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ROLE OF CYP2B6 IN PERFLUOROOCTANESULFONIC ACID (PFOS)-INDUCED TOXICITY AND NON ALCOHOLIC FATTY LIVER DISEASE (NAFLD)

A Thesis
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Master of Science
Environmental Toxicology

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by
Matthew C. Hamilton
August 2020

Accepted by:
Dr. William S. Baldwin, Committee Chair
Dr. Lisa Bain
Dr. Charles Rice

Abstract

Perfluorooctanesulfonic acid (PFOS) is a persistent, toxic fluorosurfactant foam used in firefighting foams, textiles, and other industrial products. Human CYP2B6 is predominantly expressed in the liver and responsible for metabolizing xeno- and endobiotics. CYP2B is induced by PFOS and high-fat diets in rodents and therefore it was hypothesized that CYP2B contributed to PFOS-induced steatosis. Cyp2b-9/10/13-null (Cyp2b-null) and humanized CYP2B6-Tg (hCYP2B6-Tg) mice were treated with PFOS (0, 1, or 10 mg/kg/day) by oral gavage in mice fed either a typical chow diet (ND) or a high-fat diet (HFD). Our studies show human CYP2B6 is also inducible *in vivo* by PFOS. In addition, three ND-fed hCYP2B6-Tg female mice treated with 10 mg/kg/day PFOS died during the exposure period. Similarly treated HFD-fed mice did not die. Interestingly, hCYP2B6-Tg mice retained significantly more PFOS in the serum and liver than Cyp2b-null mice presumably leading to the observed toxicity. Serum PFOS retention was significantly reduced in the HFD-fed hCYP2B6-Tg mice, which is the opposite trend observed in HFD-fed Cyp2b-null mice. Hepatotoxicity biomarkers, ALT and ALP, were higher in PFOS-treated mice and lowered by a HFD. However, PFOS combined with a HFD exacerbated hepatic lipid accumulation in all mice, especially in the hCYP2B6-Tg mice with significant disruption of key lipid metabolism genes such as *Srebp1, Pparγ, and Cpt1a*. In conclusion, CYP2B6 is induced by PFOS and protects from PFOS-mediated steatosis in ND-fed mice; however, it's presence increases hepatic triglyceride content in HFD-PFOS co-treated mice and increases toxicity in ND-fed mice.

Dedication

I would like to dedicate this thesis to my mom and dad who have always pushed me to be the best that I can be and to my brother and sister who have always been there to support me. Their love and support throughout my entire life have enabled me to be where I am today. Not only have my parents told me to work hard for what I want, but they have shown me what it takes to get there. My dad received his Master's degree in Chemistry and obtained his Juris Doctor shortly thereafter while working full time. He practiced law with a science background for nearly 30 years allowing us to have many experiences, including living abroad. My mom was a successful business woman at a young age who chose to do the hardest thing of all, leave it behind to raise three kids. Raising three happy, healthy children who all went to college was not enough for her. She found the time to do numerous volunteer activities and over the past few years has been the head accountant for a non-profit organization. The passion and drive I observed in my parents has shown me anything is possible if you work hard and desire it enough. With that said, I would also like to dedicate this to all of the friends I have made along the way.

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Chapter I: Background

Purpose of the Study

The main objective of this study was to determine the role of Cytochrome P450 2B6 (CYP2B6) in the perfluorooctanesulfonic acid (PFOS)-mediated sublethal toxicity and development of NAFLD, *in vivo.* We used two mouse models, Cyp2b9/10/13-null (Cyp2b-null) and a humanized CYP2B6 mouse (hCYP2B6-Tg) that lacks the murine hepatic Cyp2b's but contains human CYP2B6, previously generated by Dr. Melissa Heintz in our laboratory. In addition, we will investigate whether a Western-like high-fat diet (HFD) significantly escalates PFOS-induced fatty liver disease and if CYP2B6, similar to Cyp2b9 and Cyp2b10, is inducible by PFOS *in vivo*.

Routes of PFOS Contamination and Ingestion

PFOS is a chemical that was produced in the manufacturing of many products ranging from durable stain and water repellent used on fabrics, carpets, and furniture to being a key chemical in aqueous film foaming form (AFFF) used in firefighting foams (OECD, 2006). Other consumer and industrial products include paper and cardboard food packaging, insecticides, electronics, paints, and non-stick cooking surfaces (EPA, 2016a). PFOS is manufactured through two different methods; electrochemical fluorination (ECF) and telomerization (EPA, 2016b). During the more common technique used to manufacture PFOS, ECF, both branched and linear PFOS chains are produced (30% and 70%, respectively) whereas only linear chains are produced by telomerization. Both forms are able to leach into the environment and bioaccumulate (Beesoon & Martin,

2015; Yu et al., 2015). In 2002, the top manufacturer of PFOS, 3M, phased out its production in the United States (US) and the next top 8 manufacturers of PFOS in the US followed shortly behind (Wan et al., 2012). Although PFOS has been phased out of production in the US and Europe, it is still manufactured in China to produce consumer goods and the exact amount of PFOS produced is unknown due to a lack of regulation (Lim et al., 2011). PFOS is able to bioaccumulate in the environment because it is poorly degraded under standard environmental conditions due to its stable structure (Yamashita et al., 2005).

The majority of PFOS ingestion occurs while eating contaminated food or drinking contaminated water; however, PFOS can also be inhaled if the soil or dust is contaminated (EPA, 2017). Recent testing shows that cities in the US, including Brunswick County (NC) and Quad Cities (IA), have higher levels of PFOS in the tap water (Evans et al., 2020) than the EPA health advisory risk of 70 ppt (0.07 ng/mL) (EPA, 2017). There are still places around the world with PFOS levels higher than 70 ppt in their drinking water; particularly those near a factory that produced or still produces fluorinated compounds such as Veneto Region, Italy as well as Jiangsu Province, China (Ingelido et al., 2018; Yu et al., 2015). PFOS is not only persistent in the environment but also persistent in humans with a half-life of approximately 5.4 years (Olsen & Zobel, 2007). Overall, PFOS concentration detected in the environment as well as human sera has decreased over time as a result of being phased out of production in most countries but due to its stable structure, PFOS is still a concern to the environment, wildlife, and human health (Chang et al., 2016; Ingelido et al., 2018; Yu et al., 2015).

PFOS Distribution in the Human Body

PFOS is able to bioaccumulate in the body due to its high lipophilicity and partition coefficients (Ahrens et al., 2011; Allendorf et al., 2019), its prevalence in the environment and drinking water, ability to be readily absorbed in the gastrointestinal tract, stability at physiological conditions in humans, and its poor clearance rate (Seacat et al., 2002). PFOS was detected in over 98% of human serum samples tested (Calafat et al., 2007). People who were occupationally exposed to PFOS had serum PFOS levels of approximately 1,000 ng/mL, and the general US population had serum PFOS concentrations ranging from 9-30 ng/mL (Frisbee et al., 2009; Kato et al., 2011; Olsen et al., 2012; Olsen & Zobel, 2007).

PFOS is primarily eliminated in the urine but can be eliminated from the body in feces through bile mediated intestinal secretions (Chang et al., 2012; Harada et al., 2007). The renal clearance of PFOS in humans is extremely low due to its high renal reabsorption into the blood (Zhang et al., 2013). PFOS is also readily absorbed in the GI tract through the apical-sodium dependent bile salt transporter (ASBT) (Zhao et al., 2017). PFOS probably enters the liver through the Na^{+}/taurocholate cotransporting polypeptide (NTCP), a sodium transporter located on the sinusoidal side of hepatocytes and has been shown to mediate the uptake of PFOS into liver cells (Zhao et al., 2017).

PFOS primarily binds electrostatically to albumin as well as a few other proteins in the blood that allows PFOS to travel readily throughout the body (Weiss et al., 2009; Zhang et al., 2009). PFOS is able to bind more strongly to albumin when it is a linear

chain and not branched (Beesoon & Martin, 2015). The two primary compartments for PFOS accumulation in humans are the blood and the liver (Olsen et al., 2003); however, PFOS has also been shown to accumulate in kidney and lung tissues with trace amounts in brain and bone tissue (Pérez et al., 2013).

General PFOS Toxicity

PFOS exposure is associated with hepatotoxicity. PFOS ingestion has been shown to decrease body weight and increase liver weight (Fletcher et al., 2013; Lau et al., 2007; Wang et al., 2014). In addition, PFOS exposure is associated with peroxisome proliferation, decreased serum triglyceride levels, decreased serum cholesterol levels, and increased expression of genes involved with fatty acid oxidation (Kim et al., 2011; Qazi et al., 2010; Rosen et al., 2009; Wang et al., 2014). The impact that PFOS has on fatty acid oxidation within the liver perturbs lipid metabolism by affecting natural biological processes. These data taken together show the extreme effects on the liver from PFOS exposure and how levels of important biomarkers, such as cholesterol and triglycerides, are affected by PFOS as well. It is important to continue to study PFOS to better understand the mechanism by which PFOS toxicity is mediated for therapeutic purposes; toxicity is correlated to the concentration of PFOS.

Since PFOS is able to bioaccumulate, several studies have looked at whether PFOS levels in humans increase with age (Frisbee et al., 2009; Ji et al., 2012; Olsen et al., 2003; Zeng et al., 2015). There are varying reports about the dependence of age on PFOS levels (Ji et al., 2012; Zeng et al., 2015) but overall increased age has been associated

with lower PFOS concentration (Frisbee et al., 2009; Olsen et al., 2003); possibly due to an increase in renal clearance as a result of less renal reabsorption (Ingelido et al., 2018). PFOS exposure led to an increase in renal tubular apoptosis, in mice, by inducing Sirt1which deacetylated p53. The deacetylation of p53 resulted in an increased interaction between p53 and Bax; ultimately, resulting in an increase in cytosolic cytochrome C and caspase 3 activation (Wen et al., 2016), possibly suggesting toxicity associated with prolonged PFOS exposure and supporting the increased renal clearance because of decreased renal reabsorption due to apoptosis theory.

Research studies continue to find that PFOS has damaging effects on the body (Geng et al., 2019; Zarei et al., 2018). Although PFOS is primarily detected in the liver and blood, with some accumulation in the lungs and kidney, its toxicity has been observed throughout the human body.

PFOS Effects on NAFLD and Peroxisome Proliferator-Activated Receptors

NAFLD is a common liver disorder defined as the presence of at least 5% of the liver being steatotic (5% weight is lipid) (Younossi et al., 2016). Approximately 30% of Americans are currently suffering from it (Younossi et al., 2016). Due to the high prevalence of NAFLD in humans, there are many *in vivo* studies to determine the different mechanisms involved with this disease (Bagley et al., 2017; George et al., 2003). There are a variety of different genes associated with the progression of NAFLD, such as *CYP4A14* and *CYP2E1*, and the prevention of NAFLD, such as *CYP3A4* and murine *Cyp2b9&10*, that are regulated by ligand activated transcription factors including

PPARs, CAR, and PXR (Cobbina & Akhlaghi, 2017; Heintz et al., 2019; Zhang et al., 2017). PFOS has been shown to promote NAFLD through direct interaction with peroxisome proliferators in addition to independently of peroxisome proliferation; the exact mechanisms of PFOS toxicity have not fully been elucidated yet (Andersen et al., 2008; Jin et al., 2020; Kersten & Stienstra, 2017; Rosen et al., 2010; Skat-Rørdam et al., 2019).

Peroxisome proliferator-activated receptors (PPARs), alpha (-α) and gamma (-γ), are transcription factors that regulate gene expression in response to endogenous and exogenous ligands including fatty acids, eicosanoids, pyrethrins, and trichloroethylene (Corton, 2008; Liss & Finck, 2017; Takeuchi et al., 2006). PFOS has been shown to activate peroxisome proliferators (Shipley et al., 2004; Wolf et al., 2012; Zhang et al., 2014). PPARα is expressed in human liver and highly expressed in murine liver and is responsible for regulating genes involved in fatty acid beta-oxidation and plays a vital role in energy homeostasis (Corton et al., 2014; Klaunig et al., 2012; Peraza et al., 2006; Rosen et al., 2010). PFOS activates mouse and human PPARα, more so mouse, in a COS-1 cell based luciferase reporter *trans*-activation assay (Shipley et al., 2004; Wolf et al., 2012). The downstream response of activating PPARα by PFOS was evaluated using FAO-PPARα cells and an upregulation in genes involved in fatty acid oxidation, including peroxisomal 3-ketoacyl-CoA thiolase, peroxisomal bifunctional enzyme, acyl-CoA oxidase, and urate oxidase was observed (Shipley et al., 2004). Using wild type (WT) and PPARα-null mice, PFOS was shown to be a weak activator of PPARα *in vivo* and that PFOS is able to elicit hepatotoxicity independent of PPARα (Rosen et al., 2010).

These data show that PFOS can activate PPARα to some extent; however, it is probably not the predominant mechanism of PFOS induced steatosis.

PPARγ has been a therapeutic target for diabetes and NAFLD; rosiglitazone, a potent PPARγ inducer, showed significant decreases in hepatotoxicity biomarkers and increased sensitivity to insulin in patients with diabetes but elicited unwanted side effects such as obesity (Liss & Finck, 2017; Neuschwander-Tetri et al., 2003). PPARγ is expressed in many tissues, such as liver, heart, and most abundantly in adipose tissue, and plays a role in lipid metabolism, adipocyte differentiation, and inflammation (Zhang et al., 2014). HepG2 cells, a human liver cancer cell line, transfected with human PPARγ showed that PFOS is a weak agonist of PPARγ *in vitro*; however, PFOS induces the disruption of lipid homeostasis and inflammation by activating PPARγ (Zhang et al., 2014). PFOS exposure showed little to no change in *Ppar*γ gene expression in animals (Fang et al., 2012; Wen et al., 2016). Since PFOS is a weak PPARγ agonist *in vitro* and does not elicit much change in *Ppar*γ expression *in vivo,* PPARγ mediated PFOS toxicity is probably less of an issue than PPARα.

PFOS Effects on Lipid Metabolism and Mitochondrial Function

PFOS is a hydrophobic compound that has a polar head and nonpolar tail, resembling a biological fatty acid (Fletcher et al., 2013). The structure of PFOS allows it to bind the hepatocyte nuclear factor 4-alpha (HNF4α) which is a nuclear transcription factor responsible for metabolic regulation, including glucose and lipid metabolism, and

hepatocyte differentiation (Beggs et al., 2016; Bonzo et al., 2012). The ability of PFOS to directly bind HNF4α was shown using *in silico* docking simulations (Beggs et al., 2016) supporting the possibility of direct interaction of PFOS and HNF4α. HNF4α is highly expressed in the liver and is repressed by PFOS, resulting in the downregulation of genes involved in lipid metabolism and markers of hepatocellular differentiation, such as cholesterol 7α-hydroxylase (CYP7A1) and alcohol dehydrogenase 1-beta (ADH1B), respectively (Beggs et al., 2016). The activation of HNF4α in mice has been shown to upregulate genes involved in mitochondrial β-oxidation, ketogenesis, and lipid transport protecting against NAFLD progression (Huang et al., 2020). It is important to note that HNF4a also transcriptionally regulates PPARα which could explain the observed PFOS hepatotoxicity in PPARα-null mice (Beggs et al., 2016; Rosen et al., 2010). These data suggests a decrease in lipid metabolism due to the repression of HNF4α expression by PFOS, resulting in increased hepatic steatosis and the progression of NAFLD.

PFOS has been shown to decrease serum glucose, triglycerides, and cholesterol in mice (Bijland et al., 2011; Wang et al., 2014) but increase hepatic triglyceride accumulation by downregulating enzymes, such as carnitine palmitoyl transferase 1A (CPT1A), in addition to decreasing LDL/VLDL production (Cheng et al., 2016; Eisenberg, 1990). CPT1A and CYP7A1 are key enzymes in lipid metabolism and are induced by a HFD but down-regulated by PFOS (Wang et al., 2014). The observed down-regulation by PFOS decreased the amount of fatty acids transported into the mitochondria as well as decrease the amount of fatty acids converted into cholesterol. Very low density lipids (VLDL) are cholesterol that transport fatty acids out of the liver

to peripheral tissues to help prevent the buildup of hepatic triglycerides (Eisenberg, 1990) and prevent NAFLD progression. PFOS has been shown to decrease VLDL in the liver resulting in an increase in retention of fatty acids ultimately leading to steatosis and hepatomegaly (Bijland et al., 2011; Cui et al., 2017). The inhibition of CPT1A indicates a decrease in the transport of fatty acids into the mitochondria for mitochondrial β-oxidation resulting in fatty acid accumulation within the liver (Cheng et al., 2016).

PFOS was found to have the highest inhibitory effect on mitochondrial function of all fluorinated compounds tested on isolated rat liver mitochondria (Wallace et al., 2013). The respiratory inhibition was a result of PFOS altering the mitochondrial membrane fluidity (Wallace et al., 2013). The increase in mitochondrial membrane fluidity as a result of PFOS exposure is similar to how cholesterol increases mitochondrial membrane fluidity by inserting itself into the lipid bilayer (Matyszewska et al., 2008; Wallace et al., 2013). PFOS exposure, both in mice and zebrafish, significantly decreases mitochondrial β-oxidation and increases peroxisomal β-oxidation within the liver as well as decreases LDL/VLDL production; ultimately, resulting in poor fatty acid utilization and distribution, leading to hepatic steatosis (Cheng et al., 2016; Cui et al., 2017; Wan et al., 2012).

PFOS Immunotoxicity

PFOS has been associated with suppressed immune function *in vitro* and *in vivo* (Brieger et al., 2011; Peden-Adams et al., 2008; Qazi et al., 2009; Zheng et al., 2009). Human leukocytes treated with PFOS and stimulated with lipopolysaccharide (LPS)-

initiated a reduction in the number of natural killer (NK) cells, an important part of the innate immune system, produced as well as a decrease in the amount of tumor necrosis factor-alpha (TNF-α), a pro-inflammatory cytokine, *in vitro* (Brieger et al., 2011; Trinchieri, 1989). Lower levels of PFOS exposure (≤ 5 mg/kg/day) have been shown to not affect NK cell counts in mice (Peden-Adams et al., 2007); however, elevated levels of PFOS (20 mg/kg/day) tend to decrease the amount of NK cells in mice (Zheng et al., 2009).

