-}$/*Ldlr*$^{-/-}$ mice. *Pemt*$^{+/+}$/*Ldlr*$^{-/-}$ (SKO) and *Pemt*$^{-/-}$/*Ldlr*$^{-/-}$ (DKO) mice were injected with an adeno-associated virus (AAV) expressing green florescent protein (GFP) or human PEMT, and fed a western diet (40% calories from fat, 0.5% cholesterol, 3g/kg choline) for 8 weeks. In a separate experiment, SKO and DKO mice were fed the western diet containing 3 or 10g/kg choline for 12 weeks. The results demonstrated that DKO mice have low plasma lipids and were protected against atherosclerosis compared to SKO mice. AAV-PEMT administration increased plasma lipids and TMAO in DKO mice. Furthermore, AAV-PEMT injected DKO mice developed atherosclerotic lesions similar to SKO mice. In the second study, choline supplementation in DKO mice did not increase atherosclerosis or plasma lipids, but did increase plasma TMAO levels.

Next, we sought to investigate whether **reducing** dietary choline influences TMAO production and development of atherosclerosis in SKO mice. We found that reducing dietary choline attenuated atherosclerosis in SKO mice compared to mice fed a high choline diet. However, decreasing choline did not alter plasma lipids or TMAO production. In summary, this thesis focused on the role of dietary choline and PEMT enzyme in the development of atherosclerosis. We found reintroducing hepatic PEMT expression reversed the atheroprotective phenotype of DKO mice while choline supplementation did not increase atherosclerosis or plasma lipids. Our data suggests that plasma TMAO levels do not always correlate with atherosclerosis and plasma lipids. Furthermore, this is the first report suggesting that *de novo* choline synthesis alters TMAO metabolism.

Overall this research work contributes significantly towards our understanding of the complex relationship of dietary choline, de novo choline and TMAO production and how these factors influence the development of atherosclerosis and lipid metabolism.

Preface

This thesis is original work by Yumna Zia. My research work involved animals that received ethics approval from the Committee of Animal care and use committee (ACUC) Health Sciences at the University of Alberta. There are two main research projects covered in this thesis.

Chapter 3 entitled "Hepatic expression of Pemt, but not dietary choline supplementation, reverses the protection against atherosclerosis in *Pemt*$^{-/-}$/*Ldlr*$^{-/-}$ mice" is a manuscript submitted for publication. The authors of this manuscript are the following: Yumna Zia, Ala Al Rajabi, Si Mi, Ting Ting Ju, Kelly-Ann Leonard, Randal Nelson, Ben Willing, Catherine J. Field, Jonathan M. Curtis, Jelske N. van der Veen, René L. Jacobs.

I was responsible for the experimental work, data collection, statistical analysis and writing the manuscript for this project. Dr. Rajabi was involved in designing the study, establishing the protocol for atherosclerosis assessment, as well as data analysis and statistics involved in the choline supplementation experiment. Our collaborators, Dr. Willing and Ms. Tingting, performed the gut microbiota analysis. Susanne Lingrell performed the PEMT activity assays; Dr. Van der Veen performed AAV injections and Dr. Curtis and Mi measured choline metabolites.

Chapter 4 entitled "Switching from a high choline to a low choline diet attenuates atherosclerosis" was a pilot project performed by Yumna Zia. I was involved in experimental design and data collection statistical analysis and writing the manuscript for this experiment.

Both projects required technical support from Audric Moses (lipid analysis), Nicole Coursen, Kelly Leonard and Randy Nelson (animal handling procedures).

Dedication

To my daughter, the great big love of my life, Inayah Waqas. In the face of all hardships, I hope you always remember that there is nothing you can't achieve, through dedication and hard work

Acknowledgement

I would like to thank everyone who contributed towards the completion of this project and supported me during my master's program. First and foremost, my biggest thank you to an amazing supervisor Dr. René Jacobs, who was always my strongest critic and my strongest advocate. I can't thank you enough for supporting me throughout my Master's program, pushing me to do my very best and providing me excellent training environment for scientific research. I'm also so grateful for his supportive attitude during my pregnancy and after. I also want to give a special thanks to Dr. Spencer Proctor, whose criticism was something I always dreaded but found most helpful. I would like to extend my gratitude to Dr. Catherine Field, who has always taken a keen interest in my progress and given me helpful advice whenever I needed. Dr. Jelske Van der Veen, who helped me improve my critical thinking and writing skills, and Dr. Dennis Vance and Dr. Jean Vance, who inspire me to always remain passionate about science even when I get old.

I would also like to thank all my lab members: JP, Paulina, Kelly, Nicole for helping me with my experiments and presentations.

I want to thank all my friends particularly Jacqueline, Melanie and Ana from the graduate room who played a huge role in bringing out the nerd in me.

Lastly, I want to thank my husband Waqas for encouraging me to go to graduate school, and Inayah for simply being my baby.

This research was supported by grants to R.L.J. from the Alberta Livestock and Meat Agency, the Natural Sciences and Engineering Research Council of Canada and Canadian Foundation for Innovation and the Alberta Ministry for Advanced Education and Technology. R.L.J. was a new investigator of the CIHR.

Table of Contents

List of Tables

List of Figures

List of Appendix Figures

List of abbreviations

ALT	Alanine transferase
AAV	Adeno-associated virus
Apo	Apolipoprotein
CD	Choline deficient
CE	Cholesterol esters
CKD	Chronic Kidney Disease
CM	Chylomicron
CS	Choline supplemented
CVD	Cardiovascular Diseases
DKO	$Pemt^{-/-}/Ldlr^{-/-}$
FMO	Flavin monoxygenase
GFP	Green Florescent Protein
HDL	High Density lipoprotein
HF/HC	High Fat/ High Choline
HF/LC	High Fat/ Low Choline
HFD	High Fat Diet
LDLR	Low density Lipoprotein
LPL	Lipoprotein Lipase
MACE	Major Adverse Cardiac event
NAFLD	Non-alcoholic fatty liver disease
PC	Phosphatidylcholines
PE	Phosphatidylethanolamines
PEMT	Phosphatidylethanolamine *N*-methyltransferase
SAH	S-adenosyl homocysteine
SAM	S- adenosyl methionine
SKO	$Pemt^{+/+}/Ldlr^{-/-}$ or $Ldlr^{-/-}$
TG	Triglycerides
TMA	Trimethylamine
TMAO	Trimethylamine-N-oxide
VLDL	Very low density Lipoprotein

1 Chapter 1: Introduction and Literature Review

1.1 Definition of Atherosclerosis

The word atherosclerosis is derived from the Greek word *"athera"* which means "gruel" or accumulation of yellow fatty deposition and sclerosis means "hardening". Atherosclerosis is a chronic vascular disease characterized by build-up of lipids and cellular debris also referred to as 'plaque' in the intima of the arterial walls[1]. The underlying pathology of plaque formation is the imbalance of lipids that promotes a maladaptive immune response resulting in a chronic inflammatory condition [2]. According to Heart Disease and Stroke Statistics 2017 by American Heart Association (AHA), the underlying cause reported for one in every three deaths in the US is reported to be due to cardiovascular disease[3]. It is the leading of morbidity and mortality in the western world, contributing towards 31% of all deaths in United States[4]. The economic burden of treating diagnosed cases of atherosclerosis continues to rise, from $128 billion per year in 1994[5] to a more recent estimate of about $195.6 billion in 2016[6]. The AHA recognizes seven key factors that influence the progression of the chronic condition of atherosclerosis which include: Smoking, hypertension, diabetes, obesity, diet, physical activity and plasma cholesterol levels[3].

1.2 Role of lipids in atherosclerosis

Well-established risk factors of atherosclerosis include: genetic predisposition, diabetes, obesity, smoking, hypertension, chronic inflammation, diet rich in saturated fats, sedentary lifestyle, and age (>55 years in men, >65 years in women)[2]. Disorders of lipids

and lipoprotein metabolism can lead to alterations in plasma cholesterol and triglyceride (TG) levels leading to increased risk for cardiovascular diseases (CVD)[7]. Total plasma cholesterol is strongly associated with CVD. However, it is often confounded by its distribution in different lipoproteins. Low-density lipoprotein (LDL) has the highest cholesterol percentage compared to other lipoproteins and is a known risk factor of atherosclerosis[8]. Hence total plasma cholesterol and plasma LDL cholesterol are primary measures for CVD assessment and often correlate with each other[9–11]. Particularly, more atherogenic forms of LDL include oxidized LDL particles and small dense LDL. Overall, all apoB containing lipoproteins including LDL, VLDL, and IDL, tend to promote atherosclerosis but these particles differ in their apolipoprotein and TG content[9].

High Density Lipoprotein (HDL) also contains cholesterol, however, it is inversely correlated with atherosclerosis. HDL decreases atherosclerosis by two main mechanisms; it plays an important role in reverse cholesterol transport by removing excess cellular cholesterol and transporting it to the liver[12]. HDL also has antioxidative properties, which help prevent oxidative modification of lipids[13]. Raising HDL has been shown to cause a 2-3% reduction in CVD[14]. The lipid parameters used for assessing CVD risk include measurement of fasting plasma low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), total cholesterol (TC) and non-fasting triglycerides (TG)[15]. The role of TG in atherosclerosis is controversial since it has minor contribution in plaque formation, however; post-prandial and non-fasting data on TG level demonstrate elevated TG levels are a sign of dyslipidemia and early hallmark of atherosclerosis in children as well as adults[16].

Remnant like particles (RLP) are TG-rich remnants lipoproteins derived from Very Low-Density Lipoprotein (VLDL) and intestinal Chylomicrons (CM) secretion. RLP have been shown to be positively associated with CVD[17]. Hence one of the primary methods for decreasing CVD risk is managing dyslipidemia by lipid lowering strategies. Current research is also focusing on identifying factors beyond lipids particularly novel risk factors that influence atherosclerosis disease progression independent of lipid.

1.2.1 Overview of lipid metabolism

Lipids in the circulation are packaged and transported around the body in the form of lipoprotein particles that have apolipoproteins on their hydrophobic surface, which enables solubility in the plasma[18]. Cholesterol present in the circulation may be derived from exogenous or endogenous sources (Figure 1-1 below illustrates cholesterol metabolism). Exogenous cholesterol referred to as dietary sources of cholesterol (eggs, prawn, meat, dairy), undergoes digestion and absorption in the intestinal epithelia. The intestinal epithelial cells secrete the digested lipids in the circulation in the form of chylomicron particles[19]. Chylomicrons (CM) are the largest lipoproteins with a density of <0.94g/mL[20] mainly composed of triglycerides, phospholipids, and cholesterol coated with a surface apolipoproteins B-48[21]. ApoBec is the enzyme present in the intestine that generates a premature stop codon generating ApoB48. In humans, ApoB48 is solely derived from the intestine; however in mice, it is secreted from intestine as well as the liver[22]. In a normal physiological condition, CM rapidly undergoes lipolysis by the action of lipoprotein lipase (LPL) present on the surface of endothelial cells that liberates the fatty acids from TG in the CM. The resulting CM-remnant particles are taken up by the liver

and utilized in synthesis of lipoproteins or bile. The CM remnants get cleared within 1-5 hours after a meal[20].

The endogenous pathway or *de novo* cholesterol synthesis is responsible for 3/4th of the total cholesterol pool of the body[23]. The rate-limiting step of *de novo* cholesterol synthesis is conversion of mevalonate, which is catalyzed by 3-hydroxy-3-methyl-CoA (HMG Co-A) reductase. HMG Co-A reductase is the key target of statin medications to lower cholesterol levels[23]. The liver is the main organ for cholesterol production and maintaining homeostatic balance. The liver produces VLDL particles (density between 0.94-1.006g/mL[20]) that transport the main source of hepatic TG and cholesterol from the hepatic source[23]. VLDL is mainly composed of triglyceride, fatty acids, cholesterol and phospholipids coated by a protein ApoB100. Like CM, VLDL undergoes lipolysis by the action of LPL. The fatty acids released are taken up by the peripheral tissues and form intermediate density lipoprotein (IDL) and then further into LDL. ApoB100 remains the major apolipoprotein on the surface of LDL, which enables its recognition and uptake by the LDL receptors (Ldlr) present on the liver and other cells. LDL has a density of 1.019-1.063g/mL and is usually cleared within 2 days[20]. Another important lipoprotein is HDL that has apo-A1, a surface protein responsible for uptake of excess cellular cholesterol regulated by ATP-binding cassette A1 transporter (ABCA1) present on the cellular surface. HDL removes excess cellular cholesterol and transports it to liver where cholesterol is secreted into bile. This is referred to as reverse cholesterol transport (RCT) and plays an important role in maintaining cellular cholesterol balance. HDL also plays an important role in transferring TG and cholesterol between lipoprotein particles. The density of HDL particles ranges between 1.063-1.21g/mL. Figure 1-2 illustrates the relative size of each

lipoprotein particle. Hence, apolipoproteins coating the surface of the major lipoproteins play an important role in their transport, cellular uptake and clearance from circulation.

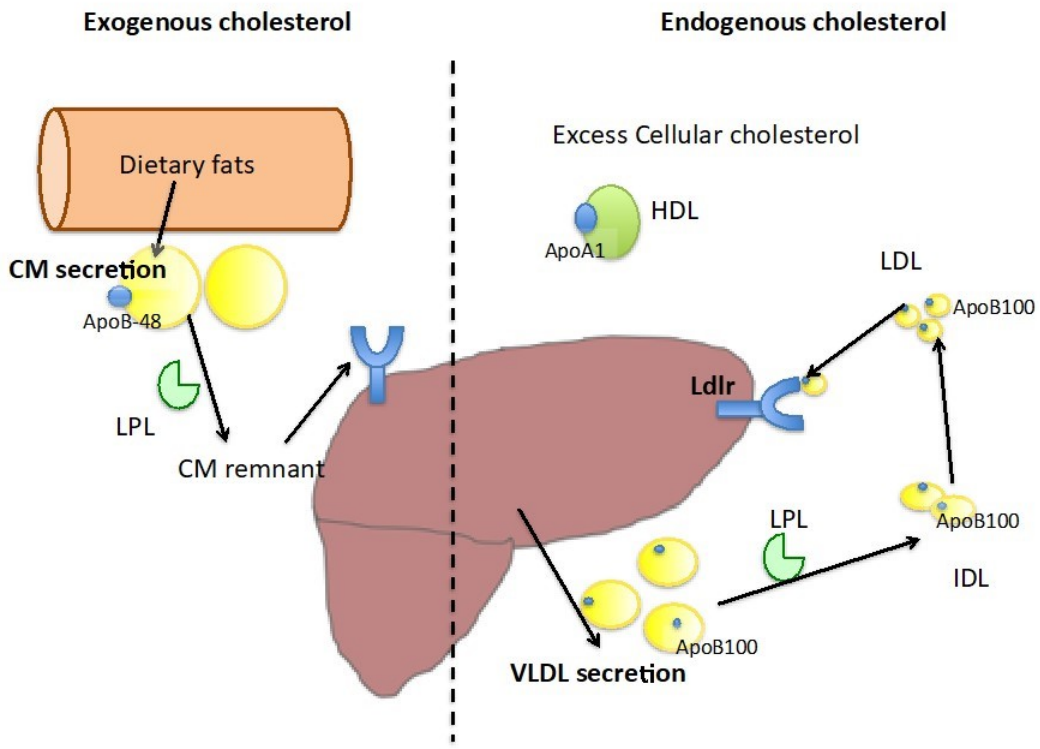


Figure 1-1 Overview of Cholesterol Metabolism in the body demonstrating two sources of cholesterol in the body: Exogenous source (diet) and Endogenous (synthesized in the liver)

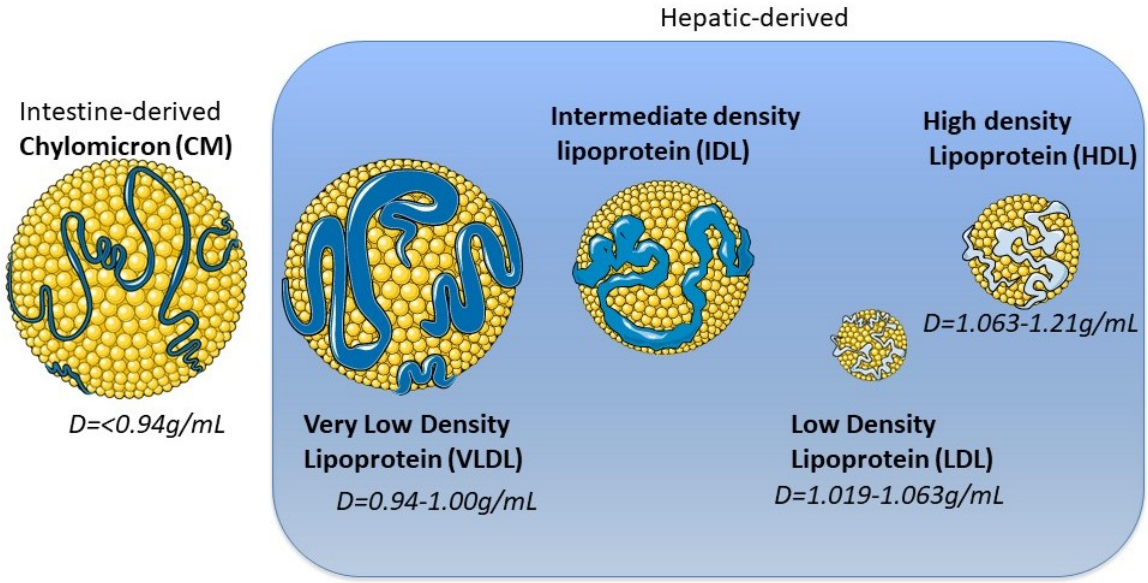


Figure 1-2 Relative Size and density of lipoprotein particles derived from intestine and hepatic source: density, D; lipoprotein assessment was done using ultracentrifugation. This figure was created in part using illustrations from ©Servier medical art "With Permissions".

1.3 Mechanism of atherosclerosis

The process of atherosclerosis is divided into three main stages beginning with the formation of the fatty streak, followed by fibrous plaque, leading to a complicated lesion. The fatty streak is mainly comprised of cholesterol, triglycerides and cholesterol esters; phospholipids (mainly phosphatidylcholine), sphingomyelin and lysophosphatidylcholine are also present in small quantities[24]. The key mechanism of lipid deposition in atherosclerosis is the retention of apoB containing lipoproteins in the arterial wall. LDL is the predominant apoB containing lipoprotein that can pass through the monolayer of endothelial cells into the extracellular space[25](Figure 1-3 illustrates the mechanism of atherosclerosis). There is also evidence to suggest that other apoB containing lipoproteins, such as chylomicron (CM) remnants and VLDL remnants, can be retained in the intimal wall in a similar manner as LDL-C[26]. Retained LDL-C particles in the arterial walls are

prone to aggregation and subsequent oxidation by free radicals, resulting in the formation of oxidized LDL (OxLDL)[27]. OxLDL signals migration and sequestration of monocytes, which take up OxLDL from the sub-endothelial space, creating foam cells. Eventually, foam cells become saturated with lipids and rupture. The spilled contents of the foam cells promote an inflammatory response, which subsequently attracts other immune cells creating a necrotic core[26]. The presence of plaque also leads to the migration of smooth muscle cells to compensate for the additional bulk in the artery. The above processes results in the narrowing of the arterial lumen, which creates blockages in major coronary vessels and may ultimately result in a heart attack, or stroke.

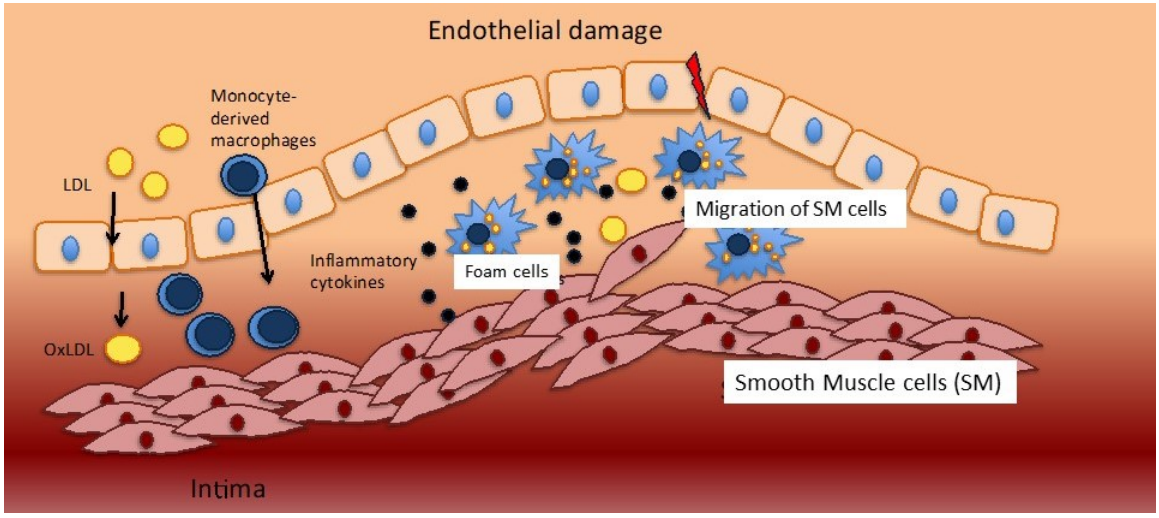


Figure 1-3: Mechanism of atherosclerosis: LDL gets readily retained by arterial walls, converts into OxLDL. Monocyte-derived macrophages uptake OxLDL converting into foam cells which promote inflammation apoptosis and necrosis at the site resulting in plaque formation.

1.3.1 Role of Phospholipids in atherosclerosis

The outer lipoproteins of plaque are composed of phospholipids (PL) that play an important structural and signaling role in the progression of atherogenesis. A study of human plaque composition collected during vascular surgery revealed that 70% of the atherosclerotic lesion is composed of cholesterol, while the remaining 30% was primarily

composed of phospholipids (PL), mainly phosphatidylcholine (PC)[28]. *In vitro* studies have found that the PL of LDL in epithelial cells of atherosclerotic lesions may undergo oxidative modification, which promotes monocyte binding to endothelial cells and monocyte chemotaxis. For example: lyso PC (oxidized PL) promotes the expression of adhesion molecules on endothelial cells in the arterial walls, macrophage aggregation and monocyte chemotaxis[28]. Oxidized PL of LDL also increases the vascular permeability for white blood cells and platelets, promoting a pro-inflammatory state[29]. Consequently, PLs are increasingly being recognized as important players in lipid metabolism and atherosclerosis due to their structural role in plaque formation and their ability to promote inflammation[26].

1.3.2 Atherosclerosis mouse models (*Apoe* and *Ldlr)*

Transgenic mice models are widely used in cardiovascular research due to their feasibility and cost-effectiveness. Two most commonly used mouse models in cardiac research include the low density lipoprotein receptor knockout mice (*Ldlr*$^{-/-}$) and ApoE lipoprotein knockout mice (*Apoe*$^{-/-}$)[30]. The process of atherosclerosis in both these mice models is driven by different lipoprotein retention in the arterial walls. ApoE is an apolipoprotein mainly synthesized by the liver and macrophages and is mainly present on chylomicron CM and IDL[31]. It plays an important role in the catabolism of TG rich lipoprotein by acting as a ligand for receptors to clear CM and VLDL remnants. At the atherosclerotic lesion site, the lesional ApoE interacts with plasma ApoA-1 and promotes uptake of cellular cholesterol efflux from the foam cell macrophages[32]. Lastly, it is atheroprotective by direct interactions with T and B lymphocyte-mediated immune responses that contribute towards chronic inflammation[32]. Mice lacking ApoE develop

atherosclerosis even on a chow diet with plasma cholesterol level around >500mg/dL. While on a western diet, these values increase 4-fold. The cholesterol fractions observed in *Apoe*$^{-/-}$ mice are mainly VLDL and CM remnants[31].

Another widely used atherogenic mouse model is the *Ldlr*$^{-/-}$ mouse. The LDL receptor plays an important role in clearance of ApoB and ApoE containing lipoprotein. When fed a chow diet, the *Ldlr*$^{-/-}$ mice develop little or no atherosclerosis with moderate cholesterol levels (around >200mg/dL)[31]. However, when fed a Western diet (high fat/high cholesterol), cholesterol levels increased to >1500mg/dL, associated with a significant increase in VLDL, IDL, and LDL cholesterol and decrease in HDL cholesterol[33]. These mice also exhibit skin xanthomas and aortic thickening[31,33]. Although the two mouse models are largely similar due to their ability to develop atherosclerotic lesions in hypercholesterolemic conditions; the variables such as study design, length of the study, the dietary composition, gender of the mice used, protocol for sampling lesions and assessing atherosclerosis and influence of the gut microbiome between the vivarium all contribute towards the differences observed in the literature between the two mouse models[30]. The

Table 1-1 below summarizes the main differences between these two commonly used genetic modified mice models used in atherosclerosis research.

Table 1-1 Main characteristics of two-mouse model of atherosclerosis: *Apoe*$^{-/-}$ and *Ldlr*$^{-/-}$

Genotype	***Apoe*$^{-/-}$**	***Ldlr*$^{-/-}$**
Cause	Deficiency on ApoE; major ligand for binding of CM and VLDL, ApoE has anti inflammatory properties	Deficiency in LDL receptor, predominantly in the liver

Atherosclerosis	Occurs spontaneously even on chow diet	Requires western diet to develop atherosclerosis
Cholesterol levels	~400mg/dl	~200-275mg/dl
Litter size	6-8 pups	4-6 pups
Western diet induced plaques	Large abundant at 14 weeks	Medium plaques at 12 weeks
Predominant lipoprotein	VLDL remnants (ApoB48)	LDL (ApoB100)
HDL cholesterol	Low levels of HDL	No difference compared to controls

Table derived from[30,34–36]

1.4 Importance of Dietary Choline

The nutritional importance of choline was first recognized in 1930s when rodents developed a fatty liver when fed a choline deficient diet. In later years, similar findings were demonstrated in many mammals including dogs, rats, chickens, pigs and monkeys[37]. In these animals, the common manifestation of choline deficiency was accumulation of hepatic TG but some demonstrated more adverse physiological effects including liver cancer, hemorrhagic kidneys and other organ dysfunction[37]. Controlled feeding studies in healthy men demonstrated that choline deficiency led to development of fatty liver and liver damage which was reversed by choline replenishment[38]. Parenteral nutrition studies also demonstrated choline deprivation causes liver damage. Despite the animal research, the importance of choline in our diet was not recognized until much later. In 1998, choline was recognized as an essential nutrient by the Institute of Medicine and the recommended adequate intake of choline was established (425mg/day for women, 550mg/day for men)[39]. Today, choline is recognized as an essential water-soluble nutrient required for cell structure and membrane biogenesis, synthesis of neurotransmitter acetylcholine, bile synthesis, VLDL secretion and cell signaling as well as one-carbon-cycle metabolism[39]. A

small portion of choline is also utilized in the synthesis of platelet activating factor (PAF), plasmalogen, glycerophosphocholine, lysophosphocholine and betaine[40].

1.4.1 Dietary sources of choline

One of the best sources of choline is the beef liver containing up to 418mg/100g of liver. Other important sources include chicken liver (290), eggs (251), wheat germ (152) bacon (125) soy (116) and pork (103). Choline is widely distributed in our diet but choline derived from animal source is a more concentrated source of choline than plant based material[41]. There are water-soluble forms of choline that include free choline, phosphocholine, glycerophosphocholine and lipid-soluble form that include sphingomyelin, lysophosphatidylcholine and phosphatidylcholine[41]. Figure 1-4 below shows the chemical formula of different choline containing compounds.

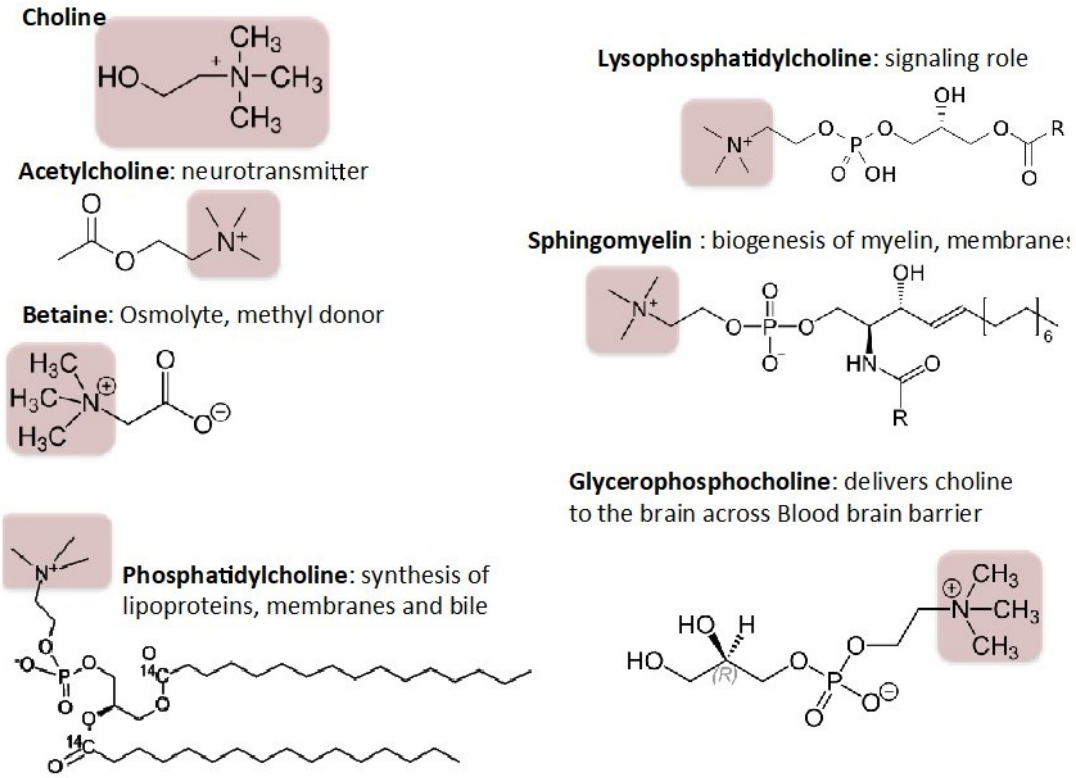


Figure 1-4: Different forms of choline and their respective chemical formulae

1.4.2 Fate of dietary choline

Dietary choline, usually in the form of phosphatidylcholine, reaches the lumen of the intestine where it is broken down to lyso-PC and fatty acids primarily by pancreatic phospholipase A2(PLA_2)[42]. Lyso-PC gets reacylated to PC in enterocytes, where it is utilized for chylomicron assembly and secretion[42]. CM are the major lipoproteins secreted by the enterocytes following a lipid-rich meal[43].

The remaining PC in the enterocytes gets hydrolyzed to glycerophosphocholine and subsequently to glycerophosphate and free choline both of which are distributed to liver and other tissues[44]. Free choline is absorbed in the jejunum and ileum by two distinct transport systems: sodium-dependent and sodium-independent transports which are either saturable (high affinity) or non-saturable (low affinity) [45]. In the enterocytes, a portion of choline can get oxidized in the mitochondria to betaine. Betaine serves as a major osmolyte in kidney function[46] (Figure 1-5 illustrates the major fate of dietary choline). Recently, it has been shown that unabsorbed dietary choline that reaches the large intestine gets metabolized to trimethylamine (TMA) by the gut microbiota. TMA gets excreted in the urine or oxidized by the hepatic flavin monooxygenase (FMO) enzymes to TMAO. TMAO can also be readily excreted from the kidneys or in the feces[47]. Lastly, it is important to know that choline uptake occurs via three main mechanism: (1) sodium-dependent high affinity choline uptake that occurs in the synaptosomes for the synthesis of acetylcholine (2) sodium independent low affinity choline uptake for membrane biogenesis and lastly a unique choline uptake mechanism for the brain[48].

