The Effect of Perfluorooctane Sulfonate (PFOS) and Choline Supplementation on Hepatic Steatosis in Sprague Dawley Rats

A Dissertation

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Abstract

Perfluorooctane sulfonate (PFOS) is bioaccumulative and prevalent in the human population. PFOS induces hepatic steatosis in male rats at dietary exposures of 100 ppm via an unknown mechanism. *In vitro*, PFOS creates a choline ion complex. Choline deficiency induces hepatic steatosis in rats by decreasing VLDL secretion. The primary hypothesis was that a hepatic PFOS:choline ion complex causes steatosis that could be prevented by dietary choline supplementation. PFOS activation of steatosis related nuclear receptors (i.e., LXR, PXR, CAR, and PPAR-gamma) was investigated as a secondary hypothesis. To identify a choline dietary concentration, Sprague Dawley rats (5-6/sex/group) were fed control diet or 5X, 10X, or 15X basal choline diets for four weeks. The 5X diet was selected based on decreased body weights and body weight gains in the 10X (females only) and 15X groups. Sprague Dawley rats (12/sex/group) were fed control, choline supplemented (CS), 100 ppm PFOS, or 100 ppm PFOS + CS diets for three weeks. The male PFOS (±CS) rats developed hepatic steatosis, decreased mean serum cholesterol, and increased liver choline concentrations; the supplemented diet did not prevent hepatic steatosis. Female rats did not have these findings, even though serum and liver PFOS concentrations were similar to the males. *In vitro*, 400 µM PFOS did not inhibit choline kinase activity, which does not support the primary hypothesis. Regarding the secondary hypothesis, there was no activation (LXR, PXR, and CAR) or very weak activation (PPAR-gamma) by PFOS in a luciferase-linked assay. Also, liver mRNA activated by these nuclear receptors were not upregulated in rats fed PFOS. There are no clear data from this project that support the primary or secondary hypothesis. However, increased hepatic choline concentrations in the male PFOS rats correlates with the primary hypothesis. This finding and the sex-related difference in PFOS-induced hepatic steatosis warrant further investigation.

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List of Abbreviations

Abbreviation	Full Name
AC	Adenylate cyclase
ACC	Acetyl-CoA carboxylase
ACL	ATP citrate lyase
ACOX1	Acyl-coenzymeA oxidase 1
ALT	Alanine aminotransferase
AMPK	AMP-activated protein kinase
ANOVA	Analysis of variance
apoB-100	Apolipoprotein B-100
AS	Acyl CoA synthetase
ATP	Adenosine triphosphate
cAMP	Cyclic adenosine monophosphate
CAR	Constitutive androstane receptor
CD36	Cluster of Differentiation 36 also known as fatty acid transporter (FAT)
ChORE	Carbohydrate response element
ChREBP	Carbohydrate response element binding protein
CPT1	Carnitine palmitoyl transferase I
CPT2	Carnitine palmitoyl-transferase II
CT	Carnitine acylcarnitine translocase
CV	Coefficient of variation
CYP2B1/2	Cytochrome P450 2B1/2
CYP3A1	Cytochrome P450 3A1
CYP4A1	Cytochrome P450 4A1
CYP4A22	Cytochrome P450 4A22
EHHADH	Enoyl-CoA hydratase and 3-hydroxyacyl CoA dehydrogenase

Abbreviation	Full Name
FA-carn	Fatty acid carnitine
FA-CoA	Fatty acid Coenzyme A
FAS	Fatty acid synthase
FFA	Free fatty acids
FSP27	Cell death-inducing DFFA-like effector c (fat-specific protein)
FXR	Farnesoid X receptor
GCR	G-coupled receptor
GSST1	Glutathione S-transferase theta 1, transcript variant X2
HDL	High density lipoprotein
IDL	Intermediate density lipoprotein
LDL	Low density lipoprotein
HSL	Hormone-sensitive lipase
LXR-alpha	Liver X receptor alpha
LXR-beta	Liver X receptor beta
MTP	Microsomal triglyceride transfer protein
N-Et-FOSE	N-ethyl-N-(2-hydroxyethyl)perfluorooctanesulfonamide
OAA	Oxaloacetate
PFOS	Perfluorooctane sulfonate
POSF	Perfluorooctane sulfonyl fluoride
PPAR-alpha (PPARA)	Peroxisome proliferator-activated receptor alpha
PPAR-gamma	Peroxisome proliferator-activated receptor gamma
PPRE	Peroxisome proliferator response element
PXR	Pregnane X receptor
Pyr	Pyruvate
RXR	Retinoid X receptor
SCAP	Sterol regulatory element binding protein cleavage activating protein
SCD1	Stearoyl-CoA desaturase

Abbreviation	Full Name
SHP	Small heterodimer partner
SRE	Sterol regulatory element
SREBP-1c	Sterol response element binding protein 1-c
TCA	Tricarboxylic acid cycle
TG	Triglycerides
TK	Tyrosine kinase receptor
VLDL	Very low density lipoprotein

Chapter 1: Overview of Perfluorooctane Sulfonate (PFOS) and Hepatic Lipid Accumulation

1.1 Introduction

Fluorocarbons have been used for decades as industrial intermediates and surfactants [1]. Molecules containing perfluorinated carbon chains as part of their structure have been identified as bioaccumulative and/or environmentally persistent [2, 3]. One of these molecules is perfluorooctane sulfonate (PFOS). PFOS is an eight-carbon, fully fluorinated chemical with a sulfonate head group that can be linear or have internal monomethyl, isopropyl or other branches (Figure 1). The PFOS used for this dissertation (3M PFOS, lot 217) contains 86.9% PFOS isomers where 69.3% of the isomers are linear PFOS, 16.5% are monomethyl branched PFOS, 10.2% are isopropyl branched PFOS, and ≤1.7% are of other branch types based on the certificate of analysis [4].

In 1997, PFOS was identified in the serum of 3M workers [5], and since that time, its potential toxicity and environmental impact have been extensively investigated. One identified effect is the potential for hepatocellular vacuoles to form in rats [6, 7] and monkeys [8] after ingesting high dose PFOS; for example, rats fed 100 ppm PFOS in the diet for at least four weeks [6]. It is possible that these vacuoles represented lipid accumulation (i.e., hepatic steatosis).

This project confirmed the putative hepatic steatosis effect in rats following dietary high dose PFOS exposure and investigated two potential mechanisms for this effect. The primary hypothesis was that PFOS formed an ion complex with choline leading to hepatic steatosis similar to the effects of choline deficiency. The secondary hypothesis was that PFOS activated nuclear receptor(s) that can cause hepatic steatosis, for example the pregnane X receptor (PXR).

1.2 Background on Lipid Metabolism

Lipid metabolism is the key to energy homeostasis throughout the body. The low energy (i.e., fasting) state is the common state of the body, thus the lipid catabolism pathway is critical to maintaining energy levels in cells between meals. In high energy (i.e., fed) states, lipid anabolism allows the body to store high energy triglyceride (TG) molecules in the adipocytes for future use. Understanding the balance between lipid catabolism (Figure 2) and lipid anabolism and VLDL secretion (Figure 3) is critical to understanding how perturbations in these systems can lead to pathology (e.g., hepatic steatosis).

When circulating serum glucose concentrations are low (e.g., fasted conditions, see Figure 2), glucagon is produced by the pancreas, activates adenylate cyclase in adipose tissue, and binds to the glucagon receptor (GCR) on the hepatocyte [9]. In the adipose tissue, the activated adenylate cyclase converts adenosine triphosphate (ATP) to cyclic adenosine monophosphate (cAMP). Cyclic AMP-dependent protein kinase (PKA) is activated by the cAMP, then phosphorylates perilipin A, a lipid droplet surface protein, and hormone-sensitive lipase (HSL) [9]. HSL moves from the cytosol to the lipid droplet and hydrolyzes triglyceride to release free fatty acid (FFA). Perilipin A must be phosphorylated for HSL to associate with the lipid droplet and this is one of the regulatory steps controlling the release of FFA. Once liberated from the triglyceride, the FFA is released from the adipocyte into the blood where it binds to serum albumin and is transported throughout the body. In the hepatocyte, glucagon signals the inhibition of sterol response element binding protein-1c (SREBP-1c), the key regulatory enzyme in lipid synthesis, and the conversion of ATP to cAMP [9]. The increased cAMP activates adenosine monophosphate kinase (AMPK) to phosphorylate and inactivate acetyl-CoA carboxylase (ACC). Phosphorylated ACC cannot catalyze the reaction of acetyl-CoA to malonyl-CoA, the key regulatory step in lipid synthesis [9]. AMPK and free fatty acids (FFA) activate the nuclear membrane bound peroxisome proliferator activated receptor alpha (PPAR-alpha) to enter the nucleus, bind with retinoid X receptor (RXR), and then bind to the peroxisome proliferation response elements (PPRE) of target genes [10]. PPAR-alpha upregulates transcription of CD36, the key membrane transport molecule for FFA uptake from the bloodstream by hepatocytes [10]. PPAR-alpha also upregulates transcription of mRNA for cytochrome P450 4A1 (CYP4A1), the first enzyme of the fatty acid omega oxidation pathway. Long and medium chain fatty acids can be first

subjected to omega oxidation in the endoplasmic reticulum by cytochromes of the 4A family (i.e., oxidized from the omega carbon, the carbon distal to the carboxylic acid); however, this is generally a minor oxidation pathway in rats and humans with alpha and beta oxidation being more important [9, 11]. PPAR-alpha also upregulates transcription of peroxisomal acyl-coenzyme A oxidase 1 (ACOX) and enoyl-CoA hydratase and 3-hydroxyacyl CoA dehydrogenase (EHHADH), two enzymes in the peroxisome for lipid catabolism [10]. Peroxisomal oxidation leads to hydrogen peroxide formation, whereas mitochondrial oxidation leads to energy production in the form of NADH and $FADH_2$. In the peroxisome, each cycle of peroxisomal oxidation reduces the length of the FFA chain by two carbons resulting in shorter chain acyl CoA molecules (e.g., 8-carbon acyl CoA) [12]. Mitochondrial beta oxidation requires the transport of the FFA into the mitochondria through the carnitine palmitoyl transferase I (CPT 1) and carnitine palmitoyl-transferase II (CPT 2) pathway [10]. CPT 1 and CPT2 are also upregulated by PPAR-alpha [10]. CPT1 catalyzes the addition of carnitine to the acyl-CoA to permit transport of the acyl-CoA-carnitine (FA-carn) into the mitochondria by carnitine acylcarnitine translocase (CT) [13]. In the interior of the mitochondria CPT2 removes the carnitine from the FA-carn [13] to make the acyl-CoA available for mitochondrial beta oxidation. Every round of mitochondrial beta oxidation removes two carbon molecules from the acyl-CoA as acetyl-CoA [9]. The released acetyl-CoA is metabolized in the tricarboxylic acid cycle (TCA) to ultimately produce ATP through the electron transport chain. The widespread effects of PPAR-alpha make it the key regulatory nuclear receptor for activation of lipid catabolism and maintaining energy homeostasis during fasting conditions, i.e., the most common state.

When circulating serum glucose concentrations are high (e.g., fed conditions, see Figure 3), the pancreas produces insulin that binds to the tyrosine kinase receptor (TK) in the hepatocyte starting a signaling cascade that cleaves SREBP-1c from sterol regulatory element binding protein cleavage activating protein (SCAP) in the endoplasmic reticulum [14]. The SREBP-1c migrates to the Golgi body and becomes activated where it can then travel to the nucleus and bind to the sterol response element (SRE) [14]. SREBP-1c upregulates transcription of pyruvate kinase (PK), ACC, and fatty acid synthase (FAS) [15]. In addition, a separate insulin signaling cascade activates phosphatase that dephosphorylates ACC producing active ACC that can metabolize acetyl-CoA to

malonyl-CoA [14]. The increased concentrations of malonyl-CoA act as a negative feedback loop and inhibits CPT1, thus preventing the creation of FA-carn and inhibiting fatty acid uptake by the mitochondria as previously described [14]. Similarly, the high serum glucose is taken up by the glucose 2 (GLUT2) transporter. The glucose enters the glycolysis pathway and, following a series of metabolic steps, also activates the carbohydrate response element binding protein (ChREBP) by a series of dephosphorylations [14]. The activated ChREBP can bind to the carbohydrate response element (ChORE) and upregulate the transcription of similar genes [14]. The increased pyruvate is metabolized to citrate through the TCA cycle and citrate can also allosterically promote the metabolism of acetyl-CoA to malonyl-CoA by ACC. Malonyl-CoA is metabolized to palmitate by FAS through repeated additions of two carbon units. Adding an additional two carbon unit to palmitate yields stearate that can be converted to oleate by the addition of a double bond at the C9 position by stearoyl-CoA desaturase (SCD1) [16]. The resulting fatty acids can undergo further metabolism to acyl-CoA units and with the addition of glycerol-3-phosphate (gly-3-P) be converted to triglyceride (TG). TG is packaged into apolipoprotein B100 particles (apoB100) by microsomal triglyceride transfer protein (MTP) to produce nascent very low density lipoprotein (VLDL) particles that are transported to the Golgi body and ultimately secreted as mature VLDL [17]. The secreted VLDL is then transported through the circulatory system to the adipose tissue, where the TG is stored for use during future fasting states. As the circulating VLDL releases TG to the adipose tissue, the VLDL is converted to intermediate density lipoprotein (IDL), low density lipoprotein (LDL) and ultimately high density lipoprotein (HDL), resulting in the four circulating forms of lipoprotein.

The formation of apoB100 particles is dependent on the production of phosphatidylcholine [18]. Phosphatidylcholine (PC) production is dependent on the uptake of choline and metabolism through the CDP-pathway or metabolism of ethanolamine to phosphatidylcholine through the phosphatidylethanolamine N-methyltransferase (PEMT) pathway [17]. The committed step in the CDP pathway is the metabolism of choline to phosphocholine (P-Cho) by choline kinase (CK) [19]. The PEMT pathway is dependent on the availability of methionine which is metabolized to S-adenosyl methionine (S-Ado-Met) and used in the formation of PC from phosphatidylethanolamine (PE) by PEMT [17]. Thus, the formation and secretion of

VLDL particles is dependent, in part, on the proper functioning of the CDP and PEMT pathways.

1.3 Species and Sex-related Differences in Lipid Metabolism

Humans are less responsive to PPAR-alpha agonists compared to rats and mice [20]. Humans produce a splice variant of PPAR-alpha mRNA that lacks exon 6 and results in an early stop codon and a truncated PPAR-alpha protein that is inactive [21]. This truncated PPAR-alpha protein is present in human liver at 3- to 5-fold lower concentrations than the full PPAR-alpha protein [21] and can act as an endogenous inhibitor of native PPAR-alpha [22]. The presence of this truncated PPAR-alpha has been suggested to be the source of the decreased PPAR-alpha agonist response in humans relative to rodents [22]. Humans also have differences in the ACOX gene promoter region relative to rodents that may lead to less effective PPAR-alpha activation even in the presence of native PPAR-alpha [23].

Fatty acids can be oxidized by omega oxidation, for long-chain fatty acids, and beta oxidation, for short to very long chain fatty acids [12]. Omega oxidation takes place in the endoplasmic reticulum by CYP4A enzymes and can be followed by peroxisomal beta oxidation or mitochondrial beta oxidation [12]. Humans can also perform alpha oxidation in the peroxisomes, releasing formyl-CoA in each round of oxidation rather than the acetyl-CoA produced by beta oxidation [12]. Based on the affinity of CPT1 for short-chain fatty acids (e.g., C8 acyl-CoA), fatty acid oxidation of long chain fatty acids must occur prior to entry into the mitochondria. Since, humans are less responsive to peroxisome proliferation than rats [20], humans generally conduct more beta oxidation in the mitochondria; whereas rats conduct generally more peroxisomal oxidation.

Female rats are less sensitive than male rats to the hepatic steatosis effects of choline deficiency [24]. Female mice favor the PEMT pathway for PC production; whereas males favor the CDP pathway for PC production [25]. Estrogen upregulates hepatic PEMT expression and protein content in rats [26]. The increased usage of the PEMT pathway in females relative to males can decrease the effects of choline deficiency in females.

It is the balance of lipid uptake, catabolism, anabolism, and secretion that maintains appropriate energy homeostasis in cells. Cellular lipid accumulation (i.e., steatosis) can only occur when the balance is shifted towards cellular lipid retention through increased uptake, increased anabolism, decreased catabolism, or decreased secretion or some combination thereof. The potential for PFOS to affect the proper formation and secretion of VLDL particles was investigated. If PFOS sequesters choline through a choline:PFOS ion complex causing decreased VLDL secretion, then that would alter this homeostasis leading to lipid accumulation. Similarly, if PFOS upregulated key nuclear receptors to favor lipid uptake (i.e., PXR activation upregulating CD36), then that would alter lipid homeostasis and cause lipid accumulation. The effects of PFOS on lipid homeostasis in the presence and absence of dietary choline supplementation were investigated.

1.4 Sources of PFOS

3M manufactured perfluorooctane sulfonyl fluoride (POSF), its derivatives, and PFOS as industrial intermediates and surfactants [27]. POSF can be reacted with methyl or ethyl amines, which can be further reacted to form functionalized perfluorooctanesulfonamido derivatives that were used in a wide variety of applications. N-methyl- (2-hydroxyethyl)perfluorooctanesulfonamide (N-Me-FOSE) and N-ethyl-N-(2-hydroxyethyl)perfluorooctanesulfonamide (N-Et-FOSE) were major derivatives that were reacted to form various molecular structures. POSF-derived fluorochemicals like these were used in multiple applications including carpet protection, food packaging, and fire extinguishing products [1]. These derivatives are considered the precursors of PFOS in the environment [27].

PFOS is a terminal metabolite of N-Et-FOSE and other POSF derivatives [28], such that once the metabolite is formed there are no known biochemical processes (e.g., biodegradability, hydrolysis, or photolysis) that can alter the structure of PFOS [27]. For example, [^{14}C]PFOS was recovered unchanged in the liver of male CD rats at two- to three-weeks after intravenous injection of 3.5 mg/kg [^{14}C]PFOS. 3M discontinued the production of POSF and related derivatives, including PFOS, by the end of 2002 based on evidence of widespread environmental distribution and persistence of PFOS [27].

1.5 PFOS in the Environment

The use of PFOS, N-Et-FOSE, and other POSF derivatives in such a wide variety of products led to the indirect release these chemicals to the environment during waste disposal and from industrial processes and products. POSF derivatives are highly hydrophobic and readily taken up by organisms in the environment where they are metabolized to PFOS. PFOS is tightly bound to serum proteins [29] and as such, it is not easily excreted. As a result, PFOS has an elimination half-life of 30 to 50 days in rats [30], 200 days in cynomolgus monkeys [30], and 5.4 years in humans [31]. These factors suggest that body burdens of PFOS may accumulate in mammalian species following repeated exposures. PFOS is then biomagnified through the food web with top predators (e.g., polar bears) accumulating concentrations of PFOS from their food sources [32-35]. As a result of its persistence and bioaccumulation properties, PFOS has spread worldwide and been identified in the blood and tissues of fish, birds, and other wildlife from Greenland [32, 33] to Antarctica [36]. The widespread environmental prevalence of PFOS and its bioaccumulation properties and environmental persistence led to it becoming listed as an Annex B (restricted) persistent organic pollutant by the Stockholm Convention [3].

Efforts to control the release of PFOS and to reduce or eliminate the production of its precursors have been underway since 2001. These efforts have correlated to decreasing PFOS concentrations in wildlife. For example, a comparative study of PFOS in Greenland polar bear dams and cubs by Bytingsvik, et al. [32] showed that average PFOS plasma concentrations decreased in dams from approximately 450 ppb to 300 ppb from 1998 to 2008 while cub average PFOS plasma concentrations remained relatively stable from approximately 380 ppb to 280 ppb over the same period. Similarly, a prospective study by Rigét, et al. [37] showed that average PFOS liver concentrations peaked in ringed seals and polar bears in 2006 at 397 ppb and 2966 ppb, respectively, and decreased by 2010 to 16 ppb and 1248 ppb, respectively. However, as production by 3M ended, production of PFOS and its precursor molecules began in China in 2002 [38]. PFOS reached a peak production volume in China of 236 tons in 2006 and then decreased to a stable 100 tons/year from 2008 to 2012 [38]. Thus, the issue of PFOS in the environment may continue to be a concern for years to come.

1.6 PFOS and Human Exposure

Human exposure to PFOS can occur through direct exposure to PFOS, or POSF derived materials that can be metabolized to PFOS, from environmental sources (e.g., food), commercial products, industrial products, and from occupational exposures prior to 2002 [39]. The PFOS serum and liver body burden in humans has been an ongoing topic of research.

In 1997, the average PFOS serum concentration in 3M workers at one production facility was 1,960 ppb with 95% of the tested workers having serum concentrations of ≤6,000 ppb [5]. In 2000, the US general population had an average PFOS serum concentration of 34.9 ppb [40]. Similarly, in 2002, the Australian general population had an average PFOS serum concentration 25.9 ppb [41]. Similar to other mammals, humans accumulate PFOS primarily in the liver [42]. A review of paired human serum and liver samples by Olsen, et al. [43] showed that the mean PFOS liver to serum ratio in non-occupationally exposed humans was 1.3:1, which was similar to the liver to serum ratios in monkeys that had 100-fold the serum concentrations of the human donors. This makes the liver a potential target organ for toxic effects (see Sections 1.5 and 1.6 below).

Over time, PFOS serum concentrations have steadily decreased in all populations. As of 2010, average PFOS serum concentrations in the general public have decreased to 8.3 ppb in the US [40] and 10.2 ppb in Australia [41]. This decrease has generally conformed to the measured PFOS elimination half-life of 5.4 years in retired perfluorinated production workers reported by Olsen, et al. [31]. A steady decline in PFOS concentrations in humans is to be expected, since PFOS, and the precursors to PFOS, haven't been manufactured in the US since 2002. However, as previously stated, the production of perfluorinated precursors and PFOS in China will influence environmental exposures, the food web, and quite possibly PFOS serum concentrations in the general public in the future.

1.7 PFOS and Serum Cholesterol Effects in Humans

PFOS serum concentrations in the general public were measured as part of a cross-sectional epidemiology study on perfluorooctanoic acid, i. e., the C8 Health Project [44].

Some studies have used the C8 Health Project data to suggest correlations between increased PFOS serum concentrations and increased serum concentrations of alanine aminotransferase (ALT) [45], serum total cholesterol [46], and non-high density lipoprotein cholesterol (non-HDL) [47]. However, these correlations are being disputed by Kerger, et al. [48] because the C8 Health Project is 1) cross-sectional, 2) the magnitude of mean serum cholesterol increases across the exposures was small (i. e., 12 mg/dL), and 3) the lowest exposure quartile in the C8 Health Project had a lower prevalence of high cholesterol than the general US population, which may have biased the outcome towards an apparent dose-response where one does not actually exist.

A four-year longitudinal study was performed by Fitz-Simon, et al. [49] by following-up with 560 subjects from the C8 cohort in 2010 who were not taking lipid lowering medications during the interim from 2006-2010. Fitz-Simon, et al. showed these subjects had an overall 50% decrease in mean serum PFOS concentration with only a slight increase in mean serum LDL concentration ($1.8 \pm 26.6\%$). Even with this apparent increase in mean LDL concentration following reduction in serum PFOS concentration, the authors suggested a trend that a greater decrease in serum PFOS concentration produced a greater decrease in serum LDL cholesterol whereby a subject with a 50% decrease in serum PFOS concentration would be predicted to have a 5% decrease in serum LDL concentration. Fitz-Simon, et al. concluded that this longitudinal study supported the previous cross-sectional findings. However, even if the proposed trend were an accurate reflection of the population, the toxicological relevance of a 5% decrease in mean serum cholesterol is questionable given that a controlled dietary intake can produce a 15% decrease in serum total cholesterol [50] and statin drug therapies can reduce serum LDL cholesterol by 25% to 60% [50].

A two-year longitudinal study was performed by Olsen, et al. [51] using 179 workers who were demolishing perfluoroalkyl manufacturing facilities from 2008 to 2010 and were not taking lipid lowering medications. Olsen, et al. showed no adverse associations between serum concentrations of PFOA, PFOS, non-HDL cholesterol, HDL, or hepatic clinical chemistries (i.e., ALT, aspartate aminotransferase (AST), alkaline phosphatase, and total bilirubin). This study demonstrated that PFOS exposure did not lead to increased serum non-HDL cholesterol.

Based on a review of the longitudinal study data and the deficiencies noted in the cross-sectional studies above, it is unlikely that there are adverse hepatic or cholesterol effects in humans at serum PFOS concentrations measured in the general population or workers. Also, as described below, high serum PFOS concentrations in animals are associated with decreased serum cholesterol concentrations and hepatic lipid accumulation rather than increased serum cholesterol concentrations.

1.8 PFOS and Hepatic Effects in Rats and Monkeys

Goldenthal, et al. [52] demonstrated that PFOS caused hepatic vacuolation and hepatocellular hypertrophy in rats fed ≥100 ppm K^+ PFOS in the diet for 90 days. This result was further investigated by Seacat, et al. [7] who showed that dietary exposure of 5 or 20 ppm K^+ PFOS for 14 weeks in Sprague Dawley rats (5/sex/group) caused hepatic vacuolation, decreased serum total cholesterol, and/or increased serum ALT (Table 1). No hepatic biomarker or histopathology changes were noted in rats fed the same diets for four weeks [7].

Similarly, Curran, et al. [6] demonstrated that Sprague Dawley rats fed 50 or 100 ppm K^+ PFOS in the diet had hepatic "cytoplasmic homogeneity," decreased serum triglycerides (values not reported), and decreased serum cholesterol (Table 2). Cytoplasmic homogeneity is generally caused by peroxisome proliferation or expansion of the smooth ER and can be associated with hepatocellular vacuolation. Curran, et al. did not report hepatocellular vacuolation; however, it appears to be present in the rat fed 100 ppm K^+PFOS in Figure 3B of the Curran, et al. publication [6]. Regardless, Curran, et al. [6] also reported changes in hepatic fatty acid profiles including increased total monounsaturated fatty acid and linoleic acid and decreased total polyunsaturated fatty acids and longer chain fatty acids. These fatty acid changes are consistent with increased hepatic peroxisomal metabolism of long-chain fatty acids and possibly the accumulation of short-chain fatty acids. PFOS is a known peroxisome proliferator and promotes omega oxidation in the endoplasmic reticulum and beta oxidation in the peroxisomes [53, 54]. The increased mRNA expression of cytochrome P450 4A22 (CYP4A22), promoting omega oxidation, and acyl-coenzymeA oxidase 1 (ACOX1), promoting beta oxidation, reported by Curran, et al. [6] is supporting evidence for

peroxisome proliferation in this study. Peroxisome proliferation increases oxidation of fatty acids and would not account for the lipid accumulation in this study.

Additional support for hepatic lipid accumulation was shown by Seacat, et al. [8] in male and female cynomolgus monkeys (at least 4/sex/group) administered 0.75 mg/kg-day PFOS via daily oral gavage for 183 days. Hepatocellular vacuolation was confirmed by scanning electron microscopy in the 0.75 mg/kg-day male and female groups on day 183 (Figure 4). This finding was not observed at the lower PFOS doses tested (i.e., 0.03 or 0.15 mg/kg-day PFOS) or in the control group. The vacuolation was reversed in the 0.75 mg/kg-day group following 7 months of recovery. Similar to the findings in rats, decreased serum total cholesterol and HDL cholesterol were also observed in this study (Table 3). The recovery data support the conclusion that the observed hepatic vacuolation is not evidence of hepatocyte damage, but rather related to lipid metabolism and/or secretion.

The potential for decreased lipid secretion following PFOS exposure was investigated by Bijland, et al. [55] in APOE*3-Leiden CETP mice. Wild-type mice generally circulate almost all lipoproteins as HDL with lower concentrations of circulating VLDL and LDL relative to humans. However, APOE*3-Leiden CETP mice have poor clearance of apoB-containing lipoproteins (e.g. LDL) and when fed a Western diet their serum lipoprotein profile becomes similar to a human lipoprotein profile with higher serum LDL/VLDL concentrations than HDL concentrations [56]. APOE*3-Leiden CETP mice (at least 6/group) were fed a Western diet with or without 30 ppm PFOS for 4- or 6-weeks. PFOS caused a marked decrease in plasma triglycerides (TG), HDL, and non-HDL cholesterol. The deceased very low density lipoprotein (VLDL) was caused mainly by decreased production of VLDL-TG and VLDL apolipoprotein B by as much as 80% in PFOS-treated animals relative to the control group. The authors concluded that the decreased plasma VLDL was a result of decreased hepatic secretion.

The findings in rats, mice, and monkeys clearly show that hepatic vacuolation is caused by repeated high-dose oral exposure to PFOS and it appears related to lipid accumulation. Lipid accumulation is a result of changes in lipid anabolism, catabolism, or secretion.

1.9 Primary Hypothesis: PFOS forms an ion complex with choline causing a functional choline deficiency and thus hepatic lipid accumulation in rats

A schematic for the hypotheses regarding PFOS interference with lipid metabolism leading to hepatic steatosis is provided in Figure 5. Choline is an essential nutrient required for the proper formation and secretion of VLDL particles [57]. Rats fed a diet deficient in methionine and choline develop hepatic steatosis (i.e., lipid accumulation) at week 2, hepatic inflammation at week 5, and hepatic pericellular fibrosis that progresses from weeks 12 through 17 [58]. The intracellular vacuoles produced by choline deficiency in rats (Figure 6) appear similar to the vacuoles produced by PFOS exposure in monkeys (Figure 4). In addition, these rats developed increased ALT and decreased VLDL secretion. The methionine- and choline-deficient diets have been used for years to create hepatic steatosis in animal models [58-60]. The primary hypothesis is based on this mechanism whereby hepatic choline deficiency leads to hepatic steatosis.

PFOS has been shown to create an ion complex with choline (Figure 7) in an *in vitro* methanol solution and in *ex vivo* liver samples [61]. PFOS was added to increasing concentrations of choline in methanol *in vitro*. As the molar ratio of choline:PFOS increased, the LC/MS-MS detection of the free PFOS concentration decreased, which supports the hypothesis that PFOS is complexing with choline *in vitro* (Table 4). PFOS also formed a complex with choline in a liver matrix *ex vivo*. The complex was not detected initially at room temperature using LC/MS-MS; however, when the samples were incubated at 37°C for 48 hours the complex was detected in the sample. It is possible that heating the matrix caused increased enzyme activity and released additional choline molecules into the matrix resulting in the formation of a stable ion complex. The detection of this ion complex both *in vitro* and *ex vivo* supports the hypothesis that sufficiently high hepatic concentrations of PFOS could sequester choline *in vivo* leading to a functional choline deficiency and hepatic steatosis.

The choline ion complex hypothesis was evaluated by a 3-week study in rats fed control diet or diet containing 120 ppm K^+ PFOS (i.e., 100 ppm PFOS when accounting for salt and purity) both with and without choline supplementation (Chapter 3). The choline supplementation dose was selected based on the NOAEL in a 4-week choline

supplementation rangefinder study in rats (Chapter 2). The hypothesis was also evaluated by testing the potential for PFOS to inhibit choline kinase activity by sequestering choline *in vitro* (Chapter 3).

1.10 Secondary hypothesis: PFOS activates nuclear receptors known to cause hepatic steatosis

Peroxisome proliferator-activated receptor gamma (PPAR-gamma), constitutive androstane receptor (CAR) and PXR are nuclear receptors that have been implicated in the induction of hepatic steatosis [62-64]. The effects of PPAR-gamma and PXR are somewhat linked, because activated PXR upregulates PPAR-gamma mRNA production [65]. Rifampicin, a potent PXR agonist, has been known for decades to cause hepatic steatosis in rats [66, 67] and humans [68]. PXR is a xenobiotic sensing nuclear receptor that, in addition to other functions, upregulates the *Cd36* gene [69], and overexpression of CD36 leads to hepatic lipid accumulation and storage in mice [70]. If PFOS is an agonist of these nuclear receptors *in vivo*, it would be expected to cause hepatic steatosis.

A four-week dietary study by Elcombe, et al. [71] showed that PFOS induced hepatic protein expression and activity of cytochrome P450 4A1 (CYP4A1), cytochrome P450 2B1/2 (CYP2B1/2), and cytochrome P450 3A1 (CYP3A1) relative to the control group in male Sprague Dawley rats (10/timepoint/group) fed 20 ppm or 100 ppm PFOS in the diet for 1, 7, or 28 days. CYP4A1, CYP2B1/2, and CYP3A1 were used as markers for the activation of the peroxisome proliferator-activated receptor alpha (PPAR-alpha), constitutive androstane receptor (CAR), and pregnane X receptor (PXR) nuclear receptors, respectively. The mean increased activities induced in each PFOS group were lower than the increased activity caused by the positive controls used on the study (i.e., 50 ppm Wy 14,643 for CYP4A and 500 ppm phenobarbital for CYP2B1/2 and CYP3A1). However, these data suggest that CAR or PXR activation may occur following PFOS dietary exposure in rats.

Bjork, et al. [72] showed that 25 µM PFOS administered to primary rat hepatocytes upregulated mRNA production of CD36 (Table 5). Multiple other genes were affected by PFOS administration in this study including genes that are downstream of PPAR-

gamma, PXR, and CAR activation as well as liver X receptor alpha (LXR-alpha) and PPAR-alpha activation. The mRNA profile in Bjork, et al. [72] demonstrates that the liver is under tremendous lipid metabolic stress as pathways for beta-peroxidation and lipid transport are both upregulated. In addition, PFOS was identified as an agonist for human PXR, human CAR3, and mouse, rat, and human PPAR-alpha and PPAR-gamma in luciferase assays at concentrations of ≥100 µM [73, 74].

The nuclear receptor activation hypothesis was tested by an evaluation of the hepatic mRNA profile for key genes (e.g., CD36) in the 3-week PFOS rat study and the potential for PFOS to activate rat PXR, CAR, LXR-beta, and PPPAR-gamma as well as human LXR-alpha was evaluated in a luciferase assay (Chapter 3).

Chapter 2: Investigation of Four-week Dietary Choline Supplementation in Sprague Dawley Rats

2.1 Overview

The following study was performed to investigate the maximum tolerated dose or no observed adverse effect level following four-week dietary choline supplementation in Sprague Dawley rats. This rangefinder provided the information to select the appropriate choline supplementation level for the PFOS and choline supplemented study (Chapter 3). This chapter has been submitted for publication.

2.2 Synopsis

Choline is an essential nutrient utilized for phosphatidylcholine biosynthesis, and lipoprotein packaging and secretion. Recently, choline supplementation has been used by athletes and the public for weight loss. However, the potential toxicological impact of high-level choline dietary supplementation has not been investigated since 1945. This study examined the effects of choline dietary supplementation in Sprague Dawley rats for four weeks. Rats were fed diets containing choline at basal levels (control, AIN-93G, 2.5 mg choline bitartrate/kg diet) or 5, 10, or 15 times (5X, 10X, or 15X) the basal diet concentration. In groups fed choline-supplemented diets, there were no toxicologically relevant findings in clinical observations, food intake, clinical chemistry, mean liver weights (absolute and relative), or liver histopathology. However, decreased average body weights (8.5% to 10.2%) and body weight gains (24% to 31%) were noted for the 10X choline-supplemented (females only) and 15X choline-supplemented (both sexes) groups relative to the control groups from day 3 onward. These body weight effects were not related to a persistent reduction in average food intake and thus were considered evidence of a maximum tolerated dose. Serum cholesterol was increased in the 15X choline supplemented male rats relative to the controls, an expected effect of choline supplementation; however, there were no changes in the serum cholesterol of female rats. Serum choline concentrations were increased in female rats relative to the male rats without regard to the dietary concentration of choline. **Conclusion.** The maximum tolerated dose for male and female rats were the 15X and 10X choline

supplements, respectively, based on decreased body weight and body weight gains. This study supported the conclusions of a clinical trial that showed a high choline diet can decrease body weight.

