Effects of PFMOAA, An Understudied PFAS, on Developing Mice

By

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Abstract

Perfluoro-2-methoxyaacetic acid (PFMOAA) was recently the most prevalent emerging per- and polyfluoroalkyl substance (PFAS) detected in the Cape Fear River of North Carolina (NC). A byproduct of fluorochemical manufacturing, PFMOAA is one of the shortest short-chain PFAS and is understudied with respect to its toxicity although it has been linked with immunotoxicity in mice. Well-studied PFAS are known to be immunosuppressive in both adult and developing organisms. The immune system is sensitive to disruptions and insults during development and may have an influence on immune-related conditions later in life. This study investigated the developmental effects of PFMOAA in mice to better understand the immunotoxicity of this short-chain PFAS on developing organisms. Pregnant C57BL6 mice were given PFMOAA (0.0, 0.05, 0.5, or 1.0 mg/kg with 0.5% Tween) or a 5.0 mg/kg perfluorooctane sulfonic acid (PFOS) positive control via gavage throughout gestation. B6C3F1 offspring were evaluated at postnatal days (PND) 21, 28, and 56 for organ weights, lymphoid organ cellularity was evaluated on PND 28 and 56, and T-cell dependent antibody responses (TDAR) and uterine

wall thickness were evaluated on PND 56. Dams were euthanized after weaning, liver weights were recorded, and the thickness of the uterine wall of dams was evaluated. Liver weights of dams and PND 21 female offspring exposed to PFOS were increased by 17.9% and 9.9%, respectively, compared to controls. There were increases in several relative organ weights across dose groups as well as a decrease in relative kidney weight in the 0.05 mg/kg dose group at PND 21 for female offspring. There were increases in relative heart, kidney, and brain weights at PND 56 in males of the 0.5 mg/kg dose group and increases in relative heart, kidney, and spleen weights as well as a decrease in relative uterus weight for 0.05 mg/kg group females. At PND 28, both sexes had an increase in thymus cellularity in the 0.5 mg/kg dose group as did females in the PFOS dose group when compared to controls. Females in the 0.5 mg/kg dose group had decreases in spleen cellularity at PND 28 and 56, and males in the 0.5 mg/kg dose group had a decrease in spleen cellularity at PND 56. These data suggest that at the administered doses, PFMOAA has potential to induce developmental toxicity and additional studies are warranted to determine further health effects of gestational exposure to PFMOAA and other emerging short-chain PFAS.

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Chapter 1: Introduction

1. Water Regulation

The safe drinking water act (SDWA) was introduced by the US Environmental Protection Agency (EPA) in 1974, with the goal of protecting the public from contaminants that can affect their health (EPA, 2020b). As summarized by the US EPA (EPA, 2020b), the SDWA covers all public water systems in the US but does not apply to private wells. The SDWA also allows for states to issue drinking water standards that are stricter than those provided at the federal level and to set standards for contaminants that are not federally regulated at all (EPA, 2020b). It provides safety standards for microorganisms, disinfectants, disinfection byproducts, radionuclides, inorganic chemicals, and organic chemicals (EPA, 2020b). For many contaminants covered by the safe water drinking act, the EPA establishes a Maximum Contaminant Level (MCL) or a treatment technique (TT) to ensure that contaminant levels in drinking water do not go above what is considered a safe level (EPA, 2020b).

An MCL is determined by reviewing the research data regarding the health effects of a particular contaminant. For chemical contaminants that are not suspected to be carcinogenic, this means reviewing toxicology and epidemiology studies to establish a reference dose - the amount of a toxicant that an individual may be exposed to on a daily basis across a lifetime that is not expected to induce adverse health effects (EPA, 2020a). The reference dose considers populations of people that may be more sensitive to the toxicant's effects, such as infants or those with compromised immune systems, and is used to establish a Drinking Water Equivalent Level (DWEL; EPA, 2020a). The EPA then sets a Maximum Contaminant Level Goal (MCLG) that takes into account the DWEL and the typical level of exposure for the population (EPA, 2020a). For contaminants with evidence of carcinogenicity, there is no level that is considered

safe; the EPA sets a MCLG of zero (EPA, 2020a). Once they have an MCLG, the EPA does a cost-benefit analysis to set an enforceable MCL that reduces health risk while also justifying the cost of instituting the standard (EPA, 2020a). In some cases, it may not be feasible to reliably maintain concentrations of certain contaminants below levels that would cause health risk. In these cases, the EPA may establish a TT to treat the water (EPA, 2020a). TT typically involve special filtration of the water or disinfection (CDC, 2015).

Public water systems are required to monitor their water on a regular basis to make sure their contaminant levels do not rise above the MCL, or to make sure that the TT they are using is effective at removing the contaminant (EPA, 2020a). They are also required to publish a Consumer Confidence Report available to the community that details any contaminants in the water and their potential health impacts (EPA, 2020c). When these safety regulations are violated and contaminant levels are too high, the public water system is required to notify its customers to the safety hazard and may also be fined (EPA, 2020c).

Every five years, the EPA publishes an updated Contaminant Candidate List (CCL) and an updated Unregulated Contaminant Monitoring Rule (UCMR; EPA, 2021a; EPA, 2021d). The CCL is a list of contaminants that are not currently regulated but are known or suspected to be in public water systems (EPA, 2021a). The EPA is required to make a regulatory decision on at least five contaminants from the CCL once it is published (EPA, 2021a). The UCMR is a list of up to 30 unregulated contaminants that are considered a priority for regular monitoring (EPA, 2021d). The data that are collected under the UCMR are used for future regulatory determinations (EPA, 2021d).

2. Per- and Polyfluoroalkyl Substances (PFAS)

One class of contaminants in public drinking water that is federally unregulated and that is of particular concern to public health is per- and polyfluoroalkyl substances (PFAS). Due to concerns about health effects, the EPA published a lifetime health advisory for two members of the class, perfluorooctanoic acid (PFOA) and perfluorooctane sulfonic acid (PFOS), in drinking water at a concentration of 70 ng/L; this health advisory level is not an enforceable standard but is designed to provide guidance to purveyors of public water for levels of these PFAS (EPA, 2016). PFAS are synthetic chemical compounds, consisting of multiple fluorine atoms bonded to a carbon backbone with a charged headgroup (Buck et al., 2011). There are now thousands of PFAS identified, some more well studied than others. One subclass, the perfluoroalkyl acids (PFAAs), has been more extensively studied than others due to their widespread use and prevalence in the environment and are generally divided into two major groups - perfluorosulfonic acids with a sulfonic acid headgroup, and perfluorocarboxylic acids with a carboxylic acid headgroup (Buck et al., 2011; Wang et al., 2017). Several of these types of PFAAs are considered “legacy PFAS” for their persistence in the environment and their historic use and include PFOA and PFOS, which were first put into product applications by the company 3M in the 1950s (3M, 2021). Since their introduction, many other unique PFAS, including additional PFAAs and other subclasses, have been created and put into use in a wide variety of applications (3M, 2021). PFAS carbon-fluorine bonds exhibit a strength that make them extremely useful in industrial and commercial products. Today PFAS are used in food packaging, nonstick cookware, water-repellent fabrics, fire-fighting foams, paints, cleaning products, and many other common items we encounter on a regular basis (Glüge et al., 2020). For the sake of modern convenience and comfort, PFAS have become ubiquitous in our daily

lives. Unfortunately, the strength and stability of the carbon-fluorine bonds that give products containing PFAS longevity are also what makes them a large part of the problem for the environment and human health.

PFAS can be found in varying concentrations in soil and groundwater all over the world, even in the far remote regions of the earth (Casal et al., 2017; Kim et al., 2007; Mussabek et al., 2019). Nicknamed "forever chemicals," PFAS have a very long environmental half-life and are therefore considered persistent - meaning they are highly resistant to environmental degradation and will not break down for many years (Washington et al., 2019). Many PFAS are still being used and deposited into the environment and as they do not break down, their presence may lead to accumulation (Mussabek et al., 2019). The unique structure of PFAS also allows them to be highly mobile in soil and water (Kwiatkowski et al. 2020). In many locations where groundwater and drinking water are tested, PFAS are found in concentrations higher than the EPA 70 ng/L health advisory for PFOA and PFOS (Crone et al. 2019).

Although the EPA published provisional health advisories for PFOA and PFOS in 2009 and an updated lifetime health advisory in 2016, there has been nothing published for the many other PFAS contaminating public drinking water (EPA, 2009; EPA, 2016). Six unique PFAS were first listed on the third UCMR in 2012, and 29 PFAS are listed on the current proposed UCMR 5 (EPA, 2021b; EPA, 2020e). In 2020 the EPA decided to regulate PFOS and PFOA although that process will likely take years. Whether or not to regulate other PFAS will for now remain a state decision (EPA, 2020d). Currently, 10 states in the US have drinking water guidelines for other PFAS, and 9 states have guidelines for PFOS and PFOA that are more stringent than the federal advisory level of 70 ng/L (Bridges and Oren, 2021; Post, 2021).

3. Human Health Impacts

As people come into contact with numerous PFAS in their daily lives, one of the primary sources of PFAS exposure is through drinking water (Crone et al., 2019). Even in locations with lower relative concentrations in the water, this is of concern due to the bioaccumulative nature of some PFAS (Burkhard, 2020). Through their National Health and Nutrition Examination Survey (NHANES), the Centers for Disease Control and Prevention conducts ongoing testing of people representative of the general US population and has found that PFAS are reliably found in serum as they bind to albumin and other proteins, and with continued exposure, serum concentrations may continue to build (Kato et al., 2011). As the half-life of PFOA and PFOS in serum of retired production workers was determined to be about 4-5 years (Olsen et al., 2007), ongoing exposures are likely to contribute to a consistent body burden. For manufacturing workers and communities living near fluorochemical manufacturing facilities, exposures are higher than that of the general population due to higher levels of PFAS contamination in their surrounding environment (ATSDR, 2021). Some PFAS are known to accumulate in human blood and some have been found to accumulate in tissues as well, including the brain, lungs, liver, kidneys, and in bone (Perez et al., 2013). In the body, PFAS induce a wide variety of toxic effects, some of which are still being discovered. In 2010, it was found that PFOA exposure in women and PFOS exposure in men was positively associated with increased instances of thyroid disease (Melzer et al., 2010). High serum concentrations of PFOA have also been associated with higher mortality rates from liver cancer (Girardi and Merler, 2019) and PFAS have also been linked to kidney disease (Shankar and Ducatman, 2011; Watkins et al., 2013). There is also evidence that PFAS exposure during pregnancy induces developmental toxicity and low infant birth weight (Wikström et al., 2019; Johnson et al., 2014). One more notable effect of exposure to PFAS is

immunosuppression. In humans, PFAS-linked immunosuppression is shown through a reduced response to vaccines (Grandjean et al., 2017; Kielsen et al., 2016), and in lab animals is demonstrated by reduced natural killer (NK) cell activity and a lowered IgM response to antigens (Dong et al., 2009; Rushing et al. 2017; DeWitt et al., 2008).

