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# Human Health Effects of Perfluorinated Compounds: Assessing Exposure in the Alaska Maternal Organic Monitoring Study and Testing Reproductive Toxicity in a Human Spermatogenesis Stem Cell Model

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B.S. Biology
Tufts University
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## Abstract

Human Health Effects of Perfluorinated Compounds:
Assessing Exposure in the Alaska Maternal Organic Monitoring Study and
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By Danielle Clarkson-Townsend

**Background:** Perfluorinated compounds (PFCs) are persistent pollutants commonly used as surfactants. Human health effects of chronic exposure to PFCs are not well understood, especially for fertility, but previous studies have found associations with blood lipids.

**Objective:** I examined the association of total cholesterol and PFC exposure among Alaska Native pregnant women (2005–2006, 2010–2012) and compared PFC levels to pregnant women surveyed in National Health and Nutrition Examination Survey (NHANES) during 2005–2006. Reproductive toxicity of PFCs were also tested using a novel *in vitro* human spermatogenesis stem cell model.

**Methods:** Mean PFC exposures (PFHxS, PFOS, PFOA, Me-PFOSA-AcOH, PFNA, and PFDeA) were compared within AK MOMS cohorts and with pregnant women in NHANES (2005-2006). The association between PFC exposures and total cholesterol was calculated using multivariable linear regression. Reproductive toxicity of PFOS, PFOA and PFNA on human spermatogenesis was tested using an *in vitro* human spermatogenesis model. Impacts to apoptosis, cell cycle, gene expression, and spermatogonial differentiation were evaluated using flow cytometry, qPCR, and immunostaining for PLZF after chronic exposure.

**Results:** Mean PFNA and PFDeA exposures were significantly higher in women from AK MOMS compared to NHANES. PFC exposures overall decreased in women from AK MOMS from 2005–2006 to 2010–2012, except PFHxS, which increased. PFOS, PFDeA, and PFHxS were significantly associated with elevated total cholesterol. *In vitro* PFC exposure resulted in increased cell death, decreased haploid cells, and impacted gene expression related to lipid metabolism and spermatogenesis. These results suggest that PFOS, PFOA and PFNA impair male fertility.

**Discussion:** This analysis supports an association with PFCs and cholesterol. Some PFCs may be higher in AK MOMS because of bioaccumulation patterns and dietary exposure. PFHxS exposure may have increased over time because it is a newer replacement compound. Further studies are needed to confirm these results in other populations and examine the potential health impacts of PFNA, PFDeA, and PFHxS exposure. The *in vitro* analysis suggests that exposure negatively impacts spermatogenesis and parameters related to fertility. Future studies should focus on assessing other lipid-related outcomes, as well as the reproductive impacts of newer PFCs and PFC mixtures.

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# INTRODUCTION

## Perfluorinated Compounds: Brief History and Background

Perfluorinated compounds (PFCs), also called perfluoroalkyl or polyfluoroalkyl substances (PFAS), are a man-made class of chemicals with carbon-fluorine bonds in place of carbon-hydrogen bonds. Interest in the industrial production of PFCs began in the 1940's when they were synthesized during the Manhattan Project; however, their use on an industrial scale did not begin until the 1950's (Okazoe, 2009; Prevedouros, Cousins, Buck, & Korzeniowski, 2006).

The chemical composition of PFCs makes them stable, hydrophobic compounds. Among their many applications, they have been used to make emulsions, stain-proof and waterproof materials (Vierke, Staude, Biegel-Engler, Drost, & Schulte, 2012). The carbon chain portion of PFCs resembles a hydrocarbon chain, but with fluorine substituted for hydrogen (Figures 1-3). Similar to a hydrocarbon chain, the non-polar fluorocarbon chain of PFCs gives them hydrophobic, or "water fearing", properties. However, compared to hydrocarbons, fluorocarbons are even more hydrophobic because the "fatness" of the molecule decreases its van der Waals interactions with water (Dalvi & Rossky, 2010). This property makes them ideal as stain or water repellants. PFCs are also relatively unreactive and thermally stable. Together, these chemical properties made PFCs useful in the production of non-stick cookware such as Teflon, stain-proof carpeting, sprays like Scotchgard, and fire-fighting foams (Prevedouros, Cousins, Buck, & Korzeniowski, 2006). Some of the properties that make PFCs so useful also contribute to their biopersistance.

The industrial use of PFCs, and subsequent emissions, is believed to be the largest contributor to the environmental burden of PFCs (Prevedouros, Cousins, Buck, & Korzeniowski, 2006). When DuPont started using polytetrafluoroethylene (PTFE) in the production of its non-stick cookware, Teflon, they used perfluorooctanoic acid (PFOA) to act as a surfactant in the application of PTFE to cookware. Widespread environmental contamination of PFOA from DuPont's Washington Works plant in West Virginia and the ensuing concerns about health effects spurred a large epidemiological investigation, called the C8 project, into the effects of exposure on human health (Steenland, Tinker, Frisbee, Ducatman, & Vaccarino, 2009).

In addition to DuPont, 3M was also a large producer of PFCs in the United States. 3M manufactured and used perfluorosulfonic acid (PFOS) for approximately four decades as the main ingredient in Scotchgard, a spray stain repellant, until phase-out began in 2000 (Olsen et al., 2007a). PFCs such as PFOA and PFOS are also used in Aqueous Film Forming Foams (AFFF) to put out oil or grease fires. The use of AFFF in battling large petroleum fires, such as those from planes and other vehicles, has also caused soil and groundwater contamination of military bases and fire or crash training sites (Arias, Mallavarapu, & Naidu, 2015; Anderson, Long, Porter, & Anderson, 2016).

**Perfluorinated Compounds in the Ecosystem**

Today, PFCs are ubiquitous in the environment. The C-F bonds confer stability to PFCs, and they do not readily break down from weathering processes such as sunlight exposure (Organization for Economic Co-operation and Development [OECD], 2002). They have long half-lives. For example, the Environmental Protection Agency (EPA)

estimated that PFOS can persist for more than 41 years and PFOA can persist for more than 92 years in 25$^{o}$C water (EPA, 2014). In humans, the half-life of PFCs is uncertain, but PFOS is believed to have a half-life of approximately 4.8 years, PFOA with a half-life of 3.5 years, and perfluorohexane sulfonate (PFHxS) with a half-life of 7.3 years (Olsen et al., 2007b). Because of the strength of the carbon-fluorine bond and the rarity of natural fluorinated metabolites, there are not many microorganisms that have evolved to biodegrade PFCs (Parsons, Saez, Dolfing, & de Voogt, 2008); hence, PFCs such as PFOA are considered to be "microbiologically inert" (Liou, Szostek, DeRito, & Madsen, 2010).

While a strain of *Pseudomonas aeruginosa*, an anaerobic bacteria, was initially believed to degrade PFOS under alkaline conditions in a laboratory setting, the researchers only found shorter-chain compounds such as PFHxS and perfluorobutanesulfonic acid (PFBS) as by-products (Kwon et al., 2014). This finding suggests that the bacteria were cleaving C-C bonds rather than performing defluorination by cleaving C-F bonds, and thus not truly breaking down PFCs in a considerable way (Kwon et al., 2014). Additionally, some microorganisms are able to catalyze the formation of PFOS from perfluorooctane sulfonamide (PFOSA); they may also catalyze PFOA and perfluorononanoic acid (PFNA) from precursors (Murakami et al., 2013). PFOA may also be a breakdown product of some other perfluorinated compounds (Vierke, Staude, Biegel-Engler, Drost, & Schulte, 2012). Therefore, these compounds are biopersistent and resist degradation processes.

PFCs are subject to oceanic transport and atmospheric transport and deposition. For example, they have been found in water samples from the Northwest Pacific Ocean,

the Arctic Ocean, and the Bering Sea, as well as in Arctic ice samples (Cai et al., 2011). This suggests that PFCs are capable of long-range transport because they are not generally used in the Arctic environment. The mobility of PFCs, combined with their long half-lives and ability to bioaccumulate, pose a considerable health risk to humans and wildlife.

PFCs have been found to bioaccumulate in wildlife such as caribou and fish (Ostertag, Tague, Humphries, Tittlemier, & Chan, 2009), killer whales (Gebbink et al., 2016), dolphins (Fair et al., 2012), Baikal seals (Ishibashi et al., 2008), arctic foxes (Aas, Fuglei, Herzke, Yoccoz, & Routti, 2014), white-tailed eagles (Sletten et al., 2016), and beluga whales (Reiner et al, 2011). In the marine food web, PFCs are found to bioaccumulate in organisms of the upper trophic levels rather than in a consistent way throughout the food chain (B. C. Kelly, Ikonomou, Blair, Morin, & Gobas, 2007). It has been postulated that there is greater biomagnification of PFCs in air-breathing animals, compared to animals that use water for gas exchange, because of the high protein-air partition coefficient ($K_{PA}$) and low protein-water partition coefficient ($K_{PW}$) of PFCs such as PFOS, PFOA and PFNA (B. C. Kelly et al., 2009). However, because marine mammals tend to be long-lived, these findings could also be due to sampling bias.

Bioaccumulation in wildlife is concerning because exposure has been linked to adverse health effects *in vivo.* In salmon, PFOA and PFOS exposure led to lipid peroxidation and oxidative stress, notably in the kidneys and liver (Arukwe & Mortensen, 2011). In developmental toxicity testing of PFCs in zebrafish, researchers found that PFCs caused growth and developmental abnormalities; PFCs with longer chain lengths tended to be more harmful (Zheng et al., 2011; Hagenaars, Vergauwen, De Coen, &

Knapen, 2011). PFNA was found to affect lipid metabolism and cause oxidative stress in zebrafish larvae (Yang, Liu, Ren, Jiao, & Qin, 2014). Therefore, exposure also poses health risks to wildlife.

In a study of white-beaked dolphins, harbor porpoises and harbor seals of the North Sea, different PFAS profiles between species suggest that pinnipeds like harbor seals may metabolize these compounds differently than cetaceans such as dolphins and porpoises. The seals had an overall higher body burden of PFASs and also seemed to metabolize PFOSA more readily to PFOS than the cetaceans (Galatius et al., 2013). Another study of PFCs in marine mammals found body burdens of long-chain PFCs such as PFNA and PFDeA to be increasing over the 20-year period from 1984-2009 in cetaceans and pinnipeds living in the Arctic and North Atlantic (Rotander et al., 2012).

PFCs have been found globally; some of these exposed populations rely more heavily on wild food sources, such as in the Greenlandic Inuit population (Ostertag, Tague, Humphries, Tittlemier, & Chan, 2009). Following the phase-out of PFCs from many consumer products, PFCs appear to be decreasing in some human and animal populations (Gribble et al, 2015; Kratzer, Ahrens, Roos, Backlin, & Ebinghaus, 2011). However, while some PFCs such as PFOA appear to be decreasing over time, some studies have found that longer-chain PFCs such as perfluorononanoate (PFNA) and perfluorodecanoic acid (PFDeA) are increasing in concentration over time (Kishi et al., 2015; Kato, Wong, Jia, Kuklenyik, & Calafat, 2011), possibly because they have higher trophic magnification properties (B. C. Kelly, Ikonomou, Blair, Morin, & Gobas, 2007). Some of the newer PFCs that are used as replacement compounds for PFOS and PFOA,

such as PFHxS, are also being found in increasing concentration in the general U.S. population (Kato, Wong, Jia, Kuklenyik, & Calafat, 2011).

**Routes of Exposure**

People are mainly exposed to PFCs through ingestion of drinking water and food, in consumer products, and through occupational exposure. PFCs are increasingly recognized as a pollutant of public drinking water in the United States. For example, recent discoveries of PFOA-contaminated groundwater contamination in Hoosick Falls, NY and North Bennington, VT have mobilized communities, local government and public health agencies to assess and decrease exposure (McKinley, 2016; Yee, 2016). They are not removed by current water-treatment processes, and can be found in concentrated amounts in sludge. Water filters that use an activated carbon filter can decrease the amounts of PFCs in drinking water (Arvaniti & Stasinakis, 2015); however, these filters may need to be frequently changed to remain effective.

Because of their repellant properties, PFCs have also been used in food packaging, such as on the inside of microwave popcorn bags and pizza boxes to prevent oils from seeping through the packaging. PFCs can leach from this packaging into foods, especially oily or high-protein foods. Additionally, PFCs are used in consumer goods such as floor and ski waxes, leather goods, water or stain repellant clothing, baking paper, outdoor fabrics, cookware coated with Teflon, and waterproofing sprays (Kotthoff, Müller, Jürling, Schlummer, & Fiedler, 2015). While exposure through the skin is possible, ingestion of foods or water containing PFCs or inhalation of sprays are the most likely routes of exposure. Additionally, hand-to-mouth contact with PFC-laced materials

also causes exposure. Because PFCs have been found in house dust, inhalation and ingestion of dust are also relevant sources of exposure (Ericson Jogsten, Nadal, van Bavel, Lindström, & Domingo, 2012). Occupational exposure is also a route of exposure for those working in industries that use PFCs and their families.

**Perfluorinated Compounds in Epidemiological Studies: Human Health**

PFCs present a chronic environmental exposure to the U.S. population and occupationally exposed workers because they are distributed globally and have long half-lives. Blood is one of the easiest matrices to measure PFCs. When measured in blood, levels of PFOS tend to be higher than levels of PFOA, and concentrations tend to be higher in males compared to females. The difference in accumulation between males and females may be because males tend to have more muscle mass and serum albumin, and women experience regular blood loss during menses.

According to the 2003-2004 National Health and Nutrition Examination Survey (NHANES) data, PFCs were detected in >99% of Americans' blood (Calafat, Wong, Kuklenyik, Reidy, & Needham, 2007). From this same dataset, the mean PFOA concentration in U.S. males aged 12 and older was 4.5 ng/ml, with a 50$^{th}$ percentile of 4.6 ng/ml and a 95$^{th}$ percentile of 10.4 ng/ml (n=1,053); likewise, the female mean PFOA concentration was 3.5 ng/ml, with a 50$^{th}$ percentile of 3.6 ng/ml and a 95$^{th}$ percentile of 8.4 ng/ml (n=1,041) (Calafat, Wong, Kuklenyik, Reidy, & Needham, 2007). The mean PFOS concentration in U.S. males aged 12 and older was 23.3 ng/ml, with a 50$^{th}$ percentile of 23.9 ng/ml and a 95$^{th}$ percentile of 62.7 ng/ml (n=1,053); likewise, the female mean PFOS concentration was 18.4 ng/ml, with a 50$^{th}$ percentile of 18.2 ng/ml

and a 95th percentile of 45.7 ng/ml (n=1,041) (Calafat, Wong, Kuklenyik, Reidy, & Needham, 2007).

Occupational exposure can occur in PFC manufacturing plants or in facilities that apply PFCs to their products, such as outdoor textile manufacturing plants. For example, in a 3M study of occupationally-exposed workers in Decatur, Alabama, serum levels of PFOA ranged from 0.04 to 12.7 μg/mL (40 ng/mL to 12,700 ng/mL) and PFOS ranged from 0.06 to 10.1 μg/mL (60 ng/mL to 10,100 ng/mL) (Olson, Burris, Burlew and Mandel, 2003). A sample of 3,713 workers enrolled in the C8 study found a mean PFOA blood concentration from 2005-2006 of 325 ng/mL (K. Steenland, Zhao, Winquist, & Parks, 2013). A sample of 1,881 workers that enrolled in the C8 project were found to have a median PFOA blood concentration from 2005-2006 of 112.7 ng/mL, with a range of 0.25-22,412 ng/mL (Barry, Winquist, & Steenland, 2013). Another study found serum PFOA concentrations up to 12,700 ng/mL and PFOS concentrations up to 10,060 ng/mL (Olsen et al., 2007). A study of a ski-waxing facility found a median serum PFOA concentration of 50 ng/ml in professional waxers; this concentration is more than 10 times higher than the male 2003-2004 NHANES serum median PFOA concentration of 4.6 ng/ml (Freberg et al., 2010).

In studies of animal and human exposure, PFOA has been linked to numerous health outcomes, such as: metabolic dysfunction (Lin et al., 2010), immunotoxicity (Corsini, Luebke, Germolec, & DeWitt, 2014), weight dysregulation (Halldorsson et al., 2012), high cholesterol (Nelson, Hatch, & Webster 2010), ulcerative colitis (Steenland, Zhao, Winquist & Parks, 2013), and kidney cancer (Steenland & Woskie, 2012). PFOA is proteinophilic and can be measured in protein-rich tissues, such as the liver, as well as in

the blood, where it is bound by albumin (Kuklenyik, 2005). PFCs may play a causative role in these health outcomes by causing oxidative stress, altering metabolic pathways, and binding to receptors that set off signaling cascades. For example, PFCs are able to bind to and activate human peroxisome proliferator-activated receptor (PPAR) alpha and PPARγ, which normally bind fatty acid and set off signaling cascades (Zhang, 2014). PFCs have also been implicated in thyroid disease. A study of NHANES data from 1999-2006 found an association between current thyroid disease and exposure to PFOA and PFOS in both men and women (Melzer, 2010). Experiments with zebrafish and human adrenocarcinoma cells have found that PFOS exposure caused increased expression of genes related to thyroid growth, such as PAX8 (Du et al., 2013).

There are also anecdotal accounts of health effects seen in occupationally-exposed workers. For example, DuPont conducted their own experiments on the health effects of PFC exposure using human volunteers; volunteers smoked cigarettes laced with varying doses of PTFE and nearly all of them subsequently experienced chills, fever, coughing and backache (Zapp, 1962). Researchers coined the term "polymer-fume fever" to describe these flu-like symptoms that PTFE workers sometimes experienced; it was more common among workers who smoked, because if a tiny amount of PTFE residue got onto their cigarette it would be burned and inhaled (CDC, 1987).

**Perfluorinated Compounds and Lipid Metabolism**

One possible biological explanation for some of the observed health effects following exposure is that PFCs impact lipid metabolism pathways. In a murine model of PFOA exposure, mice that were developmentally exposed to PFOA showed an inverse

relationship between exposure and weight; the low exposure group had increased body weight, while the high exposure group had decreased body weight (Hines et al., 2009). The researchers also found that high doses of PFOA caused the mice to have an increased amount of brown fat (Hines et al., 2009). A study of murine adipocytes found that exposing preadipocytes to PFCs increased total triglycerides, decreased cell size, increased cell number, and affected the expression of lipid metabolism genes (Watkins, Wood, Lin, & Abbott, 2015). A mouse study also found that PFOA exposure led to the production of reactive oxygen species (ROS) and endoplasmic reticulum (ER) stress (Yan, Zhang, Wang, Zheng, & Dai, 2015). In another recent study, PFOS exposure increased the differentiation of mouse preadipocytes into adipocytes and increased the expression of nuclear factor (erythroid-derived 2)-like 2 (Nrf2) signaling in adipocytes; the researchers also found that human preadipocytes exposed to PFOS had increased adipogenesis and fat accumulation, activation of the peroxisome proliferator-activated receptor gamma (PPARγ), and greater binding of Nrf2 to the antioxidant response element (ARE) (Xu, Shimpi, Armstrong, Salter, & Slitt, 2016). Because the Nrf2-ARE pathway is induced in response to oxidative stress (Nguyen, Nioi, & Pickett, 2009), these findings suggest that PFOS exposure causes oxidative stress. These findings add to the growing evidence of PFOS as an endocrine disruptor, obesogen and inducer of oxidative stress (Grun & Blumberg, 2009; Reistad, Fonnum, & Mariussen, 2013).

