Perfluoroalkyl and Polyfluoroalkyl Substances and Women's Health:
A Possible Etiology for Earlier Menopause and Accelerated Reproductive Aging

by

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Dedication

This work is wholeheartedly dedicated to my mother and father, Hong Wang and Zhili Ding, who are always positive role models in my life. They have not lived in the easiest of lives. And yet in the face of challenges and difficulties along the way, they have always pushed through and did what they had to do to make things work. They have taught me the importance of hard work, honesty and integrity, and always being humble in every situation, and never giving up.

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Abstract

Perfluoroalkyl and polyfluoroalkyl substances (PFAS) are ubiquitous environmental toxicants used in consumer products and industry, such as non-stick cookware, food packaging materials, personal care products, and aqueous fire-fighting foams. PFAS have received unprecedented attention recently due to nationwide drinking water contamination that impacts up to 110 million residents in the United States. Due to the widespread use and chemical stability of these compounds, more than 98% of the general population in the United States likely has at least one PFAS detectable in their blood. Growing evidence suggests that ovaries may be a potential target for PFAS toxicity. The ovary is a primary regulator of reproductive and endocrine function in females. Menopause marks the cessation of ovarian function, and its timing has physiologic impacts beyond the reproductive system. As age at the final menstrual period reflects a woman's overall health, investigation of the role of potential endocrine-disrupting chemicals in ovarian aging is warranted.

The overall goal of my dissertation was to explore the impact of PFAS on ovarian aging and timing of natural menopause. The following specific aims were tested using a community-based, longitudinal cohort of midlife women, the Study of Women's Health Across the Nation (SWAN).

Aim 1 examined temporal variations in serum concentrations of PFAS and determined assessed whether the time trends differed by race/ethnicity, menstruation status and parity to better understand longitudinal changes in PFAS across the menopausal transition. We observed

longitudinal declines in serum concentrations of legacy PFAS including perfluorooctanoic acid (PFOA), perfluorooctane sulfonic acid (PFOS) and their precursors but increases in other PFAS compounds such as perfluorononanoic acid (PFNA) from 1999 to 2011. Menstruating women had consistently lower concentrations than non-menstruating women. Temporal trends in PFAS concentrations were not uniform across race/ethnicity and parity groups.

Previous epidemiologic studies of PFAS and menopausal timing conducted in cross-sectional settings were limited by reverse causation bias because PFAS serum concentrations increase after cessation of menstrual bleeding. Aim 2 examined the associations between PFAS exposure and incident natural menopause among 1120 midlife women aged 45-56 years at baseline from 1999 to 2017. Higher serum concentrations of PFOA and PFOS were associated with earlier onset of natural menopause, a risk factor for adverse health outcomes in later life. Women were classified into four clusters based on their overall PFAS concentrations as mixtures: low, low-medium, medium-high, and high. Compared to the low cluster, the high cluster had a HR of 1.66 (95% CI: 1.17-2.36), which is interpreted as 1.8 years earlier experience of natural menopause which is equivalent to the impact of smoking tobacco.

Aim 3 explored the mediating role of follicle-stimulating hormone (FSH) in the associations between PFAS exposure and incident natural menopause to understand potential underlying mechanisms. The proportion of the effect mediated through FSH was 26.9% (95% CI: 15.6%, 38.4%) for linear PFOA and 13.2% (95% CI: 0.0%, 24.5%) for branched PFOS. The effect of PFAS on natural menopause may be partially explained by their association with variations in FSH concentrations.

Overall this dissertation provides evidence that PFAS exposure may accelerate ovarian

aging, possibly through endocrinologic mechanisms associated with changing serum concentrations of FSH. Future studies that can confirm these findings and steps to limit exposure to these chemicals appear warranted.

Chapter I. Perfluoroalkyl and Polyfluoroalkyl Substances (PFAS) and Their Effects on the Ovary

This chapter was invited and is currently under review by *Human Reproduction Update*.

Abstract

Background: Perfluoroalkyl and polyfluoroalkyl substances (PFAS) are found widespread in drinking water, foods, food packaging materials, and other consumer products. Several PFAS have been identified as endocrine-disrupting chemicals based on their ability to interfere with normal reproductive function and hormonal signaling. Experimental models and epidemiologic studies suggest that PFAS exposures target the ovary and represent major risks for women's health.

Rationale/Objectives: This review summarizes human population and toxicological studies on the association between PFAS exposure and ovarian function.

Search methods: A comprehensive review was performed by searching PubMed. Search terms included an extensive list of PFAS and health terms ranging from general keywords (e.g., ovarian, reproductive, follicle, oocyte) to specific keywords (including menarche, menstrual cycle, menopause, primary ovarian insufficiency/premature ovarian failure, steroid hormones), based on the authors' knowledge of topic and key terms.

Outcomes: Clinical evidence demonstrates the presence of PFAS in follicular fluid and their ability to pass through the blood-follicle barrier. Although some studies found no evidence associating PFAS exposure with disruption in ovarian function, numerous epidemiologic studies have identified associations of higher PFAS exposure with later menarche, irregular menstrual cycles, longer cycle length, earlier age of menopause, and reduced levels of estrogens and androgens, mostly in cross-sectional study designs. Adverse effects of PFAS on ovarian folliculogenesis and steroidogenesis have been confirmed in experimental models. Based on laboratory research findings, PFAS could diminish ovarian reserve and reduce endogenous

hormone synthesis through activating peroxisome proliferator-activated receptors, disrupt gap junction intercellular communication between oocyte and granulosa cells, induce thyroid hormone deficiency, antagonize ovarian enzyme activities involved in ovarian steroidogenesis, or inhibit kisspeptin signaling in the hypothalamus.

Wider implications: The published literature supports associations between PFAS exposure and adverse reproductive outcomes; however, the evidence remains insufficient to infer a causal relationship between PFAS exposure and ovarian disorders. Thus, more research is warranted. PFAS are of significant concern because these chemicals are ubiquitous and persistent in the environment and in humans. Moreover, susceptible groups, such as fetuses and pregnant women, may be exposed to harmful combinations of chemicals that include PFAS. However, the role environmental exposures play in reproductive disorders has received little attention by the medical community. To better understand the potential risk of PFAS on human ovarian function, additional experimental studies using PFAS doses equivalent to the exposure levels found in the general human population and mixtures of compounds are required. Prospective investigations in human populations are also warranted to ensure temporality of PFAS exposure and health endpoints and to minimize the possibility of reverse causality.

Key words: Perfluoroalkyl and polyfluoroalkyl substances (PFAS), endocrine-disrupting chemicals (EDCs), ovary, folliculogenesis, steroidogenesis

1. Introduction

According to the definition adopted by the Endocrine Society Scientific Statement, an endocrine-disrupting chemical (EDC) is "a compound, either natural or synthetic, which, through environmental or inappropriate developmental exposures, alters the hormonal and homeostatic systems that enable the organism to communicate with and respond to its environment" (Diamanti-Kandarakis *et al.*, 2009). A variety of EDCs are used in industrial and consumer applications, such as solvents and lubricants (e.g., polychlorinated biphenyls), flame retardants (e.g., polybrominated diethyl ethers), pesticides (e.g., dichlorodiphenyltrichloroethane and chlorpyrifos), and plasticizers (e.g., phthalates and bisphenol-A) (Burger *et al.*, 2007; Caserta *et al.*, 2011). Among them, perfluoroalkyl and polyfluoroalkyl substances (PFAS) have received unprecedented attention recently due to nationwide drinking water contamination and widespread use that impacts up to 110 million residents in the United States (Environmental Working Group, 2018).