High levels of PFOS (20 mg/kg/day) exposure over a short period of time (7-10 days) caused a decrease in the spleen and thymus weight of mice (Qazi et al., 2009; Zheng et al., 2009); however, no change was observed in these immune organs in mice treated with ≤ 5 mg/kg/day PFOS (Peden-Adams et al., 2008). One group showed that PFOS suppressed humoral immunity and identified B-cells or antigen presenting cells as the potential target for PFOS mediated immunotoxicity (Peden-Adams et al., 2008). The data suggests that the immune system is suppressed by PFOS at higher concentrations and thus the immune system cannot appropriately respond to foreign harmful cells when exposed to increased amounts of PFOS.

PFOS Neurotoxicity

PFOS interfered with the cell viability and cell differentiation of the neurotypical cell line, PC12 (Slotkin et al., 2008). 250 μmol of PFOS decreased cell viability and increased the number of cells differentiating into the acetylcholine neurotransmitter phenotype instead of the dopamine neurotransmitter phenotype (Slotkin et al., 2008).

Increased acetylcholine neurotransmitters has been shown to affect the cholinergic system resulting in a hypoactive response to nicotine in mice (Johansson et al., 2008).

PFOS causes neurological issues such as memory, learning, and motor behavior disabilities potentially due to increased apoptosis in the brain (Long et al., 2013). Mice exposed to 2.15 or 10 mg/kg/day PFOS took significantly longer to escape the Morris water maze test and spent less time in the target quadrant indicating an issue with learning and motor behavior (Long et al., 2013). These mice also had a significant increase in the number of apoptotic cells within their hippocampi compared to the control mice (0 mg/kg/day PFOS). In the mice treated with 10 mg/kg/day PFOS, dopamine and one metabolite of dopamine, dihydrophenolacetic acid (DOPAC), levels were significantly decreased whereas glutamate levels increased indicating brain damage (Long et al., 2013). Dopamine plays a role in locomotor activity (Hall et al., 2014) and a surge in glutamate levels is detrimental to nearby neurons as high levels of glutamate can impair learning and memory (Greene & Greenamyre, 1996). Taken together, these data suggest that PFOS perturbs neurotransmitter levels and can cause brain damage affecting memory, learning, and motor behavior.

PFOS Reproductive and Developmental Toxicity

PFOS causes reproductive and developmental effects in many studies (Lau et al., 2003; Thibodeaux et al., 2003; Yahia et al., 2008). PFOS (20 mg/kg/day) significantly decreased thyroxine levels in mice (T4; important for growth and metabolism); while triiodothyronine (T3) and thyroid stimulating hormone (TSH) were unaffected

(Thibodeaux et al., 2003). PFOS (10 or 15 mg/kg/day) lead to extreme birth defects such as reduced fetal birth weights, cleft palate, ventricular septal deficit, and an enlargement of the right atrium. No pups survived 24 hours from the mothers treated with 15 or 20 mg/kg/day PFOS and only 50% of the pups whose mothers were treated with 10 mg/kg/day PFOS survived longer than 24 hours. All of the pups whose mothers were treated with 5 mg/kg/day PFOS or more and survived experienced a significant delay in opening their eyes as well as a significant increase in liver weights (Lau et al., 2003). Another study found similar developmental PFOS toxicity (Yahia et al., 2008). All pups from mice treated with 20 mg/kg/day PFOS were born pale, weak, inactive, and died within 24 hours and 45% of pups from mice treated with 10 mg/kg/day PFOS died within 24 hours. These data provide strong support that in utero exposure to PFOS has extreme birth defects and severely compromises the pup's survival.

Cytochrome P450 2B6

CYPs are a super family of metabolic enzymes responsible for metabolizing xenobiotics and endogenous compounds. CYP families 1-3 are responsible for 90% of all phase l metabolism of xenobiotics (Wang & Tompkins, 2008). CYP3A4 is the most abundant CYP in human livers and is the most well-known metabolizer of xenobiotics and has been estimated to be responsible for about 50% of all drug metabolism (Achour et al., 2014; Wolbold et al., 2003). Historically CYP2B6 was thought to play a minor role in drug metabolism and has recently been identified as making up 6-10% of CYP expression in the liver and responsible for approximately 8% of all drug metabolism

(Desta & Flockhart, 2017; Nolan et al., 2006). Humans only have the one CYP2B gene, CYP2B6, which is predominantly found in the liver and is responsible for metabolizing environmental chemicals, steroids, and polyunsaturated fatty acids (PUFA's) (Nelson et al., 2004). CYP2B6 is regulated by Pregnane X Receptor (PXR), Constitutive Androstane Receptor (CAR), and FoxA2; all crucial xenobiotic or metabolic transcription factors (Hashita et al., 2008; Kretschmer & Baldwin, 2005). In models where CAR and FoxA2 are inhibited or knocked out the mice become obese or develop fatty liver disease (Bochkis et al., 2013; Dong et al., 2009). PFOS moderately activates CAR (Rosen et al., 2010) and there are many genes induced by PFOS that are known to be regulated by PXR suggesting that PFOS may activate PXR in addition to CAR (Bijland et al., 2011). CYP2B6 has been shown to be induced by PFOS *in vitro* but not *in vivo* (Rosen et al., 2013).

Mice have 5 Cyp2b members (Cyp2b9/10/13/19/23), where Cyp2b23 is only expressed in the liver of younger mice, Cyp2b19 is primarily expressed in the skin, and Cyp2b9/10/13 are expressed primarily in the liver (Finger et al., 2011). Interestingly, male Cyp2b9/10/13-null mice are diet induced obese (Heintz et al., 2019) as are humans exposed to PFOS (Liu et al., 2018). PFOS also induces fatty liver disease in mice while increasing murine Cyp2b members such as Cyp2b9 and Cyp2b10 (Das et al., 2017). Furthermore, Cyp2b9 is the most induced gene in several HFD studies (Heintz et al., 2019; Hoek-van den Hil et al., 2015; Leung et al., 2016) and Cyp2b10 is highly induced in several recent PFOS studies (Martell et al., 2018; Rooney et al., 2019; Rosen et al., 2010). These data show that murine hepatic Cyp2b's respond to factors that progress the

development of NAFLD, including PFOS, and begs the question whether or not human CYP2B6 plays a protective role in the progression of PFOS mediated NAFLD and if a HFD exacerbates NAFLD progression.

Mouse Models

A Cyp2b9/10/13 null mouse model was generated by removing a 287 kb section of murine chromosome 7 containing three hepatic Cyp2b genes to aid in determining the role of murine hepatic Cyp2b's in xenobiotic and endobiotic metabolism (Kumar et al., 2017). This was done by using the Crispr-Cas9 system and 3 short guided RNAs specific for each murine hepatic Cyp2b. Through non homologous end joining one of the resultant mice lacked Cyp2b9/10/13 (Kumar et al., 2017). The mouse lacking the murine hepatic Cyp2b's was crossed with a CYP2B6/2A13/2F1-Tg mouse generated by Dr. Xinxin Ding and containing the promoter regions of each CYP (Wei et al., 2012) to yield a mouse that possessed the human CYP2B6 gene and its regulatory regions while completely lacking the primarily hepatic Cyp2b's; Cyp2b9/10/13 (Heintz, 2020; Wei et al., 2012). The purpose of producing this mouse was to reduce the uncertainty associated with extrapolating data from murine to human CYPs. This will allow the human CYP2B6 gene to be studied *in vivo* and provide a better understanding of the role that CYP2B6 plays in obesity, fatty acid metabolism, and xenobiotic metabolism.

Hypothesis and Aims

We hypothesize that humanized CYP2B6 mice will be less susceptible to PFOS toxicity, including PFOS-mediated NAFLD in comparison to Cyp2b-null mice regardless of diet. Furthermore, PFOS will prove to be a potent CYP2B6 inducer *in vivo*.

Aim 1: To test whether CYP2B6 mitigates PFOS-induced NAFLD and whether a HFD exacerbates the development of PFOS-induced NAFLD.

Experimental Design

110 mice between the ages of 10-12 weeks were split into 19 different groups based on their gender, genotype, diet, and PFOS exposure. The mice were either exposed to 0 mg/kg/day, 1 mg/kg/day, or 10 mg/kg/day PFOS (0-, 1-, or 10-PFOS) via oral gavage every morning for 21 days. After the three-week exposure, the mice were euthanized and liver, serum, white adipose tissue (WAT), brown adipose tissue (BAT), kidneys, testes, brain, lower trapezius muscle, and gastrocnemius muscle were harvested and weighed. The tissues were snap frozen before being stored at -80$_{o}$C.

To evaluate the role CYP2B6 plays in PFOS mediated NAFLD we first examined whether CYP2B6 was inducible by PFOS or not. Microsomes and RNA were isolated from liver tissue and CYP2B6 protein and gene expression was determined via Western blot and qPCR. Oil Red O staining was conducted and analyzed to visualize the progression of NAFLD. Relative hepatic expression of genes related to NAFLD, peroxisome proliferation (*Pparγ* and *Cyp4a14*) and inflammation (*Cd68*), were measured using qPCR (Chistiakov et al., 2017; Skat-Rørdam et al., 2019; Zhang et al., 2017).

Important serum biomarkers, including ALT, ALP, glucose, cholesterol, triglycerides, and LDH, were analyzed to determine the different tissues and metabolic processes affected by PFOS.

Based on the literature, it was expected to see an increased expression of genes that promote NAFLD (*Cyp4a14*) and a decrease in expression of genes that repress NAFLD (*Ppar*γ). An increase in NAFLD was expected in groups treated with PFOS and potentiated by a HFD.

Aim 2: To compare PFOS's effects on lipid metabolism in CYP2B6 and Cyp2b-null mice.

To further investigate the effect of PFOS on lipid metabolism more qPCR was performed. Relative hepatic gene expression of enzymes and proteins involved in lipid metabolism (*Cpt1a*, *Hmgcr*, and *Srebp1*) were quantified by qPCR (Jiang et al., 2018; Ruiz et al., 2014; Schlaepfer & Joshi, 2020). It is expected to see a down regulation in expression of these genes as a result of PFOS.

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Chapter II: Increased toxicity and retention of Perflourooctane Sulfonate (PFOS) in humanized CYP2B6-Transgenic mice compared to Cyp2b-null mice is relieved by a High-Fat Diet (HFD).

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Abstract

Perfluorooctanesulfonic acid (PFOS) is a persistent, toxic fluorosurfactant foam used stain repellents, firefighting foams, textiles, and other industrial products. Human CYP2B6 is predominantly expressed in the liver and responsible for metabolizing xeno- and endobiotics. CYP2B is induced by PFOS and high-fat diets in rodents and therefore it was hypothesized that CYP2B contributed to PFOS-induced steatosis. Cyp2b-9/10/13-null (Cyp2b-null) and humanized CYP2B6-Tg (hCYP2B6-Tg) mice were treated with PFOS (0, 1, or 10 mg/kg/day) by oral gavage in mice fed either a typical chow diet (ND) or a high-fat diet (HFD). Our studies show human CYP2B6 is also inducible in vivo by PFOS. In addition, three ND-fed hCYP2B6-Tg female mice treated with 10 mg/kg/day PFOS died during the exposure period. Similarly treated HFD-fed mice did not die. Interestingly, hCYP2B6-Tg mice retained significantly more PFOS in the serum and liver than Cyp2b-null mice presumably leading to the observed toxicity. Serum PFOS retention was significantly reduced in the HFD-fed hCYP2B6-Tg mice, which is the opposite trend observed in HFD-fed Cyp2b-null mice. Hepatotoxicity biomarkers, ALT and ALP, were higher in PFOS-treated mice and lowered by a HFD. However, PFOS combined with a HFD exacerbated hepatic lipid accumulation in all mice, especially in the hCYP2B6-Tg mice with significant disruption of key lipid metabolism genes such as *Srebp1, Pparγ, and Cpt1a*. In conclusion, CYP2B6 is induced by PFOS and protects from PFOS-mediated steatosis in ND-fed mice; however, it's presence increases hepatic triglyceride content in HFD-PFOS co-treated mice and increases toxicity in ND-fed mice.

Introduction

Perflurooctanesulfonic acid (PFOS) is a chemical used in stain repellents, varnishes, cleaning products, semiconductors, and firefighting foams (OECD, 2006). PFOS was phased out of production in the United States (US) and Europe for most uses; however, it is still manufactured in China. PFOS is persistent and bioaccumulates in the environment (Yamashita et al., 2005), and therefore is found in surface, ground, and drinking water (Chang et al., 2016; EPA, 2017; OECD, 2006). PFOS is also persistent in humans with a half-life of approximately 5.4 years (Olsen & Zobel, 2007), and in turn is present in the majority of US citizens where obesity related illnesses are the leading causes of death (Gleason et al., 2015; Heron, 2019). It is detected in the blood of 98% of Americans with serum PFOS concentrations of approximately 1,000 ng/mL in occupationally exposed personnel and 9-30 ng/mL in the general US population (Frisbee et al., 2009; Gleason et al., 2015; Kato et al., 2011; Olsen et al., 2012; Olsen & Zobel, 2007).

PFOS leads to a decrease in body weight and an increase in liver weight in part due to non-alcoholic fatty liver disease (NAFLD) (Fletcher et al., 2013; Lau et al., 2007; Wang et al., 2014), while simultaneously decreasing serum glucose, triglycerides, and cholesterol in mice (Bijland et al., 2011; Wang et al., 2014). NAFLD is defined by the accumulation of hepatic fats that account for at least 5% of the liver weight, and can progress to non-alcoholic steatohepatitis (NASH) and ultimately lead to the development of fibrosis and cirrhosis (Cobbina & Akhlaghi, 2017; Zhang et al., 2017). PFOS has been shown to induce hepatic steatosis by interfering with key processes such as lipid

synthesis, lipid hepatic export process, and β-oxidation (Cheng et al., 2016). Reduced mitochondrial β-oxidation would and suppression of LDL/VLDL transport would lead to excess fatty acids in the liver (Cheng et al., 2016); resulting in a buildup of hepatic triglycerides.

CYPs, especially within families 1-3, are a superfamily of metabolic enzymes responsible for metabolizing xenobiotics and endogenous compounds (Kretschmer & Baldwin, 2005). Perturbations in several CYPs have been shown to increase NAFLD, including Cyp3a members, Cyp2b members, Cyp2j members, and Cyp2e1 (Heintz et al., 2019; Kumar et al., 2018; Olona et al., 2018; Seth et al., 2014). CYP2B6 is the only CYP2B member in humans. CYP2B6 metabolizes a large array of pharmaceuticals, environmental chemicals, steroids, and polyunsaturated fatty acids (PUFA's) (Desta & Flockhart, 2017; Hodgson & Rose, 2007; Wang & Tompkins, 2008), including pesticides, polyaromatic hydrocarbons, and industrial chemicals such as chlorpyrifos, benzo(a)pyrene, and styrene, respectively (Hodgson & Rose, 2007; Kim et al., 1997; Tang et al., 2001). CYP2B6 is regulated by the pregnane X-receptor (PXR), constitutive androstane receptor (CAR), and forkhead box A2 (FoxA2); all crucial xenobiotic and/or metabolic transcription factors (Hashita et al., 2008; Kretschmer & Baldwin, 2005).

PFOS moderately activates CAR and PXR in mice (Rosen et al., 2010) and induces murine Cyp2b9 and 10 (Das et al., 2017). It also activates peroxisome proliferator activated receptors (PPARs) that plays vital roles in the regulation of lipid metabolism,

distribution, energy homeostasis, and adipocyte maturation (Corton et al., 2014; Huck et al., 2018; Klaunig et al., 2012; Xu et al., 2016). PFOS induces human CYP2B6 *in vitro* but this has not been verified *in vivo* (Rosen et al., 2013).

Our laboratory has recently developed a Cyp2b-null mouse lacking the primarily hepatic Cyp2b9, 10, and 13 genes found in tandem repeat (Heintz et al., 2019; Kumar et al., 2017). In addition, we recently developed a humanized CYP2B6-Tg (hCYP2B6-Tg) mouse on the Cyp2b-null background to reduce some of the uncertainty when extrapolating from mice to humans. The CYP2B6 mouse will allow the human CYP2B6 gene to be studied *in vivo* and provide a better understanding of the role it plays in PFOS toxicity and the co-treatment of PFOS and a HFD. Cyp2b9/10/13-null mice are diet-induced obese in males (Heintz et al., 2019); however, these effects are not observed in females and in fact Cyp2b-null females are protected from NASH (Heintz et al., 2020). Cyp2b9 and Cyp2b10 are highly inducible in the liver by high-fat diets, and in several cases Cyp2b9 was the most highly induced gene (Heintz et al., 2019; Hoek-van den Hil et al., 2015; Leung et al., 2016). Whether CYP2B6 is induced by a HFD is not known.

Because murine Cyp2b members are highly induced by PFOS, the purpose of this study was to determine if human CYP2B6 is induced by PFOS and/or a HFD, to test whether CYP2B6 protects from the development of PFOS-mediated NAFLD, and determine whether the combination of a HFD and PFOS (similar to the condition of many global and US citizens) increases the hepatotoxicity of PFOS and escalates PFOS-induced

NAFLD. To determine these aims, we compared male and female mice treated with 0, 1, or 10 mg/kg/day PFOS in both Cyp2b-9/10/13-null (Cyp2b-null) and hCYP2B6-Tg mice. This study will help identify CYP2B6's and a HFD's role in PFOS-mediated hepatotoxicity and NAFLD.