In a study of PFOS exposure in rhesus monkeys, monkeys were exposed to PFOS at doses of 10-300 mg/kg/day; all dosed monkeys died within 20 days of testing, and all of them were found to have an exhaustion of lipids in the adrenal cortex (OECD, 2002). Because the adrenal cortex is responsible for synthesizing hormones such as

glucocorticoids and androgens from cholesterol, an interference with cholesterol and lipid metabolism may have resulted in disruption of adrenal activity and death.

The relationship between PFCs and lipids is intriguing. One explanation for this observed relationship could be the structural similarity of PFCs to fatty acids. For example, the peroxisome proliferator-activated receptor (PPAR), usually activated by fatty acids, is activated by PFCs (Takacs & Abbott, 2007). PFOS, PFOA, and PFNA are all known agonists of PPARs (Zhang, Ren, Wan, & Guo, 2014; Das et al., 2015). A study in mice found that PFOA and PFOS activated uncoupling protein-1 (UCP1) in brown fat, normally activated by fatty acids (Shabalina et al., 2015). PFCs were also shown *in vitro* to bind to human liver fatty acid binding protein (hL-FABP), which functions to bind fatty acids such as C6-C12 medium chain fatty acids (Zhang, Ren, & Guo, 2013). L-FABP, expressed in the kidney, liver and intestines, are important because they help transport fatty acids to the endoplasmic reticulum and lipid droplets for storage or to mitochondria and peroxisomes where they are oxidized (Atshaves et al., 2010).

Multiple epidemiologic studies have found associations between PFOA exposure and high total cholesterol levels (Zeng et al, 2015; Geiger et al, 2014). A longitudinal study of a highly exposed population in Ohio and West Virginia found that as serum PFOA and PFOS levels decreased over approximately 4 years, serum lipid levels also decreased (Fitz-Simon et al., 2013). Previous studies that have used NHANES data to examine the correlation between PFCs and total cholesterol have found a positive association with the analytes PFOA and PFOS (Nelson, Hatch, & Webster, 2010). However, the study did not stratify on sex and excluded pregnant women. Additionally, a previous study that modeled the relationship between hypercholesterolemia and

PFOA/PFOS exposure in women aged 20-39 using a Cox proportional hazards model did not find any significant relationship between high cholesterol and PFOA or PFOS exposure in this group (Winquist & Steenland, 2014). Cholesterol may not be the best endpoint to measure lipid metabolism, and PFCs may be affecting metabolism through other pathways.

**Perfluorinated Compounds and Reproduction**

Not much is known about the effects of PFCs on fertility and reproduction, but researchers are beginning to evaluate the reproductive toxicity of PFCs. Reproductive toxicity is an important endpoint to study because PFCs have been found in seminal fluid, can be transferred *in utero* and are believed to be endocrine disruptors; therefore, it is possible that they pose a risk to reproductive health. PFCs may affect fertility and reproduction by disrupting lipid metabolism and signaling. As endocrine disrupting chemicals, PFCs could interfere with hormone signaling and fertility. Because PFCs have been found to cause oxidative stress, they may also damage male gametes via the production of reactive oxygen species.

An *in vivo* toxicity study of mice exposed to PFOA found that *in utero* exposure led to decreased body weights for male and female offspring; when fed a high fat diet, the male mice showed gains in body weight, but female mice remained with decreased body weight (van Esterik et al., 2016). PFOS exposure was also found to disrupt endocrine hormones in adult female mice; PFOS exposure decreased the acetylation of histones in a promoter region for the steroidogenic acute regulator protein (StAR), thereby decreasing the quantity of StAR mRNA (Feng et al., 2015). This led to a decreased production of

estrogen and negatively affected reproductive organs (Feng et al., 2015). Because the StAR protein helps move cholesterol from the cytoplasm to the mitochondria for steroidogenesis, a decrease in this protein could cause decreased synthesis of steroid hormones (Caron et al., 1997). PFNA was found to be lethal at doses of 10 mg/kg to developing mice, and lower doses caused mice to die shortly after birth or have developmental delays (Das et al., 2015).

PFCs can easily cross the placenta and are found in breastmilk, representing a developmental route of exposure to PFCs. Many epidemiological studies have been conducted on the effects of PFCs such as PFOA on infant health outcomes and fetal growth. For example, in the Avon Longitudinal Study of Parents and Children (ALSPAC), a large prospective cohort, female infants born to mothers in the upper tertile of PFOS exposure weighed about 140g less than female infants born to mothers in the lower tertile; however, at 20 months old, the female infants born to mothers in the upper tertile of PFOS exposure weighed 580g more than female infants in the lower tertile (Maisonet et al., 2012). This growth inversion phenomenon is possibly due to impacts by PFCs on genes that regulate fat storage and usage. Recent systematic reviews, using the infrastructure of the newly developed Navigation Guide, of human data and animal data of PFC exposure *in utero* supported the hypothesis that *in utero* PFOA exposure can cause reduced fetal growth (Johnson et al., 2014; Koustas et al., 2014; Lam et al., 2014).

In a prospective birth cohort study of women and infants in Hokkaido, Japan, there was an inverse association between PFOS levels and the amount of polyunsaturated fatty acids (FA) in the women's blood; this suggests that PFCs could compete with FA when transported by albumin or cause disruptions in lipid regulation and/or nutrient

mobilization (Kishi et al., 2015). Additionally, the researchers found that birth weight of female infants, but not male infants, was negatively correlated to PFOS levels. They also measured PFOA, but it did not have any significant relationships with the outcome parameters, possibly because PFOA concentrations were very low in these women (Kishi et al., 2015).

The Maternal-Infant Research on Environmental Chemicals (MIREC) Study in urban areas of Canada found an association between PFOA and PFHxS exposure and reduced fecundity, assessed as time to pregnancy (Velez, Arbuckle, & Fraser, 2015). A similar association between PFOA/PFOS exposure and subfecundity was found in women from the Danish National Birth Cohort (Fei et al., 2009). In the Longitudinal Investigation of Fertility and the Environment (LIFE) study, researchers found that women with higher levels of PFOSA had decreased fertility (Buck Louis et al., 2013). However, some of these findings may be related to maternal age or amenorrhea.

PFCs may affect fertility and spermatogenesis because the process of mammalian spermatogenesis relies heavily on lipid signaling and metabolism (Keber, Rozman, & Horvat, 2013; Saez, Ouvrier, & Drevet, 2011). Because PFCs closely resemble fatty acids, and fatty acids are the main source of energy for germ cells, PFCs may be mistaken for fatty acids by germ cells and disrupt normal processes (Whitmore & Ye, 2015). Additionally, cholesterol is believed to affect spermatid differentiation and sperm capacitation (Keber, Rozman, & Horvat, 2013). Therefore, if PFCs affect blood lipids such as cholesterol, they may also impact gametogenesis.

In a study of the reproductive toxicity of PFOA *in vivo*, researchers dosed male mice with PFOA and found that the exposure caused oxidative stress and reduced sperm

count (Liu et al., 2015). Although PFCs aren't considered to be lipophilic chemicals, they can still pass the blood-testis-barrier (BTB) and have been found in seminal fluid (Guruge et al., 2005). Using a mouse model, researchers found that PFOA exposure affected proteins that are important for cell adhesion, weakening the BTB (Lu, Luo, Li, & Dai, 2016). Similarly, in male rats, PFOS exposure was found to weaken the BTB by disrupting F-actin and decreasing the connection of Sertoli cells (Wan, Mruk, Wong, & Cheng, 2013, 2014).

Recent epidemiological research of male participants in the LIFE study, which followed a cohort of couples actively trying to become pregnant in Michigan and Texas, found that PFC exposure was associated with changes in sperm morphology and quality (Louis et al., 2015). However, a 3M study of semen samples from the Duke IVF clinic (n=256) did not find any significant impact of PFOA or PFOS on semen quality; additionally, the median concentration of PFOS in plasma was 32.3 ng/ml (n=252>LOD), compared to a much lower median concentration of 0.6 ng/ml in semen (n=171>LOD) (Raymer et al., 2012). Conversely, a study of young men in Denmark found a median PFOS concentration of 24.5 ng/ml in semen (n=105>LOD) (Joensen et al., 2009).

In another recent study, researchers recruited the (now adult) sons of a 1988-1989 Denmark pregnancy cohort to assess the *in utero* effects of PFC exposure on future male offspring fertility with a case-control study. Researchers measured the levels of PFCs in semen samples, and also estimated what the *in utero* exposure to PFCs was by analyzing the mothers' banked blood samples. The researchers found that males with greater PFOA exposure *in utero* had significantly higher follicle stimulating hormone (FSH) levels, necessary for spermatogenesis, compared to males with lower PFOA exposure. They did

not find any associations with *in utero* and semen parameters (Vested et al., 2013). However, the study may have been largely affected by biases and estimated *in utero* exposure might not be the best exposure measure of how PFCs affect male fertility. Because the findings are still few and split when it comes to PFCs and reproductive toxicity, there is a need to examine the direct effects of PFC exposure on spermatogenesis.

**Modeling PFC Exposure**

Well-controlled studies are needed to assess the effects of PFCs on reproductive health and fertility. While commonly utilized, animal models of fertility may not be reliable because of differences in metabolism and spermatogenesis between species. For example, there are significant differences in gene expression and function between spermatogenesis in mice compared to humans (Zhu et al., 2016). A few studies have used murine-derived stem cells and human stem cells, but none have assessed the impact of PFCs on human spermatogenesis using an *in vitro* model.

While observational human studies offer correlations between exposures and outcomes, cell-based toxicity studies could clarify the toxicodynamics and mechanisms of action for PFCs. During the process of spermatogenesis, spermatogonial cells stem cells (spermatogonia) develop into spermatocytes and then haploid spermatids. By differentiating stem cells into spermatogenic cells *in vitro*, we can also study how PFC affects the progression of each stage of spermatogenesis and assess windows of susceptibility to PFC exposure (Easley et al., 2012; Easley et al., 2015). The study of

toxic exposures to germ cells also allows for the investigation of the epigenetic impacts of chemical exposures which could have transgenerational impacts.

**Human Spermatogenesis Model**

Approximately half of all infertility is due to a male factor (Wan, Mruk, Wong, & Cheng, 2013, 2014). As societies have become more industrialized, there is a global trend of decreasing sperm quality (Merzenich, Zeeb, & Blettner, 2010); it is possible that these declines in fertility are caused by exposure to environmental pollutants. When environmental pollutants are assessed for reproductive toxicity, they are commonly assessed using an animal model. While animal models can be useful and show the effects of exposure on an entire system, human models are ideal when evaluating the effects of toxicants on human systems.

Mouse spermatogenesis models are used for toxicity testing, but there are several important differences between mouse spermatogenesis and human spermatogenesis. For example, there are several extra cellular stages that occur during mouse spermatogenesis that do not occur during human spermatogenesis (Figure 4)(Easley et al., 2015). Murine models may also fail to identify a reproductive toxicant. Unfortunately, this occurred with the evaluation of 1, 2, dibromo-3-chloropropane (DBCP), a nematocide. In a rat model, reproductive effects were not seen; in a mouse model, effects were only seen at levels just below the lethal doses. Additionally, spermatogonial cell death was not seen in either rodent model. However, for the human field workers that were exposed to DBCP, many of them experienced difficulties conceiving and many were rendered sterile (Teitelbaum, 1999). It was found that DBCP exposure in humans affected meiosis during

spermatogenesis and caused a loss of spermatogonia; it did not target Leydig or Sertoli cells, but did target the spermatogonial stem cells, haploid spermatids and sperm (Easley et al., 2015). Therefore, the commonly used murine models failed to positively identify DBCP as a reproductive toxicant in humans; a model of human spermatogenesis would have been better suited to identify this toxicant. Using a novel, *in vitro* human spermatogenesis stem cell model, DBCP was found to be a reproductive toxicant and mimicked the clinical effects that were seen in workers exposed to DBCP (Easley et al., 2015).

In this validated human spermatogenesis model, human embryonic stem cells (hESCs) are able to develop into spermatogonia, primary and secondary spermatocytes, up to elongated spermatids (Figure 5)(Easley et al., 2012).

**Regulation**

Given the health implications of PFC exposure and the biopersistence of these chemicals, steps are being taken to globally reduce and restrict their use. For example, in 2009, PFOS was added under Annex B (Restriction) in the Stockholm Convention; however, the United States is not a member. Despite the possible health and environmental risks posed by PFOS, there are still a large number of exemptions under which PFOS may be used, ensuring continual routes of exposure. For example, PFOS may still be used in the semi-conductor industry, hydraulic fluids, fire-fighting foam, metal plating, ant insecticides, textiles, plastics, carpets, coatings, paper, and apparel (Stockholm Convention, 2010). While PFOS is the only PFC currently listed under the Stockholm Convention, PFOA is currently under review (Stockholm Convention, 2010).

There is also a growing movement to regulate and reduce the use of all perfluoroalkyl substances (PFASs), such as other PFCs like PFNA and PFHxS, under the recent Madrid Statement. The Madrid Statement aims to unite researchers, government and industry in an effort to better understand PFCs, to decrease their use, and to use safer unfluorinated alternatives (Blum et al., 2015).

**Objectives**

Aim 1: Determine whether PFC exposure is associated with total cholesterol in AK MOMS participants.

Hypothesis 1: Exposure to PFCs will be positively or negatively associated with total cholesterol in the Alaska Maternal Organic Monitoring Study.

Null hypothesis 1: Exposure to PFCs will be not be associated with total cholesterol in the Alaska Maternal Organic Monitoring Study.

Aim 2: Determine relative concentrations of PFCs in AK MOMS and how the mean concentrations compare to exposure of pregnant women sampled in NHANES.

Hypothesis 2a: Women included in the cholesterol and PFC analysis of AK MOMS will have different PFC body burden trends than the pregnant subset of the NHANES population from the same time period.

Null hypothesis 2a: Women included in the cholesterol and PFC analysis of AK MOMS will not have different PFC body burden trends than the pregnant subset of the NHANES population from the same time period.

Hypothesis 2b: Women in the AK MOMS sampling period 2005-2006 will have different mean serum concentrations of PFCs compared to women in the 2010-2012 sampling period.

Null hypothesis 2b: Women in the AK MOMS sampling period 2005-2006 will have similar mean serum concentrations of PFCs compared to women in the 2010-2012 sampling period.

Aim 3: Determine if chronic exposure to PFOS, PFOA and PFNA affects differentiation in an *in vitro* human spermatogenesis model.

Hypothesis 3a: Exposure to PFOS, PFOA and PFNA will affect apoptosis and cell death.

Null hypothesis 3a: Exposure to PFOS, PFOA and PFNA will not affect apoptosis and cell death.

Hypothesis 3b: Exposure to PFOS, PFOA and PFNA will result in differential production of PLZF.

Null hypothesis 3b: Exposure to PFOS, PFOA and PFNA will not result in differential production of PLZF.

Hypothesis 3c: Exposure to PFOS, PFOA and PFNA will cause differential distribution of cellular stages.

Null hypothesis 3c: Exposure to PFOS, PFOA and PFNA will not cause differential distribution of cellular stages.

Hypothesis 3d: Exposure to PFOS will result in differential expression of genes related to spermatogenesis and fertility.

Null hypothesis 3d: Exposure to PFOS will not result in differential expression of genes related to spermatogenesis and fertility.

Aim 4: Determine if chronic exposure to PFOS affects lipid metabolism.

Hypothesis 4: Exposure to PFOS will cause differential expression of genes related to lipid metabolism.

Null hypothesis 4: Exposure to PFOS will not cause differential expression of genes related to lipid metabolism.

**METHODS**

**Alaska Maternal Organic Monitoring Study Data**

The analysis of PFCs and total cholesterol was conducted using a subset of participants from the Alaska Maternal Organic Monitoring Study (AK MOMS). AK MOMS was a cross-sectional study conducted to monitor the health of mothers and infants, specifically in relation to persistent organic pollutants (POPs) and health effects. There were a total of 3 sampling periods; the first was conducted from 2000-2003, the second from 2005-2006, and the third from 2010-2012. Biological samples were taken at the time of enrollment and lifestyle questionnaires were given.

Pregnant women were enrolled in the AK MOMS study in the Alaskan town of Bethel (Figure 6). Bethel, AK is a small community located on the Kuskokwim River within the Yukon Delta close to the Bering Sea. Alaska Native women were considered to be the primary population and community stakeholder for this study. While ethnic background was not obtained, Yupik Native Americans make up the majority of the population of Bethel (U.S. Census Bureau, 2014). The Alaska Native Tribal Health Consortium (ANTHC) worked with the National Center for Environmental Health (NCEH) of the Centers for Disease Control and Prevention (CDC) to manage the study. Consent documents and protocols were developed in consultation with local Alaska Native corporations. The Environmental Protection Agency (EPA) funded the study and the CDC/NCEH provided laboratory analysis and epidemiology support.

Pregnant women who elected to participate in the study enrolled during their first prenatal visit to the community health clinic and gave blood samples. Consent was obtained during enrollment [CDC protocol #:2320 and AK IRB#: 2003-06-013 Maternal Organics Monitoring Study (MOMS)].

Eight PFCs were measured in serum: Perfluorohexane sulfonate (PFHxS), perfluorooctane sulfonate (PFOS), perfluorooctanoate (PFOA), perfluorooctane sulfonamide (*PFOSA*), 2-(N-Methyl-perfluorooctane sulfonamido) acetate (Me-PFOSA-AcOH), 2-(N-ethyl-perfluorooctane sulfonamide) acetate (et-PFOSA-AcOH), perfluorononanoate (PFNA), and perfluorodecanoate (PFDeA). Serum was treated with formic acid and then sonicated. Samples were measured using on-line and off-line solid-phase extraction with high performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS) (Kuklenyik, Needham, & Calafat, 2005). All blood samples from AK MOMS and NHANES were analyzed by the same laboratory at CDC.

**Inclusion and Exclusion Criteria**

For entry into the analysis, the following inclusion criteria were required: total cholesterol data, the woman's date of birth, PFC analyte concentration, date of blood draw, and weeks pregnant at blood draw. With these specifications, the subset of data from AK MOMS used for this study consisted solely of women from Bethel who enrolled in the years 2005-2006 and 2010-2012. One participant was excluded from the analysis because her total cholesterol was recorded in the database as 24 mg/dL, which, physiologically, is extremely unlikely.

Weeks pregnant at blood draw data was a necessary variable because, during the course of pregnancy, blood cholesterol levels tend to rise, increasing by approximately 50% (Potter & Nestel, 1979). The use of weeks pregnant as a variable in the model could help account for this progressive rise of cholesterol and increase the precision of the

estimates. When this data was missing, it was calculated assuming a 40 weeks gestation for women with delivery date information.

**Data Analysis of AK MOMS**

The concentrations for each PFC analyte were log-transformed when included in the linear regression models. Total cholesterol was analyzed as a continuous outcome, as was age and weeks pregnant at blood draw. Before building a full model, each PFC analyte (log-transformed) was separately run as the exposure of interest (continuous) and assessed for correlation with total cholesterol as the outcome of interest (continuous). If the predictor was significantly correlated ($p<0.05$), a multiple linear regression (MLR) model was built to analyze the relationship, controlling for maternal age at blood draw and weeks pregnant at blood draw. The models controlled for maternal age at blood draw because the bioaccumulative nature of PFCs may cause older women to have higher body burdens; additionally, as the use of different types of PFCs has changed over time, older women may have a different spectrum of PFC analytes and concentrations compared to younger women.