When the immune system is suppressed, a person may have a less robust response to a pathogen, slowing the recovery process of an illness and increasing the severity of symptoms (DeWitt et al., 2018). With PFAS exposure, a reduced response to vaccines is particularly evident in children, which can reduce the vaccine's efficacy and increase the likelihood of contracting the disease – a concern that is more appreciable at this time than in recent decades (Grandjean et al., 2012).

In light of these concerns for environmental and human health, the EPA initiated the 2010/2015 Stewardship Program in 2006 and in partnership with eight companies that contributed largely to PFAS pollution (EPA, 2021c). The goal of the Stewardship program was to attain a 95% reduction from the year 2000 by 2010 in emissions and product content of PFOA and PFOA precursors, and to fully eliminate their use and emissions by 2015 (EPA, 2021c). All eight companies reported success in these endeavors, however many switched to using "short-chain" PFAS under the claim that they have a reduced toxicological profile relative to the long-chain predecessors that were phased out (EPA, 2017). While it has been shown that in general short-chain PFAS tend to have a shorter half-life in the human body, they are still environmentally persistent and there currently is little data regarding their potential for toxicity (Brendel et al., 2018; Xu et al., 2020).

4. Water Treatment

Typically in the US, public drinking water is treated in several ways. The initial method is coagulation, wherein alum or ferric chloride are added to the water, which helps precipitate dissolved contaminants in the water. The precipitate clumps together into flocs that either float to the surface or sink to the bottom where they can be removed through filtration (CDC, 2015). This treatment process is moderately successful when it comes to removing the legacy PFAS compounds PFOS and PFOA but shows very little effectiveness at removing short-chain PFAS, potentially due to a lesser affinity for the air/water interface (Xiao et al., 2013; Appleman et al., 2014). The second treatment method for public drinking water is disinfection. This is accomplished by adding disinfectants to the water, such as chlorine, or through oxidation via ultraviolet treatment or hydrogen peroxide; neither chemical disinfectants nor oxidation is notably successful at removing any PFAS from the water (Appleman et al., 2014). Outside of traditional water treatment techniques, anion-exchange treatment and reverse osmosis systems may potentially be utilized in the removal of PFAS (Appleman et al., 2014). Anion exchange, typically used to treat arsenic contaminated water, uses an ion resin to separate contaminants from solution; this technique is able to remove at moderate efficacy some long-chain PFAS including PFOA but exhibits little capability to isolate short-chain compounds (Gagliano et al. 2020). Reverse osmosis uses a semipermeable membrane and high pressure to separate contaminants from solution and is extremely effective at removing even short-chain PFAS (Appleman et al., 2014; Tang et al., 2006). It is unlikely, however, for reverse osmosis treatment to be implemented large-scale due to the cost and space required for large volume reverse osmosis systems (Tang et al., 2006; Crone et al., 2019).

5. PFAS in NC

In compliance with UCMR 3 in 2012, North Carolina public water systems began routinely testing for PFOS, PFOA, perfluorononanoic acid (PFNA), perfluorohexanesulfonic acid (PFHxS), perfluoroheptanoic acid (PFHpA), and perfluorobutanesulfonic acid (PFBS). The Cape Fear Public Utility Authority (CFPUA) found concentrations of both PFOA and PFOS and determined that a major source of these contaminants in the Cape Fear River was through discharge from a chemical plant in Bladen County (Vandermeyden and Vaughn 2020). Built by DuPont in the 1970s and operated by DuPont until a spin-off, Chemours, took over operations, the "Fayetteville Works" plant was discharging PFAS into the Cape Fear River miles upstream from the CFPUA (Vandermeyden and Vaughn, 2020). As part of the EPA Stewardship program, DuPont agreed to phase out their use of PFOA and PFOS entirely by 2015 (Chemours was formed in 2015 as a spin-off of DuPont; Turner, 2015). To replace these legacy PFAS, DuPont/Chemours introduced perfluro-2-propoxypropanoic acid, the ammonium salt of which is known by the trade name "GenX," a short-chain perfluoroether compound (Sun et al., 2017). Perfluoroether acids (PFEAs) are subgroups of PFAAs; they contain an oxygen linkage in one or more places in between the carbons on the carbon chain. In 2017, it was reported that GenX was present at high concentrations in the treated water of the CFPUA, alongside high concentrations of other short-chain alternative PFAS (Sun et al., 2017). The compound found at the highest concentration in the Cape Fear River was perfluoro-2-methoxyacetic acid (PFMOAA; Hopkins et al., 2018).

The CFPUA serves residents in the communities of New Hanover County and the city of Wilmington. In 2017, the GenX Exposure Study analyzed serum from a subset of Wilmington residents for fluoroethers and legacy PFAS (Kotlarz et al., 2020). Multiple fluoroethers and

legacy PFAS were detected in the residents' serum, a cause for major public health concern in the Cape Fear River Basin area (Kotlarz et al., 2020). Many of the compounds found in serum from the GenX Exposure study are still poorly understood with regard to their toxicity, although there is evidence linking GenX exposure with immunosuppression (Rushing et al., 2017). Research is still being done on these PFAS as individual toxicants and as components within a mixture.

6. PFMOAA

Considered a byproduct of the manufacturing process of GenX, PFMOAA is one of the shortest short-chain PFAS, a perfluorocarboxylic acid with the formula $C_3HF_5O_3$, and was the most prevalent PFAS recently detected in the Cape Fear River of North Carolina – found in concentrations in excess of 600 ng/L after treatment (Hopkins et al., 2018). PFMOAA was also recently found in relatively high concentrations in the serum of a subset of residents of Huantai County in China who live near a fluorochemical plant (Yao et al., 2020). Serum concentration of PFMOAA was correlated positively with age and was significantly higher in male residents compared to female (Yao et al., 2020). Very little, however, is known about the toxicity or potential toxicokinetics and toxicodynamics of PFMOAA, in spite of its environmental concentrations and presence in human serum. In a study of male and female C57BL6 mice orally exposed to 0, 0.00025, 0,025, and 2.5 mg/kg PFMOAA for 30 days, there were no signs of overt toxicity, nor clear evidence of effects on the immune system, through evaluation of immune cell populations and the T-cell dependent antibody response (TDAR; Woodlief et al., 2021). In another study, C57BL6 mice exposed to PFMOAA for 30 days had a significant increase in TDAR in females exposed to 0.5 mg/kg PFMOAA and a significant decrease in TDAR in males

exposed to 50 mg/kg (Tobin, *Personal Communication*). These studies highlight the need for further investigation of PFMOAA.

7. Developmental Immunotoxicology

Throughout infancy and early childhood, the immune system is still actively developing. Very early in development hematopoiesis begins and the early blood cells migrate through the embryo and finally localize to form the progenitors of red blood cells and macrophages (Ygberg and Nilsson, 2011). The hematopoietic stem cells also form the bone marrow as well as the thymus (Ygberg and Nilsson, 2011). Mature bone marrow has an active role in both the innate and adaptive immune systems, producing circulating immune cells including B-cells, T-cells, and NK-cells, as well as the migration of the circulating immune cells (Zhao et al., 2012). The thymus is a primary lymphoid organ that is a critical component of the development of the immune system in early life, although its importance wanes throughout life. The T cells produced in the bone marrow are subject to differentiation within the thymus that produce naïve T cells that may migrate to secondary lymphoid organs, the spleen and lymph nodes (Thapa and Farber, 2019). The cells of the innate immune system have low functionality at birth, producing less of a cytokine response and are less cytotoxic to invading pathogens (Ygberg and Nilsson, 2011). These continue to mature throughout childhood, reaching full functionality during adolescence (Ygberg and Nilsson, 2011). The adaptive immune response in infants and children is also actively developing until adolescence, with blunted responses to pathogens and little experience for memory cells against specific pathogens (Simon et al., 2014; Ygberg and Nilsson, 2011).

Because the immune system is still developing throughout early life, it is particularly susceptible to insults that may occur during gestation or childhood. Exposures to environmental risk factors during early life can disrupt the development of the immune system in ways that may not be immediately visible but become evident during adolescence or adulthood (Dietert et al., 2009). While it is not uncommon to include reproductive and developmental toxicity testing in regulatory determination, developmental immunotoxicity testing is not currently standard procedure and evaluation of the developing immune system is only included when epidemiological evidence calls for it or a toxicant known to be immunotoxic leads to exposure in pregnant women (Collinge et al., 2012). Many PFAS are known to suppress the immune system, and there is evidence that some are capable of crossing the placenta as well as transferring to infants through breast milk (Anderko and Pennea, 2020; Blake and Fenton, 2020; Mondal et al., 2014). In one study, pregnant C57BL6 dams were exposed orally to PFOS and the B6C3F1 offspring developed deficits in NK-cell function and antigen-specific IgM production at eight weeks of age (Keil et al., 2008). Little has been done, however, to investigate the developmental toxicity of emerging short-chain PFAS such as PFMOAA.

8. Summary

Regulation of contaminants in drinking water in the US is a process that can take many years. Although PFAS have been present in the environment for decades, the body of scientific literature that confirms their persistence and toxicity has only recently reached the point where federal regulation is being considered. Despite the lack of federal regulation, PFOS and PFOA were voluntarily phased out of production by several major US manufacturers due to concerns over public health and environmental persistence (EPA, 2021c). These were replaced with short-chain chemistries under the premise that these replacements had reduced toxicity. These short-

chain PFAS are now found in high concentrations in public drinking water and are resistant to typical water treatment techniques (Appleman et al., 2014). As PFMOAA was a short-chain PFAS most prevalent in the drinking water of the Cape Fear River Basin area (Hopkins et al., 2018), research into its toxicity is critically important. Given the potential for other PFAS to disturb the developing immune system, this project investigated the potential for developmental immunotoxic effects to the offspring of C57BL/6-C3H mice whose C57BL/6 dams were orally exposed throughout gestation to PFMOAA.

Chapter 2: Developmental Immunotoxicity Study of PFMOAA in B6C3F1 Mice

1. Introduction

Perfluoro-2-methoxyacetic acid (PFMOAA) is a recently identified short-chain per- and polyfluoroalkyl substance (PFAS) found in relatively high concentrations in the Cape Fear River of North Carolina (Hopkins et al., 2018). There are thousands of PFAS in use. PFAS consist of a carbon backbone with multiple fluorine atoms and a charged headgroup (Buck et al., 2011). The carbon-fluorine bonds give PFAS strength that makes them remarkably useful in industrial and commercial products including food packaging, nonstick cookware, water-repellent fabrics, fire-fighting foams, paints, and cleaning products (Glüge et al., 2020). PFAS are also highly mobile in soil and water and are resistant to environmental degradation, giving them a high level of environmental and biological persistence (Kim et al., 2007). As PFAS are still actively being used and released into the environment, their persistence leads to a tendency to accumulate in the environment (Mussabek et al., 2019). Many PFAS also are bioavailable and can bind to albumin and other proteins, which leads to their accumulation in living organisms (Buck et al., 2011).

