The Immunotoxicity of Two Novel Perfluoroether Acids Found in North Carolina's Cape Fear River

By

Emma E. Tobin

July 2021

Director of Thesis: Dr. Jamie DeWitt,

Department of Pharmacology and Toxicology

Abstract

Novel perfluoroether acids (PFEAs) have been identified in surface waters of North Carolina and in the blood of some North Carolina residents. As some per- and polyfluoroalkyl substances (PFAS) are presumed immune hazards to humans, markers of immunotoxicity in young adult female and male C57BL/6 mice were observed following either 30-day oral exposure to perfluoro-2-methoxyacetic acid (PFMOAA), 30-day oral exposure to Nafion by-product 2 (NBP2), or 15-day oral exposure to a mixture of PFMOAA and NBP2. In-life observations were collected and endpoints included: organ weights, immunophenotype of lymphoid organs, natural killer (NK) cell cytotoxicity, liver peroxisomal enzyme activity, and the T-cell dependent antibody response (TDAR). In animals exposed to PFMOAA orally for 30 days the following changes were observed: liver weight statistically increased, in male animals peroxisomal enzyme activity statistically increased, and in male animals the TDAR was statistically suppressed. In animals exposed to NBP2 orally for 30 days the following changes were observed: liver weight statistically increased, spleen and thymus weight statistically decreased, NK cytotoxicity statistically decreased, liver peroxisomal enzyme activity statistically

increased, and the TDAR was statistically decreased. In animals exposed to PFMOAA and NBP2 mixtures for 30 days the following changes were observed: one male group had statistically decreased body weight, some groups had alterations in organ weights. Our results indicate these novel PFEAs have immunosuppressive and immunomodulatory potential.

The Immunotoxicity of Two Novel Perfluoroether Acids Found in North Carolina's Cape Fear River

A Thesis

Presented to the Faculty of the MS Program in Biomedical Sciences

Office of Research of 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

Emma E. Tobin

July 2021

The Immunotoxicity of Two Novel Perfluoroether Acids Found in North Carolina's Cape Fear River

By

Emma E. Tobin

APPROVED BY:

Director of Thesis _____
Jamie DeWitt, Ph.D.

Committee Member _____
Tracey Woodlief, Ph.D.

Committee Member _____
Eli Hvastkovs, Ph.D.

Director, MS Program
in Biomedical Sciences _____
Richard Franklin, Ph.D.

Dean of the Graduate School _____
Paul J. Gemperline, PhD

Acknowledgements

This research was funded by the North Carolina Policy Collaboratory through an appropriation from the North Carolina General Assembly and by the Brody Brothers Endowment fund.

Table of Contents

List of Tables

List of Figures

Abbreviations

PFAS – per- and polyfluoroalkyl substances

PFAAs – perfluoroalkyl acids

PFCAs – perfluorocarboxylic acids

PFSAs – perfluoroalkyl sulfonic acids

PFOA – perfluorooctanoic acid

PFOS – perfluorooctane sulfonate

US EPA – United States Environmental Protection Agency

NC – North Carolina

CFR – Cape Fear River

PFMOAA – perfluoro-2-methoxyacetic acid

HFPO – hexafluoropropylene oxide

NBP2 – Nafion by-product 2

NC DEQ – North Carolina Department of Environmental Quality

PFC – antibody plaque forming

ELISA – enzyme-linked immunosorbent assay

NK – natural killer

SRBC – sheep red blood cell

TDAR – the T cell dependent antibody response

PFECA – perfluoroether carboxylic acid

ANOVA – analysis of variance

RPM – rotations per minute

ACOX-1 – acyl-coenzyme A oxidase 1

DCF – dichlorofluorescein

SAS – statistical analysis system

IgM – immunoglobulin M

PFEA – perfluoroether acid

ECU – East Carolina University

FBS – Fetal bovine serum

MS/MS – tandem mass spectrometry

LC-MS/MS – liquid chromatography-tandem mass spectrometry

HPLC – high performance liquid chromatography

DI – deionized

ESI – electrospray ionization

CE – collision energy

CXP – collision cell exit potential

NOAEL – no-observed-adverse-effect level

AFFF – aqueous film forming foam

PFAST – PFAS testing

Chapter 1: Introduction

1. Legacy PFAS

Per- and polyfluoroalkyl substances (PFAS) are a large class of chemicals used in myriad consumer products as well as in industrial products and processes. In recent years they have become contaminants of concern as they have been found in the blood of almost every human on earth and are distributed throughout the environment from the arctic to the deep oceans (Hansen et al. 2001; Giesy and Kannan 2001; Buck et al. 2011). In spite of their ubiquitous presence in humans and the environment only incremental progress has been made in uncovering both the distibution of PFAS and the effects they pose to environmental health, and progress in remediating and restricting their use is moving at an even slower rate due in part to the sheer number of PFAS we now know to exist.

Per- and polyfluoroalkyl substances are "chemicals with at least one aliphatic perfluorocarbon moiety"(Kwiatkowski et al. 2020). One of the most well-studied subclasses of PFAS are the perfluoroalkyl acids (PFAAs), which are often categorized as either perfluoroalkyl carboxylic acids (PFCAs) or perfluoroalkyl sulfonic acids (PFSAs) as defined by their polar head groups (Lindstrom, Strynar, and Libelo 2011). The inherent strength of the C-F bonds comprising PFAS has resulted in an enormous number of uses for PFAS since their original development in the 1950s . The usefulness of PFAS comes from their stability under a range of conditions and from their water, stain, and oil repellent qualities. They therefore have applications in waterproof, fire repellent, and nonstick coatings, as well as in fast food wrappers, textiles, implantable medical devices, cosmetics and more (Buck et al. 2011). The widespread use of PFAS has led to their extensive distribution today and sources of PFAS contamination in the environment are many – it is known PFAS are released in waste water and atmospherically,

they are transferred by wildlife, they bioaccumulate, and as the name "forever chemicals" describes, many of them do not readily or naturally degrade in the environment where to our knowledge, they will persist indefinitely (KEMI (Swedish Chemicals Agency) 2015).

Historically since the 1950s two PFAAs, a PFCA known as perfluorooctanoic acid (PFOA) and a PFSA known as perfluorooctane sulfonic acid (PFOS), have been widely used in manufacturing. Though toxicity studies were conducted internally by chemical manufacturers, for many years the general research community was unaware of the existence of these substances, their widespread distribution, or of their potential health effects (Lindstrom, Strynar, and Libelo 2011). The use and disposal of these chemicals was unregulated or lightly regulated for decades and as a result PFOA, PFOS, and other similar PFAAs were distributed in the environment worldwide via atmospheric deposition, in wastewater, and in many of the common products around us in our daily lives (Giesy and Kannan 2001; Kotthoff et al. 2015; Lindstrom, Strynar, and Libelo 2011).

In 1998 the company 3M reported evidence that PFOS was accumulating in the serum of humans not working at their manufacturing plants to the United States Environmental Protection Agency (US EPA) and in 2000 3M subsequently announced their voluntary phase out of PFOS (Lindstrom, Strynar, and Libelo 2011). Another fluorochemical manufacturer, DuPont, was exposed as having contaminated a West Virginian community with PFOA around the same time. In 2001 a class action lawsuit against DuPont was filed related to PFOA and as a result of the lawsuit, a medical monitoring program was established to determine how PFOA had impacted the health of local residents (Frisbee et al. 2009). Over 69,000 study participants had PFOA levels in their serum analyzed and had extensive demographic and medical records collected with the goal to determine "probable links" between PFOA exposure and disease; a probable link

between PFOA and a disease was defined to mean "based upon the weight of the available scientific evidence, it is more likely than not that there is a link between exposure to C-8 and a particular human disease among class members" (Frisbee et al. 2009). Probable links between PFOA exposure and six disease categories were made: high cholesterol, ulcerative colitis, thyroid disease, testicular cancer, kidney cancer, and pregnancy induced hypertension (Barry, Winquist, and Steenland 2013).

In response to this lawsuit, increasing evidence of the presence of PFOA and PFOS in the environment and in living organisms, and other actions taken by various governmental entities, fluorochemical manufacturers began to phase out the use of "legacy" PFAAs such as PFOA and PFOS, and instead sought to use alternative compounds as replacements. Replacement compounds often have a shorter carbon chain length or ether oxygen linkages in between the carbons with a goal of increasing the biological half-life in living organisms or facilitating environmental degradation, but unfortunately the toxicity of many replacement compounds is still understudied and not well understood (Strynar et al. 2015a; Wang et al. 2017). Additionally, novel PFAS, or PFAS for which no toxicological data appear to be available, recently have been identified in surface waters (Sun et al. 2016). These PFAS are theorized to have been present since the 1980s but were undiscovered due to a lack of standards or knowledge of their existence. Because many replacement and novel compounds have now been found in humans and in the environment, understanding their safety is an urgent issue.

2. Replacement and Novel PFAS in North Carolina

In North Carolina (NC) replacement and novel PFAS and have been identified in the Cape Fear River (CFR) and in the blood of eastern NC residents (Hopkins et al. 2018; Kotlarz et al. 2020). A short-chain monoether PFCA called perfluoro-2-methoxyacetic acid (PFMOAA),

thought to be a by-product of hexafluoropropylene oxide (HFPO) -dimer acid (also called GenX) production, was first identified in the CFR in 2012 (Strynar et al. 2015a). GenX was implemented into manufacturing processes at Fayetteville Works, a fluorochemical manufacturing facility in Fayetteville, NC, as a replacement compound for PFOA in the production of Teflon^{TM}, a non-stick fluoropolymer (Brase, Mullin, and Spink 2021). Though Stynar et al. (2015) first reported the presence of PFMOAA in the CFR in 2015, as analytical standards were not available at that time, the levels of PFMOAA were not quantified (Strynar et al. 2015a). Hopkins et al. (2018) quantified PFMOAA levels in the CFR in water samples collected May and June of 2017 and levels were reported to be 26,000 ng/L, which were among the highest levels reported for any PFAS measured in the CFR to date (Hopkins et al. 2018). Wastewater controls to capture GenX and its by-products were put into place in late 2017, but PFMOAA was still detectable in the CFR in February of 2018 though levels had decreased (McCord and Strynar 2019). PFMOAA was also identified by Yao et al. (2020) in serum of humans living near a fluorochemical manufacturing facility in Shandong, China, and observed significant differences in the accumulation of PFMOAA between males and females, with males accumulating more PFMOAA (Yao et al. 2020).

A short-chain PFSA with two ether oxygen linkages identified as Nafion by-product 2 (NBP2) was also identified in the CFR in 2012 (Strynar et al. 2015a). Nafion is a sulfonated tetrafluoroethylene-based fluoropolymer-copolymer used to produce energy efficient fuel cells among other applications (PubChem 2021). Because Nafion had been produced at the Fayetteville Works facility since the 1980s, and because the Nafion waste streams were managed separately from the ones being managed for PFOA and for GenX, it is unknown exactly how long its by-product NBP2 has been present in the CFR but it has been hypothesized since the

1980s (Strynar et al. 2015a; McCord and Strynar 2019). Little is known about the toxicity of NBP2 but there is concern that they may elicit similar toxic effects to those of legacy PFAS. After NBP2 was also first identified in the CFR by Strynar et al. (2015), Hopkins et al. (2018) quantified NBP2 levels and observed that NBP2 increased in the CFR between May and June of 2017. These observations prompted an agreement between The Chemours Company, owners of Fayetteville Works, and the US Environmental Protection Agency (US EPA) and NC Department of Environmental Quality (NC DEQ) to start capturing waste streams containing Nafion by-products (Hopkins et al. 2018). As a result, NBP2 levels were reduced, but NBP2 did not disappear from the environment. Guillette et al. (2020) found NBP2 in striped bass collected from the CFR in April and August of 2018 and Kotlarz et al. (2020) identified NBP2 in 99% of samples of human serum collected from individuals in the CFR watershed in November 2017. In serum samples collected in May 2018, NBP2 was still present in 65% of study participants (Guillette et al. 2020; Kotlarz et al. 2020). Lang et al. (2020) exposed Balb-c mice to 0, 0.04, 0.4, 3, or 6 mg/kg NBP2/day for seven days via oral gavage and observed a statistical increase in liver size and in serum markers of liver injury in the higher two dose groups (Lang et al. 2020). NBP2 was also identified by Yao et al. (2020) in serum of Shandong, China residents (Yao et al. 2020). Aside from the studies covered there are few known toxicity studies into the effects of PFMOAA and NBP2 at this time.

3. Timeline of PFMOAA and NBP2 in North Carolina:

2012: A Chemours PFOA groundwater monitoring report mentions PFESAs found in the area of the Fayetteville Works Nafion membrane production area (Strynar et al. 2015a)

2015: Nafion by-products identified in Cape Fear River (Strynar et al. 2015a)

2015: A mono-ether PFECA with chemical formula $C_3HF_5O_3$ (PFMOAA) identified in Cape Fear River (Strynar et al. 2015a)

2017: US EPA and NC DEQ come to agreement with Fayetteville Works to capture wastewater containing Nafion by-products (Hopkins et al. 2018)

2017: Two Nafion by-products and PFMOAA identified at drinking water intake in Cape Fear River (estimated 280,000 people impacted) (Hopkins et al. 2018)

2018: NBP2 found in surface waters (18ng/L) and found to accumulate in wildlife (levels 17x higher than surface waters) (Guillette et al. 2020)

2017/2018: NBP2 found in human serum of 99% of population sampled who source drinking water from Cape Fear River (Kotlarz et al. 2020)

2019: Nafion by-products and PFMOAA are again identified in Cape Fear River (McCord and Strynar 2019)

4. Toxicity

The consequences of exposure to legacy PFAS are relatively well understood compared to what is known following exposure to novel and replacement compounds (Lindstrom, Strynar, and Libelo 2011). Bioaccumulation of PFAS has been reported to occur, and attributes like chain length and ether linkages are thought to affect their biological half-lives; males and females also have been reported to accumulate/excreted PFAS at different rates but reasons for this difference are not clearly understood (Fenton et al. 2021). In some cases shorter chain PFAS seem to be excreted more readily, but both short-chain and long-chain PFAS have been reported to accumulate in humans and wildlife(Kannan 2011; Ng and Hungerbühler 2014).

PFAS are known to accumulate in the blood by binding to plasma proteins such as albumin and they also accumulate in other tissues (Fenton et al. 2021). It is thought the

analogous structure shared between PFAS and fatty acids may contribute to how PFAS are handled by the body – many analogous health effects between fatty acids and PFAS have been observed in animal models and humans (Ng and Hungerbühler 2014). There is still much work to be done in uncovered the mechanisms by which PFAS elicit toxicity though, mechanistic studies have only been performed with a few PFAS. A potential mechanistic targets of some PFAS is thought to be the peroxisome proliferator-activated receptor alpha (PPARα) but various other non-hepatic mechanisms have been postulated as well including mitochondrial disfunction, oxidative stress, and interfering of protein binding (Fenton et al. 2021). Hepatotoxicity is a common endpoint of PFAS exposure in rodent models but other mechanisms of action are thought to exist as rodent models lacking a functional PPARα still exhibit toxic effects. Uncovering the mechanism of PFAS toxicity is an integral step to managing PFAS in a manner protective of public health as without this knowledge it is difficult to predict the potential toxicity of novel substances and to mitigate health outcomes of exposure (Fenton et al. 2021).

In addition to probable links between PFOA and the six disease endpoints identified by the C8 Science Panel, PFAS exposure has been linked to other health effects in humans and many other health effects in animal models. In animal models, PFAS are often hepatotoxic, and evidence has shown PFAS can be developmentally toxic (Hu et al. 2012; Keil et al. 2008). Exposure to PFOS and PFOA has been negatively correlated with serum antibody concentrations after vaccination in repeated studies, and in both human and animal studies the effects of PFAS on the antibody response is strong evidence that PFAS are immunotoxicants (US NTP 2016). Evidence has shown that many PFAS can also perturb other aspects of the immune system such as immune cell subpopulations and markers of innate immunity (Grandjean et al. 2012; 2017). To predict the potential immunotoxicity of a substance, the following tests are suggested by the

US EPA: either an antibody plaque forming (PFC) assay or an enzyme linked immunosorbent assay (ELISA) to determine antibody responses after immunization with an antigen and phenotypic analysis of splenic or peripheral blood T cells, B cells, and NK cells (OPPTS 870.7800).

Because the well-studied legacy PFAS PFOA and PFOS are known to be immunotoxic, the present studies were performed to better describe the immunotoxicological outcomes of exposure to NBP2 and PFMOAA following these guidelines. It is critical the potential health effects of exposure to these two PFAS become better characterized as an estimated 280,000 residents of NC have been exposed to them with little understanding of what the potential impacts to their health could be. Additionally, as not all states in the US or other countries routinely monitor drinking water or biological samples for the presence of NBP2 or PFMOAA, it is unknown how many other people may be experiencing exposure to these two understudied PFAS. PFAS are often found as complex mixtures in the environment, further complicating the question of the toxicity of individual PFAS. As most PFAS exist as mixtures in the environment, and because the toxicity of PFAS mixtures is not well understood, a mixture study with simultaneous exposure to NBP2 and PFMOAA was also performed.

5. Study descriptions

Oral exposure to Nafion by-product 2 or PFMOAA: NBP2 or PFMOAA was administered to 6–8-week-old male and female C57BL/6 mice daily for 30 days via gavage. Administered concentrations were: 0, 0.5, 1, and 5 mg/kg NBP2/day, and 0, 0.5, 5, and 50 mg/kg PFMOAA/day. Each dose group was comprised of 6 female and 6 male animals. On the 26th day of dosing, mice were immunized with sheep red blood cells (SRBC) to elicit the T cell-dependent antibody response (the TDAR, a marker of adaptive immunity). The study terminus

was 24 hours after the 30th dose. The following endpoints were examined after terminal collections: serum concentrations of NBP2, immunophenotype of T, B, and NK cells in the spleen and of T-cells in the thymus, natural killer cell cytotoxicity, TDAR, and peroxisomal enzyme activity in the liver. Doses were chosen based on previous studies in the DeWitt lab with both legacy PFAS and preliminary studies of NBP2 and PFMOAA. A preliminary 30-day oral exposure to NBP2 including a higher dose group of 50 mg/kg was conducted but ended early as overt toxicity occurred after only a few days of dosing. The present doses were chosen to prevent overt toxicity. A preliminary 30-day oral exposure to PFMOAA using lower doses did not produce signs of overt toxicity or immunotoxicity, so the present doses were chosen to elicit sublethal responses without being overtly toxic.

Oral exposure to a Nafion by-product 2 and PFMOAA mixture: NBP2 and PFMOAA were administered to 6–8-week-old male and female C57BL/6 mice daily for 15 days via gavage. Administered concentrations were: 0/0, 0.5/0.05, 0.1/0.5, and 0.05/5 mg/kg NBP2/PFMOAA/day. Each dose group was comprised of 6 female and 6 male animals. On the 11th day of dosing, mice were immunized with sheep red blood cells (SRBC) to elicit the TDAR. The study terminus was 24 hours after the 15th dose. The following endpoints were examined after terminal collections: serum concentrations of NBP2 and PFMOAA, immunophenotype of T, B, and NK cells in the spleen and of T-cells in the thymus, natural killer cell cytotoxicity, TDAR, and peroxisomal enzyme activity in the liver. A pilot 15-day mixture study was performed using doses 10x higher than the final doses used in this study. The pilot study doses were based on doses that were not overtly toxic when administered alone, but when administered as a mixture, overt toxicity occurred as observed through rapid weight loss and mortality. The

doses were subsequently reduced 10x for the present study and no mortality or rapid weight loss occurred during the dosing period.

Chapter 2: Immunological Outcomes of Exposure to Perfluoro-2-methoxyacetic acid (PFMOAA) in C57BL/6 Mice

1. Introduction

Per- and polyfluoroalkyl substances (PFAS) are anthropogenic worldwide contaminants of concern and are defined as "chemicals with at least one aliphatic perfluorocarbon moiety" (Kwiatkowski et al. 2020). An extraordinary number of PFAS have been developed for a multitude of purposes and even more PFAS have been identified in the environment as by-products of these chemicals. The United States Environmental Protection Agency (US EPA) currently estimates the number of PFAS to be over 9,000 individual chemicals and counting ("PFAS|EPA: PFAS Structures in DSSTox" 2020). PFAS have been dispersed into the environment and have potentially made their way into the bodies of humans since their use in the 1940s first began, but it was not until almost half a century later that the general research community and public would become aware of their existence as environmental contaminants (Lindstrom, Strynar, and Libelo 2011). PFAS are useful in many applications in part due to their persistence and the strength of the carbon-fluorine bonds they all possess, but many of the qualities that make PFAS useful are also the qualities that make PFAS environmentally persistent contaminants of concern. The usefulness of these chemicals has resulted in their inclusion in a number of applications such as in nonstick cookware, waterproof coatings, and firefighting foams and the resulting accumulation of waste products containing PFAS in the environment have lead to their current worldwide distribution (Buck et al. 2011; Lindstrom, Strynar, and Libelo 2011; Giesy and Kannan 2001; Kannan et al. 2004).

One PFAS historically used in manufacturing was perfluorooctanoic acid (PFOA or C8), which DuPont's Fayetteville Works plant in Fayetteville, North Carolina (NC) began

manufacturing vinyl ether with in 1980. A class-action lawsuit against DuPont for PFOA contamination in West Virginia prompted PFOA to be substituted in manufacturing processes at the Fayetteville Works plant with a replacement compound called hexafluoroproplylene oxide dimer acid (HFPO-DA), also known as "GenX" (Clean Cape Fear, 2021). By 2012 GenX and by-products of GenX were identified in surface waters of the Cape Fear River (CFR) in NC (Strynar et al. 2015b). Although PFOA has been phased out of many products and processes due to a myriad of health outcomes being linked with exposure, there is now concern that structurally similar replacement compounds and their by-products could elicit similar toxic effects (Wang et al. 2017). These concerns have been amplified in NC as studies have emerged showing various replacement and by-product PFAS are present in rivers, finished drinking water, and in the bodies of many local residents who consumed drinking water sourced from the CFR.