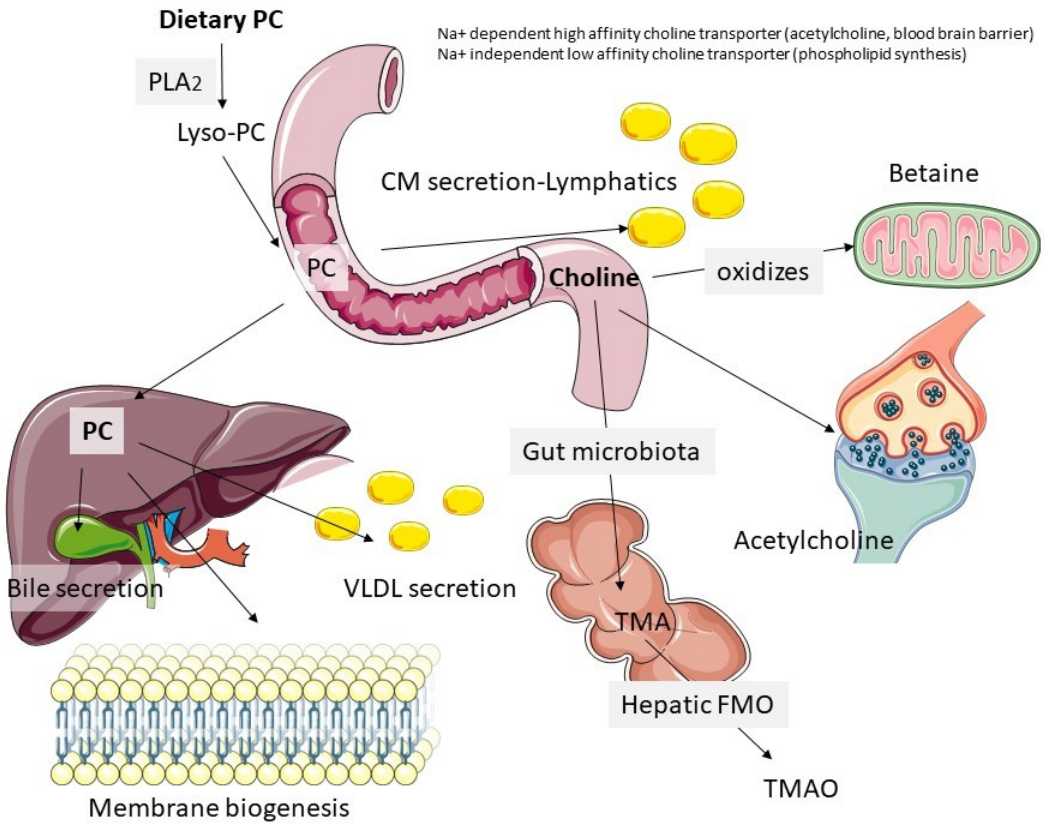


Figure 1-5 Absorption of Dietary Choline. *PLA2*, phospholipase A2, *TMA,* trimethylamine; *TMAO* trimethylamine *N*-oxide; *CM,* chylomicrons; *VLDL,* very low density lipoproteins. This figure was created in part using illustrations from ©Servier medical art "With Permissions".

1.4.3 Choline bioavailability

Generally, bioavailability of free choline is very high. A study compared the choline bioavailability of lipid soluble metabolite PC, with water soluble metabolites namely choline, phosphocholine and glycerophosphocholine and found that water soluble metabolites rapidly appeared in the blood and liver, peaking at 5 hours post prandially[49]. The lipid soluble PC took longer to appear in the blood and liver consistent with its absorption by the lymphatics versus blood portal system. However, the lipid soluble PC remained elevated for more than 24hrs in the plasma[49].

Moreover, studies often measure choline bioavailability by measuring choline metabolites[49–52]. A choline supplementation study in cows demonstrated that betaine in plasma and milk (alone or in combination with phosphocholine) is strongly associated with choline flux and is considered a strong marker of dietary choline bioavailability[52]. A recent publication demonstrated that choline bioavailability is inversely correlated with plasma TMAO levels. They found that increase in TMA-producing microbes in the gut resulted in increase in TMAO levels and decrease in plasma choline representing choline bioavailability[53].

1.4.4 Choline metabolism

Although choline comes from our diet, it is also synthesized in our body. The major form of choline is our body is PC, which is synthesized in our body by the CDP-choline pathway, also known as the Kennedy pathway, which occurs in all nucleated cells. This pathway was described by Eugene Kennedy and his coworkers in the 1950s[45]. The first step of the CDP-choline pathway involves the exogenous uptake of choline into the cell (Figure 1-6). Choline is phosphorylated to phosphocholine (P-choline) by choline kinase (CK) in the cytosol. P-choline and cytidine triphosphate is utilized by the rate limiting enzyme CTP: phosphocholine cytidylyltransferase (CT) to form CDP-choline. Lastly, the choline headgroup is added to the diacylglycerol (DAG) to form PC catalyzed by the enzyme choline/ethanolamine phosphotransferase (CPT) in the ER[54]. This is the major pathway providing about 70% of the hepatic PC supply[44]. The remaining 30% PC comes from the phosphatidylethanolamine *N*-methyltransferase (PEMT) pathway[39,40,55–57].

In the PEMT pathway, phosphatidylethanolamine (PE) undergoes three sequential methylation steps utilizing three molecules of S-adenosylmethionine to form PC and three

molecules of S-adensoylhomocysteine[58,59]. This enzyme is almost exclusively expressed in the liver (Figure 1-6 shows the two pathways for choline synthesis). PC synthesized by the PEMT pathway can further hydrolyze to produce free choline moiety and this is the only pathway through which *de novo* choline synthesis occurs in the body[45]. PC synthesized from the PEMT pathway is utilized for VLDL secretion, bile synthesis as well as hepatocyte membranes biogenesis[60]. Hence choline is obtained either from the diet or from the PEMT pathway whereby the PC synthesized undergoes catabolism to release free choline[54].

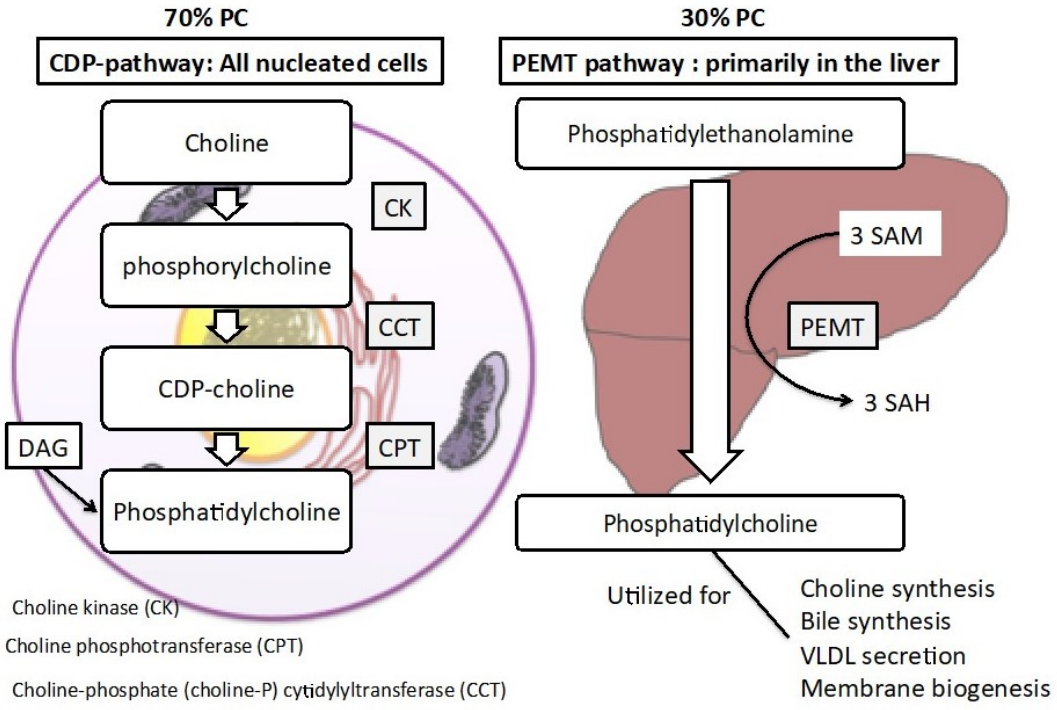


Figure 1-6 Phosphatidylcholine synthesis by the CDP-choline and PEMT pathway: Choline kinase (CK), choline-phosphate (choline-P) cytidylyltransferase (CCT), and choline phosphotransferase (CPT) catalyze reactions in the CDP-choline pathway. The final step involves addition of choline head group to Diacylglycerol (DAG) PEMT catalyzes the three-step methylation of PE to PC utilizing SAM. SAH, AdoHcy, adenosylhomocysteine; SAM AdoMet, S-adenosyl methionine.

1.4.5 Choline homeostasis

The body maintains choline homeostasis by two choline-acquisition pathways (Diet and PEMT pathway) and two choline-utilizing pathways. (Choline oxidation and biliary PC secretion) [61]. Figure 1-7 illustrates the choline homeostasis.

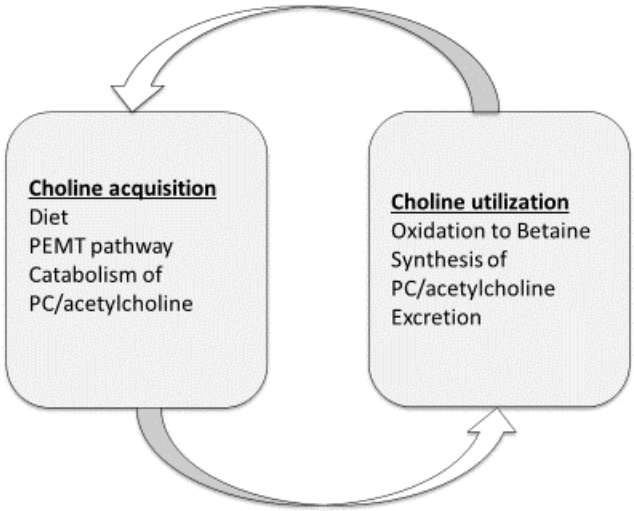


Figure 1-7: Choline homeostasis: Steady state concentrations of choline maintained through several adaptive mechanisms

If any of the pathways are perturbed an imbalance in choline concentration occurs.

There are several compensatory mechanisms for keeping choline concentration to steady-state levels. For example, livers of rats fed a choline-deficient diet have a two-fold increase in PEMT activity[62] while *Pemt*$^{-/-}$ mice fed a choline deficient diet die within 3 days because lethal low levels of hepatic PC[63]. Although there is no storage pool of choline in the body, PC is often considered long-term storage form of choline[61]. In a choline deficient state, mice maintain PC levels not only by increasing de novo choline synthesis, but also increasing the CDP-pathway in convert choline to PC, decreasing choline oxidation to betaine, increasing cellular uptake of choline and redistributing choline among tissues[64].

Similarly upon choline supplementation, there is enhanced choline oxidation and decreased PC catabolism[41].

1.5 Role of choline in lipid metabolism

Choline plays an important role in lipid metabolism in the form of PC[55]. Generally, PC is required for membrane biogenesis, lipoprotein formation, VLDL and chylomicron secretion, production of sphingomyelin (SM) and for bile secretion[54]. Moreover, PC is major source of second messenger such as diacylglycerol, phosphatidic acid and lyso-PC which can further metabolize to important signaling molecules[54].

1.5.1 Role of PC in VLDL secretion and NAFLD

Decrease in PC synthesis manifests with impaired VLDL secretion and fatty liver disease. Impaired PC synthesis is also linked to impaired liver regeneration[18,54]. Studies using hepatocyte cultures show that the absence of choline and methionine (two precursors of PC) results in impaired VLDL secretion[61]. While adding choline increases PC synthesis and restores VLDL secretion in rat hepatocytes[65]. Two important mouse models that demonstrate the importance of PC for hepatic VLDL secretion and NAFLD include $PEMT^{-/-}$ and $GNMT^{-/-}$ mice[18,66]. (See Figure 1-8 illustration) $PEMT^{-/-}$ mice have low hepatic PC and hence impaired VLDL secretion, a phenotype that is exacerbated in a choline-deficient diet. With a choline-deficient diet, $PEMT^{-/-}$ mice have limited PC synthesis (even from the CDP-choline pathway) hence these mice develop end-stage liver failure[18,67].

Contrary to $PEMT^{-/-}$, the $GNMT^{-/-}$ mice have excess of AdoMet and excess of PC synthesis via PEMT pathway. The PC catabolizes to DAG and re-esterifies to TAG thus resulting in hepatic TG accumulation and fatty liver phenotype[18]

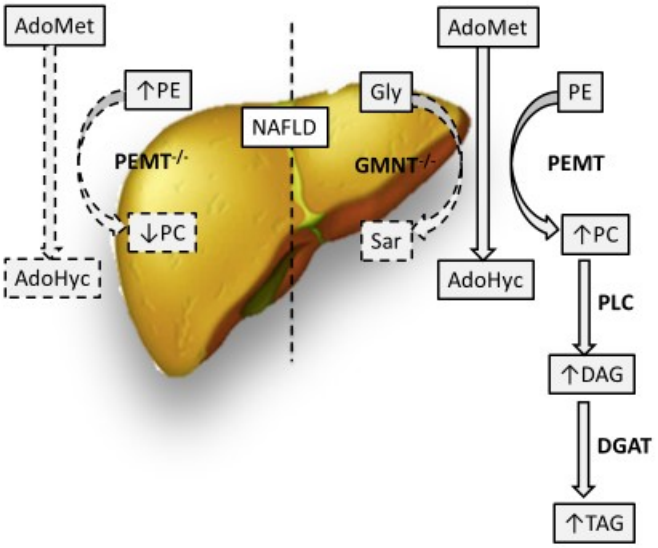


Figure 1-8 Two different mouse models demonstrating the role of PC in inducing NAFLD: Increased availability of S-adenosylmethionine (AdoMet) might stimulate flux through the PEMT pathway and increase PC content in the liver. Excess PC is converted to TAG via the action of phospholipase C (PLC) and diacylglycerol acyltransferase (DGAT). Glycine (Gly) is methylated to sarcosine (Sar) by the enzyme glycine N-methyltransferase (GNMT). Figure adapted from van der Veen et al., (2017)[18]

1.5.2 Role of PC/PE ratio in lipoproteins, liver, mitochondria

PC/PE ratio regulates the size and dynamics of the lipid droplets. Studies implicate that *Pemt*$^{-/-}$*/Ldlr*$^{-/-}$ mice have an increased PE content of lipoprotein (Or a low PC/PE ratio) and demonstrate increased rate of clearance from the plasma[68]. This increased rate of clearance may also contribute to the atheroprotective phenotype of the *Pemt*$^{-/-}$*/Ldlr*$^{-/-}$ mice[68]. Any disturbances in the hepatic PC/PE ratio can perturb the liver physiology and is associated with NAFLD. When Pemt$^{-/-}$ mice are fed a high fat diet, the livers progress from NAFLD to NASH (nonalcoholic steatohepatitis) with the hepatic PC/PE ratio decreasing from 1.6 to 1.2[18,69]. The decrease in PC/PE ratio results in increase in amount of PE on the outer leaflet of the plasma membrane of the hepatocytes, causing membrane impairment and inducing liver failure[67]. Studies have indicated that the hepatic PC/PE ratio

can regulate membrane integrity and might be a better predictor of liver failure than reduced PC concentrations alone[67].

In mitochondria, any disturbance in the PC/PE ratio has been found to have profound effects on electron transport chain and energy metabolism. The *Pemt*$^{-/-}$ mice have been found to have smaller and elongated mitochondria with decreased hepatic glucose productive[67]. Moreover, decreased PC/PE ratio resulted in increased mitochondrial respiration, increased ATP production and increase in protein activities of the electron transport chain compared to *Pemt*$^{+/+}$ mice[18].

The physiological importance of PC synthesis and all the enzymes involved in PC synthesis has been studied in mice by gene disruption. As previously mentioned first step of CDP-choline pathway involves conversion of choline to P-choline by choline kinase (CK). CK has two isoforms: CKα and CKβ. Deletion of CKα enzyme was found to be embryonically lethal, while mice lacking the CKβ enzyme developed muscular dystrophy and had impaired limbs[56]. The rate limiting reaction of CDP-choline pathway is catalyzed by CTP: phosphocholine cytidylyltransferase (CT) enzyme. In mice, CT is encoded by two genes, *Pcyt1a* and *Pcyt1b* that produces CTα (predominant form in the liver) and CTβ isoforms, respectively. CTα deficiency in mice was embryonically lethal while CTβ deficient mice had dysfunctional gonads[61]. Mice lacking PEMT enzyme survived; however, they died of steatohepatitis and liver failure when fed a choline-deficient diet[56,61].

Another choline containing lipid enriched in the membrane is SM, which is synthesized from PC and ceramide in the Golgi complex[70]. Certain tissues like brain and neurons have higher SM content in their membranes. SM provides favorable interaction with cholesterol and plays an important role in cholesterol distribution in the plasma

membrane[71]. Studies demonstrate that maintenance of a constant ratio of SM to cholesterol of the membranes supports the critical functions carried out by lipid rafts and related membrane structures[72].

1.5.3 Role of Choline in HDL metabolism

PC also plays an important role in HDL metabolism. PC is the main phospholipid in HDL. The HDL-PC is enriched in polyunsaturated fatty acid (PUFA) moieties, which is derived from hepatic (nascent lipoprotein) and extrahepatic origins[73]. The HDL-PC with the PUFA moieties also contributes towards the anti-inflammatory effect of HDL[73]. Interestingly, *Pemt*$^{-/-}$ mice have significant decrease in HDL lipids irrespective of gender. However, when *Pemt*$^{-/-}$ mice are place on a choline deficient diet, it appears the male mice exhibit liver damage while female mice are able to direct HDL associated PC to the liver, almost delaying liver damage[74]. Hence, it appears that in mice, HDL associated PC delivery to the liver plays an important role in choline homeostasis and preventing liver damage[55].

1.5.4 Role of choline in One-carbon metabolism

Choline, methionine and folate are interlinked via the 1-carbon metabolism because all three molecules influence the production of the universal donor, S-adenosylmethionine (AdoMet) (See Figure 1-9). Studies implicate that 1-Carbon metabolism plays an important role in obesity and insulin resistance[75]. In the methionine cycle, AdoMet is required for DNA methylation, post translational modification of proteins, hormone synthesis as well as PC synthesis[76]. Any impairment in the methylation can disrupt the balance between the AdoMet and AdoHcy. Hence, an important role of folate cycle is the maintenance of cellular AdoMet and AdoHcy concentrations. THF is also involved in the synthesis methyl

groups from catabolism of dimethylglycine, glycine, serine and sarcosine which are all methyl groups involved in remethylation of homocysteine[76].

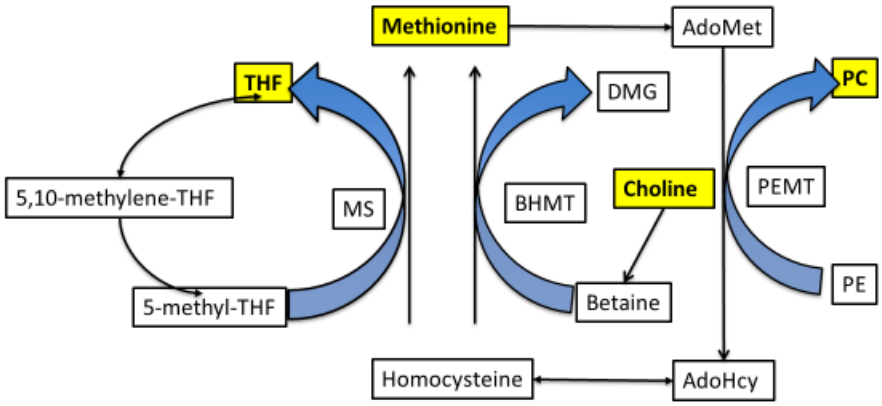


Figure 1-9 One carbon metabolism: One-carbon and folate metabolism.
Abbreviations: S-adenosylmethionine, AdoMet; S-adenosylhomocysteine, AdoHcy; Betaine: homocysteine methyltransferase, BHMT; dimethylglycine, DMG; methionine adenosyl transferase, methionine synthase, MS; phosphatidylethanolamine, PE; phosphatidylcholine, PC; Tetrahydrofolate THF. Figure adapted from da Silva et al., (2014)[76]

In the liver, SAM serves not just as a methyl donor but a key metabolite for hepatocyte growth, differentiation and death by various mechanisms including change in DNA/histone methylation, increasing glutathione and improving membrane fluidity. Consequently, depletion of SAM in the liver leads to chronic liver injury conditions spontaneous steatohepatitis and hepatocellular carcinoma[66]. Yet, excess SAM also leads abnormalities hence hepatic SAM levels needs to be maintained in a certain range[66]. Therefore, deficiency of any one of the three nutrients (choline, methionine and folate) can result in an increase in flux of the other nutrients towards methyl donation[77].

1.5.5 Role of Choline in Bile synthesis

Bile is a major source of PC in the body. Bile secretion is the major route for cholesterol elimination from the body. It is composed of 95% water, and a number of

endogenous constituents including PL and bile salts, enzymes, vitamins and heavy metals[78]. PC is the major PL in bile and cholesterol is the main sterol[78]. PC plays an important role in micelle formation, which reduces the toxicity of bile salts[77]. PC is excreted into bile by a protein called MDR2 in mice (human analog MDR3), a deficiency or mutation of which causes sever liver disease and cholestasis[78].

1.5.6 Role of choline in synthesis of Betaine

Betaine is derived by irreversible oxidation of choline that occurs in liver and kidneys. It serves as a major osmolyte and helps prevent protein misfolding. Betaine also serves as a major methyl donor for the conversion of homocysteine to methionine by Betaine-homocysteine methyltransferase (BHMT) and provides a folate-independent route for homocysteine remethylation[79]. The BHMT activity increases with choline/betaine supplementation. Studies demonstrate that betaine supplementation improves hepatic steatosis by improving insulin resistance and reducing hepatic lipids[80].

1.6 PEMT deficient mice model

In 1961 by Brenner and Greenberg were the first to demonstrate that PEMT enzyme is a membrane-bound microsomal enzyme that converts PE to PC in the liver. It is expressed predominantly in the liver and mildly in the prostate and adipose tissue. It was first isolated and purified as 20kDa molecule by Ridgway and Vance [81]. Since CDP-pathway occurs in all nucleated cells, it was previously unclear why PEMT pathway survived evolutionary mechanism. Under normal dietary conditions, PEMT enzyme is not an essential enzyme for survival. Homozygous disruption of PEMT gene demonstrated no

obvious abnormal phenotype at least on normal diet[81]. This was expected since majority PC supply comes from the CDP-pathway.

However, when *Pemt*$^{-/-}$ mice are placed on a choline deficient (CD) diet, they rapidly develop end stage liver failure. These studies revealed that PEMT pathway survived evolution to provide PC in times of decreased PC supply (e.g. starvation, pregnancy, breastfeeding)[82]. Subsequent research found unexpected roles of PEMT enzyme in obesity, nonalcoholic fatty liver disease and atherosclerosis[82]. Figure 1-10 summarizes main findings of Pemt$^{-/-}$ mice relevant to current thesis.

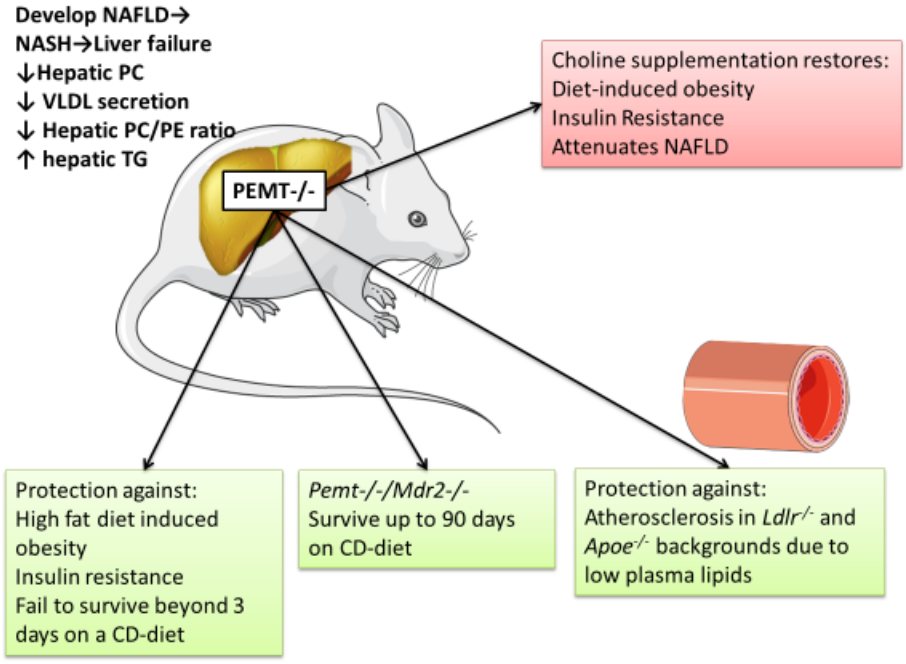


Figure 1-10 Schematic diagram demonstrating the known pluripotent effect of PEMT deficiency in mouse models: *Pemt*$^{-/-}$ mice are protected from HFD- induced obesity and insulin resistance but develop NAFLD. *Pemt*$^{-/-}$*/Ldlr*$^{-/-}$ are protected against atherosclerosis. Choline supplementation restores obesity, insulin resistance and attenuates NAFLD. Non-alcoholic fatty liver disease, NAFLD; Non-alcoholic steatohepatitis, NASH; phosphatidylcholine PC, Phosphatidylethanolamine PE; Very low density lipoprotein VLDL; triglycerides, TG; Pemt, phosphatidylethanolamine N methyl transferase, Multidrug resistant glycoprotein 2, Mdr2, choline deficient, CD; Apolipoprotein E knockout *Apoe*$^{-/-}$, Low density lipoprotein receptor knockout *Ldlr*$^{-/-}$.

The figure is created in part using illustrations from © Servier Medical Art "With Permissions".

1.6.1 PEMT and fatty liver

Nonalcoholic fatty liver disease (NAFLD) effects about 20% of the global population and 70% of diabetic patients. NAFLD represents a spectrum of liver abnormalities with key stages being hepatic steatosis, steatohepatitis (NASH), fibrosis and eventual cirrhosis[83]. The Pemt$^{-/-}$ mice provide a unique model of NAFLD, as mice develop severe liver failure with elevated ALT and die within 5 days of choline-deficient diet[84]. The fatality is due to lack of PC synthesis and decrease in biliary secretion[82,84]. In *Pemt*$^{-/-}$ mice, the production of PC is attenuated by the absence of the PEMT enzyme and with the absence of choline in the diet, the CDP-pathway cannot compensate for the lack of PC, hence *Pemt*$^{-/-}$ mice only have 50% hepatic PC compared to controls[84]. Quantitatively significant amount of PC is also utilized in bile secretion. MDR2 is a flippase protein responsible for transfer of PC from liver into bile. Hence CD-Pemt$^{-/-}$ mice rapidly die due to their small PC pool entirely getting drained into bile[85]. The hepatic PC species derived from the PEMT pathway are required for normal VLDL secretion. Radiolabelled studies have demonstrated significantly decrease in apoB100 particles in the plasma of *Pemt*$^{-/-}$ mice indicated that Pemt-derived PC is significantly important for lipoprotein secretion[86]. Moreover, *Pemt*$^{-/-}$ mice have low levels of hepatic PC with no change in PE levels. This markedly lowers the PC/PE ratio, which decreases the membrane integrity and allows leakage, which explains elevated ALT levels in CD-*Pemt*$^{-/-}$ mice[84]. Surprisingly *Pemt*$^{-/-}$ */Mdr2*$^{-/-}$ mice lacking the flippase protein MDR2, required for transfer of PC into bile, the PC/PE ratio decreased minimally[87]. So while the CD-*Pemt*$^{-/-}$ failed to survive beyond 3 days, the *Pemt*$^{-/-}$*/Mdr2*$^{-/-}$ mice survived for almost 90 days because their minimal PC supply

was conserved[67]. This strongly supported the hypothesis that low PC/PE ratio leads to fatty liver characterized by enhanced membrane permeability and leakage of hepatic enzymes into the circulation[67].

1.6.2 PEMT and obesity

In chow-fed conditions, *Pemt*$^{-/-}$ mice appear to be similar to their wild type littermates [54,55,63,67]. Jacobs and colleagues demonstrated when fed a high fat diet (HFD), *Pemt*$^{-/-}$ mice had increased energy expenditure and were protected against diet-induced obesity and insulin resistance compared to control mice[88]. Interestingly, dietary choline supplementation normalized energy expenditure, restored weight gain and insulin resistance in high fat diet fed *Pemt*$^{-/-}$ mice[88]. It was concluded from this study, that insufficient choline supply rather than decreased PC synthesis was responsible for the decrease in weight gain. Moreover, Pemt$^{+/+}$ mice when fed a CD-diet had decreased weight gain, increased oxygen consumption and improved glucose tolerance[88].

1.6.3 Pemt and atherosclerosis

Zhao et al investigated the role of impaired hepatic PC synthesis (or absence of PEMT) in the development of atherosclerosis. The DKO mice demonstrated reduced VLDL secretion, a ~60% reduction in plasma cholesterol and TG compared to SKO[68]. This decrease in plasma lipids coincided with a striking 80% reduction in atherosclerotic lesions in DKO mice compared to SKO mice[68]. Pemt deletion in mice lacking apolipoprotein E (*Apoe*$^{-/-}$) resulted in a 50% improvement in cardiac systolic function, a 30% reduction in atherosclerotic plaque formation and a significant reduction in cardiac lipids[89]. Additionally, PEMT deficiency also results in low plasma homocysteine, which is an independent risk factor for atherosclerosis[90]. Hence, PEMT deficiency in

hypercholesterolemic mice models (*Ldlr*$^{-/-}$ and *Apoe*$^{-/-}$) was found to be protective against atherosclerosis[68,89].

1.7 TMAO, an osmolyte

TMAO is a small organic colorless and odorless molecule. It is naturally abundant in fish and can also be produced by the metabolism of phosphatidylcholine and L-carnitine by the gut microbiome[91]. The TMA-producing strain in the gut microbiome belongs to the two main phyla: firmicutes and proteobacteria[53]. In fishes and deep-sea animals, TMAO serves as major osmolyte. While in humans, it is often recognized as a waste product of choline metabolism, produced by the oxidation of TMA[92,93]. Interestingly, the molecular TMA-to-TMAO ratio is an indicator of freshness of the product in the fishing industry, since some bacteria can reduce TMAO to TMA, which has a characteristic decaying-fish odor[93]. A genetic deficiency of hepatic flavin monooxygenase enzyme 3 (FMO3), the enzyme responsible for oxidizing TMA to TMAO, can result in trimethylaminuria also known as Fish-Odor syndrome; a condition characterized by increase TMA secretion in the urine and sweat[94].

In the past decade, there has been increasing interest towards identifying the biological functions of TMAO. In the intestinal anaerobic bacteria (*Enterobacteriaceae*), TMAO produced serves as an electron acceptor and supports oxidative phosphorylation[95]. It helps in protecting the renal medulla from osmotic stress and hydrostatic pressure caused by urea for concentrating urine before excretion[92]. TMAO helps inhibit ER stress by promoting protein folding by attenuating unfolding protein[96]. This property has

demonstrated improvement in diabetic peripheral neuropathy, glaucoma, asthma and in skin-blistering disease[97].

1.7.1 TMAO, Novel predictor of CVD and MACE

A recent publication demonstrated a novel association between dietary choline and development of atherosclerosis via the gut flora[98]. Using an unbiased metabolomics approach, Hazen and colleagues identified three metabolites of dietary phosphatidylcholine: choline, betaine and trimethylamine-*N*-oxide (TMAO), as novel predictors of CVD[98]. Similarly, a 3 year follow-up cohort study involving 4007 cardiac patients undergoing cardiac procedure demonstrated that after adjustment for traditional risk factors and other covariates, elevated plasma TMAO remained a significant predictor of major adverse cardiac event (MACE)[99]. In addition there appeared to be a graded increase in risk for MACE with increase in TMAO level[99]. Another publication demonstrated TMAO was significantly elevated in heart failure (HF) patients and correlated with B natriuretic peptide (BNP), hormone released by heart in response to pressure changes compared to non HF patients[100]. It was found that elevated plasma choline and betaine were predictive with MACE only with concomitant increase in plasma TMAO[101]. Hence numerous cohort studies have established elevated plasma trimethylamine-*N* oxide (TMAO), a product of choline metabolism is highly associated with CVD and was a strong predictor for myocardial infarction, stroke or death[100–104].