2.3 Introduction

Choline is an essential dietary nutrient found in leafy green vegetables, eggs, and meat [57]. It is a central molecule for the biosynthesis of phosphatidylcholine in the liver that is then used for cellular and organelle membranes as well as a surface molecule in lipoprotein packaging [19]. Recently, commercially available choline supplements have been used by individuals attempting to decrease body weight [75, 76]. However, there is limited information on the toxicity of dietary choline supplementation. For example, the Institute of Medicine tolerable upper limit [77] was selected based on a single case report of hypotension following oral administration of 7500 mg/day choline for two weeks and vomiting, salivation, sweating, diarrhea, and fishy body odor at 16,000 mg/day choline for two weeks. In 1998, the Institute of Medicine recommended additional animal studies to investigate increasing levels of dietary choline intake on organ systems including the liver [77]. Sahu, et al. [78] showed that intraperitoneal injections of 45, 150, or 225 mg/kg-day choline chloride to rats (corresponding to 0.1, 0.33, and 0.5 of the intraperitoneal LD50 in male rats, 450 mg/kg) for 5 days/week for 5 weeks (24 injections) caused decreased body weights in the 150 and 225 mg/kg-day groups relative to the control group that persisted through 26 weeks of recovery as well as splenic and thymic cellular depletion at 8 months. An additional group of rats was injected intraperitoneally with 150 mg/kg-day choline chloride for 8 weeks and at 6 months there were lymph node architecture changes, hyperreactive bronchiolar epithelium, and hepatic lymphocyte infiltration. Sahu, et al. [78] also reported that some animals died during the course of the experiment, but the timing and conditions prior to death were not reported. Recent rat studies have fed diets supplemented with up to 5 times the choline nutritional requirement in rat growth diets (750 mg choline/kg diet (0.075 %w/w) [79]) for 7 weeks [80] or 12 weeks [81] demonstrated no evidence of toxicity. However, the potential for choline toxicity following dietary intake at higher levels than the recent studies has not been investigated in rats since 1945 [82], and that study had deficiencies that limit its utility.

In 1945, Hodge [82] studied the effect of feeding rats diets containing supplemental choline at 0.01, 1.0, 2.7, 5.0, and 10.0% by weight (w/w) for three to four months. Given that standard rodent growth diets, such as AIN-93G purified diet [83], contain 0.1% w/w choline (1000 mg choline/kg diet), the diets used by Hodge contained choline at 0.1 to 100 times (0.1X to 100X) the concentration of choline in commercial rat diets. In diets supplemented with ≥2.7% w/w choline (27X), Hodge found that decreased body weight occurred with no reported change in liver weight or histology. However, there were deficiencies in this study in that only limited descriptions of the study methods were provided, the study did not contain a basal diet control group, the number of rats was reported as 5/group with no indication of sex/group, and the strain of rats was not reported. In addition, the range of low dose choline supplemented diets was too large, because there was only one group (i.e., 10X) between 0.1X and 27X choline supplement relative to a standard basal diet.

Because the available toxicological dataset did not provide a good basis to understand the toxicological effects of choline dietary supplementation at moderate levels of exposure (e.g., up to 15X basal choline), the present study was undertaken to fill this gap in knowledge. Briefly, rats were fed diets for four weeks with 5X, 10X, or 15X supplemental choline and effects were compared with rats fed the basal diet. This investigation focused on histopathology and clinical chemistry effects on the liver, because liver is important for choline metabolism and lipoprotein production. These data allowed determination of a maximum tolerated dose for choline dietary supplementation in rats and could be useful for assessing effects of human choline supplementation.

2.4 Experimental Procedures

2.4.1 Diets

Modified pellet diets based on AIN-93G purified diet [83] were obtained from Harlan Teklad containing basal choline (control, 2.5 g choline bitartrate/kg diet, 0.1% w/w choline, catalog number TD.94045, lot 989904, undyed), 5X choline supplement (12.5 g choline bitartrate/kg diet, 0.57% w/w choline, catalog number TD.120403, lot 989912, red dyed), 10X choline supplement (25.0 g choline bitartrate/kg diet, 1.15% w/w choline, catalog number TD.120404, lot 989921, yellow dyed), and 15X choline supplement (37.5

g choline bitartrate/kg diet, 1.7% w/w choline, catalog number TD.120405, lot 989939, green dyed). The diets are presented in Table 6. The diets were color-coded by the vendor using food-grade dyes to aid appropriate dosing. The amount of corn starch used in each choline supplemented diet was reduced to make up the weight-balance for the increased choline bitartrate. Calculated diet caloric content was 3.8, 3.7, 3.7, and 3.6 kcal/g for the control, 5X, 10X, and 15X diets, respectively. These slight differences in caloric content were believed to have no potential to affect the study outcome. All diets were stored at approximately 4°C during the study and used prior to the expiration dates per the vendor instructions.

Diet homogeneity was verified by choline analysis at Covance Laboratories (Madison, WI) using a method adapted from AOAC Official Method 999.14 – Choline in infant formula and milk [84]. Briefly, three samples of each diet were obtained from the bottom, middle, and top of each container. The diet samples were hydrolyzed at 70°C and, following pH adjustment to 3.75 ± 0.25 with sodium hydroxide or hydrochloric acid as appropriate, treated with phospholipase D followed by choline oxidase to produce peroxide. In the presence of peroxidase, phenol was oxidized and a chromophore was produced with 4-aminoantipyrine. Absorbance of this chromophore at 505 nm was directly proportional to the choline content of the sample. The lower limit of quantitation of this method was 0.0067% w/w choline in the diet, which was less than the lowest target dietary concentration in this study (i.e., 0.11% w/w, basal choline control group).

2.4.2 Animal Selection and Husbandry

This study was performed in a laboratory that is accredited by the International Association for the Accreditation of Laboratory Animal Care. All procedures involving laboratory rats were reviewed and approved by the Institutional Animal Care and Use Committee associated with the facility in which the laboratory rats were housed. Animal care and procedures followed the U.S. Department of Health and Human Services guidelines (ILAR, 2010) and the “Guide for the Care and Use of Laboratory Animals” prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH publication 86-23 revised 1985). The study was performed under the supervision of an attending laboratory veterinarian who was available to evaluate the rats for pain and distress as needed. Six- to eight-weeks-old adult Sprague Dawley rats

(23/sex) were obtained from Harlan Laboratories (Indianapolis, IN) at body weights ranging from 170.7 g to 193.9 g (males) and 134.0 g to 158.6 g (females). The rats were individually housed in hanging wire cages in a dedicated animal room maintained at a temperature of 72 ± 3°F, relative humidity of 30-70%, with a minimum of 10 exchanges of room air per hour and a 12 hour light/dark cycle. Cages were changed weekly. Large marbles and Nylabone® chewables were provided to each rat as enrichment. Tap water was available *ad libitum* throughout the study. All rats were acclimated to the room conditions and fed an *ad libitum* basal diet for 7 days prior to randomization and the initiation of the study. Five rats/sex were randomly assigned to the basal diet (control) and 6 rats/sex were randomly assigned to the 5X, 10X, and 15X choline supplemented groups. There were no significant differences in the initial group mean body weights (Figures 8A and 8B). Rats were fed the applicable control or experimental diet *ad libitum* for four weeks.

2.4.3 In-Life Procedures

Clinical observations, body weights, and food intake (accounting for spillage) were recorded daily. Rats were fasted overnight prior to necropsy. Fasted body weights were recorded on day 28. All rats were euthanized on day 28 by carbon dioxide asphyxiation, and blood was collected in untreated Vacutainer® tubes by exsanguination via the abdominal aorta. The collected blood was allowed to clot and was centrifuged at 1500 x g for 15 minutes at 10°C in an Avanti J30I centrifuge. The serum was collected, transferred to tubes, and stored at -70°C until analysis for choline content and clinical chemistry parameters. All euthanized rats were subjected to gross evaluation of all organs. Liver weights were recorded. Liver samples were collected from the right major lobe and left lateral lobe and fixed in neutral-buffered formalin for histopathology.

2.4.4 Serum Choline Analysis

For serum choline analysis, each standard (serial dilutions of trimethyl-d9-choline chloride, 5 ng/mL to 500 ng/mL, Sigma Aldrich, Catalog Number 492051-1G), blank (analytical grade water prepared by first passing deionized water through a Milli-Q® (Millipore Corporation, Billerica, MA, USA) water purification system with further processing through a C-18 HPLC column) and thawed rat serum sample were prepared as 100 µL of sample in 500 µL acetonitrile. The samples were allowed to stand for 10

minutes and then were vortexed for 15 to 20 seconds. An aliquot of 600 µL of 0.1N formic acid was added, and the solution was centrifuged at 3000 x g for 10 minutes. The organic layer was transferred to an autovial and used for analysis. Serum choline concentration was determined using a Sciex API 5000 mass spectrometer (Applied Biosystems / MDS-Sciex Instrument Corporation) with Turbo Ion Spray (pneumatically assisted electrospray ionization) in positive ion mode. Separation was completed on a Phenomenex polar HPLC column (150 mm x 3.0 mm, 4 µm) with a gradient elution and a flow rate of 0.25 mL/min. Buffer solutions of acetonitrile (Burdick and Jackson, distilled in glass grade, VWR) and 0.1N formic acid (J.T. Baker) were run with initial conditions of 10% acetonitrile and 90% 0.1N formic acid and then the concentrations were adjusted to 90% acetonitrile and 10% 0.1N formic acid with an analysis time of 11.0 min. The transition ions were monitored at:

Choline: 104 amu transitioned to 60 amu

Choline Internal Standard (trimethyl-d9-labelled): 113 amu transitioned to 69 amu

Quantitation was based on these transition ions. Peak areas were integrated and the ratio of the compound to the internal standard was calculated. The peak area ratio was plotted on the “Y” axis and the analyte concentration (ng/mL) was plotted on the “X” axis.

Standard curves were evaluated with either a linear regression or quadratic equation model and reviewed for best fit. All standards were weighted at 1/x for the regression analysis. The lower limit of quantitation was assigned as the lowest standard value that was included on the regression fit of the standard curve. The lower limit of quantitation for choline content analysis was 5 ng/mL (i.e., 5 ppb). The quadratic regression R^2 for the standard curve in this study was 0.9995.

2.4.5 Clinical Chemistry

Clinical chemistry analysis of the collected serum was performed by Marshfield Labs (Marshfield, WI). Serum glucose, aspartate aminotransferase, alanine transaminase, gamma-glutamyl transferase, alkaline phosphatase, total bilirubin, direct bilirubin, indirect bilirubin, total cholesterol, HDL, LDL, TG, total protein, blood urea nitrogen, creatinine, phosphorus, calcium, sodium, potassium, chloride, bicarbonate, anion gap, creatine kinase, lactate dehydrogenase, amylase, lipase, albumin, globulin, albumin/globulin ratio, urea/creatinine ratio, and sodium/potassium ratio were all

measured using a Beckman Coulter AU 5800 Clinical Chemistry System (Brea, CA, USA).

2.4.6 Histopathology

Histopathology of the liver samples was performed by Charles River Laboratories, Inc. (Durham, NC). Formalin-fixed liver samples were embedded in paraffin, 5 µm sections were cut using a microtome, and stained with hematoxylin and eosin for histopathology evaluation. The hematoxylin and eosin stained slides were examined by a board-certified veterinary pathologist using light microscopy. Findings were scored for severity using a 0 to 4 scale (unremarkable, minimal, mild, moderate, and severe). Flash-frozen samples were prepared from the formalin-fixed liver samples, 5 µm sections were cut using a microtome, and stained with Oil Red O for fat content evaluation. The Oil Red O-stained samples were scored for the degree of fat accumulation using the same 0 to 4 scale by the same board-certified veterinary pathologist.

2.4.7 Statistical Analyses

The in-life results were evaluated for statistical differences between groups within sex by one-way ANOVA with Tukey's multiple comparisons test (GraphPad Prism®, v. 6.04). The potential for sex-related differences in choline intake and serum choline concentrations was evaluated by two-way ANOVA with Bonferroni's multiple comparisons test (GraphPad Prism®, v. 6.04). The measured choline diet concentrations were compared to target choline diet concentrations by one-sample t-tests (GraphPad Prism®, v. 6.04).

2.5 Results

2.5.1 Clinical Signs

No adverse, overt clinical signs were observed in any rat. Green feces were noted in all rats in the 15X choline group from day 6 to day 28. The green feces were similar in color to the green food-grade dye used in that diet and were not considered adverse. Although the 5X and 10X diets were red and yellow colored, respectively, no color change was observed in the feces of those groups. Minor abrasions/scabs were noted sporadically throughout the study on the ventral neck region of one male and one female

rat of the 15X choline group (data not shown). This was considered an incidental finding and was not choline supplement-related.

2.5.2 Body Weights and Body Weight Gain

Male rats in the 15X group had decreased mean daily body weight from day 3 through termination as compared to the control (8.5% - 10.2%), 5X (5.9% - 7.9%), and 10X (4.8% - 7.0%) groups (Figure 8A). There were no effects on mean daily body weight of male rats in the 5X and 10X group as compared to the controls. Female rats in the 15X group had decreased mean daily body weight (10.3% – 12.8%) from day 3 through termination as compared to the control group (Figure 8B). The female 5X and 10X body weights were not different from the control group throughout the study with the exception of day 18, where the 10X group was decreased by 9% relative to the control group. There were no significant body weight differences between the female 5X, 10X, and 15X choline supplemented groups on any day.

The mean weekly body weight gain in male rats was decreased in the 15X choline group in weeks 1 and 2 (56% and 16%, respectively) relative to the control group and was decreased relative to the 5X and 10X groups as well (Figure 8C). There were no other differences between the male groups during weeks 1 through 4. Mean overall body weight gain for males during the study was significantly decreased in the 15X group by 26% relative to the control group. The mean overall body weight gains in the male 5X and 10X groups were not different from the male control group or each other. In week 1, mean female body weight gains were decreased in the 10X and 15X groups (45% and 66%, respectively) relative to the control group (Figure 8D). In week 2, mean female body weight gain in the 15X group was decreased by 33% relative to the control group. The female body weight gains for the 5X group was similar to the control group throughout the study. There were no differences in body weight gain on weeks 3 and 4 for female rats of any group. Overall body weight gain was significantly decreased in the female 10X and 15X groups relative to the control group (24% and 31%, respectively) with no significant differences between the female choline supplemented groups (Figure 8D).

2.5.3 Food Intake

Mean food intake was decreased in male rats of the 10X group (26% decrease) and the 15X group (up to 39% decrease) relative to the control group and/or the 5X group until day 5 (Figure 9A). In addition, mean food intake was decreased in female rats of the 10X group (19% decrease relative to the 5X group) and the 15X group (up to 40% decrease relative to all groups) until day 5 (Figure 9B). From day 5 onward, there were no differences in food intake for any group of either sex.

2.5.4 Dietary Choline Concentration

The mean choline concentrations were within 7% of target concentrations with the exception of the control diet, where the mean choline concentration was 18% greater than the target concentration (Table 7). The measured values were similar to the target concentrations for each diet by one-tailed t-test ($p>0.05$). The coefficient of variation (CV) among the 3 samples for each diet was less than 8%, which indicated that the diets were homogenous (Table 7).

2.5.5 Choline Intake

Mean daily choline intake over the entire study for the control, 5X, 10X, and 15X groups, respectively, was approximately 99, 434, 868, and 1168 mg/kg-day for males and 107, 468, 922, and 1273 mg/kg-day for females (data not shown). The 10X and 15X females had increased intake relative to the corresponding male groups by 6% and 9%, respectively. This increase was not considered biologically relevant.

2.5.6 Serum Choline Concentrations

A sex-related increase in mean serum choline concentration was observed without regard to choline supplement status (Figure 10). All female groups had increased mean serum choline concentrations relative to the similarly treated male group. There were no differences in mean serum choline concentrations between any control or choline supplemented groups of the same sex.

2.5.7 Clinical Chemistry

Blood for clinical chemistry analysis could not be obtained from two females in the 10X choline group because of technical difficulties during blood collection. Blood was

successfully collected from all other rats. The remaining four females in the 10X choline group provided sufficient data for evaluation of this group's clinical chemistry response.

Most clinical chemistry parameters were within the historical reference ranges for rats reported by Marshfield Labs [85]. Incidental significant differences in mean serum total cholesterol, HDL, creatinine, urea/creatinine ratio, and albumin values that at times exceeded the historical control ranges were noted for male and/or female rats fed up to 15X choline in the diet (Table 8 and Supplemental Tables 1 and 2). There were no other significant differences in any other clinical chemistry parameters for either sex including all parameters that would be correlated with potential liver toxicity (i.e., alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, bilirubin (total, indirect and direct), gamma-glutamyl transferase, or triglycerides). None of the significantly different parameters were considered toxicologically relevant.

Mean serum total cholesterol was significantly increased in the male 15X rats relative to the control group and the 10X group and exceeded the reference range by 14%. The increase was influenced by a significant increase in mean serum HDL in male 15X rats relative to the control and 5X groups; although, this increase in serum HDL did not exceed the historical control range. There were no significant differences in female serum total cholesterol concentrations. Female serum HDL was significantly increased in the 5X and 10X groups; however, this increase was within the reference range and the lack of an increase in the 15X female rats suggested that this was not a treatment-related effect. The increased serum total cholesterol in male rats was not considered toxicologically relevant because it was influenced by elevated HDL cholesterol levels.

Mean serum creatinine values appeared similar across all groups for males (0.3 mg/dL) and females (0.3 to 0.4 mg/dL). However, there was a greater incidence of 0.2 mg/dL creatinine values in the groups that were indicated as statistically significantly different from the control group (i.e., 10X males and 15X males and females). However, this minor difference was not toxicologically relevant, because it was within the historical reference range [85].

Mean serum urea/creatinine ratio was significantly increased in the male 15X rats relative to the control group and exceeded the reference range by 9.5%. There were no significant differences in this parameter for the female rats. Based on the CV of the 15X male value (15%), the creatinine values being within the reference range, and the lack of a significant increase in blood urea nitrogen, the increased male mean serum urea/creatinine ratio likely is caused by individual animal variability and was not considered toxicologically relevant.

Mean serum albumin was decreased in the 15X female group. This decrease was within the historical control range and was not considered toxicologically relevant. There were no differences in the albumin/globulin ratio which supports this conclusion.

2.5.8 Terminal Fasted Body Weights and Absolute and Relative Liver Weights

The 15X group fasted body weights were significantly decreased by 10% to 13% relative to the control group (both sexes) and/or the 5X group (males only) (Table 9). Liver weight was significantly decreased by 13% in the male 15X group relative to the control group. There were no significant differences in the liver weight of females or liver-to-body weight ratios of both sexes for any choline supplemented group.

2.5.9 Histopathology

Liver histopathology findings in all choline supplemented groups were similar to the control groups (Table 10) with the exception of minimal hepatocellular hypertrophy that was observed in 2 of 6 of the 15X choline males and 1 of 6 of the 10X choline females. No other histopathology changes or correlative clinical pathology changes (i.e., increased alanine aminotransferase) were noted, thus this finding was considered adaptive and not adverse based on the approach proposed by Hall, et al. [86]. Minimal inflammation was observed in the control and 5X groups (both sexes) and one male of the 10X group, but was absent in the 15X group. However, this findings was only present in a small portion of each section examined and was considered incidental. Oil Red O staining showed no clear changes in fat content in males and females across the treatment groups (Table 10); however, there was a trend towards decreased amount of fat content correlated to increasing choline supplementation across the groups.

2.6 Discussion

The main effects of choline supplementation were decreased body weight and body weight gain in the 10X (females only) and 15X (both sexes) groups. This effect may have been influenced by an acute reduction in dietary intake for these groups from study initiation through day 5. However, there was no subsequent increase in dietary intake during the remainder of the study. Also, there was no indication that the body weights were normalizing to control levels over the course of the study. Mean daily body weights were decreased by 8.5% to 10.2% in the 15X male rats and by 10.3% to 12.8% in the 15X female rats from day 3 to day 28. Body weight gain over the study was decreased by 24% in the 10X female rats and by 26% and 31% in the 15X male and female rats, respectively. A 10% decrease in body weight gain in a 90-day study is considered a maximum tolerated dose [87]. Thus, the body weight changes in the present study were considered suggestive of a maximum tolerated dose.

The decreased body weights in the present study are generally consistent with the data presented by Hodge [82]. In the Hodge [82] study, after one month on the choline supplemented diets, the 10X group had no apparent changes in body weight or body weight gain, the 27X group had a 20% decrease in body weight, the 50X group had a 50% decrease in body weight, and the 100X group had a 67% decrease in body weight relative to the 0.1X group. Hodge [82] maintained the rats on the diets for up to four months and showed that the rats of the 27X, 50X, and 100X groups did not recover to body weights similar to the 0.1X group. This lack of body weight recovery was also observed in the present study where the 10X (females only) and the 15X (both sexes) did not attain control body weight levels at the end of the four-week study. When considered with the results from Hodge [82], the results in the present study demonstrate that, even at lower choline supplement concentrations, there is a clear dose-dependent correlation between decreased body weight and choline supplementation.

There was no evidence of adverse effects on the liver of male or female rats based on the results for multiple clinical chemistry parameters (e.g., alanine transaminase, aspartate aminotransferase, alkaline phosphatase, bilirubin, triglycerides, etc.), average liver weights, and liver histopathology. Similarly, Hodge [82] reported no treatment-

related histopathology findings in the liver. Mean serum total cholesterol was increased in the male 15X group and exceeded the reference range. Increased serum cholesterol is expected in the presence of a high choline diet based on the well-known importance of choline for phosphatidylcholine formation and cholesterol packaging [17, 59, 88, 89]. It is likely that the increased availability of choline led to an increase in phosphatidylcholine production and thus increased lipoprotein packaging and secretion in the liver. This is supported by the trend of decreased hepatic lipid content, as shown by Oil Red O staining, correlated to increased choline supplementation. However, the lack of significant differences in serum cholesterol concentrations in female rats suggests a difference in choline handling in female rats relative to the male rats.

Baseline serum choline concentrations were higher in female rats relative to male rats (Figure 10). There were no differences between treatment groups within each sex. The sex-related difference may indicate a greater choline reservoir in female rats. In considering potential mechanisms for this observation, one could speculate that females have a higher capacity for choline storage and production. Possible evidence for this is that females of multiple species have been shown to more readily mobilize choline when fed choline deprivation diets. For example, female mice can mobilize choline from extrahepatic tissue in response to choline deprivation [19]. Similarly, when humans were fed a choline-deficient diet for up to 42 days, 77% of men and 80% of postmenopausal women developed a choline deficient phenotype (i.e., fatty liver and/or muscle damage) while only 44% of premenopausal women had this phenotype [90]. This suggests that females have a greater capacity than males to address choline deprivation and the serum choline results in this study would point to a larger homeostatic serum choline reservoir in female rats than male rats. In addition, estrogen increases phosphatidylethanolamine-N-methyltransferase activity, the key enzyme in the conversion of phosphatidylethanolamine to phosphatidylcholine [26, 91]. Phospholipases can cleave the phosphatidylcholine and generate choline [19]. Estrogen status may be an explanation for the increased serum choline concentrations in females in this study. However, a sex-related difference in serum choline has not been reported in humans [92] and given the limited dataset in rats, the observed difference may be a species-specific effect and warrants further investigation.

The choline intakes in this study (i.e., 434 to 1273 mg/kg-day across all choline supplemented groups) greatly exceed the Institute of Medicine tolerable upper limit [77] in humans (i.e., 3500 mg/day or 60 mg/kg-day in a 58-kg woman, a standard body weight according to ISO 10993-17:2002 [93]). However, even at these high intakes there were no clinical signs of toxicity or liver morphology changes and the main effect was body weight changes. Elsawy, et al. [75] showed that administration of 2500 mg/day choline to 10-12 female martial artists/group for one week caused approximately a 10.2% decrease in body fat vs. 4% decrease for the control group (placebo) and a 12.2% decrease in body mass index vs. 7.9% decrease for the control group. Data from the present study indicate that choline dietary supplementation up to 15X basal levels can result in decreased body weight with no adverse effects on the liver in rats which would support the findings by Elsawy, et al. [75].

In the absence of other clear toxicological effects, the decreased body weight and body weight gains, although not necessarily adverse, were considered treatment related and evidence of a maximum tolerated dose at 10X (females) and 15X (males) of the basal dietary levels. These levels can be used to guide future research in which dietary choline supplementation is part of the experimental procedure.

2.7 Conclusion

The 5X choline supplemented diet was selected for use in the PFOS and choline supplemented study (Chapter 3), because there were no effects on mean body weights or body weight gain in male and female rats fed this diet.

Chapter 3: Investigation of the Proposed Mechanisms for Hepatic Steatosis Induction in Sprague Dawley Rats Following PFOS Exposure

3.1 Overview

The potential for choline dietary supplementation to attenuate PFOS-induced hepatic steatosis in male rats was investigated in the following manuscript that will be submitted for publication. The potential for PFOS to inhibit the activity of choline kinase was investigated *in vitro*. Additionally, the potential for PFOS to cause hepatic steatosis through a nuclear receptor-mediated pathway was investigated as a secondary hypothesis. One key finding of this work was that 100 ppm PFOS in the diet induced hepatic steatosis in male rats, but did not induce hepatic steatosis in female rats even while the serum and liver PFOS concentrations were similar between the sexes.

3.2 Synopsis

PFOS is an environmentally persistent chemical. Dietary 100 ppm PFOS fed to male mice and rats for four weeks caused hepatic steatosis, but the mechanism of this effect is unknown. Choline deficient diets can cause similar hepatic steatotic effects. A hepatic choline:PFOS ion complex has been hypothesized to mediate this effect in mice. This study tested the hypothesis that dietary choline supplementation can attenuate PFOS-induced hepatic steatosis in rats. Sprague Dawley rats (12/sex/group) were fed control, choline supplemented (CS), 100 ppm PFOS, or 100 ppm PFOS + choline supplemented (PFOS + CS) diets for three weeks. Male rats fed both PFOS-containing diets had decreased serum cholesterol (total, HDL, and LDL) and triglycerides (TG) on days 9, 16, and/or 23 and hepatic steatosis at termination (increased hepatic free fatty acids (FFA) and TG). Female rats fed both PFOS diets had decreased serum total cholesterol and LDL on days 9 and 16 and decreased hepatic FFA and TG at termination. Liver PFOS concentrations were similar for male and female rats fed both PFOS diets. Liver choline concentrations were increased in male rats fed both PFOS diets, but the increase was lower in the PFOS + CS group. Female liver choline concentrations were not altered by any diet. These findings demonstrate a clear sex-related difference in PFOS-induced hepatic steatosis in the rat. Additional mechanisms

evaluated (i.e., nuclear receptor activation, mRNA upregulation, and choline kinase activity inhibition) did not appear to influence hepatic steatosis. *Conclusion.* Dietary PFOS at 100 ppm induced hepatic steatosis in male, but not female, rats that was not attenuated by choline supplementation at this dose level. The mechanism of lipid accumulation and the sex-related differences warrant further investigation.

3.3 Introduction

PFOS is a fully-fluorinated eight-carbon molecule that is bioaccumulative in rats [30], monkeys [30], and humans [31] and is environmentally persistent [3]. Studies have shown that dietary exposures of 100 ppm PFOS in mice [94] and rats [6] for four weeks causes hepatic subcellular vacuolation that may indicate lipid accumulation (i.e., hepatic steatosis). This level of PFOS exposure causes serum concentrations (e.g., 400 to 600 ppm[94]) that are >30-fold relative to even the highest measured human occupational serum values in 1999 (approximately 12.8 ppm [5]) and 50,000-fold the general US population serum values in 2010 (0.008 ppm [40]). However, the mechanism for hepatic steatosis induction in rats following high PFOS dietary exposure has not been established and warrants investigation because of the public concern for widespread human exposure to PFOS.

One common method for inducing hepatic steatosis in animal models is the use of methionine-choline deficient diets [58]. Choline deficiency does not occur under normal feeding states, because standard laboratory rat diets are formulated with sufficient choline and human diets generally contain sufficient choline from leafy greens. However, feeding methionine-choline deficient diets to rats causes hepatic steatosis by week 2 that progresses to hepatic inflammation by week 5 [58] and is thus a suitable model to study hepatic steatosis. Choline deficiency reduces the available phosphatidylcholine for very low density lipoprotein (VLDL) packaging, thus reducing VLDL secretion and increasing hepatic lipid retention. These findings are consistent with the “hepatic cytoplasmic homogeneity,” decreased serum cholesterol, and decreased serum triglycerides reported by Curran, et al. [6] following 100 ppm PFOS dietary exposure for four weeks in Sprague Dawley rats. Curran, et al. reported “cytoplasmic homogeneity,” but did not report hepatocellular vacuolation, which is apparent in the liver of a rat fed 100 ppm PFOS (Figure 3B of that publication). A recent study [94] showed

that male mice that were fed 120 ppm K^+PFOS (i.e., approximately 100 ppm PFOS accounting for salt and purity) in a minimal methionine-choline deficient diet had an exacerbated hepatic steatotic phenotype relative to male mice that were fed the minimal methionine-choline deficient diet alone. In addition, the results of that study [94] supported the hypothesis of the formation of a hepatic choline-PFOS ion complex causing a functional choline deficiency as a putative mechanism that could produce the observed hepatic steatosis in mice.

To further investigate this hypothesis, male and female Sprague Dawley rats were fed basal diet (control), choline supplemented diet (CS), 100 ppm PFOS diet (PFOS), or 100 ppm PFOS with choline supplement (PFOS + CS) for three weeks to investigate the potential for choline supplementation to reverse or attenuate the lipid accumulation. The choline supplement concentration was selected based on the results of a four-week choline supplement study in Sprague Dawley rats [95]. In addition, we investigated: 1) whether key liver mRNA transcripts involved in lipid accumulation (e.g., CD36) were upregulated in the PFOS-exposed rats, 2) whether PFOS can activate key nuclear receptors *in vitro* that promote lipid accumulation (e.g., pregnane X receptor (PXR)), and 3) whether PFOS can directly inhibit choline kinase activity *in vitro*, because choline kinase converts choline to phosphocholine which is the rate-limiting step in the anabolism of phosphatidylcholine from choline.

3.4 Experimental Procedures

3.4.1 PFOS

The PFOS used in this study was potassium perfluorooctanesulfonate salt (CASRN 2795-39-3, K^+PFOS, lot 217, purity 86.9%) obtained from 3M Company (St. Paul, MN). A detailed list of the impurities in this lot was presented by Seacat, et al. [8].

3.4.2 Diets

AIN-93G diets were obtained from Dyets, Inc. (Bethlehem, PA) containing basal choline (control, 4.2 g choline bitartrate/kg diet, 1700 ppm choline, catalog number d510176, lot 3183-2), choline supplemented (choline supplemented diet (CS), 12.5 g choline bitartrate/kg diet, 5125 ppm choline, catalog number d510201, lot 3183-3), basal choline with 100 ppm PFOS (100 ppm PFOS, 4.2 g choline bitartrate/kg with 120 ppm K^+PFOS

diet, 1700 ppm choline, catalog number d510179, lot 3183-4), and choline supplemented with 100 ppm PFOS (PFOS + CS, 12.5 g choline bitartrate/kg with 120 ppm K^+PFOS diet, 5125 ppm choline, catalog number d510202, lot 3183-5). The final dietary concentration of 100 ppm PFOS accounted for the potassium salt and purity. The amount of sucrose used in each applicable diet was reduced to account for the weight-balance of the increased choline bitartrate and/or added PFOS. The caloric content of each diet was 3.9 kcal/g. All diets were stored at approximately 4°C during the study and used prior to the expiration dates per the vendor instructions.

Dietary choline concentrations were verified by choline analysis at Covance Laboratories (Madison, WI) using a method adapted from AOAC Official Method 999.14 – Choline in infant formula and milk [84]. Briefly, six samples of each diet were obtained from the bottom, middle, and top of each container. The diet samples were hydrolyzed at 70°C and, following pH adjustment to 3.75 ± 0.25 with NaOH or HCl as appropriate, treated with phospholipase D followed by choline oxidase to produce peroxide. In the presence of peroxidase, phenol was oxidized, and a chromophore was produced with 4-aminoantipyrine. Absorbance of this chromophore at 505 nm was directly related back to the choline content of the sample. The lower limit of quantitation of this method was 67 ppm choline in the diet. Diet PFOS concentrations were verified using a previously described LC-MS/MS procedure [96].

3.4.3 Animal Selection and Husbandry

This study was performed in a laboratory that is accredited by the International Association for the Accreditation of Laboratory Animal Care. All procedures involving laboratory rats were reviewed and approved by the Institutional Animal Care and Use Committee associated with the facility in which the laboratory rats were housed. Animal care and procedures followed the U.S. Department of Health and Human Services guidelines (ILAR, 2010) and the “Guide for the Care and Use of Laboratory Animals” prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH publication 86-23 revised 1985). The study was performed under the supervision of an attending laboratory veterinarian.

Six- to eight-weeks-old adult Sprague Dawley rats (48/sex) were obtained from Harlan Laboratories (Indianapolis, IN) at body weights ranging from 223.9 to 258.0 g (males) and 144.1 to 175.6 g (females). The rats were individually housed in hanging wire cages in a dedicated animal room maintained at a temperature of 72 ± 3°F, relative humidity of 30-70%, with a minimum of 10 exchanges of room air per hour and a 12-hour light/dark cycle. Room light intensity was maintained at or below 500 lumens during the study. Cages were changed weekly. Large marbles and Nylabone® chewables were provided to each rat as enrichment. Tap water was available *ad libitum* throughout the study. All rats were acclimated to the room conditions and fed an *ad libitum* basal diet for 7 days prior to randomization and the initiation of the study. Twelve rats/sex were randomly assigned to the basal diet (control), choline supplemented diet (CS), 100 ppm PFOS diet (PFOS), and 100 ppm PFOS with choline supplement diet (PFOS + CS) groups. There were no significant differences in the initial group mean body weights (data not provided). Rats were fed the applicable diet *ad libitum* for three weeks.

3.4.4 In-Life Procedures

Clinical observations, body weights, and food intake (accounting for spillage) were measured daily. Blood was collected in plain Vacutainer® tubes (without anti-coagulant) via the jugular vein of non-fasted rats on days 2, 9 and 16. Rats were fasted overnight prior to necropsy. Fasted body weights were recorded on day 23. All rats were euthanized on day 23 by CO_2 asphyxiation, and blood was collected in plain Vacutainer® tubes by exsanguination via the abdominal aorta. The collected blood was allowed to clot and was centrifuged at 1500 x g for 15 minutes at 10°C in an Avanti J30I centrifuge. The resulting serum was collected, transferred to labelled tubes, and stored at -70°C until analysis for PFOS content, choline content, and clinical chemistry parameters. All euthanized rats were subjected to gross evaluation of all organs. Liver weights were recorded. Liver samples were collected from the right median lobe and left lateral lobe and fixed in neutral-buffered formalin for histopathology. Flash frozen liver samples were collected for: 1) hepatic PFOS concentration analysis; 2) hepatic cholesterol, triglyceride, and free fatty acid content; and 3) hepatic mRNA content analysis (collected in RNALater®, Applied Biosystems). The flash frozen liver samples were stored at -70°C until analysis.

3.4.5 Serum and Liver Choline Analysis

Serum choline concentrations were measured using a method described in Bagley, et al. [95]. In brief, thawed rat serum samples were prepared at a 1:6 dilution in acetonitrile, 0.1N formic acid was added, and the solution was centrifuged at 3000 x g for 10 minutes. The organic layer was removed and stored at -70 °C until analysis.

The liver samples were prepared for choline concentration analysis using a modification of the method described by Holm, et al. [97]. The frozen liver samples were prepared at 1:6 in acetonitrile and allowed to thaw for approximately 5 minutes. The samples were homogenized in tubes containing ceramic beads using a Precellys® Evolution tissue homogenizer (Bertin Instruments) set for 10 cycles of 20 seconds each at 8000 rpm with a 30 second pause between cycles. The resulting homogenate was centrifuged at 5800 x g for two minutes, and the supernatant was collected and stored at -70 °C until analysis.

The prepared liver or serum samples were run on an acetonitrile gradient using a Sciex API 5000 mass spectrometer (Applied Biosystems / MDS-Sciex Instrument Corporation) with Turbo Ion Spray (pneumatically assisted electrospray ionization) in positive ion mode. Separation was completed on a Phenomenex polar HPLC column (150 mm x 3.0 mm, 4 µm). The transition ions were monitored at:

Choline: 104 amu transitioned to 60 amu

Choline Internal Standard (trimethyl-d9-labelled): 113 amu transitioned to 69 amu

The lower limit of quantitation for choline content analysis was 5 ng/mL (i.e., 5 ppb). The quadratic regression R^2 for the standard curve in this study was 0.9995.

3.4.6 Serum and Liver PFOS Concentration Analyses

Serum and liver PFOS concentrations were determined by LC-MS/MS using previously described methods [30].

3.4.7 Clinical Chemistry

Clinical chemistry analyses of the collected serum from day 2, 9, 16, and 23 were performed by Marshfield Labs (Marshfield, WI). All serum was evaluated for glucose, aspartate aminotransferase, alanine transaminase, gamma-glutamyl transferase,

alkaline phosphatase, total bilirubin, direct bilirubin, indirect bilirubin, total cholesterol, high density lipoprotein (HDL), low density lipoprotein (LDL), triglycerides, total protein, blood urea nitrogen, creatinine, phosphorus, calcium, sodium, potassium, chloride, bicarbonate, anion gap, creatine kinase, lactate dehydrogenase, amylase, lipase, albumin, globulin, albumin/globulin ratio, urea/creatinine ratio, and sodium/potassium ratio using a Beckman Coulter AU 5800 Clinical Chemistry System (Brea, CA, USA).