Materials and Methods

Animals and Diet: All studies were reviewed and approved by Clemson University's Institution Animal Care and Use Committee (IACUC). Housing and studies were performed in a controlled environment with a temperature of 24 ± 2$_{o}$C, 60-70% relative humidity, and a 12:12 h light:dark cycle. C57BL/6J-*Cyp2b10*$_{em1(Del(7Cyp2b10-Cyp2b9))Fatso}$/Mmnc mice, often called Cyp2b9/10/13-null or abbreviated as Cyp2b-null mice, lack the murine genes *Cyp2b9, Cyp2b10, and Cyp2b13* on a C57Bl/6J (B6) background. Cyp2b-null mice were developed using Crispr/Cas9 to delete a 287 kb region of chromosome 7 (Kumar et al., 2017). Humanized CYP2B6/2A13/2F1-transgenic mice on the Cyp2b-null background were generated by breeding CYP2B6/2A13/2F1-transgenic mice generated previously (Wei et al., 2012) to our Cyp2b-null mice in order to generate Cyp2b-null mice containing the human BAC clone of CYP2B6/2A13/2F1 to what we abbreviate to hCYP2B6-Tg mice (Heintz, 2020).

Mice were genotyped from extracted genomic DNA isolated from tails using the QuantaBio (Beverly, MA USA) AccuStart II Mouse Genotyping Kit according to the manufacturer's instructions. Each mouse was genotyped to confirm that the

Cyp2b9/10/13 cluster on murine chromosome 7 was deleted using the F2/R2 primer set: (F2: 5’-gccagggtcagcatattcaccaa-3’/ R2: 5’-gcacagacatcatgaggttctggtg-3’; 59$_{o}$C) to confirm the deletion followed by a Cyp2b13 specific primer set (F1: 5’-cagactcttgttagaccggaccat-3’ / R1: 5’-ccccaaggaataaaattctacatg-3’; 59$_{o}$C) to ensure the mice were not heterozygous (Kumar et al., 2017). hCYP2B6-2A13-Tg primer set (F1: 5’-cctggacagatgcctttaactccg-3’ / R1: 5’-tggctttgcacctgcctgact-3’; 63$_{o}$C) then confirmed the presence of the human BAC clone containing CYP2B6 and the CYP2B6/2A13/2F1 P450 cluster on human chromosome 19 (Wei et al., 2012).

Table 1: Description of the different treatment groups by gender, diet and genotype with replicate numbers in parenthesis.

Males				**Females**			
Normal Diet		High Fat Diet		Normal Diet		High Fat Diet	
Cyp2b-null	hCYP2B6	Cyp2b-null	hCYP2B6	Cyp2b-null	hCYP2B6	Cyp2b-null	hCYP2B6
0 mg/kg PFOS (n = 6)	0 mg/kg PFOS (n = 7)		0 mg/kg PFOS (n = 5)	0 mg/kg PFOS (n = 5)	0 mg/kg PFOS (n = 7)	0 mg/kg PFOS (n = 5)	0 mg/kg PFOS (n = 6)
1 mg/kg PFOS (n = 6)	1 mg/kg PFOS (n = 7)			1 mg/kg PFOS (n = 5)	1 mg/kg PFOS (n = 7)		1 mg/kg PFOS (n = 5)
10 mg/kg PFOS (n = 5)	10 mg/kg PFOS (n = 7)		10 mg/kg PFOS (n = 5)	10 mg/kg PFOS (n = 5)	10 mg/kg PFOS (n = 7)	10 mg/kg PFOS (n = 5)	10 mg/kg PFOS (n = 5)

Mice, 10-12 weeks old, were separated into 19 different groups based on their genotype, PFOS (Sigma-Aldrich, St. Louis, MO, USA) dose, gender, and diet (**Table 1**). PFOS, dissolved in a solution of water and 0.5% Tween 20, was administered by gavage each morning for three weeks. Mice were either fed a normal diet (ND; 2018S-Envigo Teklad Diet, Madison, WI) consisting of 18% kcal of fat, or a high-fat diet (HFD; Envigo, TD.06414 Adjusted calorie diet) consisting of 60.3% kcal of fat. Water was provided *ad libitum*. Male Cyp2b-null male mice were not provided a HFD in conjunction with PFOS because fewer Cyp2b-null male pups were born than female pups during this round of breeding.

Necropsy: Mice were weighed prior to euthanasia. Euthanasia was performed by heart puncture to collect blood while under 3% isoflurane anesthesia followed by CO_2 asphyxiation. Whole blood was kept at room temperature for 30 minutes to allow for clotting and then centrifuged at 6,000 rpm for 10 minutes to collect serum. Liver and white adipose tissue (WAT) were harvested and weighed. Liver was diced into several pieces for lipid sampling, RNA extraction, protein preparation, and histology preparation. Samples were snap frozen in liquid nitrogen prior to storage at -80°C.

Serum Lipids and Liver Cholesterol Screening: Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), total bilirubin, direct bilirubin, blood urea nitrogen (BUN), cholesterol, creatine kinase (CK), creatinine, gamma-glutamyl transferase (GGT), glucose, lactate dehydrogenase (LDH), calcium,

magnesium, phosphorous, albumin, total protein, triglycerides, and total carbon dioxide (CO_2) were measured using a Beckmann-Coulter AU480 analyzer with the appropriate Beckmann-Coulter kits according to the manufacturer's instructions (Heintz et al., 2019). Serum HDL and LDL/VLDL cholesterol was determined using the HDL and LDL/VLDL Colorimetric Quantitation Assay from Sigma Aldrich (St. Louis, MO) according to the manufacturer's instructions. Total liver cholesterol was determined using the Cholesterol/ Cholesteryl Ester Quantitation Assay Kit from Abcam (Cambridge, MA USA) according to the manufacturer's instructions. Each measured serum and liver parameters were statistically compared across groups using GraphPad Prism 7.0 (La Jolla, CA USA). Principle component analysis (PCA) was performed using RStudio to associate serum and liver parameters with PFOS dose, diet, or genotype.

Principal Component Analysis (PCA) biplot:

Two PCA biplots were generated, male and female, to compare differences in groups based on diet, PFOS, and genotype using 19 different parameters. The PCA biplots were generated using the data visualization package, ggplot2, in RStudio. Treatment groups were color coded and overlaid on each of the plots, respectively. A normal data ellipse was drawn for each group, a scale factor of 2 was applied to observations, and a scale factor of 2 was applied to variables. An aspect ratio of 1 was used to make the plots square. The x-axis (PC1) and y-axis (PC2) show the percent of explained variability amongst the data.

Oil Red O Staining: Liver samples (n=3) from each group were sectioned and stained with Oil Red O according to standard protocol (Dong et al., 2009). Stained slides were imaged (400X magnification) on a Leica Acquire Light Microscope and analyzed using ImageJ Fiji Particle Analysis to quantify the lipids and triglycerides in the liver (Schindelin et al., 2012). Briefly, the scale bar was set to 0.05 mm and the threshold color was set to red, with red pass selected and green/blue pass deselected. The wavelength was set to 120-255 and the range of the size (mm^2) was set to 0.00001-infinity for all images (Heintz et al., 2020).

RNA Quantification and quantitative Real-time Polymerase Chain Reaction (qPCR): RNA was isolated in TRIzol (Invitrogen, Carlsbad, CA USA) according to the manufacturer's instructions, initially quantified on a NanoDrop, and stored at -80°C. DNA contamination was eliminated with the Turbo DNA-Free Kit (Invitrogen, Carlsbad, CA). RNA was then re-quantified with the Qubit RNA BR Assay (Invitrogen, Carlsbad, CA) using the Qubit 4 Fluorometer (Invitrogen, USA) for improved accuracy. qPCR was performed using primers specific for *Srebp1*, *Cpt1a*, *CYP2B6*, *Ppar*γ, *Cd68*, *Cyp2a5*, *Cyp4a14*, *Hmgcr*, *CYP2A13*, *18S* and *Gapdh* (**Supplemental Table 1**). 1 μL of cDNA was briefly mixed and incubated with 12.5 μL RT^2 SYBR Green (Qiagen Frederick, MD USA), 9.5 μL Millipore water, 1 μL of the forward and reverse primers (25 μL per well). All plates were heated to 95°C for 1 minute followed by 50 cycles of denaturation at 95°C for 30 seconds, annealing for 30 seconds (temperature varied for each primer; **Supplemental Table 1**), and elongation for 45 seconds at 72°C. To determine the melt

curve, samples were heated to 76.5$_o$C for 5 seconds and then 95$_o$C for 5 minutes. Efficiency of the reaction was determined with a standard curve performed in triplicate with a mix of samples diluted from 1:1 - 1:1024. Samples were diluted 1:20 and fluorescence measured using a Bio-Rad CFX96 Real-Time System. Gene expression was quantified and normalized to the geometric means of *18S* and *Gapdh* as the housekeepers using the inverted Muller's equation (Muller et al., 2002; Roling et al., 2004).

Western Blot: Livers were individually homogenized with a Dounce Homogenizer and microsomes isolated by differential centrifugation (Van Der Hoeven & Coon, 1974). Protein concentrations were determined using the Bradford Protein Assay according to the manufacturer's instructions (Bio-Rad, Hercules, CA, USA). Western blots were performed to determine CYP2B6 and CYP2A protein expression with 25 μg of microsomal protein. Briefly, samples were separated via gel electrophoresis, transferred to a Bio-Rad PVDF (Hercules, CA USA), and quantified using antibodies (diluted 1:1000) specific to CYP2B6 (Chemicon International, Temecula, CA USA), CYP2A (Thermo-Fisher Scientific, Rockford, IL USA), with β-actin (Sigma Aldrich, St. Louis, MO) as the reference gene (Hernandez et al., 2006). An alkaline phosphatase conjugated goat anti-rabbit IgG (Bio-Rad) secondary antibody (diluted 1:500) was used to identify the CYP2B6 and CYP2A primary antibodies. Goat anti-mouse IgG (Bio-Rad) secondary antibody (diluted 1:500) was used to recognize β-actin primary antibody. Individual proteins were detected with the chemiluminescent Immun-Star AP detection kits from

Bio-Rad, and band intensities were quantified on a Bio-Rad ChemiDoc Imaging System with iLab.

Vivid (Blue) CYP2B6 Enzyme Inhibition Assay: PFOS inhibition of CYP2B6 activity was determined and compared to nonylphenol using the Vivid CYP2B6 Blue Screening Kit from Life Technologies (Carlsbad, CA USA).

Liver PFOS Extractions: Liver PFOS was extracted using a slightly modified previously published method (Chang et al., 2017). Frozen liver tissues (~50 mg) was homogenized in 2 mL Omni Hard Tissue Homogenizing tubes containing 1.4 mm ceramic beads, with 400 µL cold, deionized water spiked with a fixed amount of a stable isotope-labeled internal standard (13C4-PFOS, Wellington Laboratories, Ontario, Canada, Product code: MPFOS). Using an Omni Bead Ruptor Elite (Omni International, Kennesaw, GA), the mixture was homogenized for 30 seconds at 4 m/s. 250 µL of homogenate was digested overnight at room temperature in 10% 1N KOH. 100 µL of digested homogenate was further treated with 100 µL of 2N HCl, 500 µL 1N formic acid, 500 µL of saturated ammonium sulfate, and 5 mL methyl tert-butyl ether (MTBE). The solution was mixed on a shaker (20-30 min at room temperature). The organic and aqueous layers were separated by centrifugation (2500 × g, 5 min), and an exact volume of MTBE (4.5 mL) was removed from the solution. The top organic layer was subsequently transferred to a new tube and evaporated. The resulting sample was reconstituted with 20 mL of acetonitrile and water (1:1) prior to LC-MS/MS analysis.

Serum PFOS Extraction: Serum collected at necropsy was prepared according to methods described by Hansen (Hansen et al., 2001). Briefly, 20 µL of sera, 2 µL of isotope-labeled internal standard (13C4-PFOS, Wellington Laboratories, Ontario, Canada, Product code: MPFOS), 200 µL of 0.5M tetrabutylammonium bisulfate (TBA; adjusted to pH 10), and 400 µL of 0.25M sodium carbonate were added to a 15-mL polypropylene tube, and thoroughly mixed. 5 mL of MTBE was added to the solution, and the mixture was placed on a shaker for 20-30 min at room temperature. The organic and aqueous layers were separated by centrifugation (2500 × g, 5 min), and an exact volume of MTBE (4.5 mL) was removed from the solution. The top organic layer was subsequently transferred to a new tube and evaporated overnight. The resulting sample was reconstituted with 20 mL of acetonitrile and water (1:1) prior to LC-MS/MS analysis

PFOS Quantification by LC-MS/MS: Liver and serum samples were vortexed for 30 s and passed through a 0.2 µm polyethersulfone membrane syringe filter (MDI Membrane Technologies, Harrisburg, PA) into an autosampler vial. Ultra-fast liquid chromatography (UFLC) was performed on a SHIMADZU Prominence UFLC system consisting of three LC-20AD pumps, a DGU-20A degassing unit, SIL-20AC autosampler, CTO-20AC column oven and CBM-20A communication bus module (Columbia, MD). Chromatographic separation was performed on a Waters XBridge® C18 column (100 mm X 4.6 mm i.d., 5 µm, Milford, MA). The mobile phase consisted of 0.1% (v/v) formic acid/water (A) and 0.1% (v/v) formic acid/acetonitrile (B) with a gradient elution

of 70% of B increased to 90% of B over 8 min; at 8 min the gradient was revered to original conditions. The column temperature was 40°C, flow rate was 0.6000 mL/min, and the injection volume was 10.00 µL. Mass spectrometry was performed on the QTRAP 4500 system coupled with an electrospray ionization (ESI) interface (AB Sciex, Framingham, MA). Nitrogen was used in all cases. The parameters were optimized as follows: negative ionization, IonSpray voltage, -4500; nebulizer gas, 40; auxiliary heater gas, 45; curtain gas, 20; turbo gas temperature, 400; declustering potential, -60; entrance potential, -10; collision energy, -122; collision cell exit potential, -15. The samples were analyzed in MRM (Multiple Reaction Monitoring) mode. The MRM ion pair used for PFOS quantification was 498.9/79.8 and compared to a standard curve prepared in either liver or serum matrix to account for matrix effects. The data were acquired using Analyst 1.6.3 software and processed using MultiQuant 3.0.1 software.

Tests of Significance: Tests of significance were performed using GraphPad Prism software 7.0 & 8.0 (LaJolla, CA USA). Student's t-tests were performed when comparing two groups, and one-way ANOVAs followed by Fisher's LSD as the *post-hoc* test were performed when comparing more than two groups. A p-value < 0.05 was considered significantly different.

Results

PFOS toxicity and effects on tissue, and body mass: Greater toxicity in the ND-fed hCYP2B6-Tg female mice given 10 mg/kg PFOS (10-PFOS) was observed. Of the 110

mice in the study, only three mice, all female treated with 10-PFOS and fed a ND, died during the 21-day exposure. Two died on day 20 and the third died on day 21 at the onset of the necropsies. Neither males nor HFD-fed females provided 10-PFOS died indicating increased sensitivity in females compared to males and protection from lethality provided by a HFD (**Fig. 1**).

Table 2: Body weights, Liver weights, and WAT weights of Male mice.

Diet	Genotype	PFOS (mg/kg)	Body Weight (g)	Liver Weight (g)	WAT Weight (g)
Normal Diet (ND)	Cyp2b-null	0	$22.58\pm0.54_{p**g**}$	$0.958\pm0.024_{p***}$	0.335 ± 0.014
		1	$23.77\pm0.70_{g***}$	$1.372\pm0.038_{g***}$	0.388 ± 0.045
		10	$18.11\pm0.37_{p**g}$	$1.820\pm0.12_{p***b}$	$0.0382\pm0.025_{p}$
	hCYP2B6	0	$23.20\pm0.55_{p**g**d**}$	$0.927\pm0.022_{p***g}$	$0.426\pm0.058_{pd***}$
		1	$23.52\pm0.50_{g***}$	$1.350\pm0.027_{g***}$	0.310 ± 0.025
		10	$18.51\pm0.81_{p**g*}$	$2.004\pm0.067_{p***gd*b}$	$0.0714\pm0.022_{p}$
High Fat Diet (HFD)		0	$27.55\pm1.87_{p***gd**}$	$1.066\pm0.10_{p***}$	$1.358\pm0.290_{p***gd***}$
		10	$20.29\pm0.70_{p***g**}$	$2.250\pm0.070_{p***g**d*}$	$0.260\pm0.051_{p***}$

Data represented as mean +/- SEM. All units are expressed in g. Statistical significance was determined by one-way ANOVA followed by Fisher's LSD as the post-hoc test.
'p' indicates difference between PFOS concentration
'g' indicates difference between gender (see Table 3)
'd' indicates difference between diet
'b' indicates difference between genotype
*Letter with no asterisk indicates $p < 0.05$, * indicates $p < 0.01$, ** indicates $p < 0.001$, and *** indicates $p < 0.0001$.*

PFOS increased liver mass and decreased body mass in a dose-dependent manner, especially at 10 mg/kg PFOS. Weight loss can be in part attributed to the complete lack of white adipose tissue in these mice (**Table 2 & 3**). The addition of a HFD increased

Table 3: Body weights, Liver weights, and WAT weights of Female mice

Diet	Genotype	PFOS (mg/kg)	Body Weight (g)	Liver Weight (g)	WAT Weight (g)
Normal Diet (ND)	Cyp2b-null	0	$18.37\pm0.34_{p*g**d**}$	$0.79\pm0.042_{p***}$	$0.212\pm0.036_{d**}$
		1	$18.15\pm0.65_{g***}$	$0.956\pm0.035_{g***}$	0.156 ± 0.037
		10	$15.15\pm0.78_{p*g}$	$1.69\pm0.039_{p***d*}$	0.024 ± 0.016
	hCYP2B6	0	$18.42\pm0.23_{p*g**d***}$	$0.73\pm0.028_{p***gd**}$	$0.1986\pm0.022_{d***}$
		1	$17.38\pm0.23_{g***d***}$	$0.8543\pm0.060_{g***d***}$	$0.1471\pm0.014_{d***}$
		10	$14.68\pm0.29_{p*d*}$	$1.763\pm0.20_{p***g}$	0.025 ± 0.025
High Fat Diet (HFD)	Cyp2b-null	0	$22.76\pm0.93_{p***d**}$	$0.902\pm0.036_{p***}$	$0.868\pm0.15_{p***d**}$
		10	$16.09\pm0.69_{p***}$	$1.954\pm0.105_{p***d*}$	$0.178\pm0.030_{p***}$
	hCYP2B6	0	$24.87\pm1.12_{p***gd***}$	$1.028\pm0.033_{p***d**}$	$1.002\pm0.24_{p***gd***}$
		1	$23.32\pm1.63_{d***}$	$1.232\pm0.074_{d***}$	$0.988\pm0.29_{d***}$
		10	$16.15\pm0.27_{p***g**}$	$1.916\pm0.030_{p***g**}$	$0.15\pm0.021_{p***}$

Data represented as mean +/- SEM. All units are expressed in g. Statistical significance was determined by one-way ANOVA followed by Fisher's LSD as the post-hoc test.
'p' indicates difference between PFOS concentration
'g' indicates difference between gender (see Table 2)
'd' indicates difference between diet
'b' indicates difference between genotype
Letter with no asterisk indicates $p < 0.05$, ** indicates* $p < 0.01$, *** indicates* $p < 0.001$, **** indicates* $p < 0.0001$.

liver, body, and WAT weights but was negated by PFOS. Furthermore, the mean liver weight of hCYP2B6-Tg male mice fed a ND and treated with 10-PFOS was 10% heavier than their Cyp2b-null counterparts ($p < 0.0001$) (**Table 2**), suggesting increased hepatic sensitivity to PFOS in the humanized mice.