One such PFAS raising concern to researchers and residents of NC is a perfluoroether carboxylic acid (PFECA) known as perfluoro-2-methoxyacetic acid (PFMOAA). PFMOAA is a short chain PFECA with three carbons and a monoether linkage and is thought to be a by-product of GenX production. PFMOAA was first identified in the CFR by Strynar et al. (2015) and was identified again by McCord and Strynar (2019) (Strynar et al. 2015b; McCord and Strynar 2019). This PFAS has also been identified in the water and in the bodies of humans living near a fluorochemical manufacturing plant in Shandong Province, China (Yao et al. 2020). To our knowledge only one peer-reviewed toxicology study can be found on PFMOAA, Woodlief et al. (2021), where C57BL/6 mice were orally exposed to low doses of PFMOAA in an exploratory immunotoxicological study and the only observed effect being a slight increase in liver weight (Woodlief et al. 2021). The present study sought to expand on the work done by Woodlief et al.

(2021) by examining the same immunological endpoints in C57BL/6 mice after exposure to higher doses of PFMOAA.

2. Methods

2.1 Experimental Design

Animals: 27 female and 27 male C57BL/6 mice were purchased from Charles River Laboratories and housed in the East Carolina University (ECU) Brody School of Medicine animal facility (accredited by the Association for Assessment and Accreditation of Laboratory Animal Care). All procedures in this study were given approval in advance by the ECU Institutional Animal Care and Use Committee. Upon arrival animals were weighed and distributed by sex into cages of 3 animals/cage and allowed a 5 day acclimation period before any further manipulation. Weights by cage were evaluated by analysis of variance (ANOVA) to ensure weights did not differ statistically at the start of the study and animals were rearranged if necessary. The polycarbonate cages were housed in temperature controlled conditions (23±3°C) on a 12 hour light:dark cycle with 50±20% relative humidity. Cages contained one nestlet, one hiding tube, and pine shaving bedding. Animals were given access to food and water *ad libitum*.

2.2 Dosing

Fresh dosing solutions were prepared from stock PFMOAA (SynQuest**)** one time per week with sterile water and 0.5% Tween. Dosing solutions were stored at 4°C when not in use to ensure dosing solutions were free from observable signs of mold or fungal contamination. Animals were weighed daily and dosing solution was administered via oral gavage for 30 consecutive days at 0.1 mL/10 g of body weight; dosing solutions were prepared so that this volume would result in the appropriate mg/kg of body weight dosage. Dosing solutions were made at the following concentrations: vehicle control water + 0.5% Tween or PFMOAA at 0.5,

5, or 50 mg/kg, A separate group of 3 females and 3 males received PFOA at 7.5 mg/kg to serve as a positive control for immunotoxicity. One day after the 30th dose animals were humanely euthanized for serum and tissue collection.

The administered doses of PFMOAA were based on our recent study investigating immunotoxic effects of PFMOAA in C57BL/6 mice given 0.00025, 0.025, or 2.5 mg/kg (Woodlief et al., 2021). In this study we observed few toxicological outcomes; terminal body weights, immunophenotype, the T-cell dependent antibody response (TDAR), and most organ weights were not altered statistically at these doses. The present doses of 0.5, 5, and 50 mg/kg reflect the range of doses of other PFAS given to C57BL/6 mice by Woodlief et al. (2021). A 7.5 mg/kg dose of PFOA was chosen to serve as a positive control for the immunotoxic effects of PFAS as this dose of PFOA has been previously shown to induce immunotoxicity but not overt toxicity in C57BL/6 mice (DeWitt et al. 2008).

2.3 Serum and tissue collection

One day after the final dose animals were anesthetized with Isoflurane and blood was collected via neck vein transection. Serum was separated from clotted blood at 4°C by centrifugation at 14,000 rotations per minute (RPM) for 7 minutes and then frozen at -80°C.

Selected organs (spleen, thymus, liver, brain, heart, kidney, and lungs) were excised and weighed following terminal blood collection. Spleen and thymus were immediately placed into RPMI + 1% FBS medium on ice. Liver was stored in aluminum foil squares and then snap frozen on dry ice and archived at -80°C for later analysis. Brain, heart, kidney, and lungs were weighed and discarded or processed to be sent to collaborators.

2.4 Measurement of the T cell-dependent antibody response (TDAR)

To elicit production of anti-sheep red blood cell (SRBC) immunoglobulin M (IgM) antibodies, animals were immunized with 4 x 10^7 SRBC in 0.2 mL of sterile saline 26 days after dosing began. Five days later, the peak of antigen-specific IgM production in C57BL/6 mice, serum was collected as described in "serum and tissue collection" and anti-SRBC antibody titers were measured as previously described (Woodlief et al. 2021) using an enzyme linked immunosorbent assay (ELISA) kit (Life Diagnostics, SRBCM-1). Briefly, serum samples and anti-SRBC IgM standards were diluted 50-fold and added in duplicate to 96-well ELISA plates pre-coated with SRBC membranes, wells containing just diluent were included as blanks. Plates were incubated at room temperature for 45 minutes on an orbital shaker at 75 RPM. Wells were washed 5x in 1x washing solution, tapped dry, and enzyme conjugate reagent was then added to each well. Plates were incubated at room temperature for 20 minutes on an orbital shaker at 75 RPM. Wells were again washed 5x in 1x wash solution and tapped dry, then TMB reagent was added to each well and plates were incubated at room temperature for 20 minutes at 75 RPM. Stop solution (1N HCL) was added to each well and the optical density at 450 nm was immediately measured on a microplate reader. The concentration of anti-SRBC IgM (units/mL) was calculated by fitting each sample onto a standard curve that was based on the absorbance of the anti-SRBC IgM standards.

2.5 Peroxisomal enzyme activity

Liver peroxisomal acyl-coenzyme A oxidase 1 (ACOX-1) activity was measured in liver homogenates as previously described (Rushing et al. 2017; McDonough et al. 2020; Woodlief et al. 2021). Briefly, an aliquot of frozen liver tissue was homogenized and protein concentrations were determined using the Bradford protein assay. Liver homogenate was then added to a 96 well plate in duplicate and the oxidation of leuco-DCF to DCF was measured in a plate reader

taking kinetic readings once/minute for 10 minutes at 502 nm excitation. The nmoles/minute rate of ACOX-1 activity was adjusted by average liver protein and final ACOX-1 activity in nmoles/min/mg was determined.

2.6 Statistical analyses

Statistical analyses were performed using the Statistical Analysis System (SAS). Daily body weights were analyzed by two-way ANOVA with the between-subjects variable being dose and the within-subjects variable being day. Within each sex a one-way ANOVA by dose was used to analyze the remaining data (organ weights, anti-SRBC IgM, peroxisomal enzyme activity). Pairwise post hoc t-tests with a Tukey's adjustment for familywise error rate were made if the F-statistic was statistically significant ($p<0.05$). One female animal in the 5 mg/kg dose group was identified as having signs of infection at the study endpoint including a white kidney and enlarged spleen, analyses were performed with and without the animals exhibiting signs of infection and final statistical analyses were performed without the inclusion of this animal.

3. Results

3.1 Terminal body weights

There was no statistical difference in terminal body weight of female or male animals when compared to controls (Figure 1).

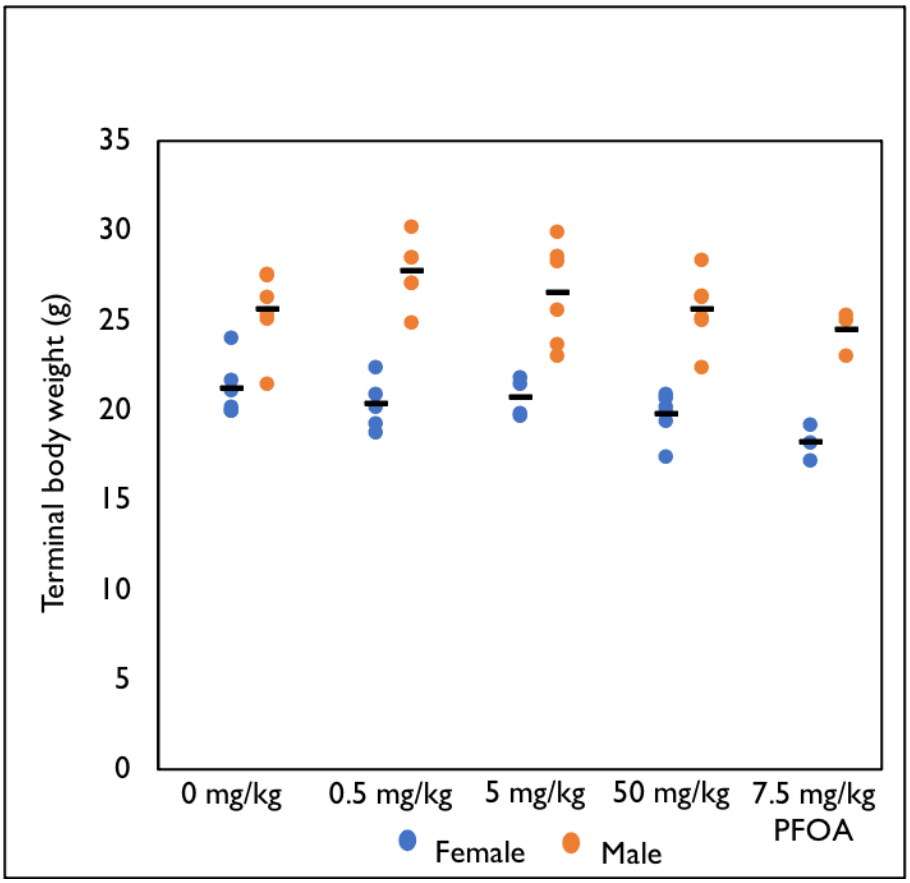

Figure 1: Terminal body weights in female and male C57BL/6 mice orally exposed either perfluoro-2-methoxyacetic acid (PFMOAA) or perfluorooctanoic acid (PFOA) for 30 days via oral gavage. Terminal weights were collected one day after dosing ended.

3.2 Lymphoid organ weights

All three female PFMOAA dose groups had an 8.9% – 18% increase, on average, in relative spleen weights compared to controls. All three male PFMOAA dose groups and the female and male PFOA dose group all had decreases in relative spleen weights compared to sex-matched controls. Only the male PFOA dose group decrease was statistically significant ($p<0.05$) (Figure 2).

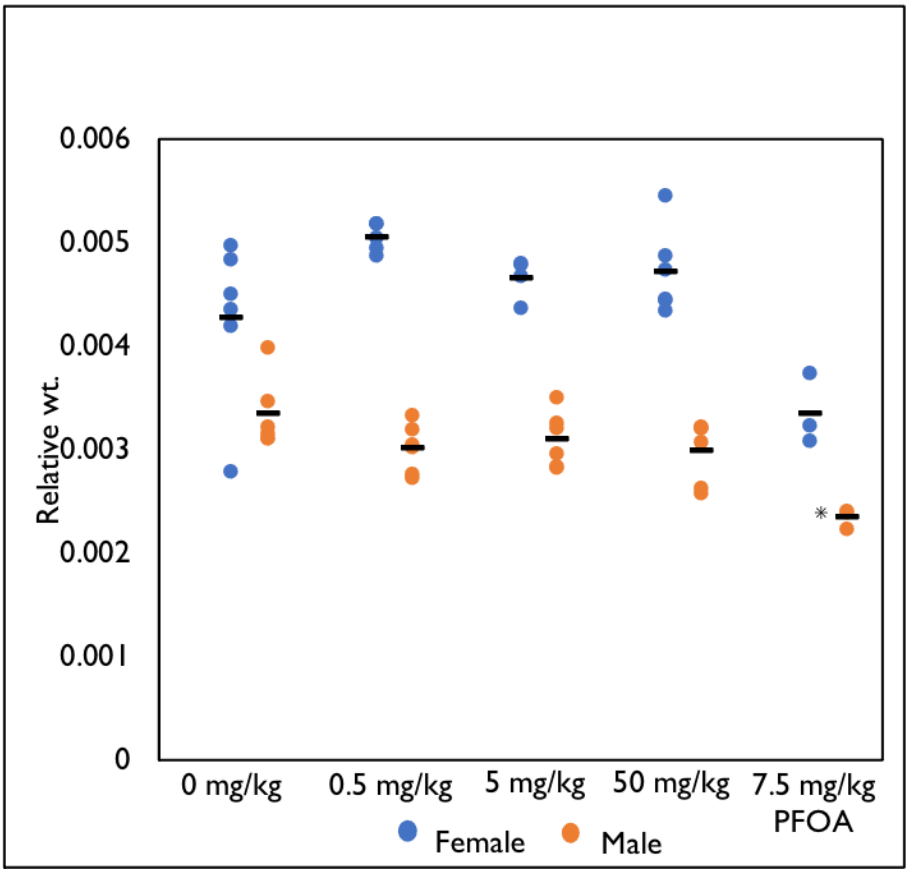

Figure 2: Relative spleen weights in C57BL/6 mice Relative spleen weights (spleen weight adjusted by body weight) in female and male C57BL/6 mice orally exposed either perfluoro-2-methoxyacetic acid (PFMOAA) or perfluorooctanoic acid (PFOA) for 30 days via oral gavage. Spleen weights were collected one day after dosing ended. *indicates statistical difference ($p<0.05$) from control

No statistically significant changes in relative thymus weights were observed. All three female PFMOAA dose groups had an 3.7% – 21% increase, on average, in relative thymus weights compared to controls. All three male PFMOAA dose groups had a 12% – 18% decrease, on average, in relative thymus weights compared to control. The relative thymus weights of female and male PFOA dose groups were within 3% of controls (Figure 3).

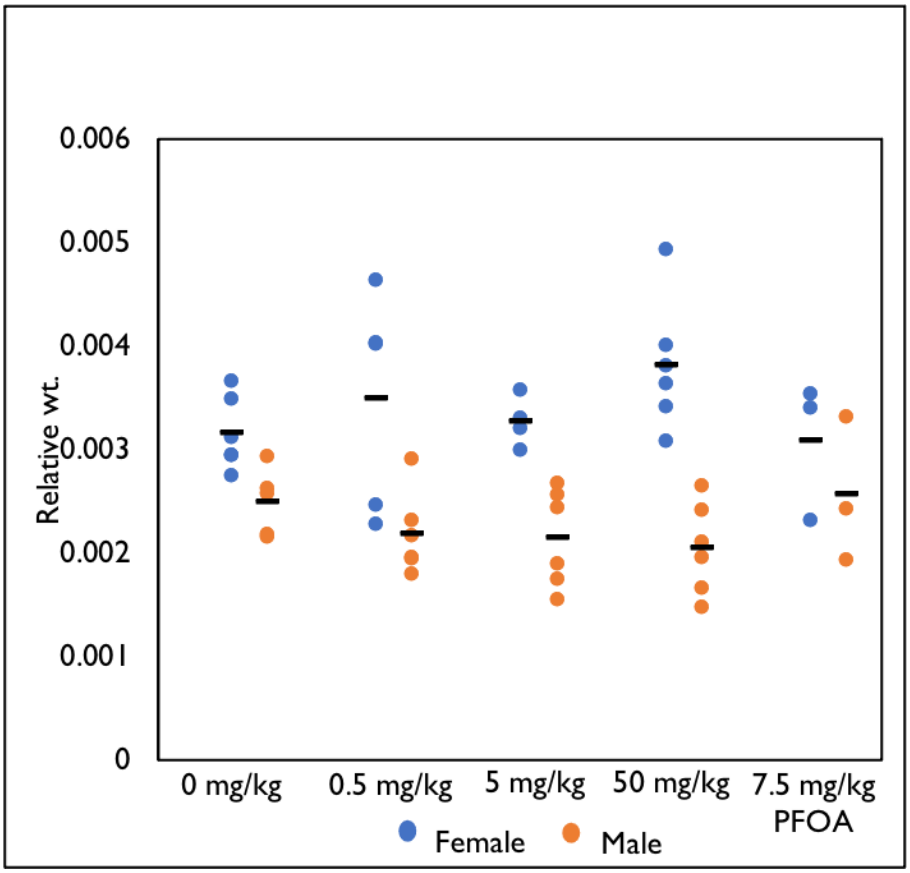

Figure 3: Relative thymus weights (thymus weight adjusted by body weight) in female and male C57BL/6 mice orally exposed to exposed to either perfluoro-2-methoxyacetic acid (PFMOAA) or perfluorooctanoic acid (PFOA) for 30 days via oral gavage. Thymus weights were collected one day after dosing ended.

3.3 Liver weights

Relative liver weights from female and male animals in the 0.5 mg/kg PFMOAA dose groups and from female animals in the 5 mg/kg dose group were not statistically different from controls. Other dose groups statistically different from sex-matched controls included: females in the 50 mg/kg PFMOAA dose group (43.9% larger than controls), males in the 5 and 50 mg/kg PFMOAA dose groups (22.5% and 153.6% larger than controls, respectively), and females and males in the 7.5 mg/kg PFOA dose groups (221.7% and 150.0% larger than controls, respectively) (Figure 4).

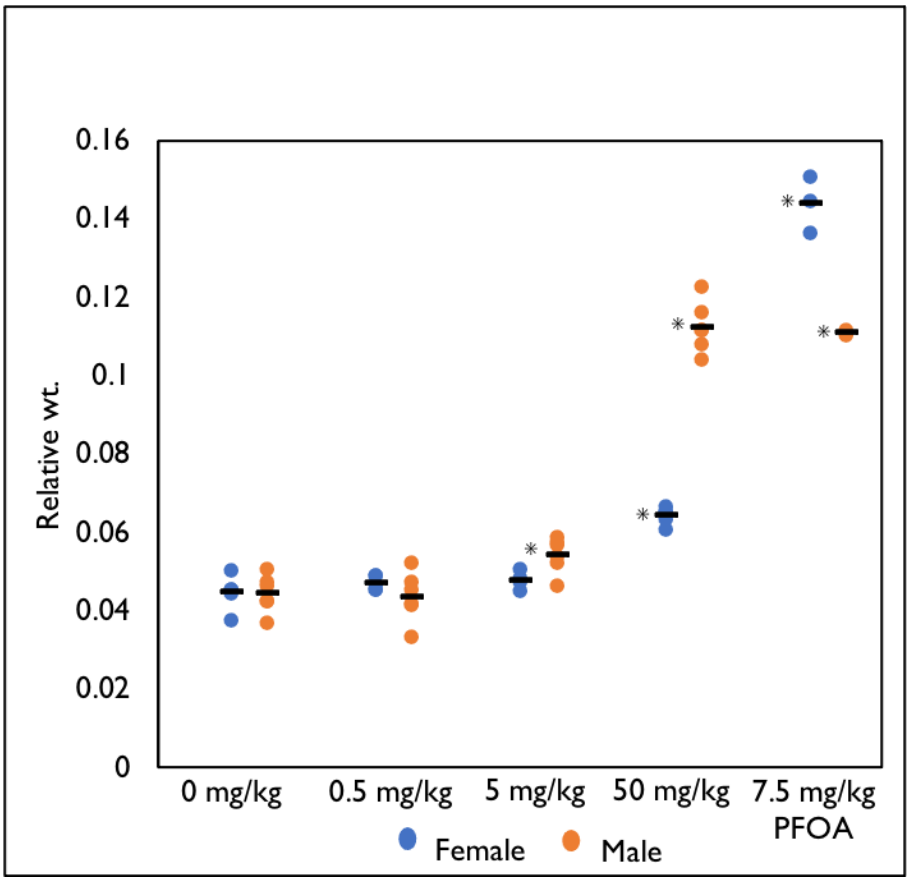

Figure 4: Relative liver weights (liver weight adjusted by body weight) in female and male C57BL/6 mice orally exposed to exposed to either perfluoro-2-methoxyacetic acid (PFMOAA) or perfluorooctanoic acid (PFOA) for 30 days via oral gavage. Liver weights were collected one day after dosing ended. *indicates statistical difference ($p<0.05$) from control

3.4 IgM antibody response

In females dosed with 0.5 mg/kg PFMOAA, anti-SRBC IgM concentrations were statistically higher than in controls and no other female dose groups were statistically different from controls (Figure 5). The female 7.5 mg/kg PFOA dose group had a 47% reduction in IgM. Males dosed with PFMOAA had decreasing IgM titers in a dose-responsive manner with the 0.5, 5, and 50 mg/kg dose groups having a 9.8%, 20.2%, and 54.6% decrease in IgM respectively relative to controls. Suppression of IgM in males was statistically significant in the 50 mg/kg PFMOAA and the 7.5 mg/kg PFOA dose groups.