Descriptive statistics for each PFC analyte were determined for the overall participants and by cohort using SAS 9.4. PFCs that were detected above the limit of detection (LOD) 50% or more of the time were included in the analysis. When a measurement was below the LOD, it was assigned the value of the LOD/($\sqrt{2}$). Geometric means of women were compared between the 2005-2006 and 2010-2012 sampling periods to see if PFC exposure was relatively similar or different between the two

sampling periods. Statistical significance for descriptive statistics was measured using either a two-sample t-test or ANOVA.

**National Health and Nutrition Examination Survey Data**

The National Health and Nutrition Examination Survey (NHANES) is a cross-sectional biomonitoring study of about 5,000 people that periodically takes place in 15 different counties throughout the United States (National Center for Health Statistics, n.d.). Each sampling cycle is generally two years in duration and the data is made publically available online. NHANES is comprised of a series of studies designed to assess the health status of adults and children across the United States (National Center for Health Statistics, n.d.). There are two main components of NHANES: a laboratory component and a questionnaire component. Biological samples are sent and analyzed through the CDC's Environmental Health Laboratory (CDC, 2009). Because NHANES purposefully oversamples certain groups, sampling weights are applied to NHANES data during statistical analysis.

**Data Analysis of NHANES**

For this data analysis, the same inclusion criteria that were used for the AK MOMS data were used for the NHANES data. Because the sample size of pregnant women with measured PFCs was very small in the NHANES dataset 2011-2012, only the 2005-2006 NHANES data and 2005-2006 AK MOMS data were compared. NHANES participants were stratified on pregnancy status and only pregnant women were included in the analysis. In order to collect all of the necessary inclusion criteria information on

each pregnant participant, the relevant data folders were downloaded and combined with SAS 9.4, using the unique participant ID as the unifier.

Due to the sampling methodology of NHANES, it is recommended that sampling weights be applied in statistical analyses. Because the analyzed women were a subsample of the overall study, the appropriate subsampling weight for the specific years of data collection were applied using SAS proc survey procedures. After applying the subsample weight, geometric means with confidence intervals were calculated for each PFC analyte. When weighted geometric mean PFC concentrations were calculated with 95% confidence intervals, the confidence intervals were compared with those of AK MOMS to determine if they were significantly different or not; where intervals did not overlap, mean concentrations were considered to be significantly different.

**Stem Cell Culture and Differentiation**

We cultured and differentiated human embryonic stem cells (hESCs) into spermatogenic lineages for an *in vitro* human spermatogenesis model (Easley et al., 2012; Easley et al., 2015). An NIH Registry approved hESC line (WA01) was used. I plated hESCs on matrigel-coated 10cm petri dishes, supplemented cells with mTeSR and passaged them every 5-7 days. When plated for differentiation, hESCs were plated on gelatin-coated dishes seeded with SIM mouse embryo-derived thioguanine- and ouabain-resistant cells (STOs). STOs were used because they secrete certain growth factors that help maintain mouse SSC cultures and induce differentiation of hESCs into spermatogenic lineages *(*West, Park, Daley, & Geijsen, 2006*)*. Cells were grown for 10 days before being harvested for data analysis. During differentiation I supplemented

hESCs with a base medium and 2 growth factors, glial-derived neurotrophic factor (GDNF, Peprotech) and human recombinant basic fibroblast growth factor (hbFGF, Peprotech). GDNF is especially important for the differentiation of hESCs into spermatogenic lineages. After cells were plated on day 0, media was replaced on every odd-numbered day. I added the experimental dose or vehicle control to media prior to pipetting into wells. On day 10 of differentiation, cells were harvested and treated for analysis.

The base mouse spermatogonial stem cell (SSC) media that was used to maintain the hESCs consisted of: minimum essential medium (MEM) alpha (Gibco), 10 mM HEPES, 0.5x Penicillin/Streptmycin (Gibco), 50 μM β-mercaptoethanol, 60 μM putrescine, 5 μg/ml insulin, 10 μg/ml transferrin, 0.2% bovine serum albumin, 2 mM L-glutamine (Invitrogen), 30 nM sodium selenite, 2.71 μM linoleic acid, 2.36 μM palmitic acid, 1.02 μM oleic acid, 0.88 μM stearic acid, 0.43 μM linolenic acid, and 0.21 μM palmitoleic acid. To this base media, 1 ng/mL of hbFGF (BD Biosciences) and 20 ng/ml GDNF (R&D Systems) was added.

During differentiation, cells were exposed to a vehicle control of dimethyl sulfoxide (DMSO) or one of three PFC doses. For each PFC, the first two doses were based on the $50^{th}$ and $95^{th}$ percentile concentrations found for males of all ages in NHANES 2003-2004 (Calafat, Wong, Kuklenyik, Reidy, & Needham, 2007). The highest dose of each PFC was approximately ten times as high as the $95^{th}$ percentile, and represented what may be found in an occupational exposure. While the doses used for cells were an order of magnitude higher than what was found in NHANES, they were still within the range of occupational exposure. Additionally, due to interfering factors, a

significantly smaller dose could be reaching the cells. For PFOS, the first dose was equivalent to 48 μM, the second dose was 126 μM, and the third dose was 200 μM. For PFOA, the first dose was 11 μM, the second dose was 25 μM, and the third dose was 242 μM. For PFNA, the first dose was 2.15 μM, the second dose was 21.5 μM, and the third dose was 215.5 μM. Stock solutions of 100mM PFOS, 100mM and 10mM PFOA, and 100mM and 10mM of PFNA were made. Cells were exposed to PFCs during days 1, 3, 5, 7, and 9 of differentiation.

**Apoptosis Assay**

I measured apoptosis using a Muse Annexin V and Dead Cell kit (EMD Millipore) with the Muse benchtop flow cytometer (EMD Millipore). For this analysis, harvested cells were transferred to labeled 15 mL centrifuge tubes. After tubes were vortexed to homogenize the cell mixture, 100 μL of the cell mixture and 100 μL of the Muse reagent were pipetted into labeled 1.5 mL tubes and left to sit in the dark for 20 minutes. Samples were vortexed prior to loading into the cleaned Muse flow cytometer and analyzed. For each set of samples, the Muse flow cytometer was set to 5000 events with a df of 2. Data were saved and analyzed using Muse software.

**Immunostaining**

Promyelocytic leukaemia zinc finger (PLZF) is used as a spermatogonial marker (Costoya et al., 2004). I quantified PLZF in cell colonies by immunostaining and fluorescence was measured with a ThermoFisher ArrayScan. Following chronic exposure to PFOA, PFOS or PFNA, cells were immunostained with PLZF antibodies and Hoescht

dye (Life Technologies). For immunostaining, cells were treated with 4% paraformaldehyde (PFA) and let to sit for 15 minutes. The PFA was aspirated and cells washed with PBS. A blocking buffer of 5% bovine serum albumin (BSA) was then added and left to sit for 30 minutes before being aspirated. The primary PLZF antibody, diluted 1:200 in 5% BSA buffer, was added to each well and left to sit overnight at 4$^{o}$C. The following day, wells were aspirated, treated with PBS containing 0.1% Triton X, and left to sit for 5 minutes. This was aspirated and repeated twice more before the secondary antibody was added and left to sit for 1 hour. Media was then aspirated, cells washed with PBS containing 0.1% Triton X, and left to sit for 5 minutes. This was repeated twice more before adding the Hoescht nuclear stain (Life Technologies) in PBS. Cells were then imaged using a ThermoFisher Arrayscan and analyzed for fluorescence using HCS Studio software. Images were analyzed by quantifying the average colony size and the average fluorescence of colonies, comparing dosed wells to the control.

**Cell Cycle Assay**

I assessed the effect of PFCs on the cell cycle by measuring cell populations using the Muse Cell Cycle Assay Kit (EMD Millipore). Harvested cells in 15mL tubes were centrifuged at 300g for 7 minutes and the supernatant aspirated. 1mL DPBS was then added to tubes and the cell pellet was re-suspended. Tubes were centrifuged again and supernatant aspirated. Cells were re-suspended with 1mL of cold 70% ethanol and left for at least 4 hours in -20$^{o}$C freezer. Then 200 uL of fixed cells were pipetted into 1.5mL tubes and centrifuged at 300g for 5 minutes. Cells were washed once with PBS and centrifuged again at 300g for 5 minutes. Supernatant was aspirated and 200uL of Muse

Cell Cycle reagent was added to each tube. Tubes were left to sit in the dark for 30 minutes. After 30 minutes, cells were re-suspended and vortexed prior to being analyzed on the Muse flow cytometer (EMD Millipore). The analysis settings were set at 5000 events.

**qPCR Assay**

I collected quantitative polymerase chain reaction (qPCR) data using custom BioRad prime PCR plates and analyzed them using a BioRad CFX96 Touch Real-Time PCR Detection System. For this analysis, harvested cells were transferred to labeled 15 mL centrifuge tubes and centrifuged at 300g for 5 minutes to pellet the cells. The supernatant was aspirated. Gloves, benchtop and pipetters were treated with RNAse. Per kit protocol, the cell pellet was then resuspended with buffer RLT plus from the RNEasy Plus Minikit (BioRad) and loaded into QIAshredder (Qiagen) tubes for homogenization and centrifuged for 30 seconds at approximately 8000g. The resuspended lysate was then transferred to a gDNA spin column placed in a 2 mL centrifuge tube. Samples were centrifuged for 30 seconds at 8000 g and column discarded. To the flow-through, 350 uL of 70% ethanol was added and protocol followed as specified in the RNEasy Plus Minikit (BioRad) protocol to collect mRNA. Once eluted, mRNA was analyzed with a nanophotometer (Kisker Biotech) and stored at -20$^{o}$C.

After mRNA collection, I created cDNA for each RNA sample using an iScript cDNA Synthesis kit (BioRad). Samples were loaded into a PCR incubating machine (BioRad) and run for 1 hour following the PrimePCR BioRad guide; 5 minutes at 25$^{o}$C, 30 minutes at 42$^{o}$C, 5 minutes at 85$^{o}$C and then held at 4$^{o}$C. Labeled cDNA samples were

then analyzed using a nanophotometer (Kisker Biotech) and stored at -20$^{o}$C for later qPCR analysis. I used custom PCR plates for the genes of interest (BioRad) and loaded the cDNA samples following BioRad instructions. The genes of interest were: *ACOX1, ACOX2, ACTB, CYP17A1, CYP51A1, FABP9, HCAR1, HCAR2, LIPE, NCEH1, PAX8, PIWIL1, PIWIL2, POMC, PON1, PPARA, PRM1, PRM2, SYCP3,* and *ZBTB16*. I ran the samples on a BioRad CFX96 Touch Real-Time PCR Detection System.

**Reagents**

For immunostaining, I used a mouse monoclonal anti-PLZF antibody (R&D Systems) as the primary antibody and an Alexa Fluor 568 goat anti-mouse IgG (Life Technologies) as the secondary antibody. I used Hoescht dye (Life Technologies) as the nuclear stain. For PFC doses, PFOA (Sigma Aldrich, CAS No. 335-67-1), PFOS (Tokyo Chemical Industry Co., CAS No. 1763-23-1) or PFNA (Sigma Aldrich, CAS No. 375-95-1) were dissolved in DMSO (Sigma). DMSO (Sigma) was used as the vehicle control. The growth factors GDNF and hbFGF (Peprotech) were added to base media.

**Statistical Analysis of Cellular Endpoints**

For each outcome of interest, experiments were run in triplicate. Cell cycle and apoptosis data were normalized to the vehicle control. As previously done when evaluating apoptosis and cell cycles in this model, I used a two-sample t-test comparing each dose to vehicle control to determine statistical significance. Gene expression data were compared to vehicle control and analyzed for statistical significance according to BioRad's instructions using BioRad CFX Manager software. PLZF data were graphed

and analyzed using SPSS software and tested for significance using 1-way ANOVA with Tukey's post-hoc test.

## RESULTS

In this analysis, the exposure trends of PFCs in Alaskan pregnant women of AK MOMS 2005-2012 were assessed for the first time. PFCs were chosen as an exposure of interest because they bioaccumulate and may have deleterious effects on human health. Because previous epidemiological studies of PFC exposure have found relationships between PFC exposure and blood lipid changes, the association between PFCs and total cholesterol was also determined in AK MOMS. To put the findings in a context of national exposure levels, mean PFC concentrations of AK MOMS 2005-2006 were compared to those of pregnant women in NHANES 2005-2006.

Additionally, the reproductive toxicity of PFOS, PFOA and PFNA were tested for the first time in a novel *in vitro* human spermatogenesis stem cell model. Given what we know about the ability of PFCs to bind to fatty acid receptors, and given the weight of evidence for their ability to interfere with lipid regulation, we sought to examine the impacts of PFCs on human spermatogenesis using our model. Because animal and other *in vitro* studies have found PFCs to impair male reproductive endpoints, the effects of PFC exposure on apoptosis, spermatogonial viability, cell cycle populations and gene expression were tested.

### Alaska Maternal Organic Monitoring Study

In AK MOMS, there were a total of 568 women enrolled in the study from 2000-2012. Of this group, 333 women had blood measurements of PFC's. 7 women of the 333 women were not included because they did not have blood measurements of total cholesterol, and 1 woman was excluded because she had a total cholesterol measurement of 24. Of the 325 women with total cholesterol data, 283 women had data available for weeks pregnant at blood draw. Following the application of the inclusion criteria, data from a total of 283 women were analyzed; 136 women were enrolled in the period 2005-2006 and 147 women were enrolled in the period 2010-2012 (Figure 7).

Before building a model, I compiled descriptive statistics of variables. The outcome, total serum cholesterol, followed a normal distribution. The mean total cholesterol level was 218.7 mg/dL with a range of 110-393 mg/dL. The mean rounded age was 26, ranging from 16 to 44 years old at the time of blood draw.

Different PFCs were measured in serum at different frequencies above the limit of detection (LOD). When the PFCs were analyzed, PFOS and PFNA were both measured 99.6% >LOD, PFOA was measured 99.3% >LOD, and PFDeA was measured 92.9%>LOD. PFHxS was measured 72.4%>LOD and Me-PFOSA-AcOH was measured 50%>LOD. PFOSA and Et-PFOSA-AcOH were both measured less than 50%>LOD (Table 4). The analyte found in the highest concentration in this population was PFOS, followed by PFNA, PFOA, PFDeA, PFHxS and Me-PFOSA-AcOH.

Weeks pregnant at blood draw was recorded for 96% of the 283 women; 12 women had missing data on weeks pregnant at blood draw but had delivery date recorded in the database. In these cases, weeks pregnant at blood draw was calculated with delivery date and assuming a 40 week gestation. Estimates derived using a 40-week

assumption were compared to the estimates derived using given weeks pregnant at blood draw. The use of a 40-week assumption led to an estimation difference of about a week for weeks pregnant at blood draw. Because more than 95% of women had weeks pregnant at blood draw data available and because weeks pregnant at blood draw was broken up into trimesters for descriptive analyses, the use of a 40 week assumption for the 12 women is not concerning.

The majority of women were enrolled in the study during their third trimester of pregnancy; 64 (22.6%) women had blood drawn during the first trimester, 88 (31.1%) women had their blood drawn during their second trimester, and 131 (46.4%) women had their blood drawn during the third trimester (Table 1). When the association between age group and trimester was analyzed, there were similar distributions of women representing each trimester for each age group (Table 2). However, of all of the women, the largest percentage was made up of women in their third trimester who were older than 26 (26%, n=74). When comparing the age groups, a greater percentage of women <26 years had blood drawn in their first and second trimesters (n=84/141, 60%) compared to women >26 (n=68/142, 48%). A greater number of women >26 had blood drawn during their third trimester.

When age was dichotomized, mean total serum cholesterol was significantly higher in women 26 years of age or older than women younger than 26 years of age (p-value <0.05). This was to be expected, as cholesterol levels tend to rise as age increases. When mean total serum cholesterol was stratified by trimester at blood draw, each subsequent trimester had significantly higher mean total serum cholesterol (Table 3). This was also to be expected, as cholesterol levels tend to increase by half over the course of

pregnancy. The mean total cholesterol for women in the first trimester was 165.8 mg/dL (95% CI 158.5-173 mg/dL), the mean cholesterol for women in the second trimester was 209.7 mg/dL (95% CI 199.7-219.7), and the mean cholesterol for women in the third trimester was 250.6 mg/dL (95% CI 241.3-259.9). Mean total cholesterol was significantly different between each of the groups when analyzed using ANOVA with Scheffe's method (p-value <0.001).

When age was dichotomized, women aged 26 and older had a significantly higher mean concentration of PFNA than women younger than 26 (p-value=0.0001); the findings for PFDeA were the same (Table 5). There was not a significant difference in the geometric means between the age groups for any of the other analytes. When stratified by trimester, there was a significant difference in the distribution of PFOS, PFDeA, PFHxS and Me-PFOSA-AcOH between different trimesters (p-value <0.05 for each using ANOVA / Scheffe's). PFOS, PFDeA, and Me-PFOSA-AcOH increased over the course of pregnancy with significantly higher serum concentrations in women in the third trimester than women in the first and second trimester. However, PFHxS showed an opposite trend, and steadily decreased over the course of pregnancy; women in the first and second trimester had significantly higher serum concentrations than women in the third trimester. Similarly, younger women were found to have higher concentrations of PFHxS, and it appeared as though PFHxS concentration decreased over trimesters; this trend is likely because there were more young women who had their blood drawn in the early stages of pregnancy.

When included in a univariable linear regression model with the logged PFC concentration as the exposure and total cholesterol as the outcome, PFOS, PFNA,

PFDeA, Me-PFOSA-AcOH, PFHxS and the summed PFC variable were each significantly associated with total cholesterol (p-value <0.05) (Table 6).

Given their significance in the univariable model, these PFCs were analyzed in a multivariable linear regression model which included age at blood draw (in years) and weeks pregnant at blood draw to control for confounders (Table 7). In this model, PFOS, PFDeA, PFHxS and the sum PFC variable were each significant variables for the outcome (each p<0.05). For PFOS, the linear regression model equation was: (y=128.01(intercept) + 8.22*(log PFOS) + 0.176*(age) + 3.05*(weeks pregnant)). For PFDeA, the linear regression model equation was: (y=160.06(intercept) + 13.14*(log PFDeA) – 0.23*(age) + 3.21*(weeks pregnant)). For PFHxS, the linear regression model equation was: (y=122.20(intercept) – 6.28*(log PFHxS) + 0.33*(age) + 3.13*(weeks pregnant)). For the sum PFC variable, the linear regression model equation was: (y=118.72(intercept) + 9.35*(log sum PFC) + 0.14*(age) + 3.15*(weeks pregnant)). For every 1-unit log increase in PFOS concentration, total cholesterol increased by 8.22 units. Another way of interpreting this relationship is that for every 10% increase in PFOS concentration, there is a +0.34 unit increase in total cholesterol. Likewise, for every 1-unit log increase in PFDeA concentration, total cholesterol increases by 13.14 units; for every 10% increase in PFDeA, there is a +0.54 increase in total cholesterol. For every 1-unit log increase in PFHxS concentration, total cholesterol decreases by 6.28 units; for every 10% increase in PFHxS, there is a -0.26 decrease in total cholesterol. For every 1-unit log increase in the sum PFC concentration, total cholesterol increases by 9.35 units; for every 10% increase in the sum PFC concentration, there is a +0.39 increase in total cholesterol. Therefore, when comparing the effects on cholesterol for

each of these PFCs, PFDeA was associated with the largest increase in total cholesterol, followed by the sum PFC measure and PFOS. Rather than increasing total cholesterol, PFHxS appeared to be inversely associated with total cholesterol.