3.4.8 Histopathology

Histopathology of the liver samples was performed by Charles River Laboratories (Durham, NC). Formalin-fixed liver was embedded in paraffin; 5 µm sections were cut using a microtome and stained with hematoxylin and eosin for histopathology evaluation. The hematoxylin and eosin slides were examined by a board-certified veterinary pathologist using light microscopy. Findings were scored for severity using a 0 to 4 scale (unremarkable, minimal, mild, moderate, and severe). Two sets of flash-frozen samples were prepared from the formalin-fixed liver samples; 5 µm sections were cut using a microtome and stained with Oil Red O or osmium tetroxide for fat content evaluation. The Oil Red O-, osmium tetroxide-, and hematoxylin and eosin-stained samples were scanned at 20X magnification using a Hamamatsu NanoZoomer® whole slide scanner. Morphometric analysis was performed using the Visiopharm® software platform[98] with 40% of the entire tissue section sampled by systematic uniform random sampling. Morphometric results are presented as the percent of the sampled area that was stained.

3.4.9 Liver Total Free Fatty Acids, Cholesterol, and Triglyceride Analyses

The total free fatty acids, cholesterol, and triglyceride concentrations in each flash frozen liver sample were analyzed by Charles River Research Animal Diagnostics Services (Wilmington, MA) using the Wako HR Series NEFA-HR(2) fatty acid ELISA, Wako Total Cholesterol ELISA kit, and the Wako Triglycerides Microtiter Assay kit (Wako Diagnostics, Mountain View, CA). The protein content of each flash frozen liver sample was determined by bicinchoninic acid assay (Pierce™, BCA Protein Assay Kit, Rockford, IL).

3.4.10 mRNA Analyses

Flash frozen liver samples were selected for mRNA analyses from rats (4/sex/group) that were fed the control and PFOS only diets. To select this subset of rats, the osmium tetroxide lipid content results for each group were stratified into quartiles and one member of each quartile was randomly selected for mRNA analysis to reduce the potential for lipid content bias to influence the mRNA results. The CS and PFOS + CS groups were not analyzed for mRNA production.

Tissue homogenization, RNA isolation, and reverse transcription PCR were performed using methods described in Bjork, et al. [72]. In brief, flash frozen liver samples (0.5 cm long) were added to 1.5 mL of DNase stock solution (RNase-Free DNase Set, Qiagen, CA) that was diluted 1:8 with the Qiagen proprietary buffer (RLT) that contained 10 µL/mL beta-mercaptoethanol. The tissue was homogenized using an UltraTurrax T25. The homogenate was centrifuged on a Qiashredder column (Qiagen, CA, catalog number 79654) for one minute at 9600 x g and then transferred to an equal volume of 50% ethanol. Then, 650 µL of the solution was transferred to RNeasy mini-columns (Qiagen, CA), centrifuged for one minute at 8000 x g, and the mRNA was isolated using the RNEasy Minikit (Qiagen, CA). The resulting RNA was quantified using a NanoDrop ND-1000 (Thermo Fischer Scientific, Watham, MA). Reverse transcription was performed with the Omniscript RT kit (Qiagen, CA), nine random primers (IDT, Coralville, IA) and approximately 1.0 µg RNA per liver sample. The resulting cDNA was stored at -20°C until quantitative PCR was performed.

Primers were designed for *Rattus norvegicus* enoyl-CoA hydratase and 3-hydroxyacyl CoA dehydrogenase (*Ehhadh*), stearoyl-CoA desaturase (*Scd1*), *Rattus norvegicus* CD36 molecule (*Cd36*), cell death-inducing DFFA-like effector c (fat-specific protein, *Fsp27*), and glutathione S-transferase theta 1, transcript variant X2 (*Gsst1*) as shown in Supplemental Table 3. These rat genes were selected because the proteins they produce are important for oxidative stress response (GSST1) and lipid synthesis (SCD1), beta-oxidation (EHHADH), and transport (FSP27 and CD36). The primers were designed using the Primer-BLAST interface [99] to produce a product with ≤250 base pairs; inclusion of at least one intron, when possible; a target melting temperature of 60°C; and less than one degree of difference in melting temperature between the

forward and reverse strands. The cDNA from the liver samples was exposed to the primers and quantitative PCR was performed with a Roche LightCycler and Sybr Green Master mix kit (Roche Applied Science, Indianapolis IN). DNA standards were prepared using previously described methods [100] and the results for each primer were compared to the results for the 18s subunit to determine the fold-change for a given mRNA.

3.4.11 *In Vitro* Nuclear Receptor Activation Analyses

A luciferase reporter cell assay was performed by Indigo Biosciences (State College, PA) using HEK 293 cells transfected with plasmid hybrids for rat constitutive androstane receptor (CAR, NR1I3) or rat pregnane X receptor (PXR, NR1I2) and Chinese hamster ovary cells transfected with plasmid hybrids for human liver X receptor alpha (LXR-alpha, NR1H3), rat liver X receptor beta (LXR-beta, NR1H3), or mouse/rat peroxisome proliferator-activated receptor gamma (PPAR-gamma, NR1C3). All of the plasmids contained a yeast Gal4 N-terminal DNA binding domain and the reporter gene, firefly luciferase, was linked to the Gal4 upstream sequence. Cytotoxicity potential was assessed using the Indigo Biosciences Live Cell Multiplex assay. Cells were treated in triplicate with 0.1% DMSO (vehicle) or 0.4, 1.3, 3.7, 11.1, 33.3, or 100 µM PFOS for a 24-hour exposure. Gene-specific reference positive controls were provided by Indigo Biosciences and were used to validate each test. The rat constitutive androstane receptor test was an inverse-agonist test to evaluate the potential for activation and inhibition, while the other reporter assays were performed as agonist assays.

3.4.12 *In Vitro* Choline Kinase Activity

The potential for PFOS to inhibit choline kinase enzyme activity was evaluated in recombinant human choline kinase alpha variant 1 (Abcam, accession number P35790), human choline kinase alpha variant 2 (OriGene, accession number NP_997634), and human choline kinase beta (R&D Systems, accession number Q9Y259). Inhibition potential was measured using the NADH-coupled choline kinase assay described by Trousil, et al.[101]. In brief, the choline kinase reaction was coupled to a pyruvate-NADH reaction as shown below and the production of NAD^+ was calculated by measuring the depletion of NADH absorbance at 340 nm:

$$\text{Choline} + \text{ATP} \xrightarrow{\text{Choline Kinase}} \text{Phosphocholine} + \text{ADP}$$

ADP + Phosphoenolpyruvate –Pyruvate Kinase → Pyruvate + ATP

Pyruvate + NADH –Lactate Dehydrogenase → Lactate + NAD^+

All reagents, pyruvate kinase (lyophilized powder), and lactate dehydrogenase (lyophilized powder) were obtained at the highest purity available from Sigma Aldrich. Triplicate wells received 400 µM PFOS in 10% DMSO, the highest soluble PFOS concentration, and choline concentrations from 2 µM to 1000 µM for a 15-minute exposure (choline kinase alpha variant 1 and choline kinase beta) or a 40-minute exposure (choline kinase alpha variant 2) that was in the linear region of NADH reduction for each enzyme. The absorbance at 340 nm was measured and the resulting data were converted to a velocity of NAD^+ formation in pmole/ng protein/min. The concentration of PFOS in the dosing preparations was confirmed by LC/MS-MS using a previously described method [96]. The results of three independent experiments for each enzyme were collated.

3.4.13 Statistical Analyses

The in-life results were evaluated for statistical differences between groups within sex by one-way ANOVA with Tukey's multiple comparisons test (GraphPad Prism®, v. 6.04). The potential for sex-related differences in serum and liver choline and PFOS concentrations were evaluated by two-way ANOVA with Bonferroni's multiple comparisons test (GraphPad Prism®, v. 6.04). The measured choline and PFOS diet concentrations were compared to the target choline and PFOS diet concentrations by one-sample t-tests (GraphPad Prism®, v. 6.04). The PFOS concentration in the PFOS diet was compared to the PFOS concentration in the PFOS + CS diet by two-sample t-test (GraphPad Prism®, v. 6.04).

3.5 Results

3.5.1 Clinical Signs

No adverse clinical signs were observed in the control or CS groups of either sex of rats. Both male and female rats that were fed PFOS (+/- CS) were sensitive to light (i.e., repeated squinting and vocalization) from day 17 to termination (three to five of 12 in each sex). One female was hyper-reactive to touch on day 22, and one male had shaking, tremors, and running into the cage sides on day 23. All rats survived to scheduled necropsy. Minor abrasions/scabs were noted sporadically throughout the

study across groups (data not provided). The observed sensitivity to light, hyper-reactivity, and seizures have been previously observed in rats at the same dietary PFOS dose after approximately four weeks of exposure in longer-term studies [52].

3.5.2 Body Weights and Body Weight Gains

Male mean body weights were decreased in the PFOS + CS group (5.1 – 8.7%) relative to the control group from day 9 through termination (Figure 11A). Female mean body weights were decreased in the PFOS (6.2 – 12.9%) and the PFOS + CS (5.7 – 11.9%) groups relative to the control group starting on day 9 and day 11, respectively, through termination (Figure 11B). There were no other differences in mean body weights among the groups for either sex.

The mean body weight gains of the male and female CS groups were similar to their respective control groups throughout the study (Figure 11C and 11D). Decreased mean body weight gains were noted in both the male and female PFOS and PFOS + CS groups relative to the control groups across the study. For the males, the largest decreases relative to the control group were noted in Week 1 (PFOS: 26%, PFOS + CS: 38%) and Week 3 (PFOS: 24%, PFOS + CS: 47%). For the females, the largest decrease relative to the control group occurred in Week 3 (PFOS: 80%, PFOS + CS: 81%). There were no differences between the PFOS and PFOS + CS groups for either sex at any timepoint.

3.5.3 Food Intake

There were no differences in mean daily food intake among the groups for male or female rats throughout the study (Figures 11E and 11F).

3.5.4 Dietary Choline and PFOS Concentrations

The mean choline and PFOS concentrations were similar to the target concentrations for each diet with the exception of the PFOS diet, which was significantly less than the target concentration (Supplemental Table 4). However, the PFOS concentrations in the PFOS and PFOS + CS diets were similar to each other. The CV among the 6 samples for each diet was less than 13%, which indicated that the diets were homogenous

(Supplemental Table 4). The diets had acceptable choline concentrations, PFOS concentrations, and homogeneity for use in the study.

3.5.5 Choline and PFOS Intake

Mean daily choline intake for the control, CS, PFOS, and PFOS + CS groups, respectively, was approximately 107, 330, 102, and 307 mg/kg-day for male rats and 123, 375, 112, and 318 mg/kg-day for female rats (data not shown). Mean daily PFOS intake for both the PFOS and PFOS + CS groups was approximately 6 mg/kg-day for male rats and 6.6 mg/kg-day for female rats. Mean daily PFOS intake for the control and CS groups was not calculated because the diets contained less than 0.05 ppm PFOS (Supplemental Table 4).

3.5.6 Serum Choline and PFOS Concentrations

Non-fasted male and female mean serum choline concentrations ranged from 4 ppm to 9 ppm for all groups with the exception of the male CS group on day 9 (Figure 12A and 12B). However, the male CS group day 9 mean was influenced by four rats with 14 to 16 ppm serum choline concentrations that may have been caused by food intake shortly prior to blood collection. The female groups had statistically significantly decreased mean serum choline concentrations relative to the male groups for all groups on day 9 and the CS groups on day 16; however, the magnitude of these differences was small and was not considered biologically relevant. On day 23, the mean serum choline concentration of the fasted male PFOS + CS group was increased relative to all other male groups. Also on day 23, the mean serum choline concentration of the fasted female PFOS group was decreased relative to the control group. Sex-related differences were noted in the fasted female control and CS groups on day 23 where mean serum choline values were increased by 57% and 76%, respectively, relative to the male groups.

All serum PFOS concentrations of the control and CS animals were below the limit of quantitation (5 ppb). Mean serum PFOS concentrations in the PFOS and PFOS + CS groups increased throughout the study with no differences between the groups by sex at any timepoint (Figures 12C and 12D). Mean serum PFOS concentrations in female rats were higher than the respective male groups on days 9, 16, and 23 (Figure 12D).

3.5.7 Liver Choline and PFOS Concentrations

Mean choline concentrations were increased in the male PFOS and PFOS + CS groups relative to the control group (Figure 12E). The male rats fed PFOS + CS had decreased liver choline concentrations relative to the PFOS group, but the concentrations were not decreased to the control or CS group levels. The CV for the male PFOS and PFOS + CS group mean liver choline concentrations were high (57% and 68%, respectively). However, the mean values of the male PFOS and PFOS + CS groups were much greater than the mean values for the male control and CS groups (10-fold and 5-fold, respectively). Although there is a high degree of variability in these data, the magnitude of the differences between the male groups suggests that these data are accurate and not an artifact of the methods. There were no differences in female liver choline concentrations among the four treatment groups.

All liver PFOS concentrations of the control and CS animals were below the limit of quantitation (0.05 ppm). Mean liver PFOS concentrations were similar between the PFOS and PFOS + CS groups. There was no difference between sexes (Figure 12F).

3.5.8 Clinical Chemistry

Most clinical chemistry parameters were within the historical reference ranges for rats reported by Marshfield Labs [85]. Selected clinical chemistry results are presented in Table 11 and the complete clinical chemistry results are presented in Supplemental Tables 5 – 12. Some incidental statistically significant differences were noted in liver (i. e., aspartate aminotransferase, alanine transaminase, alkaline phosphatase, bilirubin (total, indirect and direct), gamma-glutamyl transferase), kidney (i.e., blood urea nitrogen, creatinine, urea/creatinine ratio, albumin, globulin, and albumin/globulin ratio), and pancreas clinical chemistry biomarkers (i.e., amylase) of male and/or female rats across the evaluated timepoints, but these values were within the historical reference ranges and were not considered toxicologically relevant.

Mean serum total cholesterol, LDL, and HDL values were decreased on days 9, 16, and 23 in male rats that were fed the PFOS and PFOS +CS diets relative to the control and CS groups (Table 11). In addition, mean serum triglyceride values were decreased on

days 16 and 23 in male rats that were fed PFOS and PFOS + CS relative to the control group (Table 11). Male rats that were fed the CS diet had increased mean serum total cholesterol relative to the control group on days 9 and 16, but this effect was not observed in fasted rats on day 23. Mean serum total cholesterol and HDL values were decreased on days 9 and 16 in female rats that were fed the PFOS and PFOS + CS diets relative to the control and/or CS groups. These effects were not noted in the fasted female rats on day 23. There were no differences in mean serum triglyceride or LDL values for the female PFOS or PFOS + CS groups relative to the control group at any timepoint. Lastly, there were no differences in serum cholesterol (total, HDL or LDL) or triglycerides values between the PFOS and PFOS + CS groups of either sex at any timepoint.

Mean serum lactate dehydrogenase and lipase were consistently decreased for male and female rats that were fed PFOS and PFOS + CS relative to the control and/or CS groups on days 2, 9, and 16 (Table 11). On day 23, mean serum lactate dehydrogenase was increased in the male PFOS and PFOS + CS groups relative to the CS group, but there was no difference relative to the control group. Also, on day 23, mean serum lipase was decreased in the male PFOS and PFOS + CS groups relative to the control and CS groups. There were no significant differences in mean serum lactate dehydrogenase and lipase on day 23. While increased serum lactate dehydrogenase and lipase are associated with pancreatic effects [102, 103], decreased serum lactate dehydrogenase and lipase have no toxicological relevance and therefore are not considered adverse. This conclusion is supported by the lack of differences in mean serum amylase values.

3.5.9 Terminal Fasted Body Weights and Absolute and Relative Liver Weights

There were no differences between the control and CS groups (both sexes) in mean fasted body weights, liver weights, or liver-to-body weight ratios (Table 12). There were also no differences between the PFOS and PFOS + CS groups (both sexes) for these parameters. Mean fasted body weights were decreased by 13% to 14% in the PFOS (females only) and by 7% to 14% in the PFOS + CS group (both sexes) relative to the control group and CS group. Mean absolute liver weights were increased by 28% to

33% in female rats that were fed PFOS or PFOS + CS and by 65% to 75% in male rats that were fed PFOS or PFOS + CS relative to the control and CS groups. Similarly, mean liver-to-body weight ratios were increased in both the PFOS and PFOS + CS groups (both sexes) relative to the control and CS groups. These liver weight increases are consistent with the known hepatomegaly effects of PFOS in rats [6, 71, 104], and the choline supplemented diet did not alter or reverse this PFOS effect.

3.5.10 Histopathology

Liver histology of male and female rats that were fed the CS diet were similar to the control diet (Supplemental Table 13). Liver histopathology findings were related to PFOS exposure and were not clearly influenced by choline supplement in the PFOS + CS group (Supplemental Table 13). Representative hematoxylin and eosin-, Oil Red O-, and osmium tetroxide-stained liver sections from each group are presented in Figures 13, 14, and 15, respectively. Male rats that were fed the PFOS and PFOS + CS diets had ground-glass cytoplasmic alterations, which are consistent with the known peroxisome proliferation effects of PFOS in rats [6, 71, 104]. This ground-glass cytoplasm finding was not observed in female rats. A small incidence of minimal to moderate microscopic hepatic necrosis was noted in male and female rats that were fed PFOS and PFOS + CS. A qualitative difference in hepatocellular hypertrophy incidence and severity was noted between rats that were fed PFOS relative to rats that were fed PFOS + CS; however, this difference was not supported by the morphological evaluation of hypertrophy (Table 13). Lipid content was increased in male rats that were fed PFOS and PFOS + CS relative to the control and CS groups in both the Oil Red O- and osmium tetroxide-stained morphological analyses (Table 13). There were no reproducible differences in lipid content between the female groups when the results of both the Oil Red O- and osmium tetroxide-staining were considered.

3.5.11 Liver Free Fatty Acids (FFA), Cholesterol, and Triglyceride (TG) Concentrations

Mean hepatic FFA, cholesterol, and TG concentrations were similar between the control and choline supplemented groups for males and females (Figure 16 A – F). There were no differences in hepatic cholesterol concentrations for any group. Mean hepatic FFA and TG concentrations were increased in male rats that were fed the PFOS and PFOS +

CS diets without regard to choline supplement status. However, mean hepatic FFA and TG concentrations were decreased in female rats that were fed the PFOS and PFOS + CS diets again without regard to choline supplement status. Choline supplementation did not alter hepatic FFA or TG concentrations in male or female rats that were fed diets containing PFOS.

3.5.12 mRNA Analyses

Ehhadh was upregulated 3.7-fold in male rats and 4.1-fold in female rats that were fed PFOS relative to the control groups (Supplemental Table 14). *Scd1* was upregulated 9-fold in female rats that were fed PFOS relative to the control group. All other mRNA expression fold-changes in the PFOS groups were less than 2.5-fold and were not significantly different from the control groups.

3.5.13 *In Vitro* Nuclear Receptor Activation

PFOS did not activate rat LXR-beta, human LXR-alpha, or rat PXR at concentrations up to 100 µM (Supplemental Table 15). PFOS was a mouse/rat PPAR-gamma agonist at the 100 µM concentration only. The rat CAR inverse agonist assay did not show any clear dose-related activation or inhibition of CAR. No cytotoxicity was observed at any concentration in any test.

3.5.14 *In Vitro* Choline Kinase Activity Analysis

Based on a review of the Vmax and Km for all three forms of choline kinase, PFOS did not inhibit choline kinase *in vitro* (Figure 17). The Vmax of the control-treated choline kinase beta used in this assay was 6- to 8-fold lower than the choline kinase alpha variants.

3.6 Discussion

Based on the results of this study, male rats that were fed 100 ppm PFOS in the diet for three weeks had hepatic steatosis that was not attenuated by increased dietary choline. Zhang, et al. [94] showed that 100 ppm PFOS in the diet could exacerbate the hepatic lipid accumulation caused by a minimal methionine-choline deficient diet in male mice and demonstrated the potential for a hepatic PFOS:choline-ion complex. Supplemental dietary choline prevented PFOS-induced hepatic lipid alterations in metabolism and

hepatic oxidative stress in male mice at 30 ppm PFOS in the diet [94]. However, in this study, a similar choline supplemented diet did not attenuate the PFOS-induced lipid accumulation in male rats fed 100 ppm PFOS in the diet. The differences between these studies may indicate that at lower PFOS exposures there is a hepatic molar ratio of PFOS:choline that allows choline supplementation to decrease the hepatic steatotic effects that becomes overwhelmed at higher PFOS exposures. Some additional support for this hypothesis is the hepatic choline concentrations in this study. The hepatic steatosis observed in male rats fed both PFOS diets occurred in the presence of increased hepatic choline concentrations. Male rats fed PFOS + CS diet had decreased hepatic choline concentrations relative to the PFOS group, but it did not approach control or CS group levels and there were no differences in serum cholesterol or hepatic steatotic status. Female rats fed both PFOS diets did not have hepatic steatosis or increased hepatic choline concentrations. Taken together, these data suggest that there is a relationship between hepatic choline concentrations and the induction of hepatic steatosis caused by PFOS exposure. This may be evidence for the hypothesis that *in vivo* hepatic choline accumulation resulted in a functional choline deficiency and led to hepatic steatosis in PFOS-exposed male rats.

The PFOS-induced hepatic steatosis in this study was sex-related with no evidence of hepatic steatosis occurring in female rats based on the hepatic FFA and TG concentrations as well as the Oil Red O and osmium tetroxide staining morphometric results. These findings occurred at similar hepatic PFOS concentrations in males and females, approximately 600 ppm, and were thus not related to hepatic PFOS concentration. *Scd1* mRNA was upregulated in the female PFOS group; however, this would suggest greater FFA synthesis in the PFOS females relative to the control and there was no difference in *Scd1* mRNA content between males and females that were fed the PFOS diet. Female primary rat hepatocytes have been shown to be more efficient than male hepatocytes at both oleate [105] and palmitate [106] influx. This would suggest that females should have higher hepatic lipid concentrations than males under PFOS exposure conditions. However, the increased efficiency of female hepatic cytosolic fatty acid binding protein [105] may result in a greater mobilization of lipid to VLDL that would then be secreted to the blood and taken up by the adipose tissue. Fasted female rats of the control and choline supplemented groups had increased mean

serum choline concentrations relative to the male rats in this study, and in our previous work on choline dietary supplementation in rats [95], and may be evidence that females are mobilizing VLDL more efficiently than males. In addition, liver choline concentrations were not increased in female rats fed either PFOS-containing diet suggesting that female rats utilized the available choline, possibly through VLDL secretion, and maintained hepatic choline steady-state levels. Also, there were no differences in female serum LDL and triglyceride concentrations of the PFOS groups relative to the control group in this study, which supports this proposed mechanism. Relative adiposity was not measured in this study, but it is a potential explanation for the mobilization of hepatic lipid in females and deserves further study.

Albino rats prefer cages with lower light intensity (<100 lux) relative to cages with light intensities of 100 to 380 lux [107]. Schlingmann, et al. [108] recommended a minimum light intensity of 210 lux at working height to improve performance of the animal care personnel. In this study, the light intensity within the hanging wire cages was approximately 0 lux; however, during handling the rats were exposed to light intensities of approximately 360 – 500 lux. This rapid change in light intensity while taking the rat from the cage into the room light may have contributed to the observations of light sensitivity in the PFOS and PFOS + CS groups.

Activation of nuclear receptors (i.e., CAR, PXR, LXR-alpha, LXR-beta, and PPAR-gamma) involved in lipid accumulation and upregulation of mRNA of enzymes involved in lipid metabolism (i.e., *Scd1, Cd36*, *and Fsp27*) and oxidative stress response (i.e., *Gsst1*) were investigated as potential mechanisms for PFOS-induced hepatic steatosis. While PFOS was a weak PPAR-gamma agonist at 100 µM *in vitro*, there were no other clear effects on nuclear receptors known to promote lipid accumulation. In addition, mRNA for *Scd1*, *Cd36*, *Fsp27*, and *Gsst1* were not upregulated in male rats. *Scd1* was upregulated 9-fold in female rats that were fed the PFOS diet relative to the control group; however, there was no lipid accumulation in female rats so the relevance of this finding is unclear. *Ehhadh* was upregulated in both sexes, which was expected given the role EHHADH protein plays in peroxisomal oxidation and previous findings *in vitro* where PFOS upregulated *Ehhadh* mRNA in primary rat hepatocytes [72]. Taken together, these findings suggest that PFOS-induced hepatic steatosis in male rats is not

driven by activation of these nuclear receptors or through upregulation of mRNA involved in key hepatic steatosis pathways. Recent studies have indicated a PXR agonist potential for PFOS *in vitro* on human PXR [109] and *in vivo* in mice [55] and rats [71, 104]; however, the luciferase data in the present study do not support the notion that PFOS directly activates rat PXR..

The potential for direct inhibition of choline kinase was investigated as a potential disruption of the first step in phosphatidylcholine synthesis. No effects were observed on the activity of human choline kinase alpha (variant 1 or 2) or human choline kinase beta. Rat choline kinases were not available for evaluation. However, the rat and human variants of these enzymes are >85% homologous, and the differences are not located in the ATP or choline binding sites, so it is unlikely that there would be any differences in outcome if the rat choline kinase enzymes had been evaluated. Based on the results in this study, PFOS does not directly inhibit choline kinase *in vitro* at relative PFOS:choline molar concentrations up to 200:1.

3.7 Conclusion

In conclusion, dietary exposure of rats to 100 ppm PFOS for three weeks caused sex-related hepatic steatosis in males that was not attenuated by choline supplementation. These effects were not produced through nuclear receptor activation, upregulation of hepatic steatosis-promoting mRNA, or direct inhibition of choline kinase alpha-1, alpha-2, or beta. The sex related differences in hepatic steatosis phenotype observed in this study should be examined in greater detail.

Chapter 4: Conclusions from the Investigations

These studies clearly demonstrated that 100 ppm PFOS in the diet induced hepatic steatosis in male, but not female, rats and that this effect was not prevented by choline supplementation. The differential hepatic steatotic phenotype was typified by increased hepatic TG and free fatty acids and decreased serum TG, total cholesterol, and LDL in male rats with no observed effects on these endpoints in female rats. This occurred at similar hepatic PFOS concentrations in both sexes. These findings suggest a sex-related difference in lipid handling whereby females are not affected by PFOS and continue to mobilize hepatic lipid to the serum where males do not mobilize the hepatic lipid.

Interestingly, there was also a sex-related difference in serum choline concentrations under fasted conditions observed across the studies (Chapters 2 and 3) and in liver choline in the PFOS + CS study (Chapter 3). In the choline supplement range finding study (Chapter 2), it was clear that fasted female rats had consistently higher serum choline concentrations than fasted male rats regardless of choline dietary intake. This sex-related effect was also observed in the PFOS + CS study (Chapter 3) under fasted conditions at termination, but was not present under non-fasted conditions during the study. In addition, the mean liver choline concentrations in male rats fed 100 ppm PFOS and 100 ppm PFOS + CS were approximately 4-fold higher than the mean liver choline concentrations in similarly fed female rats. These findings support the hypothesis that female rats mobilize phosphatidylcholine and lipid more efficiently from the liver during fasted conditions than male rats.

Direct choline kinase inhibition and nuclear receptor activation potential were evaluated as potential mechanisms for the PFOS-induced hepatic steatosis. Based on the choline kinase activity data (Chapter 3), PFOS does not inhibit choline kinase activity *in vitro* at the limits of PFOS solubility (400 µM). In addition, PFOS also did not induce the CAR, PXR, or LXR nuclear receptors in the luciferase assay. PFOS weakly induced PPAR-gamma at 100 µM in the luciferase assay. The mRNA for oxidative stress response (Gsst1) and lipid desaturation (Scd1) and transport (Fsp27 and Cd36) were not

upregulated in the male rats fed 100 ppm PFOS in the diet (Chapter 3). Rather, Scd1 mRNA transcripts were upregulated in the female rats fed the PFOS diet for three weeks. It is possible that the upregulation of Scd1 in the females indicates continued throughput in the production of TG (Figure 3).

There are published data that support the potential for PFOS to activate PXR. Bijland, et al. [55] showed that PFOS increased PXR and Cyp3a11 expression and decreased Cyp7a1 expression, typical findings following PXR activation, in APOE*3-Leiden CETP mice fed 30 ppm PFOS in a Western diet for 4 to 6 weeks. In addition, increased liver CYP3A1 protein content, a marker of PXR activation, was shown in two studies by Elcombe, et al. [71, 104] using Sprague-Dawley rats fed 20 or 100 ppm PFOS in the diet for 1, 7, or 28 days. A recent study [109] indicated that 30 µM PFOS could activate human PXR (32-fold activity increase relative to the negative control) in a luciferase reporter assay using transfected HepG2 cells. However, in this study, the luciferase assay showed no evidence of activation of rat PXR at concentrations up to 100 µM PFOS (Chapter 3). However, rat PXR only shares 79% identity with human PXR [110] and this structural difference has been suggested as the source of rat and human differences in CYP3A induction [110, 111], which may account for the difference between the luciferase assays. Also, the rat PXR luciferase assay does not fully represent the *in vivo* condition where PFOS is present in the liver for the duration of a given study (e.g., up to 6 weeks). The study in Chapter 3 did not include analysis of mRNA or protein content of PXR, CYP3A11 or CYP7A11. Thus, the secondary hypothesis regarding PXR nuclear receptor activation cannot be fully excluded by the data collected in this project.

Zhang, et al. [94] identified the PFOS:choline ion pair *in chemico* (i.e., 10 µM PFOS and 10 µM choline in 60% acetonitrile containing 10 mM ammonium acetate) using a Waters G2S quadrupole time of flight mass spectrometer operating in ESI+ with tandem MS. This result was similar to the previous Exygen data (Figure 7). The LC-MS/MS methods used for the liver PFOS and choline concentrations in rats (Chapter 3) detected only the free substances, in part because the 3M method is more energetic than the Zhang et al. method and disrupts the ion complex. However, during the course of this project, we attempted to locate the choline:PFOS ion complex in liver samples using multiple

methods including the Zhang et al. [94] equipment and NMR; however, the complex could not be located in liver tissue. It is possible that the wide variety of materials available for PFOS and/or choline binding in liver tissue decreased the presence of the ion complex such that it could not be detected.

Zhang, et al. [94] showed that choline supplementation could reduce the oxidative stress induced by PFOS exposure in male mice at a lower PFOS dietary concentration (i.e., 30 ppm) than the 100 ppm PFOS concentration used in Chapter 3. In addition, Zhang, et al. [94] fed male C57BL/6 mice (5-6/group) either a control diet (i.e., diet containing 3.0 g L-methionine/kg diet and 4.2 g choline bitartrate/kg diet) or a marginal choline-methionine deficient diet (mMCD) (i.e., diet containing 0.009 g L-methionine/kg diet and 0.012 g choline bitartrate/kg diet) with or without 30, 60 or 120 ppm K^+PFOS for 21 or 23 days (two replicate experiments). In that study, two mice in the 120 ppm group were euthanized moribund in the first replicate between day 21 and 23 and one mouse in the 120 ppm group was euthanized on day 21 in the second replicate. As expected, the mMCD diet exacerbated the hepatic effects of PFOS (i.e., increased serum ALT, bile acids, and total bilirubin). The mean liver concentration of PFOS was decreased in the mice fed the 120 ppm PFOS + mMCD diet relative to the 120 ppm PFOS + control diet. The mean serum concentration of PFOS was increased for all of the PFOS groups in mice fed the mMCD diet relative to the correlated control diet groups. These serum and liver concentrations indicate that PFOS accumulated in the liver to a greater extent in the presence of a normal choline diet and suggests that the proposed choline:PFOS ion complex is contributing to the accumulation of PFOS in the liver. These data in mice support the primary hypothesis. However, as shown in Chapter 3, choline supplementation had no effect on the PFOS-induced hepatic steatosis in male rats at 100 ppm in the diet. This finding may point to a key molar ratio difference driving a difference in phenotype or it may be indicative of species-specific differences in choline handling.

The *in vivo* and *in vitro* data generated during this project do not provide clear supporting evidence for the primary or secondary hypothesis. The increased liver choline concentrations in males fed 100 ppm PFOS would support the primary hypothesis. However, the lack of choline kinase inhibition *in vitro* and the lack of hepatic steatosis in

females suggest that the increased choline concentrations in the males may be correlated to the hepatic steatosis phenotype rather than causative. In addition, no evidence was generated that supported the secondary hypothesis of PFOS activating a steatotic nuclear receptor (i.e., LXR, PXR, CAR, or PPAR-gamma) based on the lack of relevant liver mRNA transcription upregulation in male rats fed PFOS and the lack of activation or weak activation of nuclear receptors in the luciferase assay. The sex-related differences in liver choline concentrations and hepatic steatosis phenotype and the potential for species differences between mice and rats can be used to guide future research.

Chapter 5: Proposed Future Directions

5.1 Introduction

The potential for a choline supplemented diet to reduce oxidative stress in male mice fed a diet containing 30 ppm PFOS [94] and the sex-related differences noted in the 3-week PFOS study (Chapter 3) should guide the directions for future research. In each case below, the studies are proposed to be performed in the order listed.

5.2 Future Hypothesis 1: There is a molar threshold relationship between PFOS:choline whereby choline supplementation prevents the PFOS-induced hepatic steatosis at 30 ppm PFOS in the diet but not at 100 ppm PFOS in the diet

As previously stated, Zhang, et al. [94] showed that choline supplementation reduced oxidative stress induced by 30 ppm PFOS dietary exposure in male mice. Those data suggested that there may be a critical hepatic concentration threshold molar relationship between PFOS and choline. In addition, the sex-related difference in liver choline concentrations at similar liver PFOS concentrations (Chapter 3) suggests that female rats are more readily utilizing choline under PFOS exposure conditions. The developed liver choline measurement method (see Section 3.4.5) could be used to measure the choline hepatic concentrations and evaluate whether this molar ratio relative to the liver PFOS concentrations influences the hepatic phenotype.

The difference between the mouse data at 30 ppm PFOS and the rat data at 100 ppm PFOS may be due to a species specific difference in choline handling; however, no published literature were located that would support a rat-mouse species difference in choline handling. It is much more likely that this is a difference in the PFOS dose in each study (Zhang, et al. [94] and Chapter 3). However, the potential for species specific differences in choline handling can be investigated as described below. The studies for this hypothesis can also be used to further evaluate the potential for PXR nuclear receptor activation.

The following studies are proposed to investigate the molar relationship hypothesis:

1. Investigate choline partitioning under PFOS-exposed conditions: The use of d9-methyl-choline in this study would allow for tracing the fate of the dietary choline supplement. Mice (6/sex/group) and rats (6/sex/group) fed diets containing d9-methyl-choline in the basal diet (control) or choline supplemented diet each with or without 30 ppm PFOS for three weeks. Snap frozen liver and serum could be analyzed for PFOS and d9-methyl-choline analytes and mRNA and protein content for PXR, Cyp3a11, and Cyp7a1. This study would investigate whether there is hepatic retention of the administered d9-methyl-choline and whether there are species-specific differences in choline and PFOS partitioning.

2. Investigate the potential for a PFOS:choline molar threshold in male mice based on the data presented in Zhang, et al. [94]: Male mice (12/group/timepoint) fed basal diet or choline supplemented diet each with or without 30 ppm PFOS for one, two, or three weeks. The liver and serum choline concentrations could be measured at each termination to determine whether there is a critical hepatic PFOS:choline ratio that occurs across the study. The PXR activation could be investigated in this study as well through analysis of liver mRNA and protein content for PXR, Cyp3a11, and Cyp7a1. Oil Red O staining at each timepoint could establish when hepatic steatosis is observed. Since the hepatic PFOS concentration is steadily increasing during the study, one would expect a threshold effect for the presentation of hepatic steatosis. In addition, the choline concentration analyses could demonstrate a critical choline:PFOS molar relationship.

3. Investigate the potential for a PFOS:choline molar threshold in male rats: Conduct a similar experiment to experiment 2 above using male rats to evaluate whether the differences observed between the Zhang, et al. data [94] and the 3-week PFOS + CS study (Chapter 3) in this project arise from differences in PFOS exposure or if the difference was a result of choline handling differences between mice and rats.