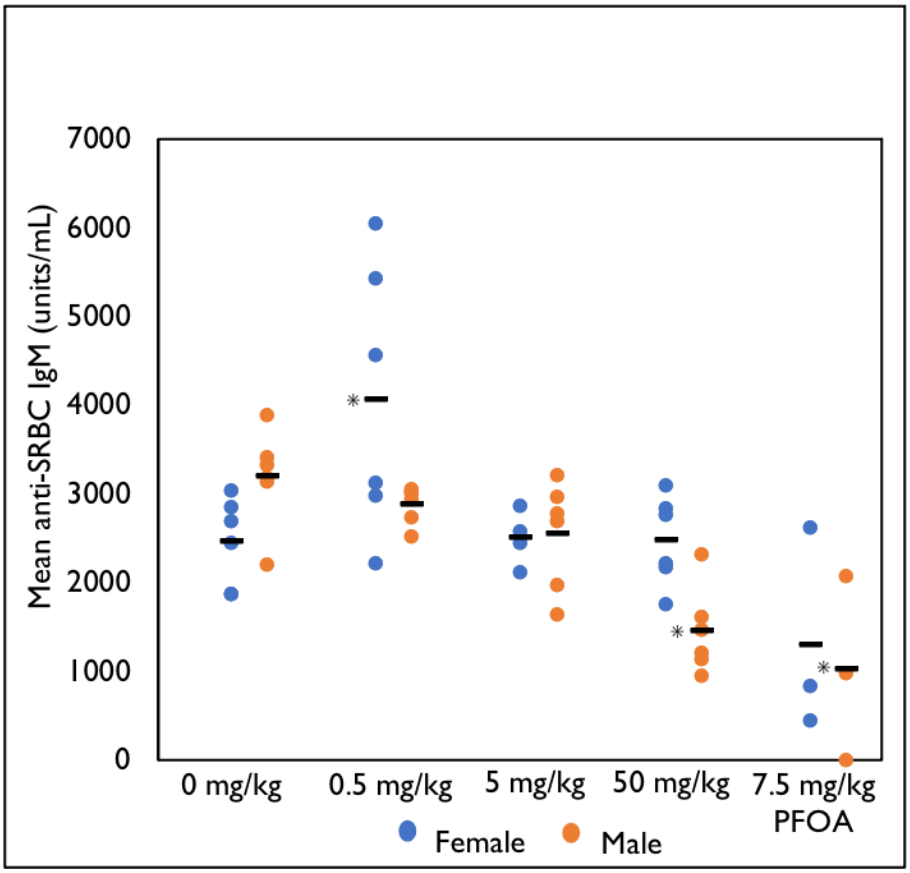

Figure 5: Anti sheep red blood cell (SRBC) immunoglobulin M (IgM) antibody titers in female and male C57BL/6 mice orally exposed to either perfluoro-2-methoxyacetic acid (PFMOAA) or perfluorooctanoic acid (PFOA) for 30 days. Concentrations were measured in sera collected one day after dosing ended and five days after animals were immunized with sheep red blood cells. *indicates statistical difference from control ($p<0.05$)

3.5 Peroxisomal enzyme activity

None of the three female PFMOAA dose groups had a statistically significant difference in peroxisome proliferation when compared to controls. The highest 50 mg/kg PFMOAA male dose group did have a statistically significant increase in peroxisomal proliferation of 263% compared to controls, and both female and male PFOA dose groups had a statistically significant increase in peroxisomal proliferation of 98% and 163% respectively compared to controls. ($p<0.05$).

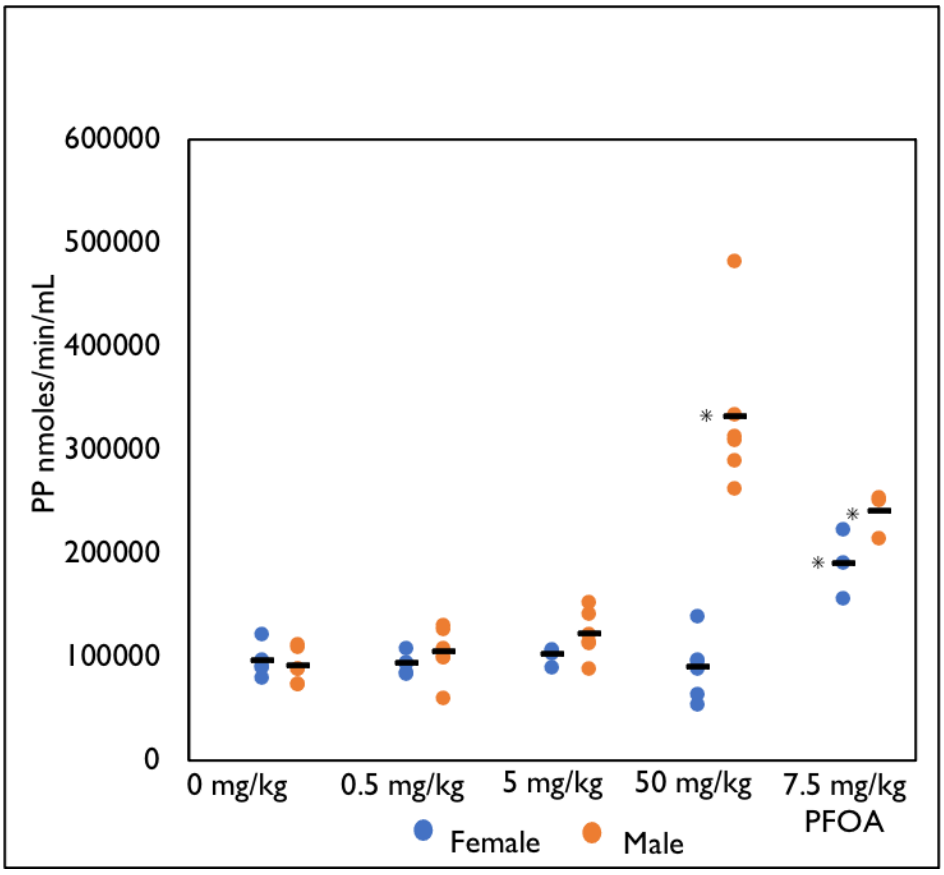

Figure 6: Peroxisome proliferation (nm/min/mL) in in female and male C57BL/6 mice orally exposed to either perfluoro-2-methoxyacetic acid (PFMOAA) or perfluorooctanoic acid (PFOA) for 30 days. Measured in liver tissue collected one day after dosing ended. *indicates statistical difference from control ($p<0.05$)

4. Discussion

To our knowledge very little data exist on the toxicity of PFMOAA and other PFECAs in the peer-reviewed literature. An exploratory immunotoxicity study in which male and female C57BL/6 mice were exposed to very low doses of PFMOAA (0, 0.00025, 0.025, or 2.5 mg/kg) reported only a slight increase in liver weight in the highest dose group and no statistical changes to immunophenotype, anti-SRBC IgM concentrations, or peroxisomal enzyme activity (Woodlief et al. 2021). PFMOAA was identified by Strynar et al. (2015) in the Cape Fear River of North Carolina (NC) using nontargeted screening methods, and PFMOAA was identified again by

McCord and Strynar (2019) in water samples taken from the CFR in June of 2018 and February 2018. Though it is thought that the short chain length of PFMOAA allows it to be eliminated rapidly from the body, a study in Shandong Province, China, identified PFMOAA in the serum of >95% of residents living near a fluorochemical plant (Yao et al., 2020). PFMOAA has a short chain length of just three carbons and Yao et al. (2020) reported it in higher concentrations in human serum than in the nearby river (median: 8.732 ng/mL). This study by Yao et al. (2020) indicates that we do not yet fully understand elimination kinetics of many novel PFAS (Yao et al. 2020). Sex-based differences in accumulation were also identified with serum PFMOAA being higher in males (median: 14.88 ng/mL) than in females (median: 10.89 ng/mL). Unlike many other PFECAs, the body burden of PFMOAA was observed to increase with age and was observed in high levels in children under 1 year old (median: 11.32 ng/mL).

As PFMOAA is known to be present in NC surface and drinking water and PFMOAA has been observed to accumulate in human serum, it is important to describe the potential toxicological outcomes of exposure as humans have already been exposed to this substance without their consent (McCord and Strynar 2019; Strynar et al. 2015b; Yao et al. 2020). Suppression of antigen-specific antibody production/vaccine responses are outcomes that have been observed after exposure to several PFAS, including PFOA, PFOS, PFHxS, and PFDA (ATSDR, 2021) and other studies have also shown that exposure to PFAS can produce sex-dependent immunological outcomes (Fenton et al. 2021; Kvalem et al. 2020; DeWitt, Blossom, and Schaider 2019). The present study found that administered doses of PFMOAA statistically increased liver weights, increased rates of peroxisomal proliferation, and statistically suppressed the TDAR in male animals. The observed increase in relative liver weight for males in the highest PFMOAA dose group (50 mg/kg) compared to controls was over 150% and was equal to

the increase in liver weight observed in PFOA-treated animals (also 150%). Alternatively, females exposed to 50 mg/kg of PFMOAA had a ~40% increase in liver weight whereas the PFOA-exposed females had liver weight increase of ~220%. A similar trend was observed for the TDAR between females and males; females exposed to PFMOAA did not have statistical suppression of the TDAR whereas males in the 50 mg/kg dose group had a ~55% decrease in IgM production. Both females and males exposed to PFOA had a ~47% and ~68% reduction in the TDAR, respectively (though only the reduction in male TDAR was statistically significant). The differential outcomes of PFMOAA exposure between females and males observed in the present study is similar to the observations made by Yao et al. (2020). Currently work is being done to quantify the PFMOAA levels in serum collected from the present study to gain a better understanding of the any sex-dependent differences in how PFMOAA accumulates in C57BL/6 mice.

The results of PFMOAA exposure in C57BL/6 mice indicate this chemical may have the potential to elicit similarly immunotoxic outcomes to legacy PFAS. A reduction in the TDAR of male animals was observed in the highest dose group, though no statistically significant alterations in lymphoid organ weights were seen in males. Male animals also had a statistically significant increase in liver weights and peroxisomal proliferation, an effect observed repeatedly after exposure to legacy PFAS in C57BL/6 mice. An additional cause of concern related to the presence of PFMOAA in surface is the difficulty water filtration methods have in removing PFMOAA. A study assessing how effective point-of-use residential filters are in removing PFAS from drinking water found PFMOAA to be the least effectively removed PFAS across filter classes (Herkert et al., 2020). Though some filter types, such as a reverse osmosis system, were found to remove >90% of PFMOAA, activated carbon countertop and whole house granular

activated carbon filters were found to increase levels of PFMOAA in filtered water by 498% and 106% respectively indicating filters can desorbing PFMOAA back into the finished water (Herkert et al. 2020).

5. Conclusions and future work

Though few studies exist into this substance based on the results of this study more research is warranted into potential health outcomes from exposure. The presence of PFMOAA in the environment in a minimum of two known geographical locations, its ability to elicit some immunotoxic effects and other sublethal effects, and the inability common water filters have in removing PFMOAA from drinking water are all concerning facts when viewed cumulatively. Further study into the distribution and toxicity of PFMOAA is needed to best protect environmental health. Other endpoints collected during the present study are still in the process of being evaluated which will contribute to our understanding of the immunotoxic outcomes associated with PFMOAA exposure, these other endpoints include; terminal serum PFMOAA concentrations, PFMOAA levels in urine collected periodically throughout the study, an evaluation of natural killer (NK) cell cytotoxicity, and the quantification of some B-cell, T-cell, and NK cells subpopulations in spleen and thymus tissue.

Chapter 3: The Immunotoxic Effects of Nafion by-product 2 in C57BL/6 Mice

1. Introduction

Per- and polyfluoroalkyl substances (PFAS) are anthropogenic worldwide contaminants of concern with over 9,000 PFAS identified ("PFAS|EPA: PFAS Structures in DSSTox" 2020). Though this large class of substances can be broken down into subclasses, PFAS can be defined as "chemicals with at least one aliphatic perfluorocarbon moiety"(Kwiatkowski et al. 2020). This basic structure imparts stability and water and oil repellent qualities, and as a result PFAS have been developed for a wide variety of uses (e.g. firefighting foams, textiles, cosmetics, medical devices, non-stick products) as they possess the desired characteristics for the manufacturing of myriad products (KEMI (Swedish Chemicals Agency) 2015).

The stability of the carbon-fluorine bond results in PFAS having long environmental half-lives as the vast majority are resistant to chemical, physical and biological degradation (Rahman, Peldszus, and Anderson 2014). Historically from the 1940s to 2000s two groups of PFAS were commonly used in manufacturing: long-chain perfluoroalkyl sulfonic acids (PFSAs, $C_n F_{2n+1}SO_3H$; $n \geq 6$), and long-chain perfluoroalkyl carboxylic acids (PFCAs, $C_nF_{2n+1}COOH$; $n \geq 7$) (Buck et al. 2011; Lindstrom, Strynar, and Libelo 2011). Mounting evidence into the toxic, persistent, and bioaccumulative qualities of the most commonly used PFSA and PFCA [perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA), respectively], and the discovery of their widespread presence in wildlife and humans has resulted in some governmental oversight of these long chain PFAS and their use being phased out by many major manufacturers in the United States (US) (Giesy and Kannan 2001; Kannan et al. 2004; KEMI (Swedish Chemicals Agency) 2015). Short-chain PFAS are now being developed and used as alternatives to the long-chain PFAS that have been phased out of many products and processes

(Sun et al. 2016). For many of the short-chain substances little is known about their persistence and toxicological effects – causing concern in communities where short-chain PFAS have been found in drinking water (Hopkins et al. 2018).

PFAS contamination in the drinking water of eastern North Carolina (NC) was first brought the public's attention in 2017 when an article in the Wilmington *Star News* reported that a short-chain perfluoroether acid (PFEA), called by the trade name GenX, was found in drinking water sourced from the Cape Fear River. The resulting public outcry increased pressure on researchers to identify and describe toxicological effects of GenX and other novel short-chain PFAS found in eastern NC drinking water. Nafion by-product 2 is a novel 6-carbon perfluoroalkyl ether sulfonic acid (PFESA) with two oxygen ether linkages. NBP2 is believed to be a by-product of a sulfonated tetrafluoroethylene-based fluoropolymer-copolymer with the trade name Nafion. Recently, Kotlarz et al. (2020) reported on a selection of legacy and novel PFAS measured in the blood of adults and children residing in Wilmington, NC. NBP2 was found in the blood of 99% of study participants, found in children as young as six, and present in residents at least five months after discharge control of the Nafion waste streams was put in place by the fluorochemical manufacturing facility from which the NBP2 was likely emitted (Kotlarz et al. 2020). An estimated 280,000 residents of eastern NC were likely impacted by the contamination (Kotlarz et al. 2020).

The knowledge that residents of eastern NC have been exposed to NBP2 makes the characterization of the toxicity of NBP2 in the best interest of public health, as at this time little toxicological data can be found on NBP2 (Guillette et al. 2020; Lang et al. 2020; Kotlarz et al. 2020). Immunotoxicity is a health effect that has emerged across multiple PFAS, and both PFOA and PFOS are presumed to be immune hazards to humans as assessed by the US National

Toxicology Program(US NTP 2016). As NBP2 is structurally similar to PFOA and PFOS in terms of chain length, it may exhibit similar immunotoxicological effects. The aims of this study were to therefore evaluate immune-specific effects of NBP2, as well as general toxicological parameters.

2. Methods

2.1 Experimental design

A total of 24 male and 24 female C57BL/6 mice aged 6-8 weeks were purchased from Charles River Laboratories (Raleigh, NC, USA) and delivered to the East Carolina University (ECU) Brody School of Medicine animal facilities (accredited by the Association for Assessment and Accreditation of Laboratory Animal Care). C57BL/6 mice have been shown to be sensitive to the immune effects of legacy PFAS (DeWitt et al. 2009). Upon arrival, animals were weighed and distributed, based on body weights, in groups of three to polycarbonate cages containing pine shaving bedding, one nestlet, and one hiding tube. Animals were housed in temperature controlled animal rooms (23±3°C) on a 12 hour light:dark cycle with 50±20% relative humidity, and were allowed access to food and water *ad libitum*. An acclimation period of at least five days was observed where no further manipulations occurred to the animals before the study began. All procedures in this study were approved in advance by the ECU Institutional Animal Care and Use Committee.

Mice were randomly assigned to one of four dose groups by cage, with six male and six female animals/dose group. Based on previous studies this is a sufficient number of mice/dose group to detect changes in immune endpoints. Animals were weighed daily and administered NBP2 (purchased from SynQuest Laboratories) at concentrations of 0, 0.5, 1.0, or 5.0 mg/kg of body weight. Dosing solutions were made fresh weekly containing the appropriate concentration

of NBP2 mixed in a solution of sterile water + 0.5% Tween-20, and dosing solutions were administered daily via oral gavage for 30 days; 0 mg/kg dose groups were gavaged with sterile water + 0.5% Tween-20. Urine was collected and pooled by cage 24 hours after the first dose was administered and on day 15 of dosing. Pooled urine samples were stored at -80°C for later analysis of NBP2 (see section 2.3). The health of the animals was monitored daily by researchers administering dosing solutions and by animal care staff.

Prior to this study a pilot study following the same experimental design was performed but that included a dose group receiving NBP2 at 50.0 mg/kg body weight. At the time of the pilot study no published rodent toxicity studies of NBP2 were found; dosages were chosen based on previous studies of other PFEAs. By day four of the study animals receiving 50 mg/kg NBP2 had experienced rapid weight loss of ~20% body weight, and the decision was made to stop dosing the animals and remove them from the study. After consultation with veterinary staff the decision was made to humanely euthanize these animals six days after dosing began as the animals had lost additional weight. At the time of euthanasia, it was noted that the animals' livers were enlarged and weighed ~4 g (on average, livers from untreated male animals weighed ~1.2 g). The doses in the current study therefore were chosen based on the pilot study results to reduce overt toxicity.

2.2 Serum and tissue collection

24 hours after dosing ended mice were anesthetized with Isoflurane. Blood was collected by neck vein transection and then held at room temperature. Serum was separated from the clotted blood within one hour of collection by centrifugation at 14,000 RPM for 7 minutes at 4°C, then was collected and frozen at -80°C. Aliquots of serum were later analyzed for NBP2 levels as described in section 2.3.

Immediately following euthanasia and serum collection, selected organs (spleen, thymus, brain, heart, kidney, lungs, and liver) were excised and weighed. Spleens and thymuses were immediately placed in RPMI + 1% fetal bovine serum (FBS) medium on ice. Livers were individually stored in aluminum foil squares, snap frozen on dry ice, and stored at -80°C for later analyses. Other organs were archived at -80°C.

2.3 Serum and urine analysis of Nafion by-product 2

NBP2 standard was directly infused to generate the tandem mass spectrometry (MS/MS) method followed injections to generate the standard liquid chromatography-tandem mass spectrometry (LC-MS/MS) response. Serum and urine samples were prepared according to the methods below and following analysis, the integrated peak area for the primary product ion transition m/z 463→216 was used to compare to the known concentration standard response. The spiked solution recovery was 100 (±6)%.

Formic acid and high performance liquid chromatography (HPLC) grade solvents were from Sigma Aldrich. Purified 18 MΩ DI water was generated using a Siemens high-purity water system. Standard NBP2 (SynQuest Laboratories) was dissolved in 50% methanol/50% deionized (DI) H_2O before dilution to desired concentrations. Serum or urine aliquots (25 μL) were vortex mixed with 50 mL formic acid for 5 minutes on ice. Acetonitrile (500 μL) was added and the samples were vortexed and placed on ice for another 5 min. Samples were then centrifuged at 2000 rpm for 3 min. The supernatant was collected, dried under a stream of N_2, and resuspended in 50% deionized water/50% methanol before diluting to a desired concentration in the same solvent. LC-MS/MS was performed using a SciEx LCMS 3200 system equipped with a Gemini 3 μM NX-C18 110 Å (50 x 2 mm) column. Isocratic chromatographic separation was performed using 70% acetonitrile/30% 2mM ammonium acetate mobile phase with a flow rate of 0.3000

mL min^{-1}, injection volume 1.00 μL, oven temperature 30°C, and analysis time of 5 min. Electrospray ionization (ESI) source conditions were negative ion mode, temperature 300°C, declustering potential -55V, entrance potential -6V, and capillary voltage -1.0 kV. Monitored mass transitions were m/z 463→213 (Collision Energy (CE) -50 V, collision cell exit potential (CXP) -2V) as quantifying transition with m/z 463→185 (CE -60 V, CXP 0V) and m/z 463→69 (CE -90V, CXP 0V) as qualifying transitions. The daily peak area responses for Nafion by-product 2 were normalized by comparing to a same day PFOA injection to compensate for any day-to-day signal variations.

2.4 Measurement of the T cell-dependent antibody response (TDAR)

On the 26^{th} day of dosing all animals were immunized with 4.0 x 10^7 sheep red blood cells (SRBC) in 0.2 mL of sterile saline. Anti-SRBC immunoglobulin M (IgM) antibody titers were determined in sera collected from animals five days after SRBC immunizations, which is the peak of antigen-specific IgM production in this strain of mice, with an enzyme-linked immunosorbent assay (ELISA) kit (Life Diagnostics, SRBCM-1). Briefly, serum samples were diluted 50-fold with diluent provided by the manufacturer and then added in duplicate, along with anti-SRBC IgM standards, to a 96-well plate pre-coated with SRBC membranes. Wells containing just the diluent were included as blanks. Plates were covered with sealing film and incubated at room temperature for 45 minutes on an orbital shaker at 75 rpm. Wells were washed 5x in 1x wash solution and tapped dry. Enzyme conjugate reagent was added to each well, and plates were re-covered and incubated at room temperature for 45 minutes on an orbital shaker at 75 rpm. After incubation, plates were washed 5x in 1x wash solution and tapped dry. TMB reagent was added to each well, plates were re-covered and incubated at room temperature for 20 minutes on an orbital shaker at 75 rpm. After incubation, stop solution (1N HCl) was added and

carefully mixed into each well and optical density at 450 nm was measured immediately on a microplate reader. Results were calculated by computing a standard curve based on absorbance of the standards and fitting each sample onto this curve to solve for the concentration of anti-SRBC IgM.

2.5 Immunophenotyping

Immunophenotyping was performed as previously described(McDonough et al. 2020). After collection, spleen and thymus were processed into single celled suspensions with RPMI+1% FBS added to each sample. The suspensions were then passed through a 70 μm cell strainer, centrifuged, decanted, and resuspended in 5 mL of red blood cell lysis buffer for 4-5 minutes with occasional shaking. 20-30 mL of 1x PBS was added, samples were again centrifuged, and supernatant was decanted. The suspensions were counted on a Nexcelom Bioscience Cellometer Auto 2000 cell counter (Nexcelom Bioscience LLC) to determine total cell count and viability, and all were adjusted to a final concentration of 2 x 10^7 cells/mL. Spleen and thymus cells were resuspended in non-specific Fc-mediated interaction blocking solutions. One aliquot of thymus cell suspension was taken for counting of T cells by flow cytometry, and two aliquots of spleen cell suspension were taken for counting of T cells and of B cells + natural killer (NK) cells by flow cytometry.