PFAS comprise a large family of man-made fluorinated chemicals that are ubiquitous environmental toxicants to which humans are exposed on a daily basis (Trudel *et al.*, 2008). At least one type of PFAS chemical was detected in the blood of nearly every person sampled in the U.S. National Biomonitoring Program (Centers for Disease Control, 2019). Many consumer products contain specific members of this family of chemicals, such as non-stick cookware (Teflon) (Bradley *et al.*, 2007; Ewan Sinclair *et al.*, 2007); food packaging materials (Begley *et al.*, 2005; Schaider *et al.*, 2017; Trier *et al.*, 2011); stain- and water-resistant coating for clothing, furniture, and carpets (Scotchgard and Gore-Tex) (Hill *et al.*, 2017; Lee *et al.*, 2017); and cosmetics and personal care products (Boronow *et al.*, 2019; Danish EPA, 2018). PFAS are also present in fire-fighting foams (or aqueous film-forming foam, AFFF) widely used in military

bases for crash and fire training (Butenhoff *et al.*, 2006; Kantiani *et al.*, 2010; Kissa, 2011; Trudel *et al.*, 2008).

Because PFAS are remarkably widespread in drinking water and groundwater in the United States and globally, especially on and near industrial sites, fire-fighting facilities and military installations, they pose a serious and immediate threat to the communities where the source of drinking water have been contaminated with PFAS. Although government and regulatory bodies have been working towards regulations that limit the production of perfluorooctane sulfonic acid (PFOS) and perfluorooctanoic acid (PFOA), two primary PFAS compounds that have been the most extensively manufactured (USEPA, 2016a, 2016b), the phase out and ban of PFOA and PFOS has led to an increased usage of alternative PFAS chemicals (Ateia *et al.*, 2019). Consequently, there is an urgent need to raise the awareness of the potential threat of PFAS to human health.

PFAS have been identified as contaminants of concern for reproductive toxicity (Jensen and Leffers, 2008). Observational studies have shown that PFAS exposure could delay menarche (Lopez-Espinosa *et al.*, 2011), disrupt menstrual cycle regularity (Zhou *et al.*, 2017), cause early menopause (Taylor *et al.*, 2014) and premature ovarian insufficiency (Zhang *et al.*, 2018), and alter the levels of circulating sex steroid hormones (Barrett *et al.*, 2015). The ovary is the site of folliculogenesis and is responsible for the proper maturation of oocytes. It is also the principle site of sex hormone steroidogenesis. Experimental studies have shown that PFAS exposure was associated with the depletion of ovarian reserve (i.e. the number of ovarian follicles and oocytes) (Bellingham *et al.*, 2009; Chen *et al.*, 2017; Domínguez *et al.*, 2016; Du *et al.*, 2019; Feng *et al.*, 2015, 2017; Hallberg *et al.*, 2019; López-Arellano *et al.*, 2019), and inhibition steroidogenic enzyme activities (Chaparro-Ortega *et al.*, 2018; Shi *et al.*, 2009; Wang, Bai, *et al.*, 2018).

Disruption of this finely controlled network may have physiologic impacts beyond the reproductive system, affecting the overall health of girls and women.

Growing evidence has suggested that ovaries may be a potential target for PFAS toxicity: however, a comprehensive review of experimental and human studies for the effects of PFAS on normal ovarian function has not previously been reported. In this review, we summarize the sources and pathways of PFAS, describe the processes of ovarian folliculogenesis and steroidogenesis, review the state of the science regarding associations between PFAS exposures and ovarian function in experimental and epidemiological studies, identify gaps in the current data and outline directions for future research.

2. Methods

A thorough search was carried out for relevant articles in order to ensure a comprehensive review on PFAS exposure and ovarian function. We searched PubMed (https://www.ncbi.nlm.nih.gov/pubmed) through August 2019. The following search terms were used, including PFAS search terms: perfluoroalkyl, polyfluoroalkyl, perfluorinated, fluorocarbons, perfluorobutanoic acid, perfluoropentanoic acid, perfluorohexanoic acid, perfluoroheptanoic acid, perfluorooctanoic acid, perfluorononanoic acid, perfluorodecanoic acid, perfluoroundecanoic acid, perfluorododecanoic acid, perfluorobutane sulfonic acid, perfluoroheptane sulfonic acid, perfluorooctane sulfonic acid, perfluorooctane sulfonamide, PFBA, PFPeA, PFHxA, PFHpA, PFOA, PFDeA, PFUnA, PFDoA, PFBS, PFHpS, PFOS, PFOSA; and outcome search terms: ovary, follicle, oocyte, menarche, menstrual cycle, menopause, primary ovarian insufficiency, premature ovarian failure, steroid hormones, polycystic ovarian syndrome, and ovarian cancer. In addition, we manually reviewed the reference lists of identified articles.

3. Basic principles of PFAS

3.1 Nomenclature

The term PFAS refers to perfluoroalkyl and polyfluoroalkyl substances, a large group of man-made chemicals with the distinguishing structure of a chain of carbon atoms (forming an "alkyl") that has at least one fluorine atom bound to a carbon. Details of PFAS terminology, classification and origins can be found elsewhere (Buck *et al.*, 2011; Interstate Technology Regulatory Council, 2017). Note that use of non-specific acronyms, such as perfluorinated compound (PFC), should be avoided in the scientific publications as it has hampered clear communications in researchers, practitioners, policy makers and the public.

Perfluoroalkyl substances are fully fluorinated molecules in which every hydrogen atom bonded to a carbon in the alkane backbone (carbon-chain) is replaced by a fluorine atom, except for the carbon at one end of the chain that has a charged functional group attached. The carbon-fluorine bond is extremely strong and renders these chemicals highly resistant to complete degradation. The basic chemical structure of perfluoroalkyl substances can be written as $C_nF_{2n+1} - R$, where "C_nF_{2n+1}" defines the length of the perfluoroalkyl chain tail with n>2, and "R" represents the attached functional group head (as shown in **Figure 1**). PFOA and PFOS (so-called C8 compounds) have been the most extensively produced and studied PFAS compounds. Perfluoroalkyl acids (PFAAs) are some of the most basic PFAS molecules and are essentially non-degradable. PFAAs contain three major groups on the basis of the functional group at the end of the carbon chain: perfluoroalkyl carboxylic acids (PFCAs), perfluoroalkane sulfonic acids (PFSAs), and perfluoroalkyl phosphonates (PFPAs) or perfluoroalkyl phosphinates (PFPiAs).

Polyfluoroalkyl substances differ from perfluoroalkyl substances by the degree of

fluorine substitution in the alkane backbone: at least one carbon must not be bound to a fluorine atom and at least two carbons must be fully fluorinated. The fluorotelomer substances are a subset of polyfluoroalkyl substances because they are oligomers with low molecular weight produced by a telomerization reaction. Some important examples fluorotelomer substances are fluorotelomer alcohol (FTOH) and perfluorooctane sulfonamidoethanol (FOSE). Since polyfluoroalkyl substances have a carbon that is lacking fluorine substitution, this weaker bond increases potential for degradation (Buck et al., 2011). For example, FTOH and FOSE can be transformed biologically or abiotically to PFOA and PFOS.

In addition to the descriptions above, PFAS can also exist as polymers. These PFAS polymers are large molecules formed by joining many identical small PFAS monomers. Current information indicates that the non-polymer PFAS constitute the greatest risk for environmental contamination and toxicity, although some PFAS polymers can be degradable to basic PFAS.