1.7.2 TMAO in atherosclerosis

The proposed mechanism by which TMAO promotes atherosclerosis remains unclear. Hazen's group demonstrated that *Apoe*$^{-/-}$ mice fed a choline-supplemented chow diet developed enhanced atherosclerotic lesions with high levels of TMAO compared to

controls. It was observed that choline required an oral-gut route to produce TMAO levels. Moreover, when mice were given the same diet with antibiotics the mice developed no atherosclerosis and presented with low plasma TMAO levels. It came to light that gut microbiota was crucial for metabolizing choline to trimethylamine (TMA). TMA gets oxidized by hepatic flavin monooxygenase 3 (FMO3) to TMAO[98]. Similarly *Apoe*$^{-/-}$ mice fed dietary TMAO or L-carnitine also yielded similar results with increased atherosclerosis and elevated TMAO levels[103].

The mechanism of how TMAO might promote atherosclerosis remains controversial. The first study demonstrated that TMAO promoted macrophage cholesterol accumulation by increasing expression of proatherogenic scavenger receptors SR-A1 and CD36, which increased foam cell formation[98] with a significant decrease in HDL cholesterol[103]. Hence, elevated TMAO was associated with inhibition of reverse cholesterol transport (RCT) in *Apoe*$^{-/-}$ mice[105]. Macrophages extracted from wild type C57BL/6J mice fed TMAO demonstrated an increased mRNA expression of ABCA1 and ABCG1, genes responsible for cholesterol efflux. Others have demonstrated that elevated TMAO down-regulates Cyp7a1, the rate-limiting enzyme for bile acid synthesis.

The link between bile acid metabolism, TMAO and atherosclerosis has been a topic of recent research interest[91,103,106,107]. Bile acids have been shown to regulate FMO3 activity, the enzyme responsible for oxidizing TMA to TMAO[108]. Another recent evidence suggests TMAO has prothrombotic effect. Elevated TMAO increases risk for thrombosis by enhancing platelet hyperactivity in atherosclerosis[109]. In animal models and cell culture studies, TMAO heightened platelet response to multiple agonist and increased Ca^{+2}signaling[109]. In a recent human cohort study plasma TMAO was found to be associated

with increased risk of thrombotic event[110] In this study, healthy vegans and omnivores were given oral dose of choline for 2 months and measured for TMAO levels and platelet aggregation. They demonstrated dose-dependent striking increase in TMAO levels and platelet aggregation; however, the prothrombotic effect of TMAO was attenuated by low dose aspirin[110].

1.7.3 TMAO in renal dysfunction

Given that renal dysfunction is common comorbidity of CVD and that TMAO is excreted from the kidneys, the association of elevated TMAO and kidney impairment was predictable. A study analyzing renal failure patients found TMAO concentration around 99.9±31.9 μM, which was significantly higher than healthy controls before hemodialysis[111]. More cohort studies have demonstrated CKD patients receiving dialysis demonstrate elevated TMAO levels[112–114]. Additionally, patients undergoing renal transplants also demonstrated increased TMAO levels pre-transplant and experienced significant reduction post-transplant[114]. Tang at el have demonstrated in mice models, increase in dietary choline or TMAO directly led to renal tubulointerstitial fibrosis and dysfunction characterized by increase in markers of renal fibrosis Smad1 and kidney injury marker (KIM) in renal tissues[113].

1.7.4 TMAO in diabetes and cancer

Elevated serum TMAO is associated with increased risk for type 2 diabetes mellitus[115]. Diabetic mice models exhibit 10-fold increase in TMAO production compared to control mice[116]. Additionally, mice fed a TMAO-supplemented diet exhibit exacerbated glucose response compared to controls with significantly increased HOMA-IR and insulin levels[115]. It also causes increase in proinflammatory cytokine MCP1 and decreases anti-

inflammatory cytokine IL-10 in the adipose tissue[115]. It appears that presence of diabetes augments the relationship of TMAO and atherosclerosis as there is a strong correlation of elevated TMAO and carotid intimal thickness in people with diabetes, gestational diabetes or impaired glucose tolerance[117]. Additionally there are studies showing correlation of TMAO levels with prostate and colorectal cancers[118]. Despite literature on TMAO, the researchers continue to debate TMAO is probably merely a bystander in a disease process rather than a mediator[93]. More importantly, there are three major confounders of plasma TMAO levels namely; kidney function, the gut microbiome and the FMO3 activity, of warrant recognition in every chronic disease condition[97]. Table 1-2 below summarizes the key properties of TMAO found in literature.

Table 1-2: The Main (patho) physiological and properties of TMAO

TMAO	**Properties**
Osmolyte	Protects renal tubules from hydrostatic pressure from urea
Protein stabilizer	Prevents protein unfolding, and helps maintains enzyme activity
Electron acceptor	Supports oxidative phosphorylation in anaerobic bacteria in the gut
Atherosclerosis	
Macrophage-derived foam cell formation	Increases scavenger receptors SRA1 and CD36 expression in macrophages promoting foam cells, associated with decrease HDL
Prothrombotic	Enhances platelet hyperactivity and thrombosis
Bile acid metabolism	Decreases Cyp71A expression, rate limiting enzyme for bile acid synthesis
Other chronic diseases	
Kidney	Increases kidney injury marker (KIM) renal fibrosis marker Smad1
Diabetes	Exacerbates impaired glucose tolerance, insulin levels, HOMA-IR
Cancer	Associated with increased risk for colorectal and prostate cancer

1.7.5 Role of Gut microbiota in atherosclerosis

Role of gut microbiota in the development of atherosclerosis has gained momentum over the past decade[119]. Studies have identified there are three main pathways by which the

gut microbiome might influence the atherosclerosis[120]. First, inflammation due to local infection can lead to atherogenesis and plaque formation. Bacteria can translocate from gut or oral cavity to the atherosclerotic plaque and influence development of heart diseases[121]. Secondly, the gut microbes can influence the metabolism of the lipid and bile acids. Bile acids not only help in cholesterol digestion but also function as signaling molecule for farnesoid X receptor (FXR), which plays a crucial role in regulating cholesterol and triglyceride metabolism[122]. Thirdly, certain dietary components namely choline, carnitine can be metabolized by the gut microbes to TMA which get oxidized to TMAO, a novel predictor of atherosclerosis[120].

A recent article identified nine different bacterial strains from two main phyla (*Firmicutes* and *Proteobacteria*) from the human gut capable of consuming choline to produce TMA by in vitro techniques[53]. Interesting, it appears that dietary choline is required for TMA accumulation; however; it does not influence the abundance of TMA-producing bacteria in mice. The gut microbiota can also promote increase in lipopolysaccharides (LPS) which promote expression of toll-like receptors in macrophages, this results in increase in foam cell formation and lipid accumulation augmenting plaque formation[119]. In contrast, certain dietary components like fiber promote probiotic bacteria (*Lactobacillus*, *Bifidobacterium*) that promote generation of short-chain fatty acids SCFA like acetate, butyrate and propionate[121]. Studies have demonstrated lactobacillus decreases cholesterol levels and atherosclerosis in *Apoe*$^{-/-}$ mice and humans[123,124]. Another example of a beneficial bacterium for atherosclerosis is *Akkermansia muciniphila*, which when supplemented in diet helps protect *Apoe*$^{-/-}$mice

from development of atherosclerosis. The bacterial strain protects the intestinal barrier lining and prevents metabolic- endotoxemia-induced inflammation[125].

Collectively, the aim of this literature review was to familiarize the reader with the underlying pathology of atherosclerosis and to introduce the role of choline and PEMT enzyme in the development of atherosclerosis. Dietary choline is essential nutrient of our diet; however, recent publications demonstrate TMAO, a metabolite of dietary choline, to be associated with increased CVD risk. This is supported by animal studies demonstrating choline supplementation induces atherosclerosis with elevated TMAO levels, while human studies demonstrate elevated TMAO to be highly predictive of CVD. However, the controversy remains that TMAO rich food like fish is known to be cardioprotective and the mechanism of how TMAO promotes atherogenesis remains ambiguous. Moreover, human studies show no correlation of dietary choline and increased CVD incidence[126]. *Pemt*$^{-/-}$*/Ldlr*$^{-/-}$ mice are protective from atherosclerosis due to significant alteration in hepatic lipid metabolism. But it is not known that this protection may also be due to decrease in choline metabolites. Hence, in view of recent findings, the effect of choline supplementation in atheroprotective mouse model warrants an investigation.

2 Chapter 2: Research Plan

2.1 Rationale

Atherosclerosis, characterized by deposition of plaque in the arterial walls, can cause narrowing or blockage. If this occurs in arteries supplying blood to heart or brain, heart attack, stroke and even death may occur[26]. Although the well-established risk factors of atherosclerosis include lipid markers such as LDL, remnant of VLDL and CM or inflammatory markers such as C-reactive protein and fibrinogen[127], there are novel risk factors emerging in this area of atherosclerosis namely, TMAO. Wang et al, (2011) demonstrated in a series of experiments that dietary choline is metabolized by the gut microbiota to TMA and oxidized by flavin monooxygenase enzymes to TMAO. Plasma TMAO is associated with increased CVD risk in humans[101–103,110,113] and has been demonstrated to be associated with increased atherosclerosis in ApoE$^{-/-}$ mice[98].

The major form of choline in our body is PC and it is synthesized by two main pathways: CDP-pathway that occurs in all nucleated cells while the Pemt pathway provides 30% PC supply and occurs almost exclusively in the liver[128]. Hepatic PC is utilized for synthesis of lipoproteins, bile and VLDL secretion. In addition, hepatic PC is also the only source of *de novo* choline synthesis (when PC undergoes catabolism to generate choline)[54]. *Pemt*$^{-/-}$ mice have a unique phenotype, which provides protection from high fat diet induced obesity and insulin resistance[88]. Interestingly, this protection provided by the absence of PEMT enzyme can be reversed upon choline supplementation[88]. Furthermore, the *Pemt*$^{-/-}$*/Ldlr*$^{-/-}$ (DKO) mice have been shown to be protected against atherosclerosis primarily due to significant reduction in VLDL secretion and plasma lipids[68,89]. Additionally, DKO mice

have increased rate of clearance due to altered lipoprotein composition[68]. Since choline supplementation partially reversed the protection against obesity and insulin resistance in *Pemt*$^{-/-}$ mice, we wanted to investigate the effect of choline supplementation on the atheroprotective phenotype of the DKO mice. Hence, the first main objective of this thesis was to investigate the role of PEMT enzyme in the development of atherosclerosis in DKO mice, secondly, to study the effect of choline supplementation on the development of atherosclerosis in this mouse model. Lastly, in a small comprehensive study, we wanted to study the effect of switching from high fat/high choline to a high fat/low choline diet on the progression of atherosclerosis, in Ldlr$^{-/-}$ mice. The specific objectives of this thesis are stated below:

2.1.1 Specific Objectives and Hypothesis

The specific objectives of this thesis and hypothesis are:

1. **To measure choline and its metabolites in chow fed-*Pemt*$^{-/-}$/*Ldlr*$^{-/-}$ (DKO) mice.** We hypothesized that DKO mice will have low plasma choline and associated metabolites due to decrease in hepatic PC synthesis.
2. **To investigate the effect of reintroducing hepatic Pemt on the development of atherosclerosis in the DKO mice.** We hypothesize that reintroducing Pemt expression in the liver would increase hepatic PC synthesis and VLDL secretion. Overall, it would increase plasma lipids and atherosclerosis. We also wanted to investigate the effect of reintroducing hepatic PEMT on plasma TMAO levels and the association of plasma TMAO levels with atherosclerotic plaque. Figure 2-1 below illustrates the expected outcome on the progression of atherosclerosis upon reintroducing hepatic PEMT in the DKO mice.

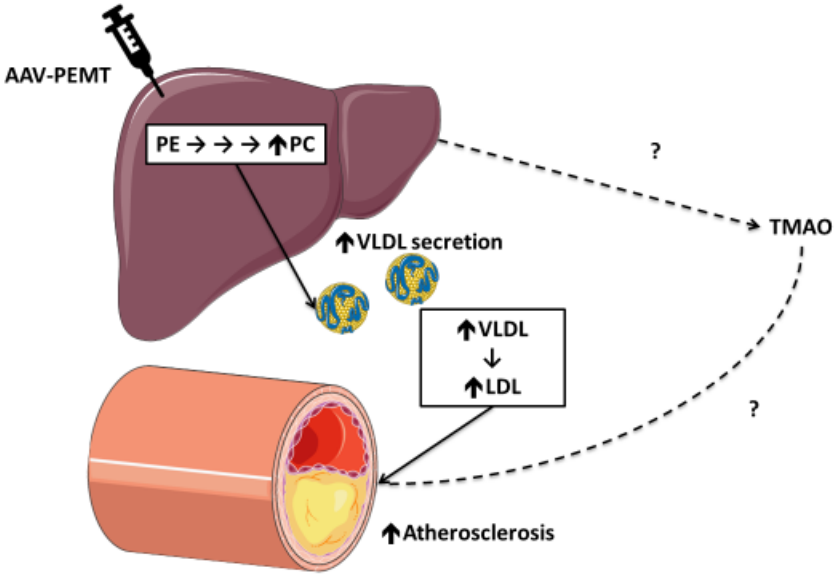


Figure 2-1 Hypothetical schematic demonstrating the effect of hepatic PEMT expression on the development of atherosclerosis in *Pemt*$^{-/-}$/*Ldlr*$^{-/-}$ mice.

Adeno-associated virus expressing human PEMT AAV-PEMT; Phosphatidylethanolamine, PE; phosphatidylcholine, PC; Very low density lipoprotein VLDL; Low density lipoprotein, LDL; Trimethylamine *N*-oxide TMAO; This figure was created in part using illustrations adapted from ©Servier Medical Art "With Permission".

3. **To determine the effect of choline supplementation on the development of atherosclerosis in DKO mice.** We hypothesized that increase in dietary choline will promote atherosclerosis by two main mechanism: It would increase plasma lipids by increasing VLDL secretion from the liver and it would also increase choline metabolism in the gut to TMAO, which is the novel risk factor for atherosclerosis. Figure 2-2 illustrates the hypothetical outcome of choline supplementation.

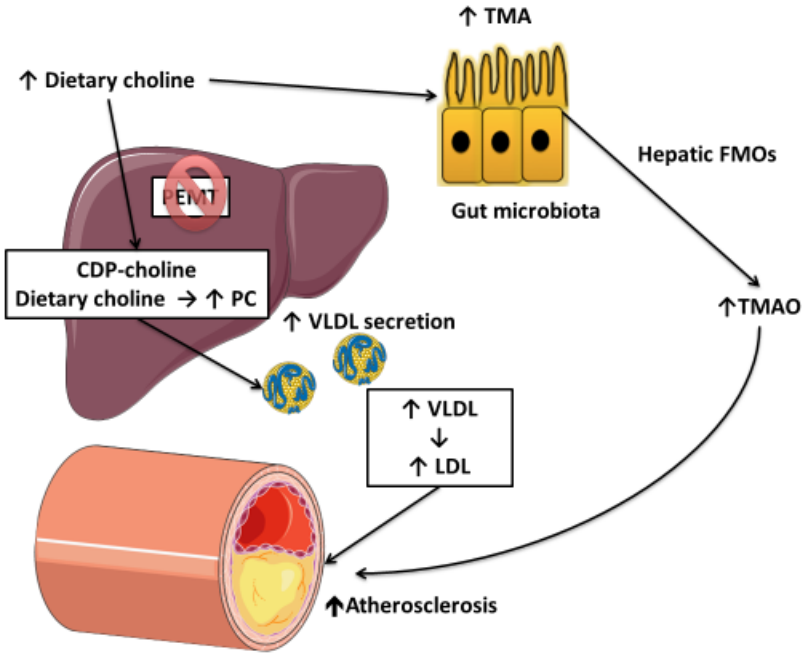


Figure 2-2 Hypothetical schematic demonstrating the effect of choline supplementation on the development of atherosclerosis in *Pemt*$^{-/-}$/*Ldlr*$^{-/-}$ mice. Phosphatidylethanolamine *N* methyltransferase, Phosphatidylethanolamine, PE; phosphatidylcholine, PC; Very low-density lipoprotein, VLDL; Low density lipoprotein, LDL; Trimethylamine, TMA; Trimethylamine *N*-oxide, TMAO; Flavin monooxygenase, FMO. This figure was created in part using illustrations adapted from ©Servier Medical Art "With Permission".

4. **To study the effect of decreasing the effect of choline in the development of atherosclerosis in *Ldlr*$^{-/-}$ mice.** We hypothesized that decreasing the amount of choline would attenuate atherosclerosis due to decrease in plasma lipids and plasma TMAO (Figure 2-3 illustrates expected outcome).

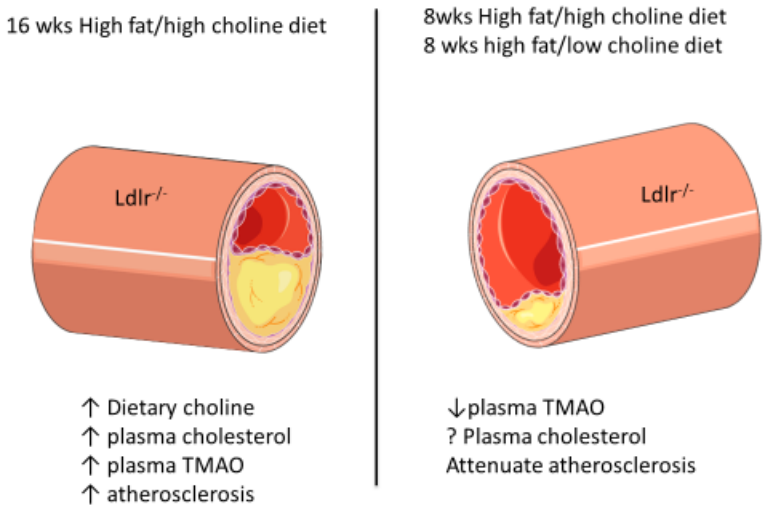


Figure 2-3 Hypothetical outcome of the switching from high fat/high choline diet to a high fat/low choline diet on the development of atherosclerosis in *Ldlr*$^{-/-}$ mice. Trimethylamine *N*-oxide, TMAO This figure was created in part using illustrations adapted from ©Servier Medical Art "With Permission".

3 Chapter 3

Title: Hepatic expression of Pemt, but not dietary choline supplementation, reverses the protection against atherosclerosis in *Pemt*$^{-/-}$/*Ldlr*$^{-/-}$ mice

3.1 Abstract:

Phosphatidylethanolamine *N*-methyltransferase (PEMT) is a hepatic enzyme that converts phosphatidylethanolamine (PE) to phosphatidylcholine (PC). *Pemt*$^{-/-}$ mice are protected from obesity and insulin resistance, a phenotype that is reversed with dietary choline supplementation. Additionally, PEMT deficiency reduces plasma lipids and is protective against atherosclerosis when crossed with the low-density lipoprotein receptor (*Ldlr*$^{-/-}$) mice. Recent studies have demonstrated that choline can be metabolized by the gut microbiota into trimethylamine-*N*-oxide (TMAO), which is an emerging risk factor for atherosclerosis. The objective of this study was to determine whether reintroducing hepatic PEMT expression or supplementing the diet with choline would promote atherosclerosis in *Pemt*$^{-/-}$/*Ldlr*$^{-/-}$ mice.

Methods: *Pemt*$^{+/+}$/*Ldlr*$^{-/-}$ (SKO) and *Pemt*$^{-/-}$/*Ldlr*$^{-/-}$ (DKO) mice were injected with an adeno-associated virus (AAV) expressing green florescent protein (GFP) or human PEMT, and fed a nutritionally complete western diet (40% calories from fat, 0.5% cholesterol, 3g/kg choline) for 8 weeks. In a separate experiment, SKO and DKO mice were fed the western diet containing 3 or 10g/kg choline for 12 weeks.

Results: DKO mice had low plasma lipids and were protected against atherosclerosis compared to SKO mice. AAV-PEMT administration increased plasma lipids and TMAO in DKO mice ($P<0.05$). Furthermore, AAV-PEMT-injected DKO mice developed atherosclerotic lesions similar to SKO mice. In the second study, choline supplementation

in DKO mice did not increase atherosclerosis or plasma lipids, but did increase plasma TMAO levels ($P<0.05$).

Conclusion: Reintroducing hepatic the PEMT enzyme reversed the atheroprotective phenotype of DKO mice. Choline supplementation did not increase atherosclerosis nor plasma lipids. Our data suggests that plasma TMAO levels do not correlate with atherosclerosis when plasma cholesterol is low. Furthermore, this is the first report suggesting that *de novo* choline synthesis alters TMAO metabolism.

3.2 Introduction

Choline, an essential water-soluble nutrient, is part of the human diet and is found in large amounts in eggs, meat and soy[39]. The major form of choline in the diet is phosphatidylcholine (PC). PC can also be synthesized *de novo* by two main pathways: the cytidine-5′-diphosphocholine (CDP-choline) pathway (using dietary choline) and the phosphatidylethanolamine *N*-methyltransferase (PEMT) pathway[40]. PEMT is expressed mainly in the liver and converts phosphatidylethanolamine (PE) into phosphatidylcholine (PC) via three methylation steps[39,55]. This is the only *de novo* pathway that synthesizes choline. PEMT activity accounts for 30% of hepatic PC synthesis while the CDP-pathway provides the remaining 70%[57]. Studies have established that the PEMT pathway evolved to provide PC in times when the dietary choline is limited (e.g starvation, pregnancy, breastfeeding)[82]. PC is required for the synthesis of cell membranes, bile, and lipoprotein secretion[55].

Chow-fed Pemt knockout (*Pemt*$^{-/-}$) mice appear to be similar to their wild type littermates with the exception of mild fatty liver due to low hepatic PC[54,55,67,]. However, when fed a high fat diet (HFD), *Pemt*$^{-/-}$ mice are protected against diet-induced obesity and insulin resistance compared to control mice[88]. Interestingly, dietary choline supplementation restores weight gain and insulin resistance in HFD-fed *Pemt*$^{-/-}$ mice[88], suggesting choline has a complex role in energy metabolism[88]. Zhao *et al.* (2009) demonstrated that Pemt deficiency in low-density lipoprotein receptor knockout (*Pemt*$^{-/-}$ */Ldlr*$^{-/-}$, DKO) mice fed a high fat/high cholesterol diet have significantly reduced plasma lipids as compared to *Pemt*$^{+/+}$*/Ldlr*$^{-/-}$ (SKO) fed the same diet. DKO mice were reported to

have reduced VLDL secretion and a ~60% reduction in plasma cholesterol and TG compared to SKO[68]. This decrease in plasma lipids manifests with a striking 80% reduction in atherosclerotic lesions in DKO mice compared to SKO mice[68]. Pemt deletion in mice lacking apolipoprotein E (*ApoE*$^{-/-}$) demonstrated a 50% improvement in cardiac systolic function, a 30% reduction in atherosclerotic plaque formation and a significant reduction in cardiac lipids[89]. Hence, PEMT deficiency in atherosclerotic mouse models (*Ldlr*$^{-/-}$ and *ApoE*$^{-/-}$) was found to be protective against atherosclerosis[68,89].

An increase in circulating lipids, either due to increased secretion or due to diminished clearance, promotes lipid accumulation in the vessel walls[27]. Low-density lipoprotein (LDL) is a primary risk factor for atherosclerosis due to its ability to permeate the arterial walls, undergo oxidative changes and promote an inflammatory response[27,129]. Although elevated lipids are well established risk factors of atherosclerosis, there is growing literature linking the gut microbiota with obesity and its associated comorbidities including diabetes, heart failure, and atherosclerosis[119,130–133]. Using a metabolomics approach, Hazen *et al.* (2011) identified three PC-derived plasma metabolites (choline, betaine and trimethylamine N-oxide (TMAO)) as being positively associated with atherosclerosis[105]. This was supported by cohort studies finding a higher risk for future major cardiac events and all-cause mortality with increased levels of TMAO in patients undergoing coronary angiography[101,117,134,135].

Certain strains of the gut microbiota metabolize dietary choline into trimethylamine (TMA), which, after being absorbed, is oxidized by hepatic flavin monooxygenase (FMOs) enzymes to TMAO[105]. A study identified eight species from Firmicutes and Proteobacteria phyla and six genera of microbiota for their ability to consume choline and produce TMA[53].

The mechanism by which TMAO causes atherosclerosis remains unclear; however, TMAO may influence many processes that induce foam cell formation, increase platelet aggregation and reduce reverse cholesterol transport[98,109]. Human cohort studies have identified plasma TMAO as a predictor of major adverse cardiac events, renal dysfunction and atherosclerosis in patients undergoing cardiac procedures[112,117,134–136].

The purpose of this study was to determine the role of dietary choline and hepatic PEMT expression in the development of atherosclerosis in *Pemt*$^{-/-}$/*Ldlr*$^{-/-}$ (DKO) mice. Choline supplementation in *Pemt*$^{-/-}$ mice abolished the protection from obesity and insulin resistance[88], but its role in the development of atherosclerosis has not been investigated. Our results showed that chow-fed DKO mice had similar plasma lipid profile compared to their SKO mice but had low plasma choline, betaine, TMA and TMAO. On a western diet, DKO mice were protected against atherosclerosis; however, reintroducing hepatic Pemt activity increased atherosclerosis, plasma lipids and TMAO production in DKO mice to levels similar to SKO mice. This indicated that PEMT plays an essential role in TMAO production. However, in a separate study, we demonstrated that dietary choline did not reverse the protection against atherosclerosis in Western diet-fed DKO mice. Interestingly, choline supplementation increased plasma TMAO, but not plasma lipids, in DKO mice. Our data suggests that plasma TMAO levels do not correlate with atherosclerosis when plasma lipids are low. Furthermore, this is the first report suggesting that *de novo* choline synthesis alters TMAO metabolism.

3.3 Materials and Methods:

All animal procedures were conducted in accordance with the Canadian Council on Animal Care Guidelines and Policies approved by the University of Alberta Health

Sciences Animal Care and Use Committee. Male C57BL/6 *Pemt*$^{+/+}$/*Ldlr*$^{-/-}$ (SKO) and *Pemt*$^{-/-}$/*Ldlr*$^{-/-}$ (DKO) mice (n=8-10 per group) were given free access to water and standard chow diet (Unpurified diet PICO Laboratory Rodent Diet 20; LabDiet). At age 8-10 weeks, SKO and DKO mice mice were injected with adeno-associated virus (AAV) expressing green florescent protein (GFP) or human PEMT ($3 \times 10e^{10}$ genome copy/mouse) and fed a western diet (Supplementary Table 1) containing 40% calories from fat, 0.5% cholesterol, 3g/kg choline bitartrate for 8 weeks. In another cohort, SKO and DKO mice were fed a western diet containing 3 or 10g/kg choline bitartrate (C1629; Sigma-Aldrich) for 12 weeks (See Figure 3-1).

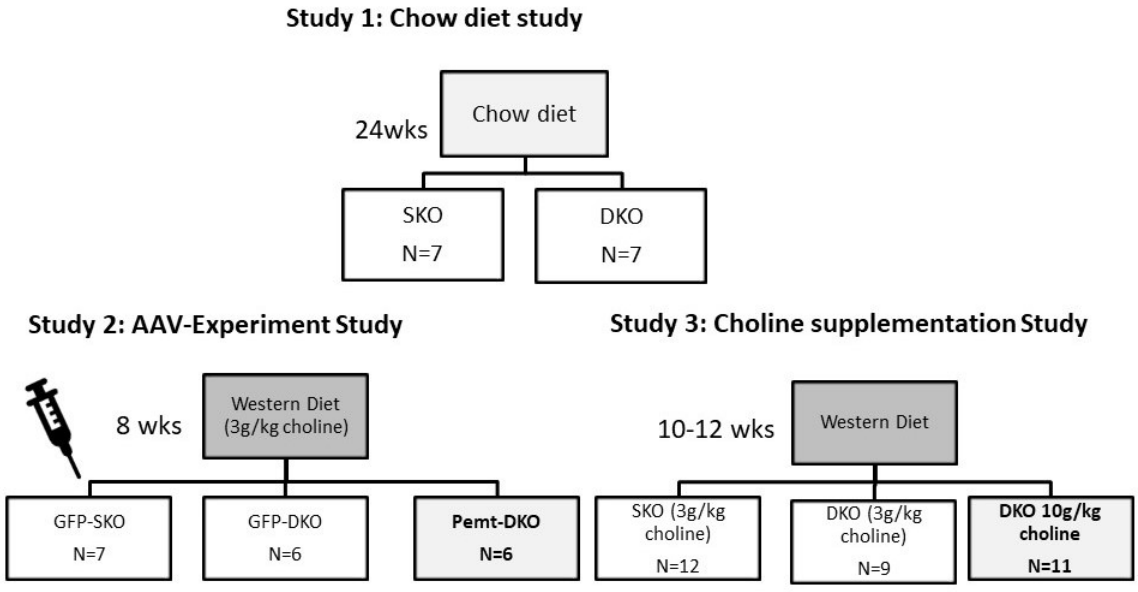


Figure 3-1 Overview of the experimental design of: Study 1, 2 and 3. *Pemt*$^{+/+}$/*Ldlr*$^{-/-}$, SKO; *Pemt*$^{-/-}$/*Ldlr*$^{-/-}$, DKO, 40% high fat diet+0.5g/kg cholesterol+3g/kg choline, Western Diet; Adeno-associated virus expressing GFP or PEMT, AAV; Green fluorescent Protein, GFP; Phosphatidylethanolamine *N*-methyl transferase, PEMT.

For all experiments, mice were fasted for 12 hours before being euthanized by exsanguination under isoflurane anesthesia. Blood was collected by cardiac puncture and

plasma was separated and frozen at -80° C. Tissues (heart and liver) were harvested, and processed as described below. Plasma cholesterol and triglycerides were measured by gas chromatography[137] or enzymatic colorimetric assay (Wako Diagnostics).

3.3.1 Generating the human PEMT AAV vector:

A codon optimized sequence for human PEMT was synthesized (GenScript, Piscataway, New Jersey) and sub-cloned into the Pst I and Not I sites of the self-complementary AAV shuttle vector pENN.AAVscTBG.PI.RBG (University of Pennsylvania Vector Core Lab). This vector allows expression from a liver-specific TBG (human thyroxine binding globulin) promoter, with an RBG **(***rabbit beta-globin***)** poly (A) sequence. This AAV8 vector was produced by the University of Pennsylvania Vector Core Lab.

3.3.2 Atherosclerotic Aortic Root Lesion Quantification

Hearts were perfused through the left ventricle with 1 mL ice-cold PBS containing 5mM EDTA. Next, they were incubated in Krebs-Henseleit buffer for 30 mins, fixed overnight in 10% phosphate buffered formalin and cut transversally perpendicular to the aortic root[138]. Top portions of the heart were embedded in optimal cutting temperature compound (OCT; Thermo Scientific™ Shandon™ Cryomatrix™), and frozen at -20 °C. Cross-sectional aortic root analysis was performed as described elsewhere[139]. Briefly, serial cryosections (10 µm thick) were collected from the apex to the base of the aortic sinus using Cryostat®, and stained with Oil Red O to detect the atherosclerotic plaque and Mayer's Hematoxylin (Sigma-Aldrich) for tissue visualization. ImageJ analysis software was used to quantify lesion area in 12 cross-sections (3 sets of 4 consecutive sections, 200 µm apart) over 520µm aortic root, and the mean lesion area per mouse was reported.

3.3.3 Liver Histology and Lipid Analysis

Livers were removed, weighed, snap-frozen in liquid nitrogen and stored at -80° C, or preserved in 10% phosphate-buffered formalin, pH 7.0. Formalin-preserved liver samples were stained with hematoxylin and eosin and evaluated by a pathologist for non-alcoholic fatty liver disease (NAFLD) histological activity using the brunt criteria[140]. Livers were homogenized with a Polytron in PBS. Protein concentrations of liver homogenate were measured using Pierce BCA Protein Assay Kit (Thermo Fisher Scientific) with bovine serum albumin as standard. Total lipid analysis of the liver homogenates was performed, as previously described[141]. To measure phospholipids, total lipids were extracted from liver homogenates using the Folch method and phospholipids were separated by TLC in chloroform:methanol:acetic acid:water, 50:30:8:4 (v:v:v:v)[142]. Amounts of PC and PE were determined by phosphorus assay[143].