**AK MOMS and NHANES**

Using the dataset of the NHANES survey cycle 2005-2006, there were 88 women who fit the inclusion criteria. Of these women, 14 (16%) were in the first trimester, 32 (36%) were in the second trimester, and 42 (48%) were in their third trimester (Table 9). This distribution is similar to that for AK MOMS, but AK MOMS had a higher percentage of women who had their blood drawn during the first trimester and fewer in their second trimester. The mean total cholesterol levels increased as trimester increased (Table 9); women in the first trimester had a mean total cholesterol of 166.4 mg/dL (95% CI 151.7-181.1), women in the second trimester had a mean total cholesterol of 223.5 (95% CI 214.8-232.2), and women in their third trimester had a mean total cholesterol of 268.1 (95% CI 227.1-309.1). Total cholesterol significantly increased as trimester at blood draw progressed; mean total cholesterol was approximately 60% higher in the third trimester compared to the first.

When the PFCs were analyzed in NHANES, PFOS and PFOA were both measured 100% >LOD, PFNA was measured 96.6% >LOD, and PFHxS was measured 88.6% >LOD. Me-PFOSA-AcOH was measured 71.6% >LOD and PFDeA was measured 69.3% >LOD. In the NHANES women, the analyte with the highest mean concentration was PFOS, followed by PFOA, PFHxS, PFNA, Me-PFOSA-AcOH, and PFDeA (Table 8). PFOS had a mean concentration of 7.42 ng/mL (95% CI 6.05-9.10),

PFOA had a mean concentration of 1.73 ng/mL (95% CI 1.22-2.45), PFHxS had a mean concentration of 0.72 ng/mL (95% CI 0.49-1.05), PFNA had a mean concentration of 0.62 ng/mL (95% CI 0.49-0.78), Me-PFOSA-AcOH had a mean concentration of 0.30 ng/mL (95% CI 0.22-0.39), and PFDeA had a mean concentration of 0.23 ng/mL (95% CI 0.18-0.29).

When exposure was stratified by trimester, concentration of PFOS, PFOA and PFNA appeared to decrease as trimester increased, although not significantly (Table 10). Concentrations of all other PFCs appeared to be similar across different trimesters. Additionally, younger women <26 appeared to have higher concentrations of all PFCs compared to older women, although not significantly.

The geometric mean of PFOS, PFOA, PFNA, PFDeA, Me-PFOSA-AcOH, and PFHxS were each calculated for AK MOMS and NHANES women (using the appropriate subsample weight) (Table 16). Geometric means were compared using 95% confidence intervals. Where confidence intervals for the mean did not overlap between the two groups, the mean was considered significantly different. Using this methodology, both mean PFOA and PFHxS concentrations were found to be higher in the NHANES group, while PFNA and PFDeA were both found to be higher in the AK MOMS group.

Next, to examine the effects of trimester, mean concentrations were stratified by trimester and compared between the two groups (Table 17). Mean PFHxS concentration was significantly higher for women in the third trimester in NHANES compared to AK MOMS. PFDeA was significantly higher for AK MOMS women in the third trimester. Likewise, PFNA was significantly higher for AK MOMS women in both the second and third trimester compared to NHANES. Mean concentrations were also stratified by age

group and compared between NHANES and AK MOMS 2005-2006 (Table 18). PFOA and PFHxS were significantly higher for both younger and older women of NHANES compared to AK MOMS. Me-PFOSA-AcOH was significantly higher for younger NHANES women compared to younger AK MOMS women. PFNA and PFDeA were significantly higher for older women in AK MOMS compared to older women in NHANES.

**PFC Exposure and Lipids**

Because previous research has found an association between PFC exposure and lipid parameters, this analysis examined two different sources for evidence to test this relationship. Using the epidemiological data, the association between PFC exposure and total cholesterol was analyzed as the lipid parameter of interest. Cholesterol and lipids are very important for reproduction and development, so the finding that PFDeA, PFOS and PFHxS were associated with cholesterol in AK MOMS may have implications for human health. Using the *in vitro* model, gene expression related to lipid metabolism was chosen as a lipid parameter of interest because lipid metabolism is necessary for spermatogenesis. Epidemiological studies can be used to inform *in vitro* studies, and vice versa. While the *in vitro* study used a spermatogenesis model, any adverse effects seen could have implications for female reproduction. Additionally, there is currently no known oogenesis *in vitro* model, so the effects of PFC exposure on human female gametogenesis cannot be evaluated at this point in time.

**PFC Exposure Causes Increased Apoptosis in Germ Cells**

I analyzed cells using the Muse Annexin V Dead Cell Assay for apoptotic stages. Data of dosed cells were normalized to the vehicle control. For PFOS-treated cells, there were no significant differences in live or apoptotic cells when compared to the control. However, there did appear to be a greater number of late apoptotic and dead cells for PFOS-dosed cells compared to control (Figure 8). Following PFOA treatment, there appeared to be an increased amount of cells in the stages of late apoptosis compared to control. However, there were no significant differences in live or apoptotic cells when comparing dosed cells to control (Figure 9). Following PFNA treatment, there appeared to be a greater amount of cells in the early and late stages of apoptosis for the highest dose; however, similar to PFOS and PFOA, there were no significant differences in live or apoptotic cells (Figure 10). Given the trends of cell death, it appears that PFOS, PFOA, and PFNA induces apoptosis at these doses.

**PFC Exposure Decreases Spermatogonial Viability**

I assessed the viability of spermatogonia following PFC exposure by immunostaining for PLZF and quantifying the fluorescence with an Arrayscan. Because gene expression does not necessarily equate to protein levels, quantification of this protein can also be compared to qPCR findings of *ZBTB16* and examined for correlation. These findings have important implications for male fertility following population and occupational exposure scenarios.

Following PFOS exposure, the average size of colonies did not appear to be affected by exposure. Per colony, PLZF was detected in less of the overall colony area for the highest dose of PFOS compared to control. Although the 126 μM PFOS dose had

a similar amount of colony area with PLZF, the actual fluorescence of PLZF was significantly weaker for both the 126 μM PFOS and 200 μM PFOS dose groups compared to the control; this means that the 126 μM dose group had a lower amount of PLZF overall (Figures 11 & 12). The average intensity, independent of area, of all PLZF+ within an identified colony showed a non-monotonic response but decreased significantly for the second and third doses; this finding shows that colonies had less of this spermatogonial marker at the higher doses of PFOS exposure. When checked for correlation with the qPCR results, these results aligned with those found for *ZBTB16* expression. This supports that the decreased expression of *ZBTB16* caused a decreased production of the PLZF. Thus, PFOS exposure interferes with the ability of spermatogonial cells to remain viable, impairing differentiation and fertility.

Following PFOA exposure the average size of colonies did not appear to be affected by exposure. Per colony, PLZF was detected in less of the overall colony area for every dose of PFOA compared to control. The actual fluorescence of PLZF was also significantly weaker for all doses of PFOA compared to the control; this means that the 126 μM dose group had a lower amount of PLZF overall (Figures 13 & 14). The average intensity, independent of area, of all PLZF+ within an identified colony decreased significantly for all doses of PFOA; this finding shows that colonies had less of this spermatogonial marker at all doses of PFOA exposure. This suggests that PFOA exposure interferes with the ability of spermatogonial cells to remain viable, impairing differentiation and fertility.

Following PFNA exposure the average size of colonies was affected at the highest dose of PFNA. Per colony, PFZF appeared to follow a non-monotonic dose response and

was detected significantly less for the highest dose of PFNA compared to control. The actual fluorescence of PLZF was also significantly weaker for the highest dose of PFNA compared to the control; this means that the 215.5 μM dose group had a lower amount of PLZF overall (Figures 15 & 16). The average intensity, independent of area, of all PLZF+ within an identified colony was very similar across the first two dose groups but decreased significantly for the highest dose of PFNA. This suggests that colonies had less of this spermatogonial marker the highest dose of PFNA. This suggests that PFNA exposure interferes with the ability of spermatogonial cells to remain viable, impairing differentiation and fertility.

**PFC Exposure Decreases Haploid Spermatids**

I used a flow cytometer to determine the distribution of cells in the different stages of the cell cycle. This analysis is useful in assessing whether exposure affects differentiation and fertility. To determine cell populations, collected cells were vortexed and approximately 5000 cells were sampled by the Muse flow cytometer for the cell cycle assay. The percentages of these cells in the haploid stage, G0/G1 phase, S phase, and G2/M phase were calculated and analyzed.

Following PFOS treatment, the highest dose had a lesser amount of cells in the haploid stage compared to the control, although not significantly. All groups had similar amounts of cells in the G0/G1 phase and G2/M phase. The control and dose 1 groups had similar amounts of cells in the S phase, but the high dose group had significantly less cells in this stage (Figure 17). This suggests that PFOS exposure affects differentiation.

Compared to PFOS exposure, PFOA treatment had a larger impact on cell populations (Figure 18). The high dose of PFOA had significantly fewer haploid cells compared to the control and the low dose of PFOA. There were also significantly fewer cells in the S and G2/M phases in the high PFOA dose group compared to the control and low dose group. The low dose of PFOA had similar cell populations compared to the control. These findings suggest that PFOA exposure negatively impacts spermatogenesis and meiosis, resulting in fewer haploid cells.

PFNA also had significant effects on the cell cycle (Figure 19). There were no haploid cells detected in the high dose group, and the low dose group appeared to have more haploid cells compared to control, although not significantly. There were significantly more cells in the G0/G1 phase for the high dose group compared to the control and low dose. Conversely, there were significantly fewer cells in the S phase and G2/M phase in the high dose group compared to the control and low dose.

The significant reduction in germ cells following PFOA and PFNA exposure and the decreasing trend in spermatids for PFOS suggest that PFC exposure negatively impacts spermatogenesis and may disrupt meiotic processes. Additionally, because fatty acids one of the main sources of energy for the developing germ cell and lipid metabolism is crucial for spermatogenesis (Whitmore & Ye, 2015), the decrease in spermatids following PFC exposure may be due to disruptions in lipid metabolism. PFC exposure may be interfering with the ability of retinoic acid to induce meiotic processes in germ cell development by affecting *LIPE*, as PFOS exposure was found to cause decreased *LIPE* expression.

**PFC Exposure Affects Gene Expression**

I assessed the expression of 20 genes with qPCR to determine how PFC exposure affected their expression. This data indicates if selected genes have increased or decreased expression after exposure by measuring mRNA. The following marker genes for spermatogenesis were measured: *PIWIL1, PIWIL2, PRM1, PRM2, SYCP3*, and *ZBTB16*. The following genes related to fertility and detoxification mechanisms were: *CYP17A1, CYP51A1, PAX8, POMC*, and *PON1*. The genes related to lipid metabolism were: *ACOX1, ACOX2, FABP9, HCAR1, HCAR2, LIPE, NCEH1*, and *PPARA*. Following chronic PFOS administration, there appeared to be dose-dependent trends for spermatogenesis genes. *PIWIL1, PRM2*, and *SYCP3* appeared to have overall decreased expression compared to control, although the findings were not significant (Figure 20). Because these genes mediate spermatogenesis and the replacement of histones with protamines during the late stages of spermatogenesis, impacts to these processes have negative implications for germ cell development. *PIWIL2* and *PRM1* also appeared to suggest a non-monotonic dose-response. *ZBTB16* expression appeared to have decreased expression as dose increased, and the highest dose had significantly less gene expression compared to the control. This finding suggests that PFOS exposure decreased the synthesis of PLZF; immunostaining for PLZF supported this conclusion. *ZBTB16* (PLZF) is a germ cell marker for spermatogonia and the early stages of spermatogenesis. The decrease in *ZBTB16* expression following PFOS exposure reflects that DNA may not be being packaged as tightly around histones during meiosis and that PFOS may be interfering with meiotic processes; therefore, decreases in the expression of this gene suggests that PFOS exposure interferes with spermatogonial viability.

The detoxification genes *CYP17A1* and *CYP51A1* did not appear to be affected by PFOS exposure, but the fertility-related genes *PAX8, POMC*, and *PON1* did appear to have dose-related trends (Figure 21). POMC expression appeared to decrease and approached statistical significance ($p=0.07$) for the highest dose of PFOS, while *PON1* expression appeared to decrease and then increase at the highest dose. POMC-derived molecules may regulate the activity of Leydig and Seroli cells in the testis and the antioxidant activity of *PON1* may serve as a marker of male infertility risk; therefore, the decrease in *POMC* has implications for normal Leydig and Sertoli cell activity and the increase in *PON1* suggests that the cells may be responding to oxidative stress induced by PFOS. *PAX8* expression increased as dose increased, and the highest dose had significantly greater expression compared to control. *PAX8* encodes a transcription factor that affects the development of the Mullerian system and thyroid gland; epigenetic alterations to *PAX8* have been associated with male infertility (Rajender, Avery, & Agarwal, 2011). This finding is also in line with another that found increased *PAX8* expression in zebrafish embryos following PFOS exposure (Du et al., 2013). This supports the findings of PFOS as an endocrine disruptor and may have implications for PFOS as a disruptive to duct formation of the seminiferous tubules, which would have negative implications for fertility.

PFOS exposure also appeared to affect genes related to lipid metabolism. Because PFCs have been found to affect lipid metabolism, genes relating to lipid metabolism were chosen for analysis. Fatty acids are one of the main energy sources for spermatogenesis and lipids are necessary for normal spermatogenesis (Whitmore & Ye, 2015; Osuga,

Ishibashi et al., 2000); thus changes to lipid metabolism are relevant for spermatogenesis. While information for the control and the lowest dose of PFOS was missing for *FABP9*, there appeared to be a dose-dependent increase in expression for the higher doses of PFOS (Figure 22). Because *FABP9* encodes a protein that binds fatty acids, it is interesting that the expression appears to increase as PFOS increases because PFOS resembles a fatty acid; increased expression may mean that more of these binding proteins are being produced following PFOS exposure. *HCAR1* also appeared to have overall decreased expression, and the highest dose had significantly less expression compared to the control. *HCAR1* (also *GPR81*) promotes lipid storage, so decreased expression suggests that PFOS exposure may cause decreased lipid storage. *LIPE* had significantly decreased expression for the highest dose of PFOS; this suggests that PFOS disrupts cholesterol conversion for steroid hormone production, which would impair spermatogenesis. Additionally, *LIPE* is activated for the use of energy stores; decreased expression suggests that PFOS may disrupts the cell's ability to mobilize stored energy. Because previous research has found that the absence of *LIPE* interferes with the retinoic acid pulse that normally occurs during spermatogenesis (Whitmore & Ye, 2015), a decrease in *LIPE* expression suggests that PFOS exposure may cause sperm defects and disrupt spermatogenesis. Because PFOS appears to be affecting both lipolytic and energy storage mechanisms, these findings support the animal and epidemiological findings that have found disrupted lipid metabolism following PFC exposure. These findings also suggest that PFOS exposure impairs spermatogenesis.

## DISCUSSION

This project examined the role of PFCs in human health and lipid metabolism from two different lenses: an epidemiological assessment and an *in vitro* experiment. This type of work sets the stage to fuse epidemiological data with laboratory-based studies in future projects. Epidemiological data can be used to develop dosing parameters and to investigate possible mechanisms of disease using *in vitro* models. While the data for this analysis came from disparate sources, it had a central goal: to evaluate lipid-related effects of PFC exposure. While the *in vitro* study uses a human male germ cell model, effects seen in this model may have implications for reproductive effects of PFC exposure in females. For example, because proteins such as hormone-sensitive lipase (*LIPE*) are found in both males and females, disruption to its activity could affect reproductive endpoints of both sexes.

In AK MOMS, PFOS, PFDeA and PFHxS were all significantly associated with the lipid parameter of total cholesterol. However, the observed differences in blood lipids were not very large. Of the women enrolled in AK MOMS, younger women sought prenatal care earlier in their pregnancies compared to older women; this difference may explain some of the effects and trends seen with PFC exposure in this analysis. Overall, when sampling periods were compared, it appears as though PFOS, PFNA, PFDeA and Me-PFOSA-AcOH exposure has decreased over time while PFHxS has increased. This change in mean concentration between the sampling periods was significant and similar to what other studies have found. The increase in PFHxS concentration probably reflects the growing use of PFHxS as a replacement compound for PFOA and PFOS.

The differences seen in PFC concentration between AK MOMS and NHANES is likely due to different exposure sources and diet between the two groups and

bioaccumulation patterns for different PFCs. Additionally, while the percent of women in each trimester was approximately equal for each age group, there were some differences between the distribution of women by age and trimester for NHANES and AK MOMS (Table 19). These differences may have contributed to the findings. For example, in AK MOMS 2005-2006 PFNA and PFDeA were found to be higher in older women; older women were also more likely to be enrolled and have blood drawn during the third trimester. When comparing the distribution of women by trimester between AK MOMS and NHANES, a larger percentage of AK MOMS were enrolled and had blood drawn compared to NHANES. Therefore, the finding that AK MOMS had higher concentrations of PFDeA in the third trimester and PFNA in the second and third trimester compared to NHANES may be because there were more older women enrolled in the later stages of pregnancy for AK MOMS.

There were some limitations to the AK MOMS analysis, however. Because the study was cross-sectional in nature, causality cannot be deduced. There were also limited lipid parameters that were measured, and it would have been interesting to have data on HDL and LDL levels. Additionally, we did not have information on some variables that are associated with lipids and liver function, such as smoking and alcohol use. It would have also been helpful to have information on whether any of the women in the analysis were taking cholesterol-lowering medication.

Chain length with the number of carbons is one of the main differences between different PFCs. PFHxS has 6 carbons in its chain, so it's considered a C6 compound; PFOA and PFOS are C8, PFNA is C9 and PFDeA is C10. PFCs with longer chain lengths may be more biopersistent and bioaccumulative (B.C. Kelly, Ikonomou, Blair,

Morin, & Gobas, 2007). Therefore, PFNA and PFDeA may have a greater bioaccumulative potential than PFHxS. When comparing food sources of the NHANES population and the AK MOMS population, wild-caught meats may be consumed more often in the AK population (Ostertag, Tague, Humphries, Tittlemier, & Chan, 2009). Because PFNA and PFDeA are believed to accumulate up the food chain to a greater extent than PFHxS, a population that consumes wild foods high on the trophic pyramid would probably have higher exposure than people who consume lower trophic level foods (Rotander et al., 2012). Additionally, AK MOMS women may be less exposed to PFHxS, a common ingredient in fire-fighting foams and carpeting.

A study that examined trends of perfluorinated compounds in the serum of a Greenlandic Inuit population found that PFNA and PFDeA concentrations increased over 1998-2005; however, when they adjusted for age, they no longer found this significant trend (Long, Bossi, & Bonefeld-Jorgensen, 2012). This may be because consumption of locally caught or foraged foods has been decreasing over time (Deutch, Pedersen, Asmund, & Hansen, 2007). Therefore, the consumption of traditional foods may differ between age groups, with consumption more common among older people.