5.3 Future Hypothesis 2: PFOS inhibits microsomal triglyceride transfer protein (MTP) in male rats thereby reducing VLDL formation leading to hepatic steatosis

Based on the findings in female rats, it is likely that female rats fed PFOS are more efficient at packaging and secreting VLDL from the liver than male rats. VLDL packaging is dependent on the proper transport of TG to the endoplasmic reticulum, uptake of TG by microsomal triglyceride transfer protein (MTP), and packaging with apolipoprotein B-100 (apoB-100) to form the growing VLDL particle (Figure 18A). MTP inhibition can block the formation of the apoB-100 VLDL particle (Figure 18B). An MTP inhibitor (8aR) caused decreased plasma ApoB-100, triglyceride, and cholesterol with increased hepatic triglyceride and cholesterol and a hepatic steatosis phenotype in C57BL/6 mice [112]. In addition, a liver-specific MTP knockout mouse model demonstrated hepatic steatosis with markedly decreased plasma apoB-100 and triglyceride relative to wild-type mice [113]. Lastly, upregulation of MTP has been suggested as a possible treatment for non-alcoholic fatty liver disease which is the clinical end-state for hepatic steatosis [114].

MTP expression is heavily influenced by estrogen levels. Hepatic MTP mRNA and protein expression are greater in Sprague Dawley female rats than male rats and are regulated by estrogen [115]. In addition, estrogen enhances secretion of apoB-100 from BeWo cells, a placental cell line, through increased mRNA transcription of the genes coding for apoB-100, MTP, and protein-disulfide isomer (PDI), an MTP structural subunit [116]. Also, ovariectomized rats have significantly decreased hepatic MTP protein and mRNA leading to increased hepatic total cholesterol and TG and decrease VLDL synthesis and secretion leading to hepatic steatosis [117]. These effects were reversed by 17-beta-estradiol administration via a subcutaneous continuous release pellet at 0.012 mg/day for 8 weeks [117]. The function of MTP as a key component of VLDL production and this sex-related difference in expression make it a potential target for the sex-related difference observed in rats fed 100 ppm PFOS in the diet for three weeks. PFOS could perturb the MTP pathway by binding to the MTP active site and acting as a competitive inhibitor. This would decrease the TG available for the growing VLDL particle and result in apoB-100 proteolysis (Figure 18B). In female rats, the increased levels of estrogen could increase the expression of MTP relative to males and result in a

normal hepatic phenotype even under PFOS exposure. In addition, if female rats are mobilizing hepatic lipid to VLDL and ultimately to the adipose tissue, the adiposity profile of female rats fed PFOS should be similar to the control group while male rats fed PFOS should have less adipose tissue than the control group.

The following studies are proposed to investigate the MTP hypothesis:

1. *In vitro* MTP Activity Assay: MTP activity could be assessed with the Sigma Aldrich MTP Activity Assay kit (Catalog number MAK110) using HepG2 cells or primary rat hepatocytes exposed to vehicle (dimethyl sulfoxide) and increasing concentrations of PFOS. If this hypothesis is correct, PFOS should act as a competitive inhibitor.
2. *In vivo* PFOS effects prevention by estradiol administration: If MTP activity is inhibited by PFOS *in vitro*, then a 3-week study using the 100 ppm PFOS diet could be performed in male rats that receive a sham subcutaneous pellet or a pellet that releases 0.012 mg 17-beta-estradiol/day which was the same dose used by Barsalani et al. [117] to increase MTP protein and mRNA in ovarectomized rats. At termination, the rats could be evaluated for free fatty acids and TG in the liver and total cholesterol and TG in the serum to verify whether estrogen supplementation can reverse the PFOS-induced hepatic steatosis.
3. Investigate the potential for female rats fed PFOS to have relative adiposity similar to the control and for male rats fed PFOS to have less relative adiposity relative to the control: Feed rats (5/sex/group) basal diet or 100 ppm PFOS in the diet for three weeks. Relative adiposity would be measured by 1) weekly dual energy X-ray absorptiometry [118] during in-life and 2) total body fat collection at necropsy and calculation of the adiposity index ((total body fat/terminal body weight) x 100) [119]. At termination, collect liver for MTP protein and mRNA content analyses and serum for total cholesterol, VLDL, and TG content.

5.4 Future Hypothesis 3: PFOS is an agonist or antagonist for the farnesoid X receptor (FXR) leading to hepatic steatosis

FXR is a nuclear receptor expressed in the liver and intestine that is involved in systemic lipid and glucose homeostasis [120]. The endogenous ligands for FXR are bile acids and FXR, when activated, has widespread effects on lipid metabolism and bile secretion [120]. For example, FXR agonists increase serum LDL cholesterol and decrease serum HDL cholesterol. FXR agonists have even been suggested as treatments for nonalcoholic fatty liver disease [121]. However, these results for FXR agonists and the exact role of FXR is somewhat controversial, because the effects of FXR antagonists are mixed. Some FXR antagonists decrease serum LDL cholesterol and increase hepatic TG and have the potential to cause hepatic steatosis as a side effect [122]. However, mice fed a high fat diet and treated with or without an intestinal FXR antagonist, glycine-beta-muricholic acid, had gut microbiota changes (e.g., reduced *Fimicutes* and increased *Bacteroidetes*) that were correlated with decreased hepatic lipid and free fatty acid relative to the control group [123]. With regard to PFOS, Bijland, et al. [55] showed that PFOS decreased mRNA from genes involved in bile acid formation (Cyp7a1) and secretion (Slc10a1, Slc10a2, and Abcb11) in male APOE*3-Leiden CETP mice, which would support this hypothesis of FXR antagonism. Given the compound-specific effects of FXR agonism and antagonism and the potential role of FXR in hepatic steatosis, the potential for PFOS to modulate hepatic and/or intestinal FXR warrants further study.

There is no evidence of a sex-related difference in FXR expression. However, estrogen receptor-alpha does promote the expression of some genes that are promoted by activated FXR [124, 125] indicating some overlap in regulatory responsibility. For example, the expression of small heterodimer partner can be induced by both estrogen receptor-alpha and activated FXR [125]. Small heterodimer partner upregulates the expression of bile acids and promotes bile acid secretion [125]. If small heterodimer partner expression is increased in female rats fed PFOS relative to male rats, then female rats may more efficiently convert cholesterol to bile acids, resulting in a normal liver phenotype.

The following studies are proposed to investigate the FXR antagonist hypothesis:

1. Measure mRNA from key FXR-regulated genes (i.e., *Cyp7a1, Slc10a1, Slc10a2, and Abcb11)* in the liver of rats from the 3-week study: If FXR antagonism is the source of the PFOS-induced phenotype in rats, then there should be decreased mRNA expression for genes induced by FXR (e.g., Cyp7a1) in male rats fed the 100 ppm PFOS diet relative to the control male rats. In addition, the mRNA expression in female rats should be similar between both diets.
2. Conduct Western Blots to evaluate the FXR-regulated proteins (i.e., Cyp7a1, Slc10a1, Slc10a2, and Abcb11) from the same rats: If FXR antagonism is implicated, then there should be decreased amounts of these proteins in male rats fed the 100 ppm PFOS diet relative to the control male rats.
3. *In vitro* co-administration assay in primary rat hepatocytes: Co-administer a known FXR agonist, like chenodeoxycholate [126], and PFOS to primary rat hepatocytes, then measure the mRNA content of known FXR targets, like small heterodimer partner and CYP7A1, and conduct a Western blot to analyze for protein content. The method will be validated by using a known FXR antagonist, like guggulsterone [126], as a positive control.
4. *In vivo* co-administration of an FXR agonist or FXR intestinal antagonist to attempt prevention of steatosis: Male rats (6/group) fed basal diet or 100 ppm PFOS diet and administered saline, GW4064, a known FXR agonist [127], or glycine-beta-muricholic acid, a known FXR antagonist [123], via daily oral gavage for three weeks. If FXR is involved *in vivo* then the co-administration of the FXR agonist or antagonist should prevent the development of hepatic steatosis.

5.5 Future Hypothesis 4: PFOS increases hepatic LDL receptor protein amounts leading to increased LDL uptake from the serum and causing hepatic steatosis

The LDL receptor (LDLR) is a cell surface protein that transports circulating LDL from the serum into hepatocytes. Hepatic LDLR is mainly regulated by circulating proprotein convertase subtilisin/kexin type 9 (PCSK9) that binds to LDLR which makes that LDLR a target for ubiquitination and subsequent proteolysis [128]. PFOS could perturb this process by inhibiting PCSK9 binding leading to reduced proteolysis of LDLR and resulting in increased hepatic LDL uptake from the serum. PCSK9 inhibitors are currently being investigated as new drugs for decreasing serum LDL concentrations

[129]. Plasma PCSK9 levels are 10% higher in human females than males [130] suggesting that females would be more resistant to the effects of PCSK9 inhibition. The gene expression of LDLR and PCSK9 in ovariectomized rats were both reduced relative to sham rats indicating the key role estrogen plays in modulating this system [131]. However, increased endogenous serum estrogen concentrations in women caused apolipoprotein B-containing particles and circulating PCSK9 levels to be reduced [132]. In rats, estradiol administration caused increased LDLR protein expression through transcriptional increase of the LDLR gene and reduced PCSK9 expression through transcriptional suppression [128]. This inverse relationship of circulating estradiol to PCSK9 concentration in humans and rats suggests that this is not a likely mechanism for the observed PFOS effects, because it runs contrary to the observed sex-related hepatic steatosis effects in rats. However, the overall increased serum concentration of PCSK9 observed in women, and potentially in rats, and the rationale of increased hepatic LDLR leading to increased steatosis make this a worthy avenue of investigation should the other hypotheses be excluded.

The following studies are proposed to investigate the LDLR hypothesis:

1. *In vitro* PCSK9-LDLR Activity Assay: The potential for PFOS to inhibit PCSK9 binding to LDLR could be measured *in vitro* using a fluorescent assay kit (BioScience Catalog# 72010).
2. Measure hepatic LDLR and serum PCSK9 content *in vivo*: Rats (4/sex/group) fed basal diet or 100 ppm PFOS diet for three weeks and measure the hepatic LDLR (mRNA and protein) and PCSK9 (mRNA) and serum PCSK9 (protein). One would expect sex-related differences in PCSK9 content as the female rats increase PCSK9 production in response to the putative PFOS effect on hepatic LDLR amounts.

5.6 Conclusions

These future directions provide multiple avenues of investigation based on the findings from this research. The results of this project cannot rule out the potential for the PFOS:choline ion complex to lead to hepatic steatosis or the potential for PFOS activation of PXR *in vivo* to lead to the same phenotype. There were no clear results that directly supported either the primary or secondary hypotheses. Future studies

should focus on the sex-related difference in hepatic steatosis induction following high dose PFOS dietary exposure.

Illustrations

Table 1. Hepatic Histopathology and Serum Biomarker Results from Following Dietary Exposure of up to 20 ppm K+ PFOS for 14-weeks in Sprague-Dawley Rats (5/sex/group) Seacat, et al. [7]

Sex	**Males**					**Females**				
Dietary K^+PFOS (ppm)	**0**	**0.5**	**2**	**5**	**20**	**0**	**0.5**	**2**	**5**	**20**
PFOS Serum Concentration (µg/mL, ppm) [a]	<LOQ [b]	4.04 ± 0.80	17.1 ± 1.22	43.9 ± 4.9	148 ± 14	2.67 ± 4.58	6.96 ± 0.99	27.3 ± 2.3	64.4 ± 5.5	223 ± 22
PFOS Liver Concentration (µg/mL, ppm) [a]	0.46 ± 0.06	23.8 ± 3.5	74.0 ± 6.2	358 ± 29	568 ±107	12.0 ± 22.4	19.2 ± 3.8	69.2 ± 3.5	370 ± 22	635 ± 49
Serum Total Cholesterol (mg/dL) [a]	63 ± 13	53 ± 17	51 ± 15	57 ± 7	37 ± 13*	75 ± 15	88 ± 27	87 ± 24	70 ± 13	66 ± 14
Serum Alanine Aminotransferase (IU/l) [a]	36 ± 7	41 ± 6	41 ± 5	44 ± 14	65 ± 53*	34 ± 2.4	36 ± 9	37 ± 18	34 ± 5	39 ± 18
Hepatocellular Cytoplasmic Vacuolation Present [c]	-	-	-	Yes	Yes	-	-	-	-	Yes

* indicates $p<0.05$ relative to the control group using Dunnett's test.
[a] Values are mean ± standard deviation.
[b] LOQ = limit of quantitation (0.046 ppm).
[c] Incidence was not reported by Seacat, et al. [7].

Table 2. Hepatic Histopathology and Serum Biomarker Results Following Dietary Exposure of up to 100 ppm K+ PFOS for 4-weeks in Sprague-Dawley Rats (15/sex/group) from Curran, et al. [6]

Sex	**Males**					**Females**				
Dietary K^+PFOS (ppm)	**0**	**2**	**20**	**50**	**100**	**0**	**2**	**20**	**50**	**100**
PFOS Serum Concentration (µg/mL, ppm) [a]	0.47 ± 0.27	0.95 ± 0.13	13.45 ± 1.48	20.93 ± 2.36	29.88 ± 3.53	0.95 ± 0.51	1.50 ± 0.23	15.40 ± 1.56	31.93 ± 3.59	43.20 ± 3.95
PFOS Liver Concentration (µg/mL, ppm) [a]	0.79 ± 0.49	48.28 ± 5.81	560.23 ± 104.43	856.90 ± 353.83	1030.40 ± 162.80	0.89 ± 0.44	43.44 ± 6.79	716.55 ± 59.15	596.75 ± 158.01	1008.59 ± 49.41
Serum Total Cholesterol (mmol/L) [a]	2.54 ± 0.63	2.46 ± 0.55	2.06 ± 0.43	1.63 ± 0.31*	0.31 ± 0.18*	2.06 ± 0.36	2.02 ± 0.51	1.66 ± 0.28	1.37 ± 0.24*	0.52 ± 0.16*
Serum Alanine Aminotransferase (U/L) [a]	29.33 ± 7.26	30.07 ± 10.82	30.73 ± 4.73	37.87 ± 16.31	48.53 ± 17.48*	22.27 ± 4.88	26.33 ± 5.16	23.47 ± 4.61	27.70 ± 6.876	28.33 ± 7.55
Hepatocellular Cytoplasmic Homogeneity [b]	0/4	0/4	0/4	1/4	3/4	0/4	0/4	0/4	1/4	3/4

* indicates $p<0.05$ relative to the control group by ANOVA and pairwise comparison.
[a] Values are mean ± standard deviation.
[b] Only four rats/sex/group were subjected to histopathology. May be a similar finding to the vacuolation reported by Seacat, et al. [7].

Table 3. Hepatic Histopathology and Serum Biomarker Results Following Daily Oral Gavage of up to 0.75 mg/kg-day K+ PFOS for 183 days in Cynomolgus Monkeys (at least 4/sex/group) from Seacat, et al. [8]

Sex	**Males**				**Females**			
K^+PFOS (mg/kg-day)	**0**	**0.03**	**0.15**	**0.75**	**0**	**0.03**	**0.15**	**0.75**
PFOS Serum Concentration (µg/mL, ppm) [a]	0.05 ± 0.01	15.8 ± 1.4	82.6 ± 25.2	173 ± 37	0.05 ± 0.02	13.2 ± 1.4	66.8 ± 10.8	171 ± 22
PFOS Liver Concentration (µg/mL, ppm) [a]	0.12 ± 0.03	17.3 ± 4.7	58.8 ± 19.5	395 ± 24	0.11 ± 0.02	22.8 ± 2.1	69.5 ± 14.9	273 ± 14
Serum Total Cholesterol (mg/dL) [a]	152 ± 28	110 ± 17**	147 ± 24	48 ± 19*,**	160 ± 47	122 ± 22	129 ± 22	82 ± 15*,**
Serum Alanine Aminotransferase (mg/dL) [a]	63 ± 11	42 ± 4**	48 ± 14	13 ± 5**	56 ± 16	42 ± 9	36 ± 12**	21 ± 7**
Hepatocellular Vacuolation [b]	-	-	-	Yes	-	-	-	Yes

* Significantly decreased from pretreatment values (day -27) by a two-tailed paired Student's t-test.
** Significantly different from control using Dunnett's t-test ($p<0.05$, 2-tailed, homoscedastic).
[a] Values are mean ± standard deviation.
[b] Incidence was not reported by Seacat, et al. [8].

Table 4. Free PFOS LC/MS-MS Peak Decreases when Choline Concentration is Increased

Sample	Choline (g)	PFOS (g)	Methanol (mL)	Choline:PFOS Molar Ratio	Intensity		
					Free Choline (+244 m/z)	Free PFOS (+578 m/z)	Complex (+707 m/z)
1	0.0124	0.05082	10	1.17	1.00E+06	5.50E+06	7.80E+06
2	0.0205	0.05053	10	1.95	1.20E+06	4.00E+06	7.50E+06
3	0.0559	0.05272	10	5.09	4.00E+06	1.80E+06	7.10E+06
4	0.1125	0.05564	10	9.70	5.20E+06	0.50E+06	6.80E+06
5	0.2393	0.05005	10	22.94	7.20E+06	Not detected	5.00E+06

Free Choline (+244 m/z) is two choline molecules + one [Cl^-] molecule
Free PFOS (+578 m/z) is [$PFOS^-$][K^+][K^+]

Table 5. Evaluation of mRNA Affected by 25 µM PFOS in Primary Rat Hepatocytes from Bjork, et al. [72]

Metabolic Pathway	Result after PFOS treatment	Rat mRNA Name	mRNA Abbr.	Fold-change	Nuclear receptor, hormone, etc. known to upregulate this gene	Nuclear receptor, hormone, etc. known to downregulate this gene
Fatty acid oxidation	Upregulated	Aldehyde dehydrogenase 1 family, member A1	Aldh1a1	1.5	PPAR-alpha [133] PXR [62] CAR [62]	Unknown
		Acyl-Coenzyme A oxidase 1, palmitoyl	Acox1	3	PPAR-alpha [72]	Unknown
		Enoyl-Coenzyme A, hydratase	Ehhadh	21.8	PPAR-alpha [72]	T3 [134]
		Cytochrome P450, family 4, sub-family A, polypeptide 1	Cyp4a1	27.3	PPAR-alpha [72]	T3 [135]

Table 5. Evaluation of mRNA Affected by 25 µM PFOS in Primary Rat Hepatocytes from Bjork, et al. [72]

Metabolic Pathway	Result after PFOS treatment	Rat mRNA Name	mRNA Abbr.	Fold-change	Nuclear receptor, hormone, etc. known to upregulate this gene	Nuclear receptor, hormone, etc. known to downregulate this gene
Fatty acid synthesis	Downregulated	Fatty acid synthase	Fasn	0.7	LXR-alpha [136]	Long chain fatty acids [137]
	Upregulated	Sterol regulatory element binding transcription factor 1	Srebf1	1.9	LXR-alpha [72]	Unknown
		Stearoyl-CoA desaturase 1	Scd1	4	PXR [65] LXR-alpha [138]	Mainly hormonal regulation through ERK 1/2 MAPK (3,3',5-triiodothyronine (T3)) or inhibition insulin stimulating effects (glucagon) [139]
Ketogenesis	Upregulated	3-Hydroxy-3-methylglutaryl-Coenzyme A synthase 2 (mitochondrial)	Hmgcs2	1.9	PPAR-alpha [140]	PXR [141]
Fatty acid influx cellular transporter	Upregulated	CD36 molecule (thrombospondin receptor)	Cd36	2.8	PXR [65] PPAR-gamma [65] LXR-alpha [65]	Unknown
Glucose influx/efflux cellular transporter	Downregulated	Solute carrier family 2 (facilitated glucose transporter), member 2	Slc2a2 also known as Glut2	0.6	FOXA2 [142]	PXR [143] PXR can inhibit of the FOXA2 dependent upregulation (e.g., Hmgcs2) [141]

Table 5. Evaluation of mRNA Affected by 25 µM PFOS in Primary Rat Hepatocytes from Bjork, et al. [72]

Metabolic Pathway	Result after PFOS treatment	Rat mRNA Name	mRNA Abbr.	Fold-change	Nuclear receptor, hormone, etc. known to upregulate this gene	Nuclear receptor, hormone, etc. known to downregulate this gene
Glycolysis	Upregulated	Pyruvate dehydrogenase kinase, isozyme 4	Pdk4	7.3	PPAR-alpha [144] FOXO1 [145]	Insulin suppresses FOXO upregulation [146]
	Downregulated	Pyruvate kinase, liver and red blood cells	Pklr	0.2	HNF-4 [147]	Possibly PXR. PXR agonists can inhibit HNF4-alpha dependent upregulation in other genes [148].
Glycerolipid synthesis	Upregulated	Glycerol-3-phosphate acyltransferase, mitochondrial	Gpam	1.5	PPAR-gamma [149] LXR-alpha [149] CCAAT/Enhancer binding protein alpha (CEBPA) [149]	FOXA1 [150]
		Glycerol kinase	Gk	3.6	PPAR-alpha [151]	Unknown
Urea Cycle	Down-regulated	Orthine carbamoyltrans-ferase	Otc	0.6	HNF-4 [152]	Possibly PXR. PXR agonists can inhibit HNF4-alpha dependent upregulation in other genes [148]
		Argininosuccinate synthetase 1	Ass1	0.6	CREB [153]	PPAR-alpha [154] Possibly PXR. Activated PXR can bind to CREB and lead to suppression of some genes (e.g. Glucose-6-phosphate [155]).

Table 6. Compositions of the Modified AIN-93G Purified Diets (Control and 5X, 10X, and 15X Basal Choline Supplemented Diets)

Ingredient / Content	Control	5X Choline	10X Choline	15X Choline
Casein (g/kg)	200.0	200.0	200.0	200.0
L-Cystine (g/kg)	3.0	3.0	3.0	3.0
Corn Starch (g/kg)	397.486	389.386	374.886	362.386
Maltodextrin (g/kg)	132.0	132.0	132.0	132.0
Sucrose (g/kg)	100.0	100.0	100.0	100.0
Soybean Oil (g/kg)	70.0	70.0	70.0	70.0
Cellulose (g/kg)	50.0	50.0	50.0	50.0
Mineral Mix, AIN-93G-MX (94046, g/kg)	35.0	35.0	35.0	35.0
Vitamin Mix, AIN-93-VX (94047, g/kg)	10.0	10.0	10.0	10.0
Choline Bitartrate (g/kg)	2.5	12.5	25.0	37.5
Tert-Butylhydroquinone, antioxidant (g/kg)	0.014	0.014	0.014	0.014
Food-grade color additive[1]	None	Red (0.1 g/kg)	Yellow (0.1 g/kg)	Green (0.1 g/kg)

Table 6. Compositions of the Modified AIN-93G Purified Diets (Control and 5X, 10X, and 15X Basal Choline Supplemented Diets)

Ingredient / Content	Control	5X Choline	10X Choline	15X Choline
Protein content (% w/w)	17.7	17.7	17.7	17.7
Carbohydrate (% w/w)	60.1	59.2	58.0	56.9
Fat (% w/w)	7.2	7.2	7.2	7.2
Energy (kcal/g)	3.8	3.7	3.7	3.6

1. The pigments used were Red = FD&C Red #40 (CAS# 25956-17-6) Yellow = FD&C Yellow #5 (CAS# 1934-21-0) and Green = FD&C Yellow #5 (CAS# 1934-21-0) and FD&C Green #3, (CAS# 2353-45-9).

Table 7. Choline Dietary Concentrations (%w/w) in the Control (Basal Choline), 5X, 10X, and 15X Choline Supplemented Diets

Diet	Target Choline Concentration (% w/w)	Bottom sample (% w/w)	Mid Sample (% w/w)	Top Sample (% w/w)	Mean Choline Concentration (% w/w, ±SD)[1]	Coefficient of Variation	Percent of Target Concentration (measured mean relative to target mean)
Control	0.11	0.134	0.136	0.120	0.13 ± 0.01	7.7%	118%
5X Choline	0.57	0.608	0.573	0.567	0.58 ± 0.02	3.4%	102%
10X Choline	1.15	1.13	1.27	1.11	1.17 ± 0.09	7.7%	102%
15X Choline	1.72	1.53	1.68	1.61	1.61 ± 0.08	5.0%	94%

Lower limit of quantitation = 0.0067% w/w choline

1. No significance difference between the mean and the target concentration for each diet based on one-sample t-tests ($p<0.05$, GraphPad Prism®, v. 6.04).

Table 8. Selected Mean (±SD) Clinical Chemistry Results From Rats that were fed Control Diet or Diets Supplemented with 5X, 10X, or 15X Basal Choline for Four Weeks[1]

Sex	Male				Female[2]			
Parameter (units)	**Control (basal choline)**	**5X Choline**	**10X Choline**	**15X Choline**	**Control (basal choline)**	**5X Choline**	**10X Choline**	**15X Choline**
Aspartate aminotransferase (U/L)	107.8 ± 21.7	97.8 ± 11.1	97.0 ± 10.1	98.5 ± 22.7	103.2 ± 13.0	109.5 ± 7.7	100.8 ± 9.5	98.8 ± 6.9
Alanine transaminase (U/L)	35.6 ± 1.1	33.7 ± 3.7	32.8 ± 6.9	39.8 ± 8.9	34.4 ± 6.8	34.5 ± 2.2	33.8 ± 6.3	34.8 ± 10.8
Total bilirubin (mg/dL)[3]	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Gamma-glutamyl transferase (U/L)[3]	<3	<3	<3	<3	<3	<3	<3	<3
Total cholesterol (mg/dL)	80.8 ± 5.9	92.3 ± 10.3	87.5 ± 7.7[d]	105.2 ± 11.3[a,c]	86.4 ± 9.4	94.5 ± 9.9	100.0 ± 24.7	89.2 ± 14.9
High density lipoprotein (mg/dL)	27.6 ± 1.5	27.0 ± 2.0[d]	29.7 ± 2.3	34.7 ± 5.1[a,b]	27.8 ± 2.7	32.7 ± 1.2[a]	33.5 ± 3.7 [a]	32.3 ± 3.2

Table 8. Selected Mean (±SD) Clinical Chemistry Results From Rats that were fed Control Diet or Diets Supplemented with 5X, 10X, or 15X Basal Choline for Four Weeks[1]

Sex	Male				Female[2]			
Parameter (units)	**Control (basal choline)**	**5X Choline**	**10X Choline**	**15X Choline**	**Control (basal choline)**	**5X Choline**	**10X Choline**	**15X Choline**
Low density lipoprotein (mg/dL)	8.2 ± 1.6	9.5 ± 1.6	9.0 ± 2.4	10.7 ± 1.9	6.4 ± 0.9	6.3 ± 0.8	6.8 ± 1.0	7.3 ± 1.0
Blood urea nitrogen (mg/dL)	16.4 ± 1.5	16.3 ± 2.1	16.3 ± 1.5	16.8 ± 2.1	19.2 ± 1.8	17.3 ± 2.4	18.0 ± 1.6	17.2 ± 2.7
Creatinine (mg/dL)	0.3 ± 0.1	0.3 ± 0.0	0.3 ± 0.1[a]	0.3 ± 0.1[a]	0.4 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.1[a]
Urea/Creatinine Ratio	49.2 ± 8.3	58.7 ± 10.4	67.7 ± 14.5	69.0 ± 10.2[a]	54.0 ± 6.5	53.5 ± 12.9	56.3 ± 9.4	66.7 ± 19.3
Albumin (g/dL)	3.6 ± 0.0	3.6 ± 0.2	3.6 ± 0.1	3.5 ± 0.2	3.6 ± 0.2	3.6 ± 0.1[d]	3.5 ± 0.1	3.3 ± 0.1[a,b]
Globulin (g/dL)	3.4 ± 0.2	3.4 ± 0.2	3.5 ± 0.1	3.4 ± 0.2	3.2 ± 0.2	3.2 ± 0.2	3.3 ± 0.1	3.2 ± 0.1
Albumin/Globulin Ratio	1.1 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	1.1 ± 0.0	1.1 ± 0.1	1.1 ± 0.0	1.1 ± 0.1

Table 8. Selected Mean (±SD) Clinical Chemistry Results From Rats that were fed Control Diet or Diets Supplemented with 5X, 10X, or 15X Basal Choline for Four Weeks[1]

Sex	Male				Female[2]			
Parameter (units)	**Control (basal choline)**	**5X Choline**	**10X Choline**	**15X Choline**	**Control (basal choline)**	**5X Choline**	**10X Choline**	**15X Choline**

1. Mean (±SD) values for all evaluated clinical chemistry parameters are provided in Supplemental Table 1 (males) and 2 (females).

2. N = 5 control males and 6 males/choline supplement group. Not enough blood was not successfully collected from two females of the 10X choline group for clinical chemistry analysis due to technical difficulties. Sufficient animals remained for evaluation of this group. N = 4 female rats for the 10X Choline group, 5 control females, and 6 females/group for the remaining choline supplement groups.

3. Total bilirubin and gamma-glutamyl transferase values were at or below the limits of detection, 0.1 mg/dL and 3 U/L, respectively.

Letter code indicates significantly different parameter by group using one-way ANOVA with Tukey's multiple comparisons test ($p<0.05$, GraphPad Prism®, v. 6.04). (a) vs. control, (b) vs. 5X, (c) vs. 10X, and (d) vs. 15X.

Table 9. Fasted Body Weights, Absolute Liver Weights, and Relative Liver Weights for Male and Female Sprague Dawley Rats fed Control Diet or Diets Supplemented with 5X, 10X, or 15X Basal Choline for Four Weeks

Sex	Male				Female			
Parameter (units)	**Control (basal choline)[1]**	**5X Choline**	**10X Choline**	**15X Choline**	**Control (basal choline)**	**5X Choline**	**10X Choline**	**15X Choline**
Fasted Body Weight (g)	301.8 ± 8.9	294.0 ± 10.0[c]	291.3 ± 9.5	271.4 ± 17.7[a,b]	209.3 ± 19.9	197.8 ± 12.4	190.6 ± 8.2	182.4 ± 6.0[a]
Liver Weight (g)	10.3 ± 0.8	9.5 ± 0.5	9.7 ± 0.6	8.9 ± 0.9[a]	6.4 ± 1.0	6.4 ± 0.5	6.2 ± 0.5	5.8 ± 0.4
Liver-to-Body Weight ratio	0.034 ± 0.002	0.032 ± 0.0009	0.033 ± 0.001	0.033 ± 0.002	0.030 ± 0.002	0.032 ± 0.001	0.032 ± 0.002	0.032 ± 0.002

1. N = 5 control rats/sex and 6 choline supplement rats/sex/group.

Letter code indicates significantly different parameter by group within sex using one-way ANOVA with Tukey's multiple comparisons test ($p<0.05$, GraphPad Prism®, v. 6.04). (a) vs. control, (b) vs. 5X, (c) vs. 15X. There were no significant differences between the 10X groups and any other group for any parameter.

Table 10. Incidence and Severity of Liver Histopathology Results for Male and Female Sprague Dawley Rats fed Control Diet or Diets Supplemented with 5X, 10X, or 15X Basal Choline for Four Weeks

Sex	Male				Female			
Group	**Control (basal choline)**	**5X Choline**	**10X Choline**	**15X Choline**	**Control (basal choline)**	**5X Choline**	**10X Choline**	**15X Choline**
Number of Animals	**5**	**6**	**6**	**6**	**5**	**6**	**6**	**6**
Parameter (severity)								
Inflammation	3	3	1	0	2	1	0	0
- Minimal	3	3	1	- [a]	2	1	-	-
Necrosis, focal, hepatocytes	1	1	0	0	0	0	0	0
- Minimal	1	0	-	-	-	-	-	-
Vacuolation, hepatocellular, periportal	0	0	0	0	2	2	0	0
- Minimal	-	-	-	-	1	2	-	-
- Mild	-	-	-	-	-	-	-	-
- Moderate	-	-	-	-	1	-	-	-
Hypertrophy, hepatocellular	0	0	0	2	0	0	1	0
- Minimal	-	-	-	2	-	-	1	-

Table 10. Incidence and Severity of Liver Histopathology Results for Male and Female Sprague Dawley Rats fed Control Diet or Diets Supplemented with 5X, 10X, or 15X Basal Choline for Four Weeks

Sex	**Male**				**Female**			
Group	**Control (basal choline)**	**5X Choline**	**10X Choline**	**15X Choline**	**Control (basal choline)**	**5X Choline**	**10X Choline**	**15X Choline**
Number of Animals	**5**	**6**	**6**	**6**	**5**	**6**	**6**	**6**
Parameter (severity)								
Oil Red O Staining	5	6	6	6	5	6	6	6
- Minimal	-	2	3	4	-	-	1	4
- Mild	5	4	3	2	3	5	5	2
- Moderate	-	-	-	-	2	1	-	-

[a] – indicates there were no observations for this group at this severity.