3.3.4 *In Vivo* Hepatic VLDL Secretion

To measure hepatic TG secretion, mice were fasted overnight, then injected intraperitoneally with Poloxamer 407 (P-407, 1g/ kg body weight)[144]. Blood samples were drawn from the tail vein using heparinized capillary tubes (Fisherbrand® Microhematocrit) immediately prior to injection (t = 0) and then at 1, 2, 3 and 4hrs post injection. Four hrs post injection, animals were sacrificed by exsanguination under isoflurane anesthesia and blood was collected by cardiac puncture into blood-collection tubes (BD Microtainer®). All blood samples were centrifuged for 10 minutes at 2,000 *g* and 4° C. Plasma TG levels were measured by enzymatic colorimetric method using a commercially available kit (Sekisui Diagnostics).

3.3.5 Lipoprotein profile

For analysis of lipoprotein profiles, plasma samples from each experimental group were pooled and subjected to fast protein liquid chromatography (FLPC) gel filtration using two sequential Superose 6 columns and post-column cholesterol measurements (Sigma)[145].

3.3.6 Plasma Choline/Betaine/TMA/ TMAO Quantification

For choline and betaine analysis, plasma was mixed with methanol containing internal standards. Choline and betaine levels were determined by LC-MS/MS, as previously described [146,147]. For TMA and TMAO, plasma was pretreated with methanol and 0.1% formic acid. Samples were then incubated with ethyl bromoacetate and ammonium hydroxide to convert TMA to ethyl betaine. Ethyl betaine and TMAO were determined by LC-MS/MS analysis[148].

3.3.7 Analysis of intestinal microbiota

Total DNA from feces was extracted using the QIAamp Fast DNA Stool Mini Kit (Qiagen, Inc. Mississauga, ON, Canada) with the addition of a bead-beating step (FastPrep instrument, MP Biomedicals, Solon, OH) at speed 6 for 1 minute. Amplicon libraries were constructed from fecal DNA that amplified the V1 to V3 hypervariable region of the bacterial 16S *rRNA* gene using primer pairs 27 F: 5'-AGAGTTTGATCMTGGCTCAG-3' and 519 R: 5'-GWATTACCGCGGCKGCTG-3'. The forward primer includes the Roche 454 Titanium adaptor A (CCATCTCATCCCTGCGTGTCTCCGACTCAG) and a 10 bp multiplex identifier (MID), and the reverse primer contained adaptor B (CCTATCCCCTGTGTGCCTTGGCAGTCTCAG). The PCR was carried out in

triplicates with 20 μL reaction volumes each containing 0.2 μL Phusion high-fidelity DNA polymerase (ThermoFischer Scientific, Nepean, ON, Canada), 4 μL of 5 × HF buffer, 0.4 μL 10mM dNTPs, 1 μL of the extracted template DNA (100 ng), and 1 μL each 27F and 519R primers (10 ng/μL), following the PCR parameters: 98°C for 1 min, 35 cycles of 98°C for 10 s, 59°C for 30s and 72°C for 30s, with a final extension at 72°C for 7 min. Pooled PCR products for each sample were run on 0.8% agarose gel electrophoresis and the DNA band was excised and purified using the QIAquick gel extraction kit (Qiagen, Valencia, CA). Equal amount of purified PCR product (100ng) were pooled for subsequent 454 pyrosequencing on the Titanium platform (Roche, Branford, CT).

The sequencing data that passed Roche's quality thresholds were obtained. Next, barcodes were trimmed and sequences were filtered through a quality control pipeline, and bases with quality scores lower than 25 were removed. The QIIME 1.9.1 (Quantitative Insight into Microbial Ecology) tool kit (http://qiime.org/) and Usearch version 7.1 (http://drive5.com/uparse/) was applied for data analysis. High-quality sequences were assigned to operational taxonomic units (OTUs) based upon a threshold of 97% identity using the Uclust algorithm. The OTUs were assigned to taxonomy using the Ribosome Database Project (RDP) classifier[149].

3.3.8 Statistical Analysis

Graph Pad Prism 5 software was used for statistical analysis. All data was reported as mean ± SEM. For intestinal microbiota data, analysis of diversity and richness estimators (Shannon and Chao1) were performed through the vegan package in R (R 3.3.2)[150,151]. The permutational multivariate analysis of variance (PERMANOVA) was used to compare

overall bacterial community of SKO, DKO, and choline-supplemented DKO (CS-DKO) groups based on Bray-Curtis dissimilarities with 999 permutations using R (R 3.3.2). Differences in relative abundance of bacterial genera were performed by ANOVA on log(x+1) transformed data. Hepatic histologic grading was compared using a Kruskal-Wallis ANOVA of rank, followed by Dunn's multiple-comparison test. For all other parameters, data was analyzed using one way- ANOVA followed by a Tukey's post-hoc test or Student's T-test, when appropriate. Spearman correlation coefficient and linear regression was used to establish association between variables. Significance was set at $p<0.05$ in all analyses.

3.4 Results:

3.4.1 DKO mice have lower levels of plasma choline and choline metabolites

Our first objective was to investigate the effects of Pemt-deficiency on choline metabolism in *Ldlr*$^{-/-}$ mice fed a chow diet. We found that DKO mice had lower plasma choline, betaine, TMA and TMAO compared to SKO mice (**Figure 3-2**). Additionally, chow-fed SKO and DKO mice had similar plasma cholesterol levels and did not develop atherosclerosis. This data suggests that PEMT expression (or *de novo* choline synthesis) plays a role in regulating metabolism. Thus, we sought to determine if differences in plasma TMAO would contribute to the atheroprotective phenotype of the western diet-fed DKO mice.

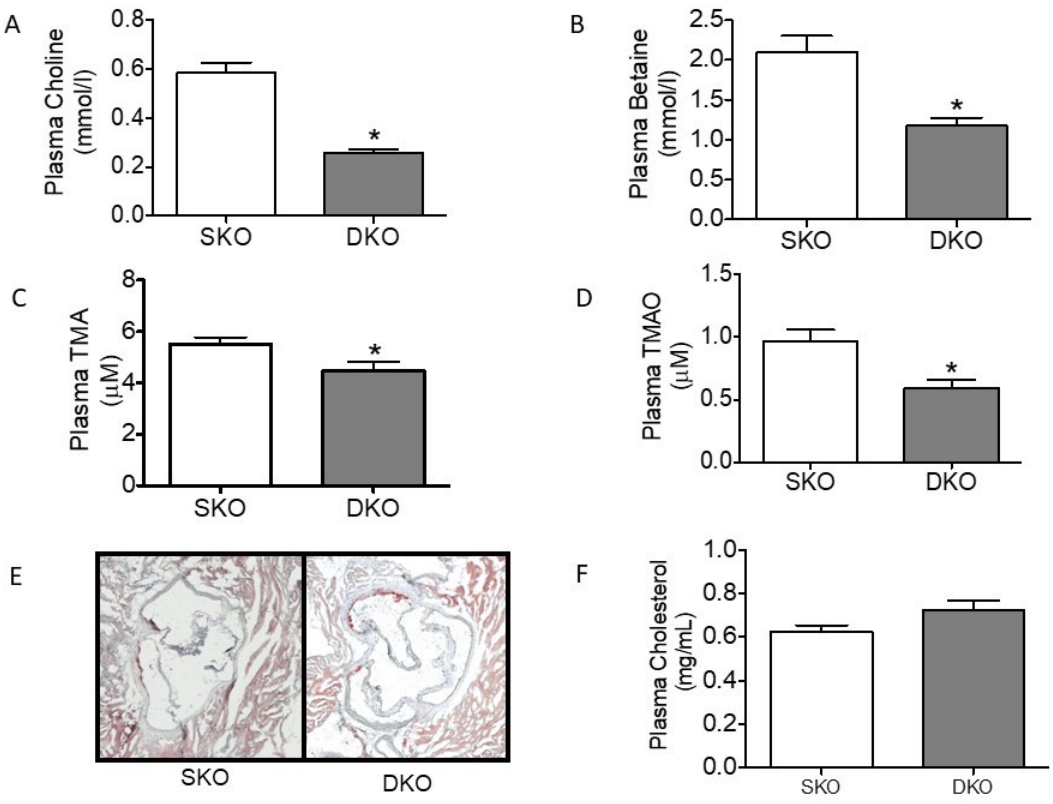


Figure 3-2 Chow-fed DKO mice have low plasma choline metabolites.
SKO and DKO mice fed a chow diet for 24 weeks. (A-D) Choline, betaine, TMA and TMAO were measured in fasting plasma samples. (E) Representative image of Oil red O staining of aortic root cross-sections. (F) Fasting plasma cholesterol. *Pemt*$^{+/+}$*/Ldlr*$^{-/-}$, SKO; *Pemt*$^{-/-}$*/Ldlr*$^{-/-}$, DKO Values are mean ± SEM, *P<0.05.

3.4.2 Restoring Pemt expression increased PC supply, VLDL secretion and cholesterol levels.

We have previously reported that DKO mice are protected from atherosclerosis when fed a western diet[68,89]. To understand whether hepatic PC and/or choline synthesis is responsible for this phenotype, we reintroduce PEMT activity via AAV-administration. As previously reported, DKO (AAV-GFP injected) mice had no detectable Pemt activity in the liver, while AAV-PEMT injected DKO mice had twice as high PEMT activity as

compared to AVV-GFP injected SKO mice (**Figure 3-3** A). DKO mice had significantly lower hepatic PC and PC/PE ratios compared to SKO mice.

Reintroducing PEMT expression increased hepatic PC and the PC/PE ratio in the DKO mice to levels observed in the SKO mice (Figure 3-3 B). We assessed hepatic VLDL secretion following P-407 injections. DKO mice had significantly impaired VLDL secretion compared to SKO mice. VLDL secretion was normalized in AAV-PEMT injected DKO mice (Figure 3-4A).

Furthermore, fasting cholesterol levels were low in DKO mice compared to SKO mice. AAV-dependent Pemt expression increased, but did not normalize, plasma cholesterol levels in the DKO mice (**Figure 3-4** B). In summary, ectopic PEMT expression resulted in a normalization of hepatic phospholipid composition, improvement in hepatic lipids (Appendix Figure 7-1) and a significant increase in plasma lipids in DKO mice compared to SKO mice.

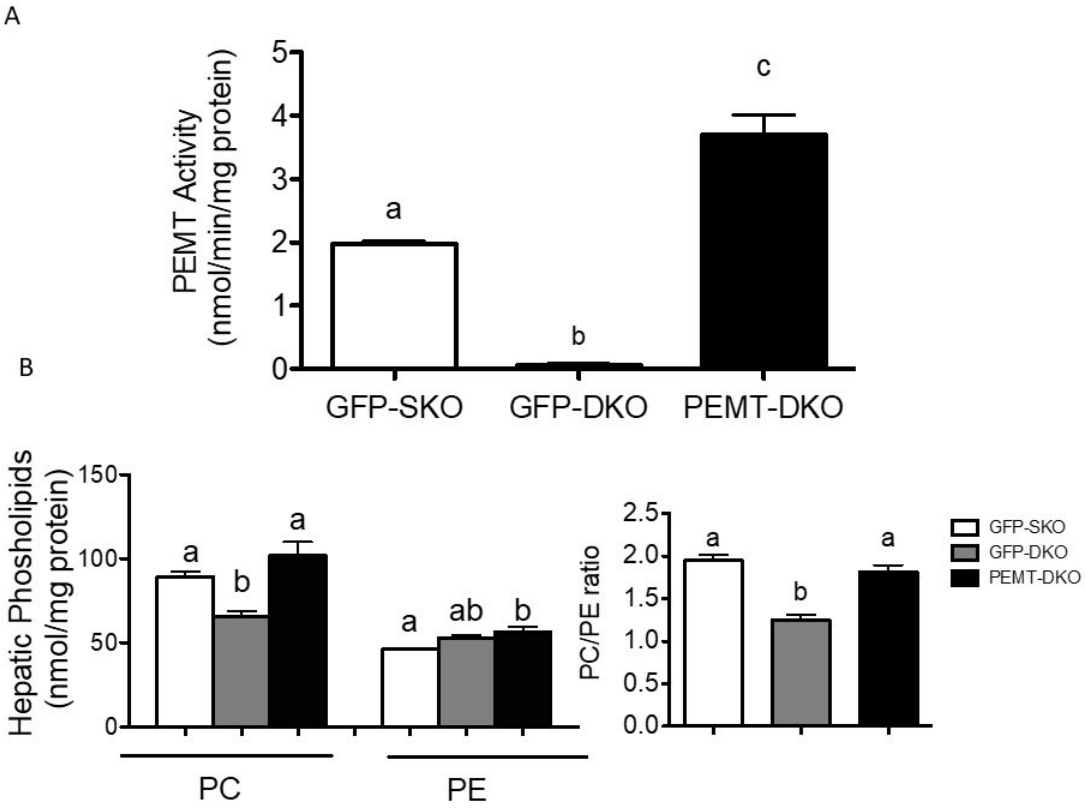


Figure 3-3 PEMT-AAV expression increased PEMT activity.
(A) Hepatic PEMT activity measured. (B) Lipids were extracted from liver (1 mg protein), and the amount of PC and PE were measured by phosphorus assay. Values are mean ± SEM, Groups without a common letter differ, $p<0.05$. *Pemt*$^{+/+}$*/Ldlr*$^{-/-}$, SKO; *Pemt*$^{-/-}$*/Ldlr*$^{-/-}$, DKO, Green Fluorescent Protein, GFP; phosphatidylethanolamine *N*-methyl transferase, PEMT; Adeno-associated virus, AAV.

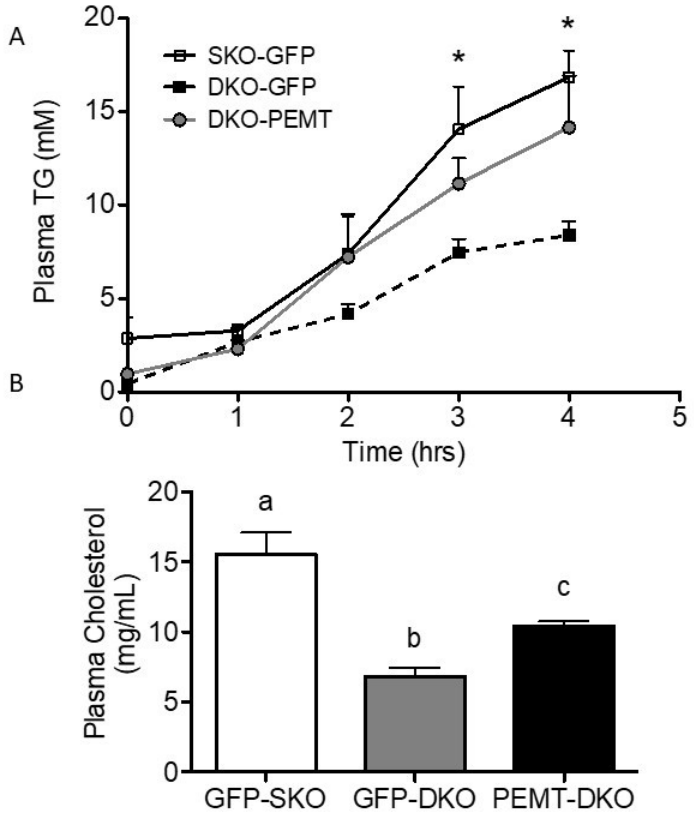


Figure 3-4 Hepatic PEMT expression increased VLDL secretion and cholesterol levels in the PEMT-DKO mice fed a western diet for 8 weeks
(A) Blood was collected at time=0 and at indicated times after intraperitoneal injection of Poloxamer 407. The accumulation of TG in plasma was measured at t=0, 1hr, 2hr, 3hr and 4hr. "*" represents significant differences between SKO-GFP and GFP-DKO at these time points (Black smooth line presents GFP injected-SKO mice, grey smooth line presents PEMT injected DKO mice and dotted line presents GFP injected DKO mice) (B) Fasting Plasma cholesterol. Values are mean ± SEM, Groups without a common letter differ, $p<0.05$ *Pemt*$^{+/+}$/*Ldlr*$^{-/-}$, SKO; *Pemt*$^{-/-}$/*Ldlr*$^{-/-}$, DKO, Green Fluorescent Protein, GFP; phosphatidylethanolamine *N*-methyl transferase, PEMT; Adeno-associated virus, AAV.

3.4.3 AAV-PEMT expression eliminated the protection against atherosclerosis and increased TMAO levels in DKO mice

Considering that reintroducing hepatic PEMT expression increased plasma lipids, we hypothesized that it would increase atherosclerosis in the DKO mice. Indeed, DKO mice were protected from development of atherosclerosis compared to SKO mice, whereas AAV-dependent hepatic Pemt expression increased plaque formation in the DKO mice to levels observed in SKO mice (**Figure 3-5**). Thus, hepatic PEMT activity abolished the atheroprotective phenotype of the DKO mice.

Both plasma TMA and TMAO (**Figure 3-6**) were lower in DKO mice compared to SKO mice. Upon reintroducing Pemt, both plasma TMA and TMAO in DKO mice increased to levels observed in SKO mice, despite no differences in dietary choline intake. In this experiment, plasma TMAO positively correlated with atherosclerosis, PEMT activity, and plasma cholesterol (Figure 3-7). It is noteworthy that plasma cholesterol also demonstrated strong correlations with atherosclerosis (Figure 3-7B). Taken together, our data suggests that alterations in hepatic PC synthesis influence the development of atherosclerosis through changes in lipoprotein metabolism and, potentially, TMAO metabolism.

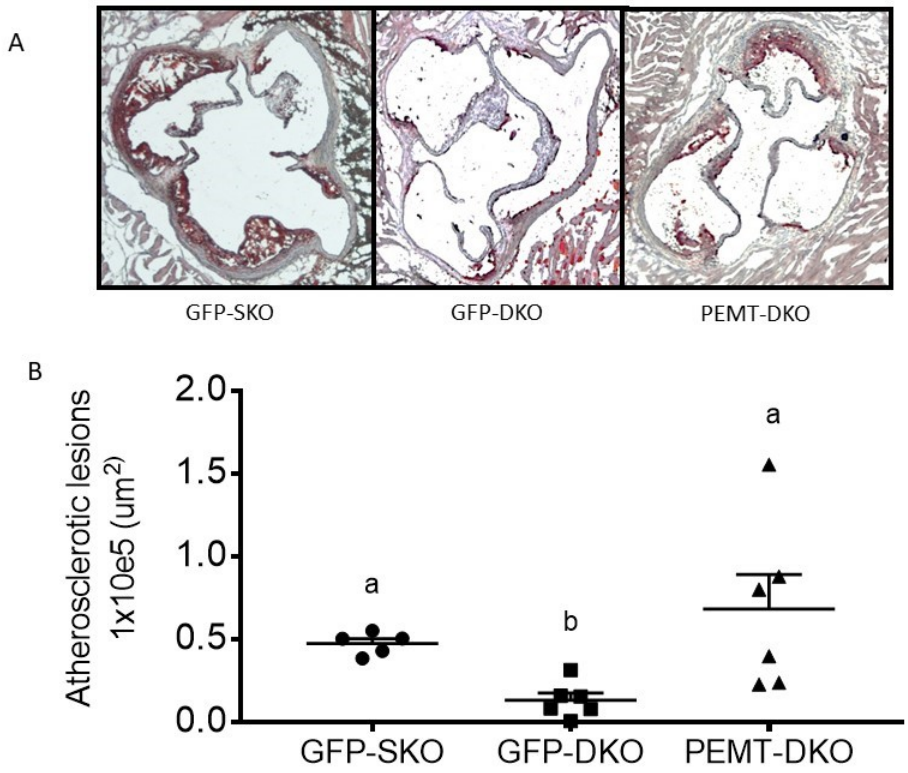


Figure 3-5 Pemt expression eliminated the atheroprotective phenotype.
(A) Representative image of Oil red O staining of aortic root section (B) Quantification of mean lesion area from cross sections of the aortic arch was performed using Image J. Each dot represents 1 mouse. Groups without a common letter differ, Values are mean ± SEM, *P<0.05.

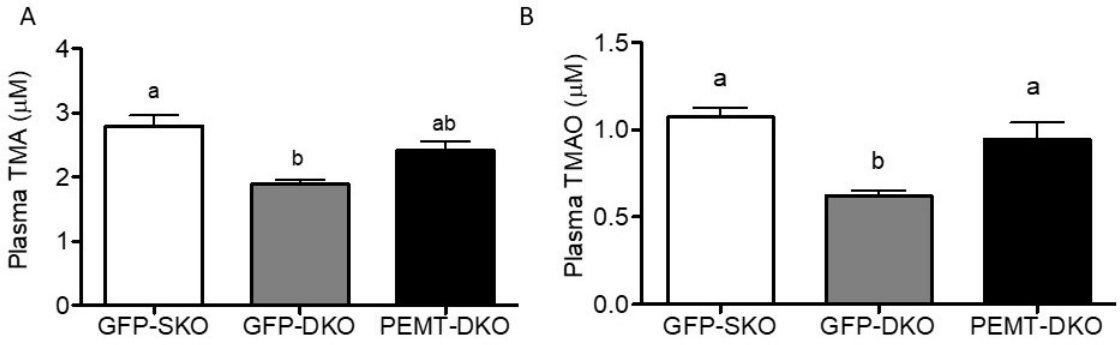


Figure 3-6 Pemt expression increased plasma TMA and TMAO. (A) Measurement of plasma TMA and (B) plasma TMAO. Groups without a common letter differ, Values are mean ± SEM, *P<0.05. *Pemt*$^{+/+}$*/Ldlr*$^{-/-}$, SKO; *Pemt*$^{-/-}$*/Ldlr*$^{-/-}$, DKO, Green Fluorescent Protein, GFP; phosphatidylethanolamine *N*-methyl transferase, PEMT; Adeno-associated

virus, AAV.

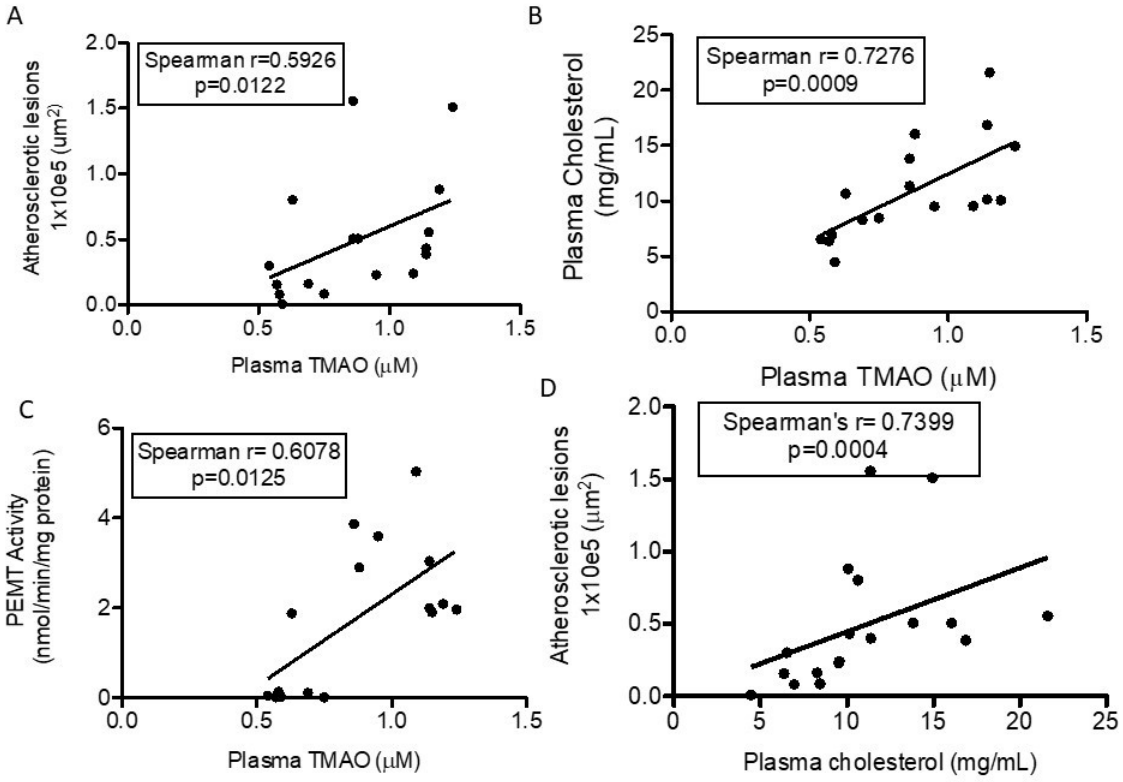


Figure 3-7 Correlations of plasma TMAO. (A) with atherosclerotic lesions, (B) plasma TMAO with plasma cholesterol, (C) plasma TMAO with PEMT enzyme activity and (D) Correlation of plasma cholesterol with atherosclerotic lesions.

3.4.4 Dietary choline increased plasma TMAO but did not increase atherosclerosis in DKO mice.

We previously demonstrated that the *Pemt*$^{-/-}$ mice are protected against diet induced obesity and insulin resistance, a phenotype that was reversed by choline supplementation[88]. However, whether choline supplementation would also reverse the atheroprotective effect of Pemt-deficiency was still unknown. After 12 weeks of Western diet feeding there was a 90% reduction in atherosclerotic plaque in the DKO compared to SKO mice; however,

choline supplementation did not increase plaque formation in the DKO mice (**Figure 3-8**A). Plasma lipoprotein analysis demonstrated DKO mice had lower cholesterol-rich VLDL and LDL particles and higher HDL particles compared to SKO mice; choline supplementation did not increase these lipoproteins in DKO mice (**Figure 3-9**A). In agreement to this, DKO mice had an 80% reduction in plasma total cholesterol levels compared to SKO mice and 70% reduction in triglycerides (**Figure 3-9** B and C). Dietary choline supplementation did not increase plasma cholesterol or triglycerides in DKO mice.

We next investigated the effect of dietary choline supplementation on choline metabolites. Plasma choline concentration was not different between SKO and DKO and plasma betaine was lower in DKO mice than in SKO mice (**Figure 3-10** A and B) To our surprise, choline supplementation did not increase either plasma choline or betaine in DKO mice. In this experiment, plasma TMA levels were not different among groups (**Figure 3-10** A- C). Although plasma TMAO was not different between SKO and DKO mice, there was a 2.5-fold increase in TMAO production upon choline supplementation (**Figure 3-10** D). Unlike the AAV-PEMT experiments (Figure 3-7 A and B), plasma TMAO levels did not correlate with atherosclerosis and plasma cholesterol (**Figure 3-11**A and B)

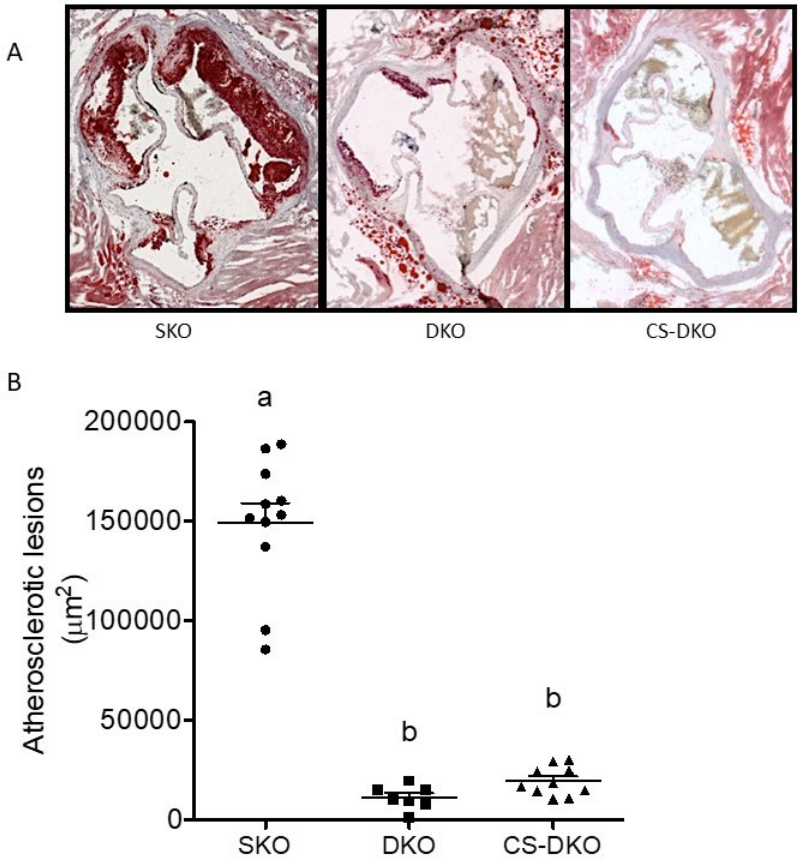


Figure 3-8 Choline supplementation did not increase atherosclerosis
SKO, DKO and CS-DKO mice were fed a western diet (40% high fat diet, 0.5% cholesterol) supplemented with either 3 (SKO and DKO) or 10 (CS-DKO) g/kg choline for 12 weeks. (A) Representative image of Oil red O staining of aortic root section (B) Quantification of mean lesion area from cross sections of aortic arch was performed using Image J. Each dot represents 1 mouse. Values are mean ± SEM, Groups without a common letter differ, *P<0.05. *Pemt*$^{+/+}$*/Ldlr*$^{-/-}$, SKO; *Pemt*$^{-/-}$*/Ldlr*$^{-/-}$, DKO. Choline supplemented DKO, CS-DKO

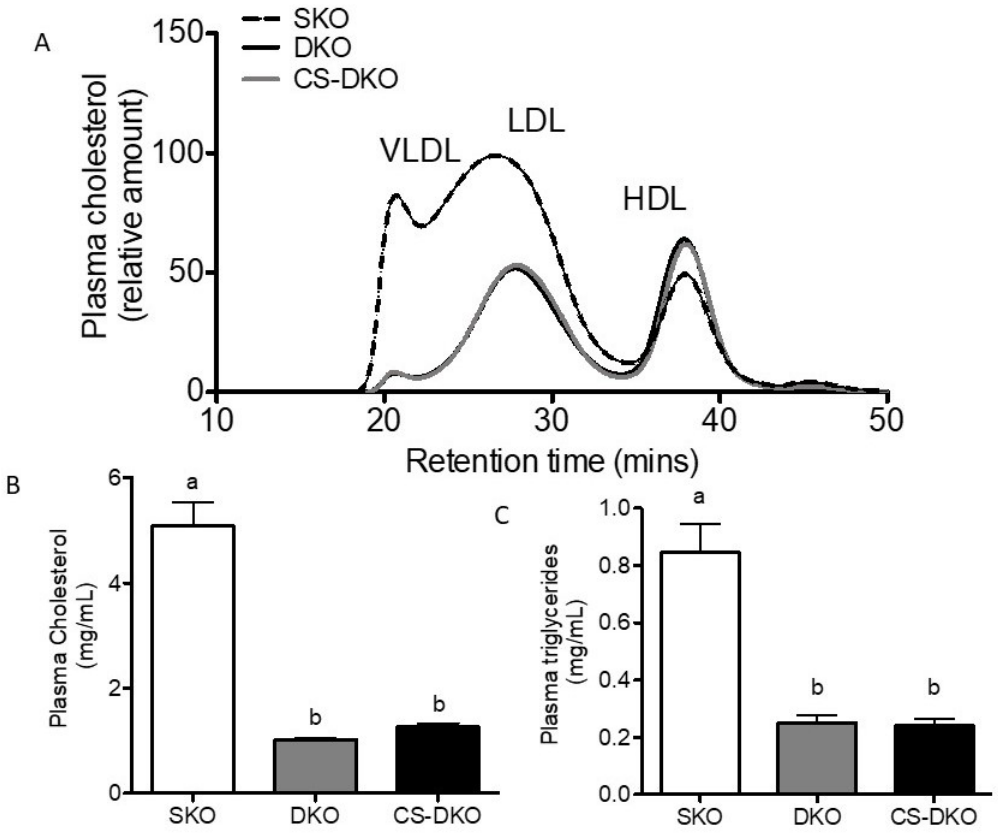


Figure 3-9 Choline supplementation did not increase plasma lipids (A) Fast Protein Liquid Chromatography of plasma shown (B) Fasting plasma cholesterol (C) plasma triglycerides. *Pemt*$^{+/+}$*/Ldlr*$^{-/-}$, SKO; *Pemt*$^{-/-}$*/Ldlr*$^{-/-}$, DKO. Choline supplemented DKO, CS-DKO