Lipid-related effects of PFC exposure were also found with the *in vitro* model. Expression of genes relating to lipid metabolism, spermatogenesis and fertility was measured. Following chronic PFOS administration, there appeared to be dose-dependent decreases in genes that mediate spermatogenesis and histone replacement during the late stages of spermatogenesis. *ZBTB16* expression appeared to have decreased expression, which suggests that PFOS exposure decreases spermatogonial viability. Other fertility-related genes *PAX8*, *POMC*, and *PON1* appeared to have dose-related trends. These

findings have implications for Leydig and Sertoli cell regulation and fertility following PFOS exposure. These results also provide support for PFOS as an endocrine disruptor. These findings are in line with animal studies and epidemiological studies that have found PFCs to affect the thyroid, Leydig and Sertoli cells, and that have suggested PFCs are endocrine disrupting chemicals (Zhao et al., 2014; Wan, Mruk, Wong, & Cheng, 2013, 2014). PFOS exposure also appeared to decreased expression of a gene that promotes lipid storage, suggesting that PFOS exposure could decrease lipid storage. However, there was also decreased expression of a gene related to energy utilization, suggesting PFOS exposure could disrupt the cell's ability to mobilize stored energy. Therefore, PFOS appears to be affecting opposite pathways of energy metabolism, which could be disrupting cellular metabolism and signaling. These findings also provide evidence that PFOS exposure could impair spermatogenesis.

There also appeared to be some increases in apoptotic stages and cell death in PFC-exposed cells compared to control. This may be because of oxidative stress or activation of other cellular signaling, such as PPAR. PPAR signaling has been shown to affect cell growth and cell death (Fajas et al., 2003); because these compounds can activate PPAR and PPAR, they may be affecting cell cycle processes and apoptosis through PPAR signaling. Therefore, these findings are consistent with underlying biological mechanisms and other studies of PFCs and apoptosis (Fang, Feng, Wang, & Dai, 2010; Cui et al., 2015).

The average intensity, independent of area, of all PLZF+ within an identified colony decreased significantly for the second and third doses of PFOS, for all doses of PFOA, and for the highest dose of PFNA. These finding match those found in the qPCR

analysis of *ZBTB16*, which show that the decreased expression of *ZBTB16* led to a decrease in the PLZF protein. These findings show that PFOS, PFOA and PFNA decrease spermatogonial viability, impairing fertility.

This is supported by the findings that PFOS, PFOA and PFNA exposure decrease the amount of haploid spermatids *in vitro*. The evidence suggests that these compounds have this effect via disrupting lipid metabolism. Therefore, it appears that PFOS, PFOA and PFNA are reproductive toxicants for developing male germ cells.

**Possible Mechanisms**

There is a large body of evidence to support the role of PFCs in dysregulation of lipid metabolism and as endocrine disruptors. Due to the similarity of their structures, PFCs may be mimicking fatty acids and handled by the body as such. This has wide-ranging implications for health because fatty acids serve as one of the main energy sources for tissues. However, because of the stability of PFCs, the body may not be able to break these compounds down, and thus they may be disrupting the processes that generally deal with fatty acid metabolism. They may also be causing oxidative stress, binding permanently to receptors, or competing with fatty acids for binding sites. While low levels may not cause outright cell death, PFCs may wreak more subtle effects on metabolic signaling.

We found significantly reduced expression of the *LIPE* gene in cells following PFOS exposure; this is interesting because *LIPE* encodes an important enzyme, hormone-sensitive lipase (HSL). HSL is believed to be responsible for increasing levels of free fatty acids. This enzyme is also expressed in elongating spermatids and believed to be

essential for the differentiation of male germ cells (Osuga, Ishibashi et al., 2000). For example, a study of HSL-knockout mice found that HSL -/- mice were sterile, not because of any effects to Leydig cells, but because of impaired spermatogenesis and oligospermia; the researchers concluded that this was because the release of FFA by HSL was crucial for spermatogenesis (Osuga, Ishibashi et al., 2000). Interestingly, they also found that HSL -/- mice had significantly increased brown adipose tissue mass and size, but not white adipose tissue mass (Osuga, Ishibashi et al., 2000); these findings are consistent with others that found PFC exposure increased brown adipose tissue in mice (Hines et al., 2009). Overall, the researchers found that HSL -/- mice were sterile but not obese (Osuga, Ishibashi et al., 2000); this is possibly because HSL is the main lipase for spermatids, so its absence would cause sterility, but other cells have multiple lipases which could be utilized in place of HSL. HSL is also believed to be a retinyl ester hydrolase and helps provide retinoids for important cell-signalling events (Ström, Gundersen et al. 2009). It is believed that a pulse of retinoic acid may help initiate differentiation in stem cells and related cell signaling cascades (Linney, Donerly et al. 2011); given the importance of retinoids in regulating spermatogenesis (Chung and Wolgemuth 2004), an interruption in the availablility of retinoids for spermatogonial cells could prevent differentiation (Whitmore & Ye, 2015). Therefore, PFOS and other PFC exposure could interfere with differentiation and decrease the amount of haploid spermatids by interfering with the ability to metabolize and mobilize FFA and by decreasing the amount of retinoids available to cells.

PFCs could also be influencing the mechanistic target of rapamycin (mTOR), which is correlated with differentiation in human spermatogenesis; as differentiation

progresses, mTOR levels rise (Easley et al., 2010). mTOR is also believed to help regulate the breakdown of fatty acids via β-oxidation and serves as an important system for lipid metabolism (Soliman, 2011). Therefore, perturbations in the amount of fatty acids available may interfere with mTOR and possibly interfere with differentiation by this pathway.

**Albumin as a Confounder?**

Despite the evidence that PFCs are linked to negative health outcomes, there is possible confounding by serum albumin. Albumin is an important carrier protein in the blood, and transports substances such as fatty acids to cells. PFCs are measured in serum because they are bound to albumin; they are quantified and identified following sonication and denaturation to release them from this blood protein. Therefore, the quantity of albumin in a blood sample could largely influence the quantities of PFCs that are collected and analyzed. For example, a recent study found that blood loss decreased serum PFC concentrations (Lorber et al., 2015). Albumin could be considered a confounder because it can be related to the health effects that have also been linked to PFC exposure. Abnormal albumin levels can be a sign of kidney disease, liver disease, hypothyroidism, malnutrition, and tend to decrease during pregnancy. Albumin adjustment could possibly address the concerns of confounding by serum albumin; however this has not become a standard procedure among researchers and it is still unclear whether or not this is necessary.

PFCs are likely being internalized into cells mainly via albumin. They are able to bind to carrying pockets in albumin, which then docks to the cell membrane and is likely

endocytosed. However, by binding to albumin, PFCs could also be preventing the transport of physiologically important materials in the blood. Therefore, PFCs could be competing with fatty acids in binding to albumin; having a high amount of PFC exposure could prevent fatty acids from being transported in the blood and reaching other tissues. Another possibility is that PFC binding could change the conformational shape of albumin, either transiently or permanently; this could prevent other molecules such as fatty acids or hormones from binding to albumin. This could possibly cause downstream signaling effects if albumin weren't able to transport these other substances as efficiently and these substances weren't meeting their cellular targets.

Additionally, because albumin is the main vehicle to shuttle PFCs into the cell, it is likely that not all of the PFCs that are added into the media are taken up by albumin. The albumin added to cell medium already has lipids and other molecules within its binding pockets, which could prevent PFCs from being taken up by albumin. Therefore, it is likely that the doses used in our *in vitro* analysis are similar to lower population levels, despite the higher dose initially added to the media. The amount of PFCs actually reaching the cells could be approximated by isolating albumin from dosed media and quantifying bound PFCs with HPLC-MS/MS.

Albumin may play some role in assessment of exposure and should be considered when conducting a study of PFC exposure. However, confounding by albumin would not explain all of the *in vitro* experiments and experiments using animal models that support causal effects of PFCs. Given the studies that have shown that PFCs can bind to and activate different receptors and affect cellular signaling, the relationship between PFCs and health is likely causal.

**Possibilities to Decrease Body Burden?**

Although it is important to measure exposures and health effects of environmental pollutants, it is also important to develop ways of decreasing body burden if persistent pollutants are found in a population. When environmental POPs are found to cause detrimental health effects, there is an ethical imperative to try to develop ways to decrease body burden and harm.

While PFCs have long half-lives, there are some possible treatments that could decrease body burdens of these compounds in heavily exposed populations. One possible treatment is cholestyramine, a drug commonly used as a medication to decrease cholesterol in the blood. Cholestyramine binds bile acid in the gut and aids in its expulsion; because PFCs can be found in considerable levels in bile, this medication may aid in the removal of PFCs (Genius, Curtis & Birkholz, 2013). Phlebotomy has also been proposed and used as a possible method to decrease PFC body burden (Genuis, Liu, Genuis, & Martin, 2014). For women, another route to decrease body burden is breastfeeding. These three methods are the only ones that have currently been proposed; each of them has risks and limits to how much can actually be removed. Additionally, blood may not accurately reflect total body burden. Organs such as the liver and kidney may harbor much higher concentrations of PFCs, and while bile cycles through the liver, once PFCs bind to these organ systems they may be nearly impossible to remove.

**CONCLUSIONS**

Despite its non-lipophilic nature, PFCs are emerging as disruptors of lipid metabolism and lipid-related processes. In this population of Alaska Native pregnant

women, PFC exposure was found to be associated with a lipid parameter, total cholesterol. Additionally, PFC exposure was also found to affect gene expression related to lipid metabolism in spermatogenic cells and negatively impact spermatogenesis. Because lipids are so important for endpoints related to fertility and development, exposure to PFCs may impair reproductive health. Fortunately, lower mean PFC concentrations were recorded for the most recently sampled AK MOMS participants, suggesting that the decreased use by industry has successfully resulted in lower body burdens. However, these compounds continue to be used in large amounts abroad and further studies are needed to evaluate how exposure presents a risk to human health. Very little research has been done to evaluate the replacement compounds, which are likely increasing. Additionally, these compound are often encountered as mixtures, which may have different health effects compared to exposure to a single compound. Advancements in *in vitro* modelling could allow for modelling of organ systems. Future studies could use a 3-D spermatogenesis model, with Leydig and Sertoli cells in addition to the human spermatogenesis model, to test the reproductive toxicity of PFCs in a somatic system. Additionally, it is hoped that future techniques would allow for *in vitro* modelling of female oogenesis to assess reproductive toxicity. Because these findings provide the first evidence for the reproductive toxicity of perfluorinated compounds in *in vitro* human spermatogenesis, future studies should focus on the toxicity of replacement compounds and assessing reproductive toxicity in occupationally-exposed populations.

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## TABLES

**Table 1. Distribution of women from AK MOMS 2005-2012 for age, total cholesterol, and trimester**

| Characteristic | All participants |
|---|---|
| Age<br>Mean (Range) | 26 (16-44) |
| Total cholesterol<br>Mean (Range) | 218.7 (110-393) |
| Trimester at blood draw (n, %)<br>1st trimester<br>2nd trimester<br>3rd trimester | <br>64 (22.6%)<br>88 (31.1%)<br>131 (46.3%) |

**Table 2. Distribution of women from AK MOMS 2005-2012 by trimester and age**

| Trimester | Age <26 | Age >26 |
|---|---|---|
| 1st | 35 (12.3%) | 29 (10.3%) |
| 2nd | 49 (17.3%) | 39 (13.8%) |
| 3rd | 57 (20.1%) | 74 (26.2%) |

**Table 3. Association and distribution of age and trimester at blood draw on total cholesterol of women from AK MOMS 2005-2012**

| Characteristic | Number | Total Cholesterol Mean (95% CI) | p-value |
|---|---|---|---|
| Age<br><26<br>>26 | <br>141<br>142 | <br>210.5 (201.5-219.5)<br>226.9 (217-237) | <br>0.016 |
| Trimester at blood draw<br>1st trimester<br>2nd trimester<br>3rd trimester | <br>64<br>88<br>131 | <br>165.8 (158.5-173)<br>209.7 (199.7-219.7)<br>250.6 (241.3-259.9) | <br><0.001 |

**Table 4. Descriptive statistics of PFC analytes in AK MOMS 2005-2012 (ng/mL)**

| Analyte | N (%)>LOD | Median (Range) | Geometric Mean (95th% CI) |
|---|---|---|---|
| PFOS | 282/283 (99.6%) | 3.1 (0.14-22.5) | 3.35 (3.00-3.73) |
| PFOA | 281/283 (99.3%) | 1.0 (0.07-3.9) | 0.90 (0.84-0.98) |
| PFNA | 282/283 (99.6%) | 0.8 (0.07-6.10) | 0.91 (0.83-0.99) |
| PFDeA | 263/283 (92.9%) | 0.3 (0.07-2.10) | 0.31 (0.28-0.33) |
| Me-PFOSA-AcOH | 172/283 (61%) | 0.12 (0.06-1.83) | 0.15 (0.14-0.17) |
| PFHxS | 205/283 (72.4%) | 0.2 (0.07-4.6) | 0.22 (0.20-0.25) |

**Table 5. Association of PFC analyte concentrations (geometric means, ng/mL) by age and trimester in AK MOMS 2005-2012**

| | Age | | | Trimester | | | |
|---|---|---|---|---|---|---|---|
| Analyte | <26 years GM (95% CI) | ≥26 years GM (95% CI) | p-value | Trimester 1 GM (95% CI) | Trimester 2 GM (95% CI) | Trimester 3 GM (95% CI) | p-value |
| PFOS | 3.22 (2.75-3.78) | 3.48 (2.99-4.05) | 0.49 | 2.39 (1.94-2.95) | 2.47 (2.06-2.96) | 4.84 (4.17-5.63) | <.0001 |
| PFOA | 0.96 (0.86-1.07) | 0.85 (0.76-0.96) | 0.13 | 0.96 (0.82-1.12) | 0.90 (0.79-1.03) | 0.88 (0.78-1.0) | 0.714 |
| PFNA | 0.76 (0.68-0.85) | 1.08 (0.95-1.22) | 0.0001 | 0.90 (0.75-1.08) | 0.83 (0.71-0.97) | 0.96 (0.85-1.08) | 0.359 |
| PFDeA | 0.26 (0.24-0.29) | 0.36 (0.32-0.40) | 0.0001 | 0.28 (0.24-0.33) | 0.28 (0.24-0.32) | 0.34 (0.31-0.38) | 0.044 |
| Me-PFOSA-AcOH | 0.15 (0.13-0.17) | 0.16 (0.14-0.18) | 0.585 | 0.13 (0.11-0.15) | 0.13 (0.11-0.15) | 0.19 (0.17-0.22) | <.0001 |
| PFHxS | 0.22 (0.19-0.26) | 0.22 (0.19-0.26) | 0.967 | 0.32 (0.26-0.39) | 0.26 (0.22-0.31) | 0.16 (0.14-0.19) | <.0001 |

**Table 6. Univariable linear regression models assessing the association between logged PFC concentrations and total cholesterol in AK MOMS 2005-2012**

| Analyte | Parameter estimate (95% CI) | p-value |
|---|---|---|
| PFOS Continuous (logged) | 192.52 + 21.67 (log_pfos) | <.0001 |
| PFOA Continuous (logged) | 218.43 + -2.88 (log_pfoa) | 0.573 |
| PFNA Continuous(logged) | 219.64 + 9.35 (log_pfna) | 0.049 |
| PFDeA Continuous(logged) | 244.04 + 21.34 (log_pfdea) | <.0001 |
| Me-PFOSA-AcOH Continuous(logged) | 240.86 + 11.77 (log_me-pfosa-acoh) | 0.0056 |
| PFHxS Continuous(logged) | 192.34 + -17.40 (log_pfhxs) | <.0001 |
| Sum PFC Continuous (logged) | 172.41 + 24.34 (log_sumPFC) | <.0001 |

**Table 7. Multivariable linear regression models for the association between PFC concentration with cholesterol while controlling for age at blood draw (years) and weeks pregnancy at blood draw in AK MOMS 2005-2012**

| Analyte | Parameter estimate (95% CI) | p-value |
|---|---|---|
| PFOS | | |
| Intercept | 128.01 (100.7-155.3) | <0.0001 |
| Log PFOS | 8.22 (2.01-14.42) | 0.0096 |
| Age | 0.176 (-.79-1.14) | 0.72 |
| Weeks Pregnant | 3.05 (2.51-3.60) | <.0001 |
| PFNA | | |
| Intercept | 135.05 (105.7-164.4) | <0.0001 |
| Log PFNA | 5.17 (-2.7-13.1) | 0.198 |
| Age | 0.06 (-1.0-1.1) | 0.905 |
| Weeks Pregnant | 3.31 (2.8-3.8) | <0.0001 |
| PFDeA | | |
| Intercept | 160.06 (125.8-194.3) | <0.0001 |
| Log PFDeA | 13.14 (4.5-21.8) | 0.003 |
| Age | -0.23 (-1.3-0.8) | 0.655 |
| Weeks Pregnant | 3.21 (2.7-3.7) | <0.0001 |
| Me-PFOSA-AcOH | | |
| Intercept | 128.22 (94.4-162) | <0.0001 |
| Log Me-PFOSA- | -0.07 (-7-6.9) | 0.984 |
| AcOH | 0.28 (-0.7-1.3) | 0.571 |
| Age | 3.32 (2.8-3.9) | <0.0001 |
| Weeks Pregnant | | |
| PFHxS | | |
| Intercept | 122.20 (94.2-150.2) | <0.0001 |
| Log PFHxS | -6.28 (-12.1- -0.4) | 0.035 |
| Age | 0.33 (-0.6-1.3) | 0.497 |
| Weeks Pregnant | 3.13 (2.6-3.7) | <0.0001 |
| Sum PFC | | |
| Intercept | 118.72 (90-147.4) | <0.0001 |
| Log Sum PFC | 9.35 (1.1-17.6) | 0.026 |
| Age | 0.14 (-0.8-1.1) | 0.780 |
| Weeks Pregnant | 3.15 (2.6-3.7) | <0.0001 |

**Table 8. Descriptive statistics of PFC analytes in NHANES pregnant women 2005-2006**

| Analyte | N (%)>LOD | Geometric Mean ($95^{th}$% CI)(weighted) |
|---|---|---|
| PFOS | 88/88 (100%) | 7.42 (6.05-9.10) |
| PFOA | 88/88 (100%) | 1.73 (1.22-2.45) |
| PFNA | 85/88 (96.6%) | 0.62 (0.49-0.78) |
| PFDeA | 61/88 (69.3%) | 0.23 (0.18-0.29) |
| Me-PFOSA-AcOH | 63/88 (71.6%) | 0.30 (0.22-0.39) |
| PFHxS | 78/88 (88.6%) | 0.72 (0.49-1.05) |

**Table 9. Association and distribution of age and trimester at blood draw on total cholesterol (weighted) of pregnant women from NHANES 2005-2006**

| Characteristic | Number | Total Cholesterol Mean (95% CI) (weighted) |
|---|---|---|
| Age | | |
| <26 | 42 (47.7%) | 220.40 (196.30-244.51) |
| >26 | 46 (52.3%) | 247.10 (192.98-301.15) |
| Trimester at blood draw | | |
| $1^{st}$ trimester | 14 (16%) | 166.4 (151.66-181.12) |
| $2^{nd}$ trimester | 32 (36%) | 223.5 (214.78-232.15) |
| $3^{rd}$ trimester | 42 (48%) | 268.10 (227.11-309.09) |