One of the primary exposure routes for PFAS is through drinking water (Crone et al., 2019). Most current drinking water treatment technologies are ineffective at removing PFAS from public water systems, and the general population is exposed to varying levels of PFAS contamination through their drinking water (Appleman et al., 2014). For manufacturing workers and communities living near fluorochemical manufacturing facilities, exposures are higher than that of the general population due to higher levels of PFAS contamination in their surrounding environment (ATSDR, 2021). For PFAS that have been well-studied, exposures in humans have been associated with increased risk of kidney and testicular cancer, increased cholesterol levels,

changes in liver enzymes, reduced infant birth weight, increased risk of pre-eclampsia or hypertension in pregnant women, and immunosuppression (ATSDR, 2021).

The US Environmental Protection Agency (EPA) has recommended a non-enforceable lifetime drinking water health advisory of 70 ng/L for two legacy PFAS, perfluorooctanoic acid (PFOA) and perfluorooctanesulfonic acid (PFOS), and several states have health advisories or enforceable maximum contaminant levels (MCLs) set at lower concentrations and often include a broader list of PFAS (Bridges and Oren, 2021; Post, 2021). In response to environmental and public health concerns, the EPA and eight major PFAS manufacturing companies joined the 2010/2015 Stewardship Program in 2006 with the goal to phase out and fully eliminate the use and emissions of PFOA and higher homologues in the US (EPA, 2021). All eight companies succeeded in this goal, although they did so by replacing PFOA with short-chain PFAS that under the assumption that they pose fewer risks to human and environmental health compared to the PFAS they replaced (EPA, 2017; Brendel et al., 2018; Xu et al., 2020). By 2002, the primary U.S. manufacturer voluntarily phased out production of PFOS (EPA, 2017b). In 2020 the EPA announced that it was moving forward with developing drinking water regulations for PFOS and PFOA; however other PFAS will remain federally unregulated but regularly monitored, in part through the Unregulated Contaminate Monitoring Rule published by the EPA every five years (EPA, 2020).

As these short-chain alternative PFAS and their by-products are still emerging and being identified, relatively little research has been done to determine their toxicity despite their continued prevalence in drinking water. In 2016, several emerging PFAS were found in the treated drinking water of the Cape Fear Public Utility Authority (CFPUA), including perfluro-2-propoxypropanoic acid, a short-chain alternative PFAS, the ammonium salt of which is known as

"GenX" (Sun et al., 2016). GenX has been found in the serum of a subset of Wilmington, North Carolina residents living downstream of a fluorochemical plant in Bladen County, along with several other PFAS (Kotlarz et al., 2020). Although many of the PFAS found in the drinking water and serum of Wilmington residents are still being studied and are currently poorly understood with regard to their toxicity, there is evidence linking GenX exposure with immunosuppression (Rushing et al., 2017).

The PFAS found in the highest concentration in the Cape Fear River in 2018 was PFMOAA and was found in treated water at concentrations above 600 ng/L (Hopkins et al., 2018). Considered a by-product of the manufacturing process of GenX, PFMOAA is one of the shortest short-chain PFAS, consisting of only a three-carbon backbone, five fluorine atoms, and a carboxylic acid head group (Hopkins et al., 2018). It was recently found in the serum of a subset of residents living near a fluorochemical plant located in China (Yao et al., 2020). Very little is known about PFMOAA or its toxicity; one study reported no signs of overt toxicity or evidence of immunotoxicity at the doses tested (Woodlief et al., 2021) whereas another study noted alterations in endpoints commonly impacted by PFAS exposure such as increased liver weight (Tobin et al., *in preparation*). A sensitive endpoint of immunotoxicity may be found in the developing immune system. Throughout infancy and early childhood, the immune system is still actively developing and is particularly susceptible to insults from environmental risk factors, although these disruptions may not become evident until adolescence or adulthood (Dietert et al., 2009). Developmental immunotoxicity testing for regulatory determinations is not currently a standard procedure and is typically only included when epidemiological evidence calls for it or if pregnant women are exposed to a toxicant known to be immunotoxic (Collinge et al., 2012). Well studied PFAS are known to suppress certain aspects of the immune system, and there is

evidence that they can cross the placenta and be transferred through breast milk (Blake and Fenton, 2020; Mondal et al., 2014). Gestational exposure to PFOS in mice has been shown to induce deficits in immune functionality in offspring evaluated at eight weeks of age, although the emerging short-chain PFAS have not been investigated for similar outcomes (Keil et al., 2008). This study aimed to investigate the potential for PFMOAA to induce developmental immunotoxic effects in B6C3F1 mouse offspring whose C57BL6 dams were orally exposed throughout gestation.

2. Materials and Methods

2.1 Animals

All experimental animal dosing and handling was carried out in accordance with procedures approved by the East Carolina University's Institutional Animal Care and Use Committee (IACUC). Two cohorts of male (10) C3H mice six to eight weeks old and four cohorts of female (20) C57BL/6 mice six to eight weeks old, were purchased from Charles River Laboratories. Males were housed two/cage. Females were assigned to groups of four/cage for a two-week long acclimation and estrous synchronization period. After acclimation, females were housed two/cage for a period of three days and each cage received a small sample of bedding from one male cage to induce the Whitten Effect prior to breeding. Animals were divided into four dams per dose group. Animals were housed with access for food and water *ad libitum* and kept at 22 ± 3 °C and 50 ± 20% humidity with a 12:12 h light-dark cycle.

2.1.1 Breeding

Breeding consisted of one overnight grouping to ensure that birth among dams occurred around the same 24-hour window. Each cage of two females was paired with one male from the

same cage used to induce the Whitten Effect. The first set of males were bred with female cohorts one and two, and the second set of males were bred with female cohorts three and four.

2.1.2 Dosing

Dosing solutions of PFMOAA (purchased from Fluoryx Labs, Carson City NV) were prepared fresh weekly in sterile water with 0.5% Tween at the following concentrations: 0.005 mg/mL, 0.05 mg/mL, and 0.1 mg/mL. Based on our previous studies, dosing solutions were administered at 0.1 mg/10 g of body weight, resulting in the appropriate 0.0, 0.05, 0.5, or 1.0 mg of PFMOAA/kg of mouse body weight, including a 5.0 mg/kg dose of PFOS as a positive control. Dosing was performed via oral gavage daily, from gestational days 1-17 based on individual daily body weights.

2.1.3 Birthing and sorting of offspring

On the day of birth, postnatal day (PND) 0, B6C3F1 pups were counted, sexed, and weighed as a litter. Litters were reduced to three males and three females per dam where possible, with cross-fostering between litters of the same dose group to achieve ideal sex/litter ratios. Litters were weighed three times/week to monitor growth. At PND 21, one male and one female per litter were randomly selected for tissue collection and remaining offspring were weaned into same-sex groups of two-four/cage. Once weaned, offspring body weights were recorded once/week until PND 56.

After weaning dams were euthanized, livers weights were recorded, and uteruses were collected, immersion fixed in 10% neutral buffered formalin for 24 hours and stored in 70% ethanol at 4°C for future analyses.

2.2 Offspring Endpoints

2.2.1 PND 21 Organ Weights

On PND 21, following anesthesia of selected offspring with Isoflurane, blood was collected by neck vein transection into collection tubes with a clot activator, allowed to sit for at least 30 minutes, and then centrifuged at 2000 x g for 15 minutes at 4°C. Serum was saved and frozen at -80°C for measurement of serum PFMOAA concentrations by collaborators. Immediately after euthanasia, brain, liver, heart, kidneys were removed and weights were recorded. All organs were frozen at -80°C for measurement of serum PFMOAA concentrations by collaborators.

2.2.2 PND 28 Lymphoid Organ Cellularity

On PND 28, one male and one female from each dam were randomly selected for lymphoid organ cellularity determination and immunophenotyping. Immediately after euthanasia, spleen and thymus were removed, placed into 3-mL of RPMI supplemented with 1% fetal calf serum (FCS), weighed, and aseptically processed into single-cell suspensions by grinding and passing through a 70 µm nylon filter with 7-mL of supplemented RPMI. Red blood cells were lysed, and an aliquot of each organ suspension was counted on a Nexcelom Bioscience Cellometer Auto 2000 cell counter (Nexcelom Bioscience LLC) to determine viability and cell counts. Cell counts were divided by the organ wet weight to calculate lymphoid organ cellularity. Cellularity is an observational marker often used as a sign of non-specific immune perturbation and can be an early warning sign of downstream immune dysfunction. Samples were adjusted to 2×10^7 cells/mL. Suspensions of spleen cells were aliquoted for T-, B-, and natural killer (NK)-cell quantification and suspensions of thymus cells were aliquoted for

T- cell quantification by flow cytometry. Optimal concentrations of antibodies and reagents were determined in previous studies (Woodlief et al. 2020). Monoclonal antibodies (eBioscience, San Diego, CA, USA) coupled to fluorochromes specific for chosen markers were used: APC anti-mouse CD3e, FITC anti-mouse CD4, and PE anti-mouse CD8a were used for enumeration of T cells from spleen and thymus and FITC anti-mouse CD45RB and PE anti-mouse NK1.1 were used for enumeration of B and NK cells in spleen. Each experimental replicate included unstained cells as a negative control and single-color samples as positive controls to determine color compensation. Flow cytometric analysis was performed and data were archived for future analysis. As the entire spleen and thymus were utilized for cellularity and flow cytometry analysis, histology was not performed on these organs.

2.2.3 PND 51 Immunizations

On PND 51, all remaining offspring in each dose group were immunized with sheep red blood cells (SRBCs) adjusted to 4×10^7 cells in 0.2-mL of sterile saline via tail vein injections.

2.2.4 PND 56

On PND 56, all remaining offspring in each dose group were anaesthetized with Isoflurane, and serum and tissues were collected and stored at -80°C as described above in *PND 2.2.5 21 Organ Weights*. Spleen and thymus were also collected for cellularity determinations and immunophenotyping as described above in *PND 28 Lymphoid Organ Cellularity*. For female offspring, uteruses were collected and immersion fixed in 10% neutral buffered formalin for 24 hours before storing in 70% ethanol at 4°C.