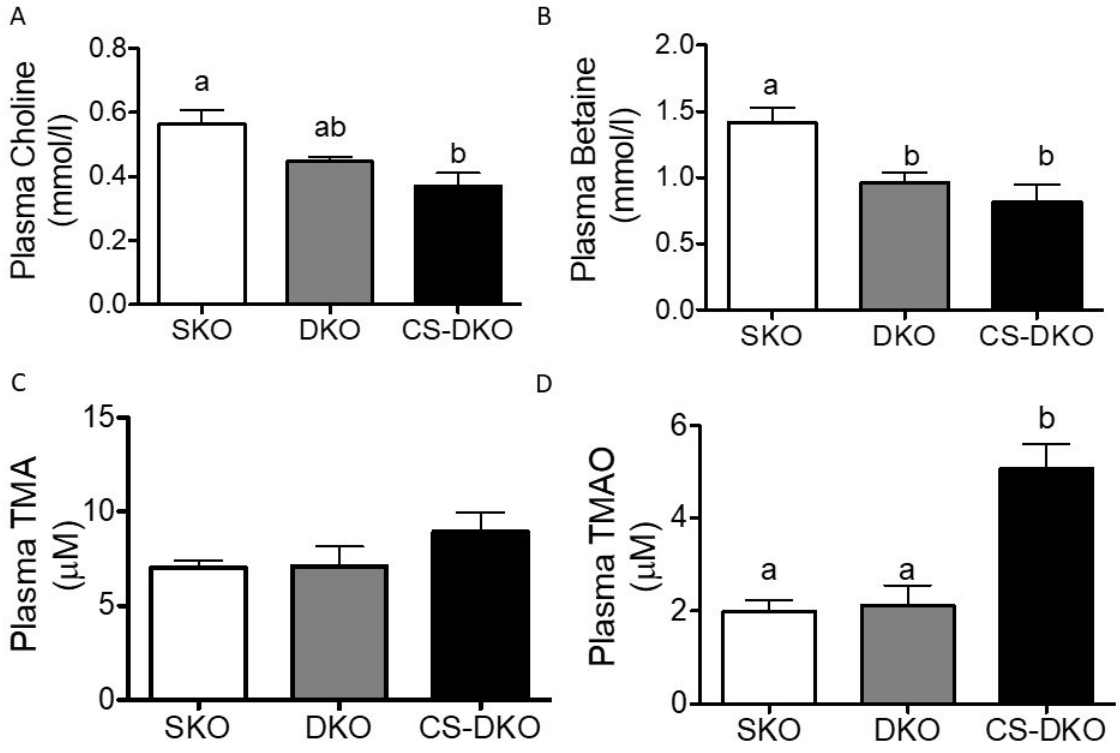


Figure 3-10 Choline supplementation increased TMAO levels (A) plasma choline (B) plasma betaine (C) Plasma TMA and (D) plasma TMAO were measured. *Pemt*$^{+/+}$*/Ldlr*$^{-/-}$, SKO; *Pemt*$^{-/-}$*/Ldlr*$^{-/-}$, DKO. Choline supplemented DKO, CS-DKO

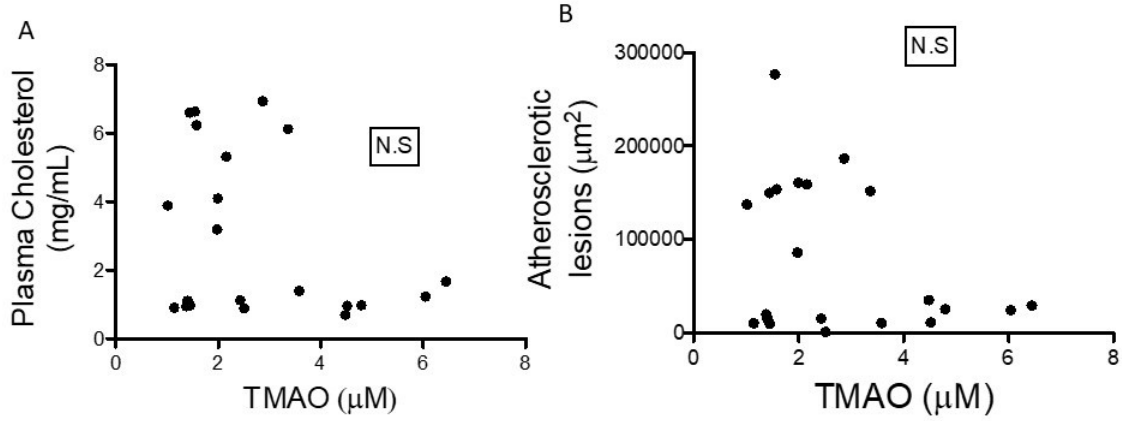


Figure 3-11 Plasma TMAO did not correlate with atherosclerosis (A) No significant correlation between Plasma TMAO with plasma cholesterol. (B) No significant correlations between plasma TMAO with atherosclerotic lesions

3.4.5 Choline supplementation attenuates NAFLD in DKO mice.

We next investigated the effect of choline supplementation on liver health. PC derived from the PEMT pathway is required for hepatic VLDL secretion[67]. Hepatic PC and the PC/PE ratio were significantly reduced in DKO mice compared to SKO mice (**Figure 3-12** A). Choline supplementation does not increase PC/PE ratio in the CS-DK mice. As a result, there was a 50% inhibition in VLDL secretion in DKO mice compared to SKO mice. To our surprise, choline supplementation did not increase VLDL secretion, nor did it increase PC or PC/PE ratio in DKO (**Figure 3-13** A and B). A consequence of reduced VLDL secretion, was a 70% increase in hepatic TG in DKO mice compared to SKO. Although choline supplementation did not restore VLDL secretion, it did reduce hepatic TG levels to levels of the SKO mice (**Figure 3-13** A and B). The hepatic size and number of lipid droplets were increased in DKO mice, both of which were reduced by choline supplementation. The average NAFLD score was clearly higher in DKO compared to SKO mice (Mean (SEM)) = 3.6 (0.67) vs. 0.6 (0.33), respectively, and was decreased significantly to 1.3 (0.49) (**Figure 3-14** A and B). Overall, choline supplementation did not alter hepatic PC nor did it alter VLDL secretion, but it did reduce hepatic TG levels and improved NAFLD score.

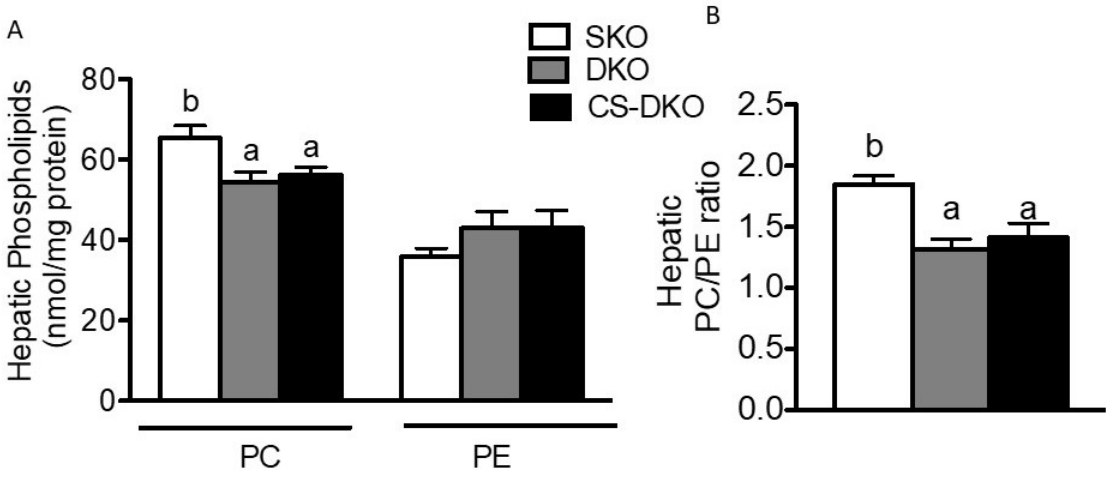


Figure 3-12 Choline supplementation did not increase PC/PE ratio
(A)Lipids were extracted from liver and the amount of PC and PE were measured by phosphorus assay. (B) hepatic PC/PE ratio. Values are mean ± SEM, Groups without a common letter differ, *P<0.05.

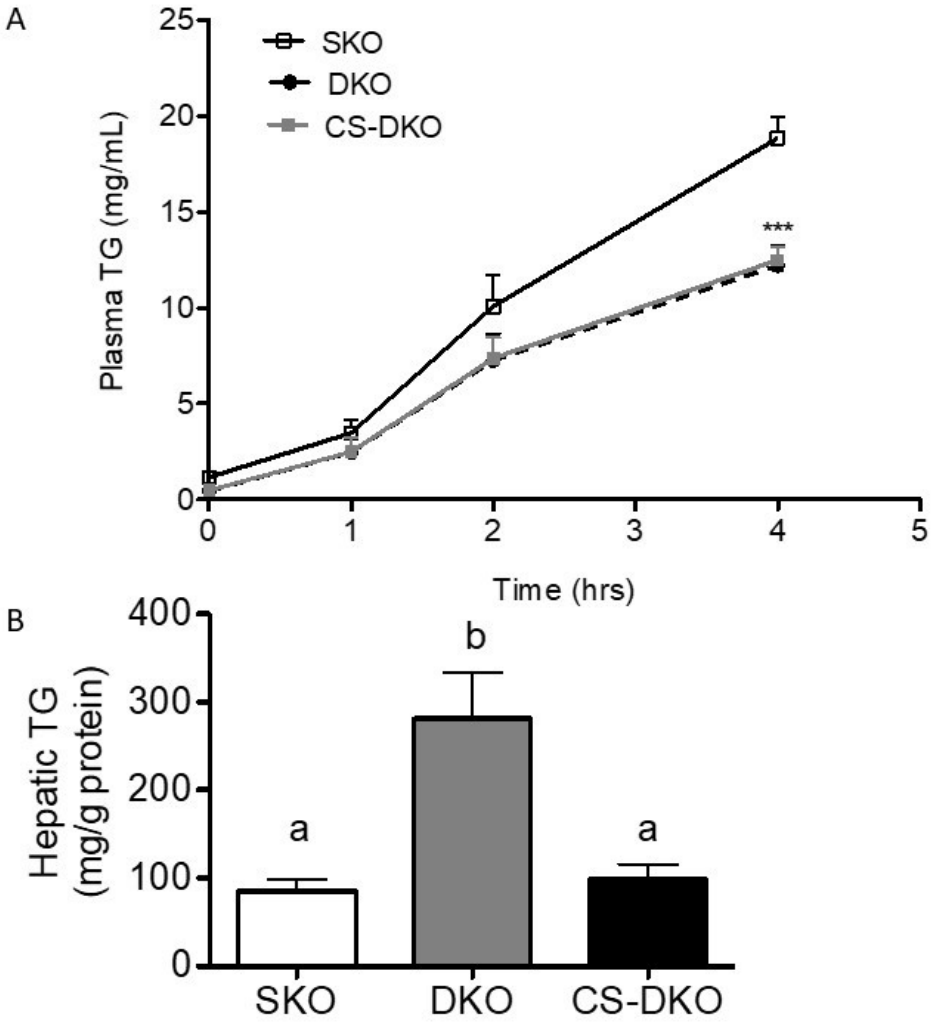


Figure 3-13 Choline supplementation did not alter VLDL secretion but decreased hepatic TG levels

(A) Blood was collected at time=0 and at indicated times after intraperitoneal injection of Poloxamer 407. The accumulation of TG in plasma, an indicator of hepatic VLDL secretion was measured at t=0, 1hr, 2hr and 4hr (B) Lipids were extracted from liver (1 mg protein), Phospholipids were removed enzymatically by phospholipase C and the mass of TG was measured by liquid Gas Chromatography. Values are mean ± SEM, Groups without a common letter differ, *P<0.05, ***P<0.001 compared to SKO.

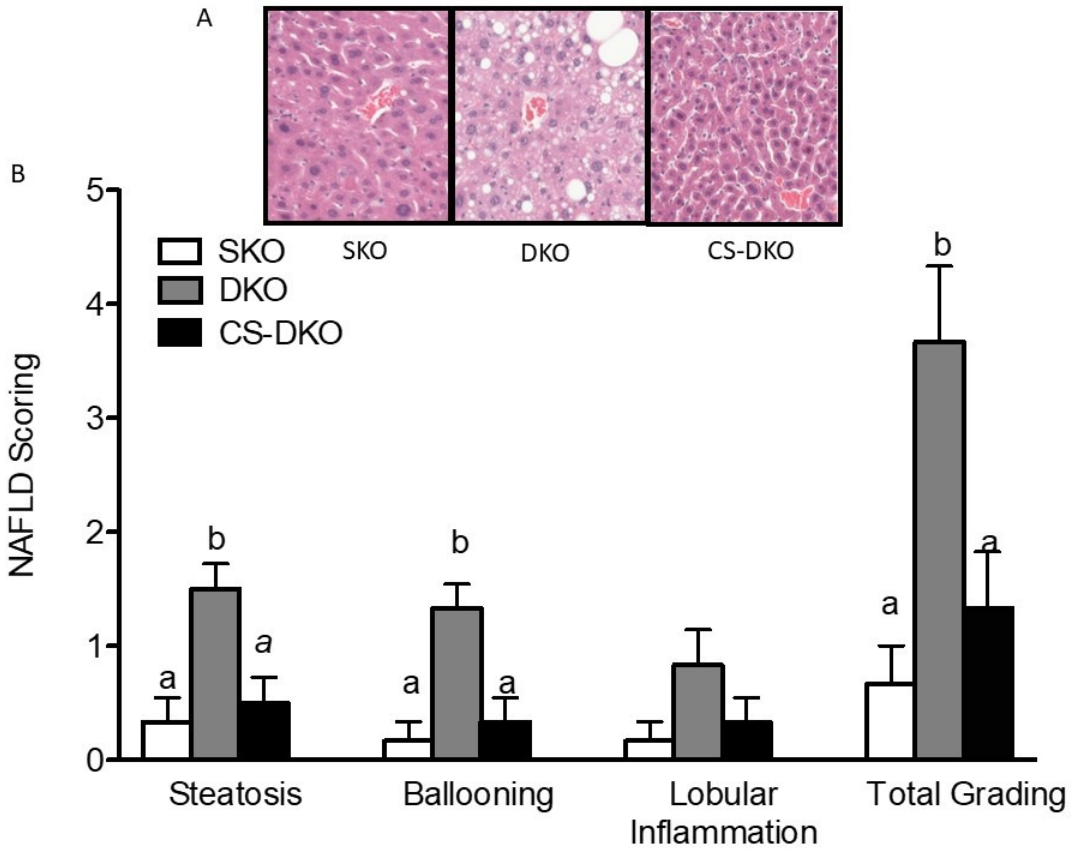


Figure 3-14 Choline supplementation attenuated NAFLD (A) Representative images of hematoxylin and eosin stained Liver histology from each group are shown with the (B) NAFLD histologic activity scores. Values are mean ± SEM, Groups without a common letter differ, *P<0.05. *Pemt*$^{+/+}$*/Ldlr*$^{-/-}$, SKO; *Pemt*$^{-/-}$*/Ldlr*$^{-/-}$, DKO. Choline supplemented DKO, CS-DKO

3.4.6 Choline supplementation did not significantly alter the gut microbiota in the DKO mice

The ability of certain strains of the gut microbes to metabolize choline to TMA appears to be linked to the development of atherosclerosis. Since choline supplementation increased plasma TMAO levels in DKO mice (Figure 4i), we investigated whether this was merely due to increased substrate availability or whether choline supplementation also induced alterations in gut microbiota composition that promote TMA and subsequent

TMAO production. We performed 16S rRNA gene sequencing on feces to study the taxonomic profiles including diversity and composition of the microbiota (Appendix Figure 7-2). Principle coordinate analysis (PCoA) of bacterial beta diversity using Bray-curtis metrics indicated that overall microbial composition was significantly different between the SKO and DKO mice and between the SKO and CS-DKO mice. However, there was no significant different between the DKO mice and CS-DKO mice (**Figure 3-15**). We also analyzed bacterial richness by Chao1 and alpha diversity by Shannon index in the three groups and found no difference (Figure 3-15).

Relative abundance of bacterial genera that were significantly different between treatment SKO, DKO and CS-DKO mice are shown in (**Figure 3-16**). There were some significant differences between SKO and DKO mice, including a substantial increase in *Parasutterella* and *Akkermansia* and DKO mice, however, there were very few differences between DKO and CS-DKO mice. In summary, there were some changes in the bacterial composition due to the genetic background of the mice but not due to dietary choline supplementation. The increase in plasma TMAO upon choline supplementation is thus most likely a result of increased substrate availability.

.

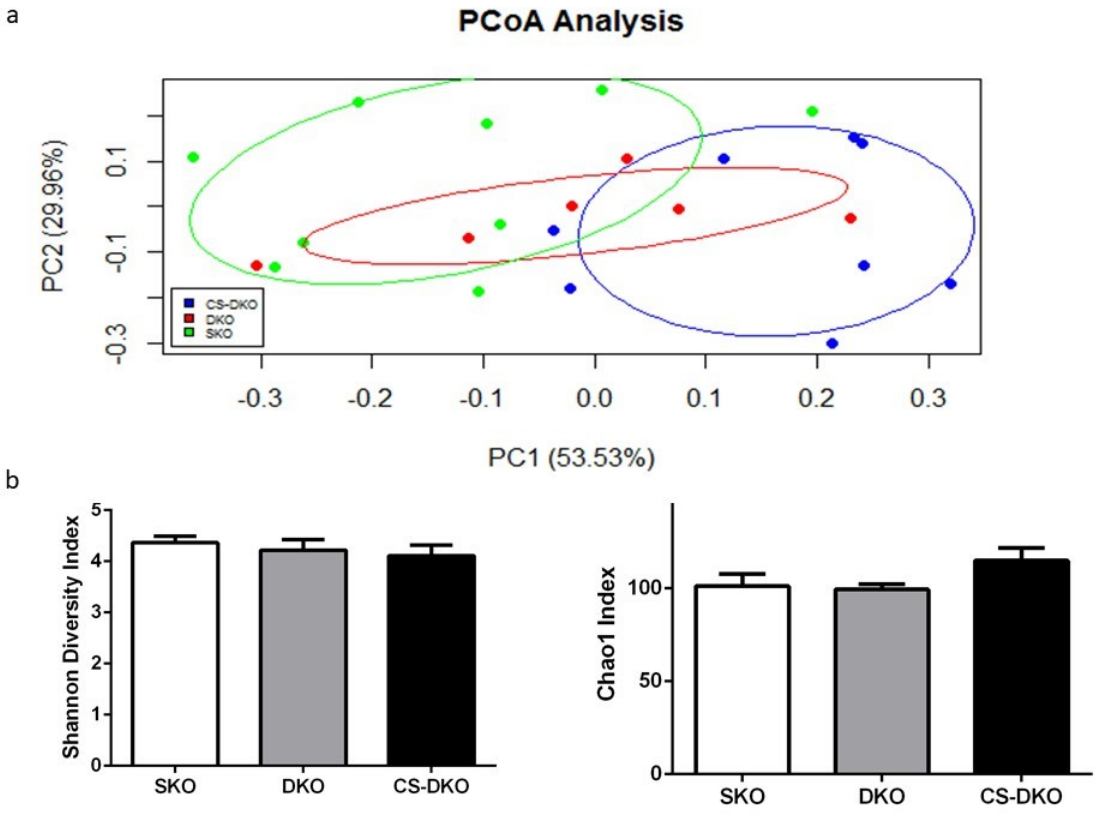
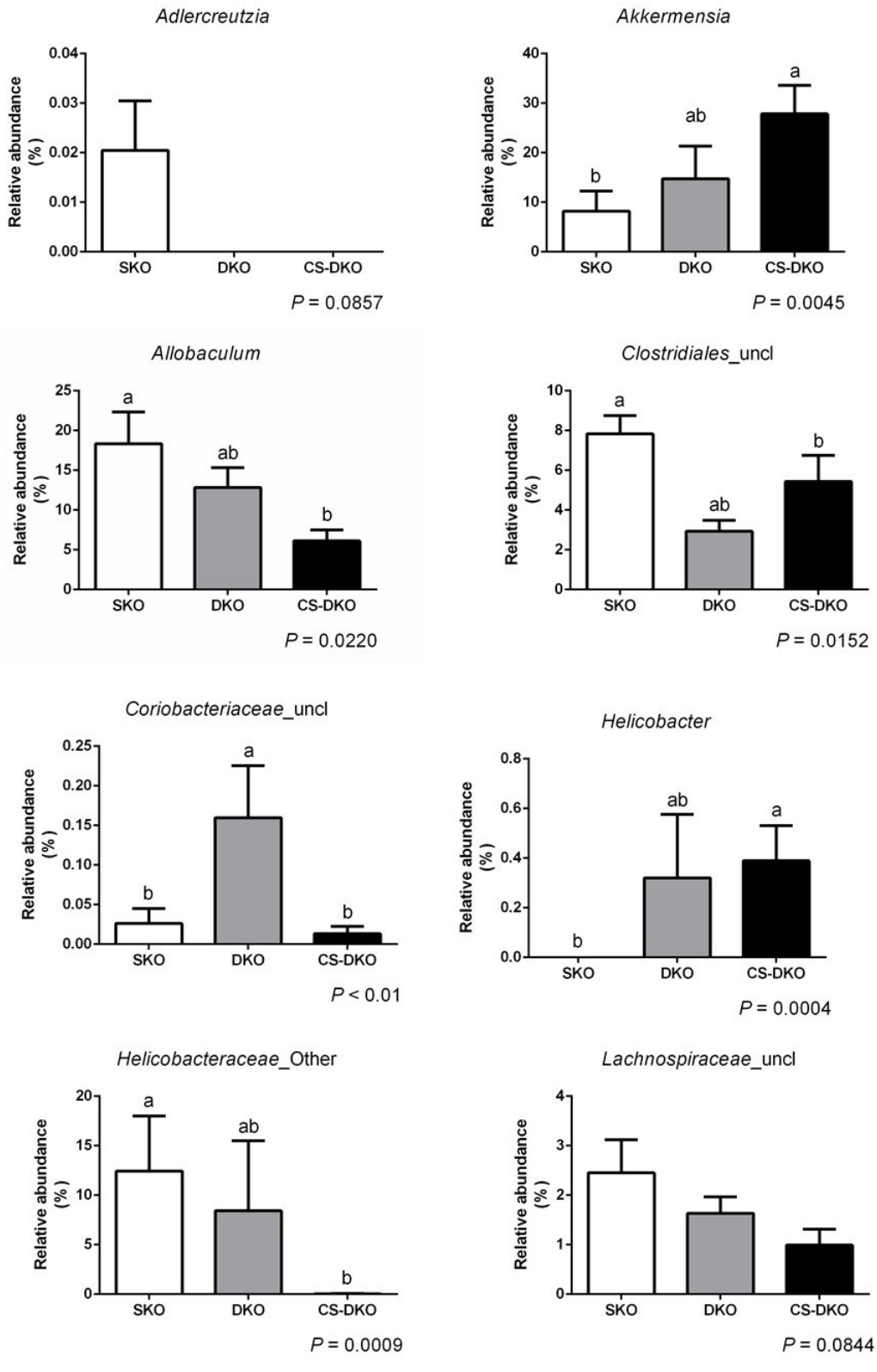


Figure 3-15 Choline supplementation did not alter the gut microbial diversity in DKO mice

(A) Principle Coordinates Analysis (PCoA) plots of the bacterial communities based on the Bray-Curtis dissimilarity. SKO were distinct from DKO based on PERMANOVA ($P< 0.05$) and CS-DKO did not differ from DKO. Each plot point represents an individual mouse. (B) Microbial alpha-diversity represented by the Shannon index and Chao1 index. Values are mean ± SEM, *Pemt*$^{+/+}$*/Ldlr*$^{-/-}$, SKO; *Pemt*$^{-/-}$*/Ldlr*$^{-/-}$, DKO. Choline supplemented DKO, CS-DKO


Adlercreutzia
Relative abundance (%)
0.04
0.03
0.02
0.01
0.00
SKO
DKO
CS-DKO
P = 0.0857
Akkermensia
Relative abundance (%)
40
30
20
10
0
a
ab
b
SKO
DKO
CS-DKO
P = 0.0045
Allobaculum
Relative abundance (%)
25
20
15
10
5
0
a
ab
b
SKO
DKO
CS-DKO
P = 0.0220
Clostridiales_uncl
Relative abundance (%)
10
8
6
4
2
0
a
ab
b
SKO
DKO
CS-DKO
P = 0.0152
Coriobacteriaceae_uncl
Relative abundance (%)
0.25
0.20
0.15
0.10
0.05
0.00
b
a
b
SKO
DKO
CS-DKO
P < 0.01
Helicobacter
Relative abundance (%)
0.8
0.6
0.4
0.2
0.0
b
ab
a
SKO
DKO
CS-DKO
P = 0.0004
Helicobacteraceae_Other
Relative abundance (%)
20
15
10
5
0
a
ab
b
SKO
DKO
CS-DKO
P = 0.0009
Lachnospiraceae_uncl
Relative abundance (%)
4
3
2
1
0
SKO
DKO
CS-DKO
P = 0.0844

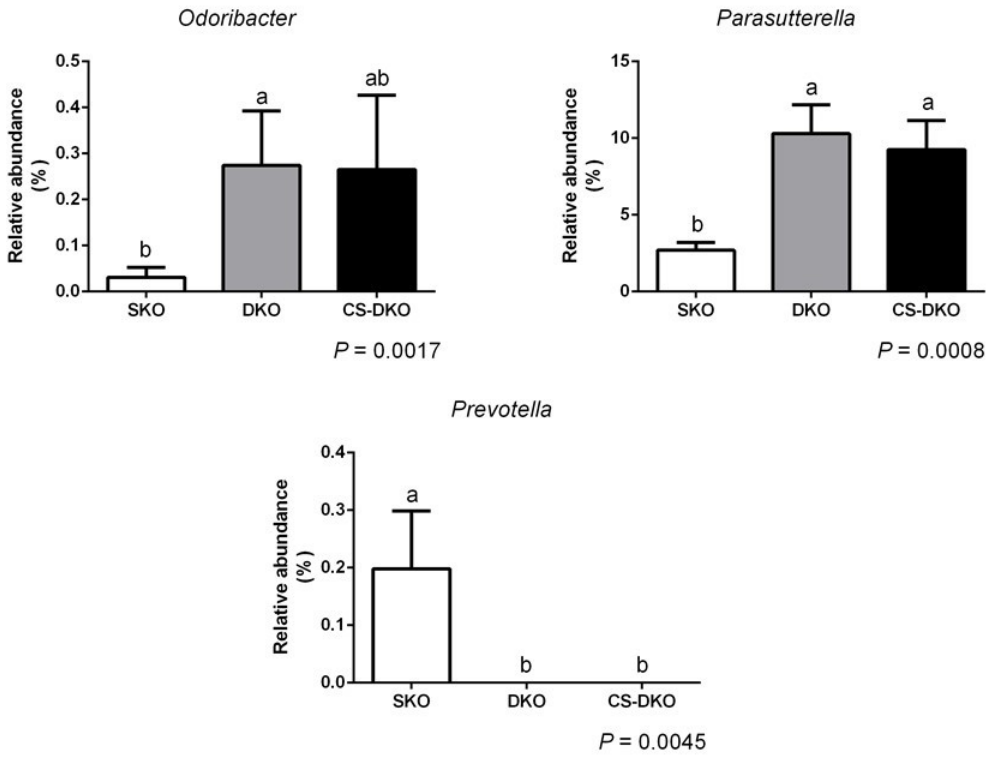


Figure 3-16 Relative abundance of bacterial genera that were significantly different between SKO, DKO, and CS-DKO mice. Data are shown as mean ± SEM. a,b,c Means that do not share a common letter are significantly different $p<0.05$. *Pemt*$^{+/+}$*/Ldlr*$^{-/-}$, SKO; *Pemt*$^{-/-}$*/Ldlr*$^{-/-}$, DKO. Choline supplemented DKO, CS-DKO

3.5 Discussion:

This study investigated the role of both dietary intake and *de novo* synthesis of choline in the development of atherosclerosis. We demonstrated that Pemt deficiency results in low plasma TMAO, which may attribute towards the atheroprotective phenotype of the DKO mice. Restoring *de novo* choline synthesis, by restoring hepatic Pemt expression, eliminated the atheroprotective phenotype most likely through an increase of both plasma cholesterol and TMAO while choline supplementation did not, despite

increase in TMAO levels. Taken together, an increase in plasma cholesterol, rather than TMAO, seems to increase atherosclerotic plaque formation.

The PEMT pathway is the only known pathway for *de novo* choline synthesis. Therefore, DKO mice have impaired *de novo* choline synthesis, which results in low choline status in chow-fed conditions. When fed a western diet, DKO mice are protected against atherosclerosis, most likely due to altered lipoprotein metabolism. DKO mice have reduced hepatic VLDL secretion, which results in a significant decrease in plasma lipids. Additionally, the phospholipid composition of lipoproteins is altered in DKO mice, which enables clearance at a faster rate compared to SKO mice[68]. Although low LDL-cholesterol levels in DKO is obviously important, altered choline metabolism may also contribute to the protection against atherosclerosis. Recent studies have shown an association between dietary choline and atherosclerosis in both animal studies and human cohorts[98,109,113,152]. It has been shown that unabsorbed dietary choline entering the large intestine is metabolized to the methylamine, TMA, by gut microbiota[153]. TMA is oxidized in the liver to TMAO and is released into circulation and excreted by the kidney in urine. Recent publications have suggested that TMAO is as a novel biomarker of atherosclerosis[103,105]. The mechanism of how TMAO promotes atherogenesis remains ambiguous; however, evidence suggests that TMAO is linked to increase in cholesterol uptake by macrophages, resulting in an increase in foam cells in the intima of the arterial walls and a reduction in reverse cholesterol transport[105]. It is also suggested to play a role in platelet aggregation[109], complex bile acid metabolic pathways via FMO3 expression[91,122,154] and renal function[113,155]. Moreover, numerous cohort studies demonstrated a significant correlations of plasma TMAO and increased risk of atherosclerosis, stroke and death in patients

undergoing cardiac procedure independent of traditional risk factors (e.g. high cholesterol)[33–35].

Recent literature emphasizes that *dietary* choline is metabolized by gut microbiota to TMA and that the resulting TMAO is associated with increased risk for CVD[102,105,135,136]. However, the current study demonstrates that chow-fed mice lacking *de novo* choline synthesis also have low TMAO levels. This indicates that diet-microbe interaction may not be the only regulator of TMAO production. It is possible that less choline is reaching the distal part of the intestine because of increased absorption in the proximal region of the intestines or because of decreased biliary PC secretion in the DKO mice, and therefore less choline would be available in the gut of DKO mice to be metabolized to TMA.

Restoring hepatic PEMT expression via AAV ameliorated the protection from atherosclerosis in DKO mice. The data clearly shows that hepatic PEMT activity is a determinant of atherosclerosis in mice. To the best of our knowledge, this is the first experiment to show that *de novo* choline synthesis modulates TMA and TMAO levels. It is unclear whether modulating hepatic PEMT influences bile secretion[156]. When fed a western diet, the DKO mice may have decreased PC reaching the bile and ultimately the gut, which can contribute to the low TMA and TMAO levels. Restoring the PEMT enzyme would possibly increase biliary PC reaching the microbiota, which could then be metabolized to TMA. In this experiment TMAO was positively correlated with cholesterol and atherosclerosis. Thus, it possible that changes in both TMAO and cholesterol play a role in the atheroprotective phenotype of DKO mice. It is noteworthy that when we included cholesterol to the multivariable regression analysis, it eliminated the significant relationship between atherosclerotic lesions and plasma TMAO (p value increased to 0.09).