Table 11. Selected Mean (±SD) Clinical Chemistry Results of Male and Female Sprague Dawley Rats (n = 12/sex/group) fed Control, Choline Supplemented (CS), 100 ppm PFOS, or 100 ppm PFOS + CS Diets for Three Weeks[1]

Sex	Male				Female			
Group	**Control (basal diet)**	**CS**[2]	**100 ppm PFOS**	**100 ppm PFOS + CS**[2]	**Control (basal diet)**	**CS**	**100 ppm PFOS**	**100 ppm PFOS + CS**
Day 2[3]								
Aspartate aminotransferase (U/L)	69 ± 7.2	61 ± 6.7[a]	58 ± 3.8[a]	61 ± 6.3[a]	65 ± 7.0	60 ± 8.1[c]	70 ± 9.9[b]	60 ± 5.0[c]
Alanine transaminase (U/L)	29 ± 4.0	27 ± 3.1	26 ± 3.0	25 ± 3.1[a]	19 ± 3.6	20 ± 1.9	21 ± 3.2	21 ± 2.3
Total bilirubin (mg/dL)	0.02 ± 0.0	0.03 ± 0.0	0.01 ± 0.03	0.01 ± 0.03	0.0 ± 0.0[4]	0.01 ± 0.03	0.01 ± 0.0	0.0 ± 0.0
Total cholesterol (mg/dL)	104 ± 9.6	103 ± 12.0	97 ± 9.7	101 ± 5.5	82 ± 15.0	93 ± 9.0	97 ± 12.9[a]	98 ± 15.7[a]
High density lipoprotein (mg/dL)	36 ± 3.3	35 ± 3.4	36 ± 3.0	37 ± 2.3	29.8 ± 3.5[4]	29.6 ± 2.5	30.8 ± 2.7	30.6 ± 2.9
Low density lipoprotein (mg/dL)	22 ± 2.7	21 ± 3.3 [c, d]	17 ± 2.3 [a, b]	17 ± 1.6 [a, b]	12.9 ± 1.8[4]	14.2 ± 2.2	14.8 ± 2.4	13.6 ± 2.5
Triglycerides (mg/dL)	117 ± 40.6	159 ± 57.6	119 ± 39.1	115 ± 51.2	50 ± 13.6	52 ± 20.2	70 ± 38.3	54 ± 18.6
Blood urea nitrogen (mg/dL)	15 ± 1.8	16 ± 2.7[c, d]	13 ± 2.5[b]	13 ± 2.1[b]	15.7 ± 3.1	14.4 ± 2.8	12.9 ± 2.3	12.4 ± 2.6[a]
Creatinine (mg/dL)	0.1 ± 0.1	0.1 ± 0.05	0.1 ± 0.04	0.1 ± 0.1	0.1 ± 0.0[4]	0.1 ± 0.03	0.2 ± 0.1	0.1 ± 0.0

Table 11. Selected Mean (±SD) Clinical Chemistry Results of Male and Female Sprague Dawley Rats (n = 12/sex/group) fed Control, Choline Supplemented (CS), 100 ppm PFOS, or 100 ppm PFOS + CS Diets for Three Weeks[1]

Sex	**Male**				**Female**			
Group	**Control (basal diet)**	**CS**[2]	**100 ppm PFOS**	**100 ppm PFOS + CS**[2]	**Control (basal diet)**	**CS**	**100 ppm PFOS**	**100 ppm PFOS + CS**
Urea/Creatinine Ratio	121 ± 47.0	142 ± 45.1	123 ± 40.8	104 ± 36.6	145.8 ± 40.8[4]	137 ± 31.8	99.6 ± 44.9	102 ± 34.5
Albumin (g/dL)	3.3 ± 0.1	3.2 ± 0.1	3.2 ± 0.1	3.3 ± 0.1	3.1 ± 0.5[4]	3.2 ± 0.2	3.3 ± 0.2	3.3 ± 0.1
Globulin (g/dL)	2.7 ± 0.1	2.7 ± 0.2	2.6 ± 0.1	2.8 ± 0.1	2.2 ± 0.4	2.3 ± 0.1	2.4 ± 0.1[a]	2.5 ± 0.1[a, b]
Albumin/Globulin Ratio	1.2 ± 0.1	1.2 ± 0.1	1.2 ± 0.1	1.2 ± 0.1	1.5 ± 0.3	1.4 ± 0.1	1.4 ± 0.1	1.3 ± 0.1
Lactate dehydrogenase (U/L)	298 ± 186	171 ± 142.6	119 ± 65.2[a]	161 ± 57.8[a]	457 ± 97.9	291 ± 185	565 ± 320[b, d]	210 ± 91[c]
Lipase (U/L)	6.4 ± 1.0	7.3 ± 1.3 [c, d]	4.1 ± 0.8 [a, b]	3.9 ± 1.4 a, b	1.9 ± 0.9	2.2 ± 1.1[c]	3.7 ± 1.2[a, b, d]	2.3 ± 1.0[c]
Day 9[3]								
Aspartate aminotransferase (U/L)	89 ± 20.0	76 ± 8.3[a]	64 ± 5.2[a]	63 ± 6.8[a]	70 ± 7.5	59 ± 5.1[a]	59 ± 5.3[a]	58 ± 3.9[a]
Alanine transaminase (U/L)	32 ± 6.3	28 ± 3.2	28 ± 3.7	26 ± 3.7[a]	26 ± 5.1	22 ± 4.3	26 ± 5.9	24 ± 3.8
Total bilirubin (mg/dL)	0.1 ± 0.1	0.0 ± 0.1 [a]	0.02 ± 0.04 [a]	0.01 ± 0.03[a]	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0

Table 11. Selected Mean (±SD) Clinical Chemistry Results of Male and Female Sprague Dawley Rats (n = 12/sex/group) fed Control, Choline Supplemented (CS), 100 ppm PFOS, or 100 ppm PFOS + CS Diets for Three Weeks[1]

Sex	Male				Female			
Group	**Control (basal diet)**	**CS**[2]	**100 ppm PFOS**	**100 ppm PFOS + CS**[2]	**Control (basal diet)**	**CS**	**100 ppm PFOS**	**100 ppm PFOS + CS**
Total cholesterol (mg/dL)	96 ± 9.4	106 ± 7.7[a, c, d]	74 ± 8.1[a, b]	81 ± 7.9[a, b]	92 ± 7.6	99 ± 12.3[c, d]	82 ± 12.0[b]	83 ± 11.4[b]
High density lipoprotein (mg/dL)	40 ± 3.4	43 ± 3.5[c, d]	31 ± 4.3[a, b]	32 ± 2.9[a, b]	33.4 ± 2.5	34.7 ± 2.3[c]	32.4 ± 3.4[b]	30.8 ± 2.1
Low density lipoprotein (mg/dL)	17 ± 2.2	16 ± 1.9[c, d]	13 ± 2.3[a, b]	13 ± 2.2[a, b]	13.0 ± 2.2	13.1 ± 2.5	10.9 ± 2.4	11.0 ± 2.7
Triglycerides (mg/dL)	116 ± 38.5	121 ± 46.6[c]	85 ± 23.6	80 ± 17.9[b]	46 ± 6.8	54 ± 23.8	50.6 ± 9.6	57 ± 13.1
Blood urea nitrogen (mg/dL)	13 ± 1.8	12 ± 1.6[d]	10 ± 2.3[a]	8.6 ± 1.6[a, b]	14 ± 2.1	15 ± 2.8[c, d]	11 ± 3.0[a, b]	11 ± 2.1[a, b]
Creatinine (mg/dL)	0.2 ± 0.1	0.2 ± 0.03	0.2 ± 0.04	0.2 ± 0.03	0.2 ± 0.04	0.2 ± 0.05	0.2 ± 0.1	0.2 ± 0.0
Urea/Creatinine Ratio	56 ± 12.4	59 ± 9.8	54 ± 22.1	47 ± 15.9	74.0 ±12.7	66.0 ± 15.4	53.5 ± 23.5	59.6 ± 27.2
Albumin (g/dL)	3.6 ± 0.1	3.5 ± 0.1[c]	3.7 ± 0.1[a, b]	3.6 ± 0.1[c]	3.7 ± 0.1	3.6 ± 0.1[c, d]	3.9 ± 0.1[a, b]	3.7 ± 0.2[b, c]
Globulin (g/dL)	2.8 ± 0.2	2.9 ± 0.1	2.8 ± 0.1	2.8 ± 0.1	2.5 ± 0.1	2.6 ± 0.1	2.6 ± 0.1	2.5 ± 0.1[b, c]
Albumin/Globulin Ratio	1.3 ± 0.1	1.2 ± 0.05 [c, d]	1.4 ± 0.1[a, b]	1.3 ± 0.1[b]	1.4 ± 0.1	1.4 ± 0.0[c, d]	1.5 ± 0.1[b]	1.5 ± 0.1[b]

Table 11. Selected Mean (±SD) Clinical Chemistry Results of Male and Female Sprague Dawley Rats (n = 12/sex/group) fed Control, Choline Supplemented (CS), 100 ppm PFOS, or 100 ppm PFOS + CS Diets for Three Weeks[1]

Sex	Male				Female			
Group	**Control (basal diet)**	**CS[2]**	**100 ppm PFOS**	**100 ppm PFOS + CS[2]**	**Control (basal diet)**	**CS**	**100 ppm PFOS**	**100 ppm PFOS + CS**
Lactate dehydrogenase (U/L)	1008 ± 584.2	789 ± 326.3 [c, d]	391 ± 142.8[a, b]	337 ± 156.2[a, b]	507 ± 196.4	282 ± 159 [a, d]	209 ± 66.3[a]	124 ± 72.9[a, b]
Lipase (U/L)	5.8 ± 1.3	7.9 ± 0.9[c, d]	4.3 ± 1.1[a, b]	4.8 ± 2.8[b]	4.4 ± 0.7	6.0 ± 1.3[a, c, d]	4.3 ± 0.9[b, d]	2.9 ± 0.7[a, b, c]
Day16[3]								
Aspartate aminotransferase (U/L)	63 ± 8.3	58 ± 4.6	62 ± 7.2	57 ± 5.5	65 ± 6.2	58 ± 5.4[a]	60 ± 4.5	60 ± 5.4[4]
Alanine transaminase (U/L)	28 ± 7.2	26 ± 3.7	30 ± 7.0	25 ± 7.2	28 ± 4.5	27 ± 2.8	27 ± 3.9	30 ± 6.4[4]
Total bilirubin (mg/dL)	0.03 ± 0.1	0.02 ± 0.04	0.02 ± 0.04	0.03 ± 0.05	0.03 ± 0.05	0.0 ± 0.0 [c]	0.04 ± 0.1 [b, d]	0.0 ± 0.0[4] [c]
Total cholesterol (mg/dL)	87 ± 10.7	105 ± 13.3[a, c, d]	54 ± 7.4[a, b]	58 ± 7.3[a, b]	89 ± 10.3	94 ± 8.0[c, d]	68 ± 7.8[a, b]	73 ± 16.7[4, a, b]
High density lipoprotein (mg/dL)	38 ± 3.9	41 ± 3.9[c, d]	26 ± 2.6[a, b]	25 ± 3.3[a, b]	36 ± 3.0	37 ± 2.2[c, d]	30 ± 3.3[a, b]	30 ± 4.9[4, a, b]
Low density lipoprotein (mg/dL)	14 ± 2.6	16 ± 2.1[c, d]	7.8 ± 1.4 [a, b]	8.1 ± 1.2[a, b]	10.3 ± 1.5	9.4 ± 1.2	9.3 ± 1.8	9.5 ± 3.0[4]

Table 11. Selected Mean (±SD) Clinical Chemistry Results of Male and Female Sprague Dawley Rats (n = 12/sex/group) fed Control, Choline Supplemented (CS), 100 ppm PFOS, or 100 ppm PFOS + CS Diets for Three Weeks[1]

Sex	Male				Female			
Group	**Control (basal diet)**	**CS**[2]	**100 ppm PFOS**	**100 ppm PFOS + CS**[2]	**Control (basal diet)**	**CS**	**100 ppm PFOS**	**100 ppm PFOS + CS**
Triglycerides (mg/dL)	120 ± 36.6	131 ± 26.5[c, d]	71 ± 19.3[a, b]	67 ± 23.6[a, b]	53 ± 15.6	54 ± 15.3	58 ± 17.3	41 ± 10.0[4]
Blood urea nitrogen (mg/dL)	14 ± 1.0	14 ± 2.1	14 ± 2.6	13 ± 1.6	16 ± 1.9	16 ± 1.7[c, d]	12 ± 2.4[a, b]	13 ± 3.3[4, a, b]
Creatinine (mg/dL)	0.2 ± 0.0	0.2 ± 0.03	0.2 ± 0.03	0.2 ± 0.03	0.2 ± 0.0	0.2 ± 0.0	0.3 ± 0.1	0.2 ± 0.0[4]
Urea/Creatinine Ratio	67 ± 10.4	66 ± 12.9	67 ± 13.1	69 ± 26.4	73 ± 19.4	71 ± 12.4[c]	49 ± 16.2[a, b]	63 ± 16.6[4]
Albumin (g/dL)	3.5 ± 0.1	3.5 ± 0.2[c]	3.8 ± 0.2[a, b]	3.6 ± 0.1[c]	3.7 ± 0.1	3.7 ± 0.1[c, d]	4.0 ± 0.1[a, b, d]	3.9 ± 0.2[4, b, c]
Globulin (g/dL)	2.8 ± 0.1	2.8 ± 0.2[c, d]	2.6 ± 0.1[a, b]	2.6 ± 0.1[a, b]	2.7 ± 0.1	2.7 ± 0.2	2.8 ± 0.2	2.7 ± 0.2[4]
Albumin/Globulin Ratio	1.2 ± 0.1	1.2 ± 0.1[c, d]	1.5 ± 0.1[a, b]	1.4 ± 0.1[a, b]	1.4 ± 0.0	1.4 ± 0.1	1.5 ± 0.1	1.5 ± 0.1[4]
Lactate dehydrogenase (U/L)	370 ± 218	163 ± 58.2[a]	167 ± 82.1[a]	187 ± 92.3[a]	419 ± 145	230 ± 117	277 ± 96.0[a]	203 ± 80.3[a]
Lipase (U/L)	6.6 ± 1.4	7.8 ± 1.5[c, d]	3.8 ± 0.9[a, b]	3.8 ± 1.6[a, b]	6.4 ± 0.9	6.1 ± 1.1[c, d]	4.6 ± 0.7[a, b, d]	3.3 ± 0.6[a, b, c]

Table 11. Selected Mean (±SD) Clinical Chemistry Results of Male and Female Sprague Dawley Rats (n = 12/sex/group) fed Control, Choline Supplemented (CS), 100 ppm PFOS, or 100 ppm PFOS + CS Diets for Three Weeks[1]

Sex	Male				Female			
Group	**Control (basal diet)**	**CS**[2]	**100 ppm PFOS**	**100 ppm PFOS + CS**[2]	**Control (basal diet)**	**CS**	**100 ppm PFOS**	**100 ppm PFOS + CS**
Day 23[3]								
Aspartate aminotransferase (U/L)	80 ± 8.9	80 ± 7.5	95 ± 15.2	96 ± 31.2	92 ± 24.5[4]	69.5 ± 5.6[a]	67 ± 7.4[a]	72 ± 14.5[a]
Alanine transaminase (U/L)	25 ± 4.7	25 ± 4.7[c, d]	54 ± 16.2[a, b]	49 ± 22.8[a, b]	32 ± 20.1	21 ± 3.8	25 ± 4.2	29 ± 6.6
Total bilirubin (mg/dL)	0.1 ± 0.1	0.1 ± 0.0[c, d]	0.3 ± 0.1[a, b, d]	0.2 ± 0.1[a, b, c]	0.1 ± 0.1	0.0 ± 0.0	0.1 ± 0.1	0.1 ± 0.0
Total cholesterol (mg/dL)	84 ± 108	89 ± 9.5[c, d]	46 ± 5.2[a, b]	47 ± 7.3[a, b]	93 ± 10.5	94 ± 13.1	79 ± 14.3	78 ± 18.5
High density lipoprotein (mg/dL)	25 ± 3.7	27 ± 3.1[c]	22 ± 2.9[b]	24 ± 3.4[b]	31.1 ± 2.8	31.0 ± 3.1	30.0 ± 4.0	31 ± 4.5
Low density lipoprotein (mg/dL)	14 ± 3.8	11 ± 1.6[a, c, d]	3.6 ± 0.7[a, b]	4.1 ± 1.3[a, b]	9.8 ± 1.7	10.8 ± 2.0[c]	8.1 ± 2.0[b]	8.5 ± 3.3
Triglycerides (mg/dL)	57 ± 9.3	53 ± 14.9[c, d]	28 ± 6.3[a, b]	31 ± 5.0[a, b]	48 ± 8.7	37 ± 3.7	36 ± 4.6	44 ± 21.6
Blood urea nitrogen (mg/dL)	15 ± 1.7	13 ± 1.0[a, c, d]	18 ± 2.1[a, b]	16 ± 2.0[b]	15 ± 1.2	14 ± 2.2[d]	19 ± 1.8	22 ± 10.4[a, b]

Table 11. Selected Mean (±SD) Clinical Chemistry Results of Male and Female Sprague Dawley Rats (n = 12/sex/group) fed Control, Choline Supplemented (CS), 100 ppm PFOS, or 100 ppm PFOS + CS Diets for Three Weeks[1]

Sex	Male				Female			
Group	Control (basal diet)	CS[2]	100 ppm PFOS	100 ppm PFOS + CS[2]	Control (basal diet)	CS	100 ppm PFOS	100 ppm PFOS + CS
Creatinine (mg/dL)	0.3 ± 0.1	0.3 ± 0.0[c, d]	0.2 ± 0.04[a, b]	0.2 ± 0.04[a, b]	0.4 ± 0.1	0.3 ± 0.1	0.3 ± 0.0[a]	0.3 ± 0.0[a]
Urea/Creatinine Ratio	52 ± 37.4	43 ± 9.3[c, d]	84 ± 14.7[a, b]	94 ± 25.7[a, b]	44.0 ± 11.0	51.8 ± 13.2[c, d]	75 ± 20.4[a, b]	86 ± 20.9[a, b]
Albumin (g/dL)	3.6 ± 0.2	3.6 ± 0.2[c, d]	3.9 ± 0.1[a, b]	3.9 ± 0.1[a, b]	3.9 ± 0.2	3.6 ± 0.1[a, c, d]	4.4 ± 0.2[a, b]	4.2 ± 0.4[a, b]
Globulin (g/dL)	2.9 ± 0.2	2.8 ± 0.2	3.0 ± 0.2	3.0 ± 0.2	2.8 ± 0.2	2.8 ± 0.2	3.0 ± 0.2	3.1 ± 0.3[a]
Albumin/Globulin Ratio	1.3 ± 0.1	1.3 ± 0.1	1.3 ± 0.1	1.3 ± 0.1	1.4 ± 0.1	1.3 ± 0.1[c, d]	1.5 ± 0.1[a, b, d]	1.4 ± 0.1[c]
Lactate dehydrogenase (U/L)	358 ± 148	270 ± 83.3[c, d]	678 ± 134[b]	627 ± 594[b]	680 ± 885	165 ± 30.8[a]	347 ± 76.4	281 ± 90.0
Lipase (U/L)	8.3 ± 2.2	9.4 ± 2.5[c, d]	5.5 ± 2.1 [a, b]	5.1 ± 2.3[a, b]	9.9 ± 2.5	7.4 ± 1.7	5.3 ± 1.1	12 ± 22.1

1. Mean (±SD) values for all evaluated clinical chemistry parameters are provided in Supplemental Tables 5 - 12.
2. CS indicates the choline supplemented diet.
3. Blood was collected from non-fasted rats on days 2, 9, and 16. The rats were fasted overnight prior to blood collection on day 23.
4. Insufficient serum was available for analysis from one to six rats for this parameter (see the Supplemental Tables for the specific number of animals per parameter). The available sera was sufficient for evaluation of this parameter.

Letter code indicates significantly different parameter by group using one-way ANOVA with Tukey's multiple comparisons test ($p<0.05$, GraphPad Prism®, v. 6.04). (a) vs. control, (b) vs. CS, (c) vs. PFOS, and (d) vs. PFOS + CS.

Table 12. Fasted Body Weights, Absolute Liver Weights, and Relative Liver Weights of Male and Female Sprague Dawley Rats fed Control, Choline Supplemented (CS), 100 ppm PFOS, or 100 ppm PFOS + CS Diets for Three Weeks

Sex	**Male**				**Female**			
Parameter (units)	**Control (basal choline)**	**Choline Supplemented (CS)**	**100 ppm PFOS**	**100 ppm PFOS + CS**	**Control (basal choline)**	**Choline Supplemented (CS)**	**100 ppm PFOS**	**100 ppm PFOS + CS**
Fasted Body Weight (g)	301.2 ± 17.2	294.6 ± 13.2	284.9 ± 22.4	274.9 ± 12.5 [a,b]	187.0 ± 8.8	189.4 ± 11.1	162.7 ± 10.4 [a,b]	163.5 ± 13.2 [a,b]
Liver Weight (g)	9.3 ± 1.2	8.9 ± 1.4	16.4 ± 1.6 [a,b]	15.1 ± 0.8 [a,b]	5.9 ± 0.6	5.7 ± 0.5	7.6 ± 0.6 [a,b]	7.7 ± 1.0 [a,b]
Liver-to-BW ratio	0.031 ± 0.003	0.030 ± 0.004	0.057 ± 0.003 [a,b]	0.055 ± 0.003 [a,b]	0.032 ± 0.003	0.030 ± 0.001	0.047 ± 0.002 [a,b]	0.047 ± 0.005 [a,b]

1. N = 12 rats/sex/group.

Letter code indicates significantly different parameter by group within sex using one-way ANOVA with Tukey's multiple comparisons test ($p<0.05$, GraphPad Prism®, v. 6.04). (a) vs. control and (b) vs. choline supplement (CS). There were no differences between the PFOS and PFOS + CS groups for either sex in any parameter.

Table 13. Mean (±SD) Percent Area of Hepatocellular Lipid (Oil Red O or Osmium Tetroxide Staining) and Hypertrophy (Hematoxylin and Eosin Staining) in Male and Female Sprague Dawley Rats fed Control, Choline Supplemented (CS), 100 ppm PFOS, or 100 ppm PFOS + CS Diets for Three Weeks

Sex	Male				Female			
Group	Control (basal diet)	Choline Suppl.[1] (CS)	100 ppm PFOS	100 ppm PFOS + CS	Control (basal diet)	Choline Suppl. (CS)	100 ppm PFOS	100 ppm PFOS + CS
Stain								
Oil Red O for lipid (% area)	2.432 ± 1.131	2.492 ± 1.239	52.424 ± 19.369 [a,b]	54.296 ± 16.988 [a,b]	6.454 ± 2.388	15.958 ± 8.240 [a]	4.179 ± 1.308 [b]	6.580 ± 2.848 [b]
Osmium tetroxide for lipid (% area)	0.283 ± 0.148	0.219 ± 0.102	9.023 ± 10.802 [a,b]	7.715 ± 4.578 [a,b]	0.290 ± 0.178	0.352 ± 0.170	0.152 ± 0.095	0.339 ± 0.343
Hematoxylin and eosin for hepatocellular hypertrophy (% area)	85.117 ± 2.179	85.433 ± 1.626	89.408 ± 1.748 [a,b]	89.232 ± 1.576 [a,b]	80.870 ± 1.905	78.334 ± 3.520	85.594 ± 1.798 [a,b]	86.606 ± 1.617 [a,b]

N = 12/sex/group.

1. Choline Supplemented.

Statistically significant differences are indicated for each group comparison vs. control (a) and vs. CS (b) using one-way ANOVA with Tukey's multiple comparisons test, $p<0.05$. There were no differences between the PFOS and PFOS + CS groups.

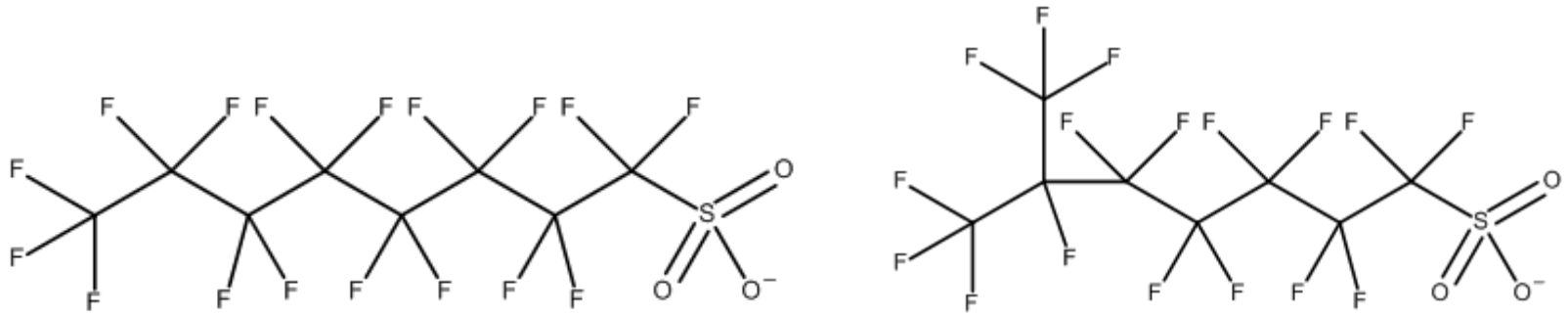

Figure 1. Structures of linear and branched PFOS. The branch can occur at multiple places along the PFOS backbone as an internal monomethyl or isopropyl branch or other branches [4].

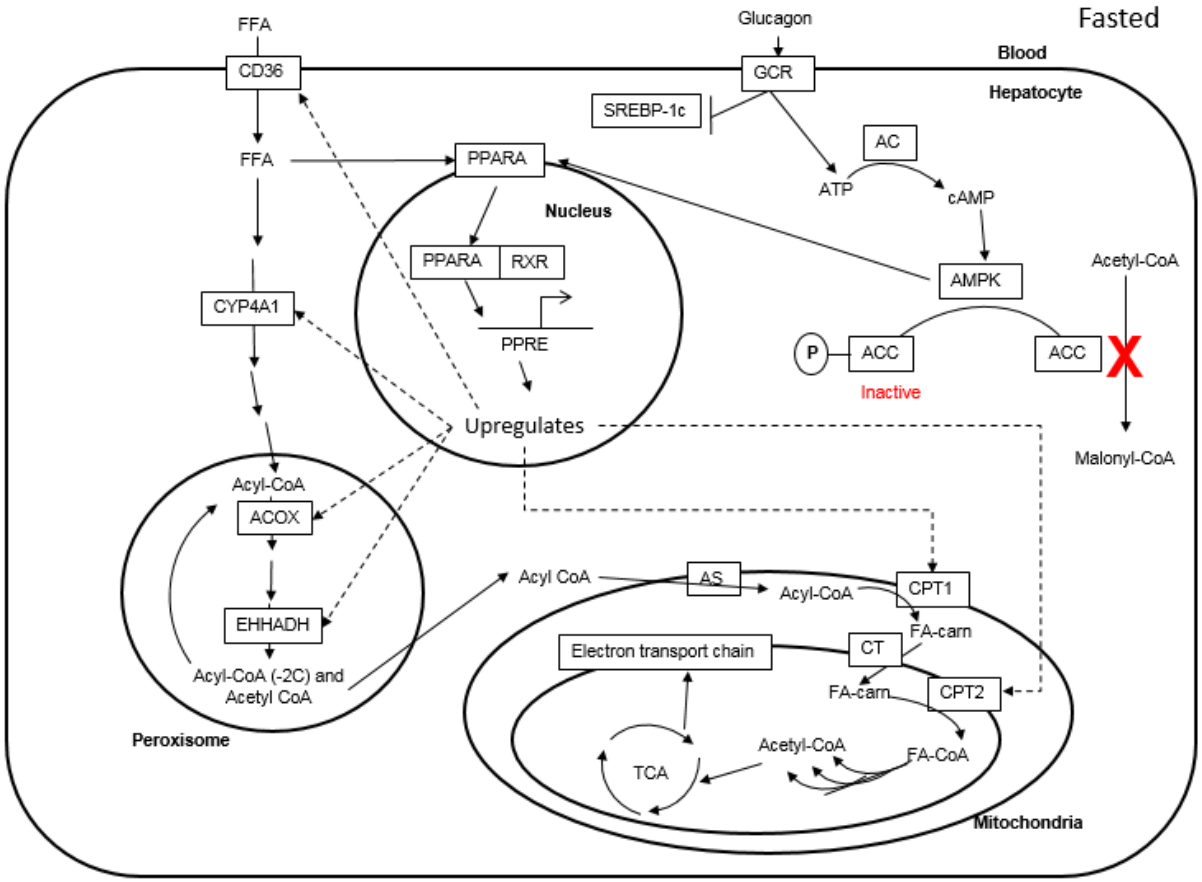

Figure 2. Lipid catabolism during low energy states (e.g., fasted conditions). Based on information in Lehninger Principles of Biochemistry [9]

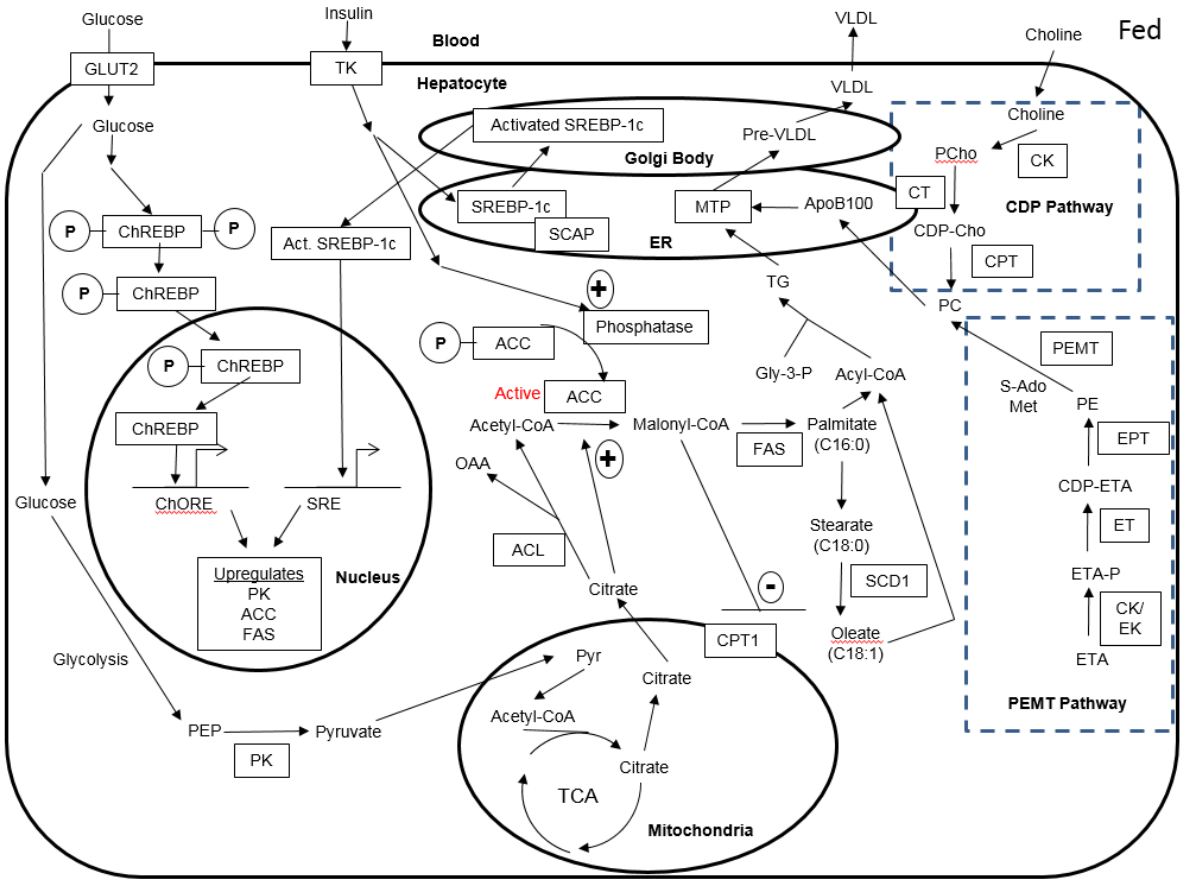

Figure 3. Lipid synthesis and VLDL secretion during high energy states (e.g., fed conditions). Based on information in Lehninger Principles of Biochemistry [14].

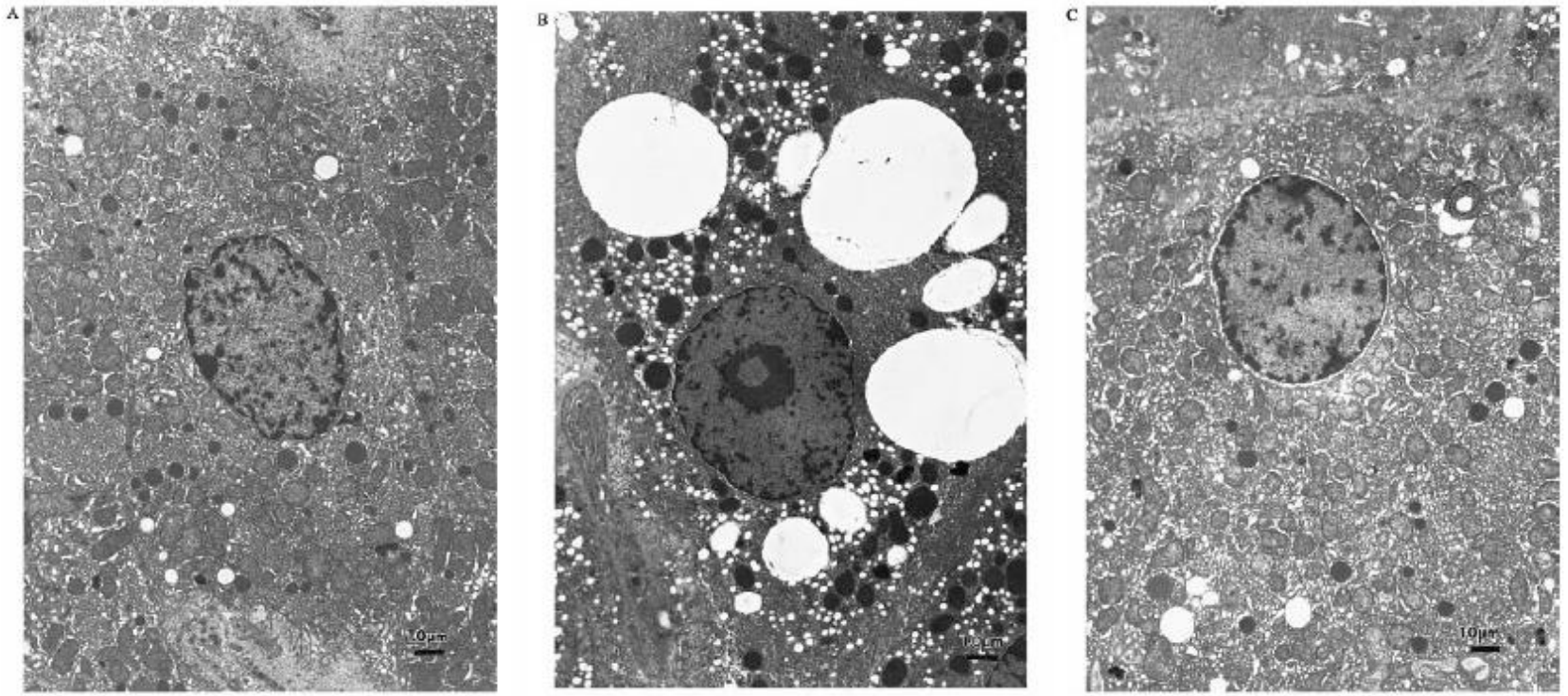

Figure 4. Scanning electron microscopy from Seacat, et al. [8] of (A) control liver on day 184, (B) 0.75 mg/kg-day PFOS-treated liver on day 184, and (C) 0.75 mg/kg-day PFOS-treated liver after 211 days of recovery (i.e., day 393) from male cynomolgus monkeys

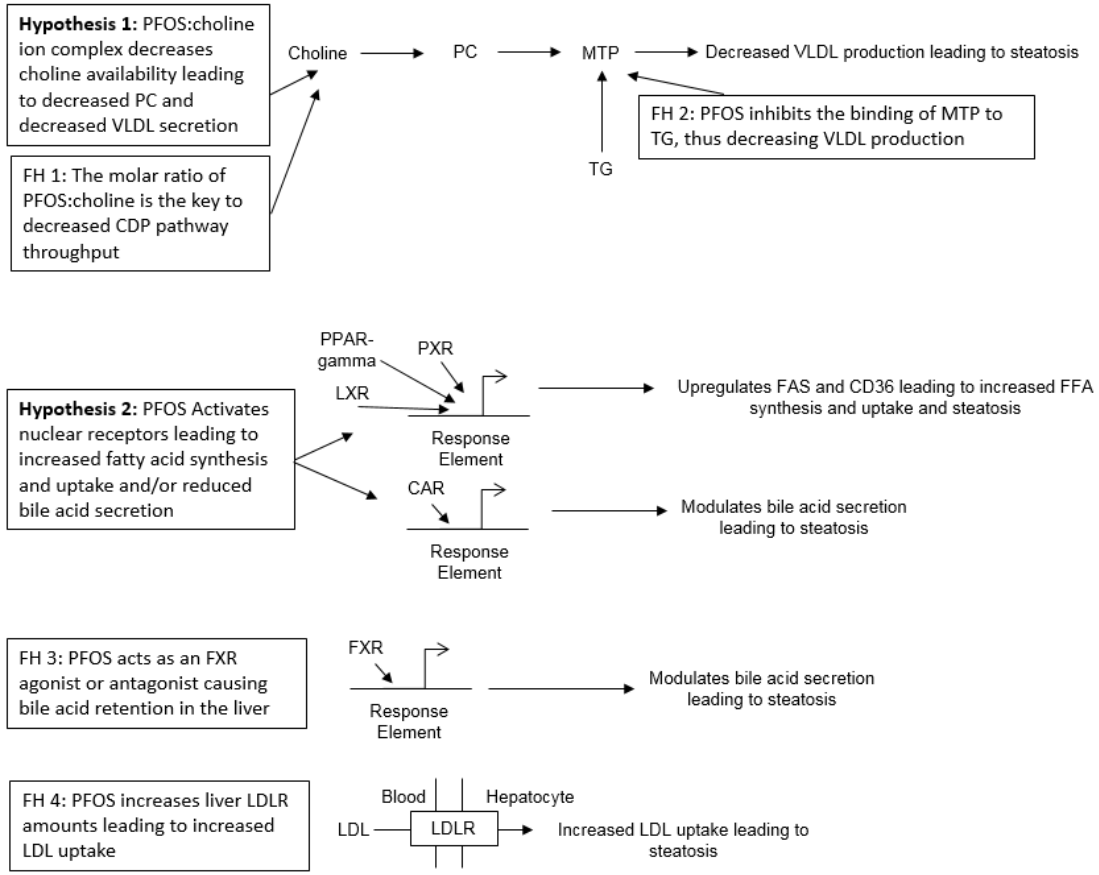

Figure 5. Hypotheses for possible PFOS mechanisms of action on lipid metabolism resulting in hepatic steatosis. Hypotheses 1 and 2 were evaluated in this project. Future hypotheses (FH) 1 through 4 are also shown (see Chapter 5).