Monoclonal antibodies (eBioscience) coupled with fluorochrome specific markers were added as follows: for spleen and thymus T cell-specific subsamples APC anti-mouse CD3e, FITC anti-mouse CD4, and PE anti-mouse CD8a primary antibodies were added. For a second spleen B and NK cell-specific subsample FITC anti-mouse CD45RB and PE anti-mouse NK1.1 primary antibodies were added. An unstained (negative) control and single stained (positive) controls were included for each set of subsamples. After the addition of antibodies, samples were

incubated, washed, and resuspended in flow cytometry staining buffer 3x for flow cytometric analysis (BD LSRII flow cytometer, BD sciences). 10,000 events were collected per sample and dead cells and debris were excluded using forward scatter and 90° light scatter to establish a gate around viable cell populations. Unstained controls and single stained controls were used to determine positive populations for T cells, B cells, and NK cells. Cells were gated based on CD3 expression for subsequent analysis of CD4/CD8 T-subpopulations, but not B and NK cell subpopulations. The specific organ cellularity was used to determine the total number of each cell type.

2.6 Peroxisomal enzyme activity

Peroxisome enzyme activity was measured in liver homogenates via acyl CoA oxidase activity as a way to indirectly estimate peroxisomal proliferation. Horseradish peroxidase was used to catalyze the H_2O_2 -dependent oxidation of leuco-DCF to DCF. Details of the procedure are as follows:

In 1.5 mL Eppendorf tubes, 100 mg of liver tissue (stored at -80°C from collection until use) + 1 mL homogenization buffer (0.25 mM sucrose, 5 mM EDTA, and 20 mM Tris-HCL) was homogenized. Tubes were kept on ice before and after homogenization. Samples were then centrifuged at 12,000*xg* at 4°C for 20 minutes and supernatant was collected. Total protein concentrations were determined by the Bradford protein assay. The following were added per well to a 96 well plate: 64 µl of potassium phosphate buffer with horseradish peroxidase, 4 µl of 0.02% Triton X-100, and 79 µl of aminotriazole stock. 44 µl of liver homogenate was then added in duplicate to the plate, control wells instead were given 44 µl of potassium phosphate buffer. 3 µl of 0.05 mM leuco-DCF was added to each well to begin the reaction, and plate was placed in dark to incubate for 3 minutes. Quickly, 6 µl of 0.001 M palmitoyl-CoA was then added to each

well, and the plate was immediately placed into a plate reader where kinetic readings were taken at 502 nm once/minute for 10 minutes. The nmoles/min rate of peroxisome enzyme activity was adjusted by average liver protein (calculated using liver weights and protein concentration determined in Bradford protein assay) to determine the final peroxisome enzyme activity in nmoles/min/mg.

2.7 Natural killer cell cytotoxicity

The cytotoxicity of NK cells present in spleen homogenates was measured via the lysis of Yac-1 (SIGMA- 86022801) target cells. Yac-1 cells stored in liquid nitrogen were revived by warming in a water bath (37°C) and then transferred to warmed (37°C) cell medium containing RMPI (RPMI 1640) with 10% FBS, 1% pen-strep antibiotic, and 2mM L-Glutamine. Cells were incubated at 37°C in 5% CO_2 atmosphere. Before 100% confluency was reached cells were passaged, with 5-6 passages occurring total and the final passage occurring the day before the NK cell cytotoxicity assay was performed one day after dosing ended. For each passage, cells are diluted 1:10 with fresh, warmed cell medium following the same recipe previously described.

One day after the end of dosing, spleens were collected and placed in RMPI+1% FBS. Spleens were processed into a single-cell suspension and adjusted to a final concentration of 2 x 10^7 cells/ml as described in *2.5 immunophenotyping*. Three aliquots of spleen suspension were taken per animal for target:effector (Yac-1:NK) ratios of 30:1, 10:1, and 5:1. After an aliquot of Yac-1 cells was removed for an unstained control, Yac-1 cells were centrifuged at 300*xg* for 5 minutes, decanted, resuspended in 300 nM MTGreen solution (MitoTracker green FM + dimethylsulfoxide) and incubated at 37°C for five minutes, cells were centrifuged, supernatant poured off, and this process was repeated until effector cells were ready. The prepared Yac-1 cells were added to each of the three target:effector cell ratio tubes per animal and incubated for

3 hours at 37°C. A positive control (total cell lysis) was included by adding 1 mL of Yac-1 target cells to 5 µL of 5% Triton x-100, and a negative control (spontaneous release) was included as 1 mL of MTGreen labeled target cells. After incubation, 15 µL of propidium iodide solution (20% 1 mg/ml propidium iodide stock + 80% phosphate buffered saline) was added, samples were vortexed, then transferred to flow cytometer specific tubes for analysis. Unstained, positive, and negative controls were read first on the flow cytometer (BD LSRII flow cytometer, BD sciences) followed by the remaining samples. Percent specific lysis was calculated using the percentage of dead cells as follows: 100 x [(%dead cells - %spontaneous release) / (%living cells + (%dead cells - %spontaneous release))]

2.8 Statistical analyses

Statistical analyses were performed using the Statistical Analysis System (SAS). Dosing body weights collected daily were analyzed by two-way analysis of variance (ANOVA) with dose as the between-subjects variable and day as the within-subjects variable. A nested ANOVA was used to determine if variation within cage produced a statistically significant sub-group effect on final body weights. Remaining data were analyzed within each sex by one-way ANOVA by dose. If the F-statistic was statistically significant ($p<0.05$) for the overall model by dose, pairwise post-hoc t-tests were made with a Tukey's adjustment for the familywise error rate. One female from the 0 mg/kg dose group, one female from the 0.5 mg/kg dose group, and two females from the 1 mg/kg dose group were identified as having infections of the thoracic cavity at the study endpoint. Lung adhesion within the thoracic cavity, congestion, or masses in the thoracic cavity were observed in these animals. It appeared these infections were not dose related but were related to gavage error. Analyses were performed with and without animals that

had signs of infection at study terminus; final statistical analyses were performed without inclusion of animals that had shown these obvious signs of infection or injury to the lungs.

3. Results

3.1 Serum and urine NBP2 concentrations

NBP2 serum concentrations (Table 1) were consistently higher in males than females of the same dose group. The percent difference between males and females in the 0, 0.5, 1.0, and 5.0 mg/kg dose groups respectively was 15.1%, 40.2%, 46.5%, and 48.6% lower in males when compared to females.

Between days 1 and 15 in the 0, 0.5, 1.0, and 5.0 mg/kg dose groups, respectively, male urine NBP2 (Table 2) concentration changed by -72%, +908%, +807%, and +936.585% and female urine NBP2 concentrations changed by -41.6%, +338%, +550%, and +1133%. In the 0.5 mg/kg dose group, the day 1 and day 15 male urine had higher NBP2 concentrations than female urine for days 1 and 15. Alternatively, in the 1.0 and 5.0 mg/kg dose group the day 1 and 15 urine of females had higher NBP2 concentrations than the respective urine concentrations from males.

Table 1: Average (±SD) serum Nafion by-product 2 (NBP2) concentrations in male and female C57BL/6 mice orally exposed for 30 days. Sera for analysis was collected one day after dosing ended. * n=4 for each dose group.

Males		**Females**	
Dose of NBP2* (mg/kg/day)	**NBP2 (ng/mL)**	**Dose of NBP2* (mg/kg/day)**	**NBP2 (ng/mL)**
0	1.57 (±1.36)	0	1.35 (±1.13)
0.5	2270 (±465)	0.5	1510 (±1120)
1	4850 (±1240)	1	3020 (±1890)
5	24800 (±6430)	5	15100 (±6280)

Table 2: Average (±SD) urine Nafion by-product 2 (NBP2) concentrations in male and female C57BL/6 mice collected after 1 and 15 days of oral exposure. *n=3 trials

Males			**Females**		
Dose of NBP2* (mg/kg/day)	**NBP2 (ng/mL)**		**Dose of NBP2* (mg/kg/day)**	**NBP2 (ng/mL)**	
	Day 1	**Day 15**		**Day 1**	**Day 15**
0	5.27 (± 0.20)	1.46 (± 0.55)	0	1.23 (± 0.45)	0.718 (± 0.045)
0.5	23.2 (± 3.2)	234 (± 38)	0.5	19.0 (± 3.2)	83.3 (± 7.1)
1	36.6 (± 4.3)	332 (± 50)	1	66.2 (± 14.1)	430 (± 38)
5	164 (± 30)	1700 (± 250)	5	146 (± 37)	1800 (± 112)

3.2 Body and select organ weights

Animal body weights collected daily throughout the dosing period did not differ statistically among dose groups (data not shown). Final body weights collected one day after dosing ended also did not differ statistically (data not shown).

One day after dosing ended, the relative weight (absolute organ weight/terminal body weight) of brains and lungs did not differ statistically among dose groups. The relative weight of hearts from females in the 5.0 mg/kg dose group decreased statistically ($p<0.05$) by 8.28% relative to females in the 0 mg/kg group, but did not differ statistically among other dose groups or in males. The absolute weight of kidneys from males and females in the 5.0 mg/kg dose groups decreased statistically ($p<0.05$) by 23.5% and 16.9% relative to sex-matched animals in 0 mg/kg dose groups. The relative weight of kidneys from males in the 5 mg/kg dose group decreased statistically ($p<0.05$) by 17.7% relative to the 0 mg/kg dose group, but relative weight of kidneys did not differ in females.

3.3 Lymphoid organs

One day after dosing ended, the mean relative and absolute spleen and thymus weights were reduced ($p<0.05$) in males and females in the 5.0 mg/kg dose groups compared to sex-matched control animals (Table 3). When comparing the 5.0 mg/kg dose group with control animals the following was observed: The relative weight of spleens in males and females decreased statistically by 39.1% and 31.7% respectively, and absolute weight of spleens decreased statistically by 43.3% and 34.2% respectively. The relative weight of thymuses in males and females decreased statistically by 36.2% and 39.9% respectively, and the absolute weight of thymuses decreased statistically by 40.5% and 41.4% respectively.

Table 3: Absolute and relative (adjusted by body weight) lymphoid organ (spleen and thymus) weights in male and female C57BL/6 mice orally exposed to Nafion by-product 2 (NBP2) for 30 days. Lymphoid organs were collected one day after dosing ended. Data are presented as mean (±SD).

Male lymphoid organ weights				
Dose of NBP2 (mg/kg/day)	**Spleen weight (mg)**	**Relative spleen weight**	**Thymus weight (mg)**	**Relative thymus weight**
0.0	91.67 (±16.48)	0.0040 (±0.0006)	63.3333 (±17.6823)	0.0026 (±0.0008)
0.5	90.00 (±18.02)	0.0038 (±0.0007)	62.1667 (±10.1472)	0.0027 (±0.0005)
1.0	82.33 (±20.61)	0.0035 (±0.0009)	52.6667 (±8.7788)	0.0023(±0.0003)
5.0	52.00 (±9.98)*	0.0024 (±0.0004)*	37.6667 (±7.7115)*	0.0018 (±0.0004)*
Female lymphoid organ weights				
Dose of NBP2 (mg/kg/day)	**Spleen weight (mg)**	**Relative spleen weight**	**Thymus weight (mg)**	**Relative thymus weight**
0.0	100.00 (±12.9357)	0.0048 (±0.0005)	84.5 (±9.4692)	0.0040 (±0.0004)
0.5	101.50 (±14.7083)	0.0048 (±0.0005)	85.25 (±8.4212)	0.0041 (±0.0006)
1.0	92.50 (±4.9497)	0.0046 (±0.0005)	73.00 (±9.8995)	0.0036 (±0.0007)
5.0	65.8333 (±6.4936)*	0.0033 (±0.0004)*	49.50 (±23.8390)*	0.0024 (±0.0012)*

*indicates statistical difference ($p<0.05$) from control

3.4 Liver weights

One day after dosing ended mean relative and absolute liver weights increased statistically ($p < 0.05$) in animals exposed to 5.0 mg/kg NBP2 when compared to control animals (Table 4). The absolute weight of livers from males and females in the 5 mg/kg dose groups increased statistically ($p<0.05$) by 276.5% and 308.7% relative to sex-matched animals in the 0 mg/kg dose groups. The relative weight of livers from males and females in the 5 mg/kg dose groups increased statistically ($p<0.05$) by 306.9% and 321.1% relative to sex-matched animals in the 0 mg/kg dose groups. In both males and females exposed to 5.0 mg/kg, the mass of the livers were ~19% of the total body weight of the animals.

Table 4: Absolute and relative liver weights (adjusted by body weight) in male and female C57BL/6 mice orally exposed to Nafion by-product 2 (NBP2) for 30 days. Livers were collected one day after dosing ended. Data are presented as mean (±SD).

Male liver weights		
Dose of NBP2 (mg/kg/day)	**Liver weight (g)**	**Relative liver weight**
0.0	1.11 (±0.26)	0.0478 (±0.0086)
0.5	1.510 (±0.08)*	0.0644 (±0.0030)*
1.0	1.95 (±0.18)*	0.08335 (±0.0064)*
5.0	4.19 (±0.36)*	0.1946 (±0.0090)*
Female liver weights		
Dose of NBP2 (mg/kg/day)	**Liver weight (g)**	**Relative liver weight**
0.0	0.98 (±0.09)	0.0470(±0.0030)
0.5	1.15 (±0.11)	0.0547 (±0.0059)
1.0	1.42 (±0.17)	0.0693 (±0.0040)*
5.0	4.01 (±0.36)*	0.1978 (±0.0098)*

*indicates statistical difference ($P<0.05$) from control

3.5 IgM antibody response

Serum anti-SRBC IgM antibodies were suppressed in males and females from 5.0 mg/kg relative to antibody levels in sex-matched control animals (Figure 7). The anti-SRBC IgM antibody response was reduced by 93% ($p < 0.05$) in males and by 53% ($p < 0.05$) in females.

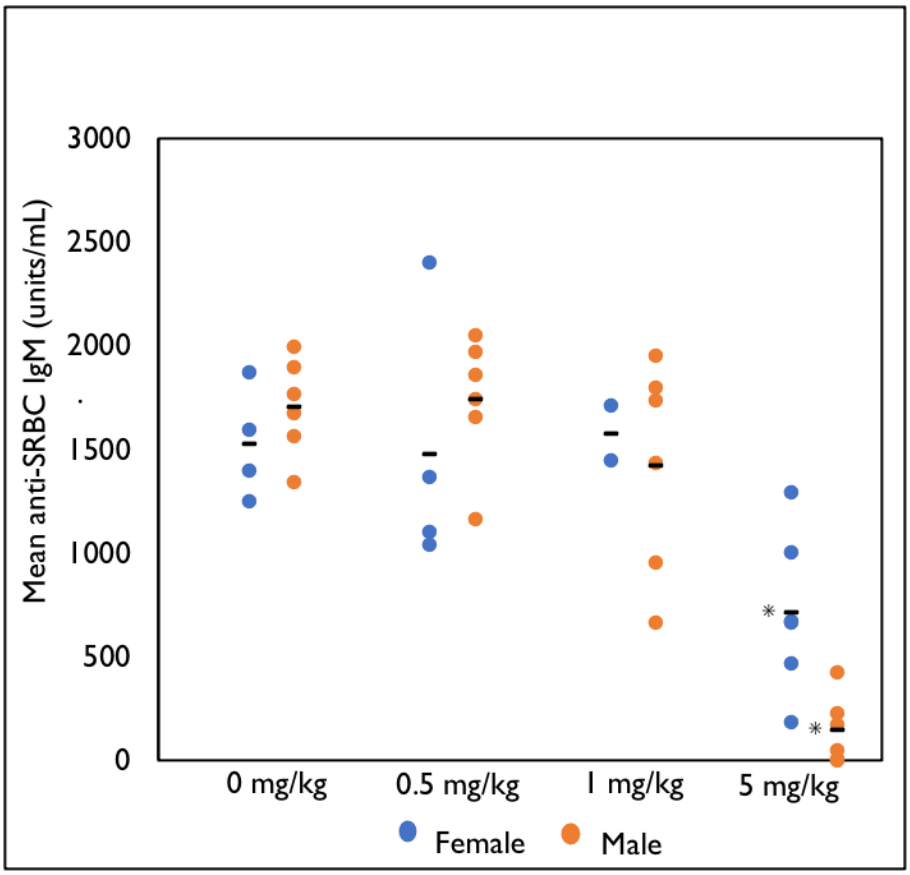

Figure 7: Anti sheep red blood cell (SRBC) IgM antibody titers in male and female C57BL/6 mice orally exposed to Nafion by-product 2 (NBP2) for 30 days. Concentrations were measured in sera collected one day after dosing ended and five days after animals were immunized with sheep red blood cells. *indicated statistical difference ($p<0.05$) from control

3.6 Immunophenotyping

3.6.1. Spleen cells

In males in the 5.0 mg/kg dose group, splenic $CD4^+$, $CD4^+/CD8^+$, and $CD4^-/CD8^-$ cell subpopulations were statistically ($p<0.5$) decreased when compared to subpopulations in control animals (Table 5). $CD4^+$ subpopulations decreased by 41.8%, $CD4^+/CD8^+$ subpopulations decreased by 58.1%, and $CD4^-/CD8^-$ subpopulations decreased by 56.5%. In females in the 5.0 mg/kg dose group, splenic $CD4^+$, $CD8^+$, andCD4-/CD8- cell subpopulations statistically ($p<0.5$) increased when compared to subpopulations in control animals. $CD4^+$ subpopulations increased

by 225.4%, $CD8^+$ increased by 266.8%, and $CD4^-/CD8^-$ increased by 133.4%. Females in the 5.0 mg/kg dose group had statistically ($p<0.5$) decreased NK and B cell subpopulations when compared to control animals, with NK cell subpopulations decreasing by 40.8%, and B cell subpopulations decreasing by 25.5%.

Table 5: Splenic lymphocyte subpopulations in male and female C57BL/6 mice orally exposed to Nafion by-product 2 for 30 days. Spleens were collected one day after dosing ended. Data are presented as mean (±SD).

Male Spleen immunophenotype											
Dose of NBP2 (mg/kg/day)	**Cellularity (cells/mg organ wt.) ($x10^5$)**	**NK cells ($x10^5$)**	**%NK cells**	**B cell ($x10^5$)**	**%B cells**	**$CD4^+$ T cells ($x10^3$)**	**%$CD4^+$T cells**	**$CD8^+$ T cells ($x10^3$)**	**%$CD8^+$ T cells**	**$CD4^-$ /$CD8^-$ cells ($x10^3$)**	**$CD4^+$ /$CD8^+$ cells**
0	4.76 (±0.75)	11.10 (±2.09)	2.6 (±0.49)	41.64 (±6.15)	9.7 (±0.48)	84.26 (±0.23)	61.42 (±1.50)	48.50 (±0.16)	34.95 (±1.60)	4.48 (±1.16)	440.37 (±146.12)
0.5	5.50 (±1.66)	12.85 (±4.39)	2.67 (±0.50)	50.84 (±15.22)	10.53 (±2.30)	96.46 (±0.38)	63.33 (±1.13)	49.88 (±0.19)	32.85 (±1.02)	5.09 (±1.60)	475.50 (±215.80)
1	2.87 (±0.74)	6.50 (±1.54)	2.85 (±0.36)	19.63 (±4.55)	8.63 (±1.16)	56.98 (±0.19)	61.17 (±0.79)	32.87 (±0.10)	35.48 (±0.39)	2.73 (±0.60)*	281.48 (±156.67)
5	2.49 (±1.04)	2.60 (±1.12)	2.03 (±0.24)	11.52 (±4.02)	9.22 (±0.57)	49.03 (±0.21)*	58.18 (±3.06)	33.35 (±0.14)	39.33 (±2.73)	1.94 (±1.00)*	184.15 (±83.20)*

Female spleen immunophenotype											
Dose of NBP2 (mg/kg/day)	**Cellularity (cells/mg organ wt.) ($x10^5$)**	**NK cells ($x10^5$)**	**%NK cells**	**B cell ($x10^5$)**	**%B cells**	**$CD4^+$ T cells ($x10^3$)**	**%$CD4^+$ T cells**	**$CD8^+$ T cells ($x10^3$)**	**%$CD8^+$ T cells**	**$CD4^-$ /$CD8^-$ cells ($x10^3$)**	**$CD4^+$ /$CD8^+$ cells**
0	8.66(±0.48)	22.78 (±2.32)	2.65 (±0.29)	73.15 (±8.42)	8.48 (±0.55)	79.47 (±87.00)	69.28 (±10.05)	40.09 (±45.73)	18.93 (±16.50)	4.81 (±5.40)	422.61 (±562.80)
0.5	11.82(±1.99)	29.33 (±7.60)	2.48 (±0.54)	80.41 (±6.91)	6.83 (±0.43)	215.72 (±49.83)*	61.60 (±0.97)	120.32 (±25.64)*	34.48 (±0.38)	12.51 (±3.68)*	1087.60 (±492.75)
1	11.20(±0.38)	24.82 (±0.70)	2.4 (±0.14)	66.03	6.35 (±0.49)	210.08 (±6.11)	63.65 (±0.64)	106.90 (±0.29)*	32.40 (±0.71)	12.04 (±.004)	990.05 (±18.89)
5	11.10(±2.07)	11.04 (±5.91)*	1.57 (±0.82)	53.27 (±8.22)	6.32 (±3.22)	258.64 (±60.58)*	61.72 (±1.36)	147.05 (±30.06)*	35.30 (±1.58)	11.231 (±2.03)*	1055.37 (±510.51)

3.6.2. Thymus cells

No statistical changes were observed for thymus cell subpopulations in males. In females from the 5.0 mg/kg dose group, thymus $CD4^+/CD8^+$ subpopulations (Table 6) statistically decreased ($p<0.5$) by 44.7% when compared to subpopulations in control animals. For thymus cell subpopulations in females, no other statistical differences were observed.