Our next objective was to determine whether dietary choline supplementation reverses the atheroprotective phenotype in the DKO mice. We found that choline supplementation failed to increase VLDL secretion, or fasting plasma cholesterol or triglycerides in the DKO mice, whereas it did increase plasma TMAO levels. Interestingly, choline supplementation did not influence plaque formation in DKO. This is most likely because plasma cholesterol was not elevated in these mice. Thus, TMAO may be atherogenic only in a hypercholesterolemic state as observed in ApoE$^{-/-}$ mice[105] or in the AAV experiment, when the DKO mice became hypercholesterolemic. It is noteworthy that levels of TMAO observed in our study are low compared to other studies[98,103]. Additionally, we used male mice in our study, whereas others have utilized both genders and have demonstrated that female mice have much higher FMO3 expression and hence increased TMAO production upon choline supplementation[98,154]. It is also well-established that female mice have higher PEMT activity compared to their male counterparts[157]. Moreover, it is unclear why there was no difference in TMAO levels between the SKO and DKO mice in the choline supplementation study (Figure 4h). It is possible that the longer feeding (12 weeks) could influence plasma choline and TMAO levels.

Another interesting finding was that an increase in dietary choline did not increase plasma choline levels in DKO mice, suggesting that fasting plasma choline is not good indicator of choline status. In a choline-deficient state as observed in DKO mice, choline gets conserved by decreasing hepatic choline oxidation to betaine, increasing cellular choline uptake, and increasing the CDP-pathway to ensure sufficient PC supply. The liver acts like a choline sink readily clearing choline from the circulation[158] This may explain the low plasma choline and other metabolites observed in DKO mice on chow diet and no

increase in plasma choline upon choline supplementation in the DKO mice. Moreover, studies have shown plasma choline is relative unresponsive to changes in dietary choline intake[159,160].

Choline deficiency in various animal species manifests with many abnormalities, most prominent of which is fatty liver and liver dysfunction[40]. Low PC results in impaired VLDL secretion and triglycerides accumulate in the liver. In severe choline-deficient states, it can alter the PC/PE ratio as observed in PEMT-deficient mice. Choline supplementation attenuated NAFLD, evident by improved liver histology and decreased hepatic triglycerides, without improving PC/PE ratio. It also did not affect VLDL secretion but reduced hepatic TG levels. The mechanism by which dietary choline improved the liver is unclear. Hepatic TG has three major fates: secretion into lipoproteins, lipolysis and oxidation to provide energy to cells, or storage in lipid droplets. We hypothesize that choline supplementation resulted in increased lipolysis and fatty acid oxidation in the liver. Similar findings have been observed in heterozygous *Pcyt2*$^{+/-}$ mice that have impaired CDP-ethanolamine pathway due to absence of CTP:ethanolaminephosphate cytidylyltransferase. *Pcyt2*$^{+/-}$ mice develop steatosis due to reduced hepatic PE synthesis, however upon choline supplementation, these mice had increased TG hydrolysis in the liver, increased expression of genes involved in mitochondrial fatty acid oxidation, and reduced fatty acid synthesis[161].

Over the past decade, there has been increasing interest in characterizing and linking the gut microbiota to the development of metabolic diseases[120]. Since choline is metabolized by the gut microbiota, we wanted to investigate whether choline supplementation would alter the gut microbiota composition and increase TMA-producing

bacteria. There was a significant difference in the relative abundance of some genera between the SKO and DKO mice. However, choline supplementation did not alter the relative abundance of different strains indicating that choline had little impact on microbial composition. Increases in TMAO production in CS-DKO mice is not explained by changes in known TMA producing bacteria[53]. This is in agreement with Romano et al., who demonstrated that the relative abundance of TMA-producing bacterial strains does not change in choline-deficient and choline-supplemented mice in a low complexity gut microbial consortium[53]. Lastly, we did observe that CS-DKO mice had more *Akkermensia* compared to SKO mice, which has been shown to be atheroprotective because of its ability to restore the gut barrier in ApoE$^{-/-}$ mice[125].

3.6 Conclusion:

Pemt$^{-/-}$*/Ldlr*$^{-/-}$ mice have impaired hepatic VLDL secretion and are protected against atherosclerosis. The role of TMAO in the development of atherosclerosis in these animals was investigated by increasing either dietary intake or PEMT-mediated *de novo* synthesis of choline. Both treatments resulted in an increase in plasma TMAO, whereas only the increase in PEMT concomitantly increased plasma cholesterol and enhanced atherosclerotic plaque. Our data suggests that plasma TMAO does not influence atherosclerosis when VLDL secretion and plasma cholesterol is low. Future work will investigate how *de novo* choline synthesis plays a role in TMAO metabolism.

4 Chapter 4

Title: Switching from high choline to a low choline diet attenuates the progression of atherosclerosis in Ldlr$^{-/-}$ mice

4.1 Introduction

Choline is nutritional component of the diet necessary for fetal brain development, synthesis of neurotransmitters, cell membranes, lipid transport and cell signaling[39]. Deficiency of choline rapidly leads to accumulation of hepatic TG accumulation resulting in fatty liver in most mammals including dogs, rabbits, rats, mice and humans[37]. A group in Cleveland clinic, Ohio demonstrated a unique link between dietary PC and atherosclerosis. Using an unbiased metabolomics approach, Hazen and colleagues identified three metabolites of dietary PC: namely choline, betaine, TMAO as novel predictors of CVD and atherosclerosis in human[105]. Mechanistic studies demonstrated that *Apoe*$^{-/-}$ mice, mouse model of atherosclerosis, fed a choline-supplemented chow diet had an increase in plasma choline, betaine and TMAO levels, and developed enhanced atherosclerosis[105]. In addition, these studies found that gut flora played a key role in conversion of choline to TMA. Elimination of gut flora by antibiotics reduced atherosclerosis and plasma TMAO[98].

A cohort study involving 4000 cardiac patients undergoing a cardiac procedure had plasma TMAO measured and divided into quartiles. After adjustment with all traditional risk factors, the 4th quartile having the highest level of TMAO exhibited the highest incidence of myocardial infarction, stroke and death. Clinical cohort studies have also demonstrated strong association of plasma TMAO with increased risk of coronary heart disease, coronary intimal thickness and cardiac event in patient populations[102,104,154].

Hence, numerous cohort studies have demonstrating plasma TMAO to be highly predictive of CVD risk. In contradiction, there is evidence suggesting that despite increase in TMAO production upon a choline consumption, or a high choline meal, the TMAO levels fall to normal levels within a few hours after a meal[162–164].

We had previously investigated the effect of choline supplementation in the DKO mice. There was no increase in atherosclerosis upon choline supplementation in the DKO mice, despite a 2.5-fold increase in plasma TMAO levels (Refer to Chapter: 3.5). We figured, it was likely due to the genotype of the DKO mice (*Pemt*$^{-/-}$/*Ldlr*$^{-/-}$), which is protected from development of atherosclerosis. Hence, we wanted to investigate the effect of high choline diet on the development of atherosclerosis in the atherogenic background of *Ldlr*$^{-/-}$ mice. The *Ldlr*$^{-/-}$ mice are like *Apoe*$^{-/-}$ mice in the way that both mice models have hypercholesterolemia with similar atherosclerotic lesions. However, the *Ldlr*$^{-/-}$ mice require a high diet/ high cholesterol diet to develop atherosclerosis while *Apoe*$^{-/-}$ mice develop atherosclerosis spontaneously even on a chow diet.

The main objective of this study was to determine whether **decreasing** dietary choline reduces the progression of atherosclerosis in *Ldlr*$^{-/-}$ mice. All *Ldlr*$^{-/-}$ mice were fed a high fat/high choline diet (60% calories from fat, 1% cholesterol, 10g/kg choline) for 8 weeks (HC^{8wks}) or 16 weeks (HC^{16wks}). However, one group of *Ldlr*$^{-/-}$ mice was switched to a high fat/low choline diet after 8 weeks (HC^{8wks}+LC^{8wks}). We found HC^{16wks} mice demonstrated significant increase in atherosclerotic lesions compared to HC^{8wks} mice. Decreasing choline in the diet after 8 weeks of high fat/high choline diet ameliorated the progression of disease process in HC^{8wks}+LC^{8wks} mice compared to HC^{16wks} mice independent of plasma lipids. Interestingly, HC^{16wks} mice had a 70% reduction in plasma

TMAO compared to HC^{8wks} mice, decreasing dietary choline also had a 70% reduction in plasma TMAO compared to HC^{8wks} mice. This implied that TMAO status is influence by age of the animal and/or length of dietary feeding.

4.2 Methodology

The details on materials and methods used in this experiment can be found in chapter 3.2 of this thesis.

4.2.1 Study Design

The purpose of the experiment was to determine whether decreasing dietary choline attenuated the progression of atherosclerosis. Hence, *Ldlr*$^{-/-}$ mice were fed a high fat /high choline diet (60% calories from fat+1% cholesterol+ 10g/kg choline) for 8 weeks (HC^{8wks}) or 16 weeks (HC^{16wks}) while a second group was switched from 8 weeks of the same diet to a high fat/low choline diet (60% calories from fat+ 1% cholesterol+ 0.5g/kg choline) for 8 more weeks (HC^{8wks}+LC^{8wks}) The aim of euthanizing HC^{8wks} mice at 8 weeks was to establish baseline characteristics (Figure 4-1), as such the results are stated and discussed as a comparison between the two HC^{8wks}+LC^{8wks} and HC^{16wks} mice.

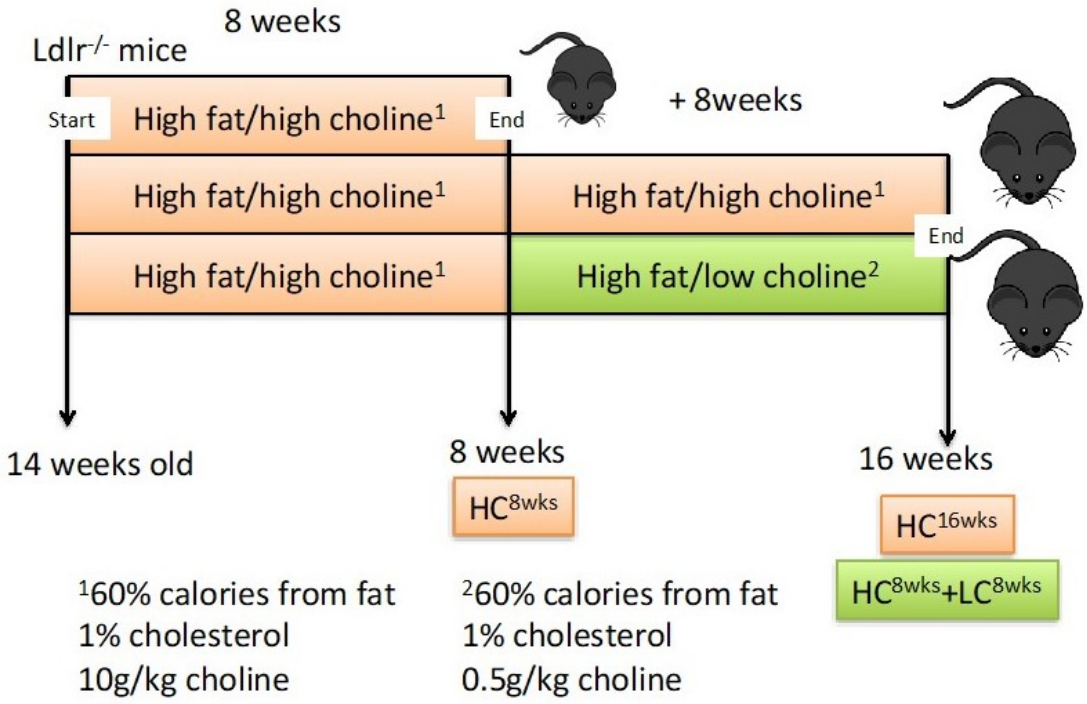


Figure 4-1 Study Design: The *Ldlr*$^{-/-}$ mice aged 14 weeks were fed a high fat/high choline diet for 8 weeks. HC^{8wks} was ended at 8 weeks. HC^{8wks}+LC^{8wks} group was switched to a low choline diet for 8 weeks while HC^{16wks} was continued on the same diet for 8 weeks.

4.2.2 Diet

The basal diet composition is summarized in Table 4-1 below. The amount of choline bitartrate was adjusted in the groups

Table 4-1: The composition of 60% High Fat Diet

Ingredients (g/kg)	High Fat/High Choline (g/kg)	High Fat/Low Choline (g/kg)
Casein	270	270
Corn Starch (Safeway)	244	244
Sucrose	126	126
Vitamin Mix (AIN-93-Vx) (Harlan TD)	19	19
Mineral Mix (Usual)	50	50
Calcium Phosphate Dibasic	3.4	3.4
Inositol	6.3	6.3
Cellulose	80	80
L-cystine	1.8	1.8
Choline Bitartrate	**10**	**0.5**
Flax Oil	7	7
Olive Oil (Safeway)	48	48
Sunflower Oil (Safeway)	67	67
Crisco canola	78	78
DHAsco	1.5	1.5
Arasco	1.5	1.5
Cholesterol	10	10

4.3 Results

Switching from high to low choline diet retards the progression of atherosclerosis independent of plasma cholesterol in Ldlr$^{-/-}$ mice

Decreasing dietary choline did not alter weight gain in HC^{8wks}+LC^{8wks} mice compared to HC^{16wks} mice (Figure 4-2). Plasma cholesterol levels in HC^{16wks} mice were similar to HC^{8wks}+LC^{8wks} mice. As was expected, HC^{8wks} mice had significantly less weight gain and plasma cholesterol compared to the 16-week diet fed groups. Next, quantification of atherosclerotic lesions revealed HC^{16wks} mice experienced a 4-fold increase in

atherosclerotic lesions compared to HC^{8wks} mice. Decreasing choline resulted in a 25% reduction in atherosclerotic lesions in HC^{8wks}+LC^{8wks} mice compared to HC^{16wks} mice.

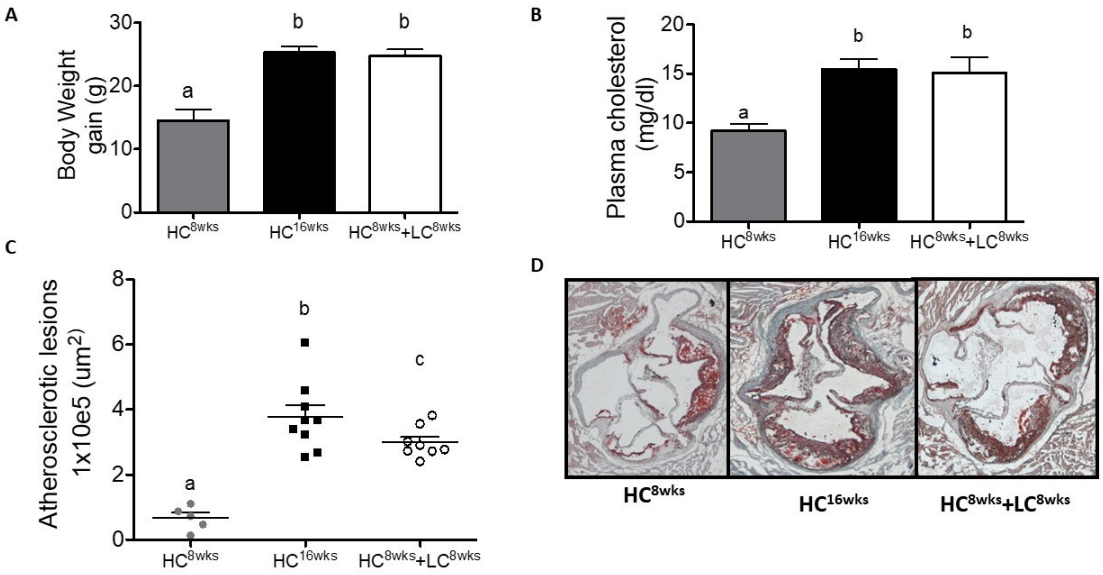


Figure 4-2 Decrease in dietary choline reduced the progression of atherosclerosis:

Weight gain calculated as final weight - initial weight (Starting Weight before the dietary intervention) (B) fasting plasma cholesterol (C) Quantification of mean lesion area from en face preparations of aortic arch was performed using Image J. Each dot represents 1 mouse (D) Representative image of Oil red O staining of aorta. All mice were $Ldlr^{-/-}$ mice fed a 60% HFD+ 1% cholesterol with either 10g/kg choline or 0.5g/kg choline. HC^{8wks} mice are fed 8 weeks of high fat/high choline diet(10g/kg choline). HC^{16wks} mice are fed 16 week of high fat/high choline diet (10g/kg choline), HC^{8wks}+LC^{8wks} mice are fed 8 weeks of high fat/high choline then 8 weeks of high fat/low choline diet (0.5g/kg choline)

Substituting to a low choline diet did not alter plasma choline, betaine, TMA and TMAO

Next, we wanted to investigate the effect of dietary intervention on plasma choline and its metabolites. Switching to a low choline diet did not significantly alter plasma choline and betaine levels in HC^{8wks}+LC^{8wks} mice compared to HC^{16wks} mice (Figure 4-3). Choline is metabolized by the gut microbiota to TMA and is oxidized by hepatic FMOs to TMAO. Interestingly, there was 70% reduction in plasma TMA and TMAO levels in the HC^{16wks} mice and HC^{8wks}+LC^{8wks} mice compared to HC^{8wks} mice (Figure 4-3 C and D). Hence decreasing choline did not significantly alter concentrations of choline, betaine, TMA and TMAO in the plasma.

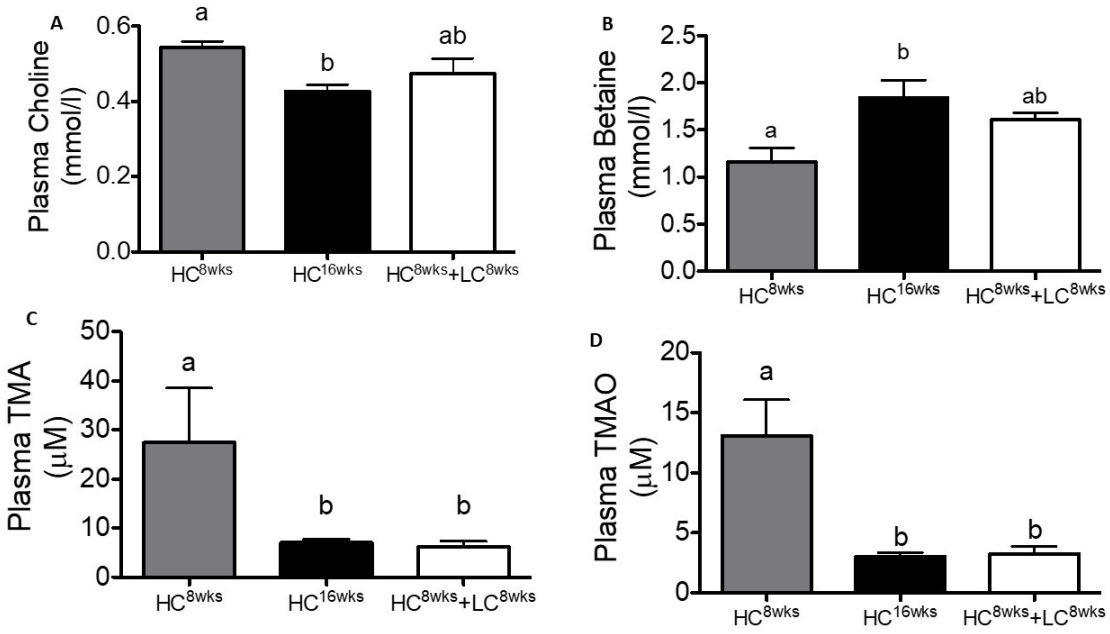


Figure 4-3 Switching to a low choline diet did not alter plasma choline and metabolites (A) Fasting plasma choline, (B) Betaine (C) TMA and (D) TMAO. Values are mean ± SEM, Groups without a common letter differ, *P<0.05

Switching from high to low choline diet did not significantly alter hepatic lipid profile

We wanted to study the effect of lowering dietary choline on hepatic lipid levels. The hepatic TG and CE levels almost doubled in HC^{16wks} mice compared to HC^{8wks} mice. (Figure 4-4 A and C). Hepatic TG and CE levels were not different between HC^{8wks}+LC^{8wks}mice compared to HC^{16wks} mice. There was a 40% increase in hepatic free cholesterol in HC^{16wks} mice compared to HC^{8wks} mice (Figure 4-4 B). Surprisingly, decreasing dietary choline decreased FC levels in HC^{8wks}+LC^{8wks} mice to baseline levels of HC^{8wks} mice.

PC and PE are the major phospholipids of the membrane and PC/PE ratio is good indicator of membrane integrity[67]. We found, that hepatic PC levels were not significantly different among the three groups. Switching to a low choline diet did not significantly alter hepatic PE levels and the PC/PE ratio was similar between the 16 weeks diet-fed mice (Figure 4-4 D and E).

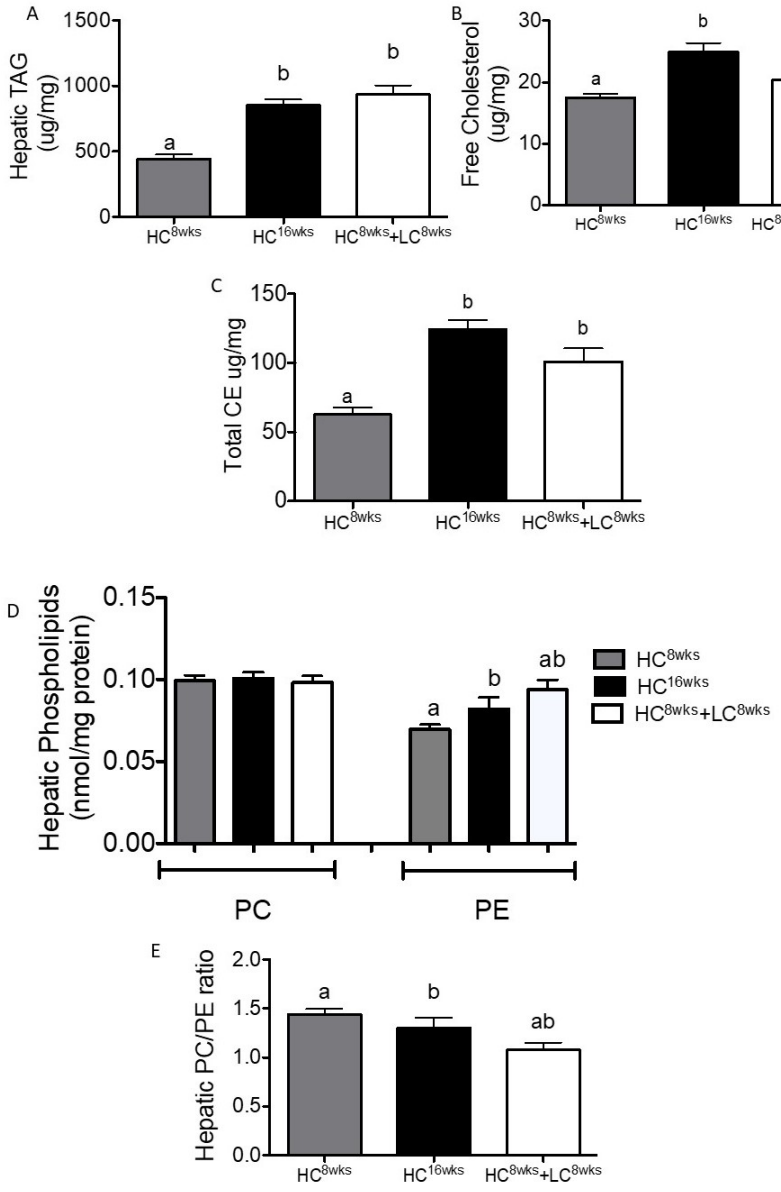


Figure 4-4 Decrease in choline decreased hepatic free cholesterol in HC^{8wks}+LC^{8wks} mice

Liver TG, Free cholesterol and CE in HC^{8wks}, HC^{16wks}, HC^{8wks}+LC^{8wks} mice, showing hepatic lipid concentrations (A-C). For phospholipid measurement, Lipids were extracted from liver (1 mg protein) and measured using folch method (D)hepatic PC and PE (E) PC and PE ratio. Values are mean ± SEM, Groups without a common letter differ, *P<0.05

All *Ldlr*$^{-/-}$ mice exhibited NAFLD

HC^{8wks} mice had lipid accumulation and an elevated NAFLD score, which was not significantly different at 16 weeks of the same diet (HC^{16wks}). Switching to a low choline diet did not change the NAFLD scoring (Figure 4-5). The kidney readily secretes TMA and TMAO in the urine. Studies have demonstrated an association of elevated plasma TMAO with renal dysfunction[155,165]. Renal histology demonstrated that all mice demonstrated significant ischemia. Notably, the 16-week fed groups appeared worse compared to HC^{8wks} mice. However, it did not reach statistical significance. Furthermore, there appeared to be no association of renal damage with TMAO levels (Figure 4-5).

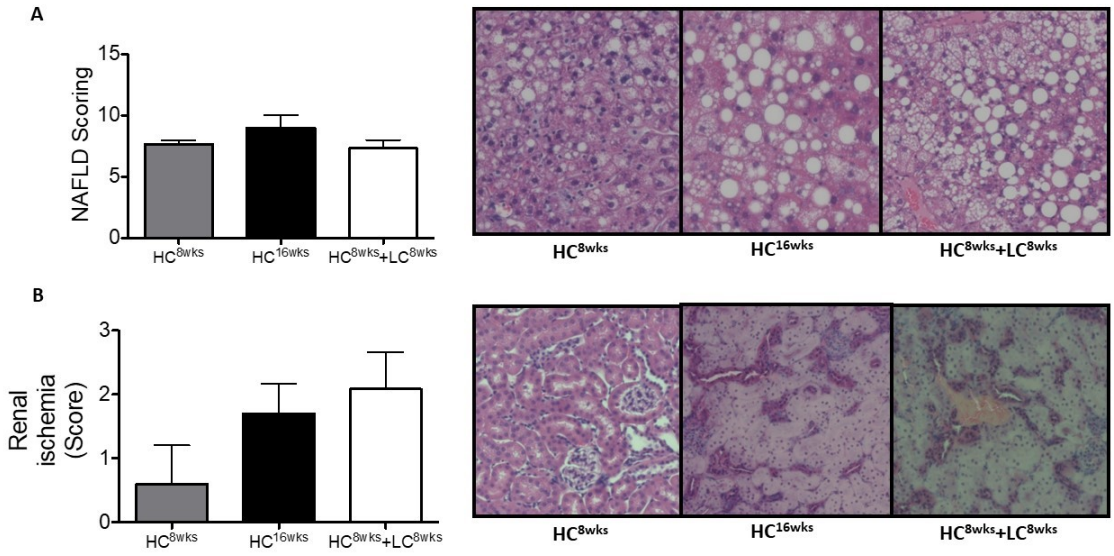


Figure 4-5 All *Ldlr*$^{-/-}$ mice exhibited NAFLD and renal pathology on high fat diet (A) NAFLD score based on the liver histology (B) Renal ischemic score based on renal histology (See Appendix for scoring criteria). Both tissue sections were stained using Hematoxylin and eosin stain.

4.4 Discussion

The purpose of this pilot project was to determine whether decrease in dietary choline ameliorates the progression of atherosclerosis. In the current study, we fed *Ldlr*$^{-/-}$ mice a high fat/high cholesterol diet supplemented with 10g/kg choline. Others have demonstrated that *Apoe*$^{-/-}$ mice fed chow diet supplemented with 1% choline (10g/kg choline) causes increase in atherosclerosis with concomitant increase in TMAO levels[105,106]. The metabolism of choline by the gut microbiota to TMA and subsequently to TMAO has been viewed as a pathogenic pathway. The mechanism of how TMAO promotes atherogenesis is unclear but evidence suggests TMAO is associated with increase in foam cell formation, decrease in HDL cholesterol[105] and increase in platelet hyperactivity[109]. Moreover, it is well established that choline is important for VLDL secretion from the liver, hence choline potentially contributes towards increase in plasma lipids[77].We found that a 95% reduction in dietary choline (from 10g/kg to 0.5g/kg choline) did not decrease plasma cholesterol but demonstrated a decrease in the progression of atherosclerosis. This decrease may be due to a reduction in ApoB particles. Studies have demonstrated that mice fed a choline-deficient diet have reduction in apo B100 and B48 compared to mice fed a choline supplemented diet for 21 days[166]. Most ApoB containing lipoproteins (LDL, Remnants of VLDL and CM) are widely accepted causal agents of atherogenesis[167].

We also hypothesized that decrease in dietary choline would decrease progression of atherosclerosis possibly by decreasing plasma TMAO levels. Unexpectedly, we found that *Ldlr*$^{-/-}$ mice at 8 weeks of high fat/high choline diet had average plasma TMAO concentration of 13μM, which decreased to 3μM at 16 weeks of the same diet. Moreover, similar TMAO levels were observed in mice that had been switched to a low choline diet.

Hence independent of choline content of the diet, there was 76% decrease in TMAO levels in both groups fed for 16 weeks. A plausible explanation could be that prolonged high fat diet may have resulted in alterations in the microbial diversity in the gut, a phenomenon which has been shown by others[168,169]. This may have resulted in a reduction in choline consuming-TMA-producing bacteria. A study demonstrated germ free mice when transplanted with "core microbiota" lacking TMA producing species had no increase in TMAO levels confirming that the *intestinal microbiota* plays an important role in modulating choline bioavailability and accumulation of TMAO in the plasma[53].

There was an increase in plasma betaine in HC^{16wks} mice compared to HC^{8wks} mice, since plasma choline is maintained in homeostatic balance by several adaptive mechanism, one of which is increasing choline oxidation to betaine[55]. Decreasing choline did not significantly alter plasma choline or betaine possibly because plasma choline remains relatively unresponsive dietary changes[170]. Moreover free choline is readily cleared from the plasma by hepatic uptake[158].

Hepatic histology demonstrated lipid accumulation and macrophage infiltration in all groups. It was not surprising that all mice developed NAFLD likely due to the high fat and cholesterol content of the diet. Likewise, renal histology demonstrated renal ischemia in all groups. The kidney readily secretes TMA and TMAO in the urine. Hence, kidney function is often considered a confounder for the total plasma TMAO level. Studies have demonstrated an association of elevated plasma TMAO with renal dysfunction[155,165]. However, in the current study the plasma TMAO levels did not correlate with the renal damage.

In conclusion, switching from high to a low choline diet may attenuate atherosclerosis. More precisely, a 95% reduction in choline content of diet resulted in 20% reduction in atherosclerosis in *Ldlr*$^{-/-}$ mice. However, this attenuation is nominal and appears to be independent to plasma cholesterol and TMAO levels, suggesting that dietary choline may not be very important factor contributing towards atherogenesis. This implies that there are other factors influenced by choline that may be important contributors towards atherosclerosis. More work is required to elucidate the mechanism.

There are studies investigating the effect of dietary choline on intestinal lipid metabolism. Intestinal PC is crucial for CM secretion[43]. Chylomicrons, also called post prandial lipoproteins are considered pro-atherogenic[171]. Choline deficiency has been demonstrated to have impaired intestinal lipid metabolism in lactating rats with significantly reduced fasting plasma TG, cholesterol and ApoB levels in fasting and fed state[172]. Hence, there is plausible reason to believe that a decrease in dietary choline decrease CM secretion which may be contributing towards decrease in the progression of atherogenesis.

5 Overall Discussion

Collectively, this research investigated the role of choline metabolism in development of atherosclerosis. DKO mice are protected against atherosclerosis due to significant reduction in plasma lipids.

The primary objective of this project was to investigate the role of hepatic PEMT expression in the development of atherosclerosis, to determine if TMAO production was altered by PEMT, and whether TMAO influenced the atherosclerosis process. Upon restoring PEMT activity in the DKO mice, we observed a reversal in the atheroprotective phenotype with an increase in VLDL secretion, cholesterol and TMAO levels, demonstrating for the first time that *de novo* choline synthesis may be an important regulator of TMAO status.

Our second objective was to determine whether choline supplementation eliminated the atheroprotective phenotype of the DKO mice. We also wanted to investigate whether choline supplementation increased TMAO production and whether it influenced atherosclerosis. When DKO mice were fed a choline supplemented western diet, the mice maintained their atheroprotective phenotype with no increase in VLDL secretion and plasma cholesterol. Choline supplementation did increase TMAO levels in DKO mice, suggesting that in the presence of low lipids, TMAO may not influence atherosclerosis process.