2.3 T Cell-Dependent Antibody Response (TDAR)

Mouse anti-SRBC IgM was evaluated in serum from PND 56 offspring (immunized with SRBC at PND 51) using pre-coated 96-well enzyme-linked immunosorbent assay (ELISA) plates (Life Diagnostics, West Chester, PA, USA). Serum samples were diluted 50-fold and were added to the plates along with anti-SRBC IgM standards in duplicate. Wells containing diluent only were included in duplicate as blanks. Plates were incubated at room temperature for 45 minutes on an orbital shaker at 75 rpm. Wells were washed five times with 1X wash solution, tapped dry, and 100 µL of enzyme conjugate was added to each well. Plates were then incubated again at room temperature for 45 minutes on an orbital shaker at 75 rpm, washed five times with 1X wash solution and tapped dry. 100 µL of TMB reagent was added to each well and incubated for 20 minutes at room temperature on an orbital shaker at 75 rpm, after which 100 µL of stop solution was added. A microplate reader was used to measure absorption at 450 nm and results were calculated by fitting each sample onto a standard curve to solve for concentration of anti-SRBC IgM (units/mL).

2.4 Uterine Wall Thickness

Uterine samples were immersion fixed in 10% neutral buffered formalin for 24 hours and vertically embedded in paraffin. Paraffin-embedded samples were sectioned on a rotary microtome in 10-µm sections; twelve sections were collected per uterus, with six being collected from each horn (Figure 5). Sections were stained with hematoxylin and eosin and average uterine wall thickness was measured in dams and mature female offspring across dose groups by using ZEN core imaging software (Zeiss, Jena, Germany; version 3.1). Uterine wall thickness was defined as the total distance between the outside of the myometrium and the inside of the endometrium. Myometrium and endometrium were combined to reduce error in measurement as the boundary between the myometrium and endometrium is often challenging to delineate but

also because both endometrium and myometrium were considered important for the metrics of this endpoint. Additionally, while endometrial thickness is a marker of fertility, the myometrium is important as it allows for the uterus to expand for proper fetal development. To determine uterine wall thickness, four measurements were collected from one region per section per uterus and an average was calculated. Average uterine wall thickness was therefore determined as a grand average of each average per section.

2.5 Statistical Analysis

All weight and immune organ cellularity data are presented as mean ± standard deviation. Relative organ weights (somatic indices; SI) were calculated by dividing organ wet weight by the terminal body weight. With the exception of litter growth, all data were analyzed using Microsoft Excel, with a one-way analysis of variance (ANOVA) to determine dose-dependent differences in body and organ weights and immune organ cellularity. Individual pairwise t-tests were also performed between each dose group and the age- and sex-matched 0.0 mg/kg control group. Litter growth data were analyzed using a repeated measures ANOVA, with a one-way ANOVA at individual time points and when one-way ANOVA indicated a statistically significant dose effect, individual t-tests were performed. Statistical significance was determined using a P value of 0.05.

3. Results

3.1 Animal Body Weights

Weights of confirmed pregnant animals did not differ statistically by dose during the dosing period (Figure 1). One animal in the 0.0 mg/kg PFMOAA dose group of the second

cohort died due to a gavage-related injury on the fifth day of dosing. N = 2-4 dams per dose group.

Average pup weight per litter in the 0.5 mg/kg dose group was increased at PND zero by 12.0% compared to the 0.0 mg/kg dose group (Figure 2). N = 2-4 litters per dose group.

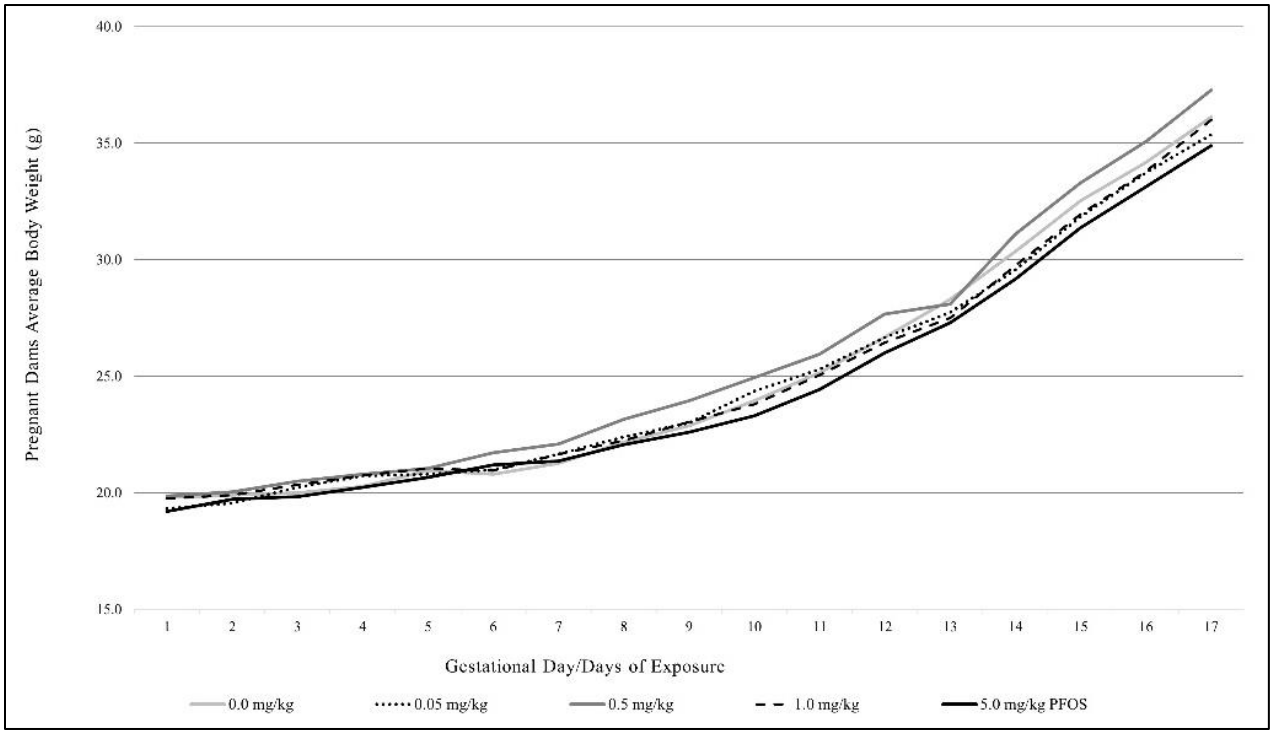
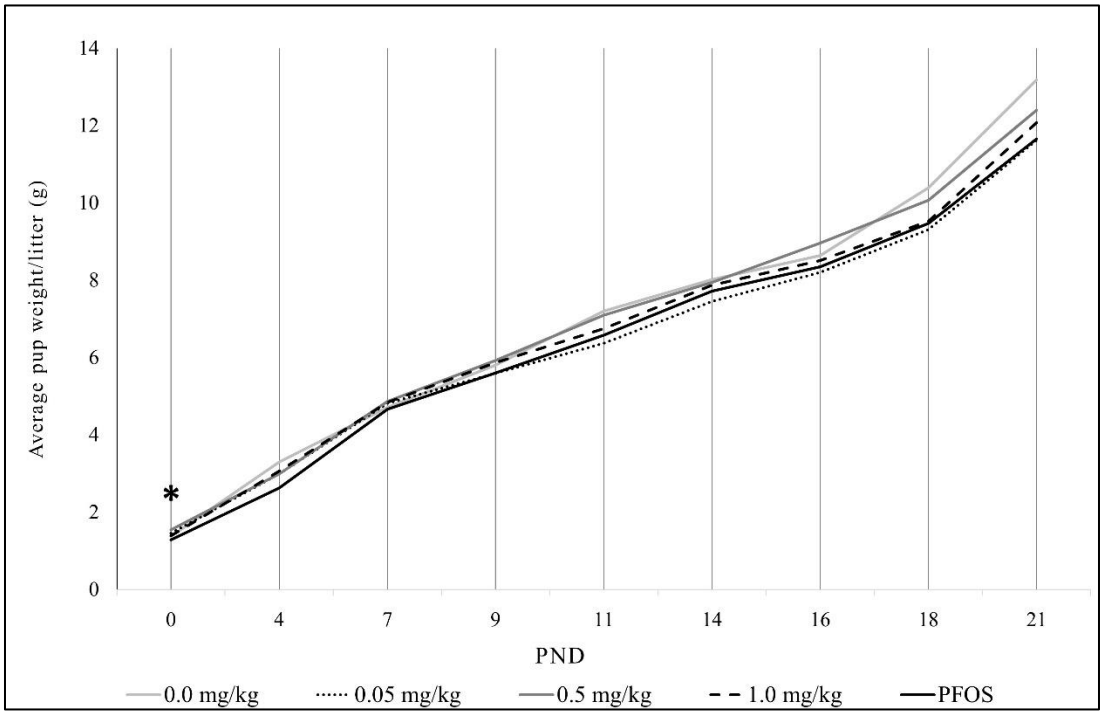


Figure 1. Average daily body weights for pregnant C57BL/6 mice exposed to PFMOAA from gestational days 1-17 (standard deviations not shown). Average body weights did not differ statistically by dose throughout the dosing period. N = 2-4 dams per dose group.

A.



B.

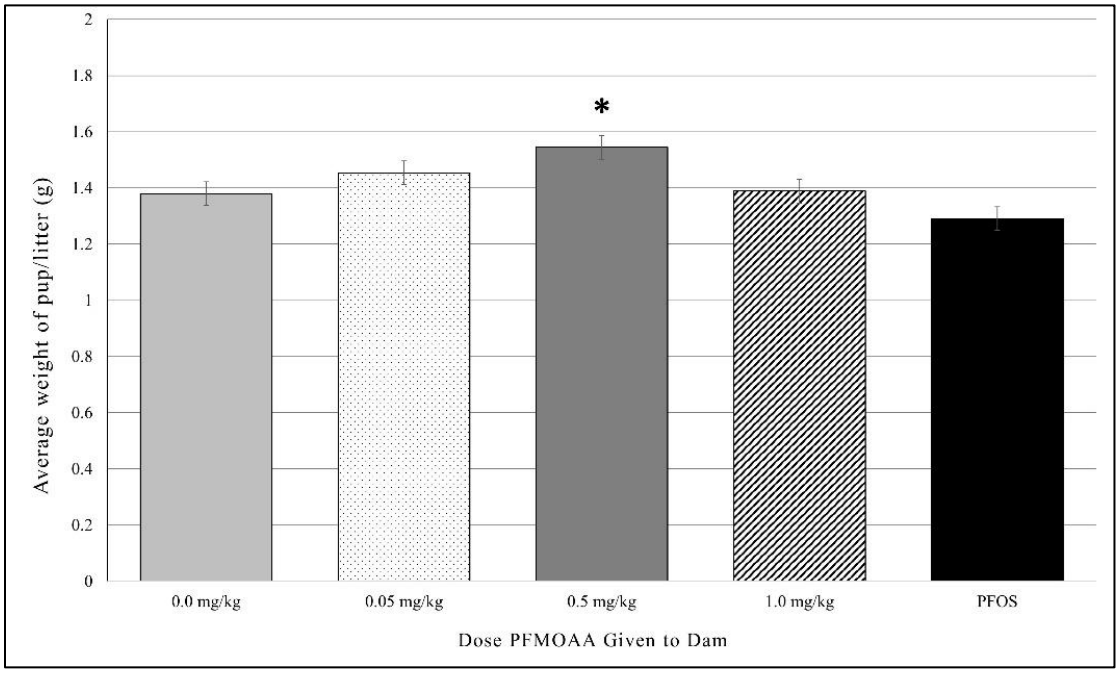


Figure 2. (A) Average body weight of B6C3F1 offspring per pup per litter from PND 0-21 (standard deviations not shown). (B) At PND 0, average pup body weight per litter increased by

12.0% for the 0.5 mg/kg dose group verses the 0.0 mg/kg control group. * indicates a statistical difference between 0.5 mg/kg group and 0.0 mg/kg group at PND0 ($p \leq 0.05$). N = 2-4 litters per dose group.