Table 6: Thymic lymphocyte subpopulations in male and female C57BL/6 mice orally exposed to Nafion Byproduct 2 for 30 days. Thymuses were collected one day after dosing ended. Data are presented as mean (±SD).

	Male thymus immunophenotype				
Dose of NBP2 (mg/kg/day)	**Cellularity (cells/mg organ wt.) (x10^5)**	**$CD4^+$ T cells (x10^5)**	**%$CD4^+$ T cells**	**$CD8^+$ T cells (x10^5)**	**%$CD8^+$ T cells**
0	11.09 (±2.51)	0.54 (±0.34)	8.33 (±2.79)	4.83 (±1.94)	76.40 (±4.52)
0.5	12.74 (±2.50)	0.99 (±0.55)	9.90 (±2.52)	5.72 (±1.34)	64.68 (±13.21)
1	13.49 (±2.07)	1.14 (±0.49)	10.42 (±2.01)	6.82 (±1.73)	65.73 (±7.14)
5	11.16 (±3.94)	0.84 (±0.52)	10.47 (±2.56)	5.13 (±1.77)	69.33 (±8.63)
	Female thymus immunophenotype				
Dose of NBP2 (mg/kg/day)	**Cellularity (cells/mg organ wt.) (x10^5)**	**CD4+ T cells (x10^5)**	**%CD4+ T cells**	**CD8+ T cells (x10^5)**	**%CD8+ T cells**
0	12.68 (±0.52)	1.19 (±0.35)	10.18 (±3.29)	6.32 (±0.65)	53.40 (±3.28)
0.5	13.56 (±2.35)	1.80 (±0.33)	14.25 (±1.01)	6.65 (±1.12)	52.90 (±4.27)
1	11.38 (±1.43)	1.47 (±0.15)	13.80 (±0.28)	5.56 (±0.98)	52.10 (±2.69)
5	10.30 (±2.36)	1.13 (±0.37)	11.95 (±2.15)	5.65 (±1.26)	60.23 (±3.95)

3.7 Natural killer cell assay

NK activity was decreased by 15.2%, 25.8%, and 17.7% respectively, in the 5:1, 10:1, and 30:1 effector:target cell ratio groups in males exposed to 5.0 mg/kg when compared to controls (Figure 8). NK cell activity was decreased by 29.8%, 28.4%, and 8.7% respectively, in the 5:1, 10:1, and 30:1 effector:target cell ratio groups in females exposed to 5.0 mg/kg when compared to controls (Figure 8). The decrease in activity was statistically significant ($p<0.05$) in 5.0 mg/kg groups compared to controls for the male 5:1 and 30:1 effector:target cell ratio groups, and in the female 10:1 and 30:1 effector:target cell ratio groups.

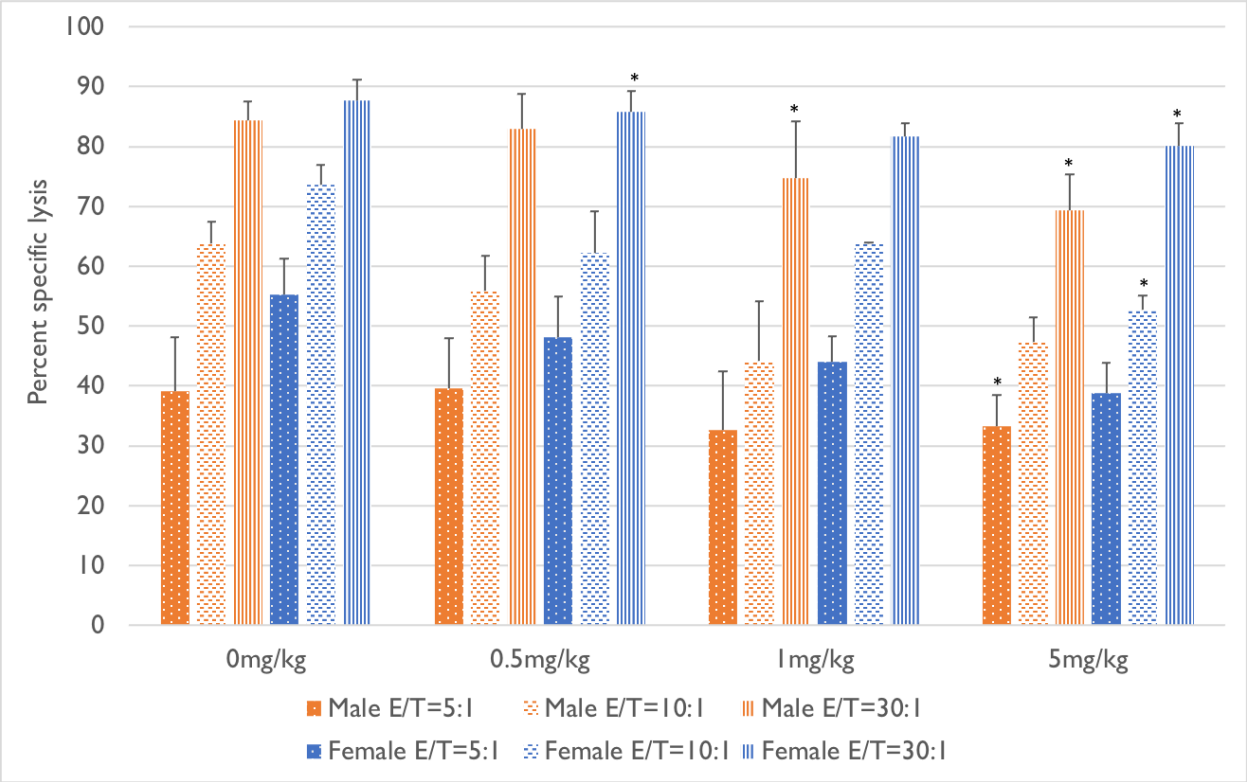

Figure 8: Natural killer (NK) cell percent specific lysis in male and female C57BL/6 mice orally exposed to Nafion by-product 2 (NBP2) for 30 days. NK cell activity was measured at 5:1, 10:1, and 30:1 effector:target cell ratios. *indicates statistical difference ($p<0.05$) from control. Data are presented as mean (±SD).

3.8 Peroxisome proliferation

Peroxisomal enzyme activity increased statistically by 475% in males ($p < 0.05$) and increased by 75% in females in the 5.0 mg/kg dose groups relative to sex-matched control groups (Figure 9).

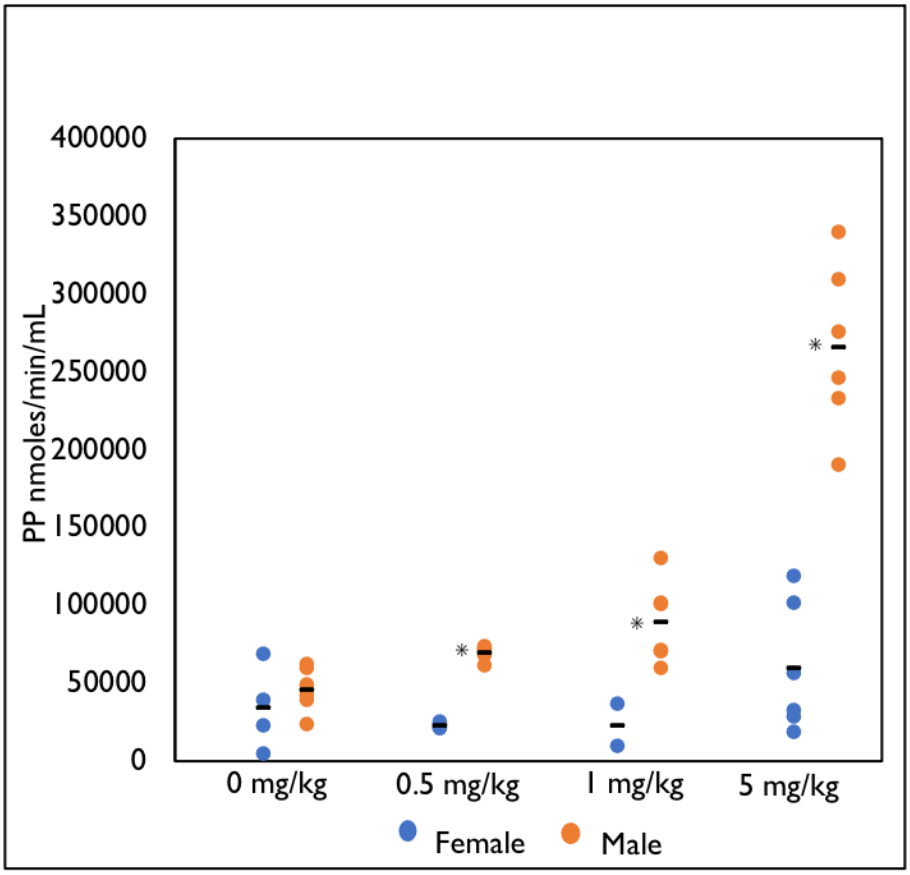

Figure 9: Peroxisome proliferation (nm/min/mL) in male and female C57BL/6 mice orally exposed to Nafion By-product 2 (NBP2) for 30 days. *indicates statistical difference ($P<0.05$) from control.

4. Discussion

4.1 Background

Strynar et al. (2015) (Strynar et al. 2015a) was the first to provide evidence of Nafion by-product emissions into the Cape Fear River (CFR). They identified short-chain PFOA replacement compounds in the CFR and two of PFEAs identified by Strynar et al. (2015) were suspected to be oxidation species of Nafion. In 2013, an onsite PFOA groundwater monitoring report from a Chemours facility located within the CFR watershed known as the Fayetteville Works plant referenced the production of Nafion membranes at the facility; it is assumed the waste streams from this production process were the source of NBP2 in the CFR. In 2018 Hopkins et al. (Hopkins et al. 2018) also identified two by-products of Nafion at a drinking water intake in the CFR believed to have originated from the Nafion membrane production area of Fayetteville Works. It was observed that NBP2 increased in concentration in the CFR between June 19, 2017 and July 15, 2017, causing the North Carolina Department of Environmental Quality and US EPA to increase their focus on the waste stream of Nafion by-products, ultimately leading to an agreement with the Fayetteville Works facility to capture wastewater containing Nafion by-products. Though the total PFEA concentration decreased in the CFR after wastewater controls were implemented, it was hypothesized by Hopkins et al. (2018) that surrounding river sediment, groundwater, and stormwater runoff would continue as sources of NBP2 contamination into the future. These fears were confirmed when in 2019 Nafion by-products were found again in the CFR identified by McCord and Strynar (2019)(McCord and Strynar 2019) using nontargeted screening methods. In 2020, Nafion by-products were again identified in the CFR by Guillette et al. (Guillette et al. 2020) with surface water concentrations of 18 ng/L, and Guillette et al. (2020) also found evidence that NBP2 accumulating in wildlife

with NBP2 being found in 78% of sampled fish and at serum concentrations in striped bass that were 17x higher than surface water concentrations. The NBP2 levels in striped bass observed by Guillette et al. were also associated with changes in liver biomarkers as well as immune system alterations and were negatively correlated with length and weight of the animals. NBP2 was found in human serum by Kotlarz et al. (2020)(Kotlarz et al. 2020) in a population who sources their drinking water from the CFR; NBP2 was found in 99% of study participants with a median concentration of 2.7 ng/mL. Though NBP2 has been identified continuously in the CFR, in wildlife, and in humans by the research community since 2015, there are a limited number of studies on the toxicological outcomes of exposure to NBP2 and to our knowledge this is the first immunotoxicity study performed with NBP2.

Due to NBP2 and PFOS having a similar total chain length (counting both C and O atoms, NBP2 is eight atoms long; PFOS has eight C), and both being sulfonic acids, there is a possibility that they may act through similar mechanisms. It has been established that in legacy PFAS, a longer perfluoroalkyl chain is associated with higher bioaccumulation potential (Ng and Hungerbühler 2014). Though NBP2 does not technically fit the definition of a long-chain PFAS developed by the US EPA under the PFOA Stewardship Agreement, it is a large molecule with a long chain length when ether-oxygen linkages are considered (Figure 10).

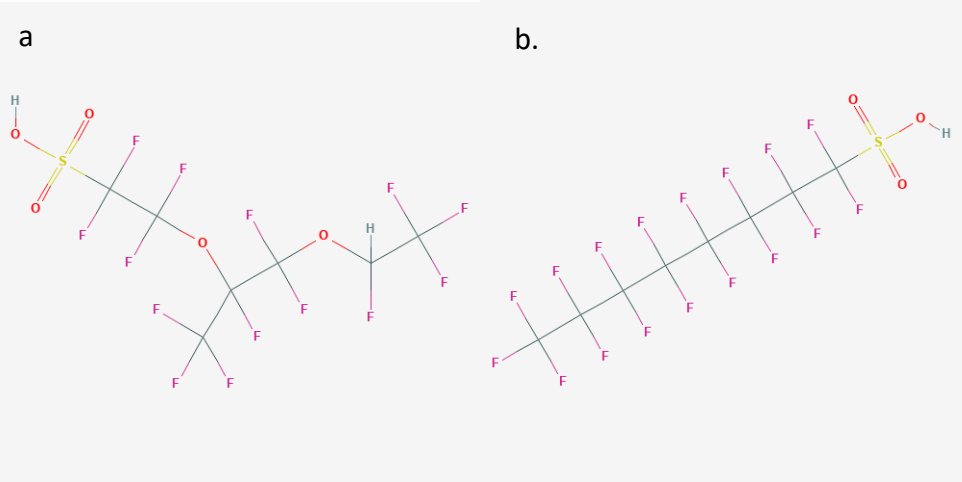

Figure 10: a. Structure of Nafion By-product 2 ("PubChem Compound Summary for CID 14317645, Nafion by-Product 2" 2021) b. Structure of perfluorooctane sulfonic acid ("PubChem Compound Summary for CID 74483, Perfluorooctanesulfonic Acid" 2021)

Lang et al. (2020)(Lang et al. 2020) published a toxicity study of NBP2 in which Balb-c mice were orally exposed to NBP2 at concentrations of 0, 0.04, 0.4 3.0, and 6.0 mg/kg/day. They found NBP2 to significantly increase the liver size of animals in the 3.0 and 6.0 mg/kg dose groups with livers almost doubling in size; changes to serum markers of liver injury also were observed. Accumulation of NBP2 occurred in the Lang et al. (2020) study, which we also observed, but while Lang et al. (2020) observed males and females accumulated a similar amount of NBP2, we observed a large difference in the amount of NBP2 males accumulated when compared to females, with females accumulating almost 50% less NBP2 by the end of the study in the 5.0 mg/kg groups. In this manner, the elimination rate between sexes and the bioaccumulation potential of NBP2 may be consistent with our current understanding of short-chain PFEA toxicokinetics as short-chain PFAS like GenX and perfluorobutane sulfonic acid (PFBA) have also been shown to have different elimination rates between male and female mice.(Han et al. 2012; Rushing et al. 2017). Accumulation of NBP2 in urine still differed between sexes, but at low doses males had higher NBP2 concentrations in their urine than

females, while at the higher two doses males had lower concentrations of NBP2 in their urine than females. It seems females in higher dose groups excreted more NBP2 than males, leading to a possible explanation for why females had lower NBP2 serum concentrations than males. Short-chain compounds are being developed as replacements to long-chain compounds as they are thought to have a reduced ability to bioaccumulate to more rapid elimination, but the limited evidence we have on NBP2 points to it having bioaccumulative potential as well.

4.2 Immunotoxicity

As the main goal of this study was to describe how NBP2 impacts the immune system, we chose to look at multiple indicators of immune system health which, when taken in totality, are robust predictors of the immune system's overall functionality (Luster et al. 1992). The TDAR is a robust, sensitive assay and the response elicited by immunization with an antigen such as SRBCs is analogous to the antibody response vaccines elicit in humans (Luster et al. 1992). A measurable suppression of the TDAR indicates that a substance can suppress the immune system's ability to respond to a challenge, and a deficit observed in the TDAR in experimental animals is a useful predictor of potential immunotoxicity in exposed humans (Selgrade 2007). It has been observed that increased serum concentrations of legacy PFAS are associated with reduced vaccine efficacy in humans and it is known that in experimental animals, legacy PFAS and GenX can suppress the TDAR (DeWitt et al. 2008; DeWitt, Blossom, and Schaider 2019; Rushing et al. 2017; Grandjean et al. 2012; 2017). In the present study, the TDAR was also found to be profoundly suppressed in C57BL/6 male and female mice exposed to NBP2 (Figure 7). The TDAR of male animals in our highest dose group was suppressed by over 90% and in females, it was suppressed by over 50%.

Data from Dong et al. (2009) was recently used by the states of New Jersey and Michigan upon which to base health protective guidelines for PFOS in drinking water (Dong et al. 2009). Dong et al. (2009) observed statistical decreases in the TDAR after exposure to 5 mg/kg/day of PFOS in C57BL/6 mice. In Michigan and New Jersey, drinking water limits of 8 ng/L and 13 ng/L, respectively, were subsequently adopted. While we have limited data on current NBP2 concentrations in the CFR, Guillette et al. (2018) reported NBP2 surface water concentrations of 18 ng/L. In C57BL/6 mice, doses of 5 mg/kg/day of NBP2 and PFOS were both associated with a statistically reduced TDAR. In the present study, the male serum concentration of NBP2, at 24,800 ng/mL, was much higher than that of PFOS reported by Dong et al. (2009), at 7,132 ng/mL . This suggests that the accumulative potential of NBP2 may be greater than that of PFOS, and that NBP2 poses a similar risk in reduction of the TDAR. The drinking water guidelines established in Michigan and New Jersey for PFOS are based on suppression of the TDAR; if NBP2 is of at least a similar potency, the surface water concentrations of 18 ng/L in the CFR may reflect a concentration that poses an immunotoxicological risk for consumers.

Like the TDAR, NK cell cytotoxicity is an immune functional measure. NK cytotoxicity was suppressed in our high dose group (Figure 8), indicating that this concentration of NBP2 suppresses this aspect of innate immunity. Other immunological outcomes we measured also indicate immune suppression: Lymphoid organ weights were statistically suppressed (Table 3), and some lymphoid cell subpopulations (Table 5, Table 6) also were statistically suppressed in our high dose group. These reductions in lymphoid organ weights and lymphoid cell subpopulations are consistent with findings reported for other PFAS, indicating NBP2 has similar immunomodulary effects (DeWitt et al. 2009; Q. Yang, Xie, and Depierre 2000; Qian Yang et al. 2002).

Hepatomegaly and hepatic peroxisome proliferation are common toxicological effects reported after exposure to long-chain PFAS such as PFOA and PFOS (Q. Yang, Xie, and Depierre 2000; Qian Yang et al. 2001). Increases in peroxisomal enzyme activity has been attributed to activation of the peroxisome proliferator activated receptor alpha (PPARα) and some studies have identified this receptor as a potential mechanism of PFAS immunotoxicity (DeWitt et al. 2009). Measurements of peroxisomal enzyme activity were included in the current study to determine if NBP2 also activated the PPARα. After treatment with NBP2, hepatomegaly was observed, with the livers in the highest dose group weighing ~4x more than control livers and making up ~20% of the total body weight. Lang et al. (2020) also observed hepatomegaly in Balb-c mice exposed to 3 or 6 mg/kg/day of NBP2 for only seven days, with livers weighing 2x more than control livers (Lang et al. 2020). Increased markers of peroxisome proliferation have been observed after exposure to PFOA and PFOS (DeWitt et al. 2009) as well as after exposure to GenX (Rushing et al. 2017). We found increased peroxisomal enzyme activity in higher dose groups compared to controls, and it was observed in the highest dose group that male animals had higher rates of peroxisomal enzyme activity (increase of 475%) than females at the same doses (increase of 75%) (Figure 9). In a study of GenX by Rushing et al. (2017),(Rushing et al. 2017) male animals exposed to GenX had a 222% increase in peroxisomal enzyme activity and female animals had a 100% increase at a 100 mg/kg/day dose of GenX. Our findings with NBP2 and the findings of Rushing et al. (2017) suggest that the sensitivity of male and female mice may differ with respect to the ability of short-chain PFAS to induce peroxisomal enzyme activity. Our findings with respect to NBP2 show similar effects on liver weight and peroxisomal enzyme activity as legacy PFAS.

5. Conclusion

NBP2, a PFAS found in drinking water of an estimated 280,000 people, has immunomodulary effects in C57BL/6 mice. At the doses administered, NBP2 suppressed lymphoid organ weights, increased liver size, suppressed the TDAR, altered immunophenotype of cells from lymphoid organs, suppressed natural killer cell activity, and increased peroxisomal enzyme activity. Though the body of evidence into the toxicity of NBP2 is small, the data thus far indicate that it produces adverse health outcomes in mice across a range of doses and exposure durations. Effects of NBP2 on the TDAR is especially concerning as it is translatable to the human vaccine response and suggests that NBP2 has the potential to produce immunotoxicity in humans. Further study into the toxicological effects of NBP2 is needed to manage and mitigate contamination and best protect human health.

Chapter 4: Immunological Outcomes in C57BL/6 Mice Following Exposure to Nafion by-product 2 (NBP2) and Perfluoro-2-methoxyacetic Acid (PFMOAA) Mixtures

1. Introduction

Per- and polyfluoroalkyl substances (PFAS) are a class of persistent anthropogenic chemicals used in a myriad of common manufacturing and industrial processes. PFAS are chemicals "with at least one aliphatic perfluorocarbon moiety" and the strength of the carbon-fluorine bonds in PFAS contributes to their stability and to their water and oil repellent qualities (Kwiatkowski et al. 2020). An ever-growing number of PFAS have been developed for a variety of uses (e.g. non-stick cookware, firefighting foams, textiles, cosmetics, waterproof coatings); presently the United States Environmental Protection Agency (US EPA) estimates over 9,000 individual PFAS have been identified (KEMI (Swedish Chemicals Agency) 2015; "PFAS|EPA: PFAS Structures in DSSTox" 2020). The widespread use of PFAS in manufacturing has resulted in extensive accumulation of PFAS-containing products/PFAS-containing waste; subsequently PFAS and their waste products are now distributed worldwide in the environment, wildlife, and humans (Giesy and Kannan 2001; Kannan et al. 2004; Lindstrom, Strynar, and Libelo 2011; Buck et al. 2011).