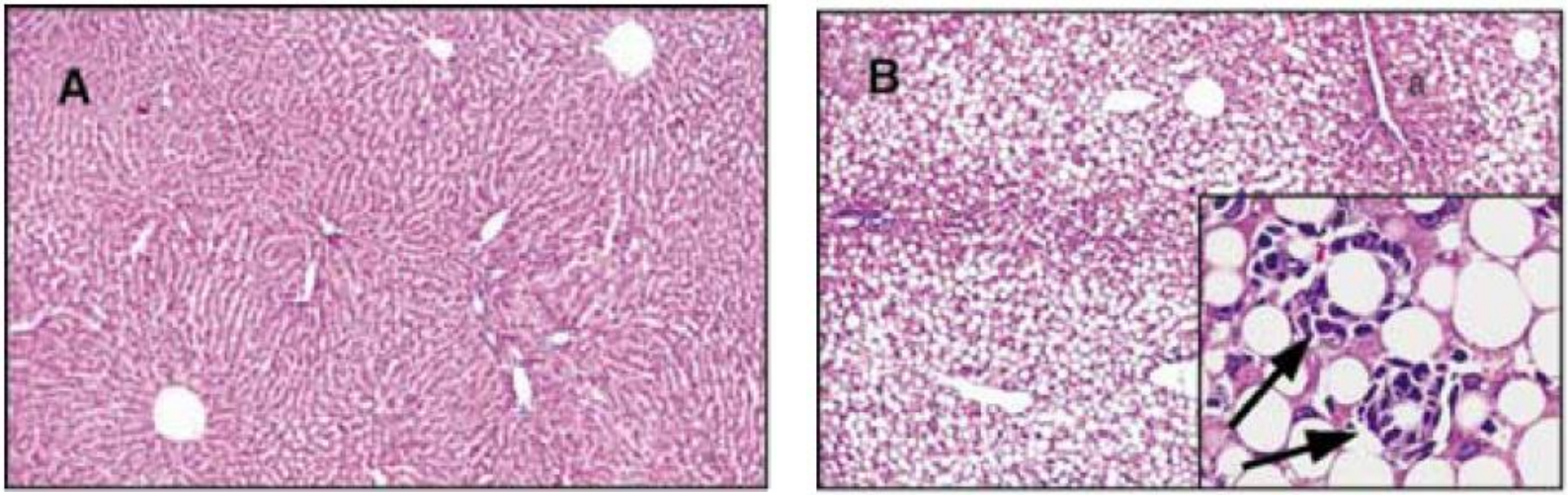

Figure 6. Comparison of light microscopy of the livers from a control diet-fed rat (A) and an MCD diet-fed rat (B) at 5 weeks from George, et al. [58]. The main images are 60X magnification and the insert is 400x magnification. Evidence of hepatic steatosis is shown in the insert with inflammatory cell infiltrate indicated by the arrows.

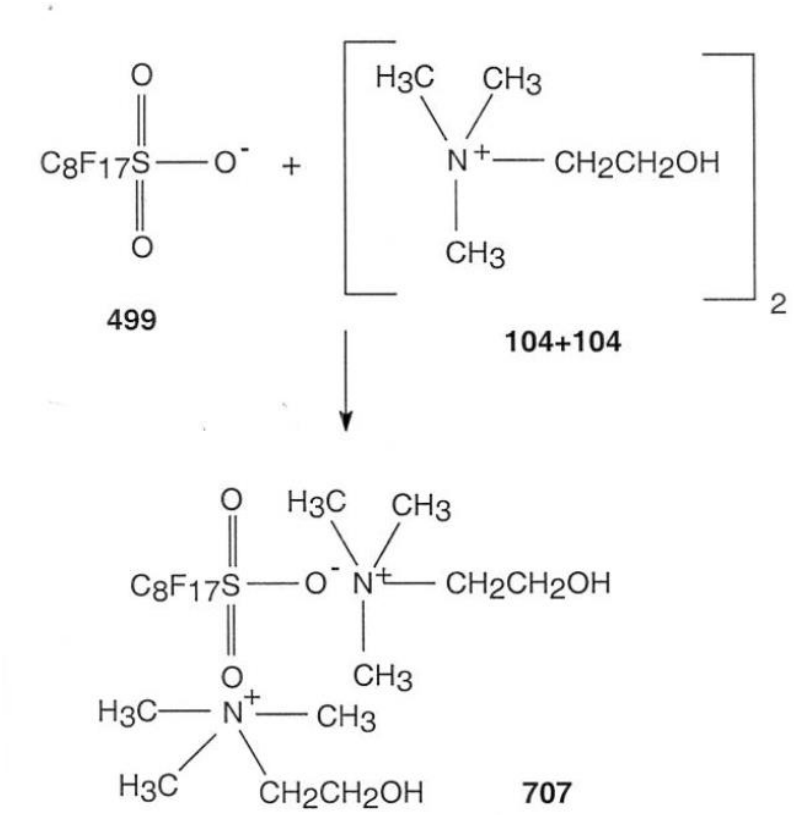

Figure 7. PFOS ion complex with choline. PFOS (molecular weight 499) forms an ion complex with two molecules of choline (molecular weight 104) to form a 707 molecular weight complex with a net +1 positive charge.

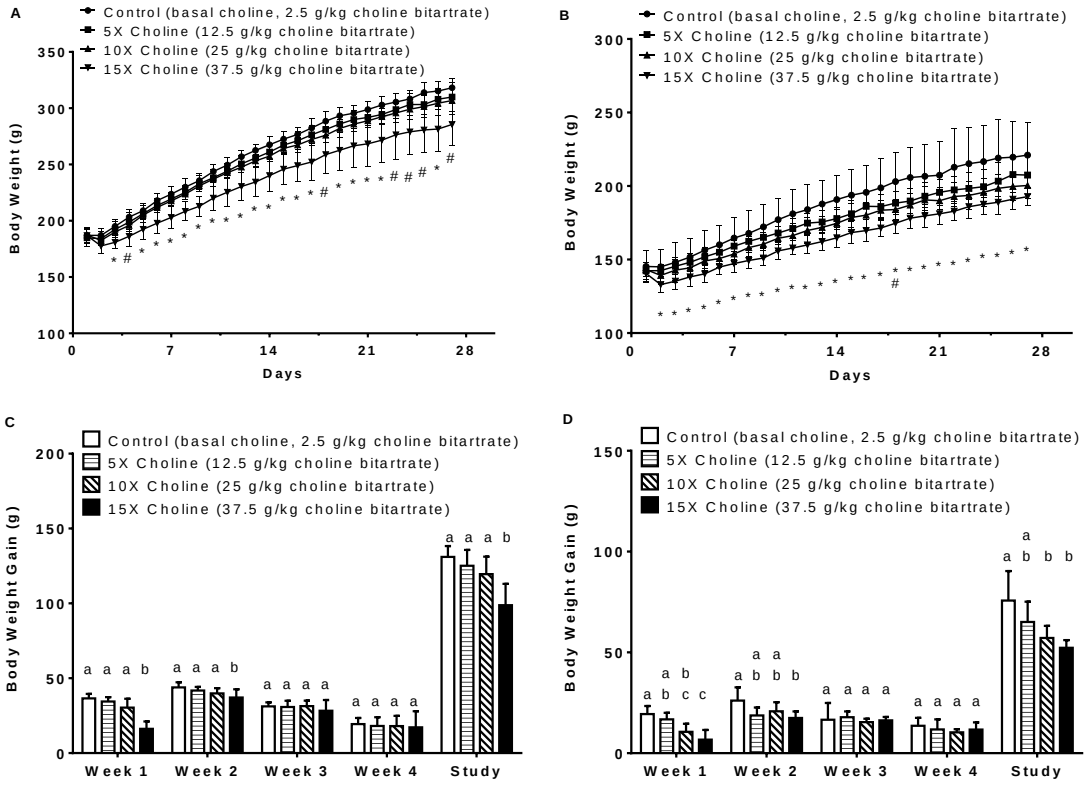

Figure 8. Mean (±SD) body weights and body weight gains in male (A and C) and female (B and D) Sprague Dawley rats fed control diet or 5X, 10X, or 15X choline supplemented diet for four weeks. N = 5 control rats/sex and 6 choline supplemented rats/sex/group. (A) 15X choline supplement male group was significantly different from all other male groups (*) and all male groups except the 10X group (#) on the indicated days, p≤0.05. (B) Mean body weights of the 10X females (#) and 15X females (*) were significantly decreased relative to the female control group on the indicated days, p≤0.05. (C and D) Letter code indicates significantly different groups by week or throughout the study, p≤0.05.

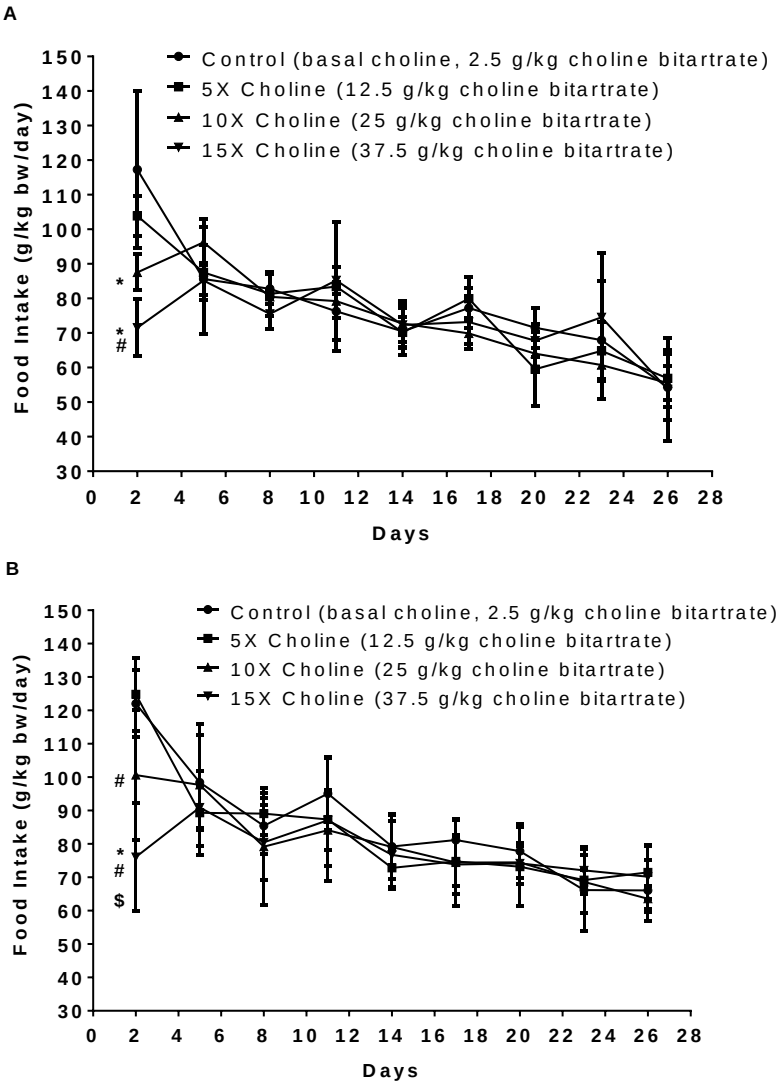

Figure 9. Mean (±SD) food intake in in male (A) and female (B) Sprague Dawley rats fed control diet or 5X, 10X, or 15X choline supplemented diet for four weeks. N = 5 control rats/sex and 6 choline supplemented rats/sex/group. Significantly decreased food intake relative to the control (*), 5X (#), and/or 10X ($) groups, p≤0.05.

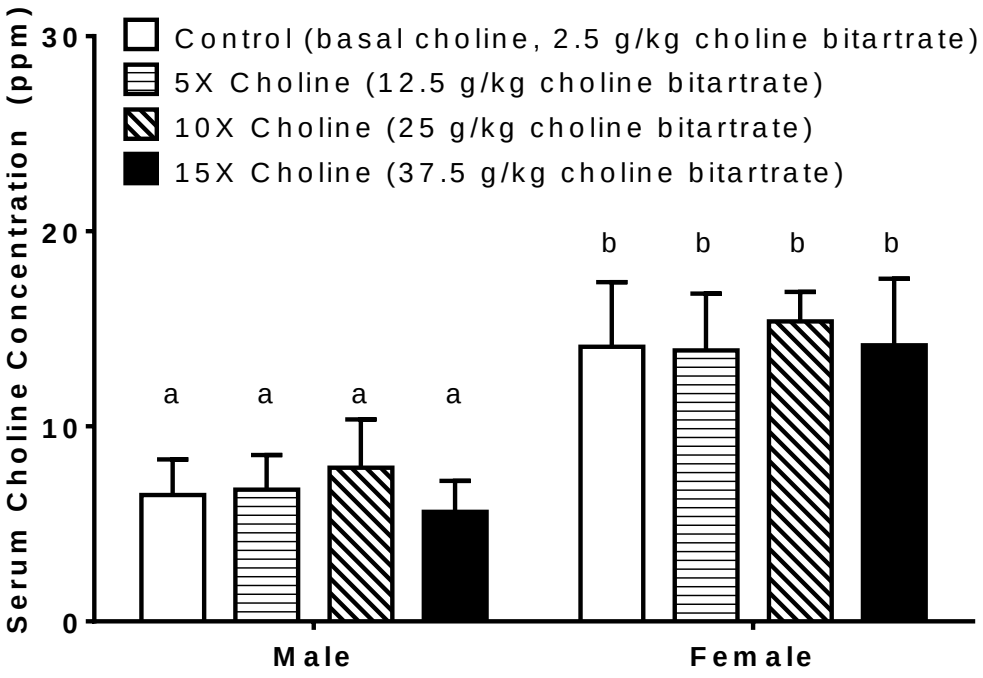

Figure 10. Mean (±SD) serum choline concentrations (ppm) at necropsy in male and female Sprague Dawley rats fed control diet or 5X, 10X, or 15X choline supplemented diet for four weeks. N = 5 control rats/sex and 6 choline supplemented rats/sex/group. Letter code indicates significant differences between groups, p≤0.05.

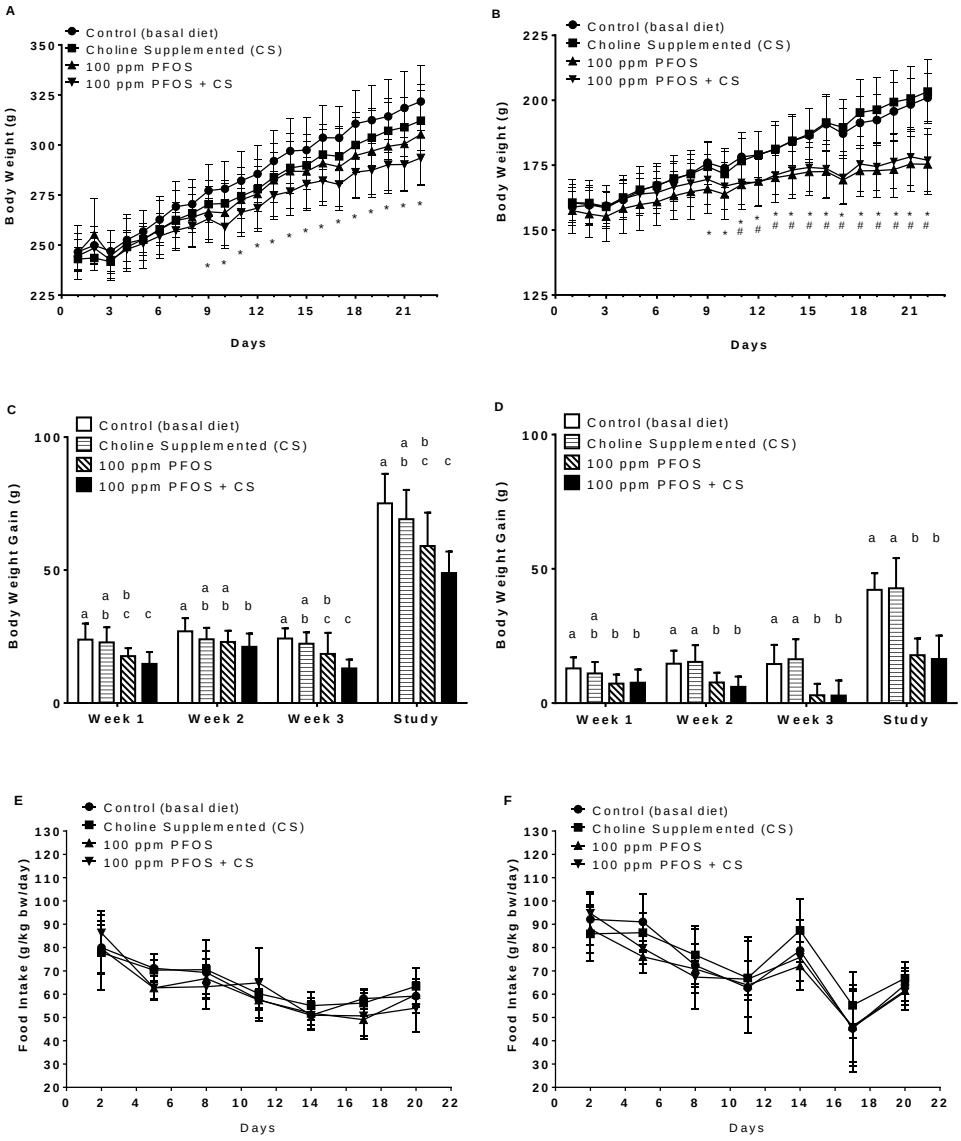

Figure 11. Effects of 3-week exposure to basal diet (control), choline supplemented diet (CS), 100 ppm PFOS diet, or 100 ppm PFOS + CS diet on male (A, C, and E) and female (B, D, and F) Sprague Dawley rat mean (±SD) body weights, body weight gains, and food intake, respectively. N = 12 rats/sex/group. (A) * PFOS + CS male group was significantly different from the male control group, p≤0.05. (B) PFOS (#) and PFOS + CS (*) females were significantly decreased relative to the female control and CS groups, p≤0.05. (C) Values with different letters are significantly different, p≤0.05. (D) Values with different letters are significantly different, p≤0.05. (E) No significant differences, p>0.05. (F) No significant differences, p>0.05.

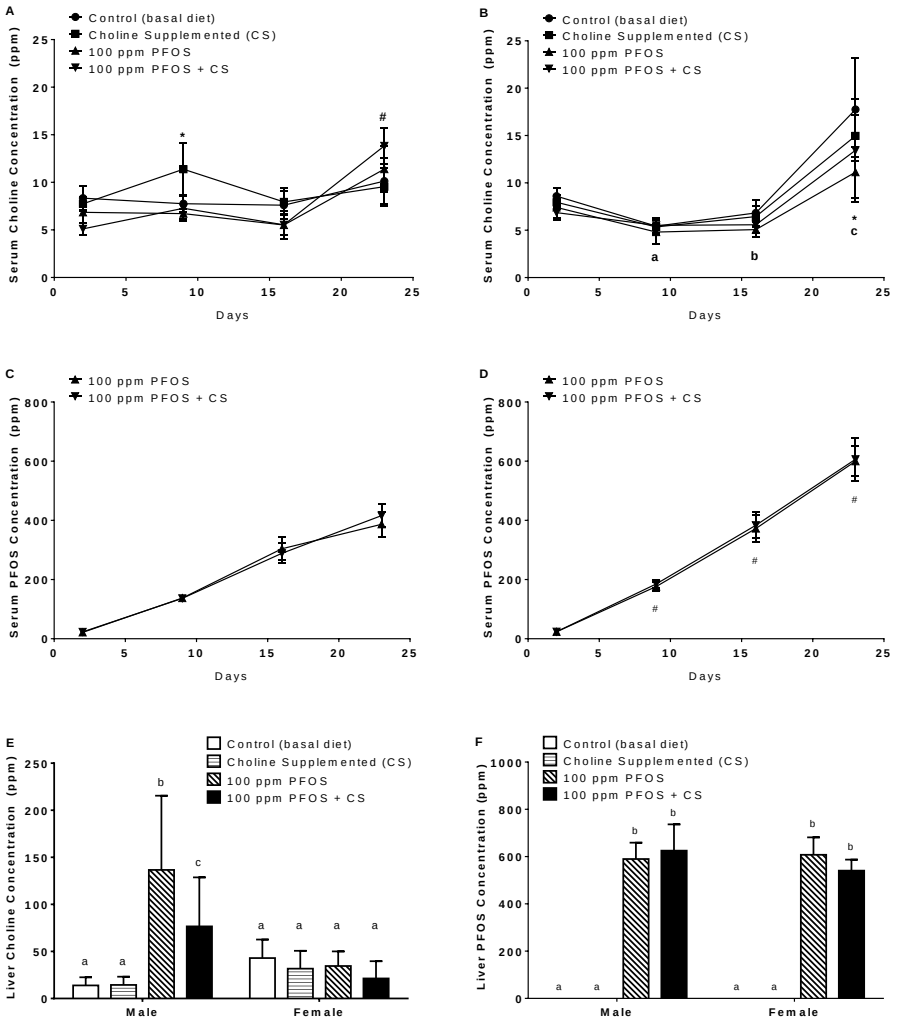

Figure 12. Effects of 3-week exposure to basal diet (control), choline supplemented diet (CS), 100 ppm PFOS diet, or 100 ppm PFOS + CS diet on male (A, C, E, and F) and female (B, D, E, and F) Sprague Dawley rat mean (±SD) serum choline, serum PFOS, liver choline, and liver PFOS concentrations, respectively. N = 12 rats/sex/group. (A) * CS group mean was increased relative all other group means, p≤0.05. # PFOS + CS group mean was increased relative all other group means, p≤0.05. (B) * PFOS group mean was decreased relative to the control group mean, p≤0.05. a. All female group means were decreased relative to the respective male group means, p≤0.05. b. Female CS group mean was decreased relative to the male CS group, p≤0.05. c. Female control and CS group means were increased relative to the respective male groups, p≤0.05. (C and D) No significant differences between groups of the same sex, p>0.05. Control and CS group values were below the limit of detection (0.05 ppm). # Female PFOS concentration was significantly increased relative to the male groups, p≤0.05. (E and F) Values with different letters are significantly different, p≤0.05.

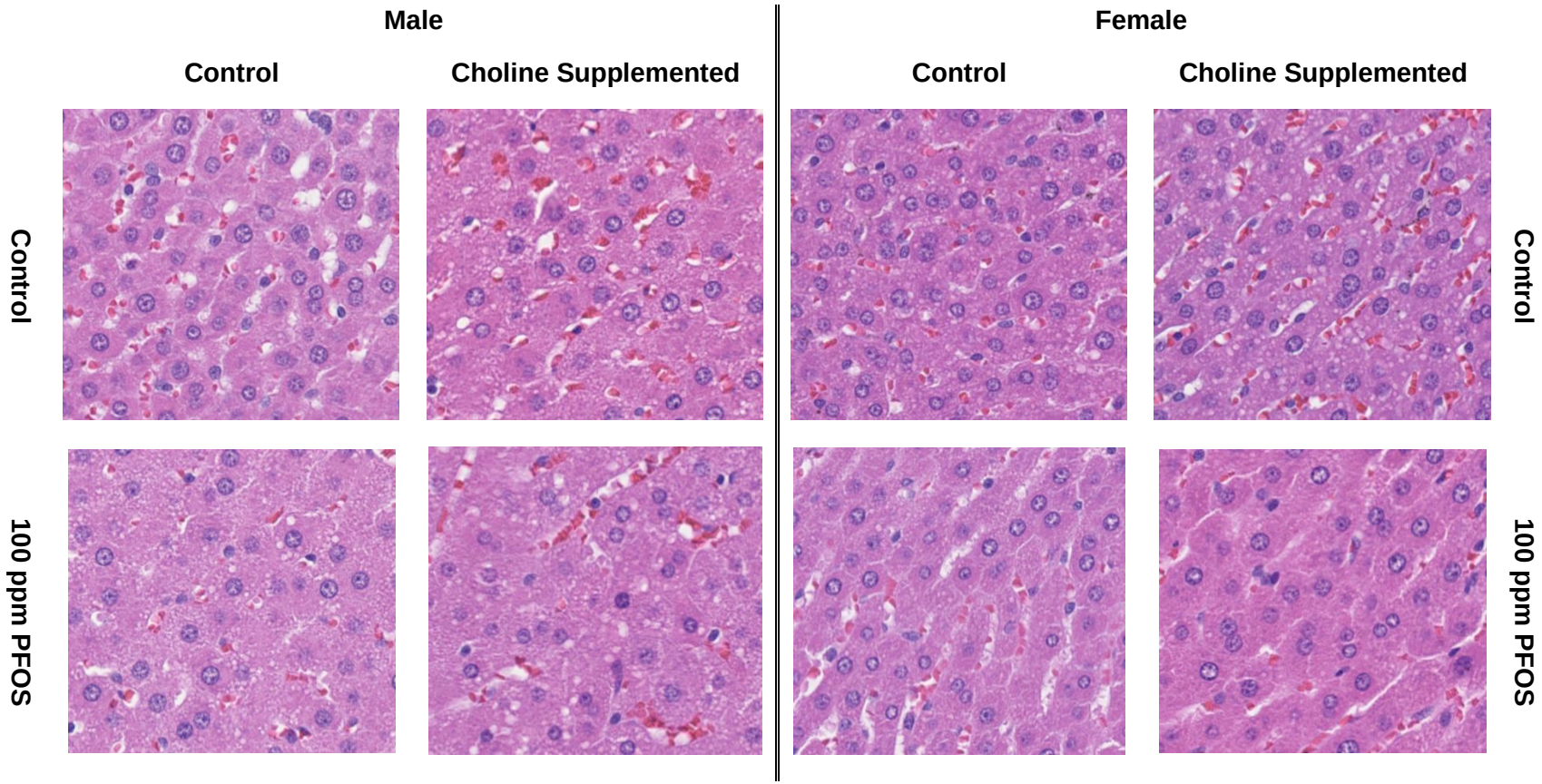

Figure 13. Representative hematoxylin and eosin-stained liver (40X magnification) at termination from male and female Sprague Dawley rats fed basal diet (control), choline supplemented diet (CS), 100 ppm PFOS diet, or 100 ppm PFOS + CS diet for three weeks.

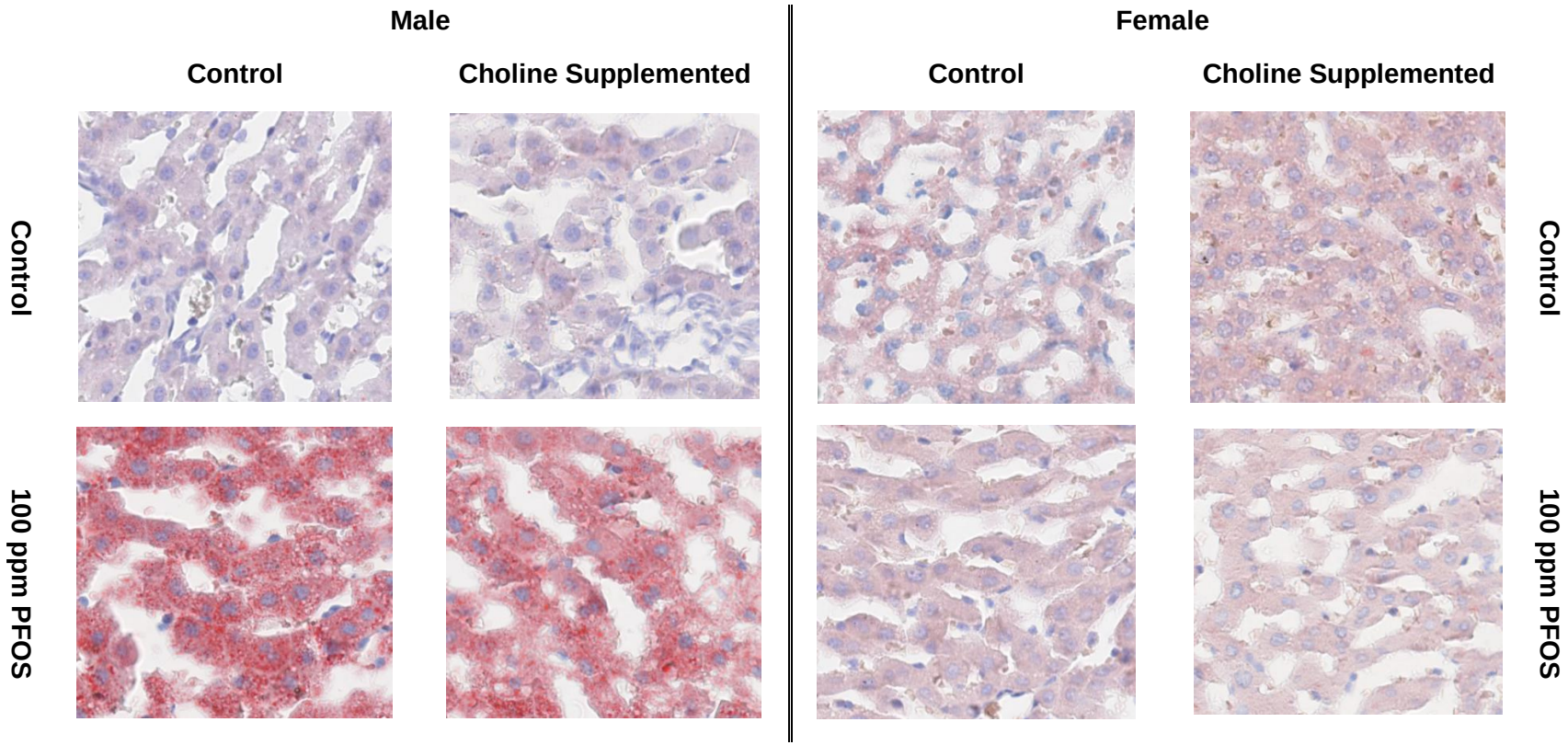

Figure 14. Representative Oil Red O-stained liver (40X magnification) at termination from male and female Sprague Dawley rats fed basal diet (control), choline supplemented diet (CS), 100 ppm PFOS diet, or 100 ppm PFOS + CS diet for three weeks. Lipid droplets are stained red by Oil Red O.

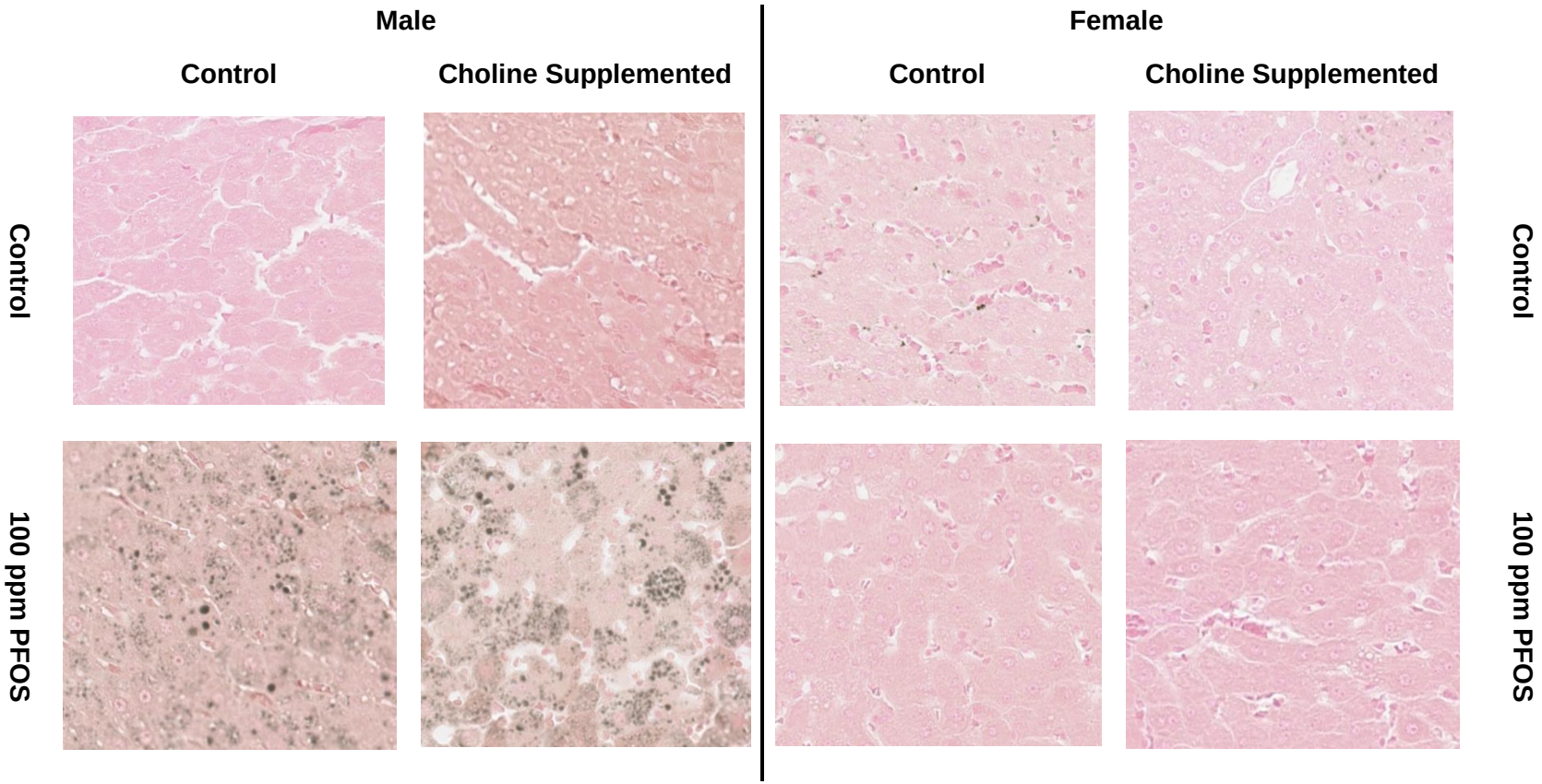

Figure 15. Representative osmium tetroxide-stained liver (40X magnification) at termination from male and female Sprague Dawley rats fed basal diet (control), choline supplemented diet (CS), 100 ppm PFOS diet, or 100 ppm PFOS + CS diet for three weeks. Lipid droplets are stained black by osmium tetroxide.

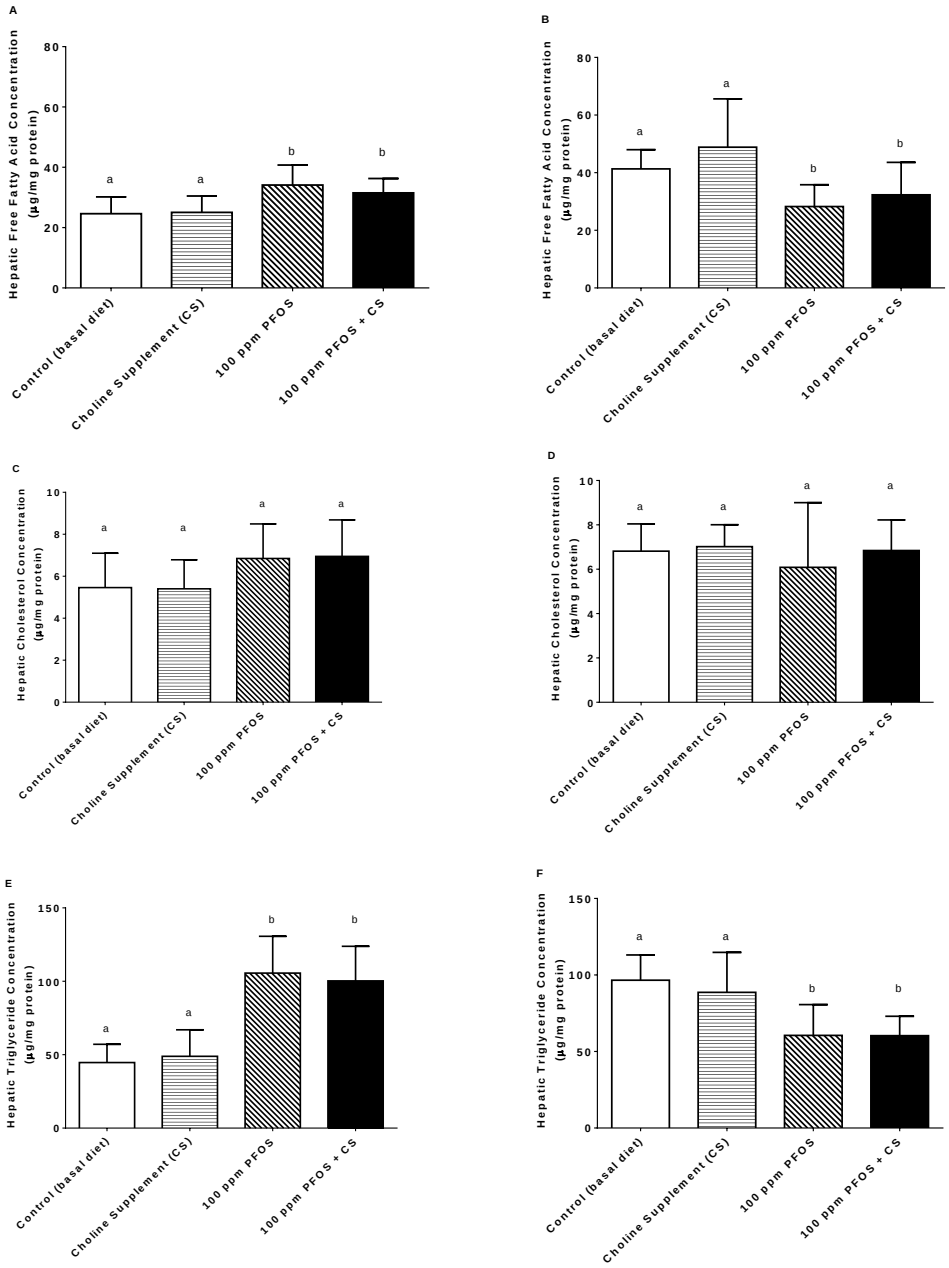

Figure 16. Effects of 3-week exposure to basal diet (control) choline supplemented diet (CS), 100 ppm PFOS diet, or 100 ppm PFOS + CS diet on male (A, C, E) and female (B, D, F) Sprague Dawley rat mean (±SD) hepatic free fatty acid, cholesterol, and triglyceride concentrations, respectively. Values with different letters are significantly different, p≤0.05. N = 12 rats/sex/group.