3.2 Organ Weights

Terminal body weights, absolute organ weights, and organ weights corrected by terminal body weight (expressed as relative weights or somatic indices; SI) are listed in Table 1. No statistical changes were observed for terminal body weights at any timepoint. Statistically significant ($P < 0.05$) changes included the following: absolute liver weight in dams exposed to PFOS were increased by 17.9% compared to control dams. At PND 21, female offspring in the 0.05 mg/kg exposure group had a 7.1% decrease in relative kidney weight compared to control offspring, whereas at the same timepoint, female offspring in the 1.0 mg/kg group had a 5.2% increase in relative kidney weight compared to control offspring. Female offspring in the PFOS group had a 41.7% increase in relative heart weight compared to control offspring and a 9.9% increase in relative liver weight compared to controls. At PND 56, female offspring in the 0.05 mg/kg dose group had 33.0%, 12.8%, and 17.2% increases in relative heart, kidney, and spleen weights, respectively, compared to controls, as well as an 8.7% decrease in the relative weight of uterus and ovaries combined. Male offspring in the 0.5 mg/kg dose group had a 17.8%, 19.4%, and 71.4% increase in relative brain, kidney, and heart weights, respectively, when compared to control offspring. N = 1-5 animals per sex per dose group.

Table 1. Average body weights, absolute organ weights, and relative organ weights (somatic indices; SI) ± standard deviation for dams and offspring at all time points. N = 1-5 animals per sex per dose group. * indicates a statistical difference between indicated group and 0.0 mg/kg dose group ($p \leq 0.05$).

Dam	**0.0 mg/kg**	**0.05 mg/kg**	**0.5 mg/kg**	**1.0 mg/kg**	**5.0 mg/kg PFOS**
Terminal body weight (g)	24.23 ± 0.95	23.8	23.6	24.9 ± 0.71	22.9
Liver weight (mg)	1596 ± 127.9	1470 ± 79.4	1295 ± 124.8	1756 ± 42.4	1881 ± 111.6*
Uterus + Ovaries (mg)	209 ± 52.8	181 ± 83.6	171 ± 37.8	180 ± 7.1	147 ± 34.5
PND 21 Offspring					
Male	**0.0 mg/kg**	**0.05 mg/kg**	**0.5 mg/kg**	**1.0 mg/kg**	**5.0 mg/kg PFOS**
Terminal body weight (g)	13.3 ± 1.7	11.9 ± 2.6	11.6 ± 2.2	11.9 ± 0.1	11.9 ± 0.4
Liver SI	5.59 ± 0.29	4.69 ± 1.17	5.15 ± 0.49	5.28 ± 0.54	5.86 ± 0.32
Brain SI	2.86 ± 0.29	2.93 ± 0.22	2.61 ± 1.02	2.82 ± 0.12	2.79 ± 0.41
Heart SI	0.63 ± 0.10	0.61 ± 0.10	0.75 ± 0.26	0.69 ± 0.05	0.82 ± 0.10
Kidney SI	1.41 ± 0.07	1.35 ± 0.01	1.49 ± 0.06	1.50 ± 0.07	1.44 ± 0.10
Female	**0.0 mg/kg**	**0.05 mg/kg**	**0.5 mg/kg**	**1.0 mg/kg**	**5.0 mg/kg PFOS**
Terminal body weight (g)	12.5 ± 1.0	11.2 ± 1.9	11.0 ± 2.4	12.3 ± 0.0	11.6 ± 0.6
Liver SI	5.33 ± 0.22	4.89 ± 0.57	5.24 ± 0.25	5.61 ± 0.15	5.86 ± 0.35*
Brain SI	2.67 ± 0.92	3.09 ± 0.48	2.95 ± 0.55	3.10 ± 0.17	2.85 ± 0.52
Heart SI	0.60 ± 0.09	0.65 ± 0.05	0.74 ± 0.13	0.66 ± 0.02	0.85 ± 0.10*
Kidney SI	1.54 ± 0.02	1.43 ± 0.06*	1.62 ± 0.07	1.62 ± 0.01*	1.56 ± 0.07
PND 28 Offspring					
Male	**0.0 mg/kg**	**0.05 mg/kg**	**0.5 mg/kg**	**1.0 mg/kg**	**5.0 mg/kg PFOS**
Terminal body weight (g)	20.15 ± 1.3	22.6	18.6	21.1 ± 0.6	20.7
Spleen weight (g)	101 ± 14.54	117 ± 6.51	109 ± 17.04	121 ± 11.31	98 ± 6.35
Thymus weight (mg)	81 ± 8.04	83 ± 9.54	90 ± 11.00	80 ± 0.71	72 ± 4.04
Female	**0.0 mg/kg**	**0.05 mg/kg**	**0.5 mg/kg**	**1.0 mg/kg**	**5.0 mg/kg PFOS**

Terminal body weight (g)	17.0 ± 1.1	19.7	15.9	17.0 ± 1.1	16.8
Spleen weight (mg)	86 ± 8.62	89 ± 5.69	94 ± 17.80	96 ± 5.66	78 ± 5.86
Thymus weight (mg)	101 ± 12.83	97 ± 16.86	92 ± 6.99	99 ± 10.61	76 ± 8.15

PND 56 Offspring					
Male	**0.0 mg/kg**	**0.05 mg/kg**	**0.5 mg/kg**	**1.0 mg/kg**	**5.0 mg/kg PFOS**
Terminal body weight (g)	27.9 ± 1.78	27.9 ± 2.40	27.1 ± 0.14	30.7 ± 1.15	27.9 ± 1.31
Liver SI	4.09 ± 2.38	5.28 ± 0.12	5.33 ± 0.16	5.21 ± 0.06	5.87 ± 0.26
Brain SI	1.46 ± 0.10	1.54 ± 0.04	1.72 ± 0.04*	1.36 ± 0.10	1.52 ± 0.14
Heart SI	0.41 ± 0.03	0.65 ± 0.22	0.70 ± 0.14*	0.43 ± 0.02	0.60 ± 0.22
Kidney SI	1.39 ± 0.03	1.49 ± 0.09	1.66 ± 0.04*	1.42 ± 0.01	1.54 ± 0.15
Spleen SI	0.34 ± 0.03	0.37 ± 0.07	0.35 ± 0.07	0.36 ± 0.02	0.31 ± 0.02
Thymus SI	0.19 ± 0.05	0.17 ± 0.02	0.17 ± 0.05	0.18 ± 0.04	0.17 ± 0.04
Female	**0.0 mg/kg**	**0.05 mg/kg**	**0.5 mg/kg**	**1.0 mg/kg**	**5.0 mg/kg PFOS**
Terminal body weight (g)	22.7 ± 1.52	21.5 ± 1.84	22.1 ± 1.78	21.6	22.3 ± 1.38
Liver SI	5.16 ± 0.31	5.15 ± 0.45	5.29 ± 0.36	5.07	5.47 ± 0.23
Brain SI	1.84 ± 0.18	2.04 ± 0.09	2.00 ± 0.06	1.92	1.92 ± 0.06
Heart SI	0.43 ± 0.02	0.58 ± 0.07*	0.50 ± 0.15	0.48	0.520 ± 0.09
Kidney SI	1.24 ± 0.04	1.40 ± 0.09*	1.35 ± 0.11	1.20	1.33 ± 0.08
Spleen SI	0.43 ± 0.03	0.50 ± 0.02*	0.48 ± 0.05	0.49	0.44 ± 0.05
Thymus SI	0.25 ± 0.05	0.29 ± 0.03	0.36 ± 0.09	0.37	0.38 ± 0.10
Uterus + Ovaries SI	0.80 ± 0.02	0.73 ± 0.01*	0.82 ± 0.28	0.81	0.81 ± 0.13

3.3 Cellularity

Spleen and thymus cellularity (Figure 3) were determined by the total cell count divided by the weight of each organ. At PND 28 female thymus cellularity was increased by 57.3% and 56.0% compared to controls in the 1.0 mg/kg dose group and PFOS dose group respectively. Male thymus cellularity for animals in the 1.0 mg/kg group was increased by 250.3% compared

to negative control male offspring. Female spleen cellularity in the 0.5 mg/kg dose group had a 27.4% decrease compared to controls. At PND 56, females and males in the 0.5 mg/kg dose group had a 41.6% and 40.0% reduction, respectively, in spleen cellularity compared to control offspring. N = 2-4 animals per sex per dose group.