CYP2B6: PFOS is a strong inhibitor of CYP2B6 *in vitro* with an IC50 of 165 nM (95% CI: 104 - 255 nM) (**Fig. 2**). PFOS shows similar, but slightly lower, potency than nonylphenol (IC50 = 77.3 nM; 95% CI: 57.5 – 103 nM), which was used as a positive control (Acevedo et al., 2005), indicating that PFOS interacts with CYP2B6 and may act as an inhibitor *in vivo*.

Previous research has indicated that PFOS is a potent murine Cyp2b inducer (Cheng & Klaassen, 2012; Das et al., 2017; Rosen et al., 2017). PFOS has been shown to induce CYP2B6 *in vitro* (Rosen et al., 2013); however whether it is an inducer of human CYP2B6 *in vivo* is in question. This transgenic model contains the CAR and FoxA2 upstream promoter and enhancer regions of CYP2B6 (Wei et al., 2012). CYP2B6 gene expression was significantly induced by PFOS in both male (7.8X) and female (163X) mice fed a ND as determined by qPCR (**Fig. 3ab**). HFD exacerbated CYP2B6 gene expression only in female mice (2.7X) compared to their ND counterparts (**Fig. 3b**). Western blots confirmed dose-dependent CYP2B6 induction at the protein level in males and females, including HFD-mediated CYP2B6 induction in the females (**Fig. 3ab**) although induction was much lower than measured by qPCR. In summary, PFOS induces

CYP2B6 in a hCYP2B6-Tg model that contains the CYP2B6 human promoter and enhancer regions (Wei et al., 2012).

CYP2A13 along with CYP2F1 were both a part of the BAC clone used to generate the original CYP2B6/2A13/2F1-Tg mouse. Both CYP2A13 and CYP2F1 are predominantly expressed in the respiratory tract and previous research indicates they are not expressed in the liver (Wei et al., 2012). qPCR was performed to confirm human CYP2A13 is not expressed in liver because members of the CYP2A subfamily are known to metabolize xenobiotics and endogenous lipids (Lu & Cederbaum, 2006). Human CYP2A13 expression was not detected in the liver by qPCR (data not shown), but genomic CYP2A13 is detected from tail snips demonstrating that our assay worked and there was no CYP2A13 hepatic expression. Murine *Cyp2a5* was also measured because it is the predominant CYP2A enzyme in adult murine liver, *Cyp2a5* and several other Cyp2a members were induced in Cyp2b-null mice or a HFD in comparison to WT mice, and it is orthologous to the human CYP2A6 hepatic enzyme (Abu-Bakar et al., 2012; Heintz et al., 2019). *Cyp2a5* gene expression was increased by 5.3X and 6.3X in ND-fed Cyp2b-null male and female mice, respectively, treated with 10-PFOS compared to Cyp2b-null controls (0-PFOS), with a 2.7X increase in HFD-fed Cyp2b-null mice provided 10-PFOS compared to HFD-fed 0-PFOS Cyp2b-null mice as well. *Cyp2a5* gene expression increased 1.9X and 2.2X in ND- and HFD-fed hCYP2B6-Tg male mice treated with 10-PFOS compared to their 0-PFOS counterparts (**Fig. 3cd**). *Cyp2a5* gene expression was not induced by 10-PFOS in hCYP2B6-Tg female mice. In contrast, PFOS repressed male

protein expression of CYP2A with slight induction in the Cyp2b-null females (**Fig. 3cd**). In summary, human CYP2A13 is not expressed in murine livers. Murine *Cyp2a5* compensates for a lack of Cyp2b expression in null mice, is responsive to PFOS by qPCR, strongly repressed by PFOS in males, and repressed by a HFD in females.

Retention of PFOS in hCYP2B6 mice: Serum and liver PFOS concentrations were determined by LC-MS/MS analysis and demonstrated concentration-dependent increases. hCYP2B6-Tg mice showed significantly increased PFOS retention compared to Cyp2b-null mice in males and females treated with 10-PFOS and fed a ND (**Fig. 4ab**). The average serum PFOS was 1.46X greater in ND-fed hCYP2B6-Tg male mice than ND-fed Cyp2b-null male mice, and 1.74X greater in ND-fed hCYP2B6-Tg female mice than identically treated Cyp2b-null female mice (**Fig. 4a**). When treated with a HFD, hCYP2B6-Tg mice showed a significant decrease in PFOS retention, unlike the Cyp2b-null mice. In humanized mice, the average serum PFOS level was 1.63X lower in HFD-fed male mice than their ND-fed counterparts and 1.13X lower in HFD-fed female mice than their ND-fed counterparts. However, in Cyp2b-null female mice, the average serum PFOS level was 1.62X greater in the HFD-fed mice than ND-fed mice at 10 mg/kg/day (**Fig. 4a**).

Liver PFOS retention followed a similar pattern to serum PFOS retention; greater PFOS retention in ND-fed hCYP2B6-Tg male and female mice compared to their Cyp2b-null counterparts (1.14X and 1.39X, respectively) (**Fig. 4b**). A HFD increased PFOS retention

within the liver of all groups with the most significant increase in the Cyp2b-null female mice compared to their ND-fed counterparts (1.3X) (**Fig. 4b**). This data suggests that a HFD may play a protective role in PFOS toxicity in hCYP2B6-Tg mice but not Cyp2b-null mice. Further, the greater PFOS retention measured in humanized mice was unexpected and may explain the decreased survival in the hCYP2B6-Tg female mice fed a ND (**Fig. 1**).

Serum markers of liver toxicity are increased by PFOS and reduced by a HFD: Alkaline phosphatase (ALP) and alanine aminotransferase (ALT), which are indicative of liver damage, were both significantly increased by PFOS in a dose-dependent manner (**Fig. 5ab**). Genotype only had an effect on ALT levels in 10-PFOS treated hCYP2B6-Tg males compared to Cyp2b-null males where the presence of CYP2B6 was protective from liver damage (**Fig. 5b**) despite greater measured PFOS concentrations (**Fig. 4**). Interestingly, ALP levels were significantly lower in HFD-fed mice and mean serum ALT levels significantly decreased in HFD-fed mice treated with 10-PFOS compared to their ND-fed counterparts (**Fig. 5**). These data indicates that a HFD decreases PFOS-mediated hepatotoxicity with CYP2B6 having a potentially protective role in males despite greater PFOS retention.

PCA biplot investigating associations between key serum parameters in different treatment groups: Other serum parameters are also perturbed by PFOS, diet or genotype, including glucose, triglycerides, total cholesterol, LDL/VLDL and HDL, and LDH

(**Supplementary Figures 1-5**). Therefore, PCA biplots examining associations between 19 different parameters were performed to compare relationships among treatment groups in males and females. In both males and females, the biplots account for approximately 60% of the variation amongst the data. The male mice treated with 0-PFOS or 1-PFOS grouped together skewing towards triglycerides (TAG) and glucose (GLU) (**Fig. 6**). The ND-fed male mice treated with 10-PFOS grouped towards biomarkers indicative of hepatotoxicity, ALT and ALP (bottom right of plot). The HFD-fed hCYP2B6-Tg male mice treated with 10-PFOS grouped towards to the 0-PFOS or 1-PFOS treated groups when compared to their 10-PFOS counterparts, consistent with data that a HFD is playing a protective role against PFOS toxicity in hCYP2B6-Tg male mice (**Fig. 6**).

The female mice treated with 0-PFOS or 1-PFOS also grouped together with TAG and GLU. HFD-fed Cyp2b-null female mice, regardless of PFOS concentration, showed lots of variation with several mice weighted toward cardiovascular toxicity biomarkers, cholesterol and lactate dehydrogenase (top left of plot), suggesting that Cyp2b-null female mice may be at an increased risk of cardiovascular disease when fed a HFD (**Fig. 7**). All of the female mice treated with 10-PFOS, regardless of diet, grouped separately from the female mice treated with 0-PFOS or 1-PFOS and towards biomarkers indicative of liver damage such as ALT and ALP (bottom left of plot) (**Fig. 7**) with surprisingly little variation between genotypes given the greater PFOS concentrations and toxicity observed in the hCYP2B-Tg females treated with 10-PFOS. Of course, serum was not collected from the mice that died and therefore their data is not reflected in the PCA

biplots. However, parameters associated with toxicity are reduced in female HFD-fed Cyp2b-null mice compared to ND-fed Cyp2b-null female mice (**Fig. 7**).

Hepatic triglycerides: Oil Red O staining indicates a significant increase in liver triglyceride concentrations in the ND-fed male and female Cyp2b-null mice treated with 1-PFOS compared to their hCYP2B6-Tg counterparts (10.33X and 50.4X, respectively) (**Fig. 8**), indicating a prominent role for CYP2B6 in protection from PFOS-mediated fatty liver when fed a ND. A HFD significantly increased liver triglyceride concentrations in every group except hCYP2B6-Tg male mice treated with 0-PFOS. In addition, NAFLD progression was exacerbated in all mice exposed to PFOS (**Fig. 8**). hCYP2B6-Tg female mice fed a HFD and treated with 10-PFOS had 1.41X greater triglyceride accumulation in the liver compared to their Cyp2b-null counterpart (**Fig. 8h**). In summary, ND-fed Cyp2b-null mice had a greater accumulation of hepatic triglycerides than their hCYP2B6-Tg counterparts treated with 1-PFOS; however, HFD-fed Cyp2b-null mice had significantly less liver triglycerides than their hCYP2B6-Tg counterparts treated with 10-PFOS (**Fig. 8**), indicating the progression of NAFLD is slowed by the presence of CYP2B6 in a ND but exacerbated by a HFD.

qPCR: qPCR was performed to identify expression changes in genes associated with peroxisome proliferation (*Pparγ*, *Cyp4a14*) (Wafer et al., 2017; Zhang et al., 2017), inflammation (*Cd68*) (Chistiakov et al., 2017), and lipid metabolism (*Hmgcr*; *Cpt1a*; *Srebp1*) (Jiang et al., 2018; Ruiz et al., 2014; Schlaepfer & Joshi, 2020). *Pparγ*

expression was significantly repressed in all PFOS-treated male mice and hCYP2B6-Tg female mice (**Fig. 9a)**, suggesting that PFOS is interfering with adipogenesis. Interestingly, *Ppar*γ was significantly increased in females by a HFD, but only in hCYP2B6-Tg mice; not Cyp2b-null mice. *Cyp4a14* gene expression was increased in all groups treated with 10-PFOS except ND-fed Cyp2b-null male mice and HFD-fed hCYP2B6-Tg female mice. *Cyp4a14* expression was significantly induced by the combination of PFOS and a HFD in hCYP2B6-Tg male mice (21.5X) and Cyp2b-null female mice (3.9X). However, PFOS-treated hCYP2B6-Tg female mice showed significantly less induction than correspondingly treated Cyp2b-null mice (**Fig. 9b**). Since *Cyp4a14* is regulated by *Ppar*α and has been associated with NAFLD (Zhang et al., 2017), this data indicates that PFOS activates *Ppar*α and promotes the progression of NAFLD, which is corroborated by the Oil Red O stains. *Cd68* gene expression was inhibited in all of the humanized mice treated with 10-PFOS except for the HFD-fed hCYP2B6-Tg male mice (**Fig. 9c**). PFOS had no significant effect on *Cd68* gene expression in Cyp2b-null mice; suggesting that although PFOS is hepatotoxic, there may be less inflammatory signaling in the liver when exposed to PFOS in the presence of the human CYP2B6 gene or untreated hCYP2B6-Tg mice are more prone to inflammation.

Hmgcr gene expression in 10-PFOS-treated hCYP2B6-Tg mice was inhibited by 60% in HFD-fed hCYP2B6-Tg male mice, 93% in ND-fed hCYP2B6-Tg female mice, and 88% in HFD-fed hCYP2B6-Tg female mice (**Fig. 10a**). These results suggest that PFOS may inhibit the metabolism of cholesterol in the liver of humanized mice potentially leading to

greater NAFLD (Jiang et al., 2018) consistent with HFD-PFOS cotreatments. *Cpt1a* gene expression was lower in all groups treated with 10-PFOS except for the HFD-fed Cyp2b-null female mice (**Fig. 10b**). *Cpt1a* gene expression was significantly repressed in all humanized groups except HFD-fed male mice potentially because they show greater constitutive expression. Because *Cpt1a* is essential for fatty acid mitochondrial transport and oxidation (Schlaepfer & Joshi, 2020), this data suggests that PFOS inhibits lipid metabolism and the humanized mice are more affected, consistent with the increased NAFLD progression observed in the humanized mice. Humanized male and female *Srebp1* gene expression was induced by a HFD (2.3X and 2.1X, respectively) and negated by 10-PFOS (1.8X and 1.6X, respectively) (**Fig. 10c**). *Srebp1* was also down-regulated by PFOS, but in a different manner depending on the genotype in females. This data is consistent with *Srebp1's* role in lipogenesis, TAG synthesis, and NAFLD (Ruiz et al., 2014), especially following a HFD and in the HFD-fed hCYP2B6-Tg mice whom are the most sensitive to NAFLD. *Srebp1* was also associated with loss of NAFLD in ND-fed PFOS treated mice.

Discussion

The purposes of this study were to determine if PFOS induces human CYP2B6, and test whether CYP2B6 protects from PFOS toxicity and NAFLD. We were able to address these questions and examine the adverse effects of co-treatment of PFOS and a HFD. First, CYP2B6 is inducible by PFOS. Surprisingly, female humanized mice are significantly more sensitive to PFOS toxicity than their Cyp2b-null counterparts,

probably due to an increase in PFOS retention in hCYP2B6-Tg mice. However, sublethal measurements, such as NAFLD, were more sensitive in Cyp2b-null mice than hCYP2B6-Tg mice unless the mice were treated with a HFD in which case hCYP2B6-Tg female mice were more sensitive to NAFLD as demonstrated by Oil Red O and several biomarkers of NAFLD.

Toxicity was significantly greater in hCYP2B6-Tg females than Cyp2b-null females. Three ND-fed hCYP2B6-Tg female mice exposed to 10-PFOS died and these were the only mice to die during the 3 week exposure period (**Fig. 1**) clearly demonstrating the sensitivity of ND-fed hCYP2B6-Tg mice to PFOS. Interestingly, their HFD-fed counterparts survived (**Fig. 1**), indicating that a HFD was protective from lethality probably due to the increased adiposity. The increased toxicity in the hCYP2B6-Tg mice is most likely due to increased PFOS retention and the subsequent effects caused by greater PFOS. LC-MS/MS analysis showed that serum and liver PFOS was significantly greater in the hCYP2B6-Tg mice compared to their counterparts when fed a ND; more so in female mice (174% and 139%, respectively) than male mice (146% and 114%, respectively). HFD significantly decreased serum PFOS concentrations in all hCYP2B6-Tg mice. In contrast, a HFD led to a significant increase in serum and liver PFOS retention in female Cyp2b-null mice compared to their ND-fed counterparts, nevertheless hCYP2B6-Tg mice still had greater liver PFOS retention.

PFOS induces human CYP2B6 in male and female mice (**Fig. 3ab**). These results confirm that CYP2B6 is inducible by PFOS in a dose dependent manner similar to murine *Cyp2b10* (Rosen et al., 2010). A HFD exacerbated CYP2B6 induction in females (**Fig. 3b**). HFD induced murine *Cyp2b9* and *Cyp2b10* in previous studies (Heintz et al., 2019; Hoek-van den Hil et al., 2015; Leung et al., 2016), potentially due to activation of CAR by PUFAs (Finn et al., 2009), or activation of FoxA2 due to insulin resistance (Wolfrum et al., 2004), glucagon or glucocorticoid signaling (Zhang et al., 2005). CYP2B6 protein induction was moderate in comparison to changes in RNA levels and it is unlikely that CYP2B6 directly regulates PFOS retention.

Hepatic CYP2A gene and protein expression was determined to ensure that one of the human genes from the original construct, *CYP2A13* (Wei et al., 2012), was not being expressed in the liver. qPCR confirmed that *CYP2A13* is not expressed in the liver in hCYP2B6-Tg mice confirming previous results (Wei et al., 2012). *Cyp2a5* is the predominant hepatic CYP2A member in adult mice and has been shown to be female predominant (Lu & Cederbaum, 2006; Poça et al., 2017). *Cyp2a5* gene expression increased significantly in all of the Cyp2b-null groups (**Fig. 3cd**), potentially as a compensatory mechanism (Kumar et al., 2017). However, CYP2A protein expression was repressed in male mice exposed to PFOS regardless of genotype and in Cyp2b-null female mice (**Fig. 3cd**). Based on these results, neither *CYP2A13* or other CYP2A members are playing roles in the increased PFOS retention and toxicity observed in hCYP2B6-Tg mice.