3.2 Sources of human exposure

Previous studies have evaluated daily exposure in populations around the world (Ericson *et al.*, 2008; Fromme *et al.*, 2007; Haug *et al.*, 2010; Heo *et al.*, 2014; Ostertag *et al.*, 2009; Renzi *et al.*, 2013; Tittlemier *et al.*, 2007; Trudel *et al.*, 2008; Zhang *et al.*, 2010). Although it is difficult to compare concentrations among populations because of differences in participant characteristics (e.g. age, sex, and geographical locations), the ranges of PFAS serum concentrations are remarkably similar worldwide. Exposure to PFAS in the general population is at lower levels compared to those affected by occupational exposure or local contaminations (ATSDR, 2018). Multiple sources of potential exposure to PFAS have been previously identified in the general population. These sources include diet (Domingo and Nadal, 2017; Haug *et al.*, 2011; Tittlemier *et al.*, 2007; Trudel *et al.*, 2008; Vestergren and Cousins, 2009), drinking water

(Domingo and Nadal, 2019; Hu *et al.*, 2016; Post *et al.*, 2009; Thompson *et al.*, 2011), air and dust (Fromme *et al.*, 2015; Goosey and Harrad, 2012; Haug, *et al.*, 2011; Karásková *et al.*, 2016; Piekarz *et al.*, 2007), and consumer products (Begley *et al.*, 2005; Boronow *et al.*, 2019; Bradley *et al.*, 2007; Ewan Sinclair *et al.*, 2007; Hill *et al.*, 2017; Lee *et al.*, 2017; Schaider *et al.*, 2017; Trier *et al.*, 2011). The widespread production of PFAS, their use in common commercial and household products, their improper disposal, and their resistance to degradation has led to daily human exposures via oral ingestion, inhalation, and dermal contact. Different sources and pathways of human exposure are summarized in **Table 1**.

The highest exposures to PFAS are often from dietary intake, particularly to PFOS and PFOA (Domingo and Nadal, 2017; Haug, Huber, Becher, *et al.*, 2011; Tittlemier *et al.*, 2007; Trudel *et al.*, 2008; Vestergren and Cousins, 2009). Fish and shellfish generally exhibit the highest PFAS concentrations and detection rates among all types of foodstuffs (Domingo and Nadal, 2017; Jian *et al.*, 2017). Other potential dietary sources of PFAS include dairy products, eggs, beverages and vegetables (Chen *et al.*, 2018; Domingo *et al.*, 2012; Eriksson *et al.*, 2013; Felizeter *et al.*, 2014; Gebbink *et al.*, 2015; Haug *et al.*, 2010; Heo *et al.*, 2014; Herzke *et al.*, 2013; Noorlander *et al.*, 2011; Vestergren *et al.*, 2012; Zhang *et al.*, 2010). However, these foodstuffs have generally low concentrations and low detection frequencies compared to fish and shellfish (Jian *et al.*, 2017). In addition, food can become contaminated with PFAS through transfer from food packaging and/or processing (Schaider *et al.*, 2017) because PFAS are used as in grease- and water-repellent coatings for food-contact materials and non-stick cookware (Begley *et al.*, 2005).

Drinking water is also a common source of PFAS in humans (Domingo and Nadal, 2019). A number of studies have detected PFAS in drinking water samples collected from

various countries (Boone *et al.*, 2019; Jin *et al.*, 2009; Mak *et al.*, 2009; Quinete *et al.*, 2009; Quiñones and Snyder, 2009; Takagi *et al.*, 2008; Thompson *et al.*, 2011; Wilhelm *et al.*, 2010). Recently, Boone et al. measured concentrations in source (untreated) and treated drinking water sampled from 24 states across the United States (Boone *et al.*, 2019): Seventeen PFAS analytes were detected in all samples and summed concentrations ranged from <1-1102 ng/L, with one drinking water treatment plant (DWTP) exceeding the health advisory of 70 ng/L for PFOA and PFOS set by the United States Environmental Protection Agency (U.S. EPA).

Some PFAS polymers such as FTOHs were frequently used for impregnation treatment of furniture and floor coverings, and as intermediates in manufacturing various household products (e.g. paints, carpet, and cleaning agents). These neutral PFAS, mainly FTOHs, FOSA, and FOSEs, are volatile compounds that are easily released into indoor environments (air and dust) due to their low water solubility and high vapor pressure (Haug, *et al.*, 2011; Langer *et al.*, 2010; Yao *et al.*, 2018). Perfluoroalkyls have also been detected in indoor air and dust (Barber *et al.*, 2007; Kubwabo *et al.*, 2005; Strynar and Lindstrom, 2008). In the study of 67 houses in Canada, carpeted home had higher concentrations of PFOA, PFOS and PFHxS in dust, possibly due to the use of stain-repellent coatings (Kubwabo *et al.*, 2005). The use of aqueous firefighting foams at military installations and the production of fluorochemicals at industrial facilities have resulted in widespread contamination in soil and sediment (Anderson *et al.*, 2016; Xiao *et al.*, 2015). Many consumer products, such as ski waxes, leather samples, outdoor textiles, and cosmetics products including hair spray and eyeliner, also contain PFAS (Danish EPA, 2018; Kotthoff *et al.*, 2015).

Previous literature has estimated the relative contributions of different exposures routes of PFOA and PFOS in adults (Gebbink *et al.*, 2015; Haug, *et al.*, 2011; Lorber and Egeghy,

2011; Trudel *et al.*, 2008; Vestergren and Cousins, 2009). Oral ingestion from diet and drinking water has been proposed as the largest source of exposure to PFOA and PFOS (around 90%) compared with inhalation or dermal contact (Gebbink *et al.*, 2015; Haug, *et al.*, 2011; Lorber and Egeghy, 2011; Vestergren and Cousins, 2009). For PFOA, Trudel et al. reported ingestion of food from PFOA-containing packaging materials (56%), inhalation of indoor air and dust (14%), and hand-to-mouth transfer of house dust (11%), as significant pathways (Trudel *et al.*, 2008). Other pathways proposed to be less important included ingestion of food prepared with PTFE-coated cookware, dermal contact from clothes and other consumer products (Trudel *et al.*, 2008).

3.3 Transport and clearance of PFAS in the human body

Whereas most persistent organic pollutants such as polychlorinated biphenyls (PCBs) and brominated flame retardants (BFRs) are lipophilic, the substitution of carbon-hydrogen bonds for the strongest carbon-fluorine counterparts coupled with a charged functional group confers unique dual hydrophobic and lipophobic surfactant characteristics to PFAS molecules (Banks and Tatlow, 1994; Kissa, 2011). Most of the available data on transport and clearance of PFAS is based on studies with PFAAs (primarily PFOA and PFOS). In contrast to other persistent organic pollutants, PFAAs are not stored in adipose tissue but undergo extensive enterohepatic circulation. The presence of PFAAs has been confirmed primarily in liver and serum (Falk *et al.*, 2015; Pérez *et al.*, 2013).

The hydrophobic nature of fluorine-containing compounds can also lead to increased affinity for proteins (Jones *et al.*, 2003). Once consumed, PFAAs tend to partition to the tissue of highest protein density, with approximately 90% to 99% of these compounds in the blood bound to serum albumin (Han *et al.*, 2003; Ylinen and Auriola, 1990). Due to the ability of albumin to pass the blood follicle barrier (Hess *et al.*, 1998; Schweigert *et al.*, 2006), it is suggested that

PFAAs can easily be transported into growing follicles. PFAAs have been detected in human follicular fluid, and could alter *in vivo* oocyte maturation and follicle development (Heffernan *et al.*, 2018; Petro *et al.*, 2014).