Associations between PFAS exposure and a multitude of toxicological effects have resulted in some governmental oversite and the resulting phase out of several PFAS in manufacturing. There are now concerns the structurally similar replacement compounds and their by-products will elicit similar toxicological effects to the phased out PFAS they are replacing (Wang et al. 2017). Additionally, as non-targeted screening methods have been employed to identify previously undescribed PFAS in the environment, it has become clear PFAS generally

exist as complex mixtures in environmental media though the toxicity of PFAS mixtures is often overlooked in toxicity studies (Strynar et al. 2015a; Wang et al. 2017).

The Cape Fear River (CFR) of North Carolina (NC) is known to be contaminated by both phased out legacy PFAS and by novel replacement PFAS (Strynar et al. 2015a; Hopkins et al. 2018). These chemicals have also been identified in wildlife of the CFR and in the blood of children and adults who source their drinking water from the CFR (Guillette et al. 2020; Kotlarz et al. 2020). In total, an estimated 280,000 people have been exposed to PFAS via their CFR sourced drinking water; this number may be even greater as more than a million people in NC obtain their drinking water from waters of the CFR basin. Many of the PFAS to which people have been exposed have little to no toxicological data available in peer-reviewed literature (Kotlarz et al. 2020).

The present study focused on immunotoxicity of exposure to a mixture containing two novel PFAS identified in the CFR: Nafion by-product 2 (NBP2) and perfluroro-2-methoxy acetic acid (PFMOAA). Both NBP2 and PFMOAA are classified as perfluoroether acids (PFEAs) and are by-products of fluorochemicals manufactured at a facility that discharges waste into the CFR. NBP2 is a six-carbon perfluoroalkyl ether sulfonic acid (PFESA) with two oxygen ether linkages and is believed to be a by-product of Nafion, the trade name of a sulfonated tetrafluoroethylene-based fluoropolymer-copolymer used in fuel cell applications. NBP2 was identified in the CFR first by (Strynar et al. 2015a) and was subsequently identified in the blood of Wilmington, NC adults and children with NBP2 being present in 99% of study participants and in children as young as age six (Kotlarz et al. 2020). PFMOAA is a three-carbon perfluoroether carboxylic acid (PFECA) believed to be a by-product of GenX production. PFMOAA was also first identified in the CFR by (Strynar et al. 2015a) and was again identified by (McCord and Strynar 2019),

though PFMOAA was not detectable in the blood of Wilmington, NC residents (Kotlarz et al. 2020).

This study aimed to expand on work done by (Tobin et al., *submitted*) and (Tobin et al., in preparation), which observed that oral exposure to either NBP2 or PFMOAA alone elicits immunosuppressive effects in C57BL/6 mice. As immunotoxicity is a health effect observed across multiple PFAS, characterizing the immunotoxicity of a PFAS mixture to which humans have been exposed is in the best interest of public health. Therefore C57BL/6 mice were exposed to NBP2-PFMOAA mixtures and immunotoxicological as well as general toxicological parameters were evaluated.

2. Methods

2.1 Experimental design

Twenty-four female and 24 male C57BL/6 mice aged six–eight-weeks-of-age were purchased from Charles River Laboratories (Raleigh, NC, USA) and were delivered to the East Carolina University (ECU) Brody School of Medicine animal facilities (Association for Assessment an Accreditation of Laboratory Animal accredited). Animals were weighed upon arrival and randomly assigned to cages of three animals/sex/cage. Animals were allowed access to food and water *ad libitum* and cages were housed in temperature controlled animal rooms (23±3°C) with 50±20% relative humidity and a 12 hour light:dark cycle. Weights of animals were analyzed by analysis of variance (ANOVA) by cage and if weights differed statistically ($p<0.05$) animals were re-housed on the day of arrival. Cages were randomly assigned to one of four dose groups with a total of six female and six male animals/dose as based on previous studies six animals is sufficient to detect statistical changes in immune endpoints. A five-day acclimation period was observed in which no further manipulations were made before dosing

began. All procedures were approved by the ECU Institutional Animal Care and Use Committee in advance.

2.2 Dosing

Fresh dosing solutions were prepared one time per week with stock PFMOAA (SynQuest) and NBP2 (SynQuest) mixed at appropriate concentrations in a solution of sterile water + 0.5% Tween-20; control animals were given sterile water + 0.5% Tween-20 only. Animals were weighed daily and dosing solutions were administered for 15 consecutive days via oral gavage once/day based on daily body weight. The dosing concentrations used in the present study were initially chosen based on the results of two immunotoxicity studies that exposed C57BL/6 mice to either NBP2 or PFMOAA as single chemical. A 30-day exposure to 5 mg/kg of NBP2 alone statistically ($p<0.05$) reduced the TDAR in female and male C57BL/6 mice, and a 30 day exposure to 50 mg/kg of PFMOAA statistically ($p<0.05$) reduced the TDAR in male C57BL/6 mice. These concentrations did not appear to be systemically toxic based on lack of statistical weight loss or increased mortality of treated animals. Therefore, in an attempt to be cautious, doses chosen for the present study were based on doses that did not appear to be systemically toxic alone: 0mg/kg PFMOAA + 0mg/kg NBP2, 0.5mg/kg PFMOAA + 5mg/kg NBP2, 5mg/kg PFMOAA + 1 mg/kg NBP2, and 50mg/kg PFMOAA + 0.5mg/kg NBP2. Animals were to be dosed for only 15 days rather than the 30-day dosing period employed in individual single chemical studies due to uncertainty about the toxicity of the mixture. However, after only three days of dosing and frequent consultations with the ECU veterinary staff, the decision was made to end the study as animals exhibited rapid weight loss, massive liver enlargement, and mortality. The study was repeated with doses reduced by a factor of ten with the aim to reduce systemically toxic effects observed at higher concentrations; final dosing

concentrations therefore were: 0mg/kg PFMOAA + 0mg/kg NBP2, 0.05mg/kg PFMOAA + 0.5mg/kg NBP2, 0.5mg/kg PFMOAA + 0.1 mg/kg NBP2, and 5.0mg/kg PFMOAA + 0.05mg/kg NBP2. No signs of overt toxicity were observed after 15 days of exposure to the reduced doses.

2.3 Serum and tissue collection

One day after the 15th dose was administered animals were anesthetized with Isoflurane and blood was collected via neck vein transection. Serum was separated from clotted blood within one hour of collection via centrifugation at 14,000 RPM for 7 minutes at 4°C and was then stored at -80°C. The TDAR was later analyzed using aliquots of serum as described in section 2.4.

Immediately following terminal blood collection, the following organs were excised and weighed: liver, spleen, thymus, brain, kidney, and lungs. Livers were individually stored in aluminum foils squares and snap frozen on dry ice, then were stored at -80°C. Peroxisomal enzyme activity was later analyzed in liver tissue as described in section 2.5. Spleens and thymuses were placed in RPMI + 1% fetal bovine serum (FBS) medium and kept on ice. Brains, kidneys, and lung were processed the meet the needs of collaborators.

2.4 Measurement of the T cell-dependent antibody response (TDAR)

On the 11th day of dosing animals were immunized with 4 x 10^7 sheep red blood cells (SRBC) in 0.2 mL sterile saline via tail vein injection. SRBC immunizations were given on the 11th day of dosing and 5 days before terminal collections as peak antigen-specific IgM production occurs after 5 days in C57BL/6 mice. The anti-SRBC IgM antibody titers were determined in serum using an enzyme-linked immunosorbent assay (ELISA) kit (Life Diagnostics, SRBCM-1). Serum samples (diluted 50-fold using diluent provided by the manufacture) were added in duplicate to a 96-well plate pre-coated with SRBC membranes.

Anti-SRBC IgM standards and wells containing only diluent as blanks were included on each plate. Plates were covered and incubated on an orbital shaker at 75 RPM for 45 minutes at room temperature, washed, and refilled with enzyme conjugate reagent. Plates were again covered and were incubated on an orbital shaker at 75 RPM for 20 minutes at room temperature. Wells were washed and TMB reagent added, and plates were incubated a final time on an orbital shaker at 75 RPM for 20 minutes at room temperature. A stop solution of 1N HCl was added to all wells and immediately the optical density at 450 nm was measured on a microplate reader. Anti-SRBC IgM concentrations in serum (units/mL) were calculated by fitting samples onto a standard curve based on the absorbance of IgM standards.

2.5 Peroxisomal enzyme activity

The activity of liver peroxisomal acyl-coenzyme A oxidase 1 (ACOX-1) was measured in liver homogenates as a way to indirectly estimate peroxisomal proliferation as previously described (Rushing et al. 2017; McDonough et al. 2020; Woodlief et al. 2021). First, aliquots of frozen liver tissue were homogenized and the protein concentrations were determined with the Bradford protein assay. Aliquots of liver homogenate were then added in duplicate to a 96-well plate containing filled with a mixture of potassium phosphate buffer containing horseradish peroxidase, 0.02% Triton X-100, and aminotriazole stock. Leuco-DCF was added to the plates, followed by palmitoyl-CoA, and then the plates were immediately placed into a plate reader taking kinetic readings at 502 nm were taken once/minute for 10 minutes. The nmoles/minute rate of ACOX-1 activity was determined by adjusting for average liver protein to calculate the final ACOX-1 activity in terms of nmoles/min/mg.

2.6 Statistical analyses

The Statistical Analysis System (SAS) was used to perform statistical analyses. Dosing body weights collected daily were analyzed by two-way ANOVA using dose as the between-subjects variable and day being the within-subjects variable. A one-way ANOVA within each sex was used to analyze the remaining data by dose group for the following endpoints: organ weights, anti-SRBC IgM concentrations, and ACOX-1 activity. If an F-statistic was statistically significant ($p<0.05$) for an overall model, then pairwise post hoc t-tests were made with a Tukey's adjustment for familywise error rate.

3. Results

3.1 Terminal body weight

The terminal body weight of male animals in the 0.5 mg/kg PFMOAA + 0.1 mg/kg NBP2 dose group was statistically ($p<0.05$) reduced by 7.6% on average when compared to controls (Figure 11). No other statistical differences in terminal body weight from control were observed for other dose groups.

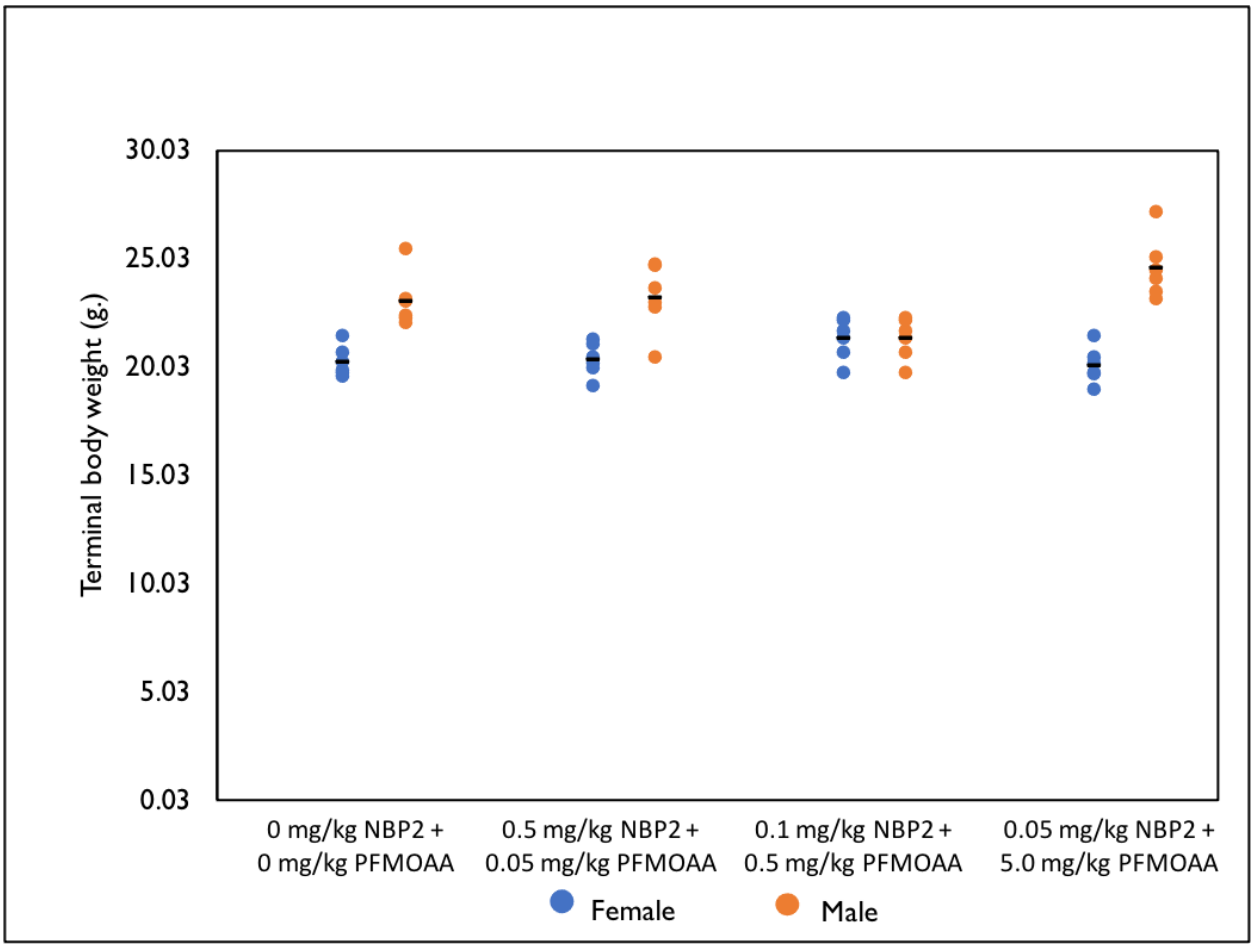

Figure 11: Terminal body weights in female and male C57BL/6 mice orally exposed to mixtures of perfluoro-2-methoxyacetic acid (PFMOAA) and Nafion by-product 2 (NBP2) for 15 days. Terminal weights were collected one day after dosing ended. *indicates statistical difference ($p<0.05$) from control

3.2 Relative liver weight

The relative liver weight of female and male animals in the 0.05 mg/kg PFMOAA + 0.5 mg/kg NBP2 dose group and of male animals in the 5.0 mg/kg PFOMAA + 0.05 mg/kg NBP2 dose group was statistically ($p<0.05$) increased on average by 22.9%, 14.5%, and 20.2% respectively when compared to controls (Figure 12).

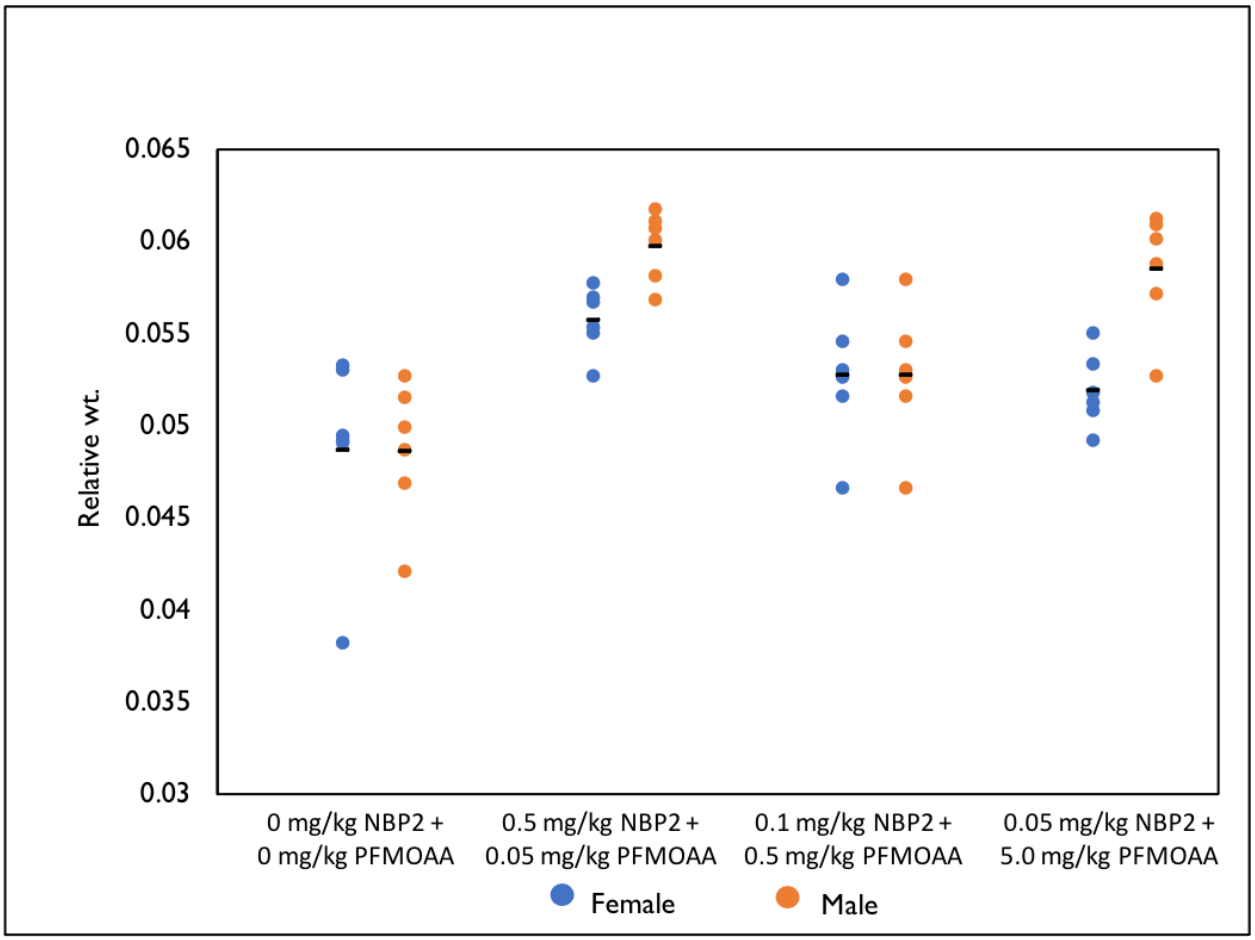

Figure 12: Relative liver weights (liver weight adjusted by body weight) in female and male C57BL/6 mice orally exposed to mixtures of perfluoro-2-methoxyacetic acid (PFMOAA) and Nafion by-product 2 (NBP2) for 15 days. Liver weights were collected one day after dosing ended. *indicates statistical difference ($p<0.05$) from control

3.3 Relative spleen and thymus weight

Relative spleen weight was statistically ($p<0.05$) reduced in the male 0.05 mg/kg PFMOAA + 0.5 mg/kg NBP2 and 5.0 mg/kg PFMOAA + 0.05 mg/kg NBP2 dose groups, on average, by 13.7% and 18.6% respectively, compared to controls. Relative spleen weight was statistically ($p<0.05$) increased in the male 0.5 mg/kg PFMOAA + 0.1 mg/kg NBP2 dose group on average by 22.2% compared to controls (Figure 13). No statistical differences from the control were observed for female relative spleen weight.

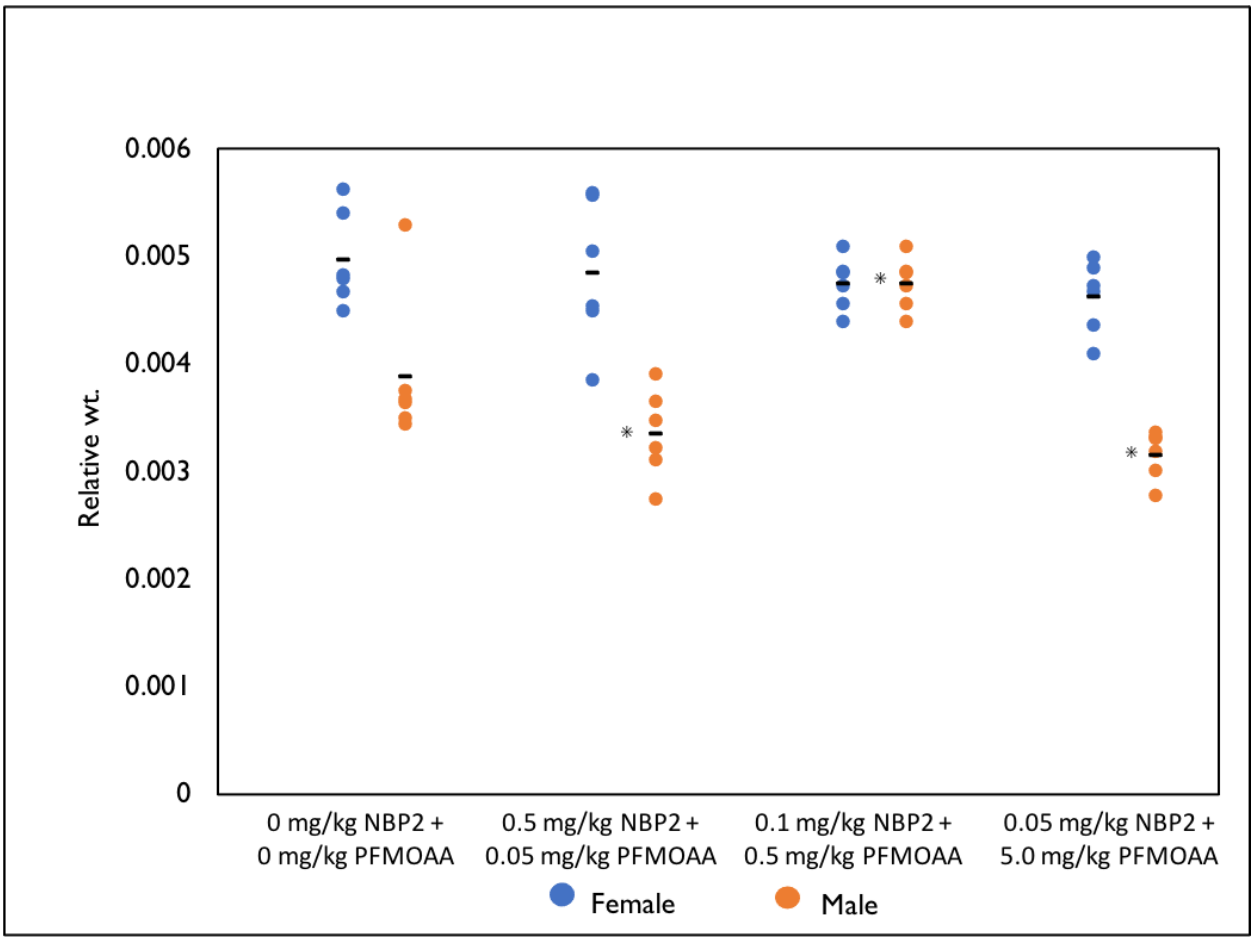

Figure 13: Relative spleen weights (spleen weight adjusted by body weight) in female and male C57BL/6 mice orally exposed to mixtures of perfluoro-2-methoxyacetic acid (PFMOAA) and Nafion by-product 2 (NBP2) for 15 days. Spleen weights were collected one day after dosing ended. *indicates statistical difference ($p<0.05$) from control

Relative thymus weight was statistically ($p<0.05$) increased in the male 0.5 mg/kg PFMOAA + 0.1 mg/kg NBP2 dose group on average by 58.7% compared to controls (Figure 14). No statistical differences from the control were observed for any other dose groups.