PFOS exhibited severe toxicity on several different tissues and metabolic pathways, which was evident by biomarkers from the serum panel. Hepatotoxicity was observed in the groups exposed to 10-PFOS based on the significantly elevated ALP and ALT levels (**Fig. 5**). However, increased toxicity caused by higher PFOS serum and liver concentrations did not always manifest themselves as higher serum markers of tissue toxicity. ALT and ALP levels indicate greater liver toxicity in ND-fed mice than HFD-fed mice, consistent with lower PFOS concentrations in the HFD-fed mice. However, hCYP2B6-Tg mice showed no difference in ALP and ND-fed male hCYP2B6-Tg had lower ALT levels than similarly fed Cyp2b-null mice (**Fig. 5**). PCA indicates a clearly protective effect of a HFD from PFOS toxicity in male mice, but not female mice (**Fig. 6-7**). This protective effect was highly pronounced in the hCYP2B6-Tg male mice as this group was found between the 0- and 1-PFOS groups and the other 10-PFOS groups.

PCA also indicates an increase in markers indicative of poor cardiovascular health such as cholesterol, triglycerides, LDH, and LDL in Cyp2b-null mice compared to hCYP2B6 mice. This may be due to differences in fatty acid metabolism as CYP2B6 and other CYPs are involved in PUFA metabolism (Bishop-Bailey et al., 2014; Heintz et al., 2019; Nelson et al., 2004). There is overlap between the genotypes in the PCA plot; however, lack of Cyp2b in both males and females pushes the groups towards these adverse cardiovascular parameters and in some cases it is clear that the combination of a Cyp2b-null genotype and a HFD are deleterious to some parameters such as LDH and

cholesterol (**Supplementary Figures 3-4**). Last, serum glucose and triglyceride levels were significantly lower in groups treated with 10-PFOS compared to 0-PFOS suggesting PFOS interferes with gluconeogenesis and fatty acid esterification (Das et al., 2017; Hagenaars et al., 2008) (**Supplementary Figure 1-2**).

PFOS increases NAFLD. Previous work has demonstrated that male Cyp2b-null mice are more susceptible to NAFLD than wildtype mice (Heintz et al., 2019), and ND-fed Cyp2b-null male and female mice were more susceptible to 1-PFOS mediated NAFLD than hCYP2B6-Tg mice. However, the combination of PFOS and a HFD potentiated NAFLD, especially in hCYP2B6-Tg mice in comparison to Cyp2b-null mice (**Fig. 8**); therefore this result appears surprising. Recent results investigating the effects of a 16-week HFD on hCYP2B6-Tg mice show that CYP2B6 is an anti-obesity CYP; however, it increases NAFLD while protecting male mice from diabetes (Heintz, 2020). Female hCYP2B6-Tg mice were more susceptible to the HFD-PFOS co-treatment than Cyp2b-null mice. New data suggests that CYP2B6 is not protective from NAFLD and possibly increases NAFLD susceptibility through increased production of oxylipins (Deol et al., 2017; Heintz, 2020). These results suggest a complicated role of CYP2B6 in metabolism of PUFAs only during a HFD in which hepatic PUFA concentrations are high.

NAFLD progression is associated with decreases in *Cpt1a* **(Fig. 10)**, a key gene involved in mitochondrial fatty acid uptake prior to β-oxidation in the liver (Schlaepfer & Joshi, 2020). There was a significant decrease of relative *Cpt1a* gene expression in HFD-fed

hCYP2B6-Tg mice treated with 10-PFOS compared to their 0-PFOS counterparts. LDL/VLDL levels in the serum were also significantly lower in PFOS treated groups (**Supplementary Figure 5**). A decrease in *Cpt1a* gene expression and oxidation coupled with lower LDL production and release may explain the exacerbated increase in hepatic triglyceride content in the liver. These findings support other studies that have suggested PFOS induced steatosis by inhibiting mitochondrial β-oxidation and decreasing LDL content that, normally, would help transport triglycerides out of the liver (Cheng et al., 2016).

Srebp1, *Pparγ*, and to a lesser extent *Cd68* all followed similar gene expression profiles following HFD or PFOS treatments with increased expression in HFD-fed hCYP2B6-Tg mice in comparison to HFD-fed Cyp2b-null mice (**Fig. 9-10**). *Srebp1* is a key regulator of lipogenesis and therefore it is not surprising that it is associated with NAFLD (Moslehi & Hamidi-Zad, 2018). *Srebp1* also regulates *Pparγ* (Fajas et al., 1999) and *Cd68* expression (Jump et al., 2013). *Hmgcr*, the rate determining step in cholesterol biosynthesis (Jiang et al., 2018), expression was slighting higher in hCYP2B6-Tg mice and repressed by PFOS which is consistent with the liver and serum cholesterol data (**Supplementary Figures 3,5**). The greater expression of these genes in the hCYP2B6-Tg mice while fed a HFD may explain their sensitivity to NAFLD.

PPARα and *PPARγ* are important in maintaining energy homeostasis and lipid metabolism (Corton et al., 2014; Klaunig et al., 2012; Peraza et al., 2006; Rosen et al.,

2010). *Pparγ* is found in most tissues and is responsible for adipocyte differentiation and plays a role in lipid metabolism (Wafer et al., 2017; Zhang et al., 2014). *Pparγ* gene expression was significantly repressed by PFOS suggesting the inhibition or inactivation of *Pparγ* by PFOS. *Pparα* regulates a wide variety of genes including certain CYPs such as *Cyp4a14* and *Cpt1a* that are associated with NAFLD but overall *Pparα* activation is associated with preventing NAFLD (Patsouris et al., 2006; Zhang et al., 2017). *Cyp4a14* levels were increased in groups treated with 10-PFOS and exacerbated by a HFD suggesting the activation of *Pparα* by PFOS and diet. Interestingly, *Cpt1a* expression increased with diet but decreased with PFOS; *Cyp4a14* was repressed by the presence of CYP2B6 in HFD-fed mice and induced by PFOS with the exception of Cyp2b-null males **(Fig. 9-10)**. The inhibition of *Pparγ,* activation of *Cyp4a14,* and inhibition of *Cpt1a* is consistent with data that PFOS promotes NAFLD. Further, these results support the Oil Red O analysis that the HFD-fed hCYP2B6-Tg mice exposed to 10-PFOS were further along in NAFLD progression.

Fatty acids such as alpha linolenic acid (ALA) and linoleic acid (LA) are known activators of *Pparα* (Huang & Chen, 2017; Kliewer et al., 1997) and there are several fatty acid metabolites that are known ligands of *Pparγ* (Straus et al., 2000). The oxylipins produced from fatty acid metabolism by CYP2B6 could act as agonists for *Pparα* in addition to PFOS which would explain the exacerbated *Cyp4a14* levels observed in HFD hCYP2B6-Tg male mice. PFOS has been shown to inactivate *Pparγ* (Wen et al., 2016).

The current study supports these findings. Since *Pparγ* gene expression is low in all groups treated with 10-PFOS.

Human CYP2B6, is inducible *in vivo* by PFOS, associated with increased PFOS toxicity and NAFLD. In addition, a HFD was protective of PFOS toxicity in male and female hCYP2B6-Tg mice and the female Cyp2b-null mice. Cyp2b-null mice also had increased levels of biomarkers indicative of cardiovascular disease. The increased toxicity observed in the hCYP2B6-Tg mice fed a ND is most likely a result of the increased serum and liver PFOS retention in these mice. It is unknown why there was an increase in PFOS retention but it is clearly a result of the presence of the *CYP2B6* gene. A HFD may have played a protective role in the humanized mice because CYP2B6 may have a higher affinity for the fats in the diet than PFOS; however, this does not explain differences in markers of toxicity associated with female Cyp2b-null mice as shown in the PCA plots. In conclusion, both CYP2B6 and a HFD effect PFOS toxicity and NAFLD with CYP2B6 enhancing toxicity and NAFLD and a HFD exacerbating NAFLD but protecting mice from toxicity.

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Graphical Abstract

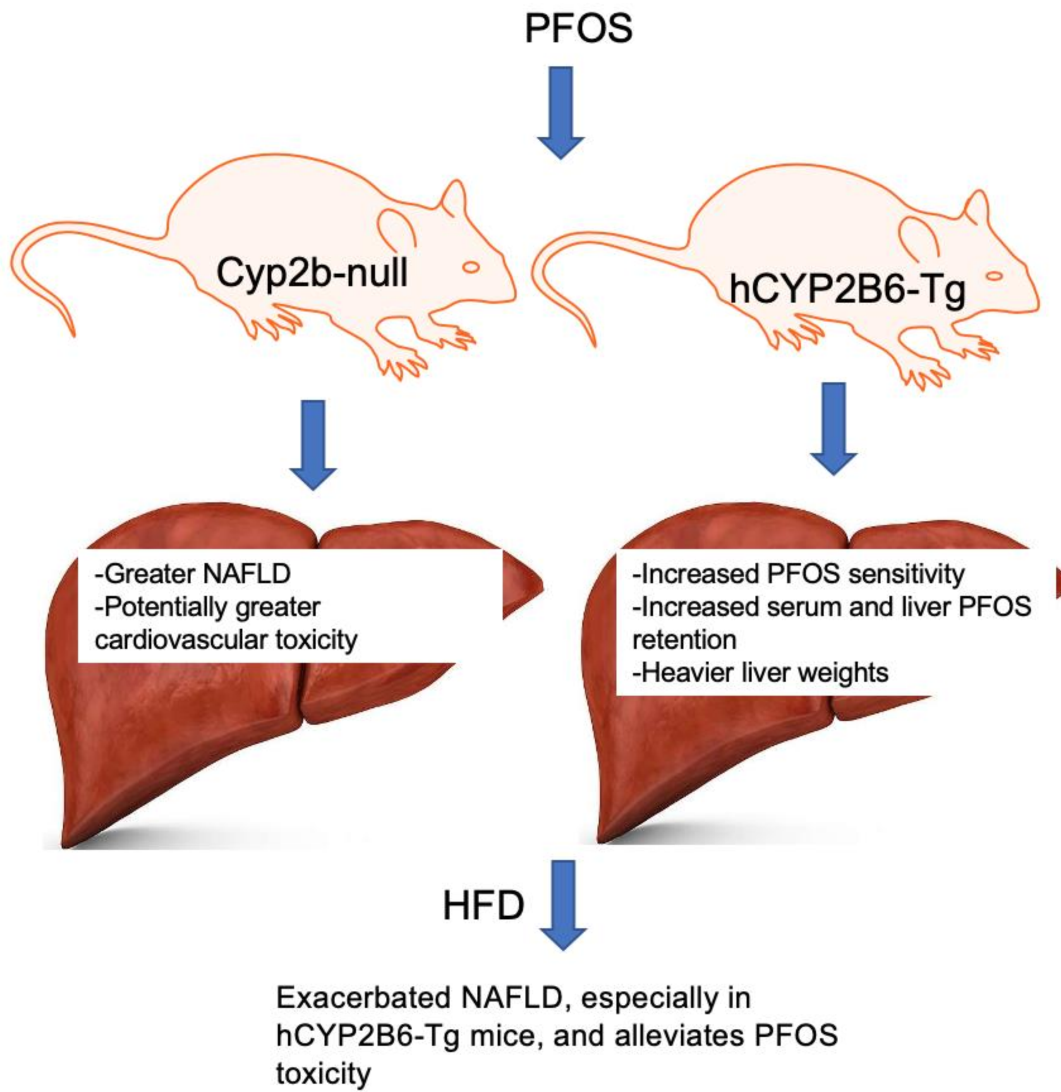


Figure Legends

Fig. 1: Survival curve of female mice treated with different concentrations of PFOS via oral gavage. This survival curve was generated using GraphPad Prism 8. Statistical differences in survival were determined by Gehan-Breslow-Wilcoxon test where ** indicates $p < 0.001$ (n = 5-7).

Fig. 2: Concentration-response curve for the inhibition of CYP2B6 by PFOS in comparison to the positive control, nonylphenol. IC50 and 95% CI values were determined using a sigmoidal dose response least squares fit test performed on GraphPad Prism 7 (n = 3). Multiple Student t-test's were performed to determine significance between PFOS and Nonylphenol. * indicates a significant difference of $p < 0.05$.

Fig. 3: CYP2B6 (A & B) and *Cyp2a5* (C & D) hepatic gene and protein expression in male and female mice. Mice were treated as described in the materials and methods and CYP2B6 and *Cyp2a5* induction measured by qPCR (n = 4-5) and Western blotting (n = 2-3). Statistical significance was determined by one-way ANOVA when comparing 3 or more groups (qPCR) and by Student's t-test when comparing two groups (Western blots). Statistical differences in qPCR were determined by One-way ANOVA where a letter indicates a $p < 0.05$, letter w/ * indicates $p < 0.01$, letter w/ ** indicates $p < 0.001$ and a letter w/ *** indicates $p < 0.0001$. * indicates a significant difference of $p < 0.05$ in Western blots.

Fig. 4: LC-MS/MS analysis of PFOS concentrations in the serum (A) and liver (B) of hCYP2B6 and Cyp2b-null mice. Data are presented as mean ± SEM. Statistical significance was determined by one-way ANOVA followed by Fisher's LSD as the post-hoc test (n=4-5). A letter indicates a $p < 0.05$, letter w/ * indicates $p < 0.01$, letter w/ ** indicates $p < 0.001$ and a letter w/ *** indicates $p < 0.0001$.

Fig. 5: Serum concentration of Alkaline Phosphatase (ALP) (A) and Alanine Aminotransferase (ALT) (B) from PFOS-treated mice. Data are presented as mean ± SEM. Statistical significance was determined by one-way ANOVA followed by Fisher's LSD as the post-hoc test (n=5). A letter indicates a $p < 0.05$, letter w/ * indicates $p < 0.01$, letter w/ ** indicates $p < 0.001$ and a letter w/ *** indicates $p < 0.0001$.

Fig. 6: Principal component analysis (PCA) biplot composed of 19 different parameters compared across the 8 male groups. Graphical representation of multivariate data. Treatment groups are color coded and the group descriptions are overlaid on the plot.

Fig. 7: Principal component analysis (PCA) biplot composed of 19 different parameters compared across the 11 female groups. Graphical representation of multivariate data. Treatment groups are color coded and the group descriptions are overlaid on the plot.

Fig. 8: Triglyceride content for M ND Cyp2b-null 0-PFOS (A), M ND hCYP2B6 0-PFOS (B), M HFD hCYP2B6 0-PFOS (C), F ND Cyp2b-null 0-PFOS (D), F ND hCYP2B6 0-PFOS (E), F HFD Cyp2b-null 0-PFOS (F), F HFD hCYP2B6 0-PFOS (G), and quantified using Image J Fiji Particle Analysis (H). Images were taken at 400x (0.05mm) magnification. Data are presented as mean ± SEM (n = 3). Statistical significance was determined by one-way ANOVA followed by Fisher's LSD as the post-hoc test. A letter indicates a $p < 0.05$, letter w/ * indicates $p < 0.01$, letter w/ ** indicates $p < 0.001$ and a letter w/ *** indicates $p < 0.0001$.

Fig. 9: Changes in hepatic gene expression caused by genotype, PFOS, HFD, or a combination of PFOS and a HFD in male and female mice. Gene expression of *Pparg* (A), *Cyp4a14* (B), and *Cd68* (C) in male and female mice was determined by qPCR as described in Material and Methods. Data are presented as mean ± SEM (n = 4-5). Statistical significance was determined by one-way ANOVA followed by Fisher's LSD as the post-hoc test. A letter indicates a $p < 0.05$, letter w/ * indicates $p < 0.01$, letter w/ ** indicates $p < 0.001$ and a letter w/ *** indicates $p < 0.0001$.

Fig. 10: Changes in hepatic gene expression caused by genotype, PFOS, HFD, or a combination of PFOS and a HFD in male and female mice. Gene expression of *Hmgcr* (A), *Cpt1a* (B), and *Srebp1* (C) in male and female mice was determined by qPCR as described in Material and Methods. Data are presented as mean ± SEM (n = 4-5). Statistical significance was determined by one-way ANOVA followed by Fisher's LSD as the post-hoc test. A letter indicates a $p < 0.05$, letter w/ * indicates $p < 0.01$, letter w/ ** indicates $p < 0.001$ and a letter w/ *** indicates $p < 0.0001$.

Supplemental Figure 1: Serum glucose levels (n = 5). A serum panel was performed as mentioned in the Materials and Methods and glucose levels were measured. PFOS decreased serum glucose. Data are presented as mean ± SEM. Statistical significance was determined by one-way ANOVA followed by Fisher's LSD as the post-hoc test. A letter indicates a $p < 0.05$, letter w/ * indicates $p < 0.01$, letter w/ ** indicates $p < 0.001$ and a letter w/ *** indicates $p < 0.0001$.

Supplemental Figure 2: Serum triglyceride levels (n=5). A serum panel was performed as mentioned in the Materials and Methods and triglyceride levels were measured. PFOS significantly decreases serum triglyceride levels. Data are presented as mean ± SEM. Statistical significance was determined by one-way ANOVA followed by Fisher's LSD as the post-hoc test. A letter indicates a $p < 0.05$, letter w/ * indicates $p < 0.01$, letter w/ ** indicates $p < 0.001$ and a letter w/ *** indicates $p < 0.0001$.

Supplemental Figure 3: Serum cholesterol levels (n=5). A serum panel was performed as mentioned in the Materials and Methods and cholesterol levels were measured. Increased serum cholesterol levels in the mice fed a high-fat diet with a significant increase in the cholesterol levels in Cyp2b-null mice fed a high-fat diet, regardless of

PFOS concentration, compared to their hCYP2B6 counterparts. Data are presented as mean ± SEM. Statistical significance was determined by one-way ANOVA followed by Fisher's LSD as the post-hoc test. A letter indicates a $p < 0.05$, letter w/ * indicates $p < 0.01$, letter w/ ** indicates $p < 0.001$ and a letter w/ *** indicates $p < 0.0001$.