The primary route of elimination of PFAAs is through the kidney in the urine (Han *et al.*, 2008). Other important clearance pathways include menstruation (Ding *et al.*, 2020; Harada *et al.*, 2005; Park *et al.*, 2019; Taylor *et al.*, 2014), pregnancy (Monroy *et al.*, 2008), and lactation (Bjermo *et al.*, 2013). Sex hormones have been identified as a major factor in determining renal clearance of PFAAs. Kudo et al. examined the role of sex hormones and transport proteins on renal clearance and concluded that, in ovariectomized female rats, estradiol could facilitate transporting of PFAAs across the membranes of kidney tubules into the glomerular filtrate, resulting in lower serum concentrations (Kudo *et al.*, 2002).

Serum concentrations of PFOA, PFOS, PFHxS and PFNA appear to be higher in males than in females across all age groups (Calafat *et al.*, 2007). Wong et al. found that approximately 30% of the PFOS elimination half-life difference between females and males was attributable to menstruation (Wong *et al.*, 2014a). The differences by sex narrows with aging, suggesting that PFAS may reaccumulate after cessation of menstrual bleeding in postmenopausal women (Dhingra *et al.*, 2017; Ruark *et al.*, 2017; Wong *et al.*, 2014b). Decreased serum concentrations have also been shown in premenopausal versus postmenopausal women and, analogously, in men undergoing venesections for medical treatment (Lorber *et al.*, 2015).

PFAAs are considered metabolically inert and remain in the human body for many years. Estimation of human elimination half-life (or population halving time) for PFOA, PFOS, PFHxS and PFNA have been reported in previous studies (Bartell *et al.*, 2010; Brede *et al.*, 2010; Ding

et al., 2020; Eriksson *et al.*, 2017; Glynn *et al.*, 2012; Li *et al.*, 2018; Olsen *et al.*, 2007, 2012; Spliethoff *et al.*, 2008; Wong *et al.*, 2014a; Worley *et al.*, 2017; Yeung *et al.*, 2013b, 2013a; Zhang *et al.*, 2013). Comparing the estimated half-lives of PFAS among populations is difficult as they differ by sampling time intervals, duration of exposure, sex and age of study subjects. Despite these challenges, most of the aforementioned studies reported that the half-life in humans of PFOA is around 2-3 years, and of PFOS is approximately 4-5 years.

4. Mechanistic evidence for ovarian toxicity of PFAS

4.1 Effects of PFAS on folliculogenesis

The ovary is the female gonad and an important endocrine organ. The ovaries consist of a surface epithelium surrounding the ovary, a dense underlying connective tissue (tunica albuginea), an outer cortex, and an inner medulla. The cortex appears dense and granular due to the presence of ovarian follicles, corpora lutea and stroma. The medulla is highly vascular with abundant blood vessels, lymphatic vessels, and nerves. The main functions of the ovary include production, maturation and release of the female gamete (oocyte), and synthesis of female sex steroid and peptide hormones that regulate reproductive and non-reproductive function. Environmental exposures can exhaust the oocyte pool and cause depletion of follicular cells, leading to earlier age at menopause, premature ovarian failure and infertility (Vabre *et al.*, 2017). The processes of oogenesis and follicle development, and the effects of PFAS exposure on folliculogenesis, are summarized in **Figure 2**.

4.1.1 Effects of PFAS on oogenesis

PFAS exposure has been shown to disrupt the earliest stage of folliculogenesis by altering oocyte development (Domínguez *et al.*, 2016; Hallberg *et al.*, 2019; López-Arellano *et*

al., 2019). The potential mechanisms include activation of peroxisome proliferator-activated receptor (PPAR) signaling pathways, disruption of intercellular communication between oocytes and granulosa cells, and induction of oxidative stress.

PPARs are family of nuclear hormone receptors that have been identified as key players in the mode of action for PFAS-induced reproductive toxicity (Desvergne and Wahli, 1999). All three known PPAR family members – α, β/δ and γ – are expressed in the ovary (Dauça *et al.*, 2014). The PPAR-α and PPAR-β/δ isoforms are expressed primarily in thecal and stromal cells in the ovary, while the PPAR-γ isoform is detected strongly in granulosa cells and the corpus lutea (Komar *et al.*, 2001). The ability of PFAS to interact with nuclear PPARs has been put forward as an explanation for metabolic disturbances associated with PFAS exposure, mainly through PPAR-α. In addition, PPAR-γ has been found to inhibit the expression of genes involved in the meiosis of oocytes (e.g. endothelin-1 and nitric oxide synthase) (Komar, 2005), implicating a role in female gamete development.

A recent study reported that administration of 10 μg/mL PFNA on bovine oocytes *in vitro* for 22 hours has a negative effect on oocyte developmental competence during their maturation (Hallberg *et al.*, 2019). This decrease in oocyte survival was attributed to PPAR-α (Hallberg *et al.*, 2019), leading to the disturbance of lipid metabolism and increased lipid accumulation in the ovaries (Bjork and Wallace, 2009; Wan *et al.*, 2012). Lending further support, another study showed that excessive lipids in the ooplasm correlated with impaired oocyte developmental competence and low oocyte survival rates (Prates *et al.*, 2014). Because PFAS can bind and activate PPARs and play an important role in PPAR signaling during ovarian follicle maturation and ovulation, it is plausible that persistent activation of ovarian PPARs through PFAS exposure could disrupt the ovarian cell function and oocyte maturation.

In addition to the impact on PPAR signaling, PFAS exposure could alter cell-cell communication within a follicle. Because the interior of an ovarian follicle is avascular, cell-cell communication among granulosa cells and between granulosa cells and the oocyte is critically dependent on bidirectional transfer of low molecular weight nutrients, signaling molecules, and waste products via gap junction intercellular communication (Clark *et al.*, 2018). When treated with an aqueous solution with 0, 12.5, 25 and 59 μM PFOS *in vitro* for a 44-hour maturation period, the number of live oocytes and the percentage of matured oocytes decreased in porcine ovaries (Domínguez *et al.*, 2016). Similarly, fetal murine oocytes exposed to 28.2 and 112.8 μM PFOA *in vitro* for 7 days exhibited increased apoptosis and necrosis (López-Arellano *et al.*, 2019). These effects are attributed due to inhibition of gap junction intercellular communication between oocytes and granulosa cells (Domínguez *et al.*, 2016; López-Arellano *et al.*, 2019).

PFAS may also induce oxidative stress with increased generation of reactive oxygen species (ROS) production, increased DNA damage, and decreased total antioxidant capacity (Wielsøe *et al.*, 2015). Pregnant mice administered 10 mg PFOA/kg/day from gestational days 1-7 or 1-13 exhibited inhibited superoxide dismutase and catalase activity, increased generation of ROS, and increased expression of p53 and Bax proteins (important in apoptotic cell death) in the maternal ovaries (Chen *et al.*, 2017; Feng *et al.*, 2015). Similarly, another study reported significantly increased ROS production in rats exposed to PFOA, which interfered with the activities of complexes I, II, III in the mitochondrial respiratory chain and led to oocyte apoptosis (López-Arellano *et al.*, 2019; Mashayekhi *et al.*, 2015).