The third objective of this thesis was to determine whether decreasing the choline content of the diet decreased the progression of atherosclerosis in *Ldlr*$^{-/-}$ mice and whether it decrease plasma TMAO levels. We found that decreasing dietary choline attenuated the

progression of atherosclerosis however; this decrease was independent of plasma cholesterol and TMAO levels.

5.1 Association of TMAO and CVD in mice and humans

Choline, usually in the form of PC is present in eggs and meat, is essential nutrient of diet and required for synthesis and secretion of lipoproteins, it is also vital for normal liver function[39]. Liver and intestine, both play a crucial role in contributing towards atherosclerosis[23]. While liver is the main contributor of LDL particles (derived from hepatic VLDL) which demonstrate a strong association with atherogenesis[9]; the CM particles secreted from the intestine are also considered pro-atherogenic[171,173]. Hence, the link that choline may be associated with CVD is consistent with this hypothesis (See Figure 5-1). However, recent literature is shedding light on a novel mechanism of choline's atherogenicity-via its metabolism by the gut flora[101,103,105,110]. Hazen's group was the first to demonstrate that *Apoe*$^{-/-}$ mice fed a choline-supplemented diet exhibited exacerbated atherosclerosis with elevated TMAO levels[105]. The gut microbiota metabolizes choline to TMA, which gets oxidized by hepatic FMO3 enzymes to TMAO, a metabolite that promotes foam cell formation, decrease in HDL cholesterol[98,103] and promotes platelet aggravation[109]. The link between TMAO and increased risk for CVD has also been supported by numerous cohort studies[99–101,134].

This thesis demonstrated that although dietary choline or *de novo* choline synthesis (via PEMT expression) increased TMAO levels, it did not always correlate with atherosclerosis, particularly when plasma cholesterol levels were low as observed in DKO mice (**Figure 3-11**). In fact, the correlation existed only when plasma cholesterol was also high (Figure 3-7).

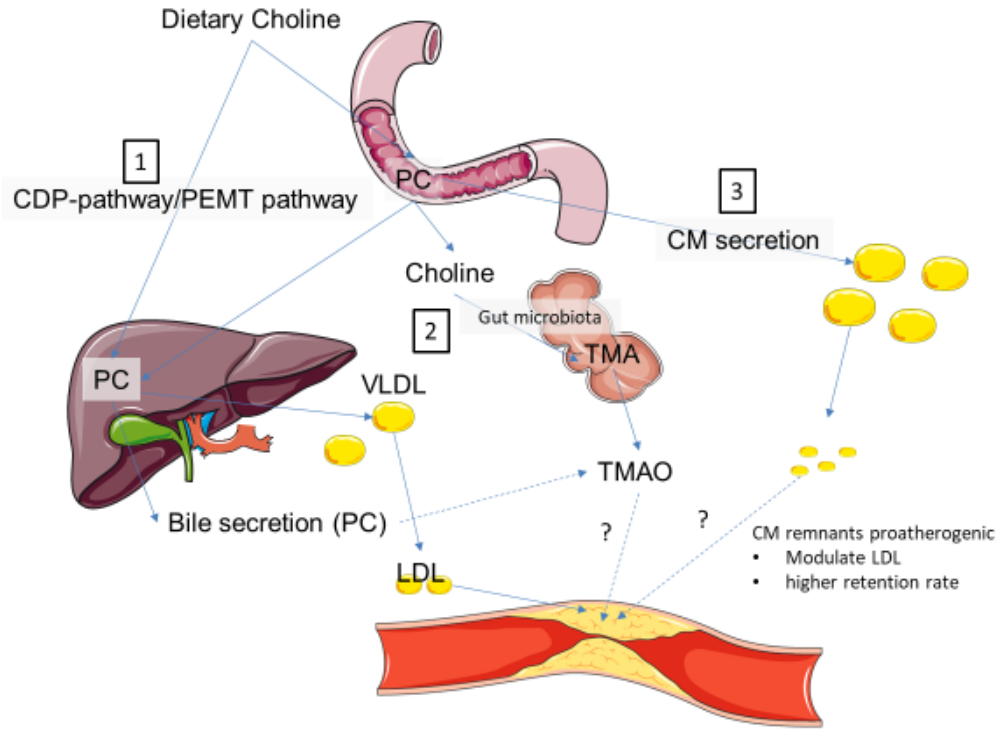


Figure 5-1 Role of choline in the development of atherosclerosis via three main mechanisms: hepatic lipid metabolism, intestinal microbial metabolism and intestinal lipid metabolism. This figure was created in part using illustrations from ©Servier medical art "With Permissions".

5.2 Atheroprotective phenotype of DKO mice

It is well established that the DKO mice are protected against atherosclerosis primarily due to impaired VLDL secretion from the liver. Impaired VLDL secretion results in significant reduction in plasma lipids[68,89]. We also know that the DKO mice have altered phospholipid composition of the VLDL particles secreted, that enables their clearance at a faster rate[68]. Furthermore, the DKO mice have decrease in plasma homocysteine which is an independent risk factor of atherosclerosis[68,89]. From current research work, we found that DKO mice also had a low TMAO status, rendering another possible atheroprotective feature to the DKO mice. When the Pemt enzyme was restored, plasma lipids and TMAO

levels were increased, eliminating the protection against atherosclerosis. Thus, *de novo* choline synthesis appears to be positively correlated with plasma TMAO levels. Moreover, plasma TMAO and atherosclerosis correlated significantly in this experiment, as has been observed in numerous cohort studies[100,102,105,114,117,134].

5.3 Choline supplementation increased TMAO, but not atherosclerosis in DKO mice

When DKO mice were supplemented with choline there was a 2.5-fold increase in TMAO production. There was no increase in hepatic VLDL secretion, plasma cholesterol and TG level. Hence, plasma TMAO did *not* correlate with atherosclerosis suggesting the correlation between TMAO and atherosclerosis is not significant in the presence of low lipids. Since choline is metabolized by the gut microbiome, it was expected that increase in dietary choline would cause alterations in the gut flora.

A recent publication demonstrated when germ-free mice were colonized with microbiota containing fewer TMA-producing species, there was a decrease in plasma TMAO[53]. Moreover, they observed that choline bioavailability, represented by elevations in serum choline, was higher in mice containing less TMA-producing microbes[53]. Hence, they concluded that higher TMAO concentrations, indicated low choline bioavailability. Although, evidence suggests that plasma choline is poor measure of choline bioavailability or of dietary choline, it is possible that CS-DKO mice have low choline bioavailability due to presence of TMA-producing microbes in the gut. However, our microbiota analysis did not identify any of the known TMA producing microbes that are identified in literature. Moreover, there was no significant change in diversity or the relative abundance of microbiota upon choline supplementation in the DKO mice. Hence, our study demonstrated

that ***gut microbiota*** is independent to the amount of choline intake, and possibly regulated choline bioavailability.

5.4 Gender differences in TMAO levels

Studies have established that gender or sex hormones could also influence TMAO levels. The hepatic FMO3 activity has been found to suppressed by androgens hence, it is found to be increased in female mice and humans[154]. Hazen research work demonstrates CS-female mice clearly have a higher TMAO production compared to CS-male mice although CS-male mice have higher TMA levels[154,174]. It is noteworthy, TMAO values measured in current thesis are 100-fold significantly lower compared to values observed in literature[105]. Although, we can't explain this discrepancy, it could be due to differences in atherogenic mouse model used. It is possible that *Apoe*$^{-/-}$ mice have higher TMA producing microbiota.

5.5 Choline supplementation did not improve PC/PE ratio but improved NAFLD in DKO mice

The hepatic PC is required for the membrane biogenesis, bile secretion and packaging and secretion of VLDL particles[65]. PC and PE are the most abundant phospholipids of the inner and outer membranes of the liver. Many studies have established that the hepatic ratio between PC and PE is crucial for maintaining liver health and function[18]. In current study, Western diet fed DKO mice had decreased PC synthesis in the liver. Hence, there was an accumulation of TG in the liver and decreased hepatic PC/PE ratio (to about 1 to 1.2). The PC/PE ratio of a healthy liver has been established to be between the range of 1.5 to 2, any value above and below this range, is associated with an

array of liver-associated pathologies[18]. We had expected that choline supplementation would increase PC supply via the CDP-pathway but we observed no increase in hepatic PC or in VLDL secretion, suggesting there was enough p-choline available for CT activity. However oddly enough, there was significant reduction in hepatic TG levels and improvement in the liver histology. The improvement was possibly due to increased oxidation in the liver, a phenomenon that has been observed by others[161,175]. Hence, by demonstrating that choline supplementation can potentially improve liver function despite a low PC/PE ratio is a very important finding. Based on the same reasoning, the *Ldlr*$^{-/-}$ mice fed a 60% high fat diet exhibited better liver histology compared to DKO mice despite high levels of hepatic TG and cholesterol esters (Figure 4). This was probably due to a fairly normal PC/PE ratio (1.5) in the *Ldlr*$^{-/-}$ mice.

5.6 Decreasing choline attenuates atherosclerosis in *Ldlr*$^{-/-}$ mice

The main purpose of the pilot project (Chapter 4) was to establish that decreasing choline content of the diet attenuated the progression of atherosclerosis. The reasoning of the study design was that at 8 weeks of high fat/high choline diet would exhibit significant atherosclerosis in *Ldlr*$^{-/-}$ mice and reducing the choline content of the same diet at 8 weeks would almost reverse the progression while continuing the same diet would exacerbate the progression of atherosclerosis. We found there was a 20% reduction in atherosclerosis in the *Ldlr*$^{-/-}$ mice upon switching from high to a low choline diet (95% reduction in choline content).

5.7 Plasma TMAO levels changes with age/ length of the diet

The kidneys are often reported as potential confounders of total plasma TMAO since, TMA and TMAO is readily excreted from the kidneys[97,155]. An impaired kidney function is often associated with elevated TMAO levels and atherosclerotic burder[114,155]. In *Ldlr*$^{-/-}$ mice, we observed that TMAO levels decreased in the 16 week fed mice compared to at 8 weeks fed mice, and choline content of the diet did not effect TMAO levels in the serum. Moreover, we observed significant renal ischemia in all groups, more so in the 16 week fed mice although it did not reach statistical significance. A study has shown that short-term high fat feeding does result in elevations in TMAO levels in humans[176]. The decrease in TMAO levels observed at 16 weeks fed mice compared to at 8 weeks fed mice may be occurring due to significant alterations in the gut microbiota. In fact, prolonged high fat diet has been shown to significantly change the microbial composition of the gut microbiota[169]. Furthermore, renal ischemia, like chronic kidney disease (CKD) may be another pathology associated with atherosclerosis[177] and may be independent to plasma TMAO levels.

5.8 Conclusion

Despite evidence suggesting choline promotes atherosclerosis in *Apoe*$^{-/-}$ mice, several large observational studies have found no significant association between choline intake and cardiovascular diseases. The Nurses Health Study, Atherosclerosis Risk in Community (ARIC) study and European Prospective investigation into Cancer and nutrition (EPIC) study have found no association of choline intake with CVD, increased risk for all-cause mortality and peripheral artery diseases[178–180]. A research suggested that

increase in dietary PC or choline rich food does **not** cause a detectable increase in TMA in healthy humans, possibly because it gets cleared from the circulation via the kidneys[153]. Others have shown dietary choline via egg consumption does increase TMAO in a dose-dependent manner in healthy subjects, but it decreases to steady state levels within 24hrs[99,162].

On the other hand, one recent study did observe higher PC consumption to be associated with increased all-cause mortality in US-based population[135] and works from Hazen and colleagues shows an association of choline/TMAO with increased CVD risk[98,101,104,109]. Hence, we must extrapolate our findings with caution. We observed that choline supplementation did increase TMAO levels in DKO mice while, in the pilot study, decreasing choline content did not decrease TMAO level in *Ldlr*$^{-/-}$ mice indicating a depletion of TMA-producing microbiota in the gut. Hence, we can conclude, altering dietary choline does alter TMAO but it may be dependent on presence of TMA-producing microbiota in the gut.

Nevertheless, dietary choline does not cause an increase in atherosclerosis in the DKO mice. Switching from high to a low choline diet content of the *Ldlr*$^{-/-}$ mice did ameliorate the progression of atherosclerosis, independent to plasma cholesterol and TMAO levels.

5.9 Future directions:

There is a still many unknowns in this research field. Certainly, the role of choline metabolism in atherosclerosis is very complex. The principle question remains does TMAO contribute towards atherosclerosis and if so what is the mechanism? Further studies

should investigate the direct mechanism by which PEMT regulates TMAO metabolism. A possible link of PEMT and TMAO may be through bile acid metabolism. Although PEMT is not quantitatively essential for biliary PC secretion[181], increasing PEMT activity may be contributing towards increased biliary PC reaching the gut microbiota and hence increasing conversion to TMA and TMAO. More work is required to understand why TMAO levels vary depending on the age of the mice and duration of the diet and how switching from a high to a low choline diet ameliorates the progression of atherosclerosis in *Ldlr*$^{-/-}$ mice. A possibility could be decreasing dietary choline alters PL composition of CM and CM remnants, which increase CVD risk. Lastly, we need to determine the mechanism of how choline supplementation decreased hepatic TG and improved NAFLD, without increasing VLDL secretion and improving PC/PE ratio. Although choline supplementation in the DKO mice has been shown to normalize hepatic cholesterol metabolism[175]; increased oxidation upon choline supplementation is a possibility that warrants an investigation. This research work opens doors to examine Pemt inhibitors as a pharmaceutical target for CVD prevention, provided choline requirements are fulfilled by supplementation.

In summary, this thesis demonstrated that dietary choline is not associated with atherosclerosis using *Ldlr*$^{-/-}$ mice and *Pemt*$^{-/-}$/*Ldlr*$^{-/-}$ mice. There seems to be an association with *de novo* choline synthesis and atherosclerosis in the *Pemt*$^{-/-}$/*Ldlr*$^{-/-}$ mice. Plasma cholesterol, rather than TMAO appears to be more important towards progression.

6 Bibliography

1. Ross R. The pathogenesis of atherosclerosis: a perspective for the 1990s. *Nature*. 1993;362(6423):801-809. doi:10.1038/362801a0.
2. Weber C, Noels H. Atherosclerosis: current pathogenesis and therapeutic options. *Nat Med*. 2011;17(11):1410-1422. doi:10.1038/nm.2538.
3. Roger V.L., Go A.S., Lloyd-Jones D.M., Benjamin E.J., Berry J.D. \emphet al. Heart {D}isease and {S}troke {S}tatistics. *Circulation*. 2017;125:e2--e225. doi:10.1161/CIR.0000000000000485.
4. Mozaffarian D, Benjamin EJ, Go AS, et al. Heart Disease and Stroke Statistics—2016 Update. *Circulation*. December 2015. http://circ.ahajournals.org/content/early/2015/12/16/CIR.0000000000000350.abstract.
5. Stone NJ. The clinical and economic significance of atherosclerosis. *Am J Med*. 1996;101(4A):4A6S-9S.
6. Fox KM, Wang L, Gandra SR, Quek RGW, Li L, Baser O. Clinical and economic burden associated with cardiovascular events among patients with hyperlipidemia: a retrospective cohort study. *BMC Cardiovasc Disord*. 2016;16:13. doi:10.1186/s12872-016-0190-x.
7. Roberts WC. Factors linking cholesterol to atherosclerotic plaques. *Am J Cardiol*. 1988;62(7):495-499.
8. Brown MS, Goldstein JL. Heart Attacks: Gone with the Century? *Science (80-)*.

1996;272(5262):629 LP-629. http://science.sciencemag.org/content/272/5262/629.abstract.

9. Carmena R. Atherogenic Lipoprotein Particles in Atherosclerosis. *Circulation*. 2004;109(23_suppl_1):III-2-III-7. doi:10.1161/01.CIR.0000131511.50734.44.

10. Parthasarathy S, Raghavamenon A, Garelnabi MO, Santanam N. Oxidized Low-Density Lipoprotein. *Methods Mol Biol*. 2010;610:403-417. doi:10.1007/978-1-60327-029-8_24.

11. Toft-Petersen AP, Tilsted HH, Aarøe J, et al. Small dense LDL particles - a predictor of coronary artery disease evaluated by invasive and CT-based techniques: a case-control study. *Lipids Health Dis*. 2011;10:21. doi:10.1186/1476-511X-10-21.

12. Barter P. The role of HDL-cholesterol in preventing atherosclerotic disease. *Eur Hear J Suppl*. 2005;7(suppl_F):F4-F8. http://dx.doi.org/10.1093/eurheartj/sui036.

13. Bandeali S, Farmer J. High-density lipoprotein and atherosclerosis: the role of antioxidant activity. *Curr Atheroscler Rep*. 2012;14(2):101-107. doi:10.1007/s11883-012-0235-2.

14. Ali KM, Wonnerth A, Huber K, Wojta J. Cardiovascular disease risk reduction by raising HDL cholesterol – current therapies and future opportunities. *Br J Pharmacol*. 2012;167(6):1177-1194. doi:10.1111/j.1476-5381.2012.02081.x.

15. Arsenault BJ, Boekholdt SM, Kastelein JJP. Lipid parameters for measuring risk of cardiovascular disease. *Nat Rev Cardiol*. 2011;8(4):197-206. http://dx.doi.org/10.1038/nrcardio.2010.223.

16. Borén J, Matikainen N, Adiels M, Taskinen M-R. Postprandial

hypertriglyceridemia as a coronary risk factor. *Clin Chim Acta*. 2014;431:131-142. doi:https://doi.org/10.1016/j.cca.2014.01.015.

17. McNamara JR, Shah PK, Nakajima K, et al. Remnant-like particle (RLP) cholesterol is an independent cardiovascular disease risk factor in women: results from the Framingham Heart Study. *Atherosclerosis*. 2001;154(1):229-236.

18. van der Veen JN, Kennelly JP, Wan S, Vance JE, Vance DE, Jacobs RL. The critical role of phosphatidylcholine and phosphatidylethanolamine metabolism in health and disease. *Biochim Biophys Acta - Biomembr*. doi:https://doi.org/10.1016/j.bbamem.2017.04.006.

19. Shepherd J. The role of the exogenous pathway in hypercholesterolaemia. *Eur Hear J Suppl*. 2001;3:2-5. doi:10.1016/S1520-765X(01)90105-1.

20. Schaefer EJ, Tsunoda F, Diffenderfer M, Polisecki E, Thai N, Asztalos B. The Measurement of Lipids, Lipoproteins, Apolipoproteins, Fatty Acids, and Sterols, and Next Generation Sequencing for the Diagnosis and Treatment of Lipid Disorders. In: De Groot LJ, Chrousos G, Dungan K, et al., eds. South Dartmouth (MA); 2000.

21. Mahmood Hussain M. A proposed model for the assembly of chylomicrons. *Atherosclerosis*. 2000;148(1):1-15. doi:https://doi.org/10.1016/S0021-9150(99)00397-4.

22. Brown AJ, Sharpe LJ. Chapter 11 - Cholesterol Synthesis A2 - Ridgway, Neale D. In: McLeod Lipoproteins and Membranes (Sixth Edition) RSBT-B of L, ed. Boston: Elsevier; 2016:327-358. doi:http://doi.org/10.1016/B978-0-444-63438-2.00011-0.

23. Tabas I. Lipids and atherosclerosis. *Biochem Lipids, Lipoproteins Membr*. 2008;224:579-605. doi:10.1016/B978-044453219-0.50023-4.

24. Strong JP, Malcom GT, Newman WP 3rd, Oalmann MC. Early lesions of atherosclerosis in childhood and youth: natural history and risk factors. *J Am Coll Nutr*. 1992;11 Suppl:51S-54S.

25. Williams KJ, Tabas I. The Response-to-Retention Hypothesis of Early Atherogenesis. *Arterioscler Thromb Vasc Biol*. 1995;15(5):551-561. http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2924812/.

26. Huff MW, Daugherty A, Lu H. Chapter 18 - Atherosclerosis A2 - Ridgway, Neale D. In: McLeod Lipoproteins and Membranes (Sixth Edition) RSBT-B of L, ed. Boston: Elsevier; 2016:519-548. doi:http://doi.org/10.1016/B978-0-444-63438-2.00018-3.

27. Grundy SM. Oxidized LDL and atherogenesis: relation to risk factors for coronary heart disease. *Clin Cardiol*. 1993;16(4 Suppl 1):I3-5. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=8472395.

28. Grimshaw MJ, Balkwill FR. Inhibition of monocyte and macrophage chemotaxis by hypoxia and inflammation--a potential mechanism. *Eur J Immunol*. 2001;31(2):480-489. doi:10.1002/1521-4141(200102)31:2<480::AID-IMMU480>3.0.CO;2-L.

29. Mcintyre TM, Zimmerman GA, Prescott SM. Biologically Active Oxidized Phospholipids*. (29).

30. Getz GS, Reardon CA. Do the Apoe-/- and Ldlr-/- mice yield the same insight on

atherogenesis? *Arterioscler Thromb Vasc Biol*. 2016;36(9):1734-1741. doi:10.1161/ATVBAHA.116.306874.

31. Meir KS, Leitersdorf E. Atherosclerosis in the apolipoprotein E-deficient mouse: A decade of progress. *Arterioscler Thromb Vasc Biol*. 2004;24(6):1006-1014. doi:10.1161/01.ATV.0000128849.12617.f4.

32. Curtiss LK, Boisvert WA. Apolipoprotein E and atherosclerosis. *Curr Opin Lipidol*. 2000;11(3):243-251.

33. Ishibashi S, Goldstein JL, Brown MS, Herz J, Burns DK. Massive xanthomatosis and atherosclerosis in cholesterol-fed low density lipoprotein receptor-negative mice. *J Clin Invest*. 1994;93(5):1885-1893. doi:10.1172/JCI117179.

34. Getz GS, Reardon CA. Animal Models of Atherosclerosis. *Arterioscler Thromb Vasc Biol*. 2012;32(5):1104 LP-1115. http://atvb.ahajournals.org/content/32/5/1104.abstract.

35. Meir KS, Leitersdorf E. Atherosclerosis in the Apolipoprotein E–Deficient Mouse. *Arterioscler Thromb Vasc Biol*. 2004;24(6):1006 LP-1014. http://atvb.ahajournals.org/content/24/6/1006.abstract.

36. Véniant MM, Withycombe S, Young SG. Lipoprotein Size and Atherosclerosis Susceptibility in Apoe-/- and Ldlr-/- Mice. 2001:1567-1571.

37. Zeisel SH. A brief history of choline. *Ann Nutr Metab*. 2012;61(3):254-258. doi:10.1159/000343120.

38. Costa DA, Franklin PD, Alexander EA, Sheard NF, Beiser A. an essential nutrient for humans. 2017;5(7):2093-2098.

39. Zeisel SH, Da Costa KA. Choline: An essential nutrient for public health. *Nutr*

Rev. 2009;67(11):615-623. doi:10.1111/j.1753-4887.2009.00246.x.

40. Zeisel SH, Carolina N, Hill C, Carolina N, Blusztajn JK. NUTRITION. 1994.

41. Zeisel SH, Mar M-H, Howe JC, Holden JM. Concentrations of choline-containing compounds and betaine in common foods. *J Nutr*. 2003;133(5):1302-1307.

42. Iqbal J, Hussain MM. Intestinal lipid absorption. *Am J Physiol - Endocrinol Metab*. 2009;296(6):E1183-E1194. doi:10.1152/ajpendo.90899.2008.

43. Nakano T, Inoue I, Katayama S, et al. Lysophosphatidylcholine for Efficient Intestinal Lipid Absorption And Lipoprotein Secretion in Caco-2 Cells. *J Clin Biochem Nutr*. 2009;45(2):227-234. doi:10.3164/jcbn.09-25.

44. Schrama AJ, de Beaufort AJ, Sukul YR, Jansen SM, Poorthuis BJ, Berger HM. Phospholipase A2 is present in meconium and inhibits the activity of pulmonary surfactant: an in vitro study. *Acta Paediatr*. 2001;90(4):412-416. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=11332933.

45. Vance DE, Vance JE. CHAPTER 8 - Phospholipid biosynthesis in eukaryotes BT - Biochemistry of Lipids, Lipoproteins and Membranes (Fifth Edition). In: San Diego: Elsevier; 2008:213-244. doi:http://doi.org/10.1016/B978-044453219-0.50010-6.

46. Kempson SA, Vovor-Dassu K, Day C. Betaine Transport in Kidney and Liver: Use of Betaine in Liver Injury. *Cell Physiol Biochem*. 2013;32(suppl 1(7):32-40. http://www.karger.com/DOI/10.1159/000356622.

47. Zeisel SH, Wishnok JS, Blusztajn JK. Formation of methylamines from ingested choline and lecithin. *J Pharmacol Exp Ther*. 1983;225(2):320-324.

48. Lockman PR, Allen DD. The Transport of Choline. *Drug Dev Ind Pharm*. 2002;28(7):749-771. doi:10.1081/DDC-120005622.

49. Cheng W-L, Holmes-McNary MQ, Mar M-H, Lien EL, Zeisel SH. Bioavailability of choline and choline esters from milk in rat pups. *J Nutr Biochem*. 1996;7(8):457-464. doi:http://dx.doi.org/10.1016/0955-2863(96)00079-4.

50. Lewis ED, Richard C, Goruk S, et al. The Form of Choline in the Maternal Diet Affects Immune Development in Suckled Rat Offspring. *J Nutr*. 2016;146(4):823-830. doi:10.3945/jn.115.225888.

51. Moukarzel S, Soberanes L, Dyer RA, Albersheim S, Elango R, Innis SM. Relationships among different water-soluble choline compounds differ between human preterm and donor milk. *Nutrients*. 2017;9(4):1-10. doi:10.3390/nu9040369.

52. de Veth MJ, Artegoitia VM, Campagna SR, Lapierre H, Harte F, Girard CL. Choline absorption and evaluation of bioavailability markers when supplementing choline to lactating dairy cows. *J Dairy Sci*. 2016;99(12):9732-9744. doi:10.3168/jds.2016-11382.

53. Romano KA, Vivas EI, Amador-noguez D, Rey FE. Intestinal Microbiota Composition Modulates Choline Bioavailability. *MBio*. 2015;6(2):1-8. doi:10.1128/mBio.02481-14.Editor.

54. Gibellini F, Smith TK. The Kennedy pathway-de novo synthesis of phosphatidylethanolamine and phosphatidylcholine. *IUBMB Life*. 2010;62(6):414-428. doi:10.1002/iub.337.

55. Li Z, Vance DE. Phosphatidylcholine and choline homeostasis. *J Lipid Res*.

2008;49(6):1187-1194. doi:10.1194/jlr.R700019-JLR200.

56. Vance DE. Phospholipid methylation in mammals: from biochemistry to physiological function. *Biochim Biophys Acta - Biomembr*. 2014;1838(6):1477-1487. doi:10.1016/j.bbamem.2013.10.018.

57. Vance DE, Walkey CJ, Yu L, Agellem LB. Evolutionary and Nutritional Significance of Phospholipid Methylation. *FASEB J*. 1998;12(8):27043-27047.

58. Ridgway ND. Chapter 7 - Phospholipid Synthesis in Mammalian Cells BT - Biochemistry of Lipids, Lipoproteins and Membranes (Sixth Edition). In: Boston: Elsevier; 2016:209-236. doi:http://doi.org/10.1016/B978-0-444-63438-2.00007-9.

59. Zhang Y-M, Rock CO. Chapter 3 - Fatty Acid and Phospholipid Biosynthesis in Prokaryotes A2 - Ridgway, Neale D. In: McLeod Lipoproteins and Membranes (Sixth Edition) RSBT-B of L, ed. Boston: Elsevier; 2016:73-112. doi:http://doi.org/10.1016/B978-0-444-63438-2.00003-1.

60. Adeli K, Bogdanov M, Bond LM, et al. Contributors BT - Biochemistry of Lipids, Lipoproteins and Membranes (Sixth Edition). In: Boston: Elsevier; 2016:xix-xxi. doi:http://doi.org/10.1016/B978-0-444-63438-2.01002-6.

61. Li Z, Vance DE. Phosphatidylcholine and choline homeostasis. *J Lipid Res*. 2008;49(6):1187-1194. doi:10.1194/jlr.R700019-JLR200.

62. Cui Z, Vance DE. Expression of Phosphatidylethanolamine N-Methyltransferase-2 Is Markedly Enhanced in Long Term Choline-deficient Rats . *J Biol Chem* . 1996;271(5):2839-2843. doi:10.1074/jbc.271.5.2839.

63. Walkey CJ, Donohue LR, Bronson R, Agellon LB, Vance DE. Disruption of the murine gene encoding phosphatidylethanolamine N-methyltransferase. *Proc Natl*

Acad Sci U S A. 1997;94(24):12880-12885.

64. Li Z, Agellon LB, Vance DE. Choline Redistribution during Adaptation to Choline Deprivation. *J Biol Chem* . 2007;282(14):10283-10289. doi:10.1074/jbc.M611726200.

65. Yao Z, Vance DE. The active synthesis of phosphatidylcholine is required for very low density lipoprotein secretion from rat hepatocytes. *J Biol Chem*. 1988;263(6):2998-3004.

66. Mato JM, Lu SC. Role of S-adenosyl-L-methionine in liver health and injury. *Hepatology*. 2007;45(5):1306-1312. doi:10.1002/hep.21650.

67. Li Z, Agellon LB, Allen TM, et al. The ratio of phosphatidylcholine to phosphatidylethanolamine influences membrane integrity and steatohepatitis. *Cell Metab*. 2006;3(5):321-331. doi:10.1016/j.cmet.2006.03.007.

68. Zhao Y, Su B, Jacobs RL, et al. Lack of phosphatidylethanolamine n-methyltransferase alters plasma vldl phospholipids and attenuates atherosclerosis in mice. *Arterioscler Thromb Vasc Biol*. 2009;29(9):1349-1355. doi:10.1161/ATVBAHA.109.188672.

69. Ling J, Chaba T, Zhu L-F, Jacobs RL, Vance DE. Hepatic ratio of phosphatidylcholine to phosphatidylethanolamine predicts survival after partial hepatectomy in mice. *Hepatology*. 2012;55(4):1094-1102. doi:10.1002/hep.24782.

70. Vance JE. Phospholipid Synthesis and Transport in Mammalian Cells. *Traffic*. 2015;16(1):1-18. doi:10.1111/tra.12230.

71. Slotte JP, Ramstedt B. The functional role of sphingomyelin in cell membranes. *Eur J Lipid Sci Technol*. 2007;109(10):977-981. doi:10.1002/ejlt.200700024.

72. Ohvo-Rekilä H, Ramstedt B, Leppimäki P, Peter Slotte J. Cholesterol interactions with phospholipids in membranes. *Prog Lipid Res*. 2002;41(1):66-97. doi:https://doi.org/10.1016/S0163-7827(01)00020-0.

73. Kontush A, Lhomme M, Chapman MJ. Unraveling the complexities of the HDL lipidome. *J Lipid Res*. 2013;54(11):2950-2963. doi:10.1194/jlr.R036095.

74. Li Z, Agellon LB, Vance DE. A role for high density lipoproteins in hepatic phosphatidylcholine homeostasis. *Biochim Biophys Acta*. 2007;1771(7):893-900. doi:10.1016/j.bbalip.2007.04.009.

75. Walker AK. 1-Carbon Cycle Metabolites Methylate Their Way to Fatty Liver. *Trends Endocrinol Metab*. 2016;xx:1-10. doi:10.1016/j.tem.2016.10.004.

76. da Silva RP, Kelly KB, Al Rajabi A, Jacobs RL. Novel insights on interactions between folate and lipid metabolism. *Biofactors*. 2014;40(3):277-283. doi:10.1002/biof.1154.

77. Corbin KD, Zeisel SH. Choline metabolism provides novel insights into nonalcoholic fatty liver disease and its progression. *Curr Opin Gastroenterol*. 2012;28(2):159-165. doi:10.1097/MOG.0b013e32834e7b4b.

78. Boyer JL. Bile Formation and Secretion. *Compr Physiol*. 2013;3(3):1035-1078. doi:10.1002/cphy.c120027.

79. Obeid R. The Metabolic Burden of Methyl Donor Deficiency with Focus on the Betaine Homocysteine Methyltransferase Pathway. *Nutrients*. 2013;5(9):3481-3495. doi:10.3390/nu5093481.

