Abstract

30-Day Immunotoxicity Study of PFMOAA in C57BL/6 Mice

by

Samuel Vance

July, 2019

Director of Thesis: Dr. Jamie DeWitt, Department of Pharmacology and Toxicology

Within the past five years, two classes of per- and polyfluoroalkyl substances (PFAS) were phased out of production in the U.S., which led to the development and production of PFAS to replace these two major classes. One family of these PFAS are perfluoro-ether carboxylic acids (PFECA), which have emerged in the public and scientific arenas due to their presence in drinking water systems across the U.S., including Wilmington, NC. Although manufacturers have touted them as having more favorable environmental and toxicological properties very little is known about the toxicity and environmental fate these emerging PFECA. One compound, perfluoro-2-methoxyacetic acid (PFMOAA), was identified as the dominant PFECA in the Cape Fear River, in concentrations as high as 35,000 ng/L. There is very little mention of PFMOAA in the publicly available scientific literature and to our knowledge, we are the first to investigate its potential for toxic effects. In this 30-day study, we orally administered 25,000, 2,500,000, or 250,000,000 ng/L of PFMOAA in water to male and female C57BL/6 mice and investigated immune and liver alterations following exposure. Mice given PFMOAA showed no signs of overt toxicity during the study and no evident changes were observed in liver mass or peroxisomal enzyme activity. While mild alterations in splenic and thymic lymphocyte sub-populations were observed in males, these results do not point to any definitive alterations in

immune function. Ultimately, we concluded that the doses administered were too low to achieve an internal dose sufficient to induce changes to immune endpoints, likely due to rapid excretion of PFMOAA in mice. Further investigation into serum and organ concentrations of PFMOAA as well its effects on antibody production will be more conclusive of immunotoxic effects.

30-Day Immunotoxicity Study of PFMOAA in C57BL/6 Mice

A Thesis Presented to

The Faculty of the MS Program in Biomedical Sciences

Office of Research and Graduate Studies at Brody School of Medicine

In Association with the Department of Pharmacology and Toxicology, Brody School of Medicine

In Partial Fulfillment of the Requirements for the Degree

Master of Science

Biomedical Science

by

Samuel Vance

July, 2019

30-Day Immunotoxicity Study of PFMOAA in C57BL/6 Mice

by

Samuel Vance

APPROVED BY:

DIRECTOR OF
THESIS: _____
Jamie DeWitt, Ph.D.

COMMITTEE MEMBER: _____
Kym Gowdy, Ph.D.

COMMITTEE MEMBER: _____
Lisa Domico, Ph.D.

COMMITTEE MEMBER: _____
Ralph Mead, Ph.D.

DIREDCOR, MS PROGRAM
IN BIOMEDICAL SCIENCES: _____
Rich Franklin, Ph.D.

DEAN OF THE
GRADUATE SCHOOL: _____
Paul J. Gemperline, PhD

ACKNOWLEDGEMENTS

We would like to thank The North Carolina Policy Collaboratory for funding this research as well as our collaborators of the PFAS Testing Network.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1: INTRODUCTION

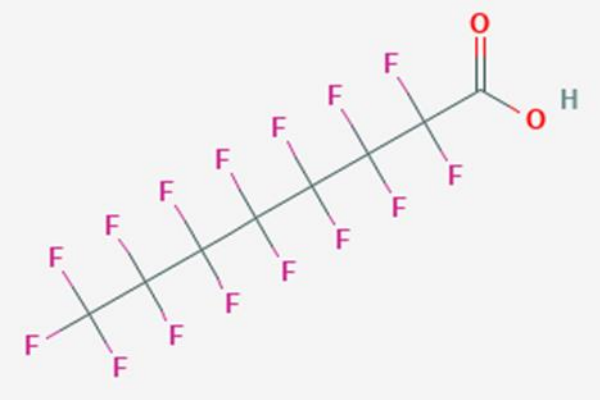
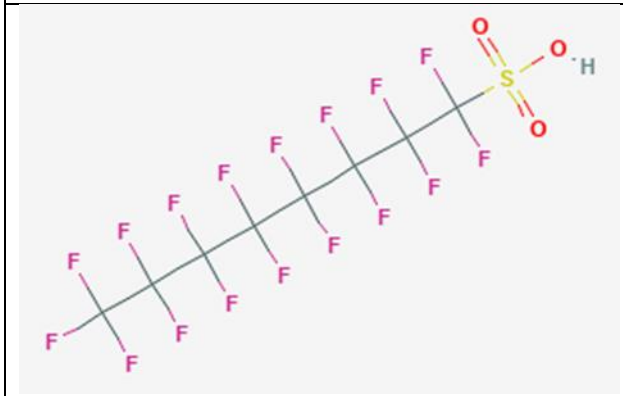
Legacy PFAS

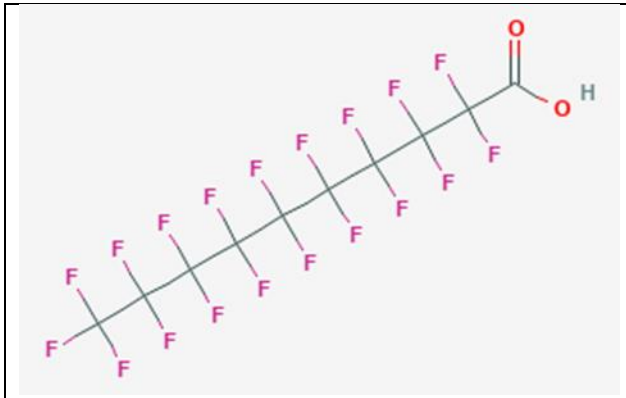
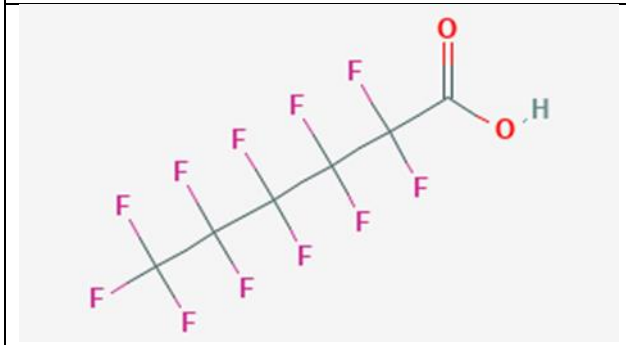
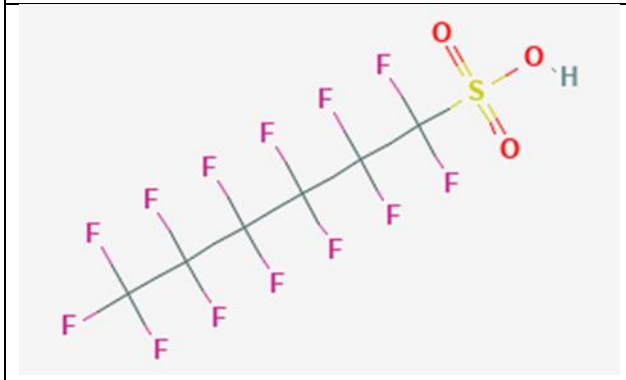
Per- and polyfluoroalkyl substances (PFAS) are a large family of amphiphilic compounds composed of alkane tails with fluorine replacing some or all the hydrogens and an active head group (Wang et al., 2017). This family of chemicals is estimated to contain over 5,000 different compounds that are widely utilized throughout the world in manufacturing and consumer products for their chemical properties that confer water, oil and stain resistance as well as temperature and friction resistance (ATSDR, 2018; US EPA, 2019). These chemical properties have made them abundant in water-resistant coatings for textiles and paper products, non-stick coatings on cookware and food packaging, and to formulate firefighting foams as well as use in aerospace, electronics and automotive industries (ATSDR, 2018; FluoroCouncil, 2014; NTP, 2016). Conversely, these same properties of the strong carbon-fluorine bond and amphiphilic characteristics have raised environmental health concerns. The carbon-fluorine bonds do not readily break down in the environment and make these compounds highly mobile in soil and water as well as bioaccumulative (US EPA, 2018; Wang et al., 2017). The Centers for Disease Control and Prevention (CDC), through their National Health and Nutrition Examination Survey (NHANES), a cross-section representative of the U.S. general population, detected PFAS in the majority of participants (ATSDR, 2018). Olsen et al., (2017) also detected PFAS in serum from American blood donors collected from 2000-2015, further contributing to the evidence of PFAS as major environmental contaminants that also get into humans.

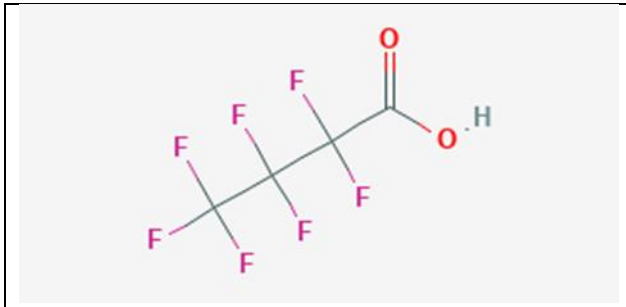
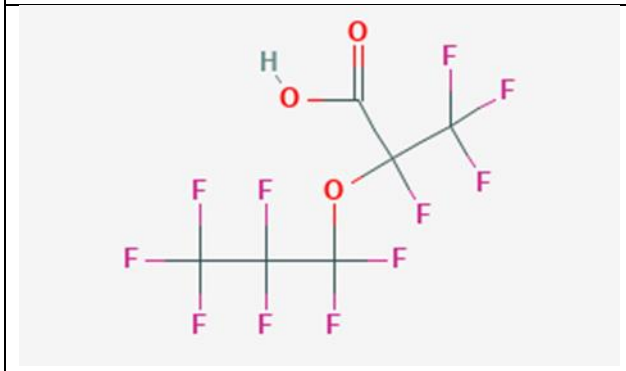
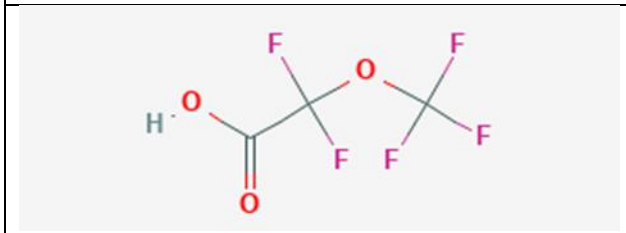
While the PFAS family of compounds is extensive, two PFAS chemicals, perfluorooctanoic acid (PFOA) and perfluorooctanesulfonic acid (PFOS) in particular, have been

heavily studied and identified by the U.S. Environmental Protection Agency (US EPA) as developmental toxicants (US EPA, 2016a, 2016b) and by the U.S. National Toxicology Program (US NTP) as presumed to immune hazards to humans (NTP, 2016). PFOA was first synthesized in the early 1940s by scientists working at DuPont; it was later primarily applied as a production aid for fluoropolymers used in non-stick coatings (Buck et al., 2011). One such fluoropolymer, Teflon™, became widely used, and its production became one of the major sources of PFAS release into the environment (Sajid et al., 2017). In the 1950s, a scientist working at the 3M Company spilled an experimental fluorochemical on a shoe and later noticed that the shoe repelled water and dirt (3M, 1999; ATSDR, 2018). This led to the invention of PFOS, which was trademarked as Scotchgard™ (ATSDR, 2018). The manufacture and use of these and other PFAS in water-repellent outerwear, food packaging, and aqueous film forming foams (AFFF), as well as many other products have led to the widespread release of PFAS into the environment.

Table 1. List of several PFAS compounds including, structure, name, abbreviation, chemical formula, and CAS number.