4.1.2 EFFECTS OF PFAS ON FOLLICLE DEVELOPMENT

Studies in laboratory rodents indicate that PFAS alters the formation and/or function of ovarian follicular cells at several stages of development. Adult female mice exposed to 0.1 mg PFOS/kg/day by gavage for 4 months had a decreased number of preovulatory follicles and increased number of atretic follicles (Chen *et al.*, 2017; Feng *et al.*, 2015). Moreover, the PFOS-exposed mice had depressed serum levels of estradiol and progesterone. Notably, PFOS reduced the mRNA expression of steroidogenic acute regulatory protein (*Star*), which codes for the StAR protein that transports cholesterol from the outer to the inner mitochondrial membrane, a critical step in steroid hormone biosynthesis: this effect on *Star* was proposed as the cause of deficits in follicle maturation and ovulation (Feng *et al.*, 2015). Similar findings were reported for pregnant mice exposed to 2.5, 5 and 10 mg PFOA/kg/day from gestational day 1–7 or 1-13, with decreased number of corpora lutea accompanied by decreased mRNA expression of *Star* in the maternal ovaries (Chen *et al.*, 2017; Feng *et al.*, 2015).

In female rats exposed as neonates to 0.1 and 1 mg PFOA/kg/day, or 0.1 and 10 mg PFOS/kg/day, there was a significant reduction in the numbers of ovarian primordial follicles, growing follicles and corpora lutea (Du *et al.*, 2019). The ovarian effects of the prior study were accompanied by down-regulated mRNA expression of KiSS-1 metastasis-suppressor (*Kiss1*) and KISS1 receptor (*Kiss1r)*, and a decrease in kisspeptin fiber intensities in the hypothalamus. Because kisspeptin signaling has a critical role in regulation of the ovarian cycle as well as initiation of puberty (Gaytán *et al.*, 2009; Hu *et al.*, 2017), the PFOA and PFOS perturbation of follicular development may have resulted from disruption of kisspeptin signaling in the hypothalamus (Bellingham *et al.*, 2009; Du *et al.*, 2019).

Pregnant mice administered oral doses of 200 and 500 mg/kg/day of perfluorobutane sulfonate (PFBS) on days 1-20 of gestation gave birth to female offspring that exhibited numerous symptoms of disrupted ovarian function: depressed ovarian size and weight, depressed size and weight, decreased number of ovarian follicles (all stages), delayed vaginal opening, delayed onset of estrus, prolonged diestrus, and reduced serum levels of estradiol (Feng *et al.*, 2017). In addition, the PFBS exposure disrupted thyroid hormone synthesis consistent with hypothyroxinemia, as indicated by depressed serum levels of the thyroid hormones triiodothyronine (T3) and thyroxine (T4) in the dams on gestation day 20 as well as in the female offspring (Feng *et al.*, 2017). Mounting evidence from animal (Chang *et al.*, 2008; Lau *et al.*, 2003; Thibodeaux *et al.*, 2003) and human studies (Dallaire *et al.*, 2009; Wang *et al.*, 2014) suggests that levels of thyroid hormones decrease with increased PFAS concentrations. Thyroid hormones play a critical role in ovarian follicular development and maturation as well as in the maintenance of other physiological functions (Fedail *et al.*, 2014; Wakim *et al.*, 1994). It is possible that thyroid hormone insufficiency could affect follicle development via an influence on the production of follicular fluid, inhibin, estrogens, and other cytokines (Dijkstra *et al.*, 1996; Tamura *et al.*, 1998).

4.2 Effects of PFAS on ovarian steroidogenesis

Another primary function of the ovary is ovarian steroidogenesis -- the production and secretion of sex steroid hormones. Ovarian steroidogenesis relies on a strict coordination of both theca cells and granulosa cells and the addition of hypothalamus and anterior pituitary gland (as shown in **Figure 3**) (Hillier *et al.*, 1994).

Thecal cells produce androgens (androstenedione, A4; and testosterone, T) via the enzyme aromatases. As the precursor to steroidogenesis, cholesterol can be transported to the

theca cell cytoplasm via the StAR protein. P450 cholesterol side-chain cleavage enzyme (CYP11A1) then catalyzes the conversion of cholesterol to pregnenolone. Pregnenolone is then converted to a precursor androgen, dehydroepiandrosterone (DHEA) that involves the enzyme 17α-hydroxylase-17, 20-desmolase (CYP17A1), or progesterone via 3β-hydroxysteroid dehydrogenase (3β-HSD). Progesterone and DHEA are then converted to an androgen, A4, via CYP17A1 or 3β-HSD, respectively. The final androgenic steroid produced in the theca cell is T using the enzyme 17β-hydroxysteroid dehydrogenase (17β-HSD).

A4 and T are androgen end-products of theca cell steroidogenesis and migrate across the basal lamina of the follicle to granulosa cells. In preovulatory follicles, granulosa cells proliferate and undergo differentiation to produce increasingly large amounts of 17β-estradiol (E2). Theca cells contain LH receptors (LHRs), and upon receptor binding, LH stimulates the transcription of theca-derived genes that encode the enzymes required for the conversion of cholesterol to androgens. Granulosa cells contain FSH receptors (FSHRs), and in response to FSH binding, the transcription of granulosa-derived genes that encode the enzymes necessary for the conversion of androgens to estrogens is stimulated.

Endocrine disruption may occur at the molecular and cellular level by interference with steroid hormone biosynthesis in ovaries (**Figure 3**). PFAS can modulate the endocrine system by up- or down-regulation of expression of proteins responsible for cholesterol transport and ovarian steroidogenesis. Oral exposure to PFDoA at 3 mg/kg/day from postnatal day 24 for 28 days significantly down-regulated the mRNA expression of ovarian luteinizing hormone/choriogonadotropin receptor (*Lhcgr*), *Star*, *Cyp11a1*, and *Hsd17b3*in pre-pubertal female rats, which led to a decrease in E2 production (Shi *et al.*, 2009). Chronic exposure of adult female rats to PFOS (0.1 mg/kg/day) suppresses biosynthesis of E2 possibly through

reduced mRNA expression of *Star* mediated by reduced histone acetylation (Feng *et al.*, 2015). Given that PFAS exposure does not change the substrate (cholesterol) supply in the ovaries (Rebholz *et al.*, 2016), a decrease in *Star* mRNA levels might account for a reduction in transport of cholesterol as a necessary precursor for ovarian steroidogenesis.

Another possible mechanism of action of PFAS as endocrine disruptors is through activation of PPARs. Exposure of isolated porcine ovarian cells *in vitro* to 1.2 μM PFOS or PFOA for 24 hours inhibited LH-stimulated and FSH-simulated secretion of progesterone, estradiol, and androstenedione in granulosa cells (Chaparro-Ortega *et al.*, 2018). PPAR-γ can inhibit the expression of aromatase, the enzyme for the conversion of androgens to estrogens, by disrupting the interaction of nuclear factor-kappa B (NF-κB) (Fan *et al.*, 2005). Rak-Mardyla and Karpeta showed that the activation of PPAR-γ caused lower expression and decreased enzymatic activity of CYP17 and 17β-HSD in porcine ovarian follicles (Rak-Mardyła and Karpeta, 2014), and thus decreased levels of progesterone and A4.

PFAS are also known to have weak estrogenic activity, and as with other weak estrogens, exposure to a combination of E2 and these compounds produced anti-estrogenic effects (Liu *et al.*, 2007). Studies have reported contradictory results using *in vitro* screening systems to assay for hormone activity by ER- or AR-mediated transactivation in the human breast adenocarcinoma cell lines MCF-7 and MVLN (Behr *et al.*, 2018; Kjeldsen and Bonefeld-Jørgensen, 2013; Maras *et al.*, 2006; Wang *et al.*, 2012), human adrenal carcinoma cell H295R (Behr *et al.*, 2018; Du, Hu, *et al.*, 2013; Du, Huang, *et al.*, 2013; Wang *et al.*, 2015), human placental choriocarcinoma cell JEG-3 (Gorrochategui *et al.*, 2014), rat Leydig cells (Biegel *et al.*, 1995; Zhao et al., 2010; Zhao et al., 2014) as well as in *in vivo* testing (Biegel *et al.*, 1995; Du,

Hu, *et al.*, 2013; Yao *et al.*, 2014). It remains unclear whether PFAS affect estrogen or androgen receptor signaling at concentrations relevant to human exposure.