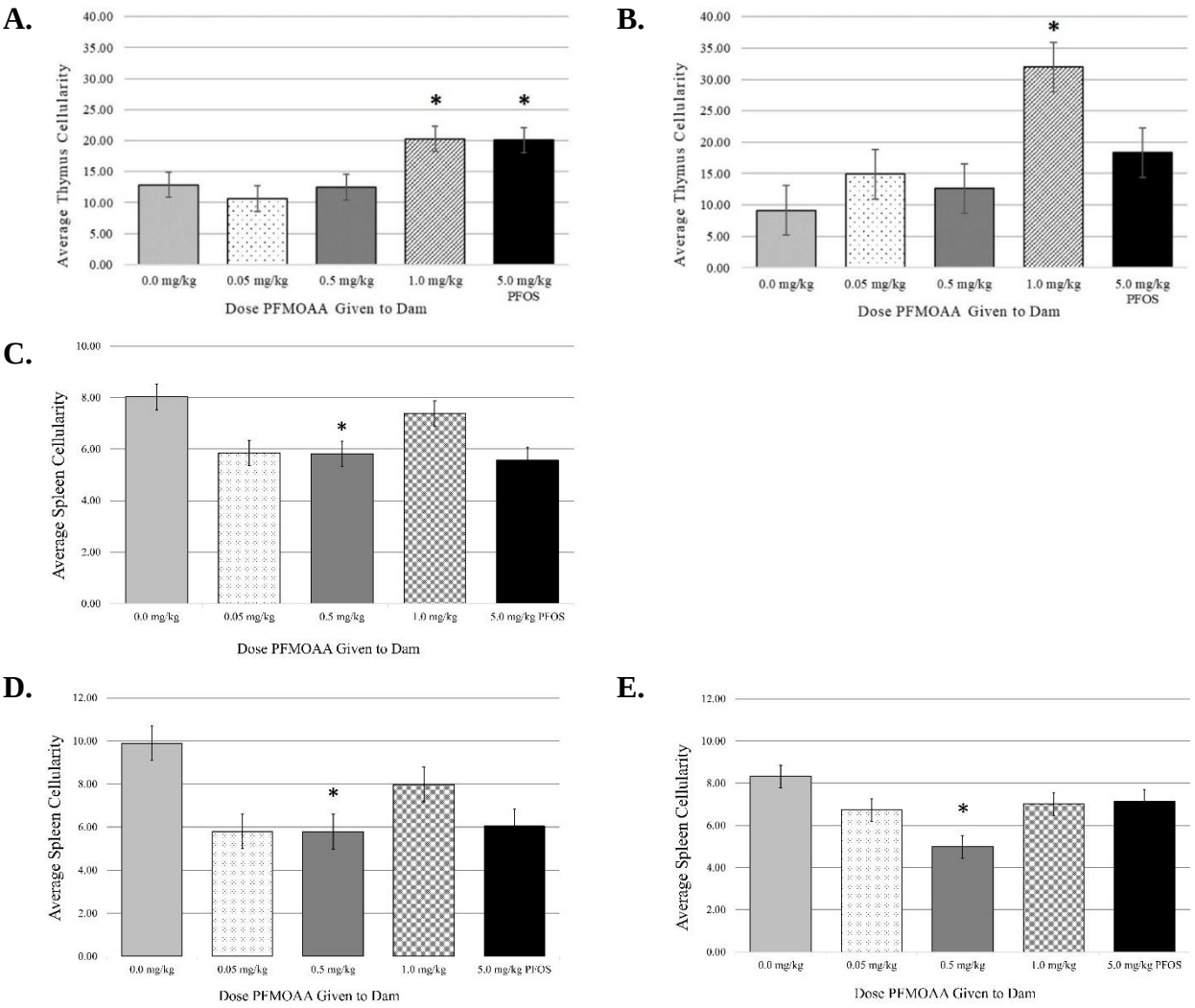


Figure 3. Average cellularity of female thymus (A), male thymus (B), and female spleen (C) at PND 28 and female spleen (D) and male spleen (E) at PND 56. * indicates a statistical difference between indicated dose group and 0.0 mg/kg dose group ($p \leq 0.05$). N = 2-4 animals per sex per dose group.

3.4 TDAR

No statistical differences were detected in the TDAR for offspring of any dose group (Figure 4). N = 1-5 animals per sex per dose group.

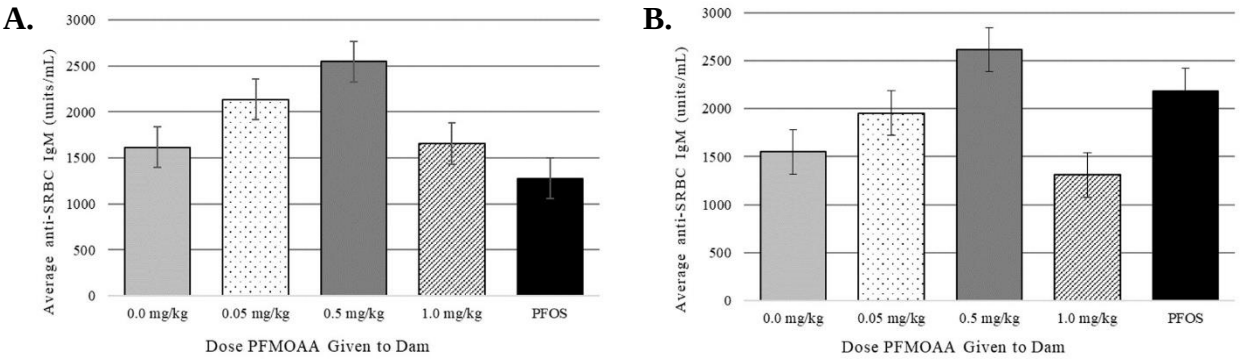


Figure 4. Mean anti-SRBC IgM (units/mL) in female (A) and male (B) offspring whose dams were exposed to PFMOAA throughout gestation. Serum was analyzed at PND 56, five days after immunizations. No statistical differences in the TDAR were detected in any dose group. N = 1-5 animals per sex per dose group.

3.5 Uterine Wall Thickness

No statistical differences were detected for the average uterine wall thickness for dams or offspring (Figure 6).

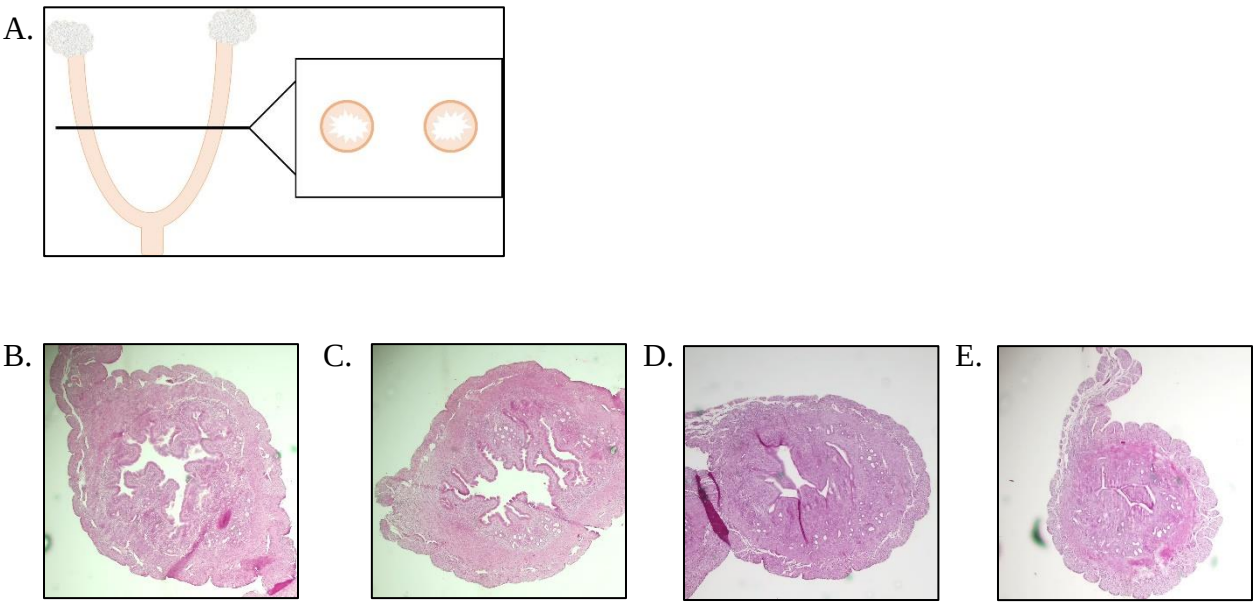


Figure 5. (A) Simplified visual representation of uterine sectioning. (B-E) Representative images of uterine sections from (B) control dam, (C) treated dam, (D) control offspring, and (E) treated offspring.

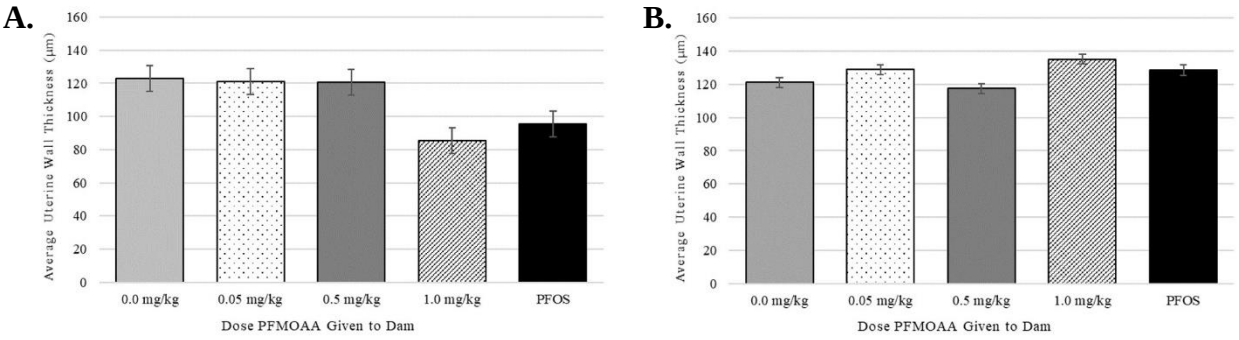


Figure 6. Average uterine wall thickness of dams (A) and female offspring at PND 56 (B) in µm. N = 2-4 dams per dose group, 1-5 female offspring per dose group.

4. Discussion

In this study, we evaluated immune endpoints in developing B6C3F1 mice known to be sensitive to PFAS exposure to determine if the emerging contaminant PFMOAA would induce

similar changes. The immune endpoints investigated were TDAR, lymphoid organ weights, and cellularity from the spleen and thymus. We also assessed multiple organ weights and evaluated uterine wall thickness of dams and mature female offspring to determine potential morphological changes in this reproductive tissue induced by PFMOAA exposure during gestation. The doses of PFMOAA and PFOS were established based on rodent studies using PFOS with the goal of identifying developmental immunotoxic effects while avoiding overt toxicity in the dams or offspring (Keil et al. 2008; Luebker et al. 2005). There were no signs of overt toxicity in the dams throughout the dosing period, and no signs of overt toxicity in the pups from birth through PND56 when they were euthanized. One dam in the 1.0 mg/kg dose group only produced two pups, which failed to thrive; however, it is unclear if this was related to PFMOAA administration. Interestingly, two dams developed nipple infections before weaning, one in the 0.05 mg/kg dose group and one in the 0.5 mg/kg dose group. However, like the dam with pups that failed to thrive, it is unclear if this was related to PFMOAA administration, although PFOA exposure has been linked to lactation impairment in mice (White et al., 2007).