Chemical Structure	Short Name	Chemical Name	Chemical Formula	CAS Number
	PFOA	Perfluorooctanoic acid	$C_8HF_{15}O_2$	335-67-1
	PFOS	Perfluorooctane sulfonic acid	$C_8HF_{17}SO_3$	1763-23-1

	PFDA	Perfluorodecanoic acid	$C_{10}HF_{19}O_2$	335-76-2
	PFHxA	Perfluorohexanoic acid	$C_6HF_{11}O_2$	307-24-4
	PFHxS	Perfluorohexane sulfonic acid	$C_6HF_{13}SO_3$	355-46-4

	PFBA	Perfluorobutanoic acid	$C_4HF_7O_2$	375-22-4
	GenX (HFPO-DA)	Perfluoro-2-propoxypropanoic acid	$C_6HF_{11}O_3$	13252-13-6
	PFMOAA	Perfluoro-2-methoxyacetic acid	$C_3HF_5O_3$	674-13-5

Chemical information obtained from NCBI PubChem chemical resource (Kim et al., 2019)

Absorption, Distribution Metabolism and Excretion

Humans can absorb PFAS through contaminated drinking water and food as well as by dust inhalation and hand to mouth contact from coated surfaces such as carpets and clothing (Fraser et al., 2013; Hopkins et al., 2018; US EPA, 2018; Wu et al., 2019). PFAS also are known to cross the placental barrier, have been measured in fetal cord blood as well as breast milk, leading to an increased exposure risk to infants (ATSDR, 2018; Impinen et al., 2019; Manzano-Salgado et al., 2015). Once absorbed into the body, they can enter circulation where PFAS primarily bind to albumin and other serum proteins (ATSDR, 2018). Binding to serum proteins leads to accumulation of PFAS in organs including the kidneys, spleen, and thymus, and in the highest extravascular concentrations in the liver (ATSDR, 2018; Gomis et al., 2018). There is no evidence that PFAS undergo metabolism in the body and are primarily excreted in the urine (ATSDR, 2018; US EPA, 2018). In females, PFAS can also be eliminated through menstrual blood, which according to Wong et al. (2014), has been shown to contribute to many of the sex differences observed in PFAS elimination in humans. It also is hypothesized that male and female rodents, especially rats, have differences in renal tubular reabsorption channels, but this still requires further investigation to determine if this also is true for humans (ATSDR, 2018; Chang et al., 2008). The long alkane chains of PFAS act similarly in the body to fatty acids, and these properties, including their distribution and excretion rates, are heavily dependent on chain length. In general, half-lives of PFAS are correlated with chain length and branching; longer chain PFAS are excreted more slowly, sulfonates are excreted more slowly than carboxylates, and branched chains are excreted more rapidly (ATSDR, 2018; Peng et al., 2013; Zhang et al., 2013). PFOA for example has a half-life in humans of approximately 3.4 years, PFOS is slightly longer at 4.3 years, perfluorodecanoic acid (PFDeA) has an approximate half-life of 8.3 years,

and perfluorobutanoic acid (PFBA) has a half-life of approximately 72 hours (ATSDR, 2018). There are exceptions to the general rule as the six carbon perfluorohexane sulfonic acid (PFHxS) has an average half-life of 8 years, longer than its eight carbon chain counterpart, PFOS (ATSDR, 2018). The similarities of the alkane chain to fatty acids are also thought to be associated with toxicity of longer chain compounds such as PFOA, which partitions to a greater degree to the liver than shorter chain molecules such as PFBA (ATSDR, 2018). Compared to humans, animal models have much faster elimination rates of PFAS with rats and mice eliminating most PFAS in a matter of hours to several days (ATSDR, 2018). It is also important to note that PFAS half-life estimates in humans may be overestimated due to persistent exposure to these environmental contaminants (Olsen et al., 2017). In other words, continual exposure from various sources means that the human body burden of PFAS also is persistent.

Toxicity

Resulting from the settlement of a class action lawsuit against DuPont in 2001, the C8 Science Panel began a massive prospective study of residents of the mid-Ohio River Valley, where a DuPont facility (Washington Works) in West Virginia had been releasing PFAS, mostly PFOA, into the river and air since 1951 (Frisbee et al., 2009). The study was conducted from 2005-2006 and collected serum samples, residential histories, and questionnaire responses from 69,000 residents (Frisbee et al., 2009; Shin et al., 2012). This became known as the C8 Health Project and data from this study highlight human health outcomes associated with PFOA, and to a degree, PFOS exposure (Frisbee et al., 2009; Steenland et al., 2009). Scientists from the C8 Health Project reported that median PFOA serum levels for over 50,000 residents in southeastern Ohio and West Virginia were six times greater than the general population while workers at the

Washington Works facility had serum levels of 1,000 ng/mL, on average, in 2004 ((Frisbee et al., 2009; Sakr et al., 2007; Shin et al., 2012). Chronic exposure to PFOA in this population has been associated with increases in pregnancy-induced pre-eclampsia and hypertension, increased diagnosis of elevated serum cholesterol, increased risk of thyroid disease, increased risk of ulcerative colitis, and increased risk of kidney and testicular cancer (Savitz et al., 2012; Steenland et al., 2010; Steenland et al., 2012). The 2018 draft Toxicological Profile for Perfluoroalkyls by the Agency for Toxic Substances and Disease Registry (ATSDR) identified additional adverse health outcomes associated with PFOA and additional PFAS. These outcomes include liver damage, increases in liver enzymes and decreases in serum bilirubin, decreased antibody response to vaccines, increased risk of asthma diagnosis, increased risk of decreased fertility, and small decreases in birth weight (ATSDR, 2018). The International Agency for Research on Cancer (IARC) has also concluded that PFOA is a possible carcinogen with evidence of increased testicular and kidney cancer observed in highly exposed humans (IARC, 2017).

Liver toxicity is a common endpoint observed in studies of experimental animals following PFAS exposure, including increases in liver weight, peroxisome proliferation, changes in liver enzymes, and eventually, liver cancer (Abbott et al., 2012; Li et al., 2017). The liver toxicity observed in rodent studies has been linked to peroxisome proliferator-activated receptor-α (PPAR-α) induced peroxisome proliferation, resulting in hepatomegaly, necrosis, and liver tumor growth at higher doses (Abbot et al. 2012). Peroxisome proliferator activated receptors (PPARs) are a family of nuclear hormone receptors that are important for lipid metabolism and inflammation and are naturally activated by fatty acids; as long chain PFAS share a similar structure to fatty acids, PFAS can also activate PPARs (Li et al., 2017). Peng et al., in a 2013

metabolomics study, showed that PFOA disrupted a variety of metabolic functions in a human immortalized liver cell line. This study demonstrated that PFOA had potent changes in lipid homeostasis, including an increase in cholesterol biosynthesis, increased biomarkers and expression of multiple genes involved in fatty acid transportation and oxidation (Peng et al., 2013). One such gene change was an induction of ACOX1 transcription, which encodes the first gene in the fatty acid beta-oxidation pathway. Interestingly, at the high dose administered in the study, (50mg/L), multiple changes were identified in biomarkers and genes involved in nucleotide and amino acid metabolism as well (Peng et al., 2013). While PPAR-α appears to be a dominant pathway of liver toxicity leading to liver tumors in rodents, as humans only have one-tenth the amount of liver PPAR-α, some in the scientific community discount the relevance of PPAR-α activation by PFAS as a relevant pathway for any toxicity in humans. The Peng et al., study however, identified that these alterations occurred without changes in PPAR-a expression/signaling in human liver cells (Peng et al., 2013). Furthermore, numerous studies of mice lacking a fully functional PPAR-α report that hepatomegaly and peroxisome proliferation still exist, although to a reduced degree, indicating that the toxic effects of PFAS on the liver are not limited to PPAR-α activation and are thus still important for human toxicity (Abbott et al., 2012; DeWitt et al., 2016; Filgo et al., 2015; Li et al., 2017; Yang et al., 2002).

The immune system appears to be sensitive to PFAS exposure, which is supported by the US NTP conclusion that PFOA and PFOS are presumed to be immune hazards in humans (NTP, 2016). This conclusion was based on data from both epidemiological studies of humans and data from experimental animal studies. One such epidemiological study was of residents of the Faroe Islands, a community where dietary intake of marine food was associated with increased exposure to PFAS (Grandjean et al., 2012). This study reported that a doubling of serum PFOA

and PFOS concentrations in five-year-old children was associated with a loss of clinical protection from tetanus and diphtheria vaccines at age seven (Grandjean et al., 2012). This study also observed that maternal PFOS serum levels with a mean of 27.3 ng/mL were negatively associated with the child's diphtheria antibody concentrations (Grandjean et al., 2012).

In another epidemiological study of families from the Norwegian Mother and Child Cohort Study (MoBa), it was observed that four PFAS at the respective median serum concentrations, PFOA (1.1ng/mL), PFOS (0.3 ng/mL), PFHxS (0.3 ng/mL), and perfluorononanoic acid (PFNA) (5.5ng/mL), were all negatively correlated with the levels of anti-rubella antibodies of the 56 children tested at three years of age (Granum et al., 2013). A follow-up study by Impinen et al., (2019) using the MoBa cohort, looked at approximately 1000 mother/child pairs over seven years to assess allergy and asthma outcomes. This study looked at six different PFAS including PFOS, PFOA, PFHxS, PFNA, perfluoroundecanoic acid (PFUnDA) and perfluoroheptanesulfonate (PFHpS), all of which were detectable in serum of the general population, with PFOS being the highest followed by PFOA. Contrary to the previous MoBa cohort, this study did not investigate antibody levels but instead focused on three types of health outcomes: doctor-diagnosed asthma, eczema, and allergies, parent-reported asthma and allergy-related symptoms, and parent-reported number of infections from age 0-3 and 6-7 (Impinen et al., 2019). While the study showed little evidence of a relationship between prenatal exposure and allergy outcomes except for an inverse relationship of PFUnDA and eczema in girls, it did support the previously identified immunosuppressive effects of PFAS with an association of increased reported airway infections and gastric flu/diarrhea (Impinen et al., 2019).

Animal studies also support the observation of immune effects in humans; numerous rodent studies indicate that PFOA and PFOS are immunotoxic. Peroxisome proliferation from PFOA exposure also appears to be partially linked to its immune-system toxicity as mice given relatively high doses of PFOA experience splenic and thymic atrophy and changes in lymphocyte subpopulations that are significantly reduced in PPAR-α null mice (DeWitt et al., 2016; Yang et al., 2002; Yang et al., 2001). While numerous studies have shown changes in lymphocyte subpopulations as well as splenic and thymic atrophy (DeWitt et al., 2016; Loveless et al., 2008), the "gold standard" for evaluation of the immunotoxicological potential of exogenous agents has long been the T-cell dependent antibody response (TDAR), which is used to evaluate the antibody response to specific antigens administered to live animals. Our lab previously identified a lowest observed adverse effect level (LOAEL) of 3.75 mg/kg for PFOA after a 15-day exposure in mice (DeWitt et al., 2008). Other studies have demonstrated that PFOS has a broader range of LOAELs, from 0.02-0.8 mg/kg, (Dong et al., 2009; Zheng et al, 2009, Peden-Adams et al., 2008). Based on their presence in drinking water supplies and growing scientific evidence of toxicity, the US EPA set a lifetime drinking water health advisory level of 70 ng/L for the combined concentration of PFOA and PFOS (US EPA, 2016a, 2016b). This value was deemed to be of minimal risk for susceptible populations, primarily based on developmental outcomes, but these levels are also supported by studies using immune endpoints.

Public Health

Due to their environmental persistence, ability to bioaccumulate, and a growing body of toxicological evidence associated with PFAS exposure, the US EPA in cooperation with the 3M Company, fostered a US-based phase-out of several PFOA and PFOS precursors and PFAS-

related products, starting with PFOS in 2002 and PFOA in 2015 (US EPA, 2015). However, this has led to a rise in the production of new PFAS alternatives, touted as having more favorable environmental and toxicological properties (Bowman, 2015; Gomis et al., 2018; Hopkins et al., 2018; Shea, 2018; Wang et al., 2017). This family of alternatives primarily consists of per- and poly-fluorinated ether carboxylic acids (PFECA). One such PFECA of importance is the PFOA replacement 2,3,3,3-tetrafluoro-2-(heptafluoropropoxy)-propanoate, known by its trade name GenX (Gomis et al., 2018; Hopkins et al., 2018). The manufacturer of GenX claims it has a favorable environmental and toxicological profile due to is shorter, branching chain, (Figure 1), and being less bio-accumulative in the environment (Shea, 2018). Despite these claims, this PFECA has become a compound of concern due to its presence in the water systems of several major cities including Wilmington NC, Parkersburg WV, and Dordrecht, Netherlands (Gomis, et al. 2015; Hopkins et al., 2018).

Emerging PFAS in North Carolina

The Chemours Company, a spin-off of DuPont, has a fluoropolymer production facility located just south of Fayetteville, NC, on the Cape Fear River, and has been producing GenX and various other fluorinated chemicals for years (Sun et al., 2016). According to a consent order with NC Department of Environmental Quality (NC DEQ), all wastewater from the facility containing GenX were to be shipped off-site for disposal (NC DEQ, 2017). Yet, in 2015, Dr. Mark Strynar and colleagues of the US EPA identified GenX downstream of the Chemours site, and later Sun et al. (2016) identified concentrations of up to 4,500 ng/L in the Cape Fear River (Strynar et al., 2015; Sun et al., 2016). Water from the Cape Fear River serves the drinking water needs of several populated areas downstream of the fluoromanufacturer, including Wilmington,

NC, and according to Chemours, GenX and other PFECA have been released as wastewater into this water source since the 1980s (NC DEQ, 2018). This raises great public concern regarding the potential health risks of being exposed to these unknown compounds. Sun et al., (2016) identified that multiple PFECA were not filtered out in water treatment plants and thus passed into the drinking water of communities downstream of the Chemours facility. Later, Hopkins et al., (2018) identified multiple PFECA present in finished drinking water, including perfluoro 2-methoxyacetic acid (PFMOAA), which is the shortest chain PEFCA as well as the dominant PFAS identified in the Cape Fear River, with an approximate concentration of 35,000 ng/L in June of 2017.