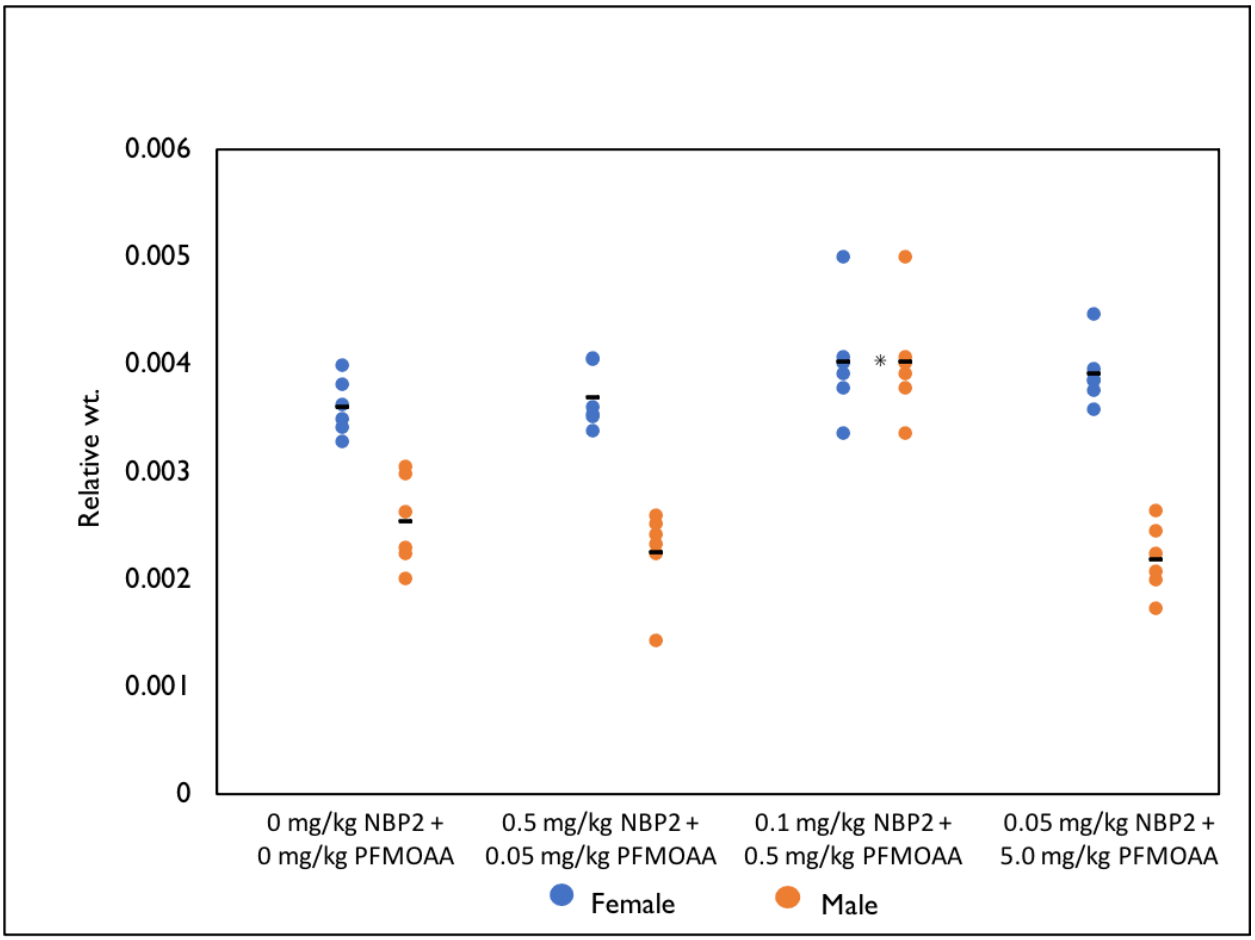

Figure 14: Relative thymus weights (thymus weight adjusted by body weight) in female and male C57BL/6 mice orally exposed to mixtures of perfluoro-2-methoxyacetic acid (PFMOAA) and Nafion by-product 2 (NBP2) for 15 days. Thymus weights were collected one day after dosing ended. *indicates statistical difference ($p<0.05$) from control

3.4 Peroxisomal enzyme activity

No statistical differences in peroxisome proliferation (nm/min/mL) were observed in treated groups compared to controls (Figure 15). Females had a 1.5% – 20% increase in peroxisome proliferation relative to controls, and males had a 2.3% – 27% increase in peroxisome proliferation relative to controls.

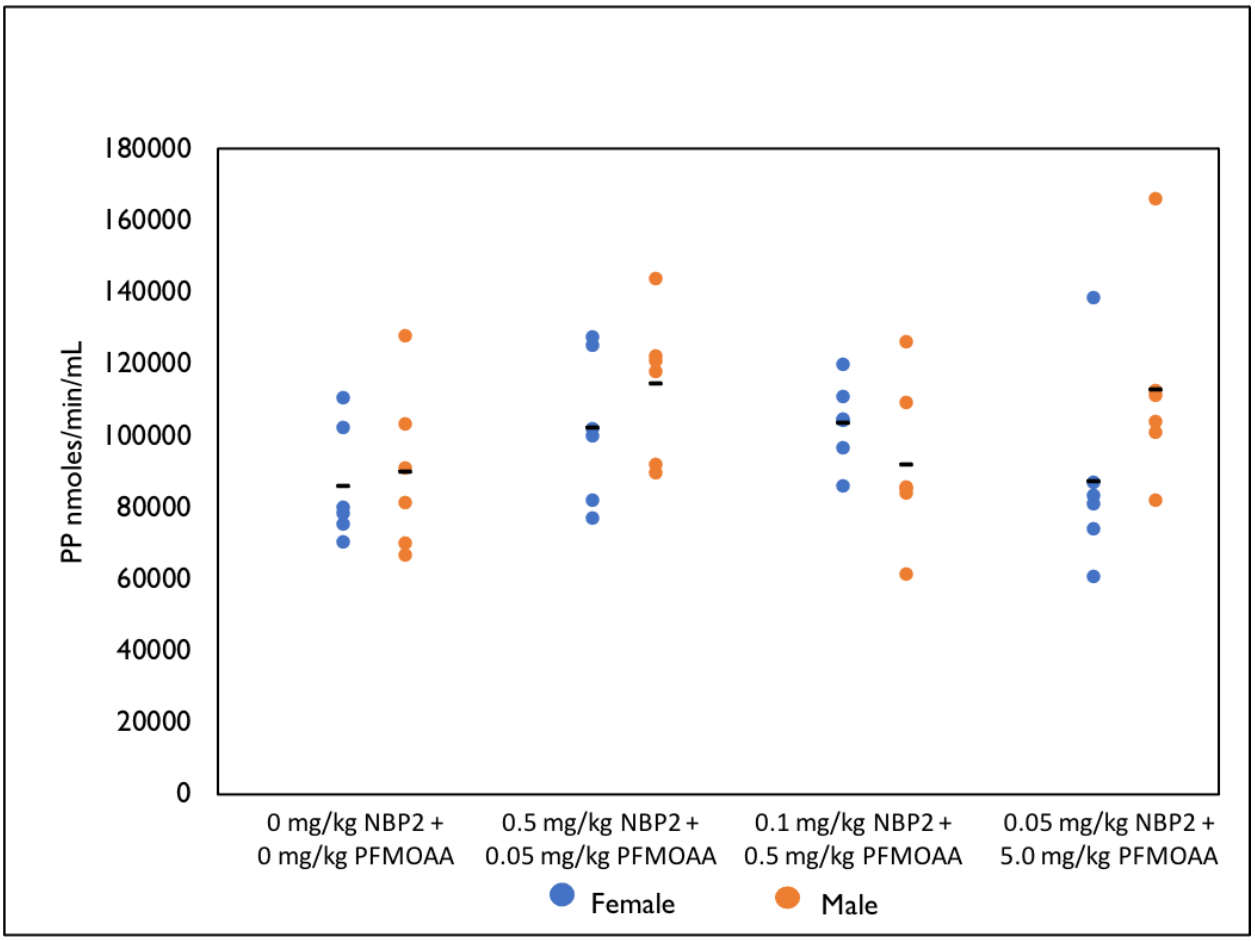

Figure 15: Peroxisome proliferation (nm/min/mL) in female and male C57BL/6 mice C57BL/6 mice orally exposed to mixtures of perfluoro-2-methoxyacetic acid (PFMOAA) and Nafion by-product 2 (NBP2) for 15 days. Measured in liver tissue collected one day after dosing ended.

3.5 T-cell dependent antibody response (TDAR)

The anti-SRBC IgM was reduced in all dose groups compared to controls but there were no statistically significant ($p<0.05$) reductions observed (Figure 16). Females had a 11% – 31% decrease in the TDAR relative to controls, and males had a 20% – 39% decrease in the TDAR relative to controls.

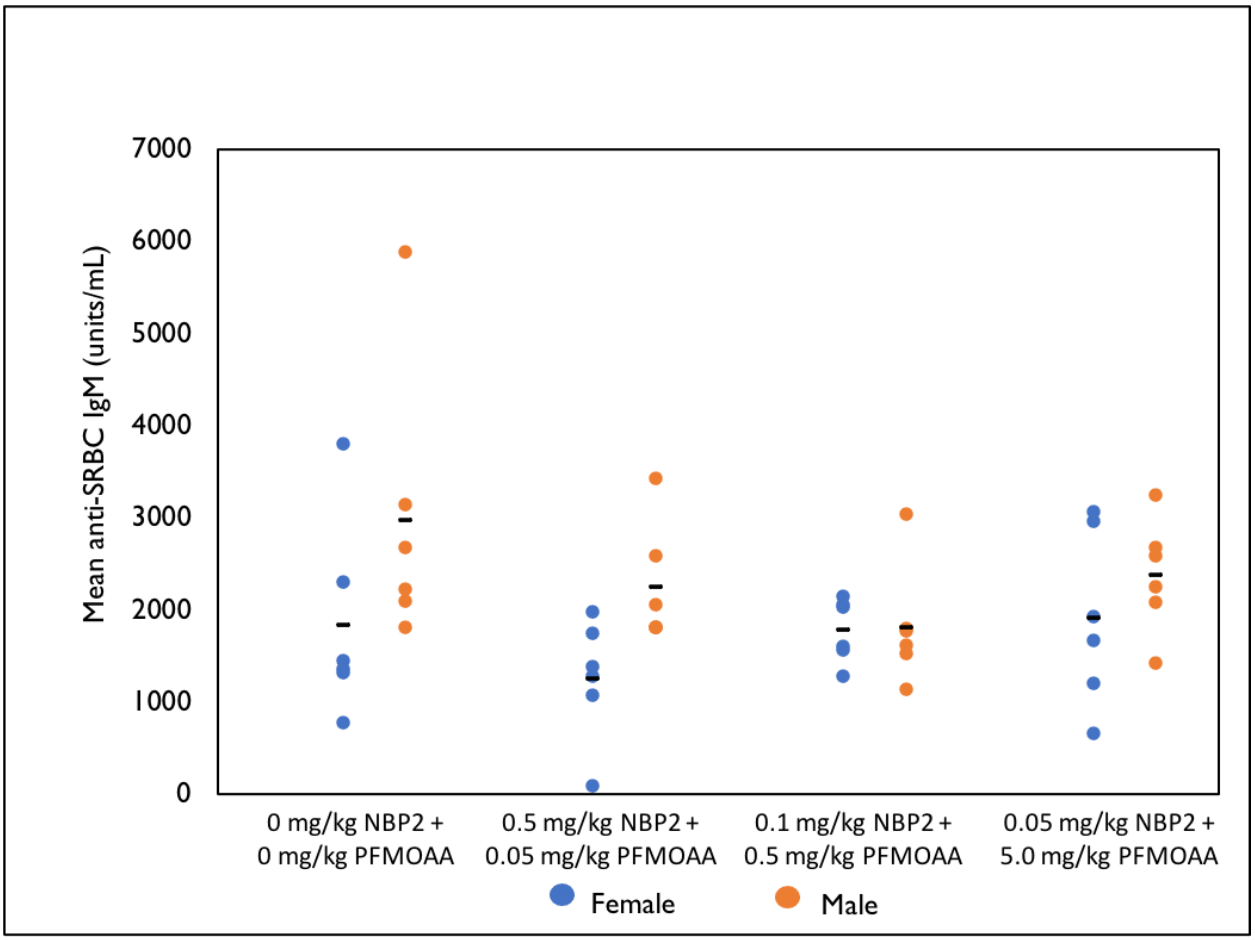

Figure 16: Anti sheep red blood cell (SRBC) immunoglobulin M (IgM) antibody titers in female and male C57BL/6 mice orally exposed to mixtures of perfluoro-2-methoxyacetic acid (PFMOAA) and Nafion by-product 2 (NBP2) for 15 days. Concentrations were measured in sera collected one day after dosing ended and five days after animals were immunized with sheep red blood cells.

4. Discussion

Doses to include in a binary mixture can be determined from systemically toxic doses, benchmark doses, toxic doses, no adverse effect levels (NOAELs), or other approaches. We chose our dose combination based on doses that produced immunotoxicity and liver hepatomegaly without signs of over toxicity. In the previously described studies, NBP2 was administered to C57BL/6 mice for 30 days at either 0.5, 1.0, or 5.0 mg/kg and PFMOAA was administered at either 0.5, 5.0, or 50.0 mg/kg. As no signs of overt toxicity (significant body

weight loss or mortality) were observed in the NBP2 and PFMOAA 30-day studies the doses initially administered in the present study were reflective of doses used in the 30 day studies: 0 PFMOAA + 0 mg/kg NBP2, 0.5 mg/kg PFMOAA + 5 mg/kg NBP2, 5 mg/kg PFMOAA + 1 mg/kg NBP2, and 50 mg/kg PFMOAA + 0.5 mg/kg NBP2. A 15-day dosing period was chosen as this duration has been associated with suppression of the TDAR from exposure to perfluorooctanoic acid (PFOA) alone and was anticipated to be tolerable if overt toxicity occurred. However, after only three days of dosing, male animals exhibited extreme weight loss and mortality and the decision to end the study was made. Hepatomegaly in two male animals in the 50 mg/kg PFMOAA + 0.5 mg/kg NBP2 dose groups was observed one day after the third and final dose was administered; livers were estimated as occupying 50%-70% of the abdominal cavities by the veterinarians who performed the necropsies.

The onset of toxic effects after administering just three consecutive days of the original doses indicates that the toxicity of PFAS mixtures may be greater than dose-additive at some concentrations. An immunotoxicity study into the effects of an aqueous film forming foam (AFFF) mixture found a similar increase in toxicity after administering the AFFF mixture to C57BL/6 mice (McDonough et al. 2020). McDonough et al. (2020) prepared dosing solutions according to the total perfluorooctane sulfonic acid PFOS+PFOA/kg/day in the mixture and at concentrations of PFOA and PFOS that were not known to be systemically toxic. Dosing with this AFFF formulation was planned to occur for 15 days, but as systemic toxicity was observed after only ten days in the highest dose group of 10 mg PFOS+PFOA/kg/day, dosing was stopped after only ten days. The toxicity of the AFFF mixture was concluded as being greater than predicted for PFOS+PFOA content alone, indicating the other PFAS present contributed to the toxicity of this complex mixture. Similarly, the present study found an unexpected increase in

toxicity with the administration of these two PFAS as a mixture compared to these PFAS when given alone.

The doses administered in the present study, which were 10-fold lower than the doses initially administered, did not statistically impact the TDAR. However, these dose combinations did reduce the TDAR relative to control levels by 11% – 31% in females and by 20% – 39% in males, on average, and alterations were observed in some body and organ weights. A statistical reduction in body weight and a statistical increase of the spleen and thymus weights of male animals receiving 0.1 mg/kg NBP2 + 0.5 mg/kg PFMOAA was observed. This male dose group also exhibited a 38.9% reduction in the TDAR which, though not statistically significant, indicates that this dose did produce effects that could be considered adverse. Liver weights statistically increased in some dose groups (Figure 12) compared to controls, and some dose groups demonstrated a statistical reduction in spleen weights (Figure 13). Though the effects on organ weights were variable across sexes and dose groups, alterations in organ weights and in the body weights of one dose group were observed. The findings of the present study and its preliminary study indicate the toxicity of this specific PFAS mixture is higher than the toxicity of these two chemicals alone. Even when doses were reduced to be 10-fold less than doses that were not systemically toxic as single compounds, statistical effects on organ and body weights were observed.

NBP2 and PFMOAA are present together in drinking water sourced from the CFR and both chemicals were also identified by Yao et al. (2020) in water samples from a river near a fluorochemical plant in Shandong Province, China, and in the serum of local residents receiving drinking water sourced from the same river (Yao et al. 2020). Until the mechanisms of PFAS toxicity are better understood, it will be challenging to predict not only the toxicity of mixtures

of PFAS to which people are exposed, but to individual PFAS as well. PFEAs have been touted as safer alternatives to legacy PFAS (Lindstrom, Strynar, and Libelo 2011) but recent studies demonstrate that PFEAs elicit similar immunomodulatory and other toxic effects to legacy PFAS. To further complicate problems decision makers must overcome to manage mixtures of PFAS, it is still not easy to identify and quantify novel PFAS in complex mixtures. Many newly identified PFAS do not have the analytical standards available needed to quantify their concentrations (Strynar et al. 2015a; Wang et al. 2017). The ubiquity and complexity of PFAS mixtures in the environment plus additional complications stemming from lack of analytical standards has made trying to manage this class of contaminants incredibly challenging. Studies like this one are important as they add to our knowledge of PFAS mixture toxicity, but the diversity and number of PFAS that exist means that other strategies must be employed if we want to better understand intricacies of PFAS toxicity in a reasonable timeframe. This study also exemplifies that care must be taken to determine appropriate doses in toxicity studies of these novel chemicals. As humans are very often exposed to complex mixtures of PFAS rather than single compounds, to better understand the potential synergies of PFAS mixture toxicity is imperative to ensure the risks posed to public health by these compounds is not underestimated.

Chapter 5: Conclusions

1. Big picture

As more unstudied PFAS are being identified in the environment and in humans the question of their potential long term impacts becomes more important. Our findings that some unstudied compounds to which people have been exposed elicit toxic effects in experimental models is concerning, and this concerning story is being repeated as more compounds are uncovered and tested. Impacted individuals not only deserve to known what chemicals they have been exposed to and for how long, they also deserve a better understanding of the health effects their exposure could cause. The disparity of resources between fluorochemical manufacturers and effected communities has resulted in the PFAS which contaminate communities being replaced in manufacturing at a faster rate than researchers can understand their toxicity. Collaboration among concerned parties is the only way forward to effectively tackle the issues public health is facing from PFAS. The work done in the present studies was funded in part by the NC Policy Collaboratory's PFAS Testing (PFAST) Network through an appropriation given by the NC General Assembly. The goal of the NC PFAST Network is to use the cumulative resources of multiple institutions in NC to address the occurrence of PFAS in NC drinking water resources (NC General Assembly 2018; NC Policy Collaboratory 2021). As part of the work done for the NC PFAST Network these studies were performed with the aims building toxicological profiles for PFMOAA and NBP2. This work investigated how these two chemicals impact the immune system of C57BL/6 mice, and collaborators are examining other endpoints from the same chemicals in other types of experimental models. To our knowledge these studies are some of the first to examine how these chemicals effect immunological endpoints and we are hoping the cumulative knowledge from the present studies and those of collaborators will allow a

better understanding of how the toxicity of replacement and novel PFAS compares to that of legacy PFAS.

2. Summaries and Implications

Exposure to PFMOAA in the present study has resulted in outcomes similar to those elicited by PFOA exposure and notably these outcomes were seen predominantly in male animals. After exposure to PFMOAA for 30 days the liver weights of males in 5 mg/kg dose group and females and males in the 50 mg/kg dose groups were statistically increased compared to controls, compared to the animals which received 7.5 mg/kg PFOA the PFMOAA treated animals saw a comparable in liver size for males (both the 50 mg/kg PFMOAA and 7.5 mg/kg PFOA males saw an increase of ~150%) but in females treated with 7.5 mg/kg PFOA the increase in liver size was much more substantial (the 50 mg/kg PFMOAA females had a ~44% increase in relative liver size while the 7.5 PFOA females had a ~222% increase). The TDAR was statistically suppressed in male 50 mg/kg PFMOAA animals to a similar extent as to the male 7.5 mg/kg dose group (~55% for PFMOAA and ~67% for PFOA animals) when compared to controls. The male 50 mg/kg dose group also had a statistical increase in peroxisomal enzyme activity when compared to controls of 263% while the male PFOA animals only had a 163% increase. In summary PFMOAA was observed as eliciting some immunomodulatory outcomes with males exhibiting a higher sensitivity to these outcomes.