**Table 10. Association of PFC analyte concentrations (geometric means, ng/mL) by age and trimester in NHANES 2005-2006**

| | Age Group | | Trimester (weighted) | | |
|---|---|---|---|---|---|
| Analyte | <26 | =>26 | Trimester 1 GM (95% CI) | Trimester 2 GM (95% CI) | Trimester 3 GM (95% CI) |
| PFOS | 9.19 (6.07-12.31) | 8.10 (6.0-10.20) | 9.40 (7.23-12.22) | 7.75 (6.10-9.86) | 6.54 (4.77-8.97) |
| PFOA | 2.89 (1.60-4.17) | 1.81 (1.18-2.44) | 1.94 (1.17-3.22) | 1.70 (1.21-2.37) | 1.66 (0.99-2.77) |
| PFNA | 0.86 (0.42-1.30) | 0.66 (0.50-0.81) | 0.90 (0.61-1.32) | 0.66 (0.52-0.85) | 0.51 (0.40-0.65) |
| PFDeA | 0.30 (0.14-0.47) | 0.26 (0.20-0.33) | 0.26 (0.16-0.43) | 0.27 (0.20-0.35) | 0.20 (0.16-0.26) |
| Me-PFOSA-AcOH | 0.49 (0.32-0.66) | 0.32 (0.24-0.41) | 0.31 (0.19-0.53) | 0.34 (0.26-0.44) | 0.28 (0.19-0.40) |
| PFHxS | 1.32 (0.73-1.91) | 1.24 (0.72-1.76) | 0.66 (0.31-1.38) | 0.50 (0.27-0.93) | 0.85 (0.49-1.47) |

**Table 11. Distribution of women from AK MOMS 2005-2006 for age, total cholesterol, and trimester**

| Characteristic | All participants |
|---|---|
| Age<br>Mean (Range) | 26.6 (17-44) |
| Total cholesterol<br>Mean (Range) | 242.9 (113-393) |
| Trimester at blood draw (N, %)<br>1st trimester<br>2nd trimester<br>3rd trimester | <br>16 (11.8%)<br>25 (18.4%)<br>95 (69.9%) |

**Table 12. Distribution of women from AK MOMS 2005-2006 by trimester and age**

| Trimester | Age <26 | Age >26 |
|---|---|---|
| 1st | 9 (6.6%) | 7 (5.2%) |
| 2nd | 12 (8.8%) | 13 (9.6%) |
| 3rd | 45 (33.1%) | 50 (36.8%) |

**Table 13. Association and distribution of age and trimester at blood draw on total cholesterol of women from AK MOMS 2005-2006**

| Characteristic | Number | Total Cholesterol Mean (95% CI) | p-value |
|---|---|---|---|
| Age | | | |
| <26 | 66 | 234.9 (221.3-248.5) | 0.12 |
| >26 | 70 | 250.5 (235.8-265.2) | |
| Trimester at blood draw | | | <0.0001 (sig different between trimester 1 vs. trimester 2 or 3) |
| 1st trimester | 16 | 172.4 (154.6-190.2) | |
| 2nd trimester | 25 | 234.2 (210.6-257.7) | |
| 3rd trimester | 95 | 257.1 (246.1-268.1) | |

**Table 14. Descriptive statistics of PFC analytes in AK MOMS 2005-2006 (ng/mL)**

| Analyte | N (%)>LOD | Median (Range) | Geometric Mean (95th% CI) |
|---|---|---|---|
| PFOS | 135/136 (99%) | 7.25 (0.14-22.5) | 7.02 (6.28-7.85) |
| PFOA | 134/136 (98.5%) | 1.10 (0.07-3.8) | 0.93 (0.81-1.07) |
| PFNA | 135/136 (99%) | 1.10 (0.07-6.1) | 1.12 (0.99-1.26) |
| PFDeA | 124/136 (46.6%) | 0.40 (0.14-2.1) | 0.41 (0.37-0.45) |
| Me-PFOSA-AcOH | 110/136 (80.9%) | 0.26 (0.12-1.83) | 0.28 (0.26-0.31) |
| PFHxS | 61/136 (44.9%) | 0.07 (0.07-4.6) | 0.152 (0.13-0.18) |

**Table 15. Association of PFC analyte concentrations (geometric means, ng/mL) by age and trimester in AK MOMS 2005-2006**

| | Age | | | Trimester | | | |
|---|---|---|---|---|---|---|---|
| Analyte | <26 years GM (95% CI) | ≥26 years GM (95% CI) | p-value | Trimester 1 GM (95% CI) | Trimester 2 GM (95% CI) | Trimester 3 GM (95% CI) | p-value |
| PFOS | 6.90 (5.74-8.28) | 7.15 (6.24-8.18) | 0.756 | 4.81 (2.75-8.40) | 7.17 (5.56-9.24) | 7.45 (6.70-8.28) | 0.046 |
| PFOA | 0.99 (0.80-1.21) | 0.88 (0.72-1.06) | 0.394 | 0.85 (0.54-1.35) | 0.96 (0.68-1.36) | 0.93 (0.80-1.10) | 0.902 |
| PFNA | 0.87 (0.73-1.03) | 1.43 (1.23-1.67) | <0.0001 | 0.97 (0.61-1.57) | 1.16 (0.87-1.56) | 1.34 (1.0-1.30) | 0.700 |
| PFDeA | 0.32 (0.28-0.37) | 0.51 (0.45-0.57) | <0.0001 | 0.33 (0.24-0.45) | 0.43 (0.33-0.55) | 0.41 (0.37-0.46) | 0.316 |
| Me-PFOSA-AcOH | 0.27 (0.23-0.31) | 0.29 (0.26-0.34) | 0.425 | 0.33 (0.25-0.44) | 0.34 (0.26-0.43) | 0.26 (0.23-0.29) | 0.069 |
| PFHxS | 0.15 (0.11-0.20) | 0.15 (0.12-0.2) | 0.866 | 0.24 (0.13-0.43) | 0.19 (0.12-0.31) | 0.13 (0.11-0.16) | 0.088 |

**Table 16. Comparison of mean analyte concentrations (geometric means, ng/mL) between NHANES pregnant women 2005-2006 and AK MOMS women 2005-2006**

| **Analyte** | **NHANES GM ($95^{th}$% CI)** | **AK GM ($95^{th}$% CI)** |
|---|---|---|
| PFOS | 7.42 (6.05-9.10) | 7.02 (6.28-7.85) |
| PFOA | 1.73 (1.22-2.45) | 0.93 (0.81-1.07) |
| PFNA | 0.62 (0.49-0.78) | 1.12 (0.99-1.26) |
| PFDeA | 0.23 (0.18-0.29) | 0.41 (0.37-0.45) |
| Me-PFOSA-AcOH | 0.30 (0.22-0.39) | 0.28 (0.26-0.31) |
| PFHxS | 0.72 (0.49-1.05) | 0.152 (0.13-0.18) |

**Table 17. Comparison of mean analyte concentrations (geometric means) between NHANES pregnant women 2005-2006 and AK MOMS women 2005-2006 by trimester**

| | NHANES Trimester | | | AK MOMS Trimester | | |
|---|---|---|---|---|---|---|
| Analyte | Trimester 1 GM (95% CI) | Trimester 2 GM (95% CI) | Trimester 3 GM (95% CI) | Trimester 1 GM (95% CI) | Trimester 2 GM (95% CI) | Trimester 3 GM (95% CI) |
| PFOS | 9.40 (7.23-12.22) | 7.75 (6.10-9.86) | 6.54 (4.77-8.97) | 4.81 (2.75-8.40) | 7.17 (5.56-9.24) | 7.45 (6.70-8.28) |
| PFOA | 1.94 (1.17-3.22) | 1.70 (1.21-2.37) | 1.66 (0.99-2.77) | 0.85 (0.54-1.35) | 0.96 (0.68-1.36) | 0.93 (0.80-1.10) |
| PFNA | 0.90 (0.61-1.32) | 0.66 (0.52-0.85) | 0.51 (0.40-0.65) | 0.97 (0.61-1.57) | 1.16 (0.87-1.56) | 1.34 (1.0-1.30) |
| PFDeA | 0.26 (0.16-0.43) | 0.27 (0.20-0.35) | 0.20 (0.16-0.26) | 0.33 (0.24-0.45) | 0.43 (0.33-0.55) | 0.41 (0.37-0.46) |
| Me-PFOSA-AcOH | 0.31 (0.19-0.53) | 0.34 (0.26-0.44) | 0.28 (0.19-0.40) | 0.33 (0.25-0.44) | 0.34 (0.26-0.43) | 0.26 (0.23-0.29) |
| PFHxS | 0.66 (0.31-1.38) | 0.50 (0.27-0.93) | 0.85 (0.49-1.47) | 0.24 (0.13-0.43) | 0.19 (0.12-0.31) | 0.13 (0.11-0.16) |

**Table 18. Comparison of mean analyte concentrations (geometric means) between NHANES pregnant women 2005-2006 and AK MOMS women 2005-2006 by age group**

| | **NHANES age** | | **AK MOMS age** | |
|---|---|---|---|---|
| Analyte | <26 years GM (95% CI) | ≥26 years GM (95% CI) | <26 years GM (95% CI) | ≥26 years GM (95% CI) |
| PFOS | 9.19 (6.07-12.31) | 8.10 (6.0-10.20) | 6.90 (5.74-8.28) | 7.15 (6.24-8.18) |
| PFOA | 2.89 (1.60-4.17) | 1.81 (1.18-2.44) | 0.99 (0.80-1.21) | 0.88 (0.72-1.06) |
| PFNA | 0.86 (0.42-1.30) | 0.66 (0.50-0.81) | 0.87 (0.73-1.03) | 1.43 (1.23-1.67) |
| PFDeA | 0.30 (0.14-0.47) | 0.26 (0.20-0.33) | 0.32 (0.28-0.37) | 0.51 (0.45-0.57) |
| Me-PFOSA-AcOH | 0.49 (0.32-0.66) | 0.32 (0.24-0.41) | 0.27 (0.23-0.31) | 0.29 (0.26-0.34) |
| PFHxS | 1.32 (0.73-1.91) | 1.24 (0.72-1.76) | 0.15 (0.11-0.20) | 0.15 (0.12-0.2) |

**Table 19. Distribution of participants in NHANES 2005-2006 and AK MOMS 2005-2006 by age and trimester**

| | **NHANES** | | **AK MOMS** | |
|---|---|---|---|---|
| Trimester | Age <26 | Age >26 | Age <26 | Age >26 |
| 1st | 7 (8%) | 7 (8%) | 9 (6.6%) | 7 (5.2%) |
| 2nd | 14 (15.9%) | 18 (20.5%) | 12 (8.8%) | 13 (9.6%) |
| 3rd | 21 (23.9%) | 21 (23.9%) | 45 (33.1%) | 50 (36.8%) |

**Table 20. Descriptive statistics of PFC exposure (geometric means) in AK MOMS 2005-2006 and AK MOMS 2010-2012**

| Analyte | AK 2005-2006 %>LOD | AK 2010-2012 %>LOD | AK 2005-2006 GM (95% CI) | AK 2010-2012 GM (95% CI) | P-value |
|---|---|---|---|---|---|
| PFOS | 99% (n=135/136) (LOD=0.2ng/mL) | 100% (n=147/147) (LOD=0.2 ng/mL) | 7.025 (6.28-7.85) | 1.687 (1.54-1.84) | <0.0001 |
| PFOA | 98.5% (n=134/136) (LOD=0.1 ng/mL) | 100% (n=147/147) (LOD=0.1 ng/mL) | 0.928 (0.81-1.07) | 0.884 (0.81-0.96) | 0.542 |
| PFNA | 99% (n=135/136) (LOD=0.1 ng/mL) | 100% (n=147/147) (LOD=0.1 ng/mL) | 1.121 (0.99-1.26) | 0.744 (0.67-0.83) | <0.0001 |
| PFDeA | 91% (n=124/136) (LOD=0.2 ng/mL) | 94.5% (n=139/147) (LOD=0.1 ng/mL) | 0.406 (0.37-0.45) | 0.237 (0.21-0.26) | <0.0001 |
| Me-PFOSA-AcOH | 81% (n=110/136) (LOD=0.174 ng/mL) | 42% (n=62/147) (LOD=0.1 ng/mL) | 0.282 (0.26-0.31) | 0.086 (0.08-0.09) | <0.0001 |
| PFHxS | 45% (n=61/136) (LOD=0.1 ng/mL) | 98% (n=144/147) (LOD=0.1 ng/mL) | 0.152 (0.13-0.18) | 0.308 (0.28-0.34) | <0.0001 |

**FIGURES**

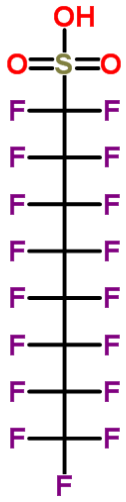


**Figure 1.** Figure depicting the chemical structure of PFOS, a sulfonated C8 compound. Retrieved from: http://www.chemspider.com/ImageView.aspx?id=67068

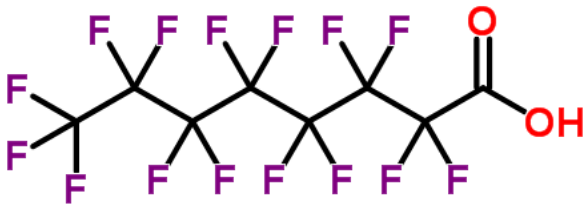


**Figure 2.** Figure depicting the chemical structure of PFOA, a carboxylated C8 compound. Retrieved from: http://www.chemspider.com/ImageView.aspx?id=9180

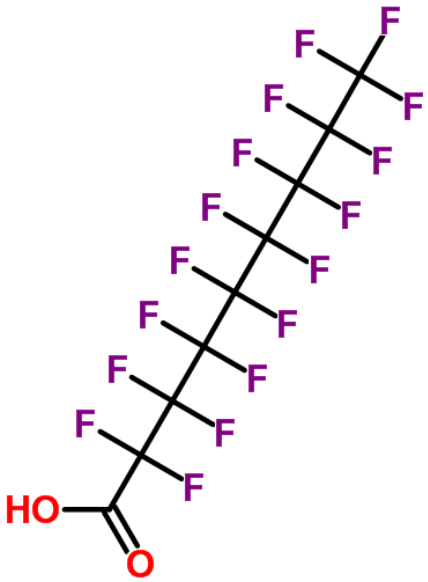


**Figure 3.** Figure depicting the chemical structure of PFNA, a carboxylated C9 compound. Retrieved from: http://www.chemspider.com/ImageView.aspx?id=61138

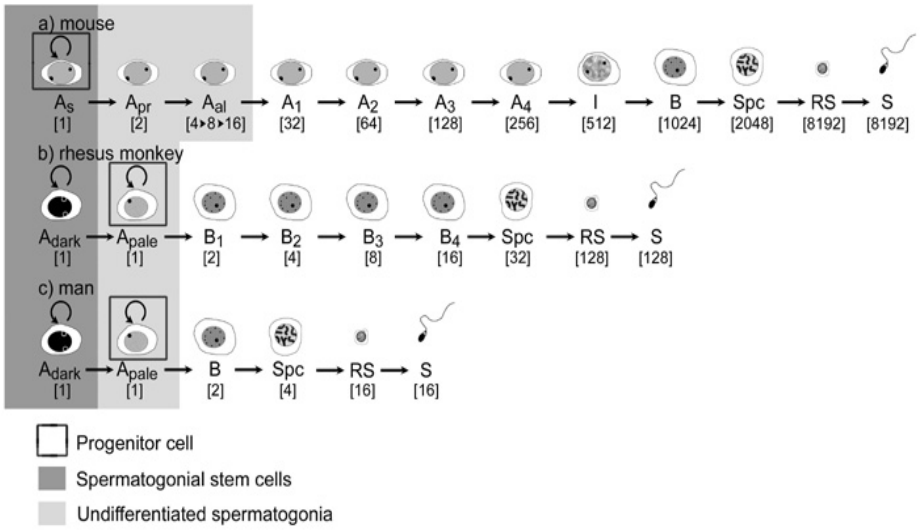


**Figure 4.** Diagram highlighting some of the major differences between human and rodent spermatogenesis. This graphical illustration shows some of the major differences between rodent and human spermatogenesis. This diagram adapted from: Spermatogonial stem cells: questions, models and perspectives. Ehmcke J, Wistuba J, Schlatt S, 2006, *Human Reproduction update*. 12(3):275-82. Epub 2006/02/01. doi: 10.1093/humupd/dmk001. PubMed PMID: 16446319

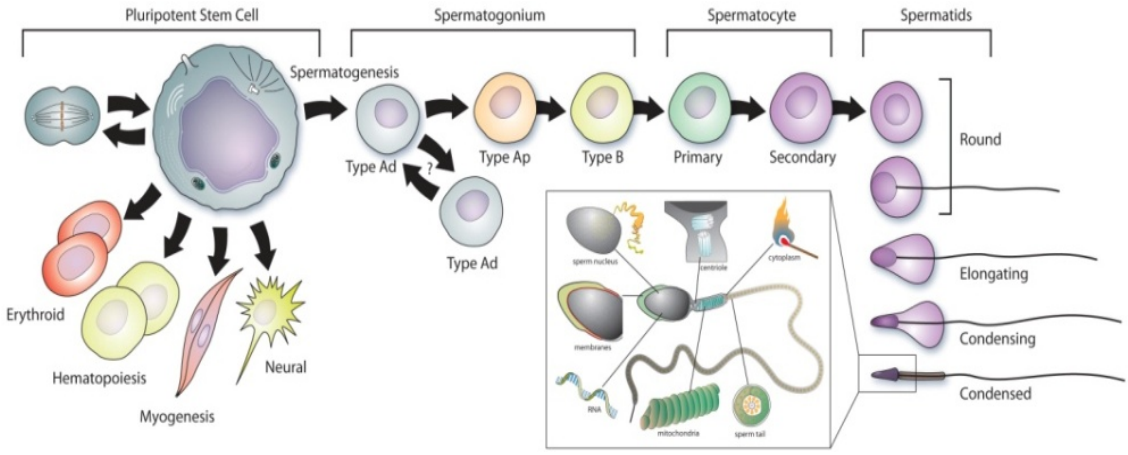


**Figure 5.** Diagram depicting the stages of spermatogenesis. This adapted from: Adult somatic cells to the rescue: nuclear reprogramming and the dispensability of gonadal germ cells. Easley CAt, Latov DR, Simerly CR, Schatten G., 2014, *Fertility and Sterility.* 101(1):14-9. Epub 2014/01/03. doi: 10.1016/j.fertnstert.2013.11.025. PubMed PMID: 24382340