Nonetheless, because gonadotropin (GnRH) neurons in the hypothalamus do not express ER, they are regulated by E2 and T primarily from kisspeptin neurons in the arcuate nucleus (ARC) and anteroventral periventricular nucleus (AVPV) that send projections to GnRH neurons (Roa *et al.*, 2009). E2 and T down-regulate *Kiss1* mRNA in the ARC and up-regulate its expression in the AVPV. Thus, kisspeptin neurons in the ARC may participate in the negative feedback regulation of GnRH secretion, whereas kisspeptin neurons in the AVPV contribute to generating the preovulatory GnRH surge in the female. *In vivo* evidence demonstrated that exposure of adult female mice to PFOS at 10 mg/kg/day for 2 weeks led to diestrus prolongation and ovulation reduction through suppression of AVPV-kisspeptin neurons, but not via ARC-kisspeptin neurons in the forebrain (Wang, Bai, *et al.*, 2018). PFAS may impair ovulation and reproductive capacity through suppression of the activation of ER-mediated AVPV-kisspeptin expression.

5. Epidemiologic evidence linking PFAS exposure and ovarian outcomes

Ovarian folliculogenesis and steroidogenesis are essential processes for normal reproductive health. Increasing evidence suggests that PFAS could adversely affect numerous aspects of these processes. Specifically, exposures to PFOA and PFOS have been shown to impact ovarian steroidogenesis (**Table 2**), delay onset of menarche (**Table 3**), disrupt menstrual cycle regularity (**Table 4**), accelerate ovarian aging (**Table 5**), and may affect other chronic conditions such as polycystic ovarian syndrome (PCOS) and ovarian cancer (**Table 6**). Other PFAS compounds may also have an impact on ovarian function (**Table 7**).

5.1 Sex hormones

Exposure to PFAS has been shown to disrupt ovarian steroidogenesis and steroidogenic-controlled processes. Although the literature on other PFAS chemicals is scant, epidemiologic evidence suggests that exposure to PFOS is associated with steroidogenic defects. Specifically in the C8 Health Project, PFOS exposure had a significant and negative relationship with serum E2 levels among women aged 42-65 years without a history of hormone contraceptive use (Knox *et al.*, 2011). The parent Energy Balance and Breast Cancer Aspects (EBBA-I) study sampled serum from healthy, naturally cycling women aged 25-35 years and found that, among nulliparous women but not parous women, PFOS exposure was negatively associated with serum E2 and progesterone (P) levels (Barrett *et al.*, 2015). Similarly, Zhang et al. suggested that PFOS exposure may lead to decreased serum E2 and prolactin (PRL) levels and increased FSH levels among premature ovarian insufficiency (POI) patients (Zhang *et al.*, 2018). McCoy et al. also found a negative correlation between PFOS concentrations and E2 levels among women undergoing in vitro fertilization (McCoy *et al.*, 2017). Moreover, Heffernan et al. observed a significant and negative association between PFOS exposure and free androgen index (FAI) among healthy women without PCOS (Heffernan *et al.*, 2018).

In contrast, no associations with hormone levels have been reported for PFOA exposure (Barrett et al., 2015; Heffernan et al., 2018; Knox et al., 2011; McCoy et al., 2017; Zhang et al., 2018). PFOS and PFOA were also not related to serum T levels among women 12-80 years of age from the NHANES 2011-2012 (Lewis *et al.*, 2015). In utero exposure to PFOA and PFOS had no impact on serum levels of total T, sex hormone binding globulin (SHBG), FAI, DHEA, FSH, LH, E2, or anti-Müllerian hormone (AMH) in female adult offspring (Kristensen *et al.*, 2013).

Other PFAS may also have the potential to disturb homeostasis of the endocrine system, although the evidence remains inconclusive. Heffernan et al. found a positive association between PFNA exposure and A4 in both PCOS cases and controls, and a positive association between PFHxS exposure and total T in healthy women (Heffernan *et al.*, 2018). Zhang et al. indicated that PFHxS exposure may increase FSH levels and decrease E2 levels in POI patients (Zhang *et al.*, 2018). No significant associations were observed in cross-sectional studies conducted among naturally cycling women in the EBBA-I study (Barrett *et al.*, 2015), general women in NHANES (Lewis *et al.*, 2015), or women receiving *in vitro* fertilization (IVF) (McCoy *et al.*, 2017).

Compared to adults, adolescents may be more susceptible to PFAS toxicity. Serum concentrations of PFOA, PFUnA, and PFOS were inversely associated with serum levels of SHBG, FSH, and T, respectively, in adolescents aged 12-17 years but not in young adults (Tsai *et al.*, 2015). Similarly, girls aged 6-9 years who enrolled in the C8 Health Project also had lower serum T levels with higher exposure to PFOS (Lopez-Espinosa *et al.*, 2016). Although no association was observed for PFOA and PFOS exposures with E2 or T in Chinese adolescent girls, serum T levels decreased by 1.2% (95% CI: -2.2%, -0.1%) with an 1 ng/mL increase in serum PFDoA concentrations (Zhou *et al.*, 2016).

5.2 Onset age of menarche

Delayed menarche is a common condition defined as the absence of physical signs of puberty by an age ≥2-2.5 standard deviations above the population mean age of menarche (typically 13 years in girls) (Palmert and Dunkel, 2012). Emerging evidence suggests that later menarche may be linked to negative physiological outcomes, and cardiovascular disease in adulthood (Zhu and Chan, 2017). Previous studies examining the associations between exposure

to PFAS and timing of menarche have yielded inconsistent results with some studies finding no association (Christensen *et al.*, 2011; Kristensen *et al.*, 2013; Lopez-Espinosa *et al.*, 2011). The latter study, a cross-sectional study of 2931 girls 8-18 years of age from the C8 Health Project, reported that PFOA and PFOS serum concentrations were associated with later age at menarche, specifically 130 and 138 days of delay comparing the highest quartile of concentrations vs. the lowest, respectively (Lopez-Espinosa *et al.*, 2011).

In addition, concern exists regarding *in utero* exposure to PFAS due to high vulnerability in this early-life stage. A Danish birth cohort established in 1988-1989 followed up 267 female offspring when they were ~20 years of age in 2008-2009. The study found that women with *in utero* exposure to higher concentrations of PFOA reached menarche 5.3 (95% CI: 1.3, 9.3) months later compared with the reference group of lower PFOA concentrations; while no associations were observed for PFOS (Kristensen *et al.*, 2013). In contrast, a study of 218 girls reporting early menarche (before age 11.5 years) and 230 controls (at or after age 11.5 years) born between 1991 and 1992 in the United Kingdom showed no association of earlier age at menarche with exposure to PFOSA, EtFOSAA, MeFOSAA, PFOS, PFHxS, PFOA, or PFNA (Christensen *et al.*, 2011).