80. Kathirvel E, Morgan K, Nandgiri G, et al. Betaine improves nonalcoholic fatty liver and associated hepatic insulin resistance: a potential mechanism for

hepatoprotection by betaine. *Am J Physiol - Gastrointest Liver Physiol*. 2010;299(5):G1068-G1077. doi:10.1152/ajpgi.00249.2010.

81. Vance DE, Walkey CJ, Agellon LB. Why has phosphatidylethanolamine N-methyltransferase survived in evolution? *Biochem Soc Trans*. 1998;26(3):337-340. http://www.ncbi.nlm.nih.gov/pubmed/9765874%5Cnhttp://www.biochemsoctrans.org/content/ppbiost/26/3/337.full.pdf.

82. Vance DE, Li Z, Jacobs RL. Hepatic phosphatidylethanolamine N-methyltransferase, unexpected roles in animal biochemistry and physiology. *J Biol Chem*. 2007;282(46):33237-33241. doi:10.1074/jbc.R700028200.

83. Sattar N, Forrest E, Preiss D. Non-alcoholic fatty liver disease. *BMJ*. 2014;349:g4596. doi:10.1136/bmj.g4596.

84. Li Z, Agellon LB, Vance DE. Phosphatidylcholine Homeostasis and Liver Failure. *J Biol Chem* . 2005;280(45):37798-37802. doi:10.1074/jbc.M508575200.

85. Kuipers F, Oude Elferink RP, Verkade HJ, Groen AK. Mechanisms and (patho)physiological significance of biliary cholesterol secretion. *Subcell Biochem*. 1997;28:295-318.

86. Noga AA, Vance DE. Insights into the requirement of phosphatidylcholine synthesis for liver function in mice. *J Lipid Res*. 2003;44(10):1998-2005. doi:10.1194/jlr.M300226-JLR200.

87. Smit JJM, Schinkel AH, Elferink RPJO, et al. Homozygous disruption of the murine MDR2 P-glycoprotein gene leads to a complete absence of phospholipid from bile and to liver disease. *Cell*. 1993;75(3):451-462. doi:10.1016/0092-8674(93)90380-9.

88. Jacobs RL, Zhao Y, Koonen DPY, et al. Impaired de novo choline synthesis explains why phosphatidylethanolamine N-methyltransferase-deficient mice are protected from diet-induced obesity. *J Biol Chem*. 2010;285(29):22403-22413. doi:10.1074/jbc.M110.108514.

89. Cole LK, Dolinsky VW, Dyck JRB, Vance DE. Impaired phosphatidylcholine biosynthesis reduces atherosclerosis and prevents lipotoxic cardiac dysfunction in ApoE-/- mice. *Circ Res*. 2011;108(6):686-694. doi:10.1161/CIRCRESAHA.110.238691.

90. Ganguly P, Alam SF. Role of homocysteine in the development of cardiovascular disease. *Nutr J*. 2015;14:6. doi:10.1186/1475-2891-14-6.

91. Wilson A, McLean C, Kim RB. Trimethylamine-N-oxide: a link between the gut microbiome, bile acid metabolism, and atherosclerosis. *Curr Opin Lipidol*. 2016;27(2):148-154. doi:10.1097/MOL.0000000000000274.

92. Yancey PH, Rhea MD, Kemp KM, Bailey DM. Trimethylamine oxide, betaine and other osmolytes in deep-sea animals: depth trends and effects on enzymes under hydrostatic pressure. *Cell Mol Biol (Noisy-le-grand)*. 2004;50(4):371-376.

93. Ufnal M, Zadlo A, Ostaszewski R. TMAO: A small molecule of great expectations. *Nutrition*. 2015;31(11-12):1317-1323. doi:10.1016/j.nut.2015.05.006.

94. Mackay RJ, McEntyre CJ, Henderson C, Lever M, George PM. Trimethylaminuria: Causes and Diagnosis of a Socially Distressing Condition. *Clin Biochem Rev*. 2011;32(1):33-43. http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3052392/.

95. Barrett EL, Kwan HS. Bacterial reduction of trimethylamine oxide. *Annu Rev Microbiol*. 1985;39:131-149. doi:10.1146/annurev.mi.39.100185.001023.

96. Ma J, Pazos IM, Gai F. Microscopic insights into the protein-stabilizing effect of trimethylamine N-oxide (TMAO). *Proc Natl Acad Sci* . 2014;111(23):8476-8481. doi:10.1073/pnas.1403224111.

97. Cho CE, Caudill MA. Trimethylamine-N-Oxide: Friend, Foe, or Simply Caught in the Cross-Fire? *Trends Endocrinol Metab*. 2016;xx:1-10. doi:10.1016/j.tem.2016.10.005.

98. Wang Z, Klipfell E, Bennett BJ, et al. Gut flora metabolism of phosphatidylcholine promotes cardiovascular disease. *Nature*. 2011;472(7341):57-63. http://dx.doi.org/10.1038/nature09922.

99. Tang WHW, Wang Z, Levison BS, et al. Intestinal Microbial Metabolism of Phosphatidylcholine and Cardiovascular Risk. *N Engl J Med*. 2013;368(17):1575-1584. doi:10.1056/NEJMoa1109400.

100. Tang WHW, Wang Z, Fan Y, et al. Prognostic value of elevated levels of intestinal microbe-generated metabolite trimethylamine-N-oxide in patients with heart failure: refining the gut hypothesis. *J Am Coll Cardiol*. 2014;64(18):1908-1914. doi:10.1016/j.jacc.2014.02.617.

101. Wang Z, Tang WHW, Buffa JA, et al. Prognostic value of choline and betaine depends on intestinal microbiota-generated metabolite trimethylamine-N-oxide. *Eur Heart J*. 2014;35(14):904-910. doi:10.1093/eurheartj/ehu002.

102. Randrianarisoa E, Lehn-Stefan A, Wang X, et al. Relationship of Serum Trimethylamine N-Oxide (TMAO) Levels with early Atherosclerosis in Humans.

Sci Rep. 2016;6(May):26745. doi:10.1038/srep26745.

103. Koeth R a, Wang Z, Levison BS, et al. Intestinal microbiota metabolism of L-carnitine, a nutrient in red meat, promotes atherosclerosis. *Nat Med*. 2013;19(5):576-585. doi:10.1038/nm.3145.

104. Senthong V, Wang Z, Li XS, et al. Intestinal Microbiota-Generated Metabolite Trimethylamine-N-Oxide and 5-Year Mortality Risk in Stable Coronary Artery Disease: The Contributory Role of Intestinal Microbiota in a COURAGE-Like Patient Cohort. *J Am Hear Assoc Cardiovasc Cerebrovasc Dis*. 2016;5(6):e002816. doi:10.1161/JAHA.115.002816.

105. Wang Z, Klipfell E, Bennett BJ, et al. Gut flora metabolism of phophatidylcholine promotes cardivovascular disease. *Nature*. 2011;472(7341):57-63. doi:10.1038/nature09922.Gut.

106. Chen M, Yi L, Zhang Y, et al. Resveratrol Attenuates Trimethylamine-N-Oxide (TMAO)-Induced Atherosclerosis by Regulating TMAO Synthesis and Bile Acid Metabolism via Remodeling of the Gut Microbiota. 2016;7(2):1-14. doi:10.1128/mBio.02210-15.Invited.

107. Noel OF, Still CD, Argyropoulos G, et al. Bile Acids, FXR, and Metabolic Effects of Bariatric Surgery. *J Obes*. 2016;2016:1-8. doi:10.1155/2016/4390254.

108. Shih DM, Wang Z, Lee R, et al. Flavin containing monooxygenase 3 exerts broad effects on glucose and lipid metabolism and atherosclerosis. *J Lipid Res*. 2015;56(1):22-37. doi:10.1194/jlr.M051680.

109. Zhu W, Gregory JC, Org E, et al. Gut Microbial Metabolite TMAO Enhances Platelet Hyperreactivity and Thrombosis Risk. *Cell*. 2016;165(1):111-124.

doi:10.1016/j.cell.2016.02.011.

110. Zhu W, Wang Z, Tang WHW, Hazen SL. Gut Microbe-Generated Trimethylamine N-Oxide From Dietary Choline Is Prothrombotic in Subjects. *Circulation*. 2017;135(17):1671 LP-1673. http://circ.ahajournals.org/content/135/17/1671.abstract.

111. Bain MA, Faull R, Fornasini G, Milne RW, Evans AM. Accumulation of trimethylamine and trimethylamine- N -oxide in end-stage renal disease patients undergoing haemodialysis . *Nephrol Dial Transplant*. 2006;21(5):1300-1304. http://dx.doi.org/10.1093/ndt/gfk056.

112. Kaysen G a., Johansen KL, Chertow GM, et al. Associations of Trimethylamine N-Oxide With Nutritional and Inflammatory Biomarkers and Cardiovascular Outcomes in Patients New to Dialysis. *J Ren Nutr*. 2015;25(4):351-356. doi:10.1053/j.jrn.2015.02.006.

113. Tang WHW, Wang Z, Kennedy DJ, et al. Gut microbiota-dependent trimethylamine N-oxide (TMAO) pathway contributes to both development of renal insufficiency and mortality risk in chronic kidney disease. *Circ Res*. 2015;116(3):448-455. doi:10.1161/CIRCRESAHA.116.305360.

114. Stubbs JR, House JA, Ocque AJ, et al. Serum Trimethylamine-N-Oxide is Elevated in CKD and Correlates with Coronary Atherosclerosis Burden. *J Am Soc Nephrol*. 2015;27(1):305-313. doi:10.1681/ASN.2014111063.

115. Gao X, Liu X, Xu J, Xue C, Xue Y, Wang Y. Dietary trimethylamine N-oxide exacerbates impaired glucose tolerance in mice fed a high fat diet. *J Biosci Bioeng*. 2014;118(4):476-481. doi:10.1016/j.jbiosc.2014.03.001.

116. Dambrova M, Latkovskis G, Kuka J, et al. Diabetes is Associated with Higher Trimethylamine N-oxide Plasma Levels. *Exp Clin Endocrinol Diabetes*. 2016;124(4):251-256. doi:10.1055/s-0035-1569330.

117. Mente A, Chalcraft K, Ak H, et al. The Relationship Between Trimethylamine-N-Oxide and Prevalent Cardiovascular Disease in a Multiethnic Population Living in Canada. *Can J Cardiol*. 2015;31(9):1189-1194. doi:10.1016/j.cjca.2015.06.016.

118. Lee JE, Giovannucci E, Fuchs CS, Willett WC, Zeisel SH, Cho E. Choline and betaine intake and the risk of colorectal cancer in men. *Cancer Epidemiol Biomarkers Prev*. 2010;19(3):884-887. doi:10.1158/1055-9965.EPI-09-1295.

119. Chistiakov DA, Bobryshev Y V., Kozarov E, Sobenin IA, Orekhov AN. Role of gut microbiota in the modulation of atherosclerosis-associated immune response. *Front Microbiol*. 2015;6(JUN). doi:10.3389/fmicb.2015.00671.

120. Jonsson AL, Bäckhed F. Role of gut microbiota in atherosclerosis. *Nat Rev Cardiol*. 2016. doi:10.1038/nrcardio.2016.183.

121. Koren O, Spor A, Felin J, et al. Human oral, gut, and plaque microbiota in patients with atherosclerosis. *Proc Natl Acad Sci U S A*. 2011;108(Suppl 1):4592-4598. doi:10.1073/pnas.1011383107.

122. Sagar NM, Cree IA, Covington JA, Arasaradnam RP. The interplay of the gut microbiome, bile acids, and volatile organic compounds. *Gastroenterol Res Pract*. 2015;2015:10-15. doi:10.1155/2015/398585.

123. Huang Y, Wang J, Quan G, Wang X, Yang L, Zhong L. Lactobacillus acidophilus ATCC 4356 prevents atherosclerosis via inhibition of intestinal cholesterol absorption in apolipoprotein E-knockout mice. *Appl Environ Microbiol*.

2014;80(24):7496-7504. doi:10.1128/AEM.02926-14.

124. Andrade S, Borges N. Effect of fermented milk containing Lactobacillus acidophilus and Bifidobacterium longum on plasma lipids of women with normal or moderately elevated cholesterol. *J Dairy Res*. 2009;76(4):469-474. doi:10.1017/S0022029909990173.

125. Li J, Lin S, Vanhoutte PM, Woo CW, Xu A. Akkermansia Muciniphila Protects Against Atherosclerosis by Preventing Metabolic Endotoxemia-Induced Inflammation in Apoe-/- Mice. *Circulation*. 2016;133(24):2434-2446. doi:10.1161/CIRCULATIONAHA.115.019645.

126. Rajaie S, Esmaillzadeh A. Dietary Choline and Betaine Intakes and Risk of Cardiovascular Diseases: Review of Epidemiological Evidence. *ARYA Atheroscler*. 2011;7(2):78-86. http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3347848/.

127. Đerić M, Kojić-Damjanov S, Čabarkapa V, Eremić N. Biochemical Markers of Atherosclerosis. *J Med Biochem*. 2008;27(2):148-153. doi:10.2478/v10011-008-0008-1.

128. Vance DE. Phospholipid methylation in mammals: from biochemistry to physiological function. *Biochim Biophys Acta - Biomembr*. 2014;1838(6):1477-1487. doi:https://doi.org/10.1016/j.bbamem.2013.10.018.

129. Nahrendorf M, Swirski FK. Lifestyle effects on hematopoiesis and atherosclerosis Matthias. 2016;116(5):884-894. doi:10.1161/CIRCRESAHA.116.303550.Lifestyle.

130. Org E, Mehrabian M, Lusis AJ. Unraveling the environmental and genetic

interactions in atherosclerosis: Central role of the gut microbiota. *Atherosclerosis*. 2015;241(2):387-399. doi:10.1016/j.atherosclerosis.2015.05.035.

131. Tremaroli V, Bäckhed F. Functional interactions between the gut microbiota and host metabolism. *Nature*. 2012;489(7415):242-249. doi:10.1038/nature11552.

132. Sitaraman R. Phospholipid catabolism by gut microbiota and the risk of cardiovascular disease. *J Med Microbiol*. 2013;62(PART6):948-950. doi:10.1099/jmm.0.053587-0.

133. Guyton K, Alverdy JC. The gut microbiota and gastrointestinal surgery. *Nat Rev Gastroenterol Hepatol*. 2016;advance on. http://dx.doi.org/10.1038/nrgastro.2016.139.

134. Senthong V, Wang Z, Li XS, et al. Intestinal microbiota-generated metabolite Trimethylamine-N-oxide and 5-year mortality risk in stable coronary artery disease: The contributory role of intestinal microbiota in a COURAGE-like patient cohort. *J Am Heart Assoc*. 2016;5(6):1-7. doi:10.1161/JAHA.115.002816.

135. Zheng Y, Li Y, Rimm EB, et al. Dietary phosphatidylcholine and risk of all-cause and cardiovascular- specific mortality among US women and men. *Am J Clin Nutr*. 2016:3-6. doi:10.3945/ajcn.116.131771.Keywords.

136. Lever M, George PM, Slow S, et al. Betaine and Trimethylamine-N-Oxide as Predictors of Cardiovascular Outcomes Show Different Patterns in Diabetes Mellitus: An Observational Study. *PLoS One*. 2014;9(12):e114969. doi:10.1371/journal.pone.0114969.

137. Harwood J. *Gas Chromatography and Lipids: A Practical Guide:* Vol 28.; 1989. doi:10.1016/0031-9422(89)80324-3.

138. Paigen B, Morrow A, Holmes PA, Mitchell D, Williams RA. Quantitative assessment of atherosclerotic lesions in mice. *Atherosclerosis*. 1987;68(3):231-240. doi:10.1016/0021-9150(87)90202-4.

139. Howatt DA. Protocols\Aortic Pathologies - Quantification Atherosclerosis -Aortic Root Sectioning and Analysis. 2013:1-5.

140. Brunt EM, Janney CG, Di Bisceglie AM, Neuschwander-Tetri BA, Bacon BR. Nonalcoholic steatohepatitis: A proposal for grading and staging the histological lesions. *Am J Gastroenterol*. 1999;94(9):2467-2474. doi:10.1111/j.1572-0241.1999.01377.x.

141. Jacobs RL, Devlin C, Tabas I, Vance DE. Targeted deletion of hepatic CTP:phosphocholine cytidylyltransferase alpha in mice decreases plasma high density and very low density lipoproteins. *J Biol Chem*. 2004;279(45):47402-47410. doi:10.1074/jbc.M404027200.

142. Lees M, Sloane GH. A simple. 1953;(3).

143. Itoh YH, Itoh T, Kaneko H. Modified Bartlett assay for microscale lipid phosphorus analysis. *Anal Biochem*. 1986;154(1):200-204. doi:10.1016/0003-2697(86)90515-4.

144. Millar JS, Cromley D a, McCoy MG, Rader DJ, Billheimer JT. Determining hepatic triglyceride production in mice: comparison of poloxamer 407 with Triton WR-1339. *J Lipid Res*. 2005;46(9):2023-2028. doi:10.1194/jlr.D500019-JLR200.

145. Vance DE, Weinstein DB, Steinberg D. Isolation and analysis of lipoproteins secreted by rat liver hepatocytes. *Biochim Biophys Acta - Lipids Lipid Metab*. 1984;792(1):39-47. doi:http://dx.doi.org/10.1016/0005-2760(84)90280-7.

146. Xiong Y, Zhao YY, Goruk S, et al. Validation of an LC-MS/MS method for the quantification of choline-related compounds and phospholipids in foods and tissues. *J Chromatogr B Anal Technol Biomed Life Sci*. 2012;911:170-179. doi:10.1016/j.jchromb.2012.10.038.

147. Zhao Y-Y, Xiong Y, Curtis JM. Measurement of phospholipids by hydrophilic interaction liquid chromatography coupled to tandem mass spectrometry: the determination of choline containing compounds in foods. *J Chromatogr A*. 2011;1218(32):5470-5479. doi:10.1016/j.chroma.2011.06.025.

148. Si Mi, Yuan-Yuan Zhao, René L. Jacobs JMC. Simultaneous determination of trimethylamine and trimethylamine N-oxide in mouse plasma samples by hydrophilic interaction liquid chromatography coupled to tandem mass spectrometry. *J Sep Sci*. 2014:1-23. doi:10.1002/jssc.201400030.This.

149. Caporaso JG, Kuczynski J, Stombaugh J, et al. QIIME allows analysis of high-throughput community sequencing data. *Nat Methods*. 2010;7(5):335-336. doi:10.1038/nmeth.f.303.

150. Edgar RC. Search and clustering orders of magnitude faster than BLAST. *Bioinformatics*. 2010;26(19):2460-2461. doi:10.1093/bioinformatics/btq461.

151. Xiao L, Feng Q, Liang S, et al. A catalog of the mouse gut metagenome. *Nat Biotech*. 2015;33(10):1103-1108. http://dx.doi.org/10.1038/nbt.3353.

152. Wang Z, Tang WHW, Buffa JA, et al. Prognostic value of choline and betaine depends on intestinal microbiota-generated metabolite trimethylamine-N-oxide. *Eur Heart J*. 2014;35(14):904-910. doi:10.1093/eurheartj/ehu002.

153. Blusztajn K, Wishnok S, STEVEN H. Z 2 JOHN, Science F. Formation of

methylamines from ingested choline and lecithin. *Pharmacology*. 1983:320-324.

154. Bennett BJ, Vallim TQDA, Wang Z, et al. Trimethylamine-N-Oxide, a metabolite associated with atherosclerosis, exhibits complex genetic and dietary regulation. *Cell Metab*. 2013;17(1):49-60. doi:10.1016/j.cmet.2012.12.011.

155. Fogelman AM. TMAO Is Both a Biomarker and a Renal Toxin. 2011:396-397. doi:10.1038/nm.3145.5.

156. Walkey CJ, Yu L, Agellon LB, Vance DE. Biochemical and Evolutionary Significance of Phospholipid Methylation. *J Biol Chem* . 1998;273(42):27043-27046. doi:10.1074/jbc.273.42.27043.

157. Noga AA, Vance DE. A Gender-specific Role For Phosphatidylethanolamine N-Methyltransferase-derived Phosphatidylcholine in the Regulation of Plasma High Density and Very Low Density Lipoproteins in Mice. *J Biol Chem* . 2003;278(24):21851-21859. doi:10.1074/jbc.M301982200.

158. Zeisel SH, Story DL, Wurtman RJ, Brunengraber H. Uptake of free choline by isolated perfused rat liver. *Proc Natl Acad Sci U S A*. 1980;77(8):4417-4419. doi:10.1073/pnas.77.8.4417.

159. Veenema K, Solis C, Li R, et al. Adequate Intake levels of choline are sufficient for preventing elevations in serum markers of liver dysfunction in Mexican American men but are not optimal for minimizing plasma total homocysteine increases after a methionine load. *Am J Clin Nutr*. 2008;88(3):685-692.

160. Abratte CM, Wang W, Li R, Moriarty DJ, Caudill MA. Folate intake and the MTHFR C677T genotype influence choline status in young Mexican American women. *J Nutr Biochem*. 2008;19(3):158-165. doi:10.1016/j.jnutbio.2007.02.004.

161. Schenkel LC, Sivanesan S, Zhang J, et al. Choline supplementation restores substrate balance and alleviates complications of Pcyt2 deficiency. *J Nutr Biochem*. 2015;26(11):1221-1234. doi:10.1016/j.jnutbio.2015.05.014.

162. Miller C a, Corbin KD, da Costa K-A, et al. Effect of egg ingestion on trimethylamine-N-oxide production in humans: a randomized, controlled, dose-response study. *Am J Clin Nutr*. 2014;(5):1-9. doi:10.3945/ajcn.114.087692.

163. Hazen Stanley L SL From the Department of Cellular and Molecular Medicine L, Hazen SL, Brown JM. Eggs as a dietary source for gut microbial production of trimethylamine-N-oxide. *Am J Clin Nutr*. 2014;100(3):741-743. doi:10.3945/ajcn.114.094458.

164. Hazen SL, Brown JM. Eggs as a dietary source for gut microbial production of trimethylamine-N-oxide. *Am J Clin Nutr*. 2014;100(3):741-743. doi:10.3945/ajcn.114.094458.

165. Mueller DM, Allenspach M, Othman A, et al. Plasma levels of trimethylamine-N-oxide are confounded by impaired kidney function and poor metabolic control. *Atherosclerosis*. 2015;243(2):638-644. doi:10.1016/j.atherosclerosis.2015.10.091.

166. Kulinski A, Vance DE, Vance JE. A choline-deficient diet in mice inhibits neither the CDP-choline pathway for phosphatidylcholine synthesis in hepatocytes nor apolipoprotein B secretion. *J Biol Chem*. 2004;279(23):23916-23924. doi:10.1074/jbc.M312676200.

167. Shapiro MD, Fazio S. Apolipoprotein B-containing lipoproteins and atherosclerotic cardiovascular disease. *F1000Research*. 2017;6:134. doi:10.12688/f1000research.9845.1.

168. Zhang H, DiBaise JK, Zuccolo A, et al. Human gut microbiota in obesity and after gastric bypass. *Proc Natl Acad Sci U S A*. 2009;106(7):2365-2370. doi:10.1073/pnas.0812600106.

169. Daniel H, Gholami AM, Berry D, et al. High-fat diet alters gut microbiota physiology in mice. *ISME J*. 2014;8(2):295-308. http://dx.doi.org/10.1038/ismej.2013.155.

170. Abratte CM, Wang W, Li R, Axume J, Moriarty DJ, Caudill MA. Choline status is not a reliable indicator of moderate changes in dietary choline consumption in premenopausal women. *J Nutr Biochem*. 2017;20(1):62-69. doi:10.1016/j.jnutbio.2007.12.002.

171. Mamo JC, Yu KC, Elsegood CL, et al. Is atherosclerosis exclusively a postprandial phenomenon? *Clin Exp Pharmacol Physiol*. 1997;24(3-4):288-293.

172. da Silva RP, Kelly KB, Lewis ED, et al. Choline deficiency impairs intestinal lipid metabolism in the lactating rat. *J Nutr Biochem*. 2015;26(10):1077-1083. doi:10.1016/j.jnutbio.2015.04.015.

173. Karpe F. Chylomicron production as a feature of atherogenic lipoproteins. *Curr Opin Lipidol*. 2012;23(4):398-399. doi:10.1097/MOL.0b013e328355ee5e.

174. Hartiala J, Bennett BJ, Tang WHW, et al. Comparative genome-wide association studies in mice and humans for trimethylamine N-Oxide, a proatherogenic metabolite of choline and L-carnitine. *Arterioscler Thromb Vasc Biol*. 2014;34(6):1307-1313. doi:10.1161/ATVBAHA.114.303252.

175. Rajabi A Al, Castro GSF, Silva RP, et al. Choline Supplementation Protects against Liver Damage by Normalizing Cholesterol Metabolism in Pemt / Ldlr

Knockout Mice Fed a High-Fat Diet 1 , 2. 2014:252-257. doi:10.3945/jn.113.185389.as.

176. Boutagy NE, Neilson AP, Osterberg KL, et al. Short-term high-fat diet increases postprandial trimethylamine-N-oxide in humans. *Nutr Res*. 2015;35(10):858-864. doi:10.1016/j.nutres.2015.07.002.

177. Chade AR, Lerman A, Lerman LO. Kidney in Early Atherosclerosis. *Hypertension*. 2005;45(6):1042 LP-1049. http://hyper.ahajournals.org/content/45/6/1042.abstract.

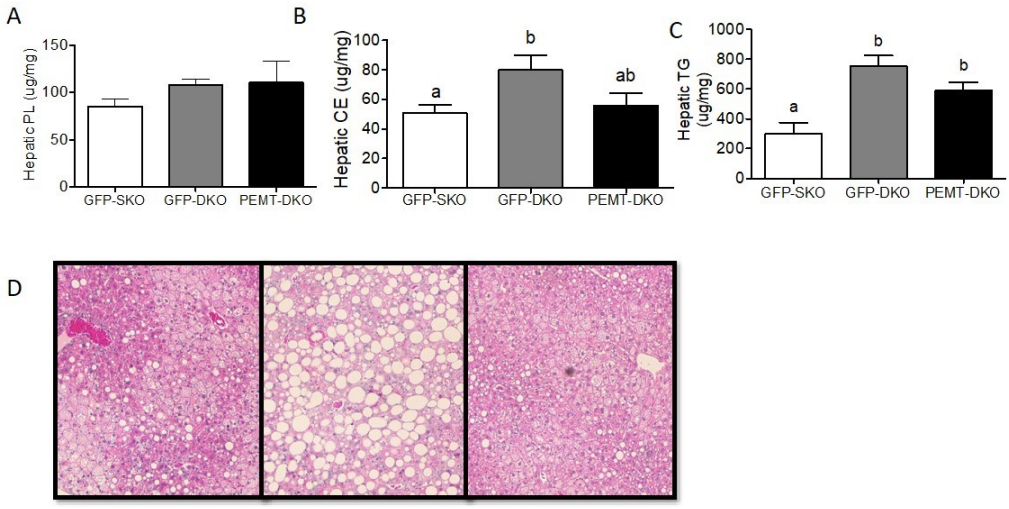
178. Dalmeijer GW, Olthof MR, Verhoef P, Bots ML, van der Schouw YT. Prospective study on dietary intakes of folate, betaine, and choline and cardiovascular disease risk in women. *Eur J Clin Nutr*. 2008;62(3):386-394. doi:10.1038/sj.ejcn.1602725.

179. Bertoia ML, Pai JK, Cooke JP, et al. Plasma homocysteine, dietary B vitamins, betaine, and choline and risk of peripheral artery disease. *Atherosclerosis*. 2014;235(1):94-101. doi:10.1016/j.atherosclerosis.2014.04.010.

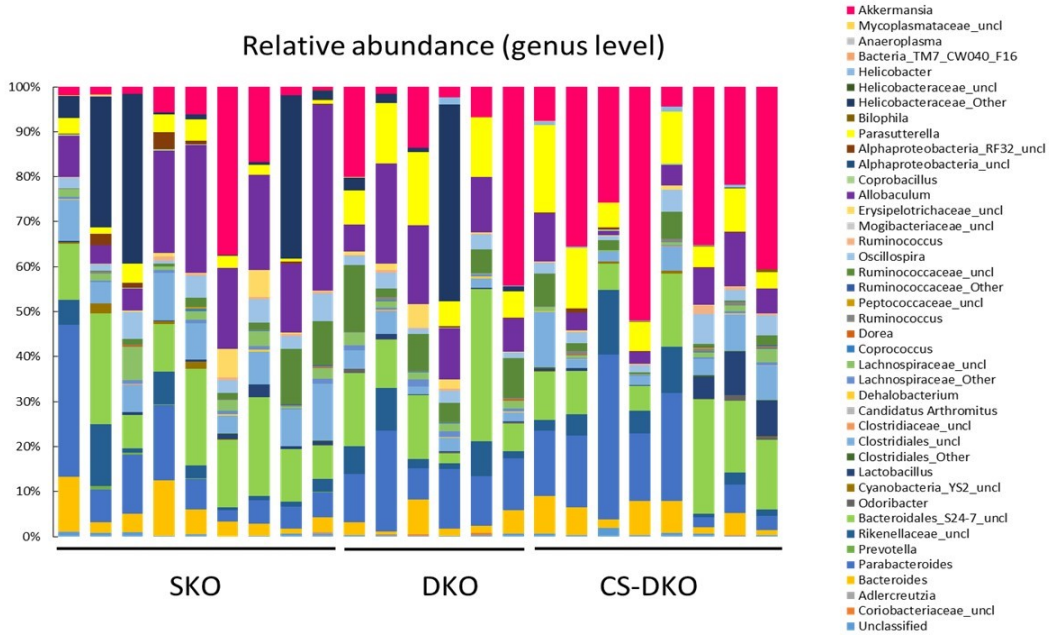
180. Bidulescu A, Chambless LE, Siega-Riz AM, Zeisel SH, Heiss G. Usual choline and betaine dietary intake and incident coronary heart disease: the Atherosclerosis Risk in Communities (ARIC) study. *BMC Cardiovasc Disord*. 2007;7:20. doi:10.1186/1471-2261-7-20.

181. Verkade HJ, Havinga R, Shields DJ, et al. The phosphatidylethanolamine N-methyltransferase pathway is quantitatively not essential for biliary phosphatidylcholine secretion. *J Lipid Res* . 2007;48(9):2058-2064. doi:10.1194/jlr.M700278-JLR200.

7 Appendix



Appendix Figure 7-1 SKO and DKO mice were injected with GFP or PEMT and fed a western diet for 8 weeks. (A) Total hepatic Phospholipids (B) hepatic CE and (C) hepatic TG in SKO and DKO mice (A-C). (D)H & E stained liver histology.



Appendix Figure 7-2 Heat map relative abundance of different microbial genus in SKO, DKO, and CS-DKO mice. SKO, DKO, and CS-DKO mice were fed a western diet containing 3g/kg or 10g/kg choline for 12 weeks.

Table 7-1 Liver Steatosis, ballooning and lobular inflammation scoring system

Grade	Brunt's Scale
0	0% biopsed hepatocytes affected
1	<33% biopsed hepatocytes affected
2	33-66% biopsed hepatocytes affected
3	>66% hepatocytes affected

Criteria based on Brunt's proposal for grading and staging Nonalcoholic fatty liver disease (NAFLD)

Table 7-2 Renal ischemic scoring system

Score	Histopathological pattern
0	Normal
0.5	Small focal injured areas
1	<10% of the cortical injured zone
2	10-25% of the cortical injured zone
3	25 to 75%
4	>75% injured

Criteria used for pathological examination of renal histology

Table 7-3 Components of the basal western diet (40% calories from fat) The western diet of the following composition was used in Pemt-AAV and choline supplementation Study.

Ingredients	**1 KG**
Casein	270
Corn Starch (Safeway)	241
Sucrose	126
Vitamin Mix (AIN-93-Vx) (Harlan TD)	19
Mineral Mix (Usual)	50
Calcium Phosphate Dibasic	3.4
Inositol	6.3
Cellulose	80
L-cystine	1.8
Choline Bitartrate	3.16 or 9.48
Fat Mixture	
Flax Oil	4
Olive Oil (Safeway)	40
Sunflower Oil (Safeway)	69.5
Crisco canola	86
DHAsco	0
Arasco	0.5
Cholesterol	5

The table above lists the ingredients in 1kg of the basal western diet. Choline bitartrate content is adjusted for purity, to achieve a concentration of 3g/kg or 10g/kg in the western diet.