To our knowledge, no published studies of the toxicity of PFMOAA exist. Several studies exist about the toxicity of GenX, including a study of chronic toxicity that identified no observed adverse effect levels (NOAELs) of 0.1 and 1 mg/kg in male and female Sprague-Dawley rats, respectively, based on liver and kidney effects (Rae et al. 2015). This study identified that male mice receiving 50 mg/kg had increased liver enzymes in circulation, evident of liver injury, as well as hepatomegaly and increased number of adenomas at 500 mg/kg, consistent with other PPAR-α agonists (Rae et al., 2015). A more recent study by Rushing et al. (2017), evaluated immune responses in mice as this is a sensitive endpoint for PFAS, as previously discussed. They also assessed common biomarkers of PFAS exposure, including liver weight and peroxisome proliferation. They identified increases in liver weight at administered doses of 10 mg/kg and higher as well as palmitoyl Co-A oxidase activity increases in males at 10 mg/kg and higher and in females at 100 mg/kg, suggesting PPAR-α activation similar to PFOA (Rushing et al. 2017). At the doses administered in this study, they also noted females given 100 mg/kg had suppressed TDAR. While males did not exhibit immunosuppression, they did have an

increase in splenic CD8+, CD4+/CD8+ and CD4-/CD8- T cell subpopulations of 74%, on average, at 100 mg/kg (Rushing et al., 2017). Gomis et al., (2015) identified that the PFECA alternatives to legacy PFAS had similar chemical properties and environmental fates as their predecessors. Another study by Gomis et al., (2018) used hepatotoxicity and internal dose as a modeling endpoint and demonstrated that the evidence of a "preferred toxicological profile" for these alternatives proposed by chemical manufacturers was negated when the excretion rate was taken out of the picture. According to this study, though GenX is excreted faster and less of it is partitioned to the liver in animal models due to shortened chain length, when compared using internal dose, it is has a greater ability to increase liver weight than, for example, PFOA (Gomis et al., 2018). This suggests that high levels of GenX exposure to those consuming contaminated water in the Cape Fear River area is a public health concern. Currently, the NC Department of Human Health (DHHS) has set a health advisory level of 140 ng/L for GenX, based on protection of the most vulnerable populations, bottle-fed infants (NC DHHS, 2017). Still, while this health advisory level is aimed to be very conservative, very little is known about the environmental fate and toxicity of these emerging contaminants. While these recent studies have shed some light on the toxicity of GenX, several other PFECA, PFMOAA, in particular, have yet to be studied. Therefore, this research project aims to investigate the immunotoxicological effects of PFMOAA in a mouse model and ask whether this newly identified PFAS water contaminant has similar effects to its longer chain counterparts.

CHAPTER 2: 30-DAY IMMUNOTOXICITY STUDY OF PFMOAA IN C57BL/6 MICE

Introduction

Per- and polyfluoroalkyl substances (PFAS) are a large family of amphiphilic compounds composed of alkane tails with fluorine replacing some or all the hydrogens respectively and an active head group (Buck et al., 2011). This family of compounds is estimated to contain over 5,000 different molecules that are widely utilized throughout the world in manufacturing and consumer products for their chemical properties that confer water, oil and stain resistance as well as temperature and friction resistance (US EPA, 2018, 2019; Wang et al., 2017). These chemical properties have made them abundant in water-resistant coatings for textiles and paper products, non-stick coatings on cookware and food packaging, and to formulate firefighting foams as well as use in aerospace, electronics and automotive industries (ATSDR, 2018; FluoroCouncil, 2014; NTP, 2016). Conversely, these same properties of the strong carbon-fluorine bond and amphiphilic capabilities have raised environmental health concerns. The strong carbon-fluorine bonds do not break down in the environment and allow these compounds to be highly mobile in soil and water as well as bioaccumulative (US EPA, 2018; Wang et al., 2017). Due to their environmental persistence, ability to bioaccumulate, and a growing body of toxicological evidence associated with PFAS exposure, the US EPA in cooperation with the 3M Company fostered a phase-out of several precursors and PFAS related products with phase out of U.S. production of PFOS in 2002 and U.S. production of PFOA in 2015 (US EPA, 2015). However, this has led to a rise in the production of new PFAS alternatives, touted as having more favorable environmental and toxicological properties (Bowman, 2015; Gomis et al., 2018; Rushing et al., 2018; Shea, 2018; Wang et al., 2017). This family of alternatives primarily consists of per- and poly-fluorinated ether carboxylic acids (PFECA). One such PFECA of importance is the PFOA

replacement 2,3,3,3-tetrafluoro-2-(heptafluoropropoxy)-propanoate, known by its trade name GenX (Gomis et al., 2018; Hopkins et al., 2018). This PFECA has become a widely studied compound due to its presence in the water systems of several major cities including the Wilmington NC, Parkersburg WV, and Dordrecht Netherlands (Gomis et al., 2015; Hopkins et al., 2018).

The Chemours Company, a spin-off of DuPont, has a fluoropolymer production facility located just south of Fayetteville, NC, on the Cape Fear River, and has been producing GenX and various other fluorinated chemicals for years (Sun et al., 2016). According to a consent order with NC Department of Environmental Quality (NC DEQ), all wastewater from the facility containing GenX were to be shipped off-site for disposal (NC DEQ, 2017). Yet, in 2015, Dr. Mark Strynar and colleagues of the US EPA identified GenX downstream of the Chemours site, and later Sun et al. (2016) identified concentrations of up to 4,500 ng/L in the Cape Fear River (Strynar et al., 2015; Sun et al., 2016). Water from the Cape Fear River serves the drinking water needs of several populated areas downstream of the fluoromanufacturer, including Wilmington, NC, and according to Chemours, GenX and other PFECA have been released as wastewater into this water source since the 1980s (NC DEQ, 2018). This raises great public concern regarding the potential health risks of being exposed to these unknown compounds. Sun et al., (2016) identified that multiple PFECA were not filtered out in water treatment plants and thus passed into the drinking water of communities downstream of the Chemours facility. Later, Hopkins et al., (2018) identified multiple PFECA present in finished drinking water, including perfluoro 2-methoxyacetic acid (PFMOAA), which is the shortest chain PEFCA as well as the dominant PFAS identified in the Cape Fear River, with an approximate concentration of 35,000 ng/L in June of 2017.

To our knowledge, no published studies of the toxicity of PFMOAA exist. Several studies exist about the toxicity of GenX, including a study of chronic toxicity that identified no observed adverse effect levels (NOAELs) of 0.1 and 1 mg/kg in male and female Sprague-Dawley rats, respectively, based on liver and kidney effects (Rae et al. 2015). This study identified that male mice receiving 50 mg/kg had increased liver enzymes in circulation, evident of liver injury, as well as hepatomegaly and increased number of adenomas at 500 mg/kg, consistent with other PPAR-α agonists (Rae et al., 2015). A more recent study by Rushing et al. (2017), evaluated immune responses in mice as this is a sensitive endpoint for PFAS, as previously discussed. They also assessed common biomarkers of PFAS exposure, including liver weight and peroxisome proliferation. They identified increases in liver weight at administered doses of 10 mg/kg and higher as well as palmitoyl Co-A oxidase activity increases in males at 10 mg/kg and higher and in females at 100 mg/kg, suggesting PPAR-α activation similar to PFOA (Rushing et al. 2017). At the doses administered in this study, they also noted females given 100 mg/kg had suppressed TDAR. While males did not exhibit immunosuppression, they did have an increase in splenic CD8+, CD4+/CD8+ and CD4-/CD8- T cell subpopulations of 74%, on average, at 100 mg/kg (Rushing et al., 2017). Gomis et al., (2015) identified that the PFECA alternatives to legacy PFAS had similar chemical properties and environmental fates as their predecessors. Another study by Gomis et al., (2018) used hepatotoxicity and internal dose as a modeling endpoint and demonstrated that the evidence of a "preferred toxicological profile" for these alternatives proposed by chemical manufacturers was negated when the excretion rate was taken out of the picture. According to this study, though GenX is excreted faster and less of it is partitioned to the liver in animal models due to shortened chain length, when compared using

internal dose, it is has a greater ability to increase liver weight than, for example, PFOA (Gomis et al., 2018). This suggests that high levels of GenX exposure to those consuming contaminated water in the Cape Fear River area is a public health concern. Currently, the NC DHHS has set a health advisory level of 140 ng/L for GenX, based on protection of the most vulnerable populations, bottle-fed infants (NC DHHS, 2017). Still, while this health advisory level is aimed to be very conservative, very little is known about the environmental fate and toxicity of these emerging contaminants. While these recent studies have shed some light on the toxicity of GenX, several other PFECA, PFMOAA, in particular, have yet to be studied. Therefore, this research project aimed to investigate the immunotoxicological effects of PFMOAA in a mouse model and ask whether this newly identified PFAS water contaminant has similar effects to its longer chain counterparts.

Materials and Methods

Animals

All experimental animal handling and dosing was carried out in accordance with procedures approved by the East Carolina University's Institutional Animal Care and Use Committee (IACUC). Male (27) and female (27) C57BL/6 mice, 6-8 weeks old, were purchased from Charles River Laboratories and after a weeklong acclimation period, were weight matched within cage and then assigned to groups of 3/cage. Animals were divided into 6 animals per dose group except for the 7.5 mg/kg PFOA positive control group that consisted of 3 animals/sex. Animals were housed with access for food and water *ad libitum*, and kept at 73°F with a 12:12 h light-dark cycle.

Dosing and Immunizations

Dosing solutions of PFMOAA (graciously gifted by Dr. Ralph Mead from the University of North Carolina at Wilmington) were prepared fresh weekly in sterile water with 0.5% Tween at the following concentrations: 25,000 ng/L, 2,500,000 ng/L 250,000,000 ng/L. Based on our previous studies, dosing solutions were administered at 0.1 ml/10g of body weight, resulting in the following mg of PFMOAA/kg of mouse body weight: 0 mg/kg, 0.00025 mg/kg, 0.025 mg/kg, 2.5 mg/kg, as well as 7.5 mg/kg of PFOA as a positive control. Dosing was performed via oral gavage, daily, for 30 consecutive days based on individual daily body weights. These dosing concentrations were based on published reports of environmental concentrations (Hopkins et al., 2018) as well as previous 15-day (DeWitt et al. 2008, 2016) and 28-day exposure studies (Rushing et al. 2017). On the 25th day after the initial dose, all mice in all dose groups were immunized with sheep red blood cells (SRBCs) via tail vein injections. SRBCs were adjusted to 4×10^7 cells in 0.2 ml of sterile saline

Organ and Body Weights

Body weights were recorded daily and evaluated for change over the course of the study. Immediately following euthanasia, spleen, thymus, brain, liver, heart, and kidneys were removed and weights were recorded. All organs except for the spleen and thymus were frozen at -80°C for later analyses. Fresh spleen and thymus were placed in 6-well plates filled with supplemented RPMI 1640 medium (supplemented with 5% fetal bovine serum) and kept on ice to be prepared for immunophenotyping.

Urine Samples

Urine samples were collected after 24 hours, after five days, and after 15 days of dosing. Pooled urine was collected from three animals per cage by placing animals in a clean, empty

cage and collecting freely deposited urine. Collected urine was frozen at -80°C for future analyses of urine PFMOAA concentrations.

Serum Collection

Following anesthesia with Isoflurane, blood was collected by neck vein transection into collection tubes with a clot activator, allowed to sit for at least 30 minutes, then centrifuged at 2000 x g for 15 minutes at 4°C. Serum that separated from the clot was then saved and frozen at -80°C for future analyses.

Liver Peroxisome Proliferation

Liver peroxisome proliferation was measured indirectly by palmitoyl CoA oxidase activity in liver homogenates, utilizing the H_2O_2 dependent oxidation of leuco-DCF to DCF. Palmitoyl CoA was used as substrate and aminotriazole was added to inhibit interference by catalase.

100 mg of frozen liver per mouse was homogenized in 1 mL of buffer containing 0.25mM sucrose, 5mM EDTA, and 20mM Tris-HCl. Homogenates were spun at 12,000 *x g* at 4°C for 20 minutes and the supernatant was collected. Protein concentration of supernatants was determined by the Bradford protein assay and palmitoyl Co-A oxidase activity was measured via florescence at one-minute intervals for 10 minutes. Data are expressed as nmoles/min/mg of liver.