5.3 Menstrual cycle characteristics

Disturbances of menstrual cycle manifest in a wide range of presentations. The key characteristics include menstrual cycle regularity, cycle length, and the amount of flow, but each of these may exhibit considerable variability. Epidemiologic data on the possible effects of PFAS on menstrual cycle regularity originate primarily from cross-sectional studies (Lyngsø *et al.*, 2014; Zhou *et al.*, 2017). Lyngsø et al. reported a statistically significant association between PFOA exposure and longer cycles (cycle length≥32 days) with an odds ratio (OR) of 1.8

(95%CI: 1.0-3.3) when comparing the highest tertile of exposure with the lowest, in 1623 fertile women enrolled in the Inuit-endocrine (INUENDO) cohort from 3 countries (Greenland, Poland and Ukraine); whereas no significant results were detected for PFOS (Lyngsø *et al.*, 2014). Moreover, a cross-sectional analysis of 950 Chinese women revealed that increased exposures to PFOA, PFOS, PFNA and PFHxS were associated with higher odds of irregular and longer menstrual cycle but lower odds of menorrhagia (Zhou *et al.*, 2017). Interestingly, women with higher concentrations of PFOA, PFNA and PFHxS were more likely to experience hypomenorrhea (Zhou *et al.*, 2017).

The relationship of PFOA and PFOS with menstrual irregularity was detected in a subset of 1240 pregnant women randomly selected from the Danish National Birth Cohort (DNBC); women had higher exposure to PFOA and PFOS tended to report having irregular period (Fei *et al.*, 2009). Lum et al. used data from 501 couples from Michigan and Texas upon their discontinuing contraception for purposes of becoming pregnant who enrolled in a prospective cohort, the Longitudinal Investigation of Fertility and the Environment (LIFE) Study (Lum *et al.*, 2017). Menstrual cycles were 3% longer among women in the second versus the lowest tertile of PFDeA serum concentrations, but 2% shorter for women in the highest versus the lowest tertile of PFOA concentrations; while no associations were observed with PFOS (Lum *et al.*, 2017). When examining the effects of prenatal exposure, a recent prospective study found no associations between maternal exposure to PFOA and PFOS and menstrual cycle length or number of ovarian follicles in their offspring (Kristensen *et al.*, 2013).

5.4 Ovarian aging

Premature ovarian insufficiency (POI) represents a gynecological disorder characterized by the absence of normal ovarian function due to depletion of the follicle pool before age 40

years with the presence of oligo/amenorrhea for at least 4 months in combination with elevated FSH levels. It should be noted that POI is the transitional stage from normal ovarian function to complete loss of ovarian function. A case-control study of 240 Chinese women found that high exposures to PFOA, PFOS and PFHxS were associated with increased risks of POI; however, no associations were observed for PFNA, PFDeA, PFUnA, PFDoA, PFHpA, and PFBS (Zhang *et al.*, 2018).

Beyond the problem of infertility in POI patients, diminished ovarian reserve and extended steroid hormone deficiency during ovarian aging have far-reaching health implications. Earlier age at natural menopause has been associated with an increased risk of overall mortality (Jacobsen *et al.*, 2003; Mondul *et al.*, 2005; Ossewaarde *et al.*, 2005), cardiovascular disease (Atsma *et al.*, 2006; Hu *et al.*, 1999) and cardiovascular death (de Kleijn *et al.*, 2002; Mondul *et al.*, 2005; van der Schouw *et al.*, 1996), low bone mineral density (Parazzini *et al.*, 1996) and osteoporosis (Kritz-Silverstein and Barrett-Connor, 1993), and other chronic conditions (Shuster *et al.*, 2010). Quality of life may be significantly decreased while risks of sexual dysfunction and neurological disease may be increased later in life (McEwen and Alves, 1999; Rocca *et al.*, 2009; Van Der Stege *et al.*, 2008).

A study of the National Health and Nutrition Examination Survey (NHANES) participants found that higher PFAS concentrations were associated with earlier menopause: a hazard ratio (HR) of natural menopause was 1.42 (95% CI: 1.08, 1.87) comparing PFHxS serum concentrations in tertile 2 versus tertile 1, and 1.70 (95% CI: 1.36, 2.12) in tertile 3 versus tertile 1; positive dose-response relationships were also detected for PFOA, PFOS, PFNA and PFHxS with hysterectomy (Taylor *et al.*, 2014). Additionally, a cross-sectional study of the C8 Health Project participants found that the odds of having already experienced natural menopause

increased with increasing exposure quintiles of PFOA and PFOS, particularly in women aged 42-65 years (Knox *et al.*, 2011).

These epidemiologic studies of PFAS and age at menopause were cross-sectional analyses in which the outcome was ascertained through an interview at the same time as a blood sample was collected to determine serum PFAS concentrations. It raises the question of reverse causation, in that measured PFAS concentrations increased with years since menopause, possibly due to the cessation of PFAS excretion via menstruation (Taylor *et al.*, 2014). Using a retrospective cohort of women recruited during 2005-2006, Dhingra et al. found no significant association between PFOA exposure (using either estimated year-specific serum concentrations during 1951 and 2011, or measured serum concentrations) and natural menopause incidence (Dhingra *et al.*, 2016).

5.5 Other conditions

PCOS is a common endocrine disorder among women of reproductive age, leading to several health complications including menstrual dysfunction, infertility, hirsutism, acne, obesity, metabolic syndrome, and an increased risk of type-2 diabetes and cardiovascular disease (Norman *et al.*, 2007). A study of 180 infertile PCOS cases and 180 healthy controls showed a significant and positive dose-response relationship between PFDoA serum concentrations and risks of PCOS-related infertility; however, no significant associations were observed for PFBS, PFHpA, PFHxS, PFOA, PFOS, PFNA, PFDeA, or PFUA (Wang, Zhou, *et al.*, 2019). In regard to cancer, only a few studies have evaluated associations between PFAS exposure and increased risks of ovarian cancer (Barry *et al.*, 2013; Vieira *et al.*, 2013). Neither of these studies observed a significant association but the number of cases in each study was small. Thus, the evidence is insufficient to assess risk of ovarian carcinogenicity.

6. Discussion

Findings from *in vitro* and *in vivo* studies suggests that PFAS exposure can target the ovary to adversely affect its two essential functional processes, i.e., folliculogenesis and steroidogenesis. PFAS exposure may alter follicle and oocyte development and diminish ovarian reserve (Bellingham *et al.*, 2009; Chen *et al.*, 2017; Domínguez *et al.*, 2016; Du *et al.*, 2019; Feng *et al.*, 2015, 2017; Hallberg *et al.*, 2019; López-Arellano *et al.*, 2019). Potential mechanisms include PPAR activation, disruption of gap junction intercellular communication, oxidative stress, and thyroid hormone disruption. Limited experimental evidence published to date also suggests PFAS can be a disruptor of ovarian steroidogenesis with independent actions on both theca and granulosa cells (Chaparro-Ortega *et al.*, 2018; Shi *et al.*, 2009; Wang, Bai, *et al.*, 2018). In addition to PPAR signaling pathways, endocrine disruption may also be facilitated by acting directly on gene coding for enzymes responsible for cholesterol transport and ovarian steroidogenesis, and a loss of kisspeptin signaling in the hypothalamus that can impact ovarian function.

In general, experimental studies were limited by the use of doses that exceed the range of estimated human exposure. For example, it is calculated that North American and European consumers had a daily uptake dose of PFOA in the range of 3-220 ng/kg bw and PFOS of 1 to 130 ng/kg bw (Trudel *et al.*, 2008). The lowest concentrations of PFOS and PFOA used in studies of animal models investigating follicle development was 0.1 mg/kg bw/day (=10^5 ng/kg bw/day) (Chen *et al.*, 2017; Feng *et al.*, 2015).