Supplemental Figure 4: Serum lactate dehydrogenase (LDH) levels (n=5). A serum panel was performed as mentioned in the Materials and Methods and LDH levels were measured. Significant increase of LDH levels in Cyp2b-null mice fed a high-fat diet regardless of PFOS concentration compared to their hCYP2B6 counterparts. Data are presented as mean ± SEM. Statistical significance was determined by one-way ANOVA followed by Fisher's LSD as the post-hoc test. A letter indicates a $p < 0.05$, and a letter w/ * indicates $p < 0.01$.

Supplemental Figure 5: Serum HDL analysis (n=4). Total serum HDL cholesterol was determined using the HDL and LDL/VLDL Colorimetric Quantitation Assay from Sigma Aldrich (St. Louis, MO). The total HDL cholesterol concentrations were determined according to the manufacturer's instructions. PFOS suppresses HDL levels in all mice. Data are presented as mean ± SEM. Statistical significance was determined by one-way ANOVA followed by Fisher's LSD as the post-hoc test (n=5). A letter indicates a $p < 0.05$, letter w/ * indicates $p < 0.01$, letter w/ ** indicates $p < 0.001$ and a letter w/ *** indicates $p < 0.0001$.

Supplemental Figure 6: Serum LDL/VLDL analysis (n=4). Total serum LDL/VLDL cholesterol was determined using the HDL and LDL/VLDL Colorimetric Quantitation Assay from Sigma Aldrich (St. Louis, MO). The total LDL/VLDL cholesterol concentrations were determined according to the manufacturer's instructions. PFOS suppresses LDL/VLDL levels in all mice. Data are presented as mean ± SEM. Statistical significance was determined by one-way ANOVA followed by Fisher's LSD as the post-hoc test (n=5). A letter indicates a $p < 0.05$, letter w/ * indicates $p < 0.01$, letter w/ ** indicates $p < 0.001$ and a letter w/ *** indicates $p < 0.0001$.

Figures

Fig. 1

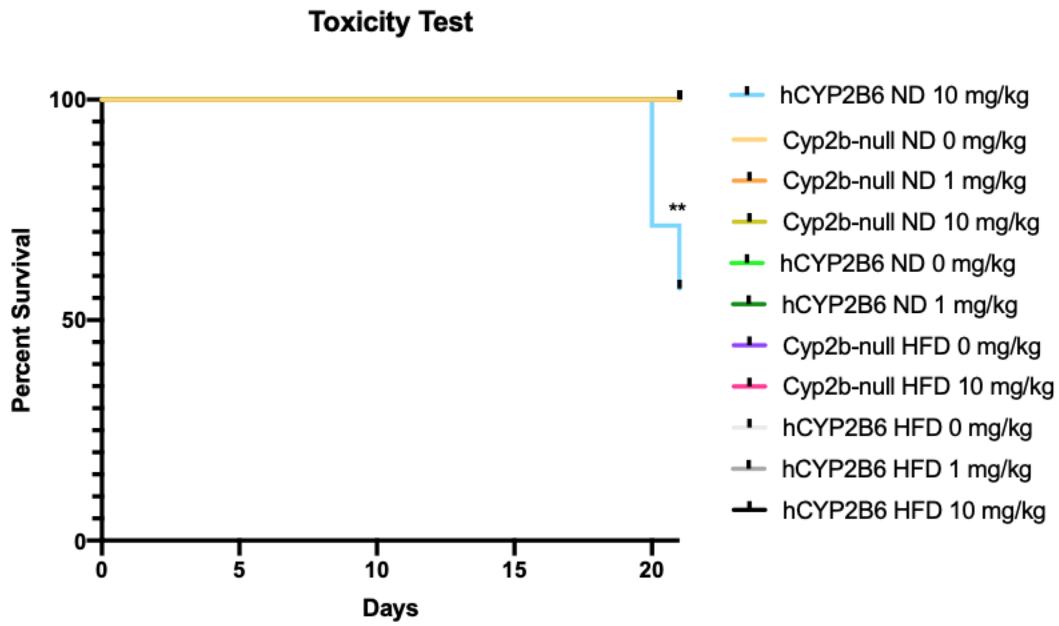


Fig. 2

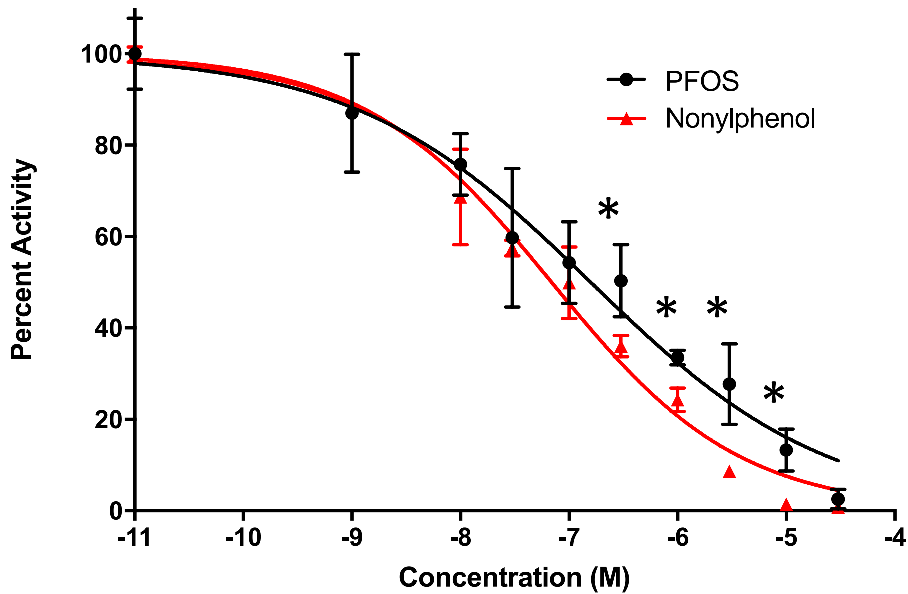


Fig. 3

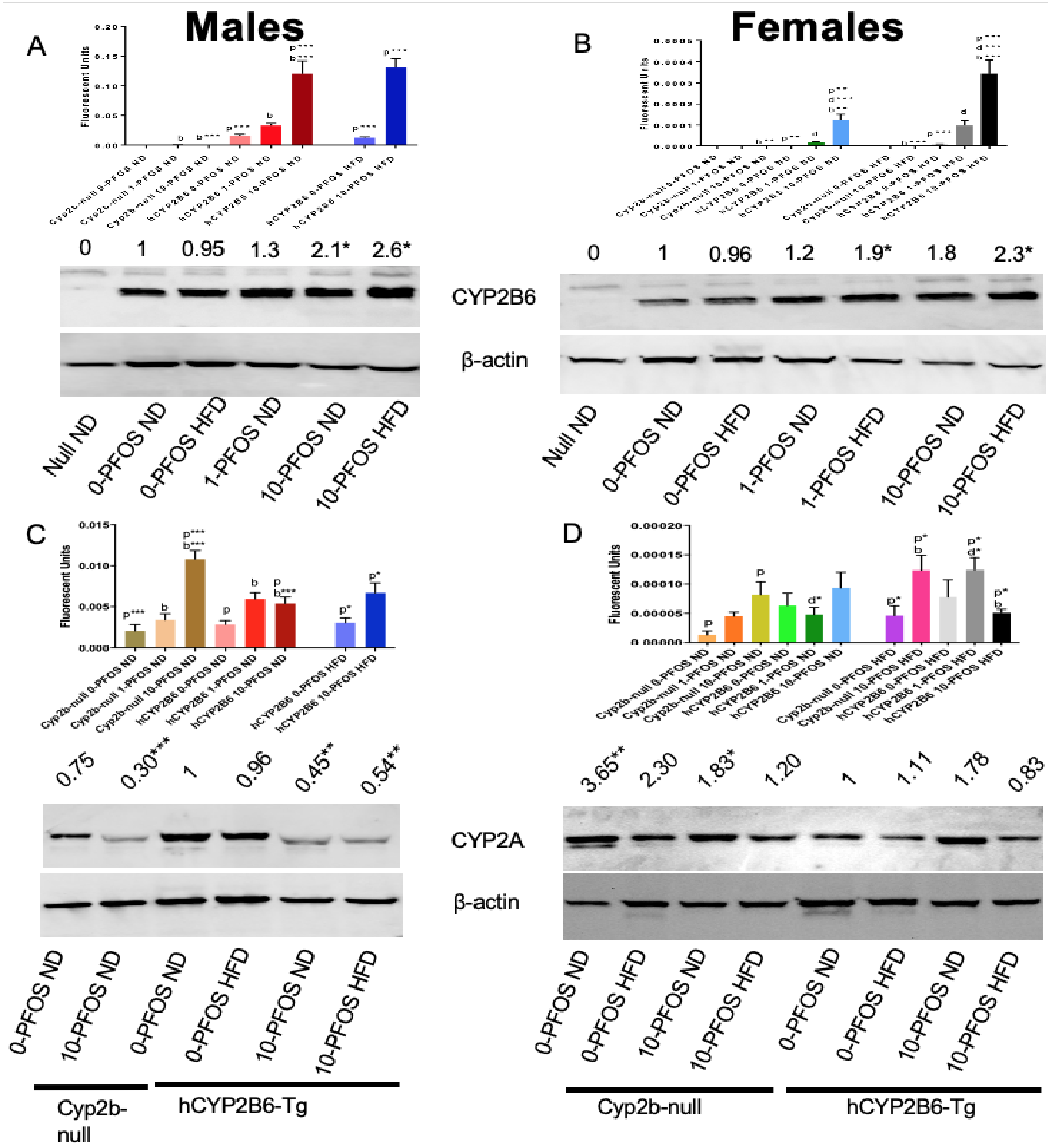


Fig. 4

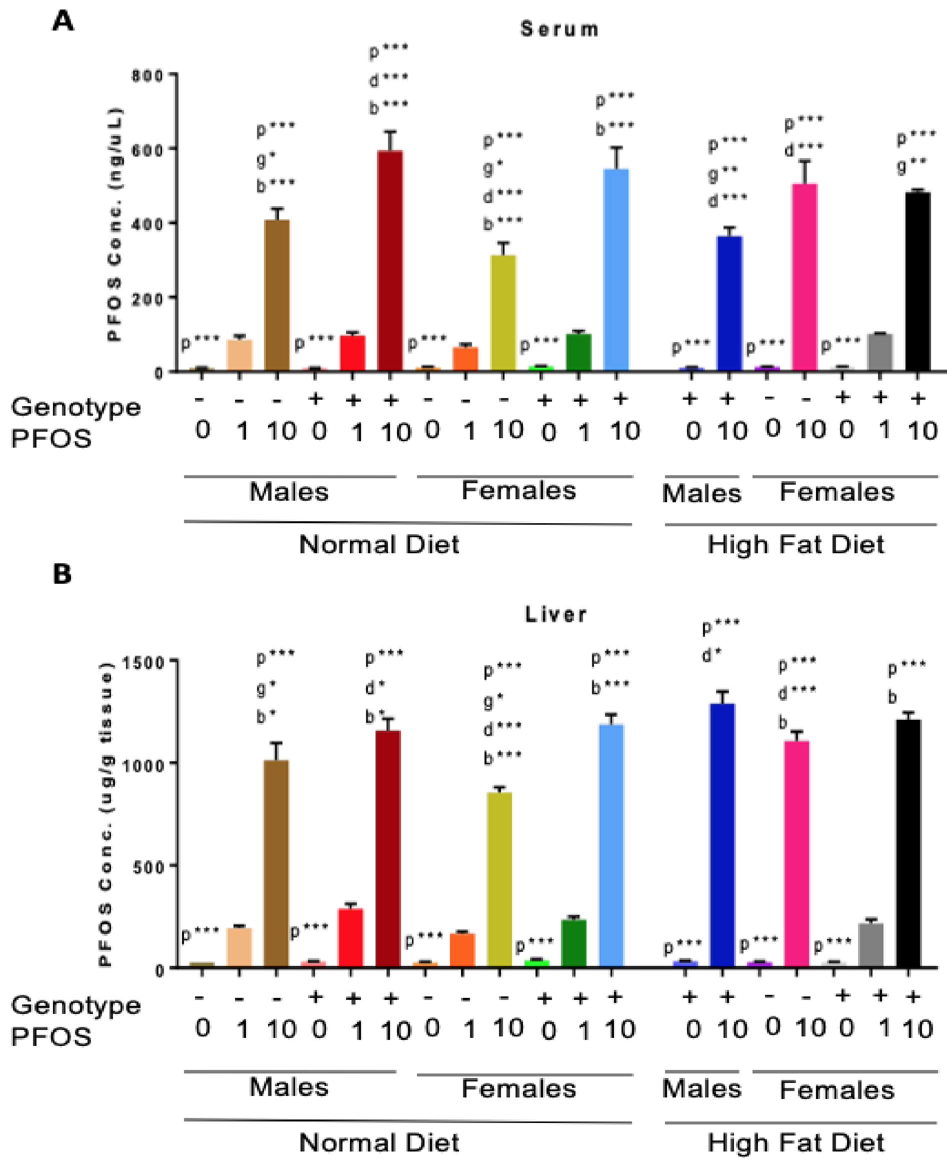


Fig. 5

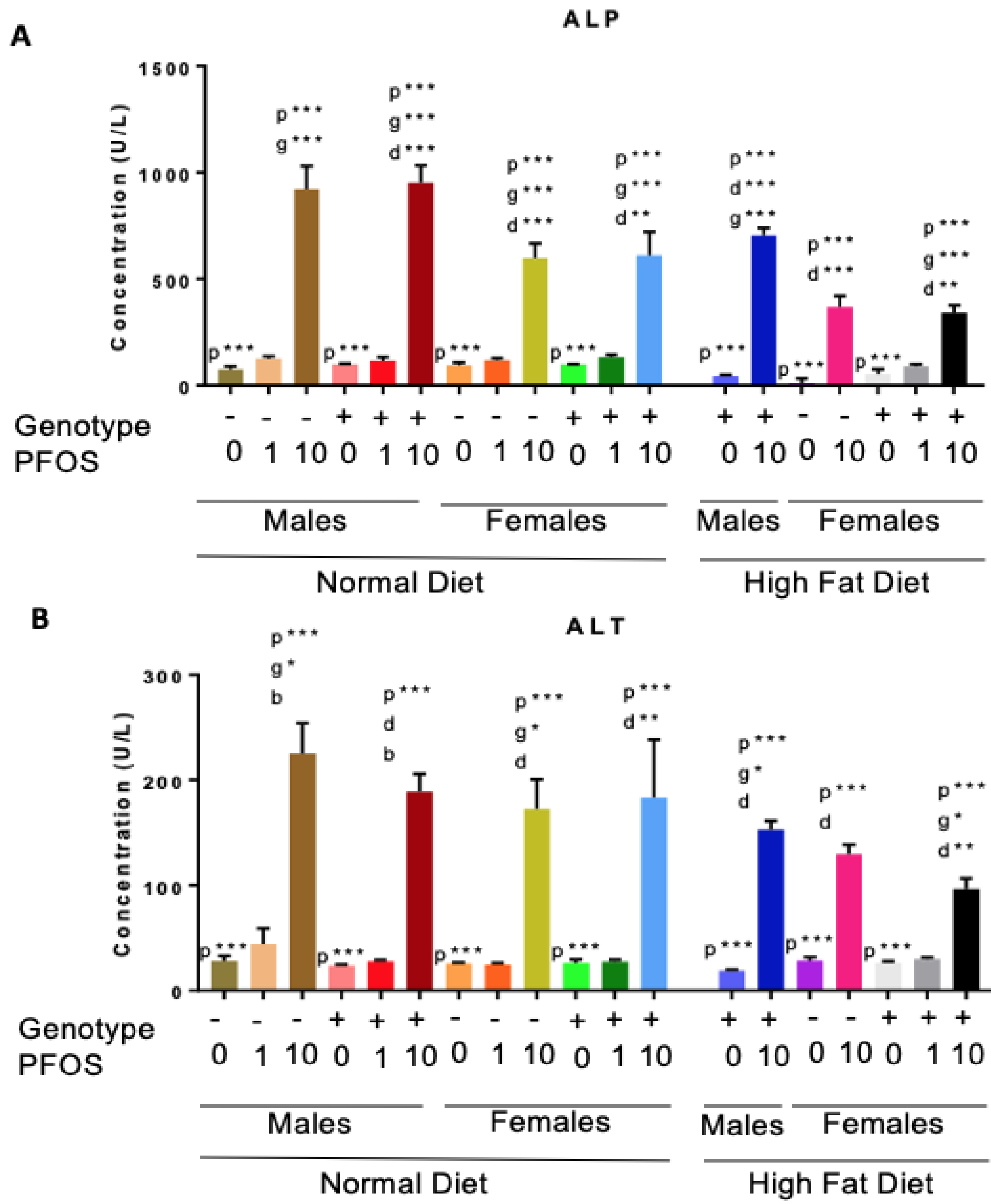
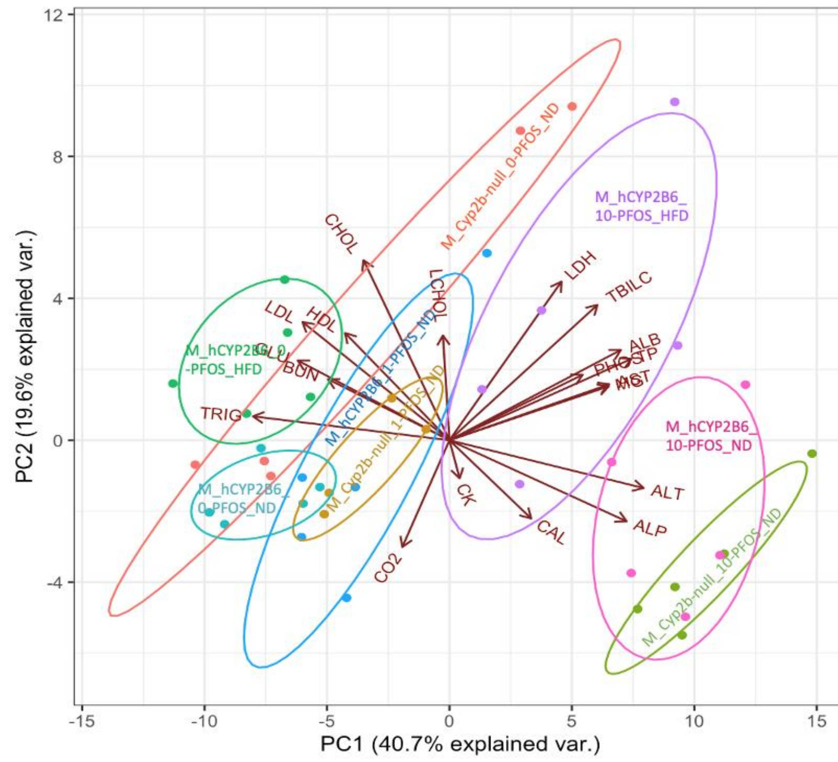