Immunophenotyping

Individual spleen and thymus were aseptically processed into single-cell suspensions by gentle grinding and passage through a 70 μm nylon filter, following by the addition of 7 mL of supplemented RPMI medium. Red blood cells were lysed, and an aliquot of each organ suspension was counted on a Nexcelom Bioscience Cellometer Auto 2000 cell counter

(Nexcelom Bioscience LLC) to determine cell counts and viability. All samples were adjusted to $2x10^7$ cells/mL. Optimal concentrations of flow antibodies, reagents, and isotype controls to estimate non-specific binding were determined in previous experiments (DeWitt et al., 2016). All experimental replicates also included unstained cells as negative controls and single-color controls as positive controls to determine color compensation. Monoclonal antibodies (ThermoFisher Scientific) coupled to fluorochromes specific for the following markers were used: APC anti-mouse CD3e, FITC anti-mouse CD4, and PE anti-mouse CD8a for both spleen and thymus samples and FITC anti-mouse CD45RB and PE anti-mouse NK1.1 for spleen samples only. Flow cytometric analysis was performed using an BD LSRII flow cytometer (BD Biosciences) and 10,000 events were collected from each sample. Dead cells and debris were excluded from analysis by using forward scatter and 90° light scatter to establish a gate around viable lymphocyte populations. Non-stained cells, isotype controls, and fluorescence minus one (FMO) controls were used to distinguish the negative populations from positive populations for B cells (spleen only) and T cells (spleen and thymus). Cells were gated based on CD3 expression for the subsequent analysis of CD4/CD8 T-subpopulations, but not B cell subpopulations. The total number of each cell type was determined from spleen and/or thymus cellularity using Microsoft Excel.

Statistical Analysis

All weight data and flow cytometry data are presented as mean ± standard deviation. All data were analyzed using SAS (SAS Institute), with a one-way analysis of variance (ANOVA) to determine dose differences in body and organ weights and immunophenotype. When ANOVA indicated a statically significant dose effect, individual Tukey's post-hoc t-tests were performed. Statistical significance was determined using a P value of 0.05.

Results

Animal Dosing Period Weights

Weights of animals dosed with PFMOAA did not differ statistically during the dosing period (Figure 1). Fluctuations in body weights of male animals on days 1-9 of dosing likely reflect the inclusion of personnel newly trained to gavaging and were likely not PFMOAA- or PFOA-related. One male animal in the PFOA group died due to a gavage-related injury on the fourth day of dosing. One female animal receiving 2.5 mg/kg PFMOAA was euthanized on day 25 after it was determined, via veterinary consultation, that she had reached a humane endpoint; she had urethral/vaginal secretions and upon necropsy, it was determined that one of her kidneys was infected. One female animal in the 2.5 mg/kg dose group also had consistently lower body weight throughout the dosing period as she had a malocclusion. At the time of euthanasia, four male mice had signs of preputial infections, including one from 0.025 mg/kg and three from the 2.5 mg/kg dose groups. All other animals remained bright, alert, and responsive throughout the dosing period.

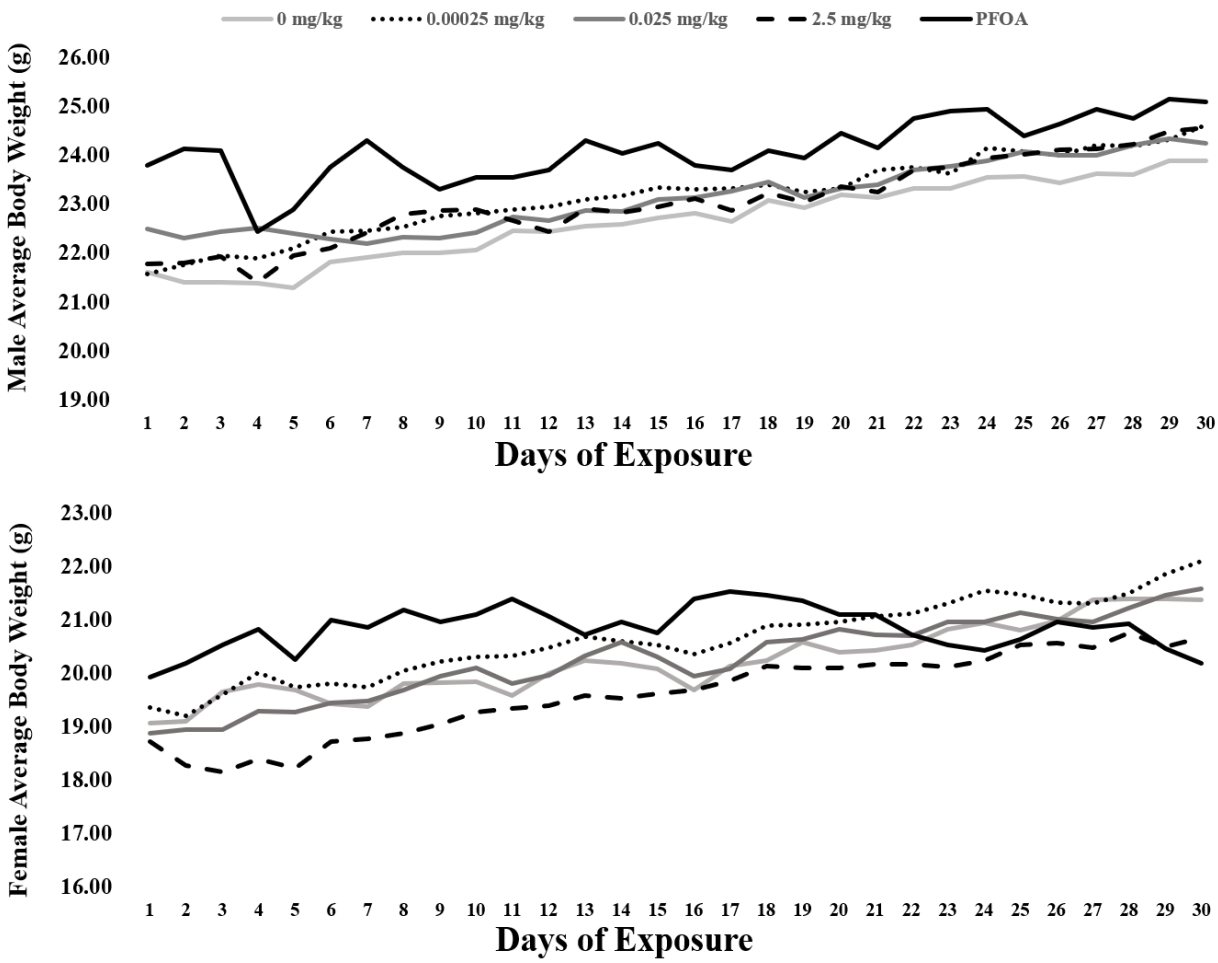


Figure 1, Average daily body weights for male (A) and female (B) C57BL/6 mice exposed to PFMOAA for 30 days (standard deviations not shown). Average body weights within sex did not differ statistically by dose throughout the dosing period.

Organ Weights

Terminal body weights and organ weights corrected by body weight are listed in Table 2. No statistical changes were observed in terminal body weights or relative organ weights of animals dosed with PFMOAA. Relative liver weights of male and female mice given 7.5 mg/kg of PFOA were increased by 135.2% and 211.7%, respectively, relative to the 0 mg/kg group. Female mice given PFOA also had a 16.7% decrease in liver-free body weight as well as a 24.2% and 37.2% decrease in relative spleen and thymus weights, respectively, relative to the 0 mg/kg group.

Table 2. Average body weights and relative organ weights (somatic indices; SI) ± standard deviation for male and female C57BL/6 mice exposed to PFMOAA for 30 days.

Male	**0 mg/kg**	**0.00025 mg/kg**	**0.025 mg/kg**	**2.5 mg/kg**	**7.5 mg/kg PFOA**
Terminal body weight (g)	23.73±1.27	24.42±2.46	24.45±1.50	24.30±1.36	24.45±1.91
Liver free body weight (g)	22.54±1.20	23.22±2.30	23.16±1.40	23.03±1.23	21.52±0.73
Liver SI	50.39±1.2	48.76±2.9	52.64±1.9	52.19±4.2	118.52±38.9
Brain SI	16.97±1.03	16.77±1.14	16.42±0.91	16.47±0.61	17.26±1.17
Heart SI	4.85±0.43	4.79±0.45	4.96±0.60	5.15±0.53	4.68±0.50
Kidney SI	12.22±0.40	11.92±0.67	12.36±0.60	12.60±0.67	12.91±0.72
Spleen SI	3.36±0.33	3.45±1.18	3.82±0.75	3.91±1.24	3.30±0.72
Thymus SI	2.29±0.39	2.00±0.26	2.19±0.34	2.49±0.44	2.05±0.27
Female	**0 mg/kg**	**0.00025 mg/kg**	**0.025 mg/kg**	**2.5 mg/kg**	**7.5 mg/kg PFOA**
Terminal body weight (g)	21.20±1.06	21.87±1.07	21.45±0.92	20.66±2.12	19.67±1.36
Liver free body weight (g)	20.23±0.98	20.81±1.00	20.41±0.85	19.65±1.94	16.86 ±1.00*
Liver SI	45.66±2.7	48.43±3.0	48.33±1.5	48.72±4.2	142.33±8.4
Brain SI	19.48±1.29	19.23±0.74	19.09±0.82	20.28±1.69	20.38±1.18
Heart SI	4.81±0.63	5.50±0.41	5.29±0.45	5.66±0.80	5.44±0.47
Kidney SI	11.95±0.92	12.36±0.77	11.80±0.72	12.35±0.61	11.97±2.24
Spleen SI	4.35±0.41	4.79±0.89	4.88±0.27	4.51±0.43	3.30±0.15 *
Thymus SI	3.48±0.44	3.30±0.54	3.14±0.36	3.41±0.79	2.18±0.79 *

*Indicates a statistical difference from the 0 mg/kg group ($p < 0.05$).

Peroxisome Proliferation

Peroxisome proliferation as indicated by increased palmitoyl-CoA oxidase activity is illustrated in Figure 2. Peroxisomal activity in male animals given PFMOAA did not appear to

be increased relative to the 0 mg/kg animals. Female mice given 0.025 mg/kg and 2.5 mg/kg of PFMOAA had a 30% and 43% increase in peroxisome enzyme activity, respectively. Male animals receiving PFOA had a 98% increase in enzyme activity and females had a 227% increase relative to the same-sex 0 mg/kg groups.

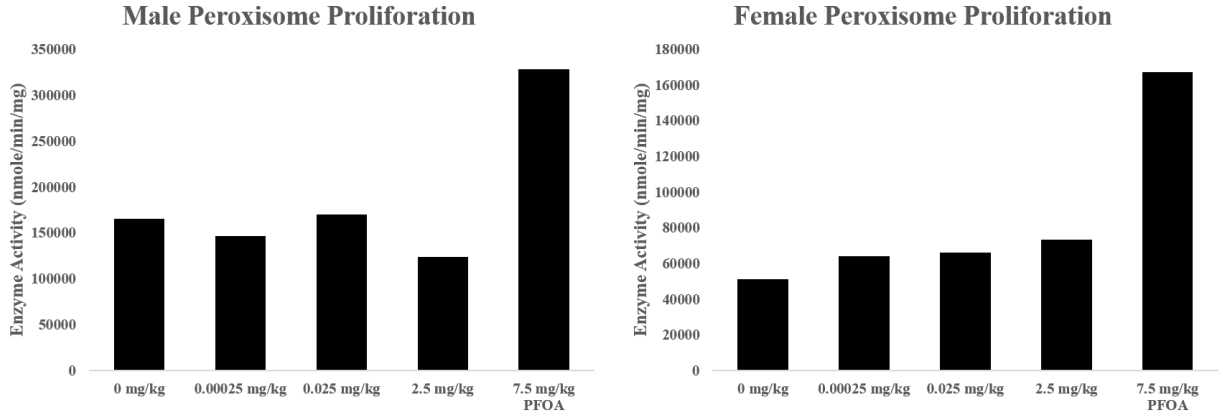


Figure 2 Average peroxisomal palmitoyl-CoA oxidase activity in nmoles/min/mg of liver for male and female C57BL/6 mice exposed to PFMOAA or PFOA for 30 days.