Dams given PFMOAA did not display hepatomegaly or changes in uterine weight, although dams exposed to 5.0 mg/kg PFOS did have a 17.9% increase in liver weight compared to controls, a response that is expected from exposure to PFOS (Lau et al., 2007). At PND 21, female offspring in the PFOS group had a 41.7% increase in relative heart weight compared to control offspring, as well as a 9.9% increase in relative liver weight. Female offspring in the 0.05 mg/kg group had a 7.1% decrease in relative kidney weight verses control offspring, in contrast to the 1.0 mg/kg group where a 5.2% increase was noted. These changes in kidney weight are challenging to interpret as they do not follow a clearly dose-responsive pattern. At PND 21, offspring are still considered prepubescent and had not yet been weaned from the dams. They

still may have been receiving PFMOAA exposure from the dams through lactation, as some PFAS are known to be excreted in breast milk (Mondal et al., 2014), although it is not known if PFMOAA is excreted in breast milk. Organs collected at PND 21 were kept frozen at -80°C and will be evaluated for PFMOAA content at a later date, which may highlight why changes in some organ weights occurred at this time point. At PND 56, female offspring in the 0.05 mg/kg group had a 33.0%, 12.8%, and 17.2% increase in heart, kidney, and spleen relative weights, respectively, as well as an 8.7% decrease in relative uterine weight. Male offspring in the 0.5 mg/kg group had a 17.8%, 19.4%, 71,4% increase in relative brain, kidney, and heart weights, respectively, when compared to control offspring. Some PFAS have been reported to accumulate in the brain, heart, and kidneys (Perez et al. 2013), however the increases of these organ weights occurred in the two lower dose groups rather than the higher 1.0 mg/kg dose group and the increases occurred at PND 56 without evidence of similar increases at PND 21. In the case of the female kidneys, the increase in the 0.05 mg/kg dose group at PND 56 occurred after a significant decrease in relative weight at PND 21. Should there be a parallel increase in PFMOAA content in these tissues upon further analyses it may help explain the increase in tissue weight but will not explain how the PFMOAA concentrations in these organs increased after 35 days without known sources of PFMOAA exposure. These contrasting changes in organ weights at different time points may be due to a possible rebound effect in response to PFAS exposure. A rebound effect may occur when there is damage or delays in development (Deniz et al., 2020; Park et al., 2016). Increased organ weights and hypertrophy can have a range of implications. One health outcome observed across multiple studies of PFAS-exposed human populations is preeclampsia (ATSDR, 2021), a condition that is thought to be caused by improper development or dysfunction of blood vessels in the placenta. There also is some evidence linking PFAS exposure

to hypertension in humans, as well as many connections between PFAS exposure and kidney hypertrophy and decline in kidney function (Pitter et al., 2020; Stanifer et al., 2018). Increases in the relative weight of some brain regions can be indicative of developmental neurotoxicity (Walker et al., 1989), and some PFAS have been linked to developmental neurotoxicity outcomes (Foguth et al., 2020). The changes in relative organ weights in this study, although they did not follow a clear dose- or age-dependent pattern, indicate that PFMOAA exposure was able to modulate physiology and suggest that some organs may be targeted differently than other organs.

At PND 28, female offspring had a 57.3% and 56.0% increase in thymus cellularity verses female controls in the 1.0 mg/kg group and PFOS group respectively, and male offspring had a 250% increase in thymus cellularity compared to control male offspring. This is challenging to interpret, as the more typical thymus response to PFAS exposure is demonstrated by atrophy and a decrease in thymus cellularity (Qazi et al., 2009). However, at PND 28 mice are mid-pubescence, at which time the thymus begins to atrophy and decline in output of naïve T cells (Brust et al., 2015; Palmer, 2013). It is possible that in this study, the offspring in the 1.0 mg/kg dose group experienced a PFMOAA-induced disruption in age-related thymus atrophy. Another possibility is a second example of a potential rebound effect of PFMOAA and PFOS exposure, resulting in higher thymus cellularity in these animals. Cellularity of the thymus is a biomarker of thymic functionality and immune system activity (Majumdar and Nandi, 2018). PFMOAA-induced thymus hyperplasia may indicate immune stimulation, which at inappropriate levels may be associated with conditions such as allergies or autoimmune diseases (Xin et al., 2017). Spleen cellularity in 0.5 mg/kg dose group females at PND 28 and 56, and in males at PND 56 had a decrease of 27.4%, 41.6%, and 40.0%, respectively, compared to controls. A

reduction in spleen cellularity may indicate decreased functionality, either through direct PFMOAA action on this organ, or impaired development from early life PFMOAA exposure.

Although there was no significant change in TDAR in offspring at PND 56, this is possibly due to low animal numbers and variability inherent in younger animals. The TDAR data suggest the potential for PFMOAA-induced changes in antibody-response as it appears as if both male and female offspring had increases in serum IgM concentrations in the 0.5 mg/kg dose group. An apparent increase in TDAR, as with increased thymus cellularity, may indicate the possibility of PFMOAA-induced immune activation, rather than immunosuppression, however there are few human studies linking PFAS exposure to immune activation (Granum et al., 2013; Timmerman et al., 2017). The developing immune system is particularly sensitive to disruptions caused by exposure to environmental risk factors (Dietert et al. 2009) In a study of gestational exposure of PFOS in B6C3F1 mice, there was a significant reduction in the TDAR at eight weeks of age for male offspring in the 5 mg of PFOS/kg dose group, demonstrating the sensitivity of the developing immune system to PFAS exposure (Keil et al. 2008). To our knowledge this is the first study to investigate the potential for developmental immunotoxicity of PFMOAA and our findings indicate that more research is warranted to fully evaluate the immune system effects of PFMOAA exposure that occurs during gestation.

Although not statistically significant, there was a 30.7% and 22.2% reduction in average uterine wall thickness for dams exposed to 1.0 mg/kg and to PFOS, respectively. Uterine wall thickness is associated with fertility outcomes, and having a thinner uterine lining is negatively correlated with fertility (Mahajan and Sharma, 2016). Exposure to PFOA has been associated with altered uterine structure in mice, including hyperplasia of uterine endometrial epithelia and hypertrophy of the layers of smooth muscle cells (Dixon et al., 2012). Some PFAS are thought to

be linked to fertility issues although the research on this is limited and varied, and for PFMOAA in particular, nonexistent (Kim et al., 2020; Ma et al., 2021; Bach et al., 2016). In this study, the 1.0 mg/kg dosed dams had the least amount of breeding success leading to viable pregnancies, although it is unknown if this outcome is related to PFMOAA administration. Further work is warranted to determine if PFMOAA may affect fertility outcomes in animal models or humans. Uterine wall thickness also changes with respect to the estrous cycle. The observed changes in uterine wall thickness in these two groups may indicate differences in estrous cyclicity for these dams but considering that uteruses were collected shortly after weaning for each dam, these changes are more likely related to exposure as during lactation, the mouse remains in anestrous and does not cycle (Liu et al., 2014).

The outcomes of this study are challenging to interpret, which suggests the need for further studies investigating the potential toxic effects gestational exposure to PFMOAA may have on the developing immune system.

5. Conclusion

At the doses of gestational PFMOAA we administered, there were some changes in organ weights in both male and female offspring at PND21 and at PND56. There were also increases in thymus cellularity at PND28 in the highest dose group for both sexes and decreases in spleen cellularity in the 0.5 mg/kg dose group at PND 28 and 56 for females and PND 56 for males. Gestational exposure to PFMOAA did not alter the TDAR for either sex. Exposure to PFMOAA did not alter uterine wall thickness for the dams or the mature female offspring. These data suggest that PFMOAA has potential for developmental immunotoxicity at the doses

administered and requires additional studies to determine further health effects on offspring gestationally exposed.

Chapter 3: Discussion

1. The Big Picture

Emerging per- and polyfluoroalkyl substances (PFAS) are a public health concern that is rapidly outpacing research and regulatory efforts. With the phase-out of legacy PFAS and replacement with short-chain alternatives, manufacturers continue to introduce novel PFAS that are slowly being identified in public drinking water alongside their by-products. With each new PFAS or by-product identified, monitoring of environmental media and exposure pathways and individual toxicity studies need to be run, as well as toxicity studies with PFAS mixtures. It can take years to accumulate enough data to prompt regulatory decisions; meanwhile novel PFAS are continuously being identified and the public are continuously being exposed to a growing number of understudied PFAS.

When regulatory decisions regarding toxicants are made, testing for developmental immunotoxicity is not currently standard procedure unless called for by epidemiological evidence or pregnant women are exposed to a known immunotoxicant (Collinge et al., 2021). The developing immune system is a sensitive target for disruptions caused by exposures to environmental risk factors, and these disruptions may not become apparent until adulthood. This putative lag between exposure and effect makes it challenging to acquire the necessary epidemiology data to institute requirements for developmental immunotoxicity testing (Dietert et al., 2009).

Although many of the emerging PFAS are understudied with respect to their developmental immunotoxicity, there is evidence linking legacy PFAS with developmental immunotoxicity in animal models and humans (Kiel et al., 2008; Granum et al., 2013). Granum

et. al found that maternal prenatal serum concentrations of PFOS, PFOA, perfluorononanoate (PFNA), and perfluorohexane sulfonate (PFHxS), were negatively associated with childhood anti-vaccine antibody levels at age three, and positively associated with the number of episodes of common cold for each child. These data indicate developmental immunosuppression from gestational exposure to legacy PFAS (Granum et al., 2013). In mice, gestational PFOS exposure has resulted in deficits in natural killer (NK)-cell function and IgM antibody production in offspring evaluated at eight weeks of age (Kiel et al., 2008).

This study aimed to investigate the potential for PFMOAA, a highly prevalent short-chain PFAS in North Carolina (NC), to induce developmental immunotoxic effects in B6C3F1 mouse offspring whose C57BL6 dams were orally exposed throughout gestation.

2. Findings and Interpretations

Gestational exposure to PFMOAA did not result in overt toxicity in the dams or the offspring from birth through postnatal day (PND) 56 when they were euthanized.