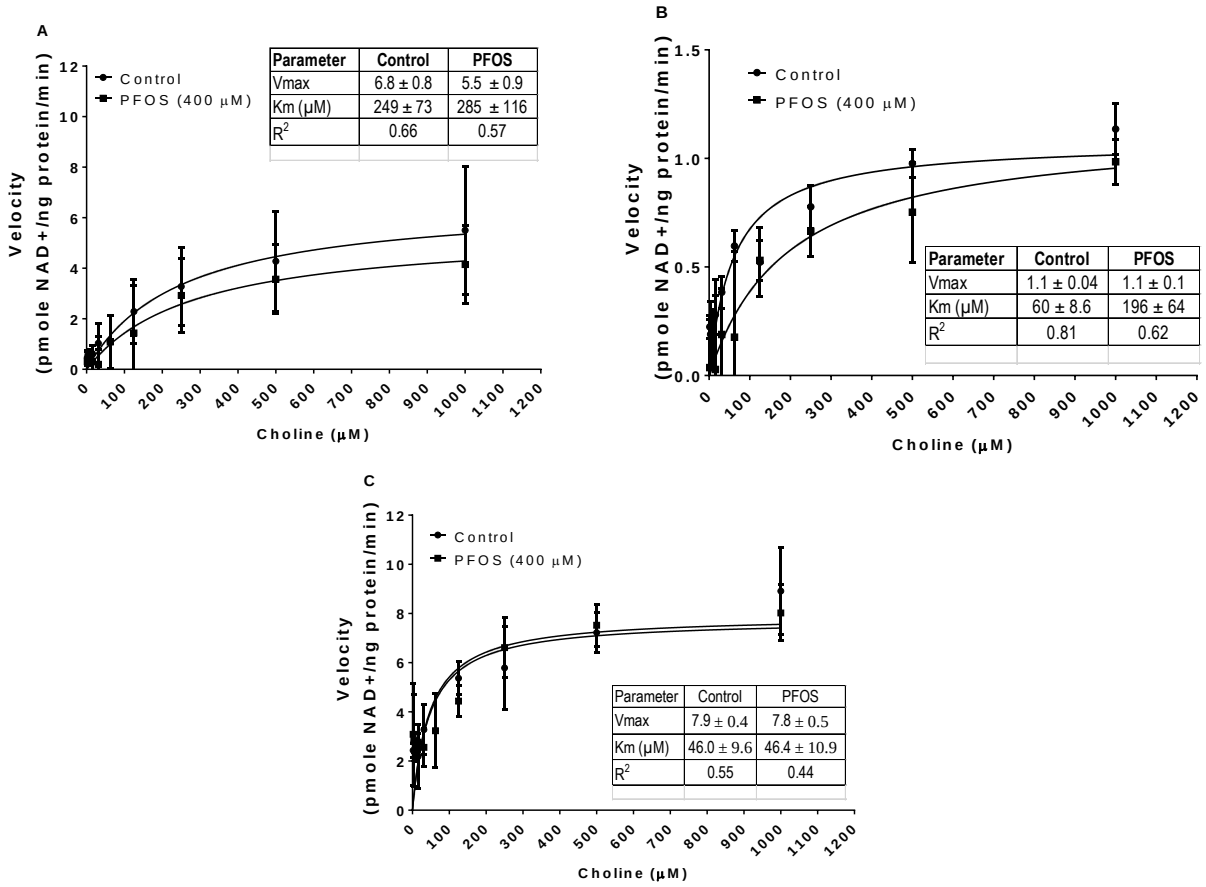

Figure 17. PFOS (400 µM) did not inhibit human choline kinase alpha variant 1 (A), human choline kinase alpha variant 2 (B), or human choline kinase beta (C) in an NADH-coupled choline kinase activity assay.

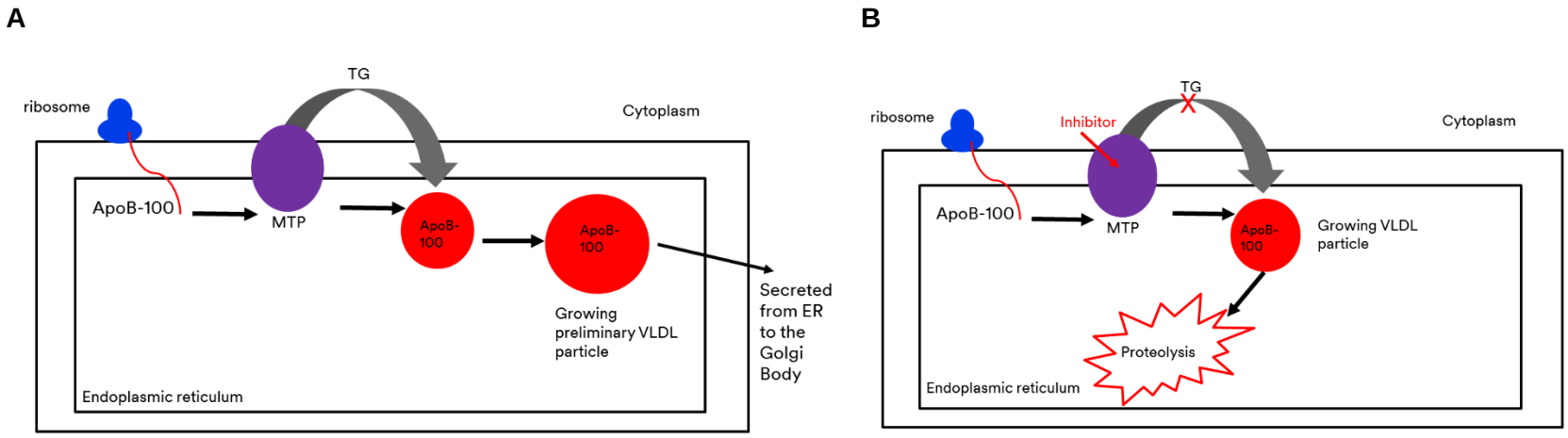

Figure 18. Very low density lipoprotein (VLDL) particle formation in the endoplasmic reticulum. (A) In the presence of ApoB-100, MTP takes up triglyceride (TG) from the cytoplasm. The growing ApoB-100 preliminary particle continues to receive TG from MTP until it is large enough to be secreted from the endoplasmic reticulum and migrate to the Golgi body to continue packaging as a VLDL particle. (B) An inhibitor can interfere with this process by competing for the TG binding site in MTP. This reduces or eliminates the formation of the growing apoB-100 preliminary VLDL particle which would then be proteolyzed. MTP inhibitors are known to cause hepatic steatosis and decreased serum TG. PFOS may induce the hepatic steatosis phenotype in male rats by inhibiting MTP.

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Appendix: Supplemental Data

Supplemental Table 1. Clinical Chemistry Results at Termination for Male Rats fed Control Diet or 5X, 10X, or 15X Choline Supplemented Diets for Four Weeks

Parameter	Units	Control (basal choline)			5X Choline			10X Choline			15X Choline		
		Mean	Standard Deviation	N	Mean	Standard Deviation	N	Mean	Standard Deviation	N	Mean	Standard Deviation	N
Glucose	mg/dL	160.4	37.1	5	147.5	33.1	6	159.8	27.8	6	182.2	78.9	6
Aspartate aminotransferase	U/L	107.8	21.7	5	97.8	11.1	6	97.0	10.1	6	98.5	22.7	6
Alanine aminotransferase	U/L	35.6	1.1	5	33.7	3.7	6	32.8	6.9	6	39.8	8.9	6
Gamma glutamyl transferase[1]	U/L	<3	-	5	<3	-	6	<3	-	6	<3	-	6
Alkaline Phosphatase	U/L	137.8	18.4	5	124.5	12.6	6	146.3	26.0	6	148.7	24.7	6
Total Bilirubin[2]	mg/dL	<0.1	-	5	<0.1	-	6	<0.1	-	6	<0.1	-	6
Direct Bilirubin[2]	mg/dL	<0.1	-	5	<0.1	-	6	<0.1	-	6	<0.1	-	6
Indirect Bilirubin[2]	mg/dL	<0.1	-	5	<0.1	-	6	<0.1	-	6	<0.1	-	6
Cholesterol	mg/dL	80.8	5.9	5	92.3	10.3	6	87.5[d]	7.7	6	105.2[a,c]	11.3	6
Triglycerides	mg/dL	54.6	15.1	5	48.3	4.2	6	50.3	13.0	6	42.5	3.4	6
Total Protein	g/dL	7.02	0.2	5	7.0	0.3	6	7.1	0.1	6	6.9	0.3	6
Albumin	g/dL	3.6	0.0	5	3.6	0.2	6	3.6	0.1	6	3.5	0.2	6
Blood Urea Nitrogen	mg/dL	16.4	1.5	5	16.3	2.1	6	16.3	1.5	6	16.8	2.1	6
Creatinine	mg/dL	0.3	0.1	5	0.3	0.0	6	0.3[a]	0.1	6	0.3[a]	0.1	6
Phosphorus	mg/dL	12.7	1.4	5	12.5	0.3	6	12.5	1.2	6	12.0	0.6	6
Calcium	mg/dL	12.12	0.6	5	12.3	0.3	6	12.4	0.5	6	12.5	0.4	6
Sodium	mmol/L	146.6	0.9	5	147.2	1.2	6	146.3	1.5	6	146.2	1.0	6
Potassium	mmol/L	8.54	1.1	5	8.4	0.7	6	8.7	1.4	6	7.9	1.0	6
Chloride	mmol/L	100.8	1.8	5	100.5	1.5	6	100.7	0.5	6	99.0	1.9	6

Supplemental Table 1. Clinical Chemistry Results at Termination for Male Rats fed Control Diet or 5X, 10X, or 15X Choline Supplemented Diets for Four Weeks

Parameter	Units	Control (basal choline)			5X Choline			10X Choline			15X Choline		
		Mean	Standard Deviation	N	Mean	Standard Deviation	N	Mean	Standard Deviation	N	Mean	Standard Deviation	N
Bicarbonate	mmol/L	29.4	2.1	5	30.7	1.6	6	32.7	3.1	6	32.5	2.2	6
Anion Gap	mmol/L	25	3.1	5	24.3	1.0	6	22.0	3.8	6	22.5	2.2	6
Creatine Kinase	U/L	463.6	624.3	5	186.7	94.4	6	166.2	33.0	6	389.2	592.7	6
Lactate Dehydrogenase	U/L	474.4	321.3	5	383.2	106.4	6	289.0	68.9	6	341.0	244.9	6
Amylase	U/L	693.2	163.9	5	661.7	97.3	6	814.0	145.3	6	717.2	84.9	6
Lipase	U/L	15.8	2.9	5	14.0	3.0	6	14.8	3.4	6	14.2	4.1	6
Globulin	g/dL	3.4	0.2	5	3.4	0.2	6	3.5	0.1	6	3.4	0.2	6
A/G Ratio	-	1.1	0.1	5	1.0	0.1	6	1.0	0.1	6	1.0	0.1	6
Urea/Creatinine Ratio	-	49.2	8.3	5	58.7	10.4	6	67.7	14.5	6	69.0[a]	10.2	6
Na/K Ratio	mmol/L	17.6	2.5	5	17.7	1.6	6	17.3	3.1	6	18.8	2.5	6
High density lipoprotein	mg/dL	27.6	1.5	5	27.0[d]	2.0	6	29.7	2.3	6	34.7[a,b]	5.1	6
Low density lipoprotein	mg/dL	8.2	1.6	5	9.5	1.6	6	9.0	2.4	6	10.7	1.9	6

Letter code indicates significantly different parameter by group using one-way ANOVA with Tukey's multiple comparisons test ($p<0.05$, GraphPad Prism®, v. 6.04). (a) vs. control, (b) vs. 5X, (c) vs. 10X, and (d) vs. 15X

1. All gamma glutamyl transferase values were below the limit of detection (3 U/L)
2. All total, direct, and indirect bilirubin values were below the limit of detection (0.1 mg/dL).

Supplemental Table 2. Clinical Chemistry Results at Termination for Female Rats fed Control Diet or 5X, 10X, or 15X Choline Supplemented Diets for Four Weeks

Parameter	Units	Control (basal choline)			5X Choline			10X Choline			15X Choline		
		Mean	Standard Deviation	N	Mean	Standard Deviation	N	Mean	Standard Deviation	N[1]	Mean	Standard Deviation	N
Glucose	mg/dL	83.2	9.3	5	79.7	8.0	6	95.0	10.6	4	92.3	13.0	6
Aspartate aminotransferase	U/L	103.2	13.0	5	109.5	7.7	6	100.8	9.5	4	98.8	6.9	6
Alanine transaminase	U/L	34.4	6.8	5	34.5	2.2	6	33.8	6.3	4	34.8	10.8	6
Gamma glutamyl transferase[1]	U/L	<3	-	5	<3	-	6	<3	-	4	<3	-	6
Alkaline Phosphatase	U/L	102.6	25.0	5	105.8	13.9	6	95.8	12.0	4	90.7	11.0	6
Total Bilirubin[2]	mg/dL	≤0.1	-	5	≤0.1	-	6	≤0.1	-	4	≤0.1	-	6
Direct Bilirubin[2]	mg/dL	<0.1	-	5	<0.1	-	6	<0.1	-	4	<0.1	-	6
Indirect Bilirubin[2]	mg/dL	<0.1	-	5	<0.1	-	6	<0.1	-	4	<0.1	-	6
Cholesterol	mg/dL	86.4	9.4	5	94.5	9.9	6	100.0	24.7	4	89.2	14.9	6
Triglycerides	mg/dL	40.4	6.6	5	48.2	10.8	6	41.3	12.4	4	36.3	3.7	6
Total Protein	g/dL	6.7	0.4	5	6.7	0.2	6	6.8	0.1	4	6.5	0.2	6
Albumin	g/dL	3.6	0.2	5	3.6[d]	0.1	6	3.5	0.1	4	3.3[a,b]	0.1	6
Blood Urea Nitrogen	mg/dL	19.2	1.8	5	17.3	2.4	6	18.0	1.6	4	17.2	2.7	6
Creatinine	mg/dL	0.4	0.1	5	0.3	0.1	6	0.3	0.1	4	0.3[a]	0.1	6
Phosphorus	mg/dL	12.4	0.7	5	11.2	0.5	6	12.6	0.6	4	12.7	1.5	6
Calcium	mg/dL	11.7	0.4	5	11.5	0.3	6	11.7	0.4	4	11.9	0.5	6
Sodium	mmol/L	145.0	1.0	5	143.8	1.5	6	143.3	1.9	4	142.7	1.2	6
Potassium	mmol/L	9.4	0.6	5	9.3	0.4	6	10.0	1.0	4	9.8	0.9	6
Chloride	mmol/L	101.2	1.1	5	101.7	1.2	6	102.0	0.8	4	101.3	1.2	6
Bicarbonate	mmol/L	31.8	1.8	5	30.3	2.5	6	29.0	0.8	4	29.7	2.2	6
Anion Gap	mmol/L	21.4	3.0	5	21.2	4.1	6	22.5	1.3	4	21.2	1.9	6

Supplemental Table 2. Clinical Chemistry Results at Termination for Female Rats fed Control Diet or 5X, 10X, or 15X Choline Supplemented Diets for Four Weeks

		Control (basal choline)			5X Choline			10X Choline			15X Choline		
Parameter	**Units**	**Mean**	**Standard Deviation**	**N**	**Mean**	**Standard Deviation**	**N**	**Mean**	**Standard Deviation**	**N[1]**	**Mean**	**Standard Deviation**	**N**
Creatine Kinase	U/L	203.2	46.1	5	209.5	49.8	6	189.5	101.6	4	131.2	18.3	6
Lactate Dehydrogenase	U/L	436.4	78.7	5	453.3	154.7	6	324.8	99.9	4	272.0	137.1	6
Amylase	U/L	316.0	40.5	5	375.8	70.5	6	382.5	54.4	4	350.0	46.0	6
Lipase	U/L	12.0	1.7	5	13.2	2.9	6	13.0	0.8	4	15.8	7.2	6
Globulin	g/dL	3.2	0.2	5	3.2	0.2	6	3.3	0.1	4	3.2	0.1	6
A/G Ratio	-	1.1	0.0	5	1.1	0.1	6	1.1	0.0	4	1.1	0.1	6
Urea/Creatinine Ratio	-	54.0	6.5	5	53.5	12.9	6	56.3	9.4	4	66.7	19.3	6
Na/K Ratio	mmol/L	15.6	1.1	5	15.3	0.5	6	14.3	1.5	4	14.8	1.2	6
High density lipoprotein	mg/dL	27.8	2.7	5	32.7[a]	1.2	6	33.5[a]	3.7	4	32.3	3.2	6
Low density lipoprotein	mg/dL	6.4	0.9	5	6.3	0.8	6	6.8	1.0	4	7.3	1.0	6

Letter code indicates significantly different parameter by group using one-way ANOVA with Tukey's multiple comparisons test ($p<0.05$, GraphPad Prism®, v. 6.04). (a) vs. control, (b) vs. 5X, (c) vs. 10X, and (d) vs. 15X

1. Blood was not successfully collected from two females of the 10X choline group due to technician difficulties. Sufficient animals remained for evaluation of this group.
2. All gamma glutamyl transferase values were below the limit of detection (3 U/L).
3. All total bilirubin values were at or below the limit of detection (0.1 mg/dL).
4. All direct and indirect bilirubin values were below the limit of detection (0.1 mg/dL).

Supplemental Table 3. mRNA Targets and Primers used to Measure Liver mRNA in Rats fed Control, Choline Supplemented (CS), 100 ppm PFOS, or 100 ppm PFOS + CS Diets for Three Weeks

Name	Abbr.	mRNA RefSeq	Forward Primer (5' → 3')	Reverse Primer (5' → 3')	Product Length (base pairs)
Rattus norvegicus enoyl-CoA hydratase and 3-hydroxyacyl CoA dehydrogenase	Ehhadh	NM_133606.1	CTGGGGATTCTTCCTGGTGC	TGTTGGGCAAGCTTGGAACT	247
Rattus norvegicus stearoyl-CoA desaturase	Scd1	NM_139192.2	CGAAACTTTCCTGCACAGCC	AAGTTGATGTGCCAGCGGTA	240
Rattus norvegicus CD36 molecule	Cd36	NM_031561.2	AGAAACCAAGTGACCGGGAA	CCACCGTTTCTTCAACTACAGA	250
Rattus norvegicus cell death-inducing DFFA-like effector c (fat-specific protein)	Fsp27	NM_001024333.2	GCTTGGGTCAGAGAAACAATGG	CCATGATGCCTTTGCGAACC	226
Rattus norvegicus glutathione S-transferase theta 1, transcript variant X2	Gsst1	XM_008772860.1	CTGCTGAGACCGAATAGCGA	TTCCAACAGCCCAGGATGAG	230

Supplemental Table 4. Dietary Choline and PFOS Concentrations in the Control, Choline Supplemented (CS), 100 ppm PFOS, and 100 ppm PFOS + CS diets used in the Three Week Study (Chapter 3)

Parameter	Control (basal choline)	Choline Supplemented (CS)	100 ppm PFOS	100 ppm PFOS + CS
Target choline concentration (ppm)	1700	5125	1700	5125
Mean (±SD) choline concentration (ppm)	1858 ± 153	4890 ± 395	1782 ± 128	4920 ± 313
Coefficient of variation for choline concentrations	8.2%	8.1%	7.2%	6.4%
Target PFOS concentration (ppm)	0	0	100	100
Mean (±SD) PFOS concentration (ppm)	<0.05[1]	<0.05[1]	92.0 ± 6.8*	94.3 ± 11.9
Coefficient of variation for PFOS concentrations	Not applicable	Not applicable	7.4%	12.7%

1. The limit of quantification for PFOS concentration in the diet was 0.05 ppm. All values were below the limit of quantification except one sample from one choline supplemented box (0.07 ppm).

* Significance difference between the mean PFOS concentration and the target concentration in the 100 ppm PFOS diet based on one-sample t-tests ($p<0.05$, GraphPad Prism®, v. 6.04). There was no significant difference in PFOS concentration between the 100 ppm PFOS and 100 ppm PFOS + CS groups based on a two-sample t-test ($p>0.05$, GraphPad Prism®, v. 6.04).

Supplemental Table 5. Day 2 Non-fasted Serum Clinical Chemistry Results in Male Rats fed Control, Choline Supplemented (CS), 100 ppm PFOS, or 100 ppm PFOS + CS Diets for Three Weeks

		Control (basal choline)			Choline Supplemented (CS)			100 ppm PFOS			100 ppm PFOS + CS		
Parameter	**Units**	**Mean**	**SD**	**N**	**Mean**	**SD**	**N**	**Mean**	**SD**	**N[1]**	**Mean**	**SD**	**N**
Glucose	mg/dL	126	11.6	12	128	13.1	12	126	8.0	12	124	15.1	12
Aspartate aminotransferase	U/L	69	7.2	12	61 [a]	6.7	12	58 [a]	3.8	12	61 [a]	6.3	12
Alanine aminotransferase	U/L	29	4.0	12	27	3.1	12	26	3.0	12	25 [a]	3.1	12
Gamma glutamyl transferase	U/L	0.7	0.7	12	0.8	0.6	12	0.6	0.5	12	0.5	0.5	12
Alkaline Phosphatase	U/L	449	27.0	12	434	57.5	12	384 [a]	56.0	12	396	50.3	12
Total Bilirubin	mg/dL	0.02	0.0	12	0.03	0.0	12	0.01	0.03	12	0.01	0.03	12
Direct Bilirubin	mg/dL	<0.1	-	12	<0.1	-	12	<0.1	-	12	<0.1	-	12
Indirect Bilirubin	mg/dL	NC[2]	-	12	NC	-	12	NC	-	12	NC	-	12
Cholesterol	mg/dL	104	9.6	12	103	12.0	12	97	9.4	12	101	5.5	12
Triglycerides	mg/dL	117	40.6	12	159	57.6	12	119	39.1	12	115	51.2	12
Total Protein	g/dL	6.0	0.2	12	6.0	0.2	12	5.8	0.2	12	6.0	0.1	12
Albumin	g/dL	3.3	0.1	12	3.2	0.1	12	3.2	0.1	12	3.3	0.1	12
Blood Urea Nitrogen	mg/dL	15	1.8	12	16 [c,d]	2.7	12	13 [b]	2.5	12	13 [b]	2.1	12
Creatinine	mg/dL	0.1	0.1	12	0.1	0.05	12	0.1	0.04	11	0.1	0.1	12
Phosphorus	mg/dL	8.5	0.6	12	8.8 [c,d]	0.5	12	9.4 [a,b]	0.6	12	10 [a,b]	0.4	12
Calcium	mg/dL	10	0.2	12	10	0.4	12	10	0.3	12	11 [a]	0.2	12

Supplemental Table 5. Day 2 Non-fasted Serum Clinical Chemistry Results in Male Rats fed Control, Choline Supplemented (CS), 100 ppm PFOS, or 100 ppm PFOS + CS Diets for Three Weeks

		Control (basal choline)			Choline Supplemented (CS)			100 ppm PFOS			100 ppm PFOS + CS		
Parameter	**Units**	**Mean**	**SD**	**N**	**Mean**	**SD**	**N**	**Mean**	**SD**	**N[1]**	**Mean**	**SD**	**N**
Sodium	mmol/L	139	4.2	12	140	3.5	12	141	2.5	12	144 [a]	4.2	12
Potassium	mmol/L	5.9	0.4	12	5.7 [d]	0.5	12	5.9	0.2	12	6.2 [b]	0.3	12
Chloride	mmol/L	99	3.4	12	100	2.5	12	100	1.8	12	104 [a]	3.6	12
Bicarbonate	mmol/L	20	4.3	12	19	2.7	12	21	1.5	12	20	5.3	12
Anion Gap	mmol/L	26	4.8	12	26	3.7	12	25	2.0	12	26	6.3	12
Creatine Kinase	U/L	142	41.8	12	115	43.8	12	107	23.2	12	127	31.2	12
Lactate Dehydrogenase	U/L	298	186.0	12	171	142.6	12	119 [a]	65.2	12	161 [a]	57.8	12
Amylase	U/L	761	60.8	12	768	93.8	12	816	80.0	12	817	67.6	12
Lipase	U/L	6.4	1.0	12	7.3 [c,d]	1.3	12	4.1 [a,b]	0.8	12	3.9 [a,b]	1.4	12
Globulin	g/dL	2.7	0.1	12	2.7	0.2	12	2.6	0.1	12	2.8	0.1	12
A/G Ratio	-	1.2	0.1	12	1.2	0.1	12	1.2	0.1	12	1.2	0.1	12
Urea/Creatinine Ratio	-	121	47.0	12	142	45.1	12	123	40.8	11	104	36.6	12
Na/K Ratio	mmol/L	24	1.1	12	25	1.9	12	24	0.9	12	23	1.0	12
High density lipoprotein	mg/dL	36	3.3	12	35	3.4	12	36	3.0	12	37	2.3	12
Low density lipoprotein	mg/dL	22	2.7	12	21 [c,d]	3.3	12	17 [a,b]	2.3	12	17 [a,b]	1.6	12

Supplemental Table 5. Day 2 Non-fasted Serum Clinical Chemistry Results in Male Rats fed Control, Choline Supplemented (CS), 100 ppm PFOS, or 100 ppm PFOS + CS Diets for Three Weeks

		Control (basal choline)			Choline Supplemented (CS)			100 ppm PFOS			100 ppm PFOS + CS		
Parameter	**Units**	**Mean**	**SD**	**N**	**Mean**	**SD**	**N**	**Mean**	**SD**	**N[1]**	**Mean**	**SD**	**N**

1. Insufficient serum was available due to technical difficulties which reduced the N for the indicated parameters. These small differences in N are not expected to have affected the outcome of the study.
2. NC indicates not calculated.

Statistically significant differences are indicated for each group comparison vs. control (a), vs. CS (b), (c) vs. PFOS, and (d) vs. PFOS + CS. using one-way ANOVA with Tukey's multiple comparisons test, $p<0.05$.

Supplemental Table 6. Day 9 Non-fasted Serum Clinical Chemistry Results in Male Rats fed Control, Choline Supplemented (CS), 100 ppm PFOS, or 100 ppm PFOS + CS Diets for Three Weeks

		Control (basal choline)			Choline Supplemented (CS)			100 ppm PFOS			100 ppm PFOS + CS		
Parameter	**Units**	**Mean**	**SD**	**N**	**Mean**	**SD**	**N**	**Mean**	**SD**	**N**	**Mean**	**SD**	**N**
Glucose	mg/dL	108	15.6	12	101	10.5	12	110 [b]	9.4	12	123	16.6	12
Aspartate aminotransferase	U/L	89	20.0	12	76 [a]	8.3	12	64 [a]	5.2	12	63 [a]	6.8	12
Alanine aminotransferase	U/L	32	6.3	12	28	3.2	12	28	3.7	12	26 [a]	3.7	12
Gamma glutamyl transferase	U/L	0.4	0.5	12	0.7	0.7	12	0.3	0.5	12	0.3	0.5	12
Alkaline Phosphatase	U/L	346	40.0	12	343	36.3	12	310	43.8	12	293	50.7	12
Total Bilirubin	mg/dL	0.1	0.1	12	0.0 [a]	0.1	12	0.02 [a]	0.04	12	0.01 [a]	0.03	12
Direct Bilirubin	mg/dL	<0.1	-	12	<0.1	-	12	<0.1	-	12	<0.1	-	12
Indirect Bilirubin	mg/dL	NC[1]	-	12	NC	-	12	NC	-	12	NC	-	12
Cholesterol	mg/dL	96	9.4	12	106 [a,c,d]	7.7	12	74 [a,b]	8.1	12	81 [a,b]	7.9	12
Triglycerides	mg/dL	116	38.5	12	121 [d]	46.6	12	85	23.6	12	80 [b]	17.9	12
Total Protein	g/dL	6.4	0.3	12	6.4	0.2	12	6.5	0.2	12	6.3	0.2	12
Albumin	g/dL	3.6	0.1	12	3.5 [c]	0.1	12	3.7 [a,b]	0.1	12	3.6 [c]	0.1	12
Blood Urea Nitrogen	mg/dL	13	1.8	12	12 [d]	1.6	12	10 [a]	2.3	12	8.6 [a,b]	1.6	12
Creatinine	mg/dL	0.2	0.1	12	0.2	0.03	12	0.2	0.04	12	0.2	0.03	12
Phosphorus	mg/dL	8.8	0.5	12	8.5	0.5	12	8.0	0.5	12	8.4	0.5	12

Supplemental Table 6. Day 9 Non-fasted Serum Clinical Chemistry Results in Male Rats fed Control, Choline Supplemented (CS), 100 ppm PFOS, or 100 ppm PFOS + CS Diets for Three Weeks

		Control (basal choline)			Choline Supplemented (CS)			100 ppm PFOS			100 ppm PFOS + CS		
Parameter	**Units**	**Mean**	**SD**	**N**	**Mean**	**SD**	**N**	**Mean**	**SD**	**N**	**Mean**	**SD**	**N**
Calcium	mg/dL	10	0.3	12	10	0.3	12	10	0.3	12	10	0.3	12
Sodium	mmol/L	143	3.7	12	141	4.4	12	142	2.6	12	142	3.7	12
Potassium	mmol/L	5.7	0.3	12	5.2 [a]	0.3	12	5.2 [a]	0.2	12	5.3 [a]	0.2	12
Chloride	mmol/L	100	2.7	12	97	3.2	12	100	1.5	12	101	2.0	12
Bicarbonate	mmol/L	19	2.5	12	20	1.7	12	22 [a]	0.7	12	20	1.9	12
Anion Gap	mmol/L	30	2.9	12	28	3.1	12	26 [a]	2.3	12	27 [a]	4.2	12
Creatine Kinase	U/L	283	235.0	12	207	60.3	12	126 [a]	30.9	12	150	63.4	12
Lactate Dehydrogenase	U/L	1008	584.2	12	789 [c, d]	326.3	12	381 [a,b]	142.8	12	337 [a,b]	156.2	12
Amylase	U/L	865	82.0	12	852	150.1	12	849	84.0	12	840	95.4	12
Lipase	U/L	5.8	1.3	12	7.9 [a,c,d]	0.9	12	4.3 [b]	1.1	12	4.8 [b]	2.8	12
Globulin	g/dL	2.8	0.2	12	2.9	0.1	12	2.8	0.1	12	2.8	0.1	12
A/G Ratio	-	1.3	0.1	12	1.2 [c,d]	0.05	12	1.4 [a,b]	0.1	12	1.3 [b]	0.1	12
Urea/Creatinine Ratio	-	56	12.4	12	59	9.8	12	54	22.1	12	47	15.9	12
Na/K Ratio	mmol/L	25	1.7	12	27 [a]	1.2	12	28 [a]	1.5	12	27	1.1	12
High density lipoprotein	mg/dL	40	3.4	12	43 [c,d]	3.5	12	31 [a,b]	4.3	12	32 [a,b]	2.9	12
Low density lipoprotein	mg/dL	17	2.2	12	16 [c,d]	1.9	12	13 [a,b]	2.3	12	13 [a,b]	2.2	12
1. NC indicates not calculated.													

Supplemental Table 6. Day 9 Non-fasted Serum Clinical Chemistry Results in Male Rats fed Control, Choline Supplemented (CS), 100 ppm PFOS, or 100 ppm PFOS + CS Diets for Three Weeks

		Control (basal choline)			Choline Supplemented (CS)			100 ppm PFOS			100 ppm PFOS + CS		
Parameter	**Units**	**Mean**	**SD**	**N**	**Mean**	**SD**	**N**	**Mean**	**SD**	**N**	**Mean**	**SD**	**N**
Statistically significant differences are indicated for each group comparison vs. control (a), vs. CS (b), (c) vs. PFOS, and (d) vs. PFOS + CS. using one-way ANOVA with Tukey's multiple comparisons test, $p<0.05$.													

Supplemental Table 7. Day 16 Non-fasted Serum Clinical Chemistry Results in Male Rats fed Control, Choline Supplemented (CS), 100 ppm PFOS, or 100 ppm PFOS + CS Diets for Three Weeks

		Control (basal choline)			Choline Supplemented (CS)			100 ppm PFOS			100 ppm PFOS + CS		
Parameter	**Units**	**Mean**	**SD**	**N**	**Mean**	**SD**	**N**	**Mean**	**SD**	**N**	**Mean**	**SD**	**N**
Glucose	mg/dL	119	11.5	12	126	11.3	12	131	22.3	12	122	15.5	12
Aspartate aminotransferase	U/L	63	8.3	12	58	4.6	12	62	7.2	12	57	5.5	12
Alanine aminotransferase	U/L	28	7.2	12	26	3.7	12	30	7.0	12	25	7.2	12
Gamma glutamyl transferase	U/L	0.2	0.4	12	0.3	0.5	12	0.0	0.0	12	0.1	0.3	12
Alkaline Phosphatase	U/L	281	36.0	12	293	24.1	12	303	36.2	12	273	43.0	12
Total Bilirubin	mg/dL	0.03	0.1	12	0.02	0.04	12	0.02	0.04	12	0.03	0.05	12
Direct Bilirubin	mg/dL	<0.1	-	12	<0.1	-	12	<0.1	-	12	<0.1	-	12
Indirect Bilirubin	mg/dL	NC[1]	-	12	NC	-	12	NC	-	12	NC	-	12
Cholesterol	mg/dL	87	10.7	12	105 [a,c,d]	13.3	12	54 [a,b]	7.4	12	58 [a,b]	7.3	12
Triglycerides	mg/dL	120	36.6	12	131 [c,d]	26.5	12	71 [a,b]	19.3	12	67 [a,b]	23.6	12
Total Protein	g/dL	6.3	0.2	12	6.3	0.3	12	6.5	0.3	12	6.2 [c]	0.2	12
Albumin	g/dL	3.5	0.1	12	3.5 [c]	0.2	12	3.8 [a,b]	0.2	12	3.6 [c]	0.1	12
Blood Urea Nitrogen	mg/dL	14	1.0	12	14	2.1	12	14	2.6	12	13	1.6	12
Creatinine	mg/dL	0.2	0.0	12	0.2	0.03	12	0.2	0.03	12	0.2	0.03	12
Phosphorus	mg/dL	7.5	0.4	12	7.6	0.6	12	7.8	0.6	12	7.6	0.6	12

Supplemental Table 7. Day 16 Non-fasted Serum Clinical Chemistry Results in Male Rats fed Control, Choline Supplemented (CS), 100 ppm PFOS, or 100 ppm PFOS + CS Diets for Three Weeks

		Control (basal choline)			Choline Supplemented (CS)			100 ppm PFOS			100 ppm PFOS + CS		
Parameter	**Units**	**Mean**	**SD**	**N**	**Mean**	**SD**	**N**	**Mean**	**SD**	**N**	**Mean**	**SD**	**N**
Calcium	mg/dL	10	0.3	12	11 [a]	0.4	12	11 [a]	0.5	12	10	0.3	12
Sodium	mmol/L	140	3.7	12	142	3.2	12	144 [a]	3.3	12	140 [c]	2.1	12
Potassium	mmol/L	5.4	0.4	12	5.1	0.4	12	4.9 [a]	0.3	12	4.8 [a]	0.3	12
Chloride	mmol/L	100	2.3	12	101	2.3	12	103 [a]	2.6	12	102	1.6	12
Bicarbonate	mmol/L	20	1.9	12	21 [c]	1.5	12	19 [b]	2.1	12	18	1.7	12
Anion Gap	mmol/L	26	3.3	12	25	1.6	12	27	2.2	12	25	2.4	12
Creatine Kinase	U/L	140	42.5	12	87 [a]	10.0	12	83 [a]	21.9	12	81 [a]	13.2	12
Lactate Dehydrogenase	U/L	370	218	12	163 [a]	58.2	12	167 [a]	82.1	12	187 [a]	92.3	12
Amylase	U/L	846	76.7	12	860	135.5	12	837	78.9	12	804	69.2	12
Lipase	U/L	6.6	1.4	12	7.8 [c,d]	1.5	12	3.8 [a,b]	0.9	12	3.8 [a,b]	1.6	12
Globulin	g/dL	2.8	0.1	12	2.8 [c,d]	0.2	12	2.6 [a,b]	0.1	12	2.6 [a,b]	0.1	12
A/G Ratio	-	1.2	0.1	12	1.2 [c,d]	0.1	12	1.5 [a,b]	0.1	12	1.4 [a,b]	0.1	12
Urea/Creatinine Ratio	-	67	10.4	12	66	12.9	12	67	13.1	12	69	26.4	12
Na/K Ratio	mmol/L	26	1.8	12	28	1.9	12	30 [a]	1.8	12	29 [a]	2.0	12
High density lipoprotein (HDL)	mg/dL	38	3.9	12	41 [c,d]	3.9	12	26 [a,b]	2.6	12	25 [a,b]	3.3	12
Low density lipoprotein (LDL)	mg/dL	14	2.6	12	16 [c,d]	2.1	12	7.8 [a,b]	1.4	12	8.1 [a,b]	1.2	12
1. NC indicates not calculated.													