Immunophenotyping

Upon collection and evaluation of thymus samples by flow cytometry, it was determined that samples from female mice were inconsistently stained, thus the data were not included here. Male animals given 2.5 mg/kg had a statistical increase in splenic CD4+/CD8+ T cells and NK cells, relative to the 0 mg/kg group; CD4+/CD8+ T cells were increased by 186% and NK cells by 83%. Female animals given PFMOAA had no statistical changes in splenic lymphocytes. Female animals given PFOA had 64% fewer NK cells relative to the 0 mg/kg group. Thymus samples from male animals given PFMOAA had several changes relative to the 0 mg/kg group. T cells in the 0.00025 mg/kg and 0.025 mg/kg dose groups were increased by 59% and 70.5% for CD4+ and 57.6% and 60.2% for CD8+, respectively. No statistical changes for splenic or thymic CD4-/8- T cells were observed. Although it was not statistically significant, total cellularity of spleens from male animals was increased above the vehicle control by 34% and

82%, on average, for the 0.025 mg/kg and 2.5 mg/kg doses of PFMOAA. This change in splenic cellularity also was associated with the changes observed in specific splenic T cell subpopulations for male animals. This pattern was not repeated in the thymuses from male animals, but female animals given 7.5 mg/kg of PFOA had a 61% decrease in spleen cellularity (p=0.045), which correlates with the changes in splenic T and B cell subpopulations observed for female animals.

Table 3 Average splenic and thymic lymphocyte subpopulations. Values are group averages ± standard deviations for the percent of the population of each subpopulation from 10,000 events adjusted by total organ cellularity.

Male Spleen	**n=**	**Organ total cellularity (e^7)**	**CD4+ (e^6)**	**CD8+(e^6)**	**CD4+/CD8+ (e^4)**	**CD4-/CD8- (e^5)**	**B cell (e^7)**	**NK cell (e^6)**
0.0 mg/kg	**6**	3.29±1.3	3.91±1.5	6.46±3.1	1.3±0.8	6.41±2.3	1.48±0.7	1.01±0.4
0.00025 mg/kg	**5,6**	2.83±1.2	3.79±1.4	5.49±2.7	1.59±1.1	6.62±1.9	1.22±0.7	1.11±0.4
0.025 mg/kg	**6**	4.42±2.2	4.95±2.5	8.48±5.1	1.02±0.3	8.50±3.9	2.33±1.3	1.22±0.4
2.5 mg/kg	**4,6**	6.01±1.9	6.23±1.2	12.6±3.5	3.73±2.3*	12.1±4.2	2.93±0.7	1.85±0.6 *
7.5 PFOA	**2**	5.81±0.001	5.79±0.3	10.6±1.4	3.07±1.6	9.5±0.4	2.23±0.1	1.66±0.2
Female Spleen	**n=**	**Organ total cellularity (e^7)**	**CD4+ (e^6)**	**CD8+(e^6)**	**CD4+/CD8+ (e^4)**	**CD4-/CD8- (e^5)**	**B cell (e^7)**	**NK cell (e^6)**
0.0 mg/kg	**2**	4.51±0.3	5.61±0.5	9.76±0.7	2.69±2.4	8.21±1.1	2.24±0.02	1.37±0.07
0.00025 mg/kg	**6**	4.83±1.9	4.94±1.9	8.71±4.2	2.72±1.3	9.13±3.1	2.17±1.0	1.35±0.4
0.025 mg/kg	**6**	3.21±0.5	3.82±0.7	4.95±0.9	2.09±1.7	6.64±0.8	1.26±0.2	1.01±0.2
2.5 mg/kg	**5**	4.16±1.7	4.92±1.8	8.15±3.5	2.78±0.9	7.73±2.2	1.86±0.9	0.89±0.1
7.5 PFOA	**3**	1.75±0.3 ɵ	2.29±0.3	2.95±0.4	1.57±0.3	3.06±0.4	0.7±0.2	0.48±0.1ɵ
Male Thymus	**n=**	**Organ total cellularity (e^7)**	**CD4+ (e^6)**	**CD8+ (e^6)**	**CD4+/CD8+ (e^6)**	**CD4-/CD8- (e^5)**		
0.0 mg/kg	**6**	6.35±1.4	0.68± 0.2	2.69±0.4	2.27±0.6	3.25±0.9		
0.00025 mg/kg	**6**	7.66±1.3	1.08±0.3*	4.24±1.0*	3.03±0.7	4.80±1.4		
0.025 mg/kg	**6**	7.73±2.1	1.16±0.3*	4.31±0.9*	3.23±1.0	4.42±1.7		
2.5 mg/kg	**6**	7.47±1.2	0.80±0.2	3.35±1.0	2.81±0.5	4.15±1.0		
7.5 PFOA	**2**	5.95±0.7	0.64±0.3	2.24±1.2	2.04±0.3	2.06±0.2		

* indicates statistically significant increase from 0 mg/kg control ($p \leq 0.05$) ɵ indicates statistically significant decrease from 0 mg/kg control ($p \leq 0.05$).

Discussion

In this study, we evaluated immune and liver endpoints known to be sensitive to PFAS exposure to determine if the emerging contaminant, PFMOAA, could also induce similar changes. These endpoints included liver weight, peroxisome proliferation, lymphoid organ weights, and lymphocyte subpopulations from the spleen and thymus. Throughout the 30-day dosing period, no signs of overt toxicity were observed in mice dosed with PFMOAA and all animals gained weight equally across dose groups. Female animals given PFOA began to show signs of overt toxicity, as indicated by a decrease in body weight, during the last 10 days of the study. In addition, upon euthanasia, these female animals also demonstrated atrophy of lymphoid organs and hepatomegaly. Male animals given PFOA also had increases in liver weight and palmitoyl-CoA oxidase activity but did not lose body weight. Although previous studies by our lab (DeWitt et al. 2008, 2016) demonstrated that 7.5 mg/kg of PFOA was immunotoxic but not overtly toxic, these previous studies had a duration of 15 and not 30 days. It is of interest to note that one male in the 0.025 mg/kg group and three males in the 2.5 mg/kg group had developed preputial infections; it is unclear whether this could be due to immunosuppression.

Unlike animals given PFOA, mice given PFMOAA did not demonstrate hepatomegaly or changes in lymphoid organ weights. Spleens from male animals had a slight increase in all cell subpopulations with an increased dose of PFMOAA and CD4+/CD8+ T cells and NK cells were statistically increased at 2.5 mg/kg. We interpret this increase in splenic lymphocyte subpopulations as associated with increases in spleen total cellularity (Table 3). Thymic lymphocytes from male animals had statistical increases in helper and cytotoxic T-cell populations at the two lower doses of PFMOAA. While these changes were statistically

significant, variability within groups make these data challenging to interpret. The increase in spleen cellularity and lymphocyte subpopulations could also have been affected by the preputial infections observed in some of the male animals. However, male animals with the infection did not have statistical elevations in spleen cellularity compared to animals in the same group that did not have a preputial abscesses. Previous studies with PFOA, for example, have shown changes in TDAR without changes in lymphocyte subpopulations (Dewitt et al., 2008, 2016; Peden-Adams et al., 2008). These changes in lymphocyte subpopulations may therefore not necessarily be indicative of changes in immune function. We plan to evaluate the TDAR in serum from these animals at a later date.

The other endpoints we investigated were based on the ability of PFAS to activate peroxisome proliferation and increase liver weight. In previous studies, PFOA and PFOS have both been shown to induce peroxisome proliferation and hepatomegaly in rodents (Yang et al. 2002, DeWitt et al. 2016). PFAS have been shown to act as ligands for PPARs, particularly PPAR-α, in part due to the similarity of PFOA and PFOS to long chain fatty acids, endogenous ligands for PPARs (Li et al., 2017; Loveless et al., 2008; Peng et al., 2013). Thus we aimed to determine if PFMOAA, a very short chain PFAS, also leads to peroxisome proliferation. At the doses of PFMOAA administered, increases in liver weight or peroxisome proliferation were not observed. We did observe a 43% increase in palmitoyl-CoA oxidase in females given 2.5 mg/kg, which may indicate some degree of peroxisome proliferation. Male animals had a slight decrease in enzyme activity at the highest dose of PFMOAA. These changes are likely negligible due to variability in the assay and the putative short half-life of PFMOAA.

One PFAS that shares structural similarity to PFMOAA is PFBA, a four-carbon PFAS that lacks the ether group contained by PFMOAA. Biomonitoring studies of 3M employees for

PFBA have identified that it has a half-life of roughly two to four days in humans (Change et al., 2008). While this study was limited by sample size (n=12) and the lack of exposure route data, it still identifies the relationship between shorter chain lengths and shorter half-lives (Chang et al., 2008). The Chang et al., (2008) study also identified that in male CD1 mice, the half-life of PFBA is approximately 12-16 hours when they were given a single dose of 10-30 mg/kg of PFBA. The excretion rate was much faster in female CD1 mice; they eliminated PFBA in approximately 2-3 hours at the three administered doses of 10, 30 and 100 mg/kg (Chang et al. 2008). A 2009 study by Foreman et al. investigated the ability of PFBA to activate PPARs. In this study, wild type (WT) mice transfected with human PPAR-α as well as PPAR-α knockout mice were administered PFBA at 35, 175 and 350 mg/kg for 28-days via oral gavage. This study identified a dose-dependent increase in relative liver weight for PFBA in both WT and human PPAR-α mice but not PPAR-α knock out mice and that PFBA induced PPAR-α transcription in both WT and humanized PPAR-α suggesting lipid modulating activity (Foreman et al., 2009). While these effects were both lower in the humanized PPAR mice than in WT, it still suggests that PFBA can activate PPAR-αrelated metabolic changes in humans. These results also suggest that even the shorter chain PFAS can lead to changes in the liver similar to PFOA and other long chain PFAS. These data suggest that short-chain compounds such as PFMOAA have toxicological potential at certain doses. However, due to the rapid excretion of PFBA in mice, this study utilized doses 10-100-fold higher than the doses we used in our study, suggesting higher doses are needed to achieve a relevant internal dose of PFMOAA. It is also important to note that in a 2018 study of PFAS in the Cape Fear River, approximately 20 ng/L of PFBA was measured; PFMOAA was identified as high as 35,000 ng/L (Geosyntec 2018, Hopkins et al 2018). This is evidence to support public health concerns for PFMOAA as even with a low

bioaccumulation potential in humans, high, chronic environmental doses could still lead to adverse health outcomes.

Conclusion

At the doses we administered, while being based on environmentally relevant levels (Hopkins et al. 2016), likely resulted in extremely low internal doses in our rodent model. Mice have been shown to excrete PFAS much more rapidly than humans and with excretion rates being inversely correlated with chain length, we suspect that the administered doses of PFMOAA led to an internal dose insufficient to induce changes in the endpoints we evaluated (Shea 2018, Gomis et al. 2015, 2018). No human data for PFMOAA levels have been published, but the high levels of exposure received by Cape Fear River communities are cause for further study into the toxicity of PFMOAA and other emerging PFAS.

CHAPTER 3: NK CELL CYTOTOXICITY ASSAY

Background

While the TDAR has long been the gold standard in immunotoxicity testing, it is a measure of the humoral adaptive immune response; this leaves out the potential for effects of exogenous agents on innate immunity. The natural killer (NK) cell is a mammalian lymphocyte-related to T and B cells but acts as a part of the innate immune system. NK cells circulate within the body and respond to numerous pathological changes. They can identify tumor cells as well as virally infected cells without the need for priming, thus being dubbed the "natural" killing cells (Abel et al., 2018). Whereas normal healthy cells express major histocompatibility complex 1 (MHC-1) which the body uses to identify self and non-self, virally infected and cancer cells often lose their MHC-1 markers, which NK cells identify (Abel et al., 2018). Once an NK cell detects a target, it releases granules containing cytokines such as tumor necrosing factor α (TNFα) and interferons (IFN), which signal the target cell to go into apoptosis as well as signaling macrophages and other immune cells to the site of infection (Abel et al., 2018). This unique natural targeting ability made them a great marker for innate immune function, as the cells can be easily harvested from an animal model and incubated with a target that leads to NK cell activation. For many years, the method for testing NK cell cytotoxicity utilized the radioactive isotope ^{51} chromium (^{51}Cr), which was taken up by target cells. When co-incubated with NK cells (effector cells), the amount of radiation released was used to calculate the number of target cells that had been killed by the NK cells (Cholujová et al., 2008). This ^{51}Cr release assay was the gold standard for testing the innate immune response but was challenging to run due to the short shelf-life of ^{51}Cr and the specialized training and equipment needed for working with radiation (Cholujová et al., 2008; Vizler et al., 2002). Due to these challenges, many scientists

have moved to using flow cytometric based assays for testing NK cell cytotoxicity. Herein we present our novel method for flow cytometric identification of NK cell cytotoxicity.

Similar to previous ^{51}Cr release assays, we used the mouse lymphoma YAC-1 cell line for our target cell as it is readily targeted by NK cells. Several fluorophores could be used, including Calcein AM, Carboxyfluorescein succinimidyl ester, or vybrant DiO; we chose to use MitoTracker Green (MTG) for labeling due to cost and efficiency. MTG is taken up by the target cells and locates to mitochondria, but when the membrane potential is lost in dead cells, it is released, making it an optimal live cell stain.