In epidemiologic studies, the associations between PFAS exposure and ovarian function across different populations and difference ranges of exposure levels were inconsistent. Despite that, most epidemiologic studies have found that exposure to PFOA, PFOS or other PFAS

compounds is associated with later menarche (Kristensen *et al.*, 2013; Lopez-Espinosa *et al.*, 2011), irregular and longer menstrual cycle (Chen *et al.*, 2017; Fei *et al.*, 2009; Lum *et al.*, 2017; Lyngsø *et al.*, 2014; Zhou *et al.*, 2017), increased risks of POI (Zhang *et al.*, 2018), and earlier onset of menopause (Knox *et al.*, 2011; Taylor *et al.*, 2014).

Methodologic problems, however, limit the causal interpretation of these findings. The observed associations between PFAS exposure and delayed menarche could be explained by reverse causation rather than a toxic effect of these substances, in that the physiological changes during reproductive growth and maturation in girls may have a considerable influence on serum PFAS concentrations (Wu *et al.*, 2015). It is also possible that the observed associations of PFAS and early onset of menopause in cross-sectional studies might be due to reverse causation related to the presence or volume of menstrual bleeding. Furthermore, information on the timing of menarche, menstrual cycle length and age at menopause were based on self-reports, and recalled data may have been imprecise particularly for users of hormonal contraceptives (Must *et al.*, 2002; Small *et al.*, 2007). Relationships between PFAS exposure and menstrual cycle length among contraceptive users may have been blurred by actions of exogenous hormones (Lum *et al.*, 2017).

Evidence from epidemiologic studies also suggests associations of PFAS exposure with lower E2 levels and higher FSH levels in female adults (Barrett et al., 2015; Heffernan et al., 2018; Knox et al., 2011; McCoy et al., 2017; Zhang et al., 2018). This is consistent with the role of PFAS in accelerating ovarian aging. Compared to adults, girls may be more vulnerable because exposures to PFAS may lead to decreased serum levels of SHBG, FSH, and total T (Lopez-Espinosa *et al.*, 2016; Tsai *et al.*, 2015; Zhou *et al.*, 2016). However, the associations have not been confirmed by longitudinal cohort studies. Results from cross-sectional studies are

also probably subject to reverse causation because sex steroid hormones could affect rates of renal clearance (Kudo *et al.*, 2002). Higher ovarian hormone levels also tend to have a more proliferative endometrial lining (Clancy, 2009) and, by extension, heavier menstrual bleeding, which could contribute to greater clearance of PFAS in menstrual blood. Therefore, we cannot rule out the possibility that fluctuations in hormone levels might impact PFAS serum concentrations in women. Given the inconsistency in previous findings and lack of longitudinal evidence, no causal inferences can be drawn at this time based on this body of literature.

7. Conclusions

The possibility of an association between PFAS exposure and abnormal ovarian function has important implications for research and public health. The ovary is a primary regulator of reproductive and endocrine function as well as general health in the female. Because millions of people worldwide are exposed to PFAS-contaminated drinking water, the public health consequences of a causal relationship could be serious. Methodologic problems limit the causal interpretation of associations between PFAS exposure and menstrual disorders in epidemiologic studies. Overall, there is insufficient evidence to determine a causal relationship between PFAS exposure and ovarian function. Experimental studies with doses relevant to human exposure and epidemiologic research with prospective study designs should be future research priorities.

8. Specific aims

Menopause is a sensitive and critical time window in a woman's life. The existing knowledge for understanding the impact of environmental pollutants on menopause is limited. This dissertation focused on PFAS which are persistent in the environment for years and can bio-accumulate in the food chain. PFAS have attracted attention in recent years for their

environmental ubiquity and their toxicity. However, few studies have addressed the role of PFASs on reproductive aging.

The overall goal of my dissertation project was to explore how exposure to PFAS affects the timing of menopause and to fill research gaps in the evidence base. This area of study will provide a new window on population-level effects from chemical exposures, help shape clinical recommendations, as well as suggest public health prevention and intervention strategies.

Aim 1: To describe the longitudinal changes in PFAS concentrations during the menopausal transition and evaluate whether time trends differed by reproductive aging (i.e. menstruation status), parity, or race/ethnicity.

Aim 2: To examine the associations between PFAS exposures and incidence of natural menopause in the multi-racial/ethnic sample of women who were premenopausal at baseline with standard approximately annual clinic visits from 1999-2017.

Sub-Aim 1: To assess whether the relationship differed by racial/ethnic groups.

Sub-Aim 2: To identify subgroups exposed to different patterns of PFAS using the k-means clustering method and evaluate the combined effects of PFAS mixtures on menopause.

Aim 3: To explore causal mediation of the relationship between exposure to PFAS, as indicated by serum PFAS concentrations, and natural menopause incidence by serum FSH levels.

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Table I. 1 Sources and pathways of human exposure to PFAS.

Sources	**Pathways**
Dietary sources	
Fish and shellfish	Environment/Ingestion
Drinking water	Ingestion
Food-packaging materials	Ingestion
Non-stick cookware	Ingestion
Others (including dairy products, eggs, beverages and vegetables)	Ingestion
Non-dietary sources	
Indoor air	Inhalation
Indoor dust	Inhalation/Ingestion
Soil and sediment	Environment
Impregnation spray (for furniture and carpet)	Inhalation/Dermal absorption
Cosmetics	Dermal absorption
Other consumer products (including ski waxes, leather samples, and outdoor textiles)	Dermal absorption

Table I. 2 Epidemiologic evidence on the associations of exposure to PFOA and PFOS with sex hormones.

1st author, Year	Study design	Population, location, and time period	Sample size	Age, year	PFOA, ng/mL	PFOS, ng/mL	Inclusion/Exclusion criteria	Hormones	Measure of association	Results	Covariate adjusted
Knox 2011	Cross-sectional	Women enrolled in the C8 Health Project from the Mid-Ohio Valley in the US during 2005-2006	25957	18-65	Median 23.6	Median 17.6	Excluding pregnant women, women on hormones or medications affecting hormones, women who had hysterectomy	Serum E2, pmol/L	β (*P* value) stratified by age groups (18-42 years, >42-51 years, and >51 years)	PFOA: no association PFOS: only in women >42-51 years, **(-) -13.4 (*P*<.0001)**; and >51 years, **(-) -3.0 (*P*=0.007)**	Age, BMI, alcohol consumption, smoking, exercise
Kristensen 2013	Birth cohort	Female offspring enrolled in a Danish population-based cohort from 1988-1989 with follow-ups in 2008-2009	267	~20	Median (IQR) maternal exposure 3.6 (2.8-4.8)	Median (IQR) maternal exposure 21.1 (16.7-25.5)	Excluding mothers with breastfeeding, or signs of premature ovarian insufficiency	Serum E2, T, SHBG, DHEA, FSH, LH, and AMH, ln(pmol/L); FAI	β (95%CI)	No association	Maternal smoking during pregnancy, household income, BMI, smoking status, menstrual cycle phase
Barrett 2015	Cross-sectional	Healthy women with natural cycling enrolled in the parent Energy Balance and Breast Cancer Aspects study from Norway during 2000-2002	178	25-35	Median by parity Nulliparous: 3.4; parous: 2.0	Median by parity Nulliparous: 14.8; parous: 12.7	Excluding women with OC use, known histories of infertility gynecological disorders, or chronic illness (e.g. type-2 diabetes or hypothyroidism)	Saliva E2 and P, ln(pmol/L) Day -7 to -1 for E2 and day +2 to +10 for P	β (95%CI) stratified by parity	Among nulliparous women, 1) ln(E2) PFOA: no association