Supplemental Table 7. Day 16 Non-fasted Serum Clinical Chemistry Results in Male Rats fed Control, Choline Supplemented (CS), 100 ppm PFOS, or 100 ppm PFOS + CS Diets for Three Weeks

		Control (basal choline)			Choline Supplemented (CS)			100 ppm PFOS			100 ppm PFOS + CS		
Parameter	**Units**	**Mean**	**SD**	**N**	**Mean**	**SD**	**N**	**Mean**	**SD**	**N**	**Mean**	**SD**	**N**
Statistically significant differences are indicated for each group comparison vs. control (a), vs. CS (b), (c) vs. PFOS, and (d) vs. PFOS + CS. using one-way ANOVA with Tukey's multiple comparisons test, $p<0.05$.													

Supplemental Table 8. Day 23 Fasted Serum Clinical Chemistry Results in Male Rats fed Control, Choline Supplemented (CS), 100 ppm PFOS, or 100 ppm PFOS + CS Diets for Three Weeks

		Control (basal choline)			Choline Supplemented (CS)			100 ppm PFOS			100 ppm PFOS + CS		
Parameter	**Units**	**Mean**	**SD**	**N[1]**	**Mean**	**SD**	**N**	**Mean**	**SD**	**N**	**Mean**	**SD**	**N[1]**
Glucose	mg/dL	107	34.3	12	117	42.2	12	116	22.4	12	112	12.0	12
Aspartate aminotransferase	U/L	80	8.9	12	80	7.5	12	95	15.2	12	96	31.2	12
Alanine aminotransferase	U/L	25	4.7	12	25 [c,d]	4.7	12	54 [a,b]	16.2	12	49 [a,b]	22.8	12
Gamma glutamyl transferase	U/L	0.1	0.3	12	0.0	0.0	12	0.0	0.0	12	0.0	0.0	12
Alkaline Phosphatase	U/L	136	104	12	114	7.2	12	146	20.6	12	143	16.5	12
Total Bilirubin	mg/dL	0.1	0.1	12	0.1 [c,d]	0.0	12	0.3 [a,b,d]	0.1	12	0.2 [a,b,c]	0.1	12
Direct Bilirubin	mg/dL	<0.1	-	12	<0.1	-	12	<0.1	-	12	<0.1	-	12
Indirect Bilirubin	mg/dL	NC[2]	-	12	NC	-	12	NC	-	12	NC	-	12
Cholesterol	mg/dL	84	10.8	12	89 [c,d]	9.5	12	46 [a,b]	5.2	12	47 [a,b]	7.3	12
Triglycerides	mg/dL	57	9.3	12	53 [c,d]	14.9	12	28 [a,b]	6.3	12	31 [a,b]	5.0	12
Total Protein	g/dL	6.5	0.4	12	6.4 [c,d]	0.3	12	6.9 [a,b]	0.3	12	6.9 [a,b]	0.2	11
Albumin	g/dL	3.6	0.2	12	3.6 [c,d]	0.2	12	3.9 [a,b]	0.1	12	3.9 [a,b]	0.1	12
Blood Urea Nitrogen	mg/dL	15	1.7	12	13 [a,c,d]	1.0	12	18 [a,b]	2.1	12	16 [b]	2.0	12
Creatinine	mg/dL	0.3	0.1	12	0.3 [c,d]	0.0	12	0.2 [a,b]	0.04	12	0.2 [a,b]	0.04	12
Phosphorus	mg/dL	12	1.3	12	12	1.2	12	12	0.8	12	11	1.0	12

Supplemental Table 8. Day 23 Fasted Serum Clinical Chemistry Results in Male Rats fed Control, Choline Supplemented (CS), 100 ppm PFOS, or 100 ppm PFOS + CS Diets for Three Weeks

		Control (basal choline)			Choline Supplemented (CS)			100 ppm PFOS			100 ppm PFOS + CS		
Parameter	**Units**	**Mean**	**SD**	**N[1]**	**Mean**	**SD**	**N**	**Mean**	**SD**	**N**	**Mean**	**SD**	**N[1]**
Calcium	mg/dL	12	0.7	12	12 [c,d]	0.4	12	13 [a,b]	0.3	12	12 [a,b]	0.3	12
Sodium	mmol/L	143	4.6	11	143 [d]	2.1	12	145	1.3	12	146 [a,b]	2.0	12
Potassium	mmol/L	9.0	1.2	11	8.8	1.0	12	8.4	0.5	12	8.6	1.4	12
Chloride	mmol/L	99	2.4	11	97 [d]	2.1	12	98	1.6	12	100 [b]	1.2	12
Bicarbonate	mmol/L	27	2.9	12	28	2.8	12	31 [a]	3.4	12	31 [a]	3.4	12
Anion Gap	mmol/L	26	2.3	11	26	3.4	12	25	4.1	12	24	3.5	12
Creatine Kinase	U/L	119	39.5	12	117	75.7	12	118	28.6	12	98	48.3	12
Lactate Dehydrogenase	U/L	358	148	12	270 [c,d]	83.3	12	678 [b]	134	12	627 [b]	594	12
Amylase	U/L	592	79.2	12	638 [c,d]	130.6	12	525 [b]	75.2	12	498 [b]	50.6	12
Lipase	U/L	8.3	2.2	12	9.4 [c,d]	2.5	12	5.5 [a,b]	2.1	12	5.1 [a,b]	2.3	11
Globulin	g/dL	2.9	0.2	12	2.8	0.2	12	3.0	0.2	12	3.0	0.2	11
A/G Ratio	-	1.3	0.1	12	1.3	0.1	12	1.3	0.1	12	1.3	0.1	11
Urea/Creatinine Ratio	-	52	37.4	12	43 [c,d]	9.3	12	84 [a,b]	14.7	12	94 [a,b]	25.7	12
Na/K Ratio	mmol/L	16	2.8	11	16	1.9	12	17	1.2	12	17	2.5	12
High density lipoprotein	mg/dL	25	3.7	12	27 [c]	3.1	12	22 [b]	2.9	12	24 [b]	3.4	12
Low density lipoprotein	mg/dL	14	3.8	12	11 [a,c,d]	1.6	12	3.6 [a,b]	0.7	12	4.1 [a,b]	1.3	12

Supplemental Table 8. Day 23 Fasted Serum Clinical Chemistry Results in Male Rats fed Control, Choline Supplemented (CS), 100 ppm PFOS, or 100 ppm PFOS + CS Diets for Three Weeks

		Control (basal choline)			Choline Supplemented (CS)			100 ppm PFOS			100 ppm PFOS + CS		
Parameter	**Units**	**Mean**	**SD**	**N[1]**	**Mean**	**SD**	**N**	**Mean**	**SD**	**N**	**Mean**	**SD**	**N[1]**

1. Insufficient serum was available due to technical difficulties during blood collection which reduced the N for the indicated parameters. These differences in the number of sera analyzed did not affect the interpretation of the study results.
2. NC indicates not calculated.

Statistically significant differences are indicated for each group comparison vs. control (a), vs. CS (b), (c) vs. PFOS, and (d) vs. PFOS + CS using one-way ANOVA with Tukey's multiple comparisons test, $p<0.05$.

Supplemental Table 9. Day 2 Non-fasted Serum Clinical Chemistry Results in Female Rats fed Control, Choline Supplemented (CS), 100 ppm PFOS, or 100 ppm PFOS + CS Diets for Three Weeks

		Control (basal choline)			Choline Supplemented (CS)			100 ppm PFOS			100 ppm PFOS + CS		
Parameter	**Units**	**Mean**	**SD**	**N[1]**	**Mean**	**SD**	**N[1]**	**Mean**	**SD**	**N**	**Mean**	**SD**	**N[1]**
Glucose	mg/dL	95.5	16.8	12	119 [a]	13.6	12	115 [a]	7.7	12	121 [a]	8.3	12
Aspartate aminotransferase	U/L	65	7.0	11	60 [c]	8.1	12	70 [b]	9.9	12	60 [c]	5.0	12
Alanine aminotransferase	U/L	19	3.6	12	20	1.9	12	21	3.2	12	21	2.3	12
Gamma glutamyl transferase	U/L	0.6	0.5	7	0.8	0.4	12	0.8	0.6	12	0.3	0.5	12
Alkaline Phosphatase	U/L	295	56.4	12	311	46.8	12	268	49.4	12	268	53.5	12
Total Bilirubin	mg/dL	0.0	0.0	8	0.01	0.03	12	0.01	0.0	12	0.0	0.0	12
Direct Bilirubin	mg/dL	<0.1	-	8	<0.1	-	12	<0.1	-	12	<0.1	-	12
Indirect Bilirubin	mg/dL	NC[2]	-	12	NC	-	12	NC	-	12	NC	-	12
Cholesterol	mg/dL	82	15.0	12	93	9.0	12	97[a]	12.9	12	98[a]	15.7	12
Triglycerides	mg/dL	50	13.6	12	52	20.2	12	70	38.3	12	54	18.6	12
Total Protein	g/dL	5.4	0.7	7	5.4	0.3	12	5.7	0.3	12	5.8[a, b]	0.2	11
Albumin	g/dL	3.1	0.5	8	3.2	0.2	12	3.3	0.2	12	3.3	0.1	11
Blood Urea Nitrogen	mg/dL	15.7	3.1	12	14.4	2.8	12	12.9	2.3	12	12.4[a]	2.6	12
Creatinine	mg/dL	0.1	0.0	6	0.1	0.03	12	0.2	0.1	12	0.1	0.0	12
Phosphorus	mg/dL	7.9	0.7	7	8.3	0.6	12	8.9 [a]	0.6	12	9.1 [a]	0.7	11

Supplemental Table 9. Day 2 Non-fasted Serum Clinical Chemistry Results in Female Rats fed Control, Choline Supplemented (CS), 100 ppm PFOS, or 100 ppm PFOS + CS Diets for Three Weeks

		Control (basal choline)			Choline Supplemented (CS)			100 ppm PFOS			100 ppm PFOS + CS		
Parameter	**Units**	**Mean**	**SD**	**N[1]**	**Mean**	**SD**	**N[1]**	**Mean**	**SD**	**N**	**Mean**	**SD**	**N[1]**
Calcium	mg/dL	9.9	0.3	6	9.7	0.4	12	9.9	0.4	12	9.8	0.3	12
Sodium	mmol/L	143	3.8	6	137[a]	2.0	12	139[a]	1.5	12	139[a]	1.8	12
Potassium	mmol/L	5.7	0.5	6	5.8	0.3	12	5.6	0.3	12	5.9	0.2	12
Chloride	mmol/L	104	3.1	6	101	1.9	12	99.8[a]	2.6	12	102[c]	1.6	12
Bicarbonate	mmol/L	20.0	2.1	6	18.3	2.9	12	19.8	2.8	12	18.2	2.8	12
Anion Gap	mmol/L	24.8	3.2	6	23.6	3.0	12	25.3	3.1	12	24.8	3.5	12
Creatine Kinase	U/L	145	30.3	8	124	37.1	12	191[b]	66.0	12	115[c]	36.9	12
Lactate Dehydrogenase	U/L	457	97.9	8	291[c]	185	12	565[b, d]	320	12	210[c]	91.0	12
Amylase	U/L	445	53.7	8	499	120	12	549[a]	84	12	460	33.7	12
Lipase	U/L	1.9	0.9	7	2.2[c]	1.1	11	3.7 [a, b]	1.2	12	2.3[c]	1.0	12
Globulin	g/dL	2.2	0.4	7	2.3[d]	0.1	12	2.4[a]	0.1	12	2.5[a, b]	0.1	11
A/G Ratio	-	1.5	0.3	7	1.4	0.1	12	1.4	0.1	12	1.3	0.1	11
Urea/Creatinine Ratio	-	145.8	40.8	6	137	31.8	12	99.6	44.9	12	102	34.5	12
Na/K Ratio	mmol/L	25.0	1.3	6	23.8[c]	1.1	12	25.2[b, d]	1.6	12	23.7[c]	0.9	12
High density lipoprotein	mg/dL	29.8	3.5	9	29.6	2.5	12	30.8	2.7	12	30.6	2.9	12
Low density lipoprotein	mg/dL	12.9	1.8	10	14.2	2.2	12	14.8	2.4	12	13.6	2.5	12

Supplemental Table 9. Day 2 Non-fasted Serum Clinical Chemistry Results in Female Rats fed Control, Choline Supplemented (CS), 100 ppm PFOS, or 100 ppm PFOS + CS Diets for Three Weeks

		Control (basal choline)			Choline Supplemented (CS)			100 ppm PFOS			100 ppm PFOS + CS		
Parameter	**Units**	**Mean**	**SD**	**N[1]**	**Mean**	**SD**	**N[1]**	**Mean**	**SD**	**N**	**Mean**	**SD**	**N[1]**

1. Insufficient serum was available due to technical difficulties which reduced the N for the indicated parameters. These small differences in N are not expected to have affected the outcome of the study.
2. NC indicates not calculated.

Statistically significant differences are indicated for each group comparison vs. control (a), vs. CS (b), (c) vs. PFOS, and (d) vs. PFOS + CS using one-way ANOVA with Tukey's multiple comparisons test, $p<0.05$.

Supplemental Table 10. Day 9 Non-fasted Serum Clinical Chemistry Results in Female Rats fed Control, Choline Supplemented (CS), 100 ppm PFOS, or 100 ppm PFOS + CS Diets for Three Weeks

		Control (basal choline)			Choline Supplemented (CS)			100 ppm PFOS			100 ppm PFOS + CS		
Parameter	**Units**	**Mean**	**SD**	**N[1]**	**Mean**	**SD**	**N**	**Mean**	**SD**	**N**	**Mean**	**SD**	**N**
Glucose	mg/dL	114	11.4	12	121	10.9	12	120	10.5	12	133 [a,b,c]	10.2	12
Aspartate aminotransferase	U/L	70	7.5	12	59[a]	5.1	12	59 [a]	5.3	12	58 [a]	3.9	12
Alanine aminotransferase	U/L	26	5.1	12	22	4.3	12	26	5.9	12	24	3.8	12
Gamma glutamyl transferase	U/L	1.0	0.0	12	0.8	0.4	12	0.6	0.5	12	0.8	0.5	12
Alkaline Phosphatase	U/L	360	47.6	12	325	94.1	12	272 [a]	46.8	12	285 [a]	28.9	12
Total Bilirubin	mg/dL	0.0	0.0	12	0.0	0.0	12	0.0	0.0	12	0.0	0.0	12
Direct Bilirubin	mg/dL	<0.1	-	12	<0.1	-	12	<0.1	-	12	<0.1	-	12
Indirect Bilirubin	mg/dL	NC[1]	-	12	NC	-	12	NC	-	12	NC	-	12
Cholesterol	mg/dL	92	7.6	12	99 [c,d]	12.3	12	82 [b]	12.0	12	83[b]	11.4	12
Triglycerides	mg/dL	46	6.8	12	54	23.8	12	50.6	9.3	12	57	13.1	12
Total Protein	g/dL	6.2	0.2	12	6.2 [c]	0.2	12	6.5 [a,b]	0.2	12	6.2 [c]	0.2	12
Albumin	g/dL	3.7	0.1	12	3.6 [c,d]	0.1	12	3.9 [a, b, d]	0.1	12	3.7 [b, c]	0.2	12
Blood Urea Nitrogen	mg/dL	14	2.1	12	15 [c,d]	2.8	12	11 [a, b]	3.0	12	11 [a, b]	2.1	12
Creatinine	mg/dL	0.2	0.04	12	0.2	0.05	12	0.2	0.1	12	0.2	0.0	12
Phosphorus	mg/dL	7.7	0.5	12	8.2 [d]	0.7	12	7.9	0.5	12	7.5 [b]	0.4	12

Supplemental Table 10. Day 9 Non-fasted Serum Clinical Chemistry Results in Female Rats fed Control, Choline Supplemented (CS), 100 ppm PFOS, or 100 ppm PFOS + CS Diets for Three Weeks

		Control (basal choline)			Choline Supplemented (CS)			100 ppm PFOS			100 ppm PFOS + CS		
Parameter	**Units**	**Mean**	**SD**	**N[1]**	**Mean**	**SD**	**N**	**Mean**	**SD**	**N**	**Mean**	**SD**	**N**
Calcium	mg/dL	10	0.3	12	10	0.3	12	11 [a]	0.2	12	11 [a]	0.3	12
Sodium	mmol/L	137	6.1	12	137 [c]	4.8	12	142 [a, b]	2.0	12	140	1.5	12
Potassium	mmol/L	5.1	0.4	12	5.0	0.4	12	5.2	0.4	12	4.8	0.3	12
Chloride	mmol/L	98.8	5.7	12	99.6 [c]	3.8	12	104 [a, b]	1.9	12	103 [a]	1.8	12
Bicarbonate	mmol/L	21.3	1.2	12	18.9	2.0	12	19.2	5.0	12	19.8	2.1	12
Anion Gap	mmol/L	22.2	2.2	12	23.8	2.3	12	24.3	5.8	12	21.6	1.7	12
Creatine Kinase	U/L	167	47.6	12	135	44.0	12	120 [a, b]	29.4	12	82.8 [a]	17.0	12
Lactate Dehydrogenase	U/L	507	196.4	12	282 [a,d]	159	12	209 [a]	66.3	12	124 [a, b]	72.9	12
Amylase	U/L	539	78.8	12	533	106	12	632 [a, b]	85.9	12	597	58.8	12
Lipase	U/L	4.4	0.7	12	6.0 [a,c,d]	1.3	12	4.3 [b, d]	0.9	12	2.9 [a, b, c]	0.7	12
Globulin	g/dL	2.5	0.1	12	2.6	0.1	12	2.6	0.1	12	2.5 [b, c]	0.1	12
A/G Ratio	-	1.4	0.1	12	1.4 [c,d]	0.0	12	1.5 [b]	0.1	12	1.5 [b]	0.1	12
Urea/Creatinine Ratio	-	74.0	12.7	12	66.0	15.4	12	53.5	23.5	12	59.6	27.2	12
Na/K Ratio	mmol/L	27.2	1.5	12	27.7	1.6	12	27.8	2.4	12	29.3 [a]	2.3	12
High density lipoprotein	mg/dL	33.4	2.5	12	34.7 [c]	2.3	12	32.4 [b]	3.4	12	30.8	2.1	12
Low density lipoprotein	mg/dL	13.0	2.2	12	13.1	2.5	12	10.9	2.4	12	11.0	2.7	12
1. NC indicates not calculated.													

Supplemental Table 10. Day 9 Non-fasted Serum Clinical Chemistry Results in Female Rats fed Control, Choline Supplemented (CS), 100 ppm PFOS, or 100 ppm PFOS + CS Diets for Three Weeks

		Control (basal choline)			Choline Supplemented (CS)			100 ppm PFOS			100 ppm PFOS + CS		
Parameter	**Units**	**Mean**	**SD**	**N[1]**	**Mean**	**SD**	**N**	**Mean**	**SD**	**N**	**Mean**	**SD**	**N**
Statistically significant differences are indicated for each group comparison vs. control (a), vs. CS (b), (c) vs. PFOS, and (d) vs. PFOS + CS using one-way ANOVA with Tukey's multiple comparisons test, $p<0.05$.													

Supplemental Table 11. Day 16 Non-fasted Serum Clinical Chemistry Results in Female Rats fed Control, Choline Supplemented (CS), 100 ppm PFOS, or 100 ppm PFOS + CS Diets for Three Weeks

		Control (basal choline)			Choline Supplemented (CS)			100 ppm PFOS			100 ppm PFOS + CS		
Parameter	**Units**	**Mean**	**SD**	**N**	**Mean**	**SD**	**N**	**Mean**	**SD**	**N**	**Mean**	**SD**	**N[1]**
Glucose	mg/dL	118	8.4	12	119	12.5	12	121	10.1	12	117	12.3	11
Aspartate aminotransferase	U/L	65	6.2	12	58 [a]	5.4	12	60	4.5	12	60	5.4	11
Alanine aminotransferase	U/L	28	4.5	12	27	2.8	12	27	3.9	12	30	6.4	11
Gamma glutamyl transferase	U/L	0.8	0.6	12	0.4	0.5	12	0.2	0.4	12	0.5	0.5	11
Alkaline Phosphatase	U/L	353	47.5	12	345 [c, d]	69.1	12	246 [a, b]	55.5	12	254 [a, b]	42.9	11
Total Bilirubin	mg/dL	0.03	0.05	12	0.0 [c]	0.0	12	0.04 [b, d]	0.1	12	0.0 [c]	0.0	11
Direct Bilirubin	mg/dL	<0.1	-	12	<0.1	-	12	<0.1	-	12	<0.1	-	11
Indirect Bilirubin	mg/dL	NC[2]	-	12	NC	-	12	NC	-	12	NC	-	11
Cholesterol	mg/dL	89	10.3	12	94 [c, d]	8.0	12	68 [a, b]	7.8	12	73 [a, b]	16.7	11
Triglycerides	mg/dL	53	15.6	12	54	15.3	12	58	17.3	12	41	10.0	11
Total Protein	g/dL	6.4	0.2	12	6.4 [c]	0.2	12	6.8 [a, b]	0.2	12	6.5	0.3	11
Albumin	g/dL	3.7	0.1	12	3.7 [c, d]	0.1	12	4.0 [a, b]	0.1	12	3.9 [b, c]	0.2	11
Blood Urea Nitrogen	mg/dL	16	1.9	12	16 [c, d]	1.7	12	12 [a, b]	2.4	12	13 [a, b]	3.3	11
Creatinine	mg/dL	0.2	0.0	12	0.2	0.0	12	0.3	0.1	12	0.2	0.0	11
Phosphorus	mg/dL	7.1	0.4	12	7.4	0.7	12	7.0	0.6	12	6.7 [b]	0.6	11

Supplemental Table 11. Day 16 Non-fasted Serum Clinical Chemistry Results in Female Rats fed Control, Choline Supplemented (CS), 100 ppm PFOS, or 100 ppm PFOS + CS Diets for Three Weeks

		Control (basal choline)			Choline Supplemented (CS)			100 ppm PFOS			100 ppm PFOS + CS		
Parameter	**Units**	**Mean**	**SD**	**N**	**Mean**	**SD**	**N**	**Mean**	**SD**	**N**	**Mean**	**SD**	**N[1]**
Calcium	mg/dL	10	0.3	12	10	0.3	12	11	0.3	12	11	0.4	11
Sodium	mmol/L	140	2.5	12	140 [c, d]	0.9	12	144 [a, b]	3.0	12	144 [a, b]	3.0	11
Potassium	mmol/L	5.0	0.3	12	5.0	0.3	12	4.9	0.3	12	4.7 [a]	0.2	11
Chloride	mmol/L	102	2.7	12	103	1.1	12	105 [a]	2.7	12	105 [a]	2.7	11
Bicarbonate	mmol/L	21.3	3.4	12	20.3 [c]	1.8	12	16.8 [a, b]	2.8	12	18.1 [a]	3.1	11
Anion Gap	mmol/L	22.6	3.8	12	22.5 [c]	1.7	12	27.5 [a, b]	4.1	12	25.4	3.9	11
Creatine Kinase	U/L	142	39.9	12	110 [a]	31.4	12	105 [a]	22.4	12	83.1 [a]	13.9	11
Lactate Dehydrogenase	U/L	419	145	12	230 [a]	117	12	277 [a]	96.0	12	203 [a]	80.3	11
Amylase	U/L	507	55.6	12	550 [c, d]	81.7	12	744 [a, b]	134	12	664 [a, b]	74.1	11
Lipase	U/L	6.4	0.9	12	6.1 [c, d]	1.1	12	4.6 [a, b, d]	0.7	12	3.3 [a, b, c]	0.6	11
Globulin	g/dL	2.7	0.1	12	2.7	0.2	12	2.8	0.2	12	2.7	0.2	11
A/G Ratio	-	1.4	0.0	12	1.4	0.1	12	1.5	0.1	12	1.5	0.1	11
Urea/Creatinine Ratio	-	73	19.4	12	71 [c]	12.4	12	49 [a, b]	16.2	12	63	16.6	11
Na/K Ratio	mmol/L	28	1.5	12	28 [d]	1.7	12	30	1.7	12	31 [a, b]	1.3	11
High density lipoprotein	mg/dL	36	3.0	12	37 [c, d]	2.2	12	30 [a, b]	3.3	12	30 [a, b]	4.9	11
Low density lipoprotein	mg/dL	10.3	1.5	12	9.4	1.2	12	9.3	1.8	12	9.5	3.0	11

Supplemental Table 11. Day 16 Non-fasted Serum Clinical Chemistry Results in Female Rats fed Control, Choline Supplemented (CS), 100 ppm PFOS, or 100 ppm PFOS + CS Diets for Three Weeks

		Control (basal choline)			Choline Supplemented (CS)			100 ppm PFOS			100 ppm PFOS + CS		
Parameter	**Units**	**Mean**	**SD**	**N**	**Mean**	**SD**	**N**	**Mean**	**SD**	**N**	**Mean**	**SD**	**N[1]**

1. Insufficient serum was available due to technical difficulties which reduced the N for the indicated parameters. These small differences in N are not expected to have affected the outcome of the study.
2. NC indicates not calculated.

Statistically significant differences are indicated for each group comparison vs. control (a), vs. CS (b), (c) vs. PFOS, and (d) vs. PFOS + CS using one-way ANOVA with Tukey's multiple comparisons test, $p<0.05$.

Supplemental Table 12. Day 23 Fasted Serum Clinical Chemistry Results in Female Rats fed Control, Choline Supplemented (CS), 100 ppm PFOS, or 100 ppm PFOS + CS Diets for Three Weeks

		Control (basal choline)			Choline Supplemented (CS)			100 ppm PFOS			100 ppm PFOS + CS		
Parameter	**Units**	**Mean**	**SD**	**N[1]**	**Mean**	**SD**	**N**	**Mean**	**SD**	**N**	**Mean**	**SD**	**N**
Glucose	mg/dL	62.8	6.1	12	83.5 [a, c, d]	10.0	12	152 [a,b]	38.9	12	177 [a,b]	53.7	12
Aspartate aminotransferase	U/L	92	24.5	11	69.5 [a]	5.6	12	67 [a]	7.4	12	72 [a]	14.5	12
Alanine aminotransferase	U/L	32	20.1	12	21	3.8	12	25	4.2	12	29	6.6	12
Gamma glutamyl transferase	U/L	0.1	0.3	11	0.2	0.4	12	0.0	0.0	12	0.0	0.0	12
Alkaline Phosphatase	U/L	87	25.6	12	85	18.6	12	89	16.0	12	92	15.7	12
Total Bilirubin	mg/dL	0.1	0.1	12	0.0	0.0	12	0.1	0.1	12	0.1	0.0	12
Direct Bilirubin	mg/dL	<0.1	-	12	<0.1	-	12	<0.1	-	12	<0.1	-	12
Indirect Bilirubin	mg/dL	NC[2]	-	12	NC	-	12	NC	-	12	NC	-	12
Cholesterol	mg/dL	93	10.5	12	94	13.1	12	79	14.3	12	78	18.5	12
Triglycerides	mg/dL	48	8.7	12	37	3.7	12	36	4.6	12	44	21.6	12
Total Protein	g/dL	6.7	0.3	12	6.4 [c, d]	0.2	12	7.4 [a, b]	0.3	12	7.3 [a, b]	0.6	12
Albumin	g/dL	3.9	0.2	12	3.6 [a, c, d]	0.1	12	4.4 [a, b]	0.2	12	4.2 [a, b]	0.4	12
Blood Urea Nitrogen	mg/dL	15	1.2	12	14[d]	2.2	12	19	1.8	12	22 [a, b]	10.4	12
Creatinine	mg/dL	0.4	0.1	12	0.3	0.1	12	0.3 [a]	0.0	12	0.3 [a]	0.1	12

Supplemental Table 12. Day 23 Fasted Serum Clinical Chemistry Results in Female Rats fed Control, Choline Supplemented (CS), 100 ppm PFOS, or 100 ppm PFOS + CS Diets for Three Weeks

		Control (basal choline)			Choline Supplemented (CS)			100 ppm PFOS			100 ppm PFOS + CS		
Parameter	**Units**	**Mean**	**SD**	**N[1]**	**Mean**	**SD**	**N**	**Mean**	**SD**	**N**	**Mean**	**SD**	**N**
Phosphorus	mg/dL	14	2.2	11	12	0.8	12	11 [a]	1.0	12	12	4.0	12
Calcium	mg/dL	12	0.8	11	12 [a, c, d]	0.4	12	13 [a, b]	0.4	12	13 [a, b]	0.5	12
Sodium	mmol/L	142	1.9	11	145 [a]	2.5	12	145 [a]	1.3	12	146 [a]	2.8	12
Potassium	mmol/L	11	1.6	11	9.4 [a]	1.1	12	8.6 [a]	0.6	12	9.6 [a]	0.9	12
Chloride	mmol/L	101	1.3	11	101	2.0	12	102	1.8	12	102	1.8	12
Bicarbonate	mmol/L	28.3	4.6	11	29.4	4.1	12	29.2	2.7	12	27.0	6.0	12
Anion Gap	mmol/L	23.5	4.4	11	24.3	5.1	12	22.6	2.6	12	26.2	8.5	12
Creatine Kinase	U/L	296	327	12	68.3 [a]	6.8	12	102 [a]	18.8	12	92.4 [a]	14.2	12
Lactate Dehydrogenase	U/L	680	885	12	165 [a]	30.8	12	347	76.4	12	281	90.0	12
Amylase	U/L	297	29.2	12	354	69.6	12	401	82.2	12	599	723.4	12
Lipase	U/L	9.9	2.5	12	7.4	1.7	12	5.3	1.1	12	12	22.1	12
Globulin	g/dL	2.8	0.2	12	2.8	0.2	12	3.0	0.2	12	3.1 [a]	0.3	12
A/G Ratio	-	1.4	0.1	12	1.3 [c, d]	0.1	12	1.5 [a, b, d]	0.1	12	1.4 [c]	0.1	12
Urea/Creatinine Ratio	-	44.0	11.0	12	51.8 [c, d]	13.2	12	75 [a ,b]	20.4	12	86 [a ,b]	20.9	12
Na/K Ratio	mmol/L	13.5	2.1	11	15.4 [a]	1.7	12	17 [a, d]	1.2	12	15 [c]	1.6	12
High density lipoprotein	mg/dL	31.1	2.8	12	31.0	3.1	12	30.0	4.0	12	31	4.5	12
Low density lipoprotein	mg/dL	9.8	1.7	12	10.8[c]	2.0	12	8.1 [b]	2.0	12	8.5	3.3	12

Supplemental Table 12. Day 23 Fasted Serum Clinical Chemistry Results in Female Rats fed Control, Choline Supplemented (CS), 100 ppm PFOS, or 100 ppm PFOS + CS Diets for Three Weeks

		Control (basal choline)			Choline Supplemented (CS)			100 ppm PFOS			100 ppm PFOS + CS		
Parameter	**Units**	**Mean**	**SD**	**N[1]**	**Mean**	**SD**	**N**	**Mean**	**SD**	**N**	**Mean**	**SD**	**N**

1. Insufficient serum was available due to technical difficulties which reduced the N for the indicated parameters. These differences in the number of sera analyzed did not affect the interpretation of the study results.
2. NC indicates not calculated.

Statistically significant differences are indicated for each group comparison vs. control (a), vs. CS (b), (c) vs. PFOS, and (d) vs. PFOS + CS using one-way ANOVA with Tukey's multiple comparisons test, $p<0.05$.

Supplemental Table 13. Incidence and Severity of Liver Histopathology Results In Male and Female Sprague Dawley Rats fed Control, Choline Supplemented (CS), 100 ppm PFOS, or 100 ppm PFOS + CS Diets for Three Weeks

Sex	Male				Female			
Group	Control (basal diet)	Choline Supplemented (CS)	100 ppm PFOS	100 ppm PFOS + CS	Control (basal diet)	Choline Supplemented (CS)	100 ppm PFOS	100 ppm PFOS + CS
Number of animals	**12**	**12**	**12**	**12**	**12**	**12**	**12**	**12**
Parameter (severity)								
Anatomic deformity	1 [a]	0	0	0	0	0	0	0
Fibrosis	1 [a]	0	0	0	0	0	0	0
- Minimal	1	- [b]	-	-	-	-	-	-
Ground-glass cytoplasmic alteration	0	0	12	12	0	0	0	0
- Mild	-	-	4	11	-	-	-	-
- Moderate	-	-	8	1	-	-	-	-
Vacuolation, microvesicular	1	0	1	0	0	0	1	0
- Minimal	1	0	1	0	-	-	1	-
Hypertrophy	0	0	9	1	0	0	7	2
- Minimal	-	-	7	1	-	-	5	2
- Mild	-	-	2	-	-	-	2	-
Necrosis	0	0	1	4	0	0	2	2
- Minimal	-	-	-	1	-	-	-	1
- Mild	-	-	-	3	-	-	2	-
- Moderate	-	-	1	-	-	-	-	1

Supplemental Table 13. Incidence and Severity of Liver Histopathology Results In Male and Female Sprague Dawley Rats fed Control, Choline Supplemented (CS), 100 ppm PFOS, or 100 ppm PFOS + CS Diets for Three Weeks

Sex	Male				Female			
Group	Control (basal diet)	Choline Supplemented (CS)	100 ppm PFOS	100 ppm PFOS + CS	Control (basal diet)	Choline Supplemented (CS)	100 ppm PFOS	100 ppm PFOS + CS
Lipid accumulation, microvesicular (Oil Red O Qualitative Evaluation)	11	12	12	12	12	12	11	12
- Minimal	11	12	-	-	12	2	10	9
- Mild	-	-	-	3	-	10	1	3
- Moderate	-	-	6	9	-	-	-	-
- Severe	-	-	6	-	-	-	-	-

[a] One control male rat had a cleft across the sampled lobe of the liver which was associated with fibrosis.
[b] – indicates there were no observations for this group at this severity.

Supplemental Table 14. Liver mRNA Fold Change of Rats that were fed 100 ppm PFOS Diet Relative to Rats that were fed the Control Diet for Three Weeks (n = 4/sex/group)

mRNA	100 ppm PFOS	
	Male	Female
Ehhadh	3.74 ± 0.26*	4.14 ± 0.58*
Scd1	2.07 ± 1.2	9.04 ± 1.8*
Cd36	1.32 ± 0.2	1.58 ± 1.0
Fsp27	1.77 ± 0.5	1.23 ± 0.2
Gsst1	2.43 ± 1.1	1.93 ± 1.0

* Significantly different from the control group within sex using one-way ANOVA with Tukey's multiple comparisons test ($p<0.05$, GraphPad Prism®, v. 6.04).

Supplemental Table 15. *In vitro* fold activation of nuclear receptors in luciferase assays

	Agonist Assays				Inverse Agonist Assay
PFOS Concentration (µM)	**Rat Liver X Receptor Beta**	**Human Liver X Receptor Alpha**	**Mouse/Rat Peroxisome Proliferator Activated Receptor Gamma**	**Rat Pregnane X Receptor**	**Rat Constitutive Androstane Receptor**
0 (DMSO vehicle)	1.0 (100%)[1]	1.0 (100%)	1.0 (100%)	1.0 (100%)	1.0 (100%)
0.4	0.98 (99%)	0.92 (98%)	0.98 (98%)	1.2 (104%)	-11 (110%)
1.2	1.1 (99%)	1.3 (102%)	1.1 (100%)	1.3 (105%)	-0.71 (107%)
3.7	1.1 (100%)	1.3 (101%)	1.0 (101%)	1.4 (105%)	1.7 (108%)
11.1	0.96 (103%)	1.3 (104%)	1.2 (103%)	1.3 (100%)	9.5 (96%)
33.3	1.1 (99%)	1.1 (96%)	1.6 (98%)	1.1 (100%)	7.6 (100%)
100.0	0.73 (100%)	1.1 (101%)	4.2 (100%)	1.5 (104%)	-11 (105%)

1. Values are presented as fold activation relative to the vehicle control. Values in ( ) are % viable cells relative to the vehicle control.