Methods

YAC-1 mouse-derived lymphomas obtained from Sigma-Aldrich (86022801) were utilized as our target cell. They were grown in RPMI 1640 culture media containing 10% fetal bovine serum (FBS), 1% L-Glutamine, and 1% Pen-Strep antibiotic. These cells grow rapidly and cell lines were started approximately five days before use to allow one to two cell passages to occur. All experiments were done on the same day of animal euthanasia and organ collection to ensure fresh spleen effector cells.

Target Cell Preparation

On the day of organ collection, YAC-1 cells were counted and diluted to a concentration of $1x10^7$ cells in two separate tubes. One tube spun and resuspended in 40 mL of RPMI containing 10% FBS and 12 µL of MTG and then incubated at 37°C for 20 minutes. The other tube was set aside to be used as an unstained control. After incubation, cells were spun, washed twice, and resuspended in RPMI containing only 10% FBS and set aside until spleen effector cells were ready.

Effector Cell Preparation

Individual spleens were aseptically processed into single-cell suspensions by gentle grinding and passage through a 70 μm nylon filter, followed by the addition of 7 mL of supplemented (+10% FBS) RPMI medium. Red blood cells were lysed, and an aliquot of each organ suspension was counted on a Nexcelom Bioscience Cellometer Auto 2000 cell counter (Nexcelom Bioscience LLC) to determine cell counts and viability. All samples were adjusted to $2x10^7$ cells/mL. After splenic effector cells were adjusted to $2x10^7$ cells/mL, they were added to three Eppendorf tubes per animal in the following amounts: 150 μL, 50 μL, and 25 μL. These tubes were spun at 300 *x g* and resuspended in 0.5 mL of RPMI+FBS media.

Co-Incubation and Reading of Results

Based on a previous study by Dr. Csava Vizler (2002) and personal contact in 2018, we utilized three effector to target cell ratios (E/T) of 5/1, 10/1 and 30/1. We added 0.5 mL of the stained YAC-1 cells to each Eppendorf tube and left them to incubate for three hours at 37°C. At this time, we also prepared two tubes containing just 1 mL of stained YAC-1 cells to serve as a spontaneous release sample and a total cell death control sample. Nothing was added to the spontaneous release control whereas 5 μL of 5% Triton X-100 was added to the total cell lysis control to lyse all of the cells. After incubation,15 μL of propidium iodide (PI) was added to all tubes to stain dead cells. Cells were counted on a BD LSRII flow cytometer (BD Biosciences) and 10,000 events were collected from each sample. Unstained control was read first to set the proper reading frame for singlet cells and samples were read on PE channels for PI and FITC-A channels for MTG. Gates were drawn and calculated around populations of MTG containing cells and dead cells without MTG. Data were presented as percent specific lysis using the formula PSL = ((% cell death of sample - %spontaneous cell death) / (% maximum cell death - %spontaneous cell death)*100).

Representative data from a test of this NK cell cytotoxicity assay are presented in Figure 3; these results are from a 30-day immunotoxicity study of 2,2,3,3-(trifluoromethoxy)propionic acid (PFMOPrA) on mice.

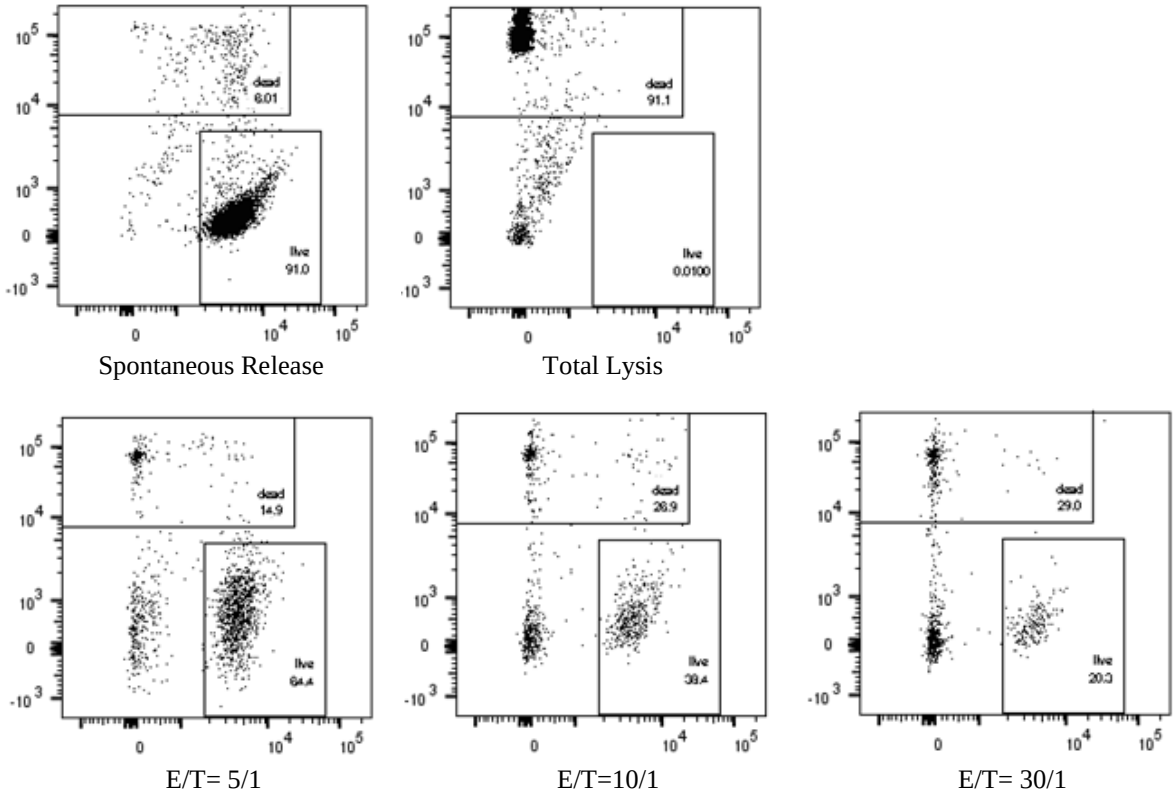


Figure 3. Representative sample of flow cytometry data from an NK cell cytotoxicity assay using MitoTracker Green and Propidium Iodide dyes. Includes spontaneous release and total cell lysis controls as well as samples from a vehicle control with 5/1 10/1 and 30/1 E/T ratios.

Conclusion

While studies have shown that the newer flow cytometric methods are less accurate than the gold standard of the ^{51}Cr release assay (Cholujová et al., 2008), the method presented here has proven to be rapid, cost-effective and simple for the battery of immunotoxicology testing performed in our lab as well as a reliable indicator of innate immune system function.

CHAPTER 4 CONCLUSIONS AND FUTURE DIRECTIONS

Emerging PFECA and other PFAS alternatives have become a prevalent subject of scientific research and public concern and though manufacturers have touted them as having more favorable environmental and toxicological properties their high concentrations in water sources around the world require us to question the safety of these contaminants. One such affected area is the Cape Fear River downstream of the Fayetteville Works Chemours facility, which has been releasing PFAS and related by-products into the river and air for more than a decade (NC DEQ, 2017; Sun et al., 2016; Wang et al., 2017). The detection of numerous PFAS compounds in the public water systems of over 200,000 residents downstream led to public attention and rising concern of those residents. In 2018 the NC General Assembly set aside $5,000,000 to fund a NC Policy Collaboratory project named the PFAS Testing Network (PFAST Network) (NC Policy Collaboratory, 2019). The PFAST Network is a state-wide research collaboration representing expertise from East Carolina University (ECU), University of North Carolina at Chapel Hill, Charlotte, and Wilmington, North Carolina State University, Duke University, and NC A&T University to examine levels of PFAS in air and drinking water and to answer other questions associated withPFAS. As part of this PFAST Network, our lab at ECU is working on building toxicological profiles of emerging PFAS contaminants identified in the Cape Fear River. We began with the dominant PFAS, PFMOAA. To our knowledge, we are the first to investigate the toxicity of PFMOAA and therefore, this study aimed to build a toxicological profile of PFMOAA and determine if it leads to similar toxic effects as legacy PFAS.

What We Found

In this study, we evaluated immune and liver endpoints known to be sensitive to PFAS exposure to determine if the emerging contaminant, PFMOAA, could also induce similar changes. These endpoints included liver weight, peroxisome proliferation, lymphoid organ weights, and lymphocyte subpopulations. Throughout the 30-day dosing period, no signs of overt toxicity were observed in mice dosed with PFMOAA and all animals gained weight equally across dose groups. Female animals given PFOA began to show signs of overt toxicity, as indicated by a decrease in body weight, during the last ten days of the study. In addition, upon euthanasia, these female animals also demonstrated atrophy of lymphoid organs and hepatomegaly. Males given PFOA also had increases in liver weight and palmitoyl-CoA oxidase activity but did not lose body weight. Although previous studies by our lab (DeWitt et al. 2008, 2016) demonstrated that 7.5 mg/kg of PFOA was immunotoxic but not overtly toxic, these previous studies had a duration of 15 and not 30 days. It is of interest to note that one male in the 0.025 mg/kg group and three males in the 2.5 mg/kg group had developed preputial infections. Whether this could be due to immunosuppression in the mice is unknown.

Unlike animals given PFOA, mice given PFMOAA did not demonstrate hepatomegaly or changes in lymphoid organ weights. Spleens from male animals had a slight increase in all cell subpopulations with an increased dose of PFMOAA and CD4+/CD8+ T cells and NK cells were statistically increased at 2.5 mg/kg. We interpreted this increase in splenic lymphocyte subpopulations as associated with increases in spleen total cellularity (Table 3). Thymic lymphocytes from male animals had statistical increases in helper and cytotoxic T-cell populations at the two lower doses of PFMOAA. While these changes were statistically significant, there was still relatively high variability within groups, which make the data challenging to interpret. The increase in spleen cellularity and lymphocyte subpopulations may

also have been affected by the preputial infections found in the higher dosed mice but this was likely not the only contributing factor. Animals with the infection did not have statistically higher spleen cellularity compared to animals in the same group that did not have preputial abscesses. Previous studies with PFOA, for example, have shown changes in TDAR without changes in lymphocyte subpopulations (DeWitt et al., 2008, 2016; Peden-Adams et al., 2008). These changes in lymphocyte subpopulations may therefore not necessarily be indicative of changes in immune function.

The other endpoints we investigated was based on the ability of PFAS to activate peroxisome proliferation and increase liver weight. In previous studies, PFOA and PFOS have both been shown to induce peroxisome proliferation and hepatomegaly in rodents (Yang et al. 2002, DeWitt et al. 2016). PFAS have been shown to act as ligands for PPARs, particularly PPAR-α. The other endpoints we investigated were based on the ability of PFAS to activate peroxisome proliferation and increase liver weight. In previous studies, PFOA and PFOS have both been shown to induce peroxisome proliferation and hepatomegaly in rodents (Yang et al. 2002, DeWitt et al. 2016). PFAS have been shown to act as ligands for PPARs, particularly PPAR-α, in part due to the similarity of PFOA and PFOS to long chain fatty acids, endogenous ligands for PPARs (Li et al., 2017; Loveless et al., 2008; Peng et al., 2013). Thus we aimed to determine if PFMOAA, a very short chain PFAS, also leads to peroxisome proliferation. At the doses of PFMOAA administered, increases in liver weight or peroxisome proliferation were not observed. We did observe a 43% increase in palmitoyl-CoA oxidase in females given 2.5 mg/kg, which may indicate some degree of peroxisome proliferation. Male animals had a slight decrease in enzyme activity at the highest dose of PFMOAA. These changes are likely negligible due to variability in the assay and the putative short half-life of PFMOAA.