We assessed relative organ weights of tissues that are known to accumulate PFAS, including the livers for both dams and offspring, as well as the brain, heart, and kidneys of the offspring (Perez et al., 2013). Organs were collected at PND 21 and 56. PFOS-exposed dams had a 17.9% increase in liver weight compared to controls, and females in the PFOS dose group at the PND 21 time point had a 9.9% increase in relative liver weight compared to controls. Increased liver weight is a common response seen in rodents exposed to PFOS (Lau et al., 2007). There was no observable change in liver weight of dams exposed to PFMOAA for the 17 day exposure period. At PND 21, a subset of offspring from each dam was euthanized and organs were weighed; remaining pups were weaned on this day as well. Female offspring in the 0.05

mg/kg group had a 7.1% decrease in relative kidney weight, and conversely female offspring exposed to 1.0 mg/kg had a 5.2% increase in kidney weight. PFOS-exposed female offspring also had a 41.7% increase in relative heart weight compared to controls. As the offspring are prepubescent at this stage and had not yet been weaned, there may have been continued PFMOAA exposure to the offspring from the dams via lactation. It is not known if PFMOAA is a PFAS that is excreted in breast milk, although some PFAS are known to be excreted in breast milk (Mondal et al., 2014). Changes in organ weights at this stage may be due to differences in PFMOAA accumulation the various tissues, although the contrasting decrease and increase in female kidney weights in different dose groups is challenging to interpret, suggesting a potential "rebound" effect at the higher dose group. A rebound effect may occur when there is damage or delays in development, resulting in hypertrophy like that observed in some PND 21 female organs. At PND 56, heart, kidney, and spleen relative weights increased by 33.0%, 12.88%, and 17.2% respectively in 0.05 mg/kg group females. There was also an 8.7% decrease in relative weight of the uterus and ovaries in 0.05 mg/kg group females compared to controls. Male offspring in the 0.5 mg/kg group had a 17.8% increase in relative brain weight, a 19.4% increase in relative kidney weight, and a 71.4% increase in relative heart weight compared to controls. At this stage in development, offspring had been weaned from the dams and had had no known source of PFMOAA exposure for 35 days. As these organ weight changes occurred at this stage without a similar increase at PND 21, it suggests that PFMOAA accumulation in these tissues was not solely responsible for the differences that occurred. Again, these changes could reflect a rebound effect from delays in development or damage that was not observable in weights of organs collected at earlier timepoints. Rebound effects can occur when an organ is subjected to stress or damage and one response seen is hypertrophy (Deniz et al., 2020; Park et al., 2016).

Some of these organ weight increases may appear small and biologically insignificant, however they may point to more serious implications in the health and functionality of the impacted systems. Increased heart weight may be indicative of ventricular hypertrophy, a symptom often resulting from chronic hypertension – an endpoint that may be linked to exposure to some PFAS (Aronow 2017; Pitter et al., 2020). Left ventricular hypertrophy is an important contributor to many serious coronary events such as heart attack or stroke (Aronow 2017). Increased relative kidney weight without PFMOAA accumulation likely points to renal hypertrophy, which is an important risk factor in developing chronic kidney disease, an outcome strongly linked to exposure to some PFAS (Tobar et al., 2013; Stanifer et al., 2018). Increased relative weight of the cerebellum may potentially be an important marker for developmental neurotoxicity (Walker et al., 1989). Some PFAS are known to accumulate in the brain, and early life PFAS exposure is also linked to developmental neurotoxicity in animal models (Gaballah et al., 2020). In human studies, serum concentration of several PFAS was correlated with cognitive impairments in eight-year-old children (Vuong et al., 2018). Increases in relative organ weights alone is not a diagnosis of serious PFMOAA induced toxicity in these organs but indicates the need for further investigation of these endpoints.

We also measured immune endpoints in the offspring that are known to be affected by exposure to some PFAS, including lymphoid organ weights and cellularity and T-cell dependent antibody response (TDAR).

At PND 28 offspring are mid-pubescence, at which time during immune development the thymus begins to atrophy and decline in output of naïve T cells (Brust et al., 2015; Palmer, 2013). PND 28 female offspring had a 57.3% increase and 56.0% increase in thymus cellularity compared to controls in the 1.0 mg/kg dose group and the PFOS dose group respectively. Male

offspring in the 1.0 mg/kg group at PND 28 had a 250% increase in thymus cellularity compared to control males. This was unexpected, as the typical response in the thymus exposed to immunotoxic PFAS is atrophy and decreased cellularity (Qazi et al., 2009). However, it is possible that in this study the typical age-related thymus atrophy was disrupted by PFMOAA exposure in the 1.0 mg/kg group offspring of both sexes and in the females of the PFOS group, resulting in higher thymus cellularity compared to controls. Another possibility is another occurrence of a rebound effect in these animals, resulting in higher thymus cellularity although there was no accompanying change in thymus weight. An increase in thymus cellularity does not always directly imply increased immune activation, however it does serve as a biomarker of immune activity and warrants further investigation of the functionality of this organ after developmental PFMOAA exposure (Majumdar and Nandi, 2018). Thymus hyperplasia and chronic inappropriate immune stimulation are linked to immune conditions that include autoimmune disorders and allergies (Xin et al., 2017), although there is conflicting evidence that these conditions may be associated with PFAS exposure (Granum et al., 2013; Timmerman et al., 2017). Also at PND 28, 0.5 mg/kg group females had a 27.4% decrease in thymus cellularity compared to controls. At PND 56, both females and males in the 0.5 mg/kg dose group had a reduction in spleen cellularity of 41.6% and 40.0%, respectively, compared to controls. PFAS exposure has typically been associated with immunosuppression (ASTDR, 2021), and a reduction in spleen cellularity could indicate a reduction in immune cell activity and total spleen functionality. This could be the result of direct action of PFMOAA on the spleen, as animal studies indicate a link between some PFAS exposure and splenic atrophy (Yang et al., 2001). Another possibility is that early life exposure to PFMOAA disrupted development of the spleen,

leading to reduced functionality. Perturbations in spleen development may lead to reduced lymphoid activity and reduced numbers of immune cell populations (Seymour et al., 2006).

At PND 56, T- and B-cell responses in the murine immune system have fully matured (Jackson et al., 2017). To test the functionality of the immune system of our gestationally exposed offspring, we immunized offspring at PND 51 with sheep red blood cells (SRBC). Five days later at PND 56, serum of immunized offspring was collected and evaluated for levels of anti-SRBC IgM. We saw no changes in anti-SRBC IgM at this time point, however there were high levels of within-group variability and low animal numbers, making it more challenging to detect a statistical difference. The TDAR data suggest that there may be potential for PFMOAA-induced changes in antibody-response, as in both female and male offspring, mean serum IgM concentrations increased from controls slightly in the 0.05 mg/kg group and peaked in the 0.5 mg/kg dose group, with little change for the 1.0 mg/kg group and variable differences in the PFOS group. Although it is not statistically significant ($p = 0.053$ in female 0.5 mg/kg dose group vs controls and $p = 0.056$ in male 0.5 mg/kg dose group vs controls), an apparent increase in IgM response in the 0.5 mg/kg dose group for both sexes was unexpected. One study that exposed adult C57BL6 mice to PFMOAA for 30 days saw a significant increase in female TDAR when exposed to 0.5 mg/kg PFMOAA, and a significant decrease in male TDAR when exposed to 50 mg/kg PFMOAA (Tobin, *Personal Communication*). In another study where B6C3F1 mice were gestationally exposed to PFOS, eight-week old male offspring had a decreased anti-SRBC IgM response in the 5 mg/kg PFOS exposure group, with no change at all in females (Kiel et al., 2008). The apparent increase in TDAR in the 0.5 mg/kg group of both sexes in this study alongside the thymus hyperplasia noted previously may indicate the possibility of immune activation rather than immune suppression following gestational exposure

to PFMOAA. This presents in epidemiological studies as increased instances of asthma, allergies, or autoimmune diseases, for example, however there are few human studies linking PFAS exposure to immune activation (Granum et al., 2013; Timmerman et al., 2017). One study using serum and survey responses from children participating in the 2013-2014 National Health and Nutrition Examination Study found weak associations between concentrations of some PFAS in serum and increased asthma risk (Jackson-Browne et al., 2020). At this time, there is very little information regarding immunotoxicity of PFMOAA and to our knowledge this is the first study to investigate the potential for developmental immunotoxicity of PFMOAA. Although these changes in TDAR are not statistically significant, they do warrant additional investigation into the potential for PFMOAA to induce developmental immunotoxicity.

In addition to organ weights and immune endpoints, we also evaluated effects of PFMOAA exposure on uterine wall thickness in dams and in the PND 56 female offspring. Although not statistically significant, these data indicate a 30.1% and 22.2% reduction in uterine wall thickness in the 1.0 mg/kg dams and PFOS-exposed dams respectively. Exposure to legacy PFAS is associated with altered puberty onset in female mice and rats, potentially due to PFAS-induced endocrine disruption (Du et al., 2019; Yang et al., 2009), as well as altered uterine structure in mice exposed to PFOA (Dixon et al., 2012). Uterine wall thickness is associated with fertility outcomes, and having a thinner endometrial lining is negatively correlated with fertility (Mahajan and Sharma, 2016). Some PFAS are considered to be linked to reduced fertility, although research on this is limited and varied (Kim et al., 2020; Ma et al., 2021; Bach et al., 2016). In this study, the 1.0 mg/kg dosed dams had the least amount of breeding success leading to viable pregnancies, although it is unknown if this outcome is related to PFMOAA

administration and PFMOAA to our knowledge has not been investigated for its impact on fertility outcomes.

3. Impacts

To our knowledge, this study is the first to investigate these endpoints in mice gestationally exposed to PFMOAA. Although the overall findings are challenging to interpret as there are no clear pattern to dose-, age- or sex-dependent outcomes, this is likely influenced by low animal numbers, and they serve to elucidate the need for repeated and further testing of the developmental effects of PFMOAA and other novel and understudied PFAS. Disruptions during immune development may be small or completely invisible at an early age and still have dramatic impacts later in life (Dietert et al., 2009). One step that could be take would be to look at immune endpoints in aging animals exposed during development or even to evaluate effects on F2 generation offspring. Regulatory determinations for drinking water are currently based on years of monitoring known contaminants through the Contaminate Candidate List and the Unregulated Contaminant Monitoring Rule, as well as large amounts of research data on each individual contaminant (EPA, 2020a). Meanwhile, more PFAS continue to be identified in drinking water and the public is continuously exposed to understudied and unregulated PFAS (Hopkins et al., 2018).

4. Conclusions and Future Work

We found that gestational exposure to PFMOAA induced changes in various organ weights at multiple developmental timepoints, induced increases in thymus cellularity in the 1.0 mg/kg group offspring of both sexes as well as the females in the PFOS group, and induced decreases in splenic cellularity at multiple timepoints and both sexes in the 0.5 mg/kg dose

group. Although not significant, gestational PFMOAA exposure also appeared to increase antigen-specific IgM antibody levels in the 0.5 mg/kg dose group for both sexes. Dams in the high dose group as well as the PFOS-exposed dams appeared to have reduced uterine wall thickness. These mixed findings make it difficult to draw specific conclusions about effects of developmental exposure to PFMOAA but do provide a basis for further testing of PFMOAA and other emerging found PFAS in public drinking water. Future work on this project will investigate the developmental immunotoxicity of other emerging PFAS and may further investigate PFMOAA developmental toxicity, in aging or F2 animals, and potentially explore the placenta as a target organ.

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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