Exposure to NBP2 in C57BL/6 mice in the present study has also resulted in immunotoxic outcomes similar to those elicited by legacy PFAS. Notably the toxicity effects of NBP2 occurred at lower doses than PFMOAA elicited toxic outcomes. It was observed male animals accumulate more NBP2 in their serum than females. The organ weights of animals were altered across both sexes; livers were so large in the 5 mg/kg dose groups liver weight comprised

~19% of the body weigh in both female and male animals, the relative thymus weights were ~41% smaller for both female and male 5 mg/kg groups, and the relative spleen weights were ~32% and ~39% lower in 5 mg/kg female and male animals. The cytotoxicity of NK cells was altered and statistically decreased in some male and female higher dose groups, and immune cell subpopulations were perturbed but in a sex differential manner; in males splenic $CD4^{+,}$ $CD4^{-}$ $/CD8^{-}$, $CD4^{+}/CD8^{+}$ T-cell subpopulations all saw statistical decreases in the 5 mg/kg dose groups when compared to controls, while T-cell subpopulations of females in the 5 mg/kg dose group saw a statistical increase in splenic $CD4^{+,}$ $CD4^{-}/CD8^{-}$, $CD4^{+}/CD8^{+}$ cells. The TDAR was statistically decreased in the 5 mg/kg groups for both females and males with females having a 53% reduction and males having a 93% reduction in serum anti-SRBC IgM concentration. The immunosuppressive effects NBP2 elicited in C57BL/6 mice were observed in both females and males, but as was observed in the 30-day PFMOAA study there was a sex-difference in the amplitude of effects. Males had a greater suppression of their TDAR and a higher rate of peroxisomal proliferation, which may be related to the higher rate of NBP2 accumulation in serum by males than by females.

The results of the PFMOAA/NBP2 mixture study are potentially very impactful for understanding PFAS mixture toxicity as the strong toxic effects observed by doses in the pilot study performed which were not overtly-toxic as single compounds were not an expected outcome. Even after reducing doses 10-fold one male dose group had a significant reduction in terminal body weight, some male and female groups had changes in liver and thymus weights, and though not statistically significant the TDAR was still suppressed in all dose groups. These results indicate the toxicity of this PFAS mixture may be greater than dose-additive, this

knowledge is impactful as PFAS generally exist as mixture in environmental media as complex mixtures.

The effects of exposure to NBP2 and PFMOAA in C57BL/6 mice indicate these chemicals have immunotoxicological potential. We know these compounds have been present in the drinking water of 280,000 NC residents since at least 2012, but sources have indicated Nafion production has been occurring in eastern NC since the 1980's making the potential time period of exposure to Nafion by-products potentially much longer (Strynar et al. 2015a). Because there is limited data available into the toxicity of these and other PFEAs recently identified in the CFR we hope these studies can guide decisions makers to better protect the public from exposure not only in eastern NC but in other areas impacted by PFAS contamination. Many of the same PFEAs present in eastern NC were also identified in Shandong, China, and as these chemicals were generally unknown until recently they could potentially be present at other contaminated sites without our knowledge (Yao et al. 2020). The present studies have given us insight into the toxicity of NBP2, a PFESA with a chain length comparable to PFOS, into PFMOAA, a short-chain PFECA which is a by-product of a replacement chemical for PFOA, and insight into the effect exposure to a PFEA mixture can have.

3. Conclusion

We found that exposure to NBP2 or to PFMOAA causes immunosuppressive and immunomodulatory outcomes at concentrations similar to the concentrations of legacy PFAS which induce immunotoxicity. We also found concentration of NBP2 and PFMOAA which did not cause signs of overt toxicity when administered alone caused rapid sign of overt toxicity to occur when administered as mixtures. Furthermore the follow-up study using NBP2/PFMOAA mixtures with a concentration reduce 10x less than the amounts which causes toxicity alone still

altered some endpoints such as lymphoid organ and liver weights. While there are still many unanswered questions into how the many PFAS present in NC could affect human health NBP2 and PFMOAA warrant further study due to their ubiquity.

References

Barry, Vaughn, Andrea Winquist, and Kyle Steenland. 2013. "Perfluorooctanoic Acid (PFOA) Exposures and Incident Cancers among Adults Living Near a Chemical Plant." *Environmental Health Perspectives* 121 (11–12): 1313–18. https://doi.org/10.1289/ehp.1306615.

Brase, Richard A., Elizabeth J. Mullin, and David C. Spink. 2021. "Legacy and Emerging Per- and Polyfluoroalkyl Substances: Analytical Techniques, Environmental Fate, and Health Effects." *International Journal of Molecular Sciences* 22 (3): 995. https://doi.org/10.3390/ijms22030995.

Buck, Robert C, James Franklin, Urs Berger, Jason M Conder, Ian T Cousins, Pim de Voogt, Allan Astrup Jensen, Kurunthachalam Kannan, Scott A Mabury, and Stefan PJ van Leeuwen. 2011. "Perfluoroalkyl and Polyfluoroalkyl Substances in the Environment: Terminology, Classification, and Origins." *Integrated Environmental Assessment and Management* 7 (4): 513–41. https://doi.org/10.1002/ieam.258.

Clean Cape Fear. n.d. "Event Flow: Timeline of Events." Accessed June 20, 2021. https://www.cleancapefear.org/new-page-3.

DeWitt, Jamie C., Sarah J. Blossom, and Laurel A. Schaider. 2019. "Exposure to Per-Fluoroalkyl and Polyfluoroalkyl Substances Leads to Immunotoxicity: Epidemiological and Toxicological Evidence." *Journal of Exposure Science & Environmental Epidemiology* 29 (2): 148–56. https://doi.org/10.1038/s41370-018-0097-y.

DeWitt, Jamie C., Carey B. Copeland, Mark J. Strynar, and Robert W. Luebke. 2008. "Perfluorooctanoic Acid–Induced Immunomodulation in Adult C57BL/6J or C57BL/6N Female Mice." *Environmental Health Perspectives* 116 (5): 644–50. https://doi.org/10.1289/ehp.10896.

DeWitt, Jamie C., Alexander Shnyra, Mostafa Z. Badr, Scott E. Loveless, Denise Hoban, Steven R. Frame, Robyn Cunard, et al. 2009. "Immunotoxicity of Perfluorooctanoic Acid and Perfluorooctane Sulfonate and the Role of Peroxisome Proliferator-Activated Receptor Alpha." *Critical Reviews in Toxicology* 39 (1): 76–94.

Dong, Guang-hui, Ying-hua Zhang, Li Zheng, Wei Liu, Yi-he Jin, and Qin-cheng He. 2009. "Chronic Effects of Perfluorooctanesulfonate Exposure on Immunotoxicity in Adult Male C57BL/6 Mice." *Archives of Toxicology. Archiv Für Toxikologie* 83 (9): 805–15. https://doi.org/10.1007/s00204-009-0424-0.

Fenton, Suzanne E., Alan Ducatman, Alan Boobis, Jamie C. DeWitt, Christopher Lau, Carla Ng, James S. Smith, and Stephen M. Roberts. 2021. "Per- and Polyfluoroalkyl Substance Toxicity and Human Health Review: Current State of Knowledge and Strategies for Informing Future Research." *Environmental Toxicology and Chemistry* 40 (3): 606–30. https://doi.org/10.1002/etc.4890.

Frisbee, Stephanie J., A. Paul Brooks, Arthur Maher, Patsy Flensborg, Susan Arnold, Tony Fletcher, Kyle Steenland, et al. 2009. "The C8 Health Project: Design, Methods, and Participants." *Environmental Health Perspectives* 117 (12): 1873–82. https://doi.org/10.1289/ehp.0800379.

Giesy, John P., and Kurunthachalam Kannan. 2001. "Global Distribution of Perfluorooctane Sulfonate in Wildlife." *Environmental Science & Technology* 35 (7): 1339–42. https://doi.org/10.1021/es001834k.

Grandjean, Philippe, Elisabeth Wreford Andersen, Esben Budtz-Jørgensen, Flemming Nielsen, Kåre Mølbak, Pal Weihe, and Carsten Heilmann. 2012. "Serum Vaccine Antibody Concentrations in Children Exposed to Perfluorinated Compounds." *JAMA* 307 (4): 391–97. https://doi.org/10.1001/jama.2011.2034.

Grandjean, Philippe, Carsten Heilmann, Pal Weihe, Flemming Nielsen, Ulla B. Mogensen, Amalie Timmermann, and Esben Budtz-Jørgensen. 2017. "Estimated Exposures to Perfluorinated Compounds in Infancy Predict Attenuated Vaccine Antibody Concentrations at Age 5-Years." *Journal of Immunotoxicology* 14 (1): 188–95. https://doi.org/10.1080/1547691X.2017.1360968.

Guillette, T.C., James McCord, Matthew Guillette, M.E. Polera, Kyle T. Rachels, Clint Morgeson, Nadine Kotlarz, et al. 2020. "Elevated Levels of Per- and Polyfluoroalkyl Substances in Cape Fear River Striped Bass (Morone Saxatilis) Are Associated with Biomarkers of Altered Immune and Liver Function." *Environment International* 136 (March): 105358. https://doi.org/10.1016/j.envint.2019.105358.

Han, Xing, Diane L. Nabb, Mark H. Russell, Gerald L. Kennedy, and Robert W. Rickard. 2012. "Renal Elimination of Perfluorocarboxylates (PFCAs)." *Chemical Research in Toxicology* 25 (1): 35–46. https://doi.org/10.1021/tx200363w.

Hansen, Kristen J., Lisa A. Clemen, Mark E. Ellefson, and Harold O. Johnson. 2001. "Compound-Specific, Quantitative Characterization of Organic Fluorochemicals in Biological Matrices." *Environmental Science & Technology* 35 (4): 766–70. https://doi.org/10.1021/es001489z.

Herkert, Nicholas J., John Merrill, Cara Peters, David Bollinger, Sharon Zhang, Kate Hoffman, P. Lee Ferguson, Detlef R. U. Knappe, and Heather M. Stapleton. 2020. "Assessing the Effectiveness of Point-of-Use Residential Drinking Water Filters for Perfluoroalkyl Substances (PFASs)." *Environmental Science & Technology Letters* 7 (3): 178–84. https://doi.org/10.1021/acs.estlett.0c00004.

Hopkins, Zachary R., Mei Sun, Jamie C. DeWitt, and Detlef R.U. Knappe. 2018. "Recently Detected Drinking Water Contaminants: GenX and Other Per- and Polyfluoroalkyl Ether Acids: JOURNAL AWWA." *Journal - American Water Works Association* 110 (7): 13–28. https://doi.org/10.1002/awwa.1073.

Hu, Qing, Jason N. Franklin, Ian Bryan, Erin Morris, Andrew Wood, and Jamie C. DeWitt. 2012. "Does Developmental Exposure to Perfluorooctanoic Acid (PFOA) Induce Immunopathologies Commonly Observed in Neurodevelopmental Disorders?" *NeuroToxicology* 33 (6): 1491–98. https://doi.org/10.1016/j.neuro.2012.10.016.

Kannan, Kurunthachalam. 2011. "Perfluoroalkyl and Polyfluoroalkyl Substances: Current and Future Perspectives." *Environmental Chemistry* 8 (4): 333. https://doi.org/10.1071/EN11053.

Kannan, Kurunthachalam, Simonetta Corsolini, Jerzy Falandysz, Gilberto Fillmann, Kurunthachalam Senthil Kumar, Bommanna G. Loganathan, Mustafa Ali Mohd, et al. 2004. "Perfluorooctanesulfonate and Related Fluorochemicals in Human Blood from Several Countries." *Environmental Science & Technology* 38 (17): 4489–95. https://doi.org/10.1021/es0493446.

Keil, Deborah E., Tracey Mehlmann, Leon Butterworth, and Margie M. Peden-Adams. 2008. "Gestational Exposure to Perfluorooctane Sulfonate Suppresses Immune Function in B6C3F1 Mice." *Toxicological Sciences* 103 (1): 77–85. https://doi.org/10.1093/toxsci/kfn015.

KEMI (Swedish Chemicals Agency). 2015. “Occurrence and Use of Highly Fluorinated Substances and Alternatives, Report 7/15,” 112.

Kotlarz, Nadine, James McCord, David Collier, C. Suzanne Lea, Mark Strynar, Andrew B. Lindstrom, Adrien A. Wilkie, et al. 2020. “Measurement of Novel, Drinking Water-Associated PFAS in Blood from Adults and Children in Wilmington, North Carolina.” *Environmental Health Perspectives* 128 (7): 077005. https://doi.org/10.1289/EHP6837.

Kotthoff, Matthias, Josef Müller, Heinrich Jürling, Martin Schlummer, and Dominik Fiedler. 2015. “Perfluoroalkyl and Polyfluoroalkyl Substances in Consumer Products.” *Environmental Science and Pollution Research* 22 (19): 14546–59. https://doi.org/10.1007/s11356-015-4202-7.

Kvalem, H.E., U.C. Nygaard, K.C. Lødrup Carlsen, K.H. Carlsen, L.S. Haug, and B. Granum. 2020. “Perfluoroalkyl Substances, Airways Infections, Allergy and Asthma Related Health Outcomes – Implications of Gender, Exposure Period and Study Design.” *Environment International* 134 (January): 105259. https://doi.org/10.1016/j.envint.2019.105259.

Kwiatkowski, Carol F., David Q. Andrews, Linda S. Birnbaum, Thomas A. Bruton, Jamie C. DeWitt, Detlef R. U. Knappe, Maricel V. Maffini, et al. 2020. “Scientific Basis for Managing PFAS as a Chemical Class.” *Environmental Science & Technology Letters* 7 (8): 532–43. https://doi.org/10.1021/acs.estlett.0c00255.

Lang, Johnsie R., Mark J. Strynar, Andrew B. Lindstrom, Amy Farthing, Hwa Huang, Judith Schmid, Donna Hill, and Neil Chernoff. 2020. “Toxicity of Balb-c Mice Exposed to Recently Identified 1,1,2,2-Tetrafluoro-2-[1,1,1,2,3,3-Hexafluoro-3-(1,1,2,2-Tetrafluoroethoxy)Propan-2-Yl]Oxyethane-1-Sulfonic Acid (PFESA-BP2).” *Toxicology* 441 (August): 152529. https://doi.org/10.1016/j.tox.2020.152529.

Lindstrom, Andrew B., Mark J. Strynar, and E. Laurence Libelo. 2011. “Polyfluorinated Compounds: Past, Present, and Future.” *Environmental Science & Technology* 45 (19): 7954–61. https://doi.org/10.1021/es2011622.

Luster, Michael I., Christopher Portier, D. Gayla Pait, Kimber L. White, Chris Gennings, Albert E. Munson, and Gary J. Rosenthal. 1992. “Risk Assessment in Immunotoxicology: I. Sensitivity and Predictability of Immune Tests.” *Fundamental and Applied Toxicology* 18 (2): 200–210. https://doi.org/10.1016/0272-0590(92)90047-L.

McCord, James, and Mark Strynar. 2019. “Identification of Per- and Polyfluoroalkyl Substances in the Cape Fear River by High Resolution Mass Spectrometry and Nontargeted Screening.” *Environmental Science & Technology* 53 (9): 4717–27. https://doi.org/10.1021/acs.est.8b06017.

McDonough, Carrie A, Chastity Ward, Qing Hu, Samuel Vance, Christopher P Higgins, and Jamie C DeWitt. 2020. “Immunotoxicity of an Electrochemically Fluorinated Aqueous Film-Forming Foam.” *Toxicological Sciences*, September, kfaa138. https://doi.org/10.1093/toxsci/kfaa138.

NC General Assembly. 2018. “FUNDING TO ADDRESS PER- AND POLY FLUOROALKYL SUBSTANCES, INCLUDING GENX/USE OF EXPERTISE AND TECHNOLOGY AVAILABLE IN INSTITUTIONS OF HIGHER EDUCATION LOCATED WITHIN THE STATE, SESSION 2017, SESSION LAW 2018-5, SENATE BILL 99.” 2018. https://ncpfastnetwork.com/wp-content/uploads/sites/18487/2019/04/NCGA-Legislation.pdf.

NC Policy Collaboratory. 2021. “North Carolina Per- and Polyfluoroalkyl Substances Testing (PFAST) Network.” 2021. https://ncpfastnetwork.com/about/.

Ng, Carla A., and Konrad Hungerbühler. 2014. “Bioaccumulation of Perfluorinated Alkyl Acids: Observations and Models.” *Environmental Science & Technology* 48 (9): 4637–48. https://doi.org/10.1021/es404008g.

“PFAS|EPA: PFAS Structures in DSSTox.” 2020. 2020. https://comptox.epa.gov/dashboard/chemical_lists/PFASSTRUCT.

PubChem. 2015 “Nafion, Pubchem.” Compound Summary: Nafion. Accessed July 20, 2021. https://pubchem.ncbi.nlm.nih.gov/compound/Nafion.

“PubChem Compound Summary for CID 74483, Perfluorooctanesulfonic Acid.” 2021. National Center for Biotechnology Information. https://pubchem.ncbi.nlm.nih.gov/compound/Perfluorooctanesulfonic-acid.

“PubChem Compound Summary for CID 14317645, Nafion by-Product 2.” 2021. National Center for Biotechnology Information. https://pubchem.ncbi.nlm.nih.gov/compound/Nafion-by-product-2.

Rahman, Mohammad Feisal, Sigrid Peldszus, and William B. Anderson. 2014. “Behaviour and Fate of Perfluoroalkyl and Polyfluoroalkyl Substances (PFASs) in Drinking Water Treatment: A Review.” *Water Research* 50 (March): 318–40. https://doi.org/10.1016/j.watres.2013.10.045.

Rushing, Blake R., Qing Hu, Jason N. Franklin, Rebecca McMahen, Sonia Dagnino, Christopher P. Higgins, Mark J. Strynar, and Jamie C. DeWitt. 2017. “Evaluation of the Immunomodulatory Effects of 2,3,3,3-Tetrafluoro-2-(Heptafluoropropoxy)-Propanoate in C57BL/6 Mice.” *Toxicological Sciences*, January, kfw251. https://doi.org/10.1093/toxsci/kfw251.

Selgrade, MaryJane K. 2007. “Immunotoxicity—The Risk Is Real.” *Toxicological Sciences* 100 (2): 328–32. https://doi.org/10.1093/toxsci/kfm244.

Strynar, Mark, Sonia Dagnino, Rebecca McMahen, Shuang Liang, Andrew Lindstrom, Erik Andersen, Larry McMillan, Michael Thurman, Imma Ferrer, and Carol Ball. 2015a. “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): 11622–30. https://doi.org/10.1021/acs.est.5b01215.

———. 2015b. “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): 11622–30. https://doi.org/10.1021/acs.est.5b01215.

Sun, Mei, Elisa Arevalo, Mark Strynar, Andrew Lindstrom, Michael Richardson, Ben Kearns, Adam Pickett, Chris Smith, and Detlef R. U. Knappe. 2016. “Legacy and Emerging Perfluoroalkyl Substances Are Important Drinking Water Contaminants in the Cape Fear River Watershed of North Carolina.” *Environmental Science & Technology Letters* 3 (12): 415–19. https://doi.org/10.1021/acs.estlett.6b00398.

US NTP. 2016. “NTP Monograph: Immunotoxicity Associated with Exposure to Perfluorooctanoic Acid or Perfluorooctane Sulfonate.” https://ntp.niehs.nih.gov/ntp/ohat/pfoa_pfos/pfoa_pfosmonograph_508.pdf.

Wang, Zhanyun, Jamie C. DeWitt, Christopher P. Higgins, and Ian T. Cousins. 2017. "A Never-Ending Story of Per- and Polyfluoroalkyl Substances (PFASs)?" *Environmental Science & Technology* 51 (5): 2508–18. https://doi.org/10.1021/acs.est.6b04806.

Woodlief, Tracey, Samuel Vance, Qing Hu, and Jamie DeWitt. 2021. "Immunotoxicity of Per- and Polyfluoroalkyl Substances: Insights into Short-Chain PFAS Exposure." *Toxics* 9 (5): 100. https://doi.org/10.3390/toxics9050100.

Yang, Q., Y. Xie, and J. W. Depierre. 2000. "Effects of Peroxisome Proliferators on the Thymus and Spleen of Mice." *Clinical and Experimental Immunology* 122 (2): 219–26. https://doi.org/10.1046/j.1365-2249.2000.01367.x.

Yang, Qian, Manuchehr Abedi-Valugerdi, Yi Xie, Xiao-Yan Zhao, Göran Möller, B Dean Nelson, and Joseph W DePierre. 2002. "Potent Suppression of the Adaptive Immune Response in Mice upon Dietary Exposure to the Potent Peroxisome Proliferator, Perfluorooctanoic Acid." *International Immunopharmacology* 2 (2–3): 389–97. https://doi.org/10.1016/S1567-5769(01)00164-3.

Yang, Qian, Yi Xie, Anna Messing Eriksson, B Dean Nelson, and Joseph W DePierre. 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*, 8.

Yao, Jingzhi, Yitao Pan, Nan Sheng, Zhaoben Su, Yong Guo, Jianshe Wang, and Jiayin Dai. 2020. "Novel Perfluoroalkyl Ether Carboxylic Acids (PFECAs) and Sulfonic Acids (PFESAs): Occurrence and Association with Serum Biochemical Parameters in Residents Living Near a Fluorochemical Plant in China." *Environmental Science & Technology* 54 (21): 13389–98. https://doi.org/10.1021/acs.est.0c02888.

Appendix A: Animal Use Protocol

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