Toxicity of Related Compounds

Although there are no studies on PFMOAA at present, we can evaluate studies of related PFAS to predict toxicological effects of PFMOAA. One such compound is the PFOA replacement, 2,3,3,3-tetrafluoro-2-(heptafluoropropoxy)-propanoate, known by its trade name GenX. GenX, while a much larger PFAS than PFMOAA, has a branched chain and ether group, suggesting similarly rapid excretion and has already shown to have significant toxic effects in animal models. (Gomis et al., 2018) One such study of chronic toxicity that identified no observed adverse effect levels (NOAELs) of 0.1 and 1 mg/kg in male and female Sprague-Dawley rats, respectively, based on liver and kidney effects (Rae et al. 2015). This study identified that male mice receiving 50 mg/kg had increased liver enzymes in circulation, evident of liver injury, as well as hepatomegaly and increased number of adenomas at 500 mg/kg, consistent with other PPAR-α agonists (Rae et al., 2015). A more recent study by Rushing et al. (2017), evaluated immune responses in mice as this is a sensitive endpoint for PFAS, as previously discussed. They also assessed common biomarkers of PFAS exposure, including liver weight and peroxisome proliferation. They identified increases in liver weight at administered doses of 10 mg/kg and higher as well as palmitoyl Co-A oxidase activity increases in males at 10 mg/kg and higher and in females at 100 mg/kg, suggesting PPAR-α activation similar to PFOA (Rushing et al. 2017). At the doses administered in this study, they also noted females given 100 mg/kg had suppressed TDAR. While males did not exhibit immunosuppression, they did have an increase in spleenic CD8+, CD4+/CD8+ and CD4-/CD8- T cell subpopulations of 74%, on average, at 100 mg/kg (Rushing et al., 2017). Gomis et al., (2015) identified that the PFECA alternatives to legacy PFAS had similar chemical properties and environmental fates as their

predecessors. Another study by Gomis et al., (2018) used hepatotoxicity and internal dose as a modeling endpoint and demonstrated that the evidence of a "preferred toxicological profile" for these alternatives proposed by chemical manufacturers was negated when the excretion rate was taken out of the picture. According to this study, though GenX is excreted faster and less of it is partitioned to the liver in animal models due to shortened chain length, when compared using internal dose, it is has a greater ability to increase liver weight than, for example, PFOA (Gomis et al., 2018).

One PFAS that shares structural similarity to PFMOAA is PFBA, a four-carbon PFAS that lacks the ether group contained by PFMOAA. Biomonitoring studies of 3M employees for PFBA have identified that it has a half-life of roughly two to four days in humans (Change et al., 2008). While this study was limited by sample size (n=12) and the lack of exposure route data, it still identifies the relationship between shorter chain lengths and shorter half-lives (Chang et al., 2008). The Chang et al., (2008) study also identified that in male CD1 mice, the half-life of PFBA is approximately 12-16 hours when they were given a single dose of 10-30 mg/kg of PFBA. The excretion rate was much faster in female CD1 mice; they eliminated PFBA in approximately 2-3 hours at the three administered doses of 10, 30 and 100 mg/kg (Chang et al. 2008). A 2009 study by Foreman et al. investigated the ability of PFBA to activate PPARs. In this study, wild type (WT) mice transfected with human PPAR-α as well as PPAR-α knockout mice were administered PFBA at 35, 175 and 350 mg/kg for 28-days via oral gavage. This study identified a dose-dependent increase in relative liver weight for PFBA in both WT and human PPAR-α mice but not PPAR-α knock out mice and that PFBA induced PPAR-α transcription in both WT and humanized PPAR-α suggesting lipid modulating activity (Foreman et al., 2009). While these effects were both lower in the humanized PPAR mice than in WT, it still suggests

that PFBA can activate PPAR-αrelated metabolic changes in humans. These results also suggest that even the shorter chain PFAS can lead to changes in the liver similar to PFOA and other long chain PFAS. These data suggest that short-chain compounds such as PFMOAA have toxicological potential at certain doses. However, due to the rapid excretion of PFBA in mice, this study utilized doses 10-100-fold higher than the doses we used in our study, suggesting higher doses are needed to achieve a relevant internal dose of PFMOAA.

Currently, the NC Department of Human Health (DHHS) has set a health advisory level of 140 ng/L for GenX, based on protection of the most vulnerable populations, bottle-fed infants (NC DHHS, 2017). Still, while this health advisory level is aimed to be very conservative, very little is known about the environmental fate and toxicity of these emerging contaminants. The recent Consent Order and public outcry have led to the Chemours Fayetteville facility to implement source control measures of wastewater contaminated with PFAS (NC DEQ, 2017; Hopkins et al., 2018). This has resulted in a significant decrease in levels of PFMOAA in the Cape Fear River as evidenced by an April 30th, 2019, report from the Cape Fear Public Utility Authority (CFPUA), which reported levels of PFMOAA in finished water of 6.11 ppt (CFPUA, 2019). Though these levels are below most health advisory levels for known PFAS, it is still the dominant PFAS present in drinking water and the length and levels of exposure before its identification in 2015 are still unknown. PFMOAA was identified as high as 35,000 ng/L (Geosyntec 2018, Hopkins et al., 2018), which suggests that historical levels of PFMOAA exposure could have been quite high and persistent, even with its low bioaccumulation potential. The health outcomes associated with such an exposure have yet to be evaluated.

Conclusions and Future Directions

At the doses we administered, while being based on environmentally relevant levels (Hopkins et al. 2016), likely resulted in extremely low internal doses in our rodent model. Mice have been shown to excrete PFAS much more rapidly than humans and with excretion rates being inversely correlated with chain length, we suspect that the administered doses of PFMOAA led to an internal dose insufficient to induce changes in the endpoints we evaluated (Shea 2018, Gomis et al. 2015, 2018). No human data for PFMOAA levels have been published, but the high levels of exposure received by Cape Fear River communities are cause for further study into the toxicity of PFMOAA and other emerging PFAS. To this end, we developed a novel NK cell cytotoxicity assay to investigate the effects of PFAS on innate immune system function. Unfortunately, we were unable to utilize this assay for the PFMOAA project, but we have employed it in subsequent projects. As a part of the PFAST Network, our lab has committed to evaluating several of the emerging PFAS contaminants identified in NC for their ability to induce adverse health outcomes in the immune system. While there are still many PFAS identified in the environment that have little to no toxicological data, a follow-up study of PFMOAA at administered doses that will better reflect internal doses experienced by chronically-exposed humans is warranted.

References

3M. (1999). *Prepared by 3M Company May 26,1999*.

Abbott, B. D., Wood, C. R., Watkins, A. M., Tatum-Gibbs, K., Das, K. P., & Lau, C. (2012). Effects of perfluorooctanoic acid (PFOA) on expression of peroxisome proliferator-activated receptors (PPAR) and nuclear receptor-regulated genes in fetal and postnatal CD-1 mouse tissues. *Reproductive Toxicology*, *33*(4), 491–505. https://doi.org/10.1016/J.REPROTOX.2011.11.005

Abel, A. M., Yang, C., Thakar, M. S., & Malarkannan, S. (2018). Natural killer cells: Development, maturation, and clinical utilization. *Frontiers in Immunology*, *9*(AUG), 1–23. https://doi.org/10.3389/fimmu.2018.01869

ATSDR. (2018). *Toxicological Profile for Per- and Polyfluoroalkyl Substances: Draft for public comment*. (June).

Bowman, J. (2015). *Perspectives | Brief Communication Fluorotechnology Is Critical to Modern Life : The FluoroCouncil Counterpoint to the Madrid Statement*. (5), 112–113. https://doi.org/10.1289/ehp.1509910.Borg

Buck, R. C., Franklin, J., Berger, U., Conder, J. M., Cousins, I. T., Voogt, P. De, … Leeuwen, V. (2011). *Perfluoroalkyl and Polyfluoroalkyl Substances in the Environment : Terminology , Classification , and Origins*. *7*(4), 513–541. https://doi.org/10.1002/ieam.258

CFPUA. (2019). *April 30th, 2019, PFAS Emerging Contaminants Report*. Retrieved from https://www.cfpua.org/761/Emerging-Compounds

Chang, S.-C., Das, K., Ehresman, D. J., Ellefson, M. E., Gorman, G. S., Hart, J. A., … Butenhoff, J. L. (2008). Comparative pharmacokinetics of perfluorobutyrate in rats, mice, monkeys, and humans and relevance to human exposure via drinking water. *Toxicological Sciences : An Official Journal of the Society of Toxicology*, *104*(1), 40–53. https://doi.org/10.1093/toxsci/kfn057

Cholujová, D., Jakubíková, J., Kubeš, M., Arendacká, B., Sapák, M., Ihnatko, R., & Sedlák, J. (2008). Comparative study of four fluorescent probes for evaluation of natural killer cell cytotoxicity assays. *Immunobiology*, *213*(8), 629–640. https://doi.org/10.1016/J.IMBIO.2008.02.006

Dewitt, J. C., Copeland, C. B., Strynar, M. J., & Luebke, R. W. (2008). Perfluorooctanoic acid-induced immunomodulation in adult C57BL/6J or C57BL/6N female mice. *Environmental Health Perspectives*, *116*(5), 644–650. https://doi.org/10.1289/ehp.10896

Dewitt, J. C., Williams, W. C., Creech, N. J., & Luebke, R. W. (2016). Suppression of antigen-specific antibody responses in mice exposed to perfluorooctanoic acid: Role of PPAR and T- and B-cell targeting. *Journal of Immunotoxicology*, *13*(1), 38–45. https://doi.org/10.3109/1547691X.2014.996682

Dong, G.-H., Zhang, Y.-H., Zheng, L., Liu, W., Jin, Y.-H., & He, Q.-C. (2009). Chronic effects of perfluorooctanesulfonate exposure on immunotoxicity in adult male C57BL/6 mice. In *Archives of toxicology* (Vol. 83). https://doi.org/10.1007/s00204-009-0424-0

Filgo, A. J., Quist, E. M., Hoenerhoff, M. J., Brix, A. E., Kissling, G. E., & Fenton, S. E. (2015).

Perfluorooctanoic Acid (PFOA)-induced Liver Lesions in Two Strains of Mice Following Developmental Exposures: PPARα Is Not Required. *Toxicologic Pathology*, *43*(4), 558–568. https://doi.org/10.1177/0192623314558463

FlouroCouncil. (2014). *Industrial Reliance on FluoroTechnology , Fluorochemicals & Fluoropolymers Industrial Reliance on FluoroTechnology , Fluorochemicals & Fluoropolymers*.

Foreman, J. E., Chang, S. C., Ehresman, D. J., Butenhoff, J. L., Anderson, C. R., Palkar, P. S., … Peters, J. M. (2009). Differential hepatic effects of perfluorobutyrate mediated by mouse and human PPAR-α. *Toxicological Sciences*, *110*(1), 204–211. https://doi.org/10.1093/toxsci/kfp077

Fraser, A. J., Webster, T. F., Watkins, D. J., Strynar, M. J., Kato, K., Calafat, A. M., … McClean, M. D. (2013). Polyfluorinated compounds in dust from homes, offices, and vehicles as predictors of concentrations in office workers' serum. *Environment International*, *60*, 128–136. https://doi.org/10.1016/J.ENVINT.2013.08.012

Frisbee, S. J., Brooks Jr, A. P., Maher, A., Flensborg, P., Arnold, S., Fletcher, T., … Ducatman, A. M. (2009). The C8 health project: design, methods, and participants. *Environmental Health Perspectives*, *117*(12), 1873–1882. https://doi.org/10.1289/ehp.0800379

Gomis, Melissa I., Vestergren, R., Borg, D., & Cousins, I. T. (2018). Comparing the toxic potency in vivo of long-chain perfluoroalkyl acids and fluorinated alternatives. *Environment International*, *113*, 1–9. https://doi.org/10.1016/J.ENVINT.2018.01.011

Gomis, Melissa Ines, Wang, Z., Scheringer, M., & Cousins, I. T. (2015). A modeling assessment of the physicochemical properties and environmental fate of emerging and novel per- and polyfluoroalkyl substances. *Science of The Total Environment*, *505*, 981–991. https://doi.org/10.1016/J.SCITOTENV.2014.10.062

Grandjean, P., Andersen, E. W., Budtz-Jørgensen, E., Nielsen, F., Mølbak, K., Weihe, P., & Heilmann, C. (2012). Serum Vaccine Antibody Concentrations in Children Exposed to Perfluorinated Compounds. *JAMA*, *307*(4), 391–397. https://doi.org/10.1001/jama.2011.2034

Granum, B., Haug, L. S., Namork, E., Stølevik, S. B., Aaberge, I. S., Loveren, H. Van, … Nygaard, U. C. (2013). *Pre-natal exposure to perfluoroalkyl substances may be associated with altered vaccine antibody levels and immune-related health outcomes in early childhood*. *6901*. https://doi.org/10.3109/1547691X.2012.755580

Hopkins, Z., Mei, S., C, D. J., & U, K. D. R. (2018). Recently Detected Drinking Water Contaminants: GenX and Other Per- and Polyfluoroalkyl Ether Acids. *Journal - American Water Works Association*, *110*(7), 13–28. https://doi.org/doi:10.1002/awwa.1073

IARC. (2017). *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans| Some Chemicals used as solvents and in Polymer Manufacture*. Retrieved from https://monographs.iarc.fr/wp-content/uploads/2018/06/mono110.pdf

Impinen, A., Longnecker, M. P., Nygaard, U. C., London, S. J., Ferguson, K. K., Haug, L. S., & Granum, B. (2019). Maternal levels of perfluoroalkyl substances (PFASs) during pregnancy and childhood allergy and asthma related outcomes and infections in the Norwegian Mother and Child (MoBa) cohort. *Environment International*, *124*, 462–472.

https://doi.org/10.1016/J.ENVINT.2018.12.041

Kim, S., Chen, J., Cheng, T., Gindulyte, A., He, J., He, S., … Bolton, E. E. (2019). PubChem 2019 update: Improved access to chemical data. *Nucleic Acids Research*, *47*(D1), D1102–D1109. https://doi.org/10.1093/nar/gky1033

Li, K., Gao, P., Xiang, P., Zhang, X., Cui, X., & Ma, L. Q. (2017). Molecular mechanisms of PFOA-induced toxicity in animals and humans: Implications for health risks. *Environment International*, *99*, 43–54. https://doi.org/10.1016/J.ENVINT.2016.11.014

Loveless, S. E., Hoban, D., Sykes, G., Frame, S. R., & Everds, N. E. (2008). Evaluation of the immune system in rats and mice administered linear ammonium perfluorooctanoate. *Toxicological Sciences : An Official Journal of the Society of Toxicology*, *105*(1), 86–96. https://doi.org/10.1093/toxsci/kfn113

Manzano-Salgado, C. B., Casas, M., Lopez-Espinosa, M.-J., Ballester, F., Basterrechea, M., Grimalt, J. O., … Vrijheid, M. (2015). Transfer of perfluoroalkyl substances from mother to fetus in a Spanish birth cohort. *Environmental Research*, *142*, 471–478. https://doi.org/10.1016/J.ENVRES.2015.07.020

NC DEQ. (2017). *NC DEQ CONCENT ORDER FOR CHEMOURS COMPANY FC, LLC,*.