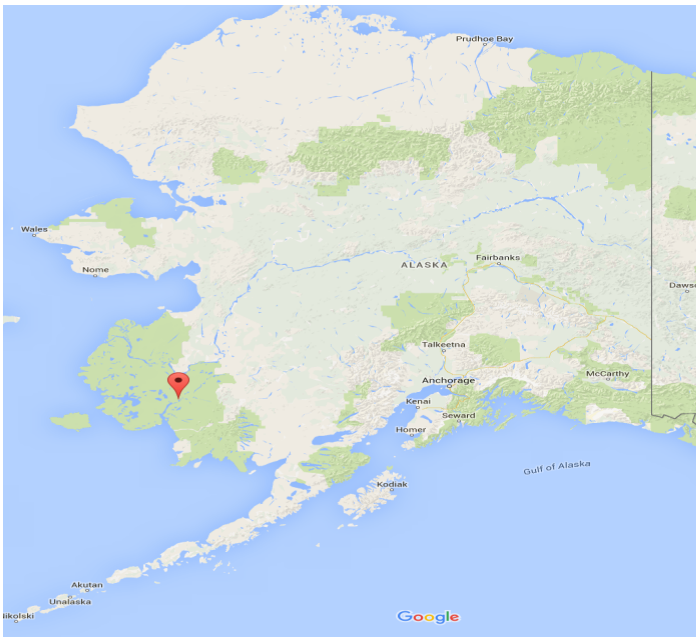


**Figure 6.** Map of Alaska with the pin depicting the location of Bethel. Image from Google Maps, 2016. Retrieved from: https://www.google.com/maps/place/Bethel,+AK/@63.6261788,-159.7502769,5z/data=!4m2!3m1!1s0x572054d63de50e35:0x22965f9a178165ef

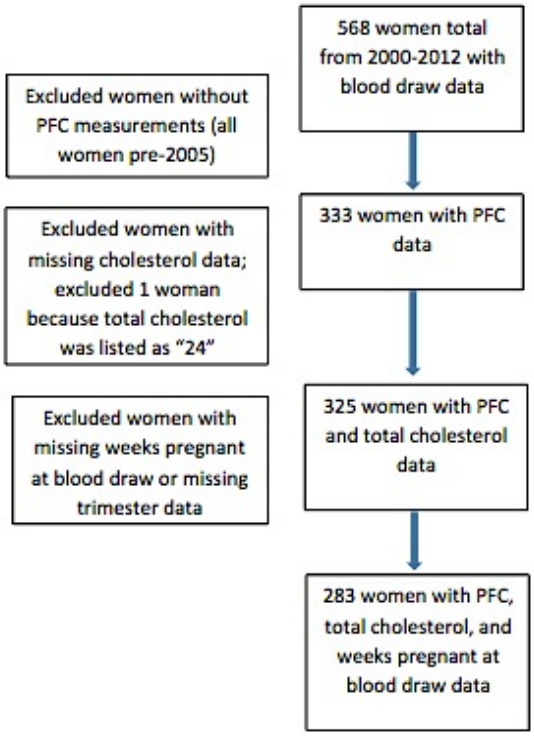


**Figure 7.** Flow chart depicting the inclusion and exclusion process for AK MOMS 2005-2012.

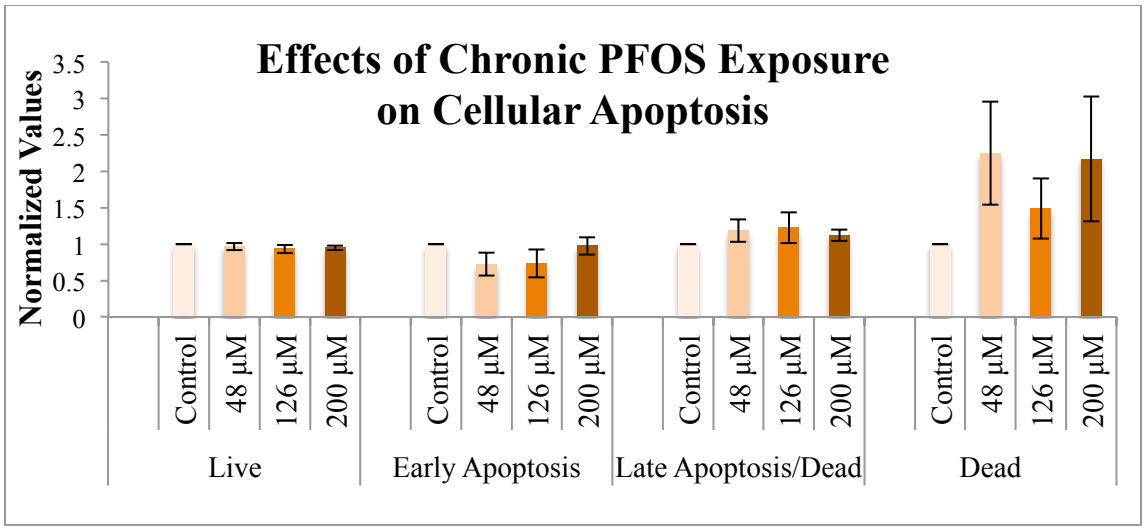


**Figure 8.** Histogram depicting the distribution of live and apoptotic cells following chronic PFOS exposure using the Muse Annexin V Assay and flow cytometer. The experiment was conducted in triplicate and data from dosed cells was normalized to the vehicle control. PFOS exposure appears to slightly increase apoptosis and cell death, although not significantly using ANOVA.

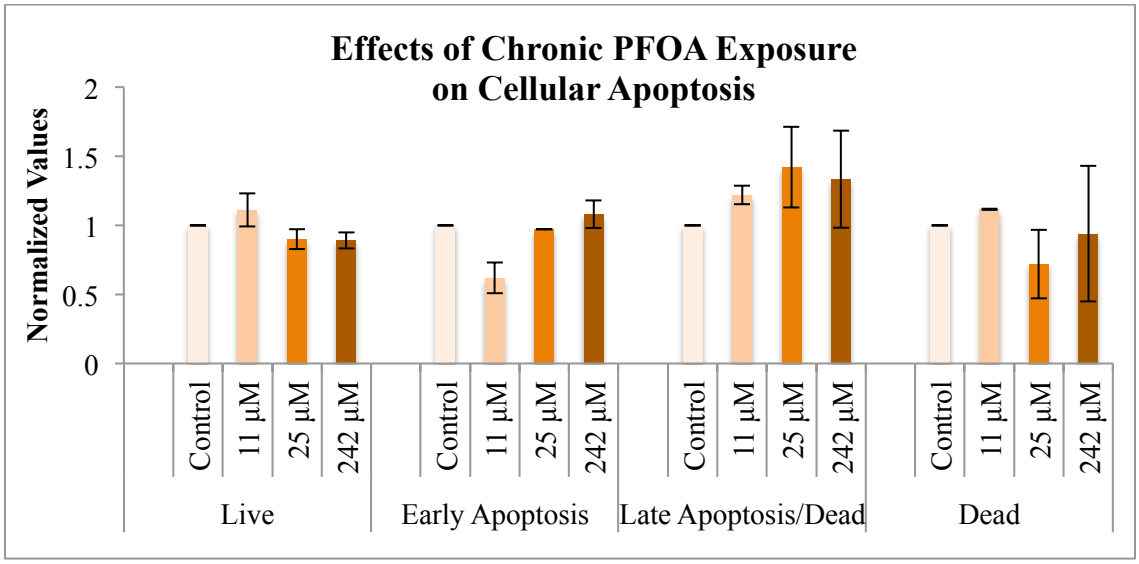


**Figure 9.** Histogram depicting the distribution of live and apoptotic cells following chronic PFOA exposure using the Muse Annexin V Assay and flow cytometer. The experiment was conducted in triplicate and data from dosed cells was normalized to the vehicle control. PFOA exposure appears to slightly increase apoptosis, although not significantly using ANOVA.

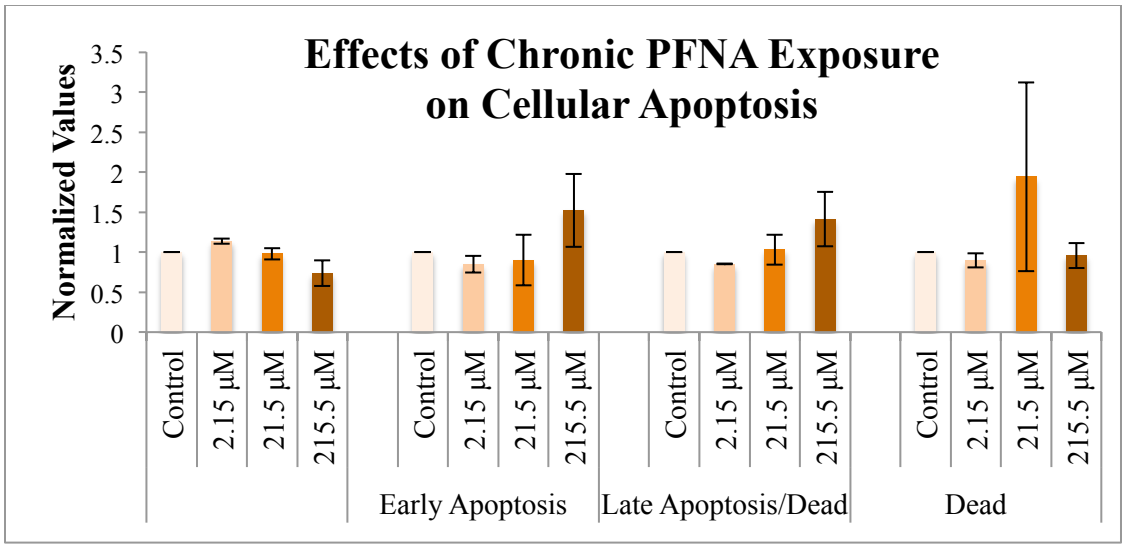


**Figure 10.** Histogram depicting the distribution of live and apoptotic cells following chronic PFNA exposure using the Muse Annexin V Assay and flow cytometer. The experiment was conducted in triplicate and data from dosed cells was normalized to the vehicle control. PFNA exposure appears to increase apoptosis and cell death, although not significantly using ANOVA.

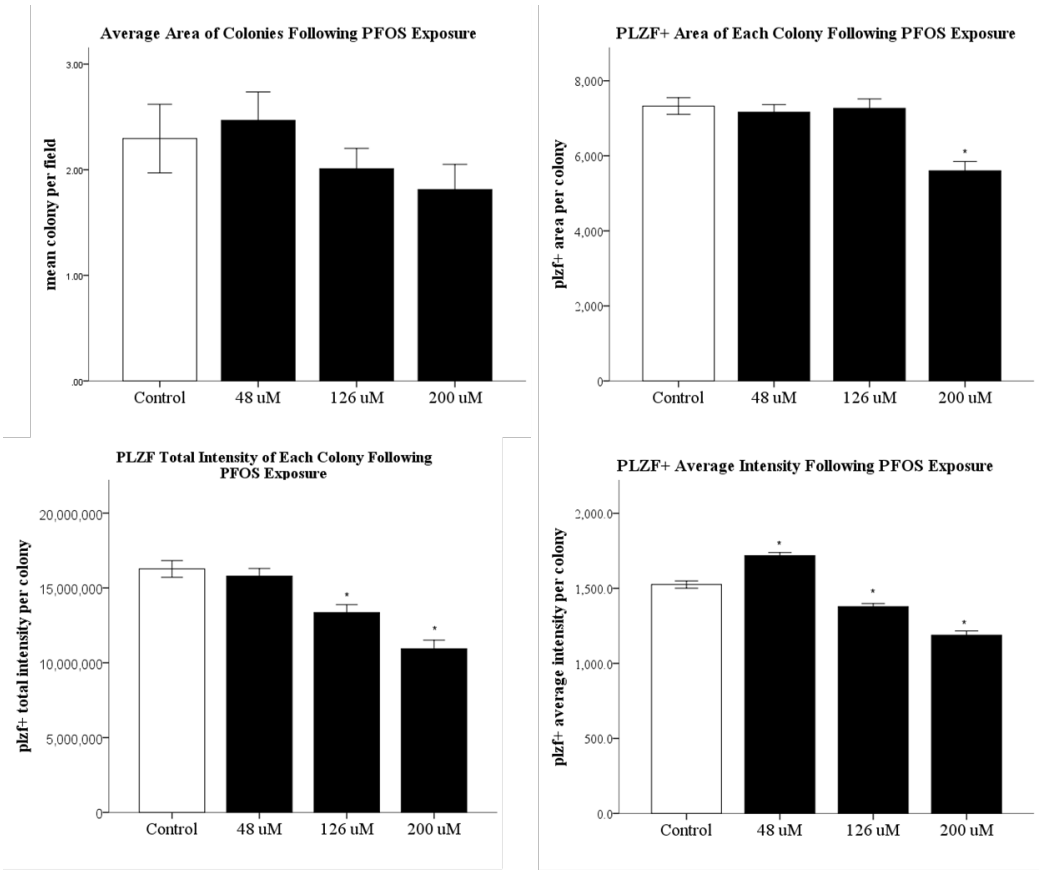


**Figure 11.** Cells were chronically dosed with 48µM, 126µM, or 200µM PFOS, respectively, immunostained for PLZF and analyzed with an Arrayscan. Colony sizes were similar across all treatment groups; per colony, there was significantly less colony area with PLZF at the highest PFOS dose and less measured fluorescence of PLZF for the medium and highest dose of PFOS compared to control. The average intensity, independent of area, for PLZF was significantly lower for the medium and high dose of PFOS compared to control. These results suggest that colonies were of similar size across all levels of PFOS exposure but less PLZF was found at the upper levels of PFOS exposure. Data were tested for statistical significance using SPSS by 1-way ANOVA with Tukey's post-hoc test ($p<0.05$).

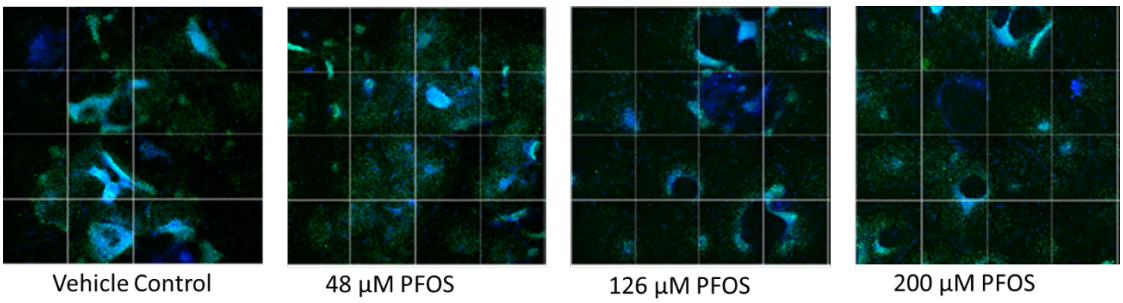


**Figure 12.** Images of immunofluorescent cells following PFOS exposure, taken with the ArrayScan.

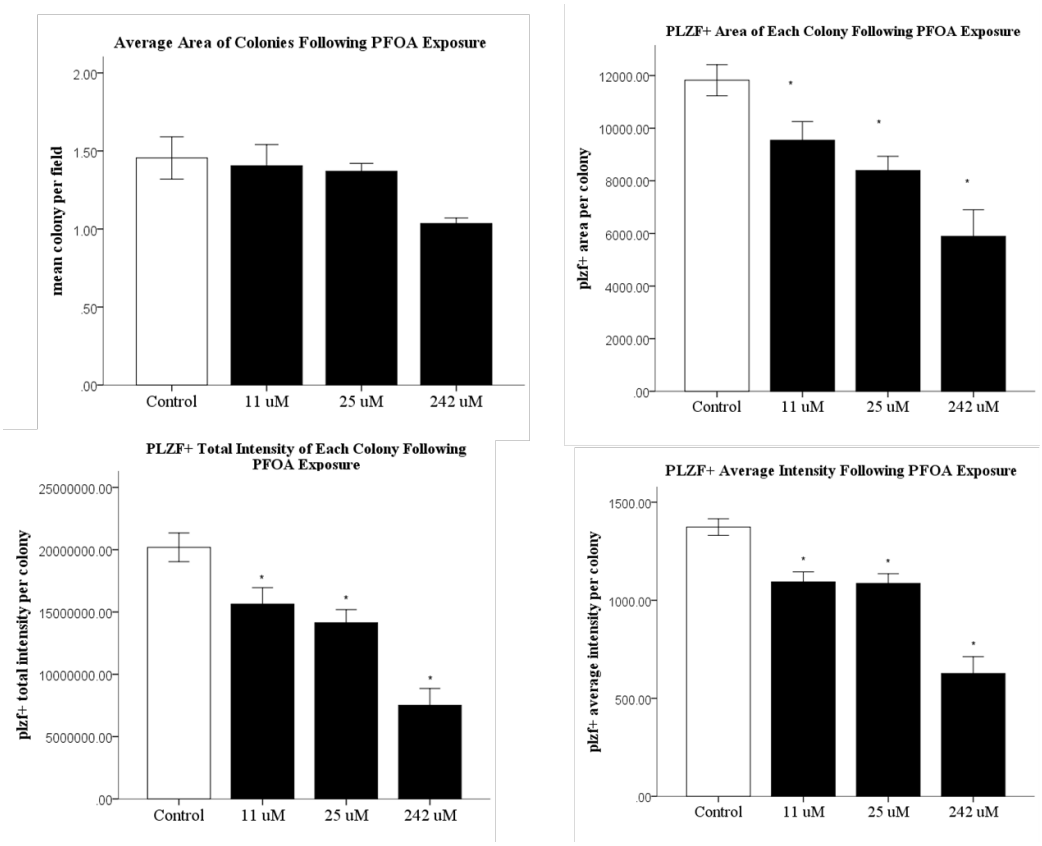


**Figure 13.** Cells were chronically dosed with vehicle control (DMSO) or 11µM, 25µM, or 242µM PFOA, respectively, immunostained for PLZF and analyzed with an Arrayscan. Colony sizes were similar across all treatment groups; per colony, there was significantly less colony area with PLZF and less measured fluorescence of PLZF for the all doses of PFOA compared to control. The average intensity, independent of area, for PLZF was significantly lower for all doses compared to control. These results suggest that colonies were of similar size across all levels of PFOA exposure but less PLZF was found across all PFOA-dosed groups. Data were tested for statistical significance using SPSS by 1-way ANOVA with Tukey's post-hoc test ($p<0.05$).