Fig. 6



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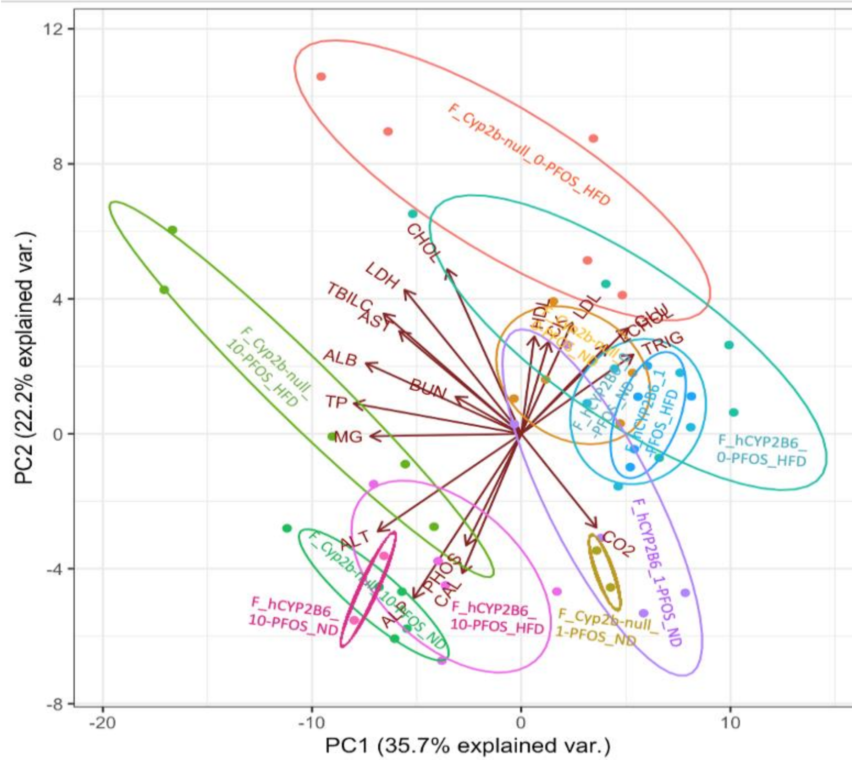
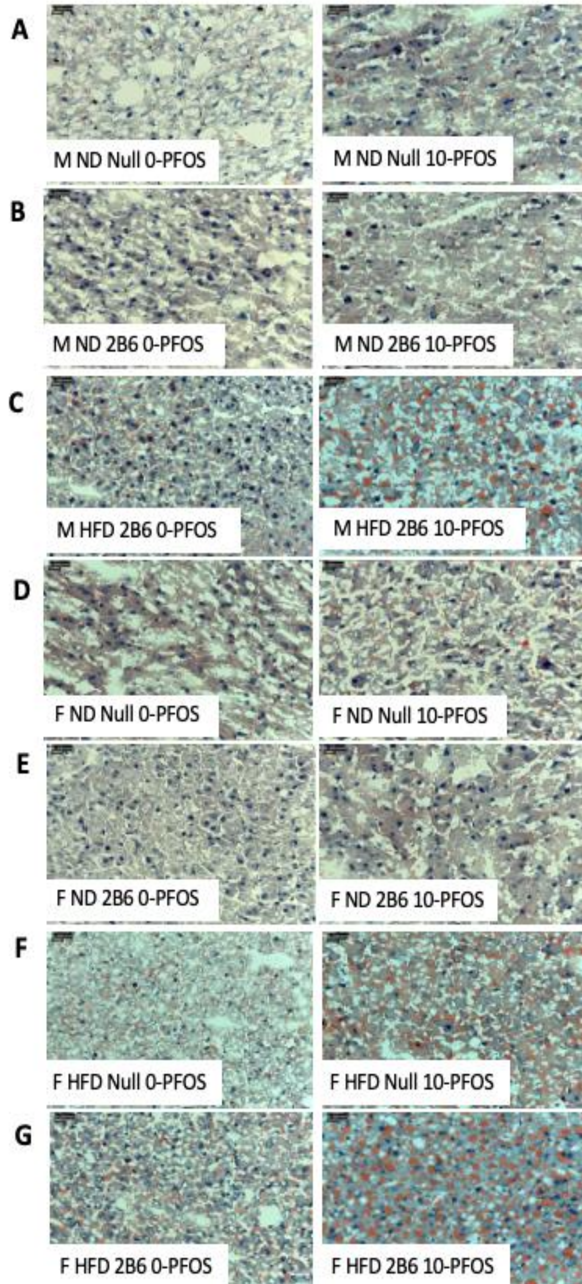


Fig. 8



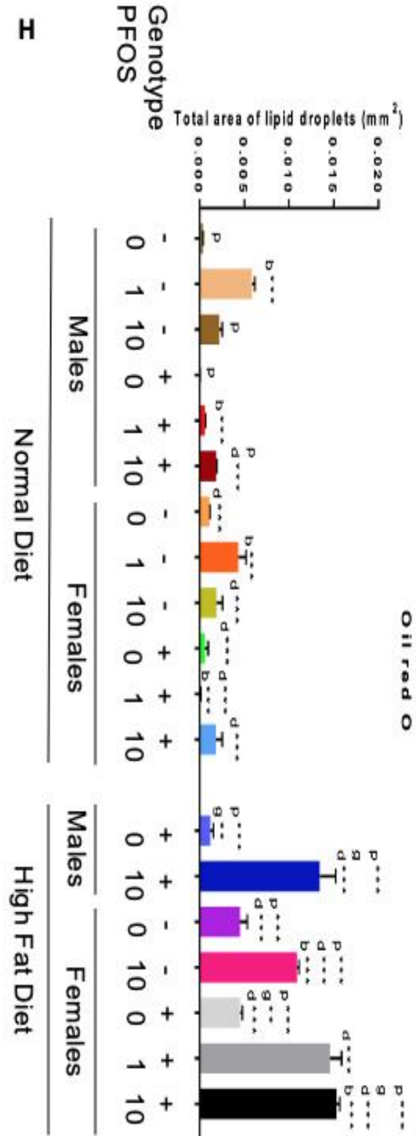


Fig. 9

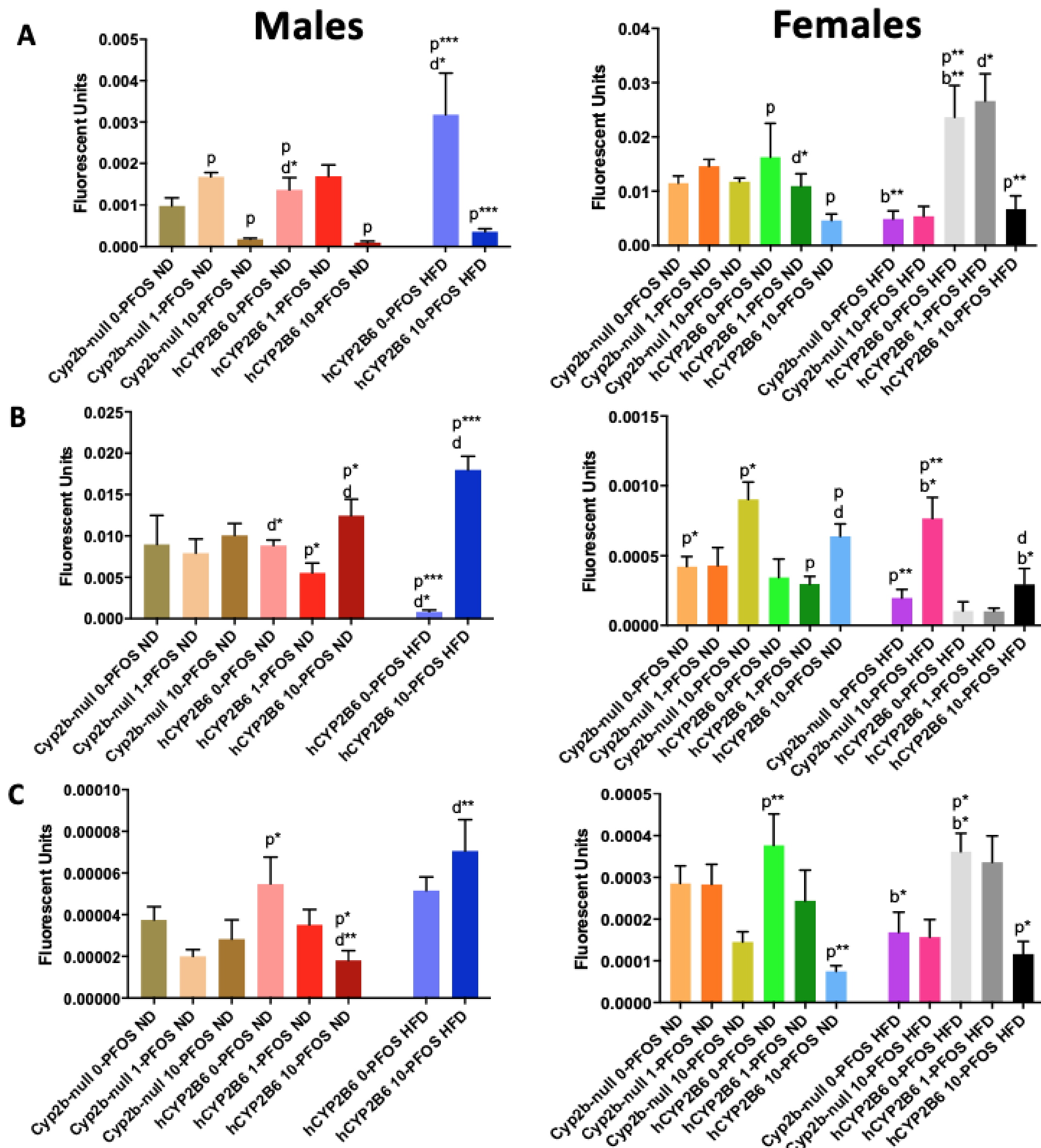
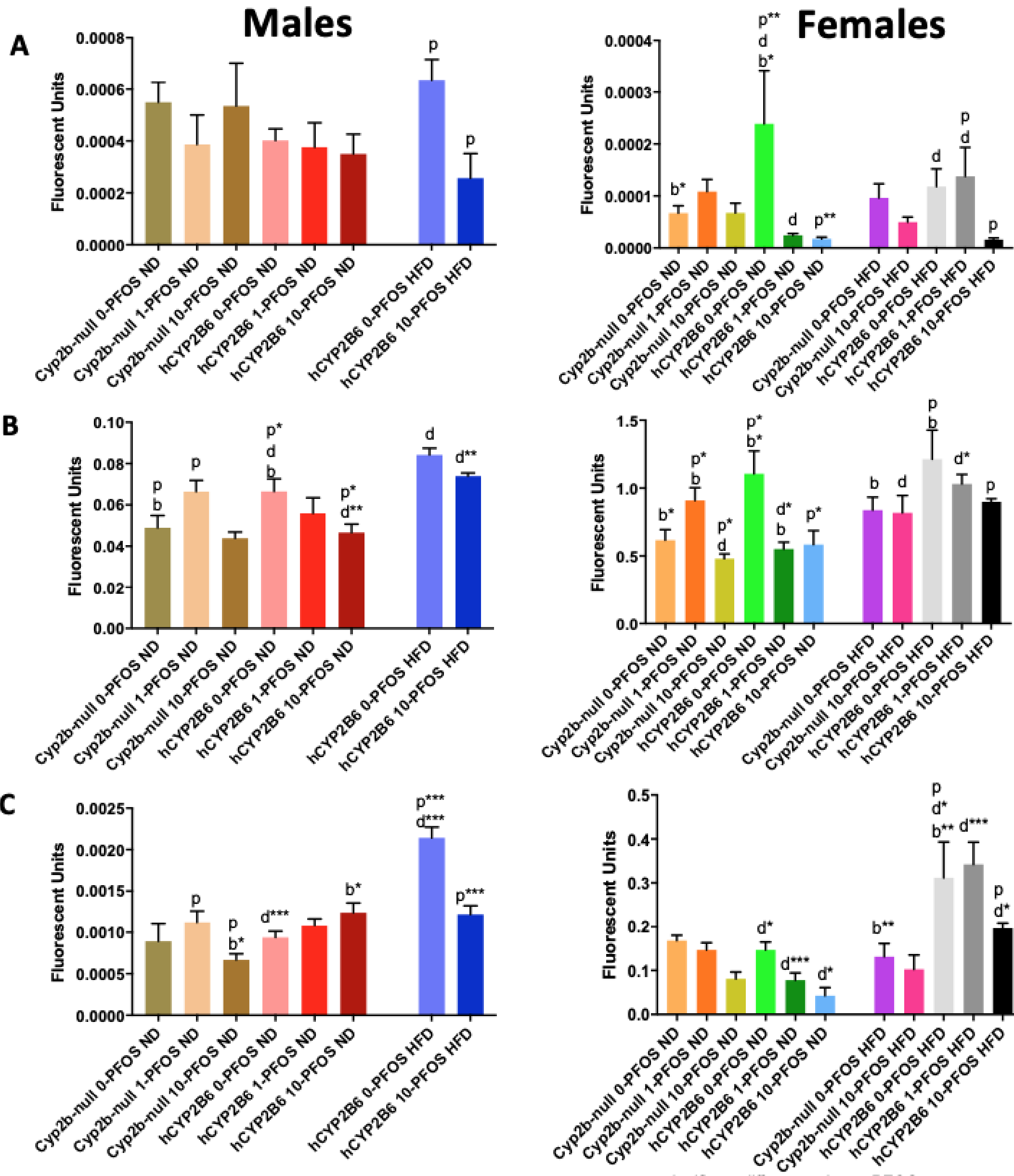


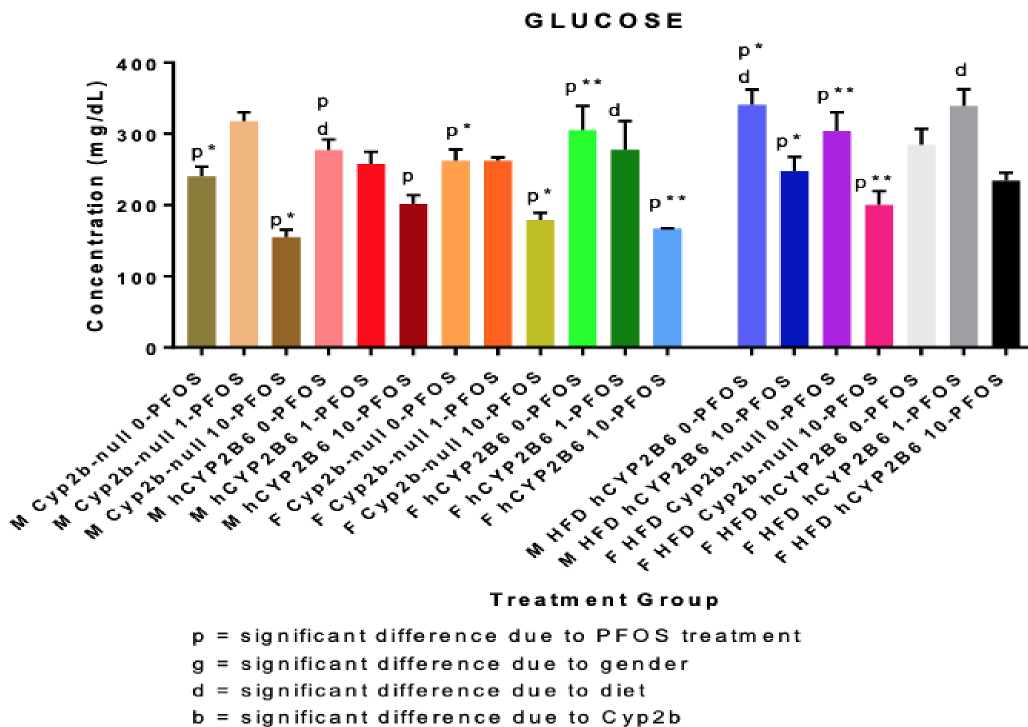
Fig. 10



Supplemental Material
Supplemental Table 1: qPCR primer information for genes of interest

Gene	Accession Number	Forward Sequence	Reverse Sequence	Annealing Temperature (℃)
SREBP1	NM_001358315.1	5'-ACGAAGTGCACACAAAAGCA-3'	5'-GCCAAAAGACAAGGGGCTAC-3'	58
CPT1A	NM_013495.2	5'-TTGATCAAGAAGTGCCGGACGAGT-3'	5'-GTCCATCATGGCCAGCACAAAGTT-3'	60
CYP2B6	KR711982.1	5'-CCATACACAGAGGCAGTCAT-3'	5'-GGTGTCAGATCGATGTCTTC-3'	54.5
CD68	NM_001291058.1	5'-CGCAGACGACAATCAACCTA-3'	5'-AGTGGCATGGTGAAGAGATG-3'	59
CYP2A5	NM_007812.4	5'-CAAAGCCAAGGAAGCAAGATG-3'	5'-AGTGGTGCTGAGTGGTAATG-3'	60
CYP4A14	NM_007822.2	5'-CCTCCTCATATTGCCCTGAATAG-3'	5'-GAGTCCATAGGCCTGAGTTATTT-3'	59
PPARG	NM_001127330.2	5'-TGGGTGAAACTCTGGGAGATTC-3'	5'-AATTTCTTGTGAAGTGCTCATAGGC-3'	60.1
HMGCR	NM_008255.2	5'-TTAGGCATGTGGTGGTGAAG-3'	5'-GCCAAGGAGGAGCAGAATAAA-3'	60
18S	NR_003278.3	5'-ATGGCCGTTCTTAGTTGGTG-3'	5'-ATGCCAGAGTCTCGTTCGTT-3'	64
GAPDH	NM_008084.3	5'-CCTTCATTGACCTCAACTA-3'	5'-CTGGAAGATGGTGATGG-3'	50
CYP2A13	NM_000766.5	5'-TCTCCCACTTCTTCCTCTGT-3'	5'-GGGAAGGAGGACAGACAATTAC-3'	59.8