NC DEQ. (2018). *Amended Complaint and Motion for Preliminary Injunctive Relief*.

NC DHHS. (2017). *Questions and Answers Regarding North Carolina Department of Health and Human Services Updated Risk Assessment for GenX (Perfluoro-2-propoxypropanoic acid)*. 2–9.

NC Policy Collaboratory. (2019). *NORH CAROLINA PER- AND POLYFLUOROALKYL SUBSTANCES TESTING (PFAST) NETWORK*.

NTP. (2016). NTP Monograph Immunotoxicity Associated with Exposure to Perfluorooctanoic Acid or Perfluorooctane Sulfonate. *National Toxicology Program*, (September). Retrieved from https://ntp.niehs.nih.gov/ntp/ohat/pfoa_pfos/pfoa_pfosmonograph_508.pdf

Olsen, G. W., Mair, D. C., Lange, C. C., Harrington, L. M., Church, T. R., Goldberg, C. L., … Ley, C. A. (2017). Per- and polyfluoroalkyl substances (PFAS) in American Red Cross adult blood donors, 2000–2015. *Environmental Research*, *157*, 87–95. https://doi.org/10.1016/J.ENVRES.2017.05.013

Peden-Adams, M. M., Keller, J. M., EuDaly, J. G., Berger, J., Gilkeson, G. S., & Keil, D. E. (2008). Suppression of Humoral Immunity in Mice following Exposure to Perfluorooctane Sulfonate. *Toxicological Sciences*, *104*(1), 144–154. https://doi.org/10.1093/toxsci/kfn059

Peng, S., Yan, L., Zhang, J., Wang, Z., Tian, M., & Shen, H. (2013). An integrated metabonomics and transcriptomics approach to understanding metabolic pathway disturbance induced by perfluorooctanoic acid. *Journal of Pharmaceutical and Biomedical Analysis*, *86*, 56–64. https://doi.org/10.1016/J.JPBA.2013.07.014

Rae, J. M. C., Craig, L., Slone, T. W., Frame, S. R., Buxton, L. W., & Kennedy, G. L. (2015). Evaluation of chronic toxicity and carcinogenicity of ammonium 2,3,3,3-tetrafluoro-2-(heptafluoropropoxy)-propanoate in Sprague-Dawley rats. *Toxicology Reports*, *2*, 939–949. https://doi.org/10.1016/j.toxrep.2015.06.001

Rushing, B. R., Hu, Q., Franklin, J. N., McMahen, R., Dagnino, S., Higgins, C. P., … DeWitt, J.

C. (2017). Evaluation of the immunomodulatory effects of 2,3,3,3-tetrafluoro-2-(heptafluoropropoxy)-propanoate in C57BL/6 mice. *Toxicological Sciences : An Official Journal of the Society of Toxicology*. https://doi.org/10.1093/toxsci/kfw251

Sajid, M., & Ilyas, M. (2017). PTFE-coated non-stick cookware and toxicity concerns: a perspective. *Environmental Science and Pollution Research*, *24*(30), 23436–23440. https://doi.org/10.1007/s11356-017-0095-y

Sakr, C. J., Leonard, R. C., Kreckmann, K. H., Slade, M. D., & Cullen, M. R. (2007). Longitudinal study of serum lipids and liver enzymes in workers with occupational exposure to ammonium perfluorooctanoate. *Journal of Occupational and Environmental Medicine*, *49*(8), 872–879. https://doi.org/10.1097/JOM.0b013e318124a93f

Savitz, D. A., Stein, C. R., Bartell, S. M., Elston, B., Gong, J., & Shin, H. (2012). *Perfluorooctanoic Acid Exposure and Pregnancy Outcome in a Highly Exposed Community*. *23*(3). https://doi.org/10.1097/EDE.0b013e31824cb93b

Shea, D. (2018). Proposed Drinking Water Health Advisory Value for GenX :2,3,3,3-tetrafluoro-2- (heptafluoropropoxy)-propanoic acid. *Chemours*. Retrieved from https://www.chemours.com/Fayetteville-Works/en-us/c3-dimer-acid/more-information/index.html

Shin, H.-M., Vieira, V., Ryan, P. B., etwiler, R., Sanders, B., Steenland, K., & Bartell, S. (2012). P47—Retrospective exposure estimation for perfluorooctanoic acid (PFOA) for participants in the C8 health project. *Reproductive Toxicology*, *33*(4), 615. https://doi.org/10.1016/J.REPROTOX.2011.11.081

Steenland, K., Fletcher, T., & Savitz, D. A. (2010). *Review Epidemiologic Evidence on the Health Effects of Perfluorooctanoic Acid (PFOA)*. (8), 1100–1108. https://doi.org/10.1289/ehp.0901827

Steenland, K., Jin, C., Macneil, J., Lally, C., Ducatman, A., & Vieira, V. (2009). *Predictors of PFOA Levels in a Community Surrounding a Chemical Plant*. *117*(7), 1083–1088. https://doi.org/10.1289/ehp.0800294

Steenland, K., & Woskie, S. (2012). Cohort Mortality Study of Workers Exposed to Perfluorooctanoic Acid. *American Journal of Epidemiology*, *176*(10), 909–917. https://doi.org/10.1093/aje/kws171

Strynar, M., Dagnino, S., McMahen, R., Liang, S., Lindstrom, A., Andersen, E., … Ball, C. (2015). Identification of Novel Perfluoroalkyl Ether Carboxylic Acids (PFECAs) and Sulfonic Acids (PFESAs) in Natural Waters Using Accurate Mass Time-of-Flight Mass Spectrometry (TOFMS). *Environmental Science & Technology*, *49*(19), 11622–11630. https://doi.org/10.1021/acs.est.5b01215

Sun, M., Arevalo, E., Strynar, M., Lindstrom, A., Richardson, M., Kearns, B., … Knappe, D. R. U. (2016). *Legacy and Emerging Per fl uoroalkyl Substances Are Important Drinking Water Contaminants in the Cape Fear River Watershed of North Carolina*. https://doi.org/10.1021/acs.estlett.6b00398

US EPA. (2015). 2010/2015 PFOA Stewardship Program. Retrieved from https://www.epa.gov/assessing-and-managing-chemicals-under-tsca/fact-sheet-20102015-pfoa-stewardship-program#meet

US EPA. (2016a). *Drinking Water Health Advisory for Perfluorooctane Sulfonate (PFOS)*. (May), 1–88.

US EPA. (2016b). *Drinking Water Health Advisory for Perfluorooctanoic Acid (PFOA)*. (May), 1–103.

US EPA. (2018). *Environmental Fate and Transport for Per-and Polyfluoroalkyl Substances*. Retrieved from https://pfas-1.itrcweb.org/wp-content/uploads/2018/03/pfas_fact_sheet_fate_and_transport__3_16_18.pdf

US EPA. (2019). PFAS Master List of PFAS Substances. Retrieved from PFAS Master List of PFAS Substances website: https://comptox.epa.gov/dashboard/chemical_lists/pfasmaster

Vizler, C., Nagy, T., Kusz, E., Glavinas, H., & Duda, E. (2002). Flow cytometric cytotoxicity assay for measuring mammalian and avian NK cell activity. *Cytometry*, *47*(3), 158–162. https://doi.org/10.1002/cyto.10066

Wang, Z., DeWitt, J. C., Higgins, C. P., & Cousins, I. T. (2017). A Never-Ending Story of Per- and Polyfluoroalkyl Substances (PFASs)? *Environmental Science & Technology*, *51*(5), 2508–2518. https://doi.org/10.1021/acs.est.6b04806

Wong, F., MacLeod, M., Mueller, J. F., & Cousins, I. T. (2014). Enhanced Elimination of Perfluorooctane Sulfonic Acid by Menstruating Women: Evidence from Population-Based Pharmacokinetic Modeling. *Environmental Science & Technology*, *48*(15), 8807–8814. https://doi.org/10.1021/es500796y

Wu, N., Cai, D., Guo, M., Li, M., & Li, X. (2019). Per- and polyfluorinated compounds in saleswomen's urine linked to indoor dust in clothing shops. *Science of The Total Environment*, *667*, 594–600. https://doi.org/10.1016/J.SCITOTENV.2019.02.287

Yang, Q., Xie, Y., Alexson, S. E. H., Dean Nelson, B., & DePierre, J. W. (2002). Involvement of the peroxisome proliferator-activated receptor alpha in the immunomodulation caused by peroxisome proliferators in mice. *Biochemical Pharmacology*, *63*(10), 1893–1900. https://doi.org/10.1016/S0006-2952(02)00923-1

Yang, Q., Xie, Y., Eriksson, A. M., Nelson, B. D., & DePierre, J. W. (2001). Further evidence for the involvement of inhibition of cell proliferation and development in thymic and splenic atrophy induced by the peroxisome proliferator perfluoroctanoic acid in mice. *Biochemical Pharmacology*, *62*(8), 1133–1140. https://doi.org/10.1016/S0006-2952(01)00752-3

Zhang, Y., Beesoon, S., Zhu, L., & Martin, J. W. (2013). *Biomonitoring of Per fl uoroalkyl Acids in Human Urine and Estimates of Biological Half-Life*. https://doi.org/10.1021/es401905e

Zheng, L., Dong, G.-H., Jin, Y.-H., & He, Q.-C. (2009). Immunotoxic changes associated with a 7-day oral exposure to perfluorooctanesulfonate (PFOS) in adult male C57BL/6 mice. *Archives of Toxicology*, *83*(7), 679–689. https://doi.org/10.1007/s00204-008-0361-3

APPENDIX: ANIMAL USE PROTOCOL LETTER

East Carolina University
Tomorrow starts here.

Animal Care and Use Committee
212 Ed Warren Life Sciences Building
East Carolina University
Greenville, NC 27834-4354

252-744-2436 office
252-744-2355 fax

September 19, 2018

Jamie DeWitt, Ph.D.
Department of Pharmacology
Brody 6S-10
East Carolina University

Dear Dr. DeWitt:

Your Animal Use Protocol entitled, "Evaluation of Immunotoxicity Induced by Exposure to Per- and Polyfluoroalkyl Substances (PFASs)" (AUP #W256) was reviewed by this institution's Animal Care and Use Committee on September 19, 2018. The following action was taken by the Committee:

"Approved as submitted"

Please contact Aaron Hinkle at 744-2997 prior to hazard use

A copy is enclosed for your laboratory files. Please be reminded that all animal procedures must be conducted as described in the approved Animal Use Protocol. Modifications of these procedures cannot be performed without prior approval of the ACUC. The Animal Welfare Act and Public Health Service Guidelines require the ACUC to suspend activities not in accordance with approved procedures and report such activities to the responsible University Official (Vice Chancellor for Health Sciences or Vice Chancellor for Academic Affairs) and appropriate federal Agencies. **Please ensure that all personnel associated with this protocol have access to this approved copy of the AUP and are familiar with its contents.**

Sincerely yours,

Susan McRae, Ph.D.
Chair, Animal Care and Use Committee

SM/jd

Enclosure