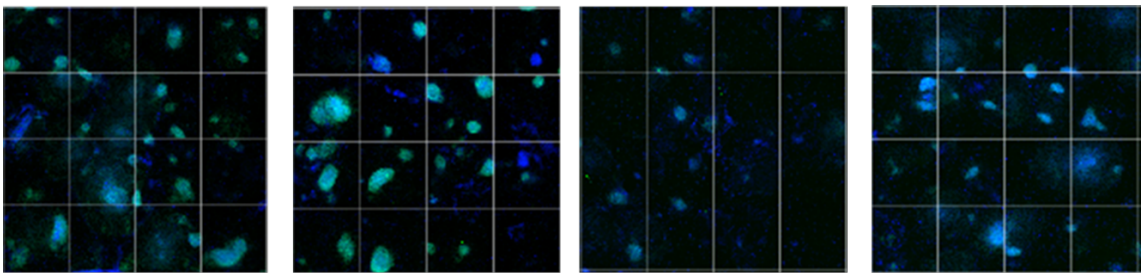


**Figure 14.** Images of immunofluorescent cells following PFOA exposure, taken with the ArrayScan.

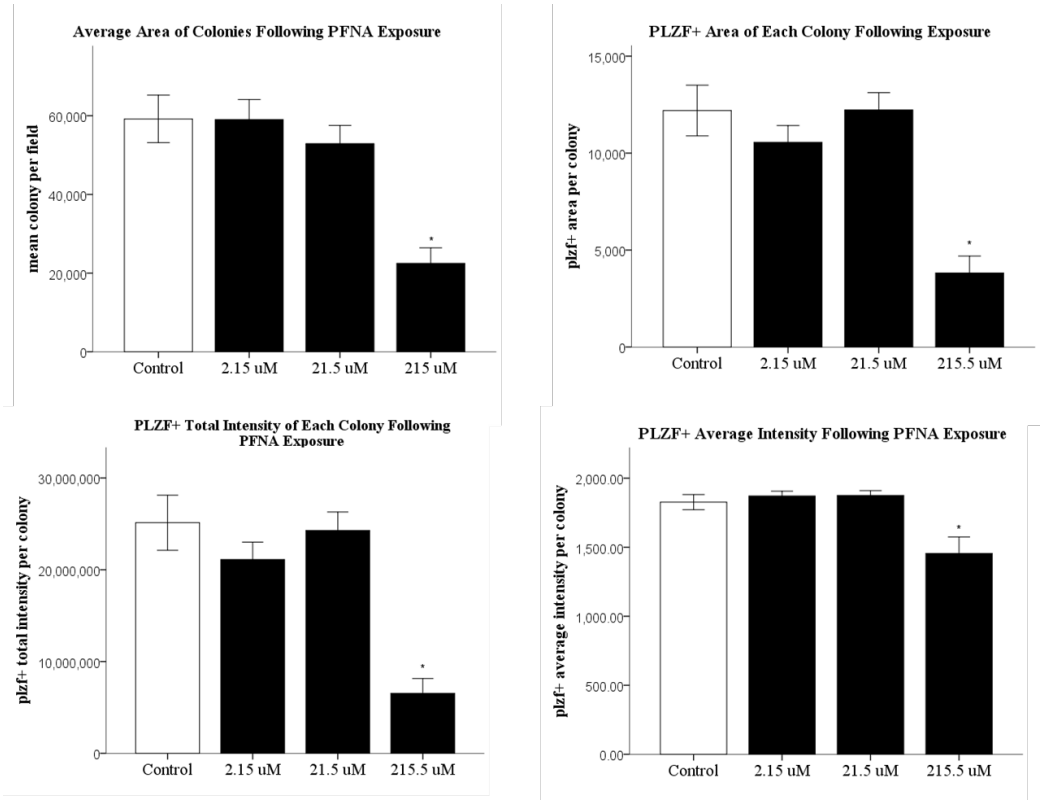


**Figure 15.** Cells were chronically dosed with vehicle control (DMSO) or 2.15µM, 21.5µM, or 215.5µM PFNA, respectively, immunostained for PLZF and analyzed with an Arrayscan. There were significantly fewer colonies in the highest dose group compared to control; per colony, there was also significantly less colony area with PLZF and less measured fluorescence of PLZF for the highest dose of PFNA. The average intensity, independent of area, for PLZF was significantly lower for the highest dose compared to control, with the first two doses showing similar intensity. These results suggest that there were fewer colonies at the highest dose of PFNA exposure and, in those colonies, there was less PLZF measured for the highest dose of PFNA. Data were tested for statistical significance using SPSS by 1-way ANOVA with Tukey's post-hoc test ($p<0.05$).

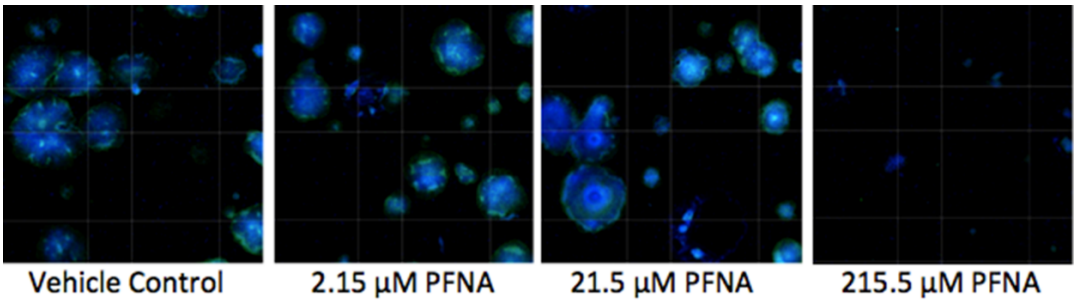


**Figure 16.** Images of immunofluorescent cells following PFNA exposure, taken with the ArrayScan.

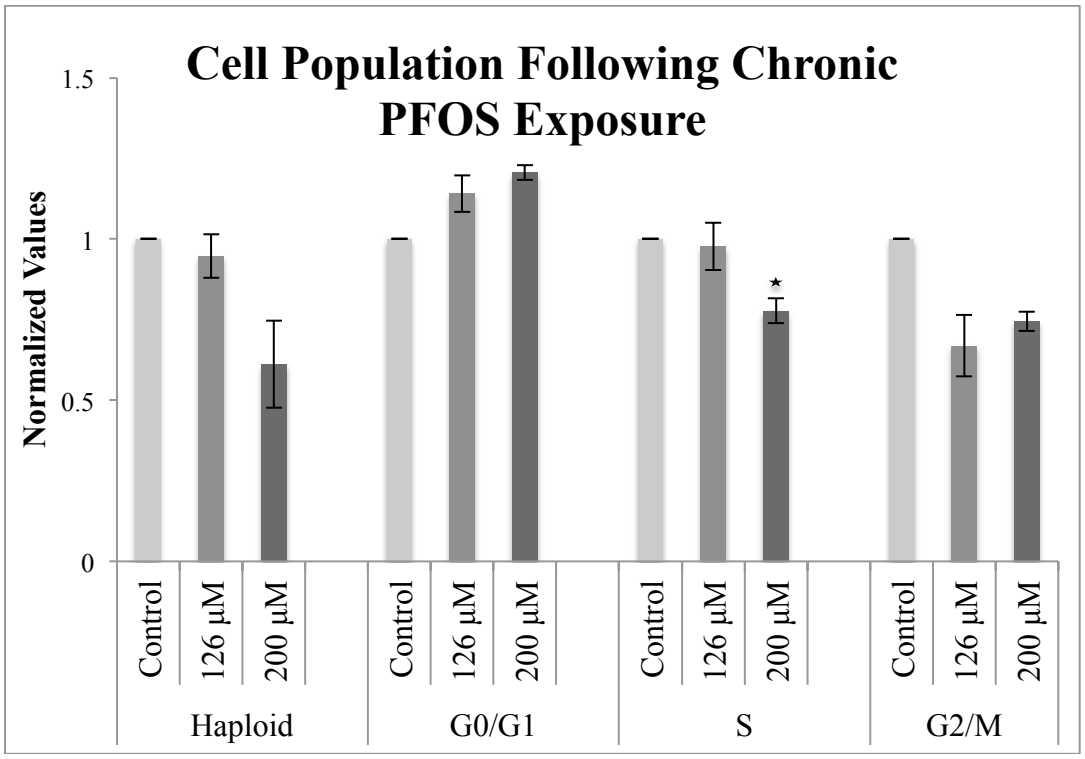


**Figure 17.** Histogram depicting the distribution of cell cycle populations from approximately 5000 cells following chronic PFOS exposure using the Muse Cell Cycle Assay and flow cytometer. The experiment was conducted in triplicate and data from dosed cells was normalized to the vehicle control. PFOS exposure appears to decrease haploid cell population and approaches significance ($p=0.055$) and 200µM PFOS had significantly decreased cells in the S phase ($p<0.05$).

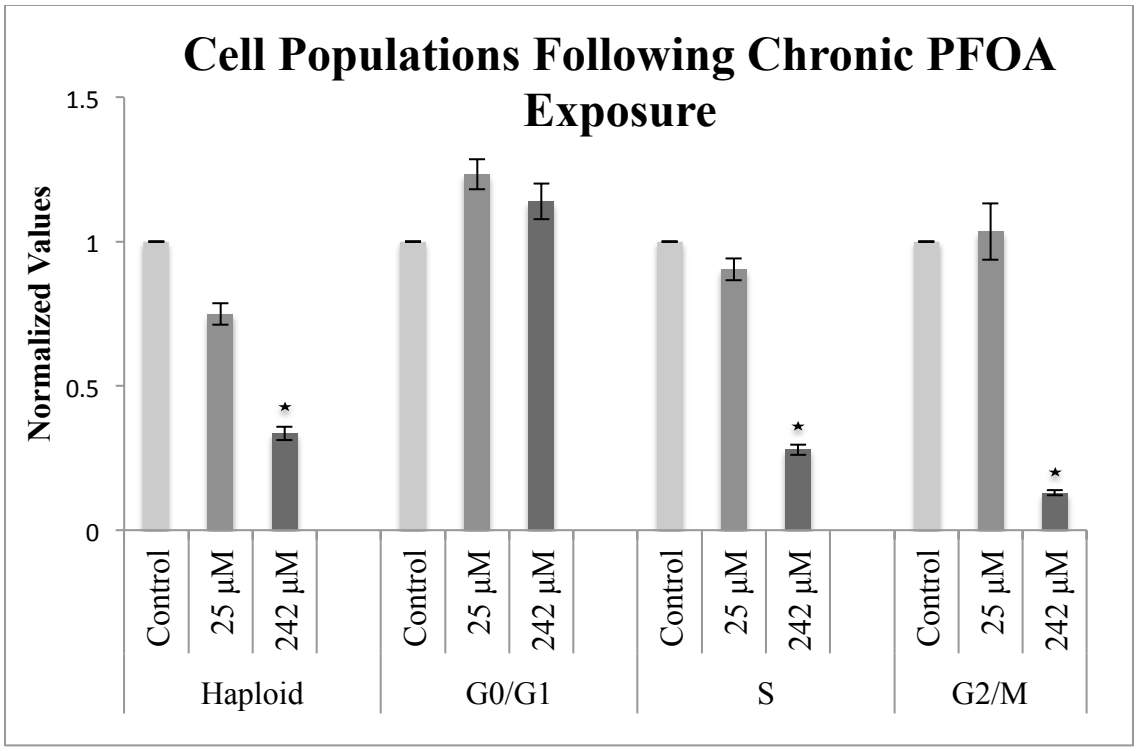


**Figure 18.** Histogram depicting the distribution of cell cycle populations from approximately 5000 cells following chronic PFOA exposure using the Muse Cell Cycle Assay and flow cytometer. The experiment was conducted in triplicate and data from dosed cells was normalized to the vehicle control. 242µM PFOA exposure significantly decreased the haploid cell population and the amount of cells in the S phase and G2/M phase ($p<0.05$, two-tailed)

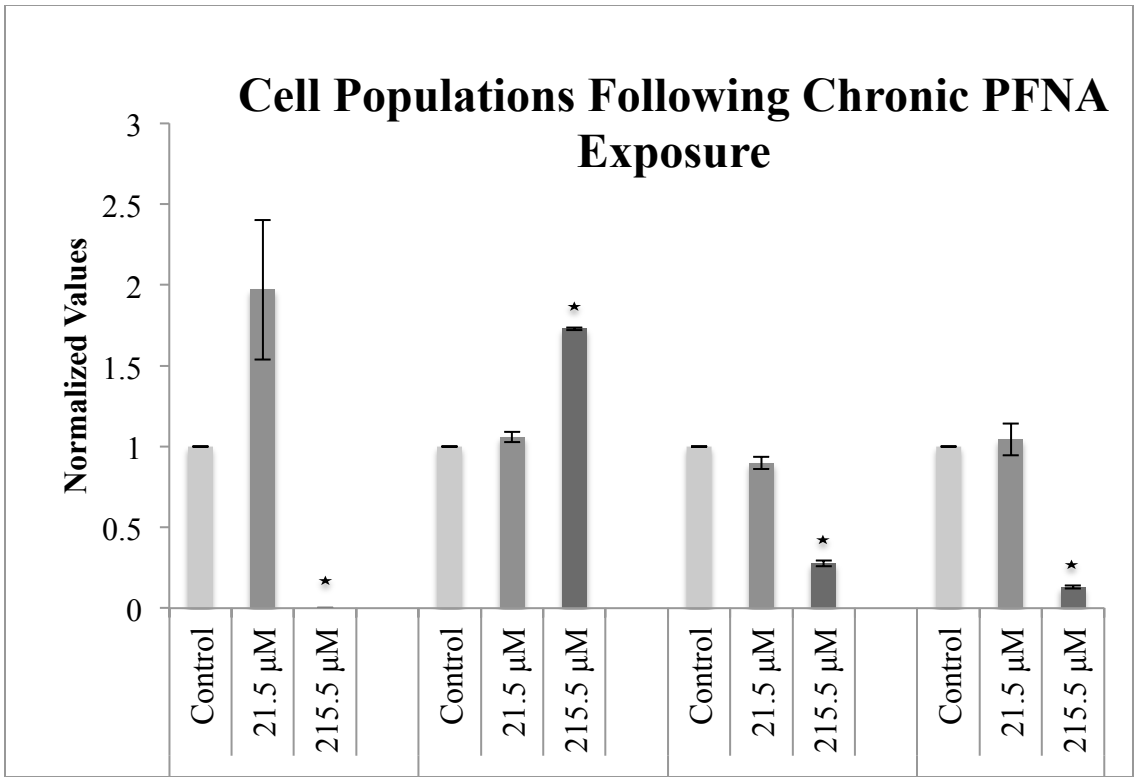


**Figure 19.** Histogram depicting the distribution of cell cycle populations from approximately 5000 cells following chronic PFNA exposure using the Muse Cell Cycle Assay and flow cytometer. The experiment was conducted in triplicate and data from dosed cells was normalized to the vehicle control. 215.5µM PFNA exposure significantly decreased the haploid cell population, as no haploid cells were found ($p<0.05$, two-tailed). 215.5µM PFNA exposure also decreased the amount of cells in the S phase and G2/M phase, and increased the amount of cells in the G0/G1 phase ($p<0.05$, two-tailed).

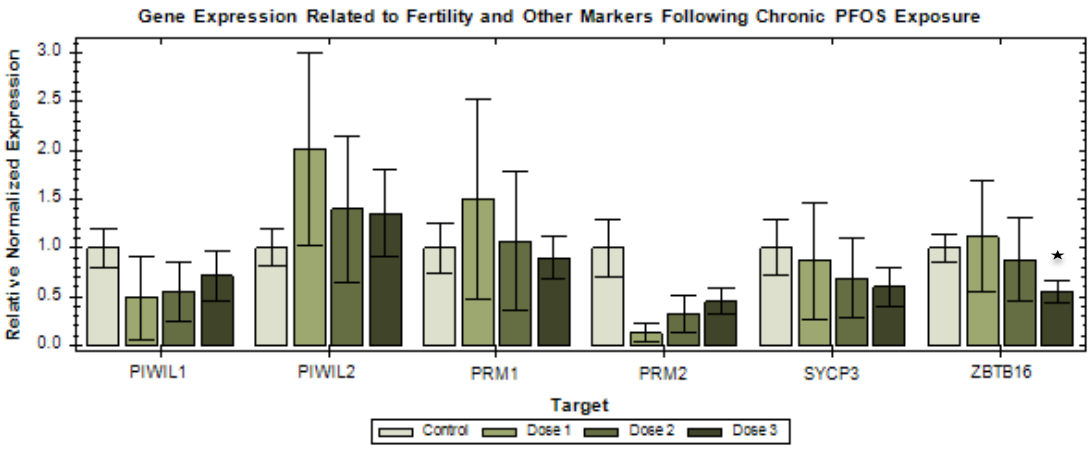


**Figure 20.** qPCR results for genes related to spermatogenesis following chronic exposure to 48µM, 126µM, or 200µM PFOS, respectively; the vehicle control is DMSO. The experiment was conducted in triplicate and data from dose cells was normalized to the control. Expression of ZBTB16 for dose 3 is significantly lower compared to control ($p<0.05$). Trends of gene expression suggest that PFOS affects meiosis.

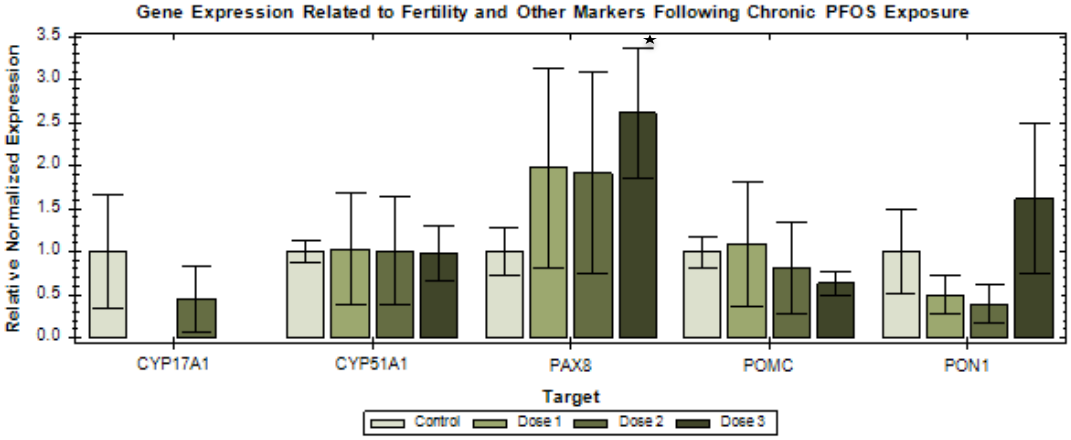


**Figure 21.** qPCR results for genes related to fertility following chronic exposure to 48µM, 126µM, or 200µM PFOS, respectively; the vehicle control is DMSO. The experiment was conducted in triplicate and data from dose cells was normalized to the control. Expression of PAX8 for dose 3 is significantly higher compared to control ($p<0.05$). Expression of POMC approaches significance ($p=0.07$). These findings suggest that PFOS exposure may impair fertility.

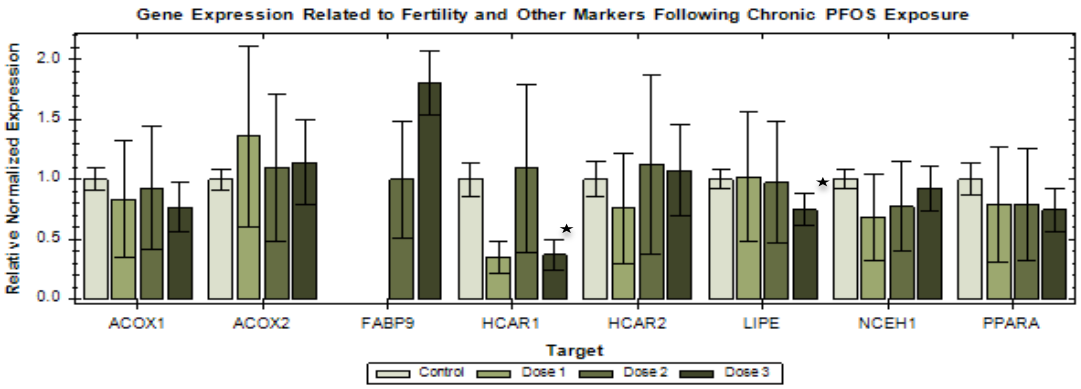


**Figure 22.** qPCR results for genes related to lipid metabolism following chronic exposure to 48µM, 126µM, or 200µM PFOS, respectively; the vehicle control is DMSO. The experiment was conducted in triplicate and data from dose cells was normalized to the control. Expression of HCAR1 for dose 1 and 3 is significantly lower compared to control ($p<0.05$). Expression of LIPE is significantly lower for dose 3 compared to control ($p<0.05$). These findings suggest that PFOS causes inflammation and disrupts cholesterol conversion. Because the absence of LIPE interferes with the retinoic acid pulse that occurs during spermatogenesis, a decrease in LIPE may impair fertility.