Supplemental Figure 1



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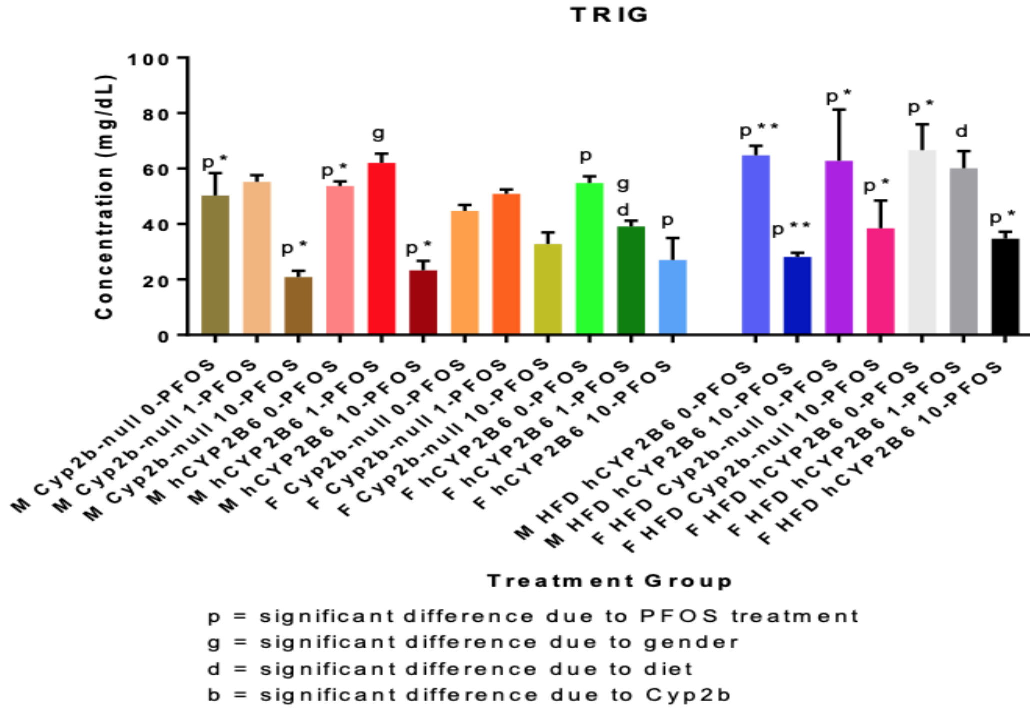
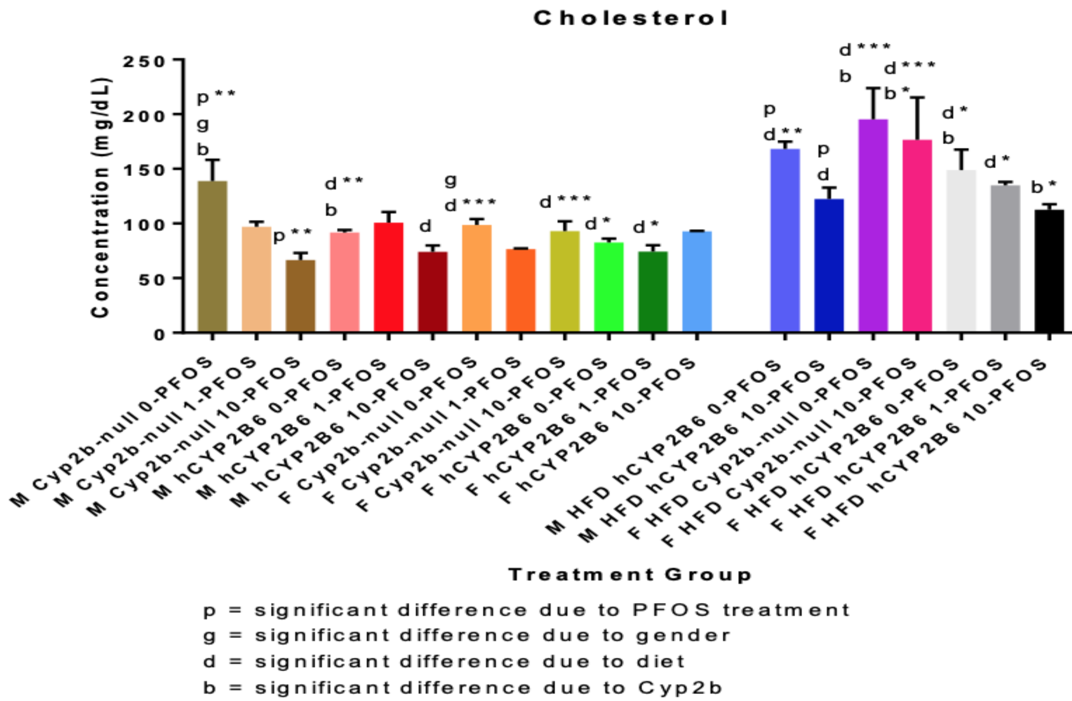


Supplemental Figure 3



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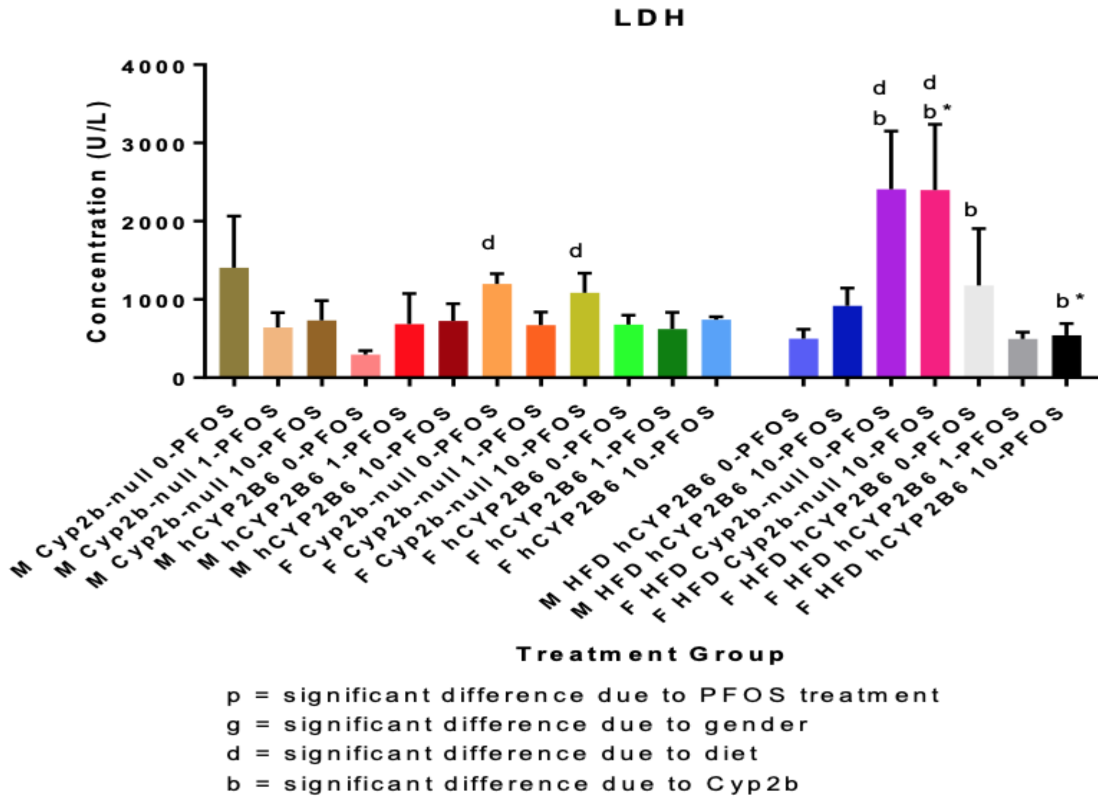
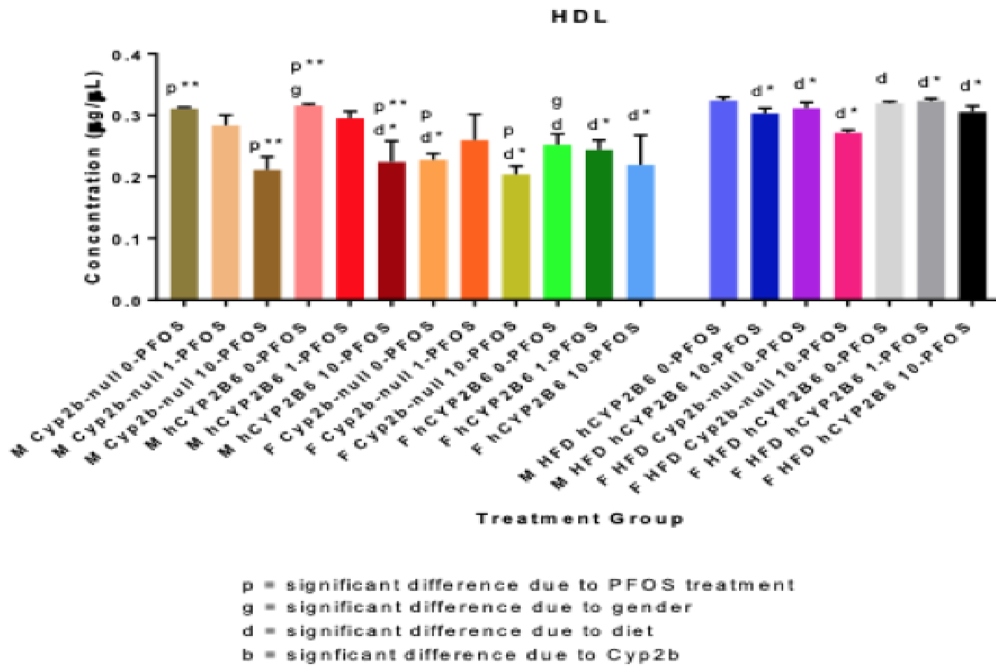
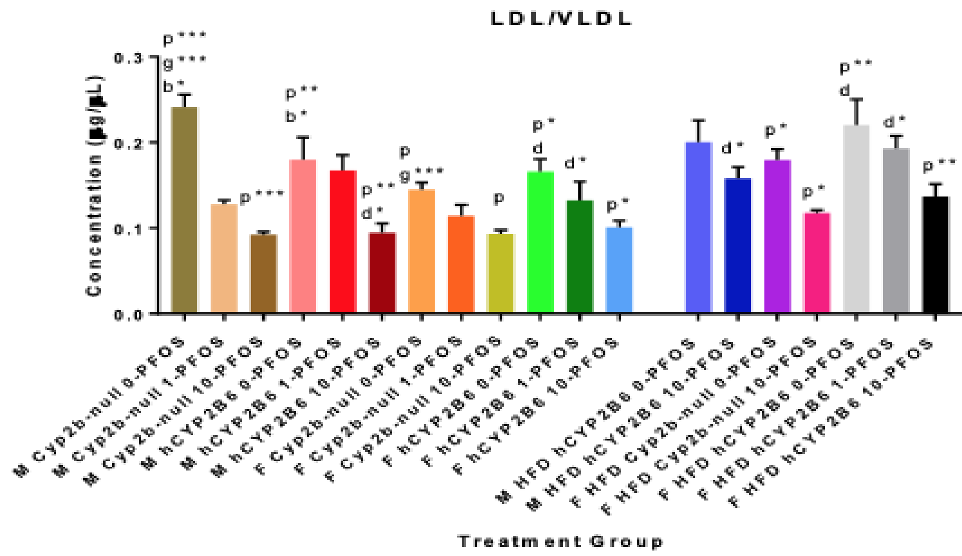


Supplemental Figure 5



Supplemental Figure 6



p = significant difference due to PFOS treatment
g = significant difference due to gender
d = significant difference due to diet
b = signficant difference due to Cyp2b

Chapter III: Summary and Conclusions

Despite PFOS being phased out of production in the US and Europe in the early 2000's, it is still produced in China (Lim et al., 2011; Wan et al., 2012) and many studies have reported that PFOS is still prevalent within the environment due to its stable structure (Beesoon & Martin, 2015; Yamashita et al., 2005). It is also prevalent in the human body with a half-life of approximately 5.4 years due to poor metabolism and elimination (Olsen & Zobel, 2007; Seacat et al., 2002). PFOS concentrations are still above the health advisory risk of 70 ppt, set by the EPA (EPA, 2017), in places around the world, such as Veneto Region, Italy (Ingelido et al., 2018) and Jiangsu Province, China (Yu et al., 2015), as well as cities within the US, such as Brunswick County, NC and Quad Cities, IA (Evans et al., 2020). Due to its prevalence and toxicity, understanding the mode of action of PFOS is vital.

CYP2B6 is the only CYP2B member in humans and is a known detoxification enzyme that is regulated by PXR, CAR, and FoxA2 in response to many endo- and xenobiotics as well as PUFA's (Hashita et al., 2008; Kretschmer & Baldwin, 2005; Nelson et al., 2004). PFOS's structure, a polar head and hydrophobic tail, resembles a biological fatty acid allowing it to perturb lipid metabolism (Fletcher et al., 2013) and cause an increase in hepatic triglyceride accumulation (Bijland et al., 2011; Cheng et al., 2016; Cui et al., 2017). Using the two unique mouse models previously generated in our lab, Cyp2b-null and hCYP2B6-Tg (Heintz, 2020; Kumar et al., 2017), the current study is the first to evaluate CYP2B6, specifically, and its role in PFOS mediated NAFLD in mice

fed a ND. This study also examined whether or not a HFD exacerbated the progression of PFOS mediated NAFLD.

Overall, this study shows that PFOS is able to significantly induce CYP2B6 *in vivo*, mice that express CYP2B6 are more sensitive to PFOS, and PFOS toxicity is relieved by a HFD in both genotypes; however, deleterious effects, such as elevated serum biomarkers indicative of cardiovascular toxicity (LDH & cholesterol), were observed in the Cyp2b-null mice fed a HFD.

The only mice to die during the three week exposure period were three ND fed hCYP2B6-Tg female mice; however, none of their HFD-fed counterparts died. The observed PFOS toxicity in the hCYP2B6-Tg mice fed a ND can likely be attributed to the increased retention of liver and serum PFOS compared to their Cyp2b-null counterparts. The average serum PFOS retention was significantly reduced in hCYP2B6-Tg mice when fed a HFD whereas a HFD increased liver and serum PFOS retention in the Cyp2b-null mice. All ND-fed mice treated with 10-PFOS exhibited significantly elevated biomarkers indicative of liver damage, ALT & ALP; surprisingly, these elevated levels were relieved by a HFD in all groups. In the current study, PFOS decreased serum glucose, triglyceride, and cholesterol levels in all mice which is consistent with previous findings (Bijland et al., 2011; Wang et al., 2014).

hCYP2B6-Tg mice had significantly elevated hepatic triglyceride levels in HFD-PFOS co-treated groups compared to their Cyp2b-null counterparts. Initially, these Oil Red O results were shocking, however, recent studies have shown that CYP2B6 is not protective of NAFLD and may increase susceptibility by producing oxylipins that could

progress NAFLD (Deol et al., 2017; Heintz, 2020). qPCR was performed to determine the relative gene expression of certain genes related to peroxisome proliferation (*Pparγ*, *Cyp4a14*) (Skat-Rørdam et al., 2019; Zhang et al., 2017) and lipid metabolism (*Cpt1a*) (Schlaepfer & Joshi, 2020). *Pparγ* prevents the progression of NAFLD whereas *Cyp4a14* increases NAFLD progression (Liss & Finck, 2017; Zhang et al., 2017). PFOS significantly repressed *Pparγ* gene expression in all groups except for HFD-fed Cyp2b-null mice and increased *Cyp4a14* gene expression. These data could explain the exacerbated levels of hepatic triglycerides in the HFD-PFOS co-treated hCYP2B6-Tg mice compared to their Cyp2b-null counterparts. *Cpt1a*, a key regulator of mitochondrial β-oxidation (Cheng et al., 2016), expression was repressed by PFOS in hCYP2B6-Tg mice suggesting that PFOS inhibits mitochondrial β-oxidation resulting in the accumulation of fatty acids within the liver of these mice. PFOS also lowered LDL/VLDL levels which are important in hepatic triglyceride export (Eisenberg, 1990). In agreement with other studies (Cheng et al., 2016; Wan et al., 2012), these findings suggest one way PFOS elicits hepatotoxicity is through the inhibition of mitochondrial β-oxidation and decreased hepatic triglyceride export. The observed hepatotoxicity was greater in hCYP2B6-Tg mice.

While these results are interesting, more research needs to be done to determine why mice that express CYP2B6 are more sensitive to PFOS toxicity but appear to be protected by a HFD. To help understand why mice that express CYP2B6 are more sensitive to PFOS, more analysis of different genes related to lipid metabolism should be performed. The current study found that PFOS down regulates a few important genes

related to lipid metabolism, including *Cpt1a* and *Srebp1* (Ruiz et al., 2014; Schlaepfer & Joshi, 2020), to a greater extent in the hCYP2B6-Tg mice compared to their Cyp2b-null counterparts. To better understand the different mechanisms of PFOS toxicity, a microarray analysis or RNA sequencing should be conducted to examine the transcriptomic response of genes, including key hepatic regulators such as HNF4α (Beggs et al., 2016), from the liver to further analyze the effects of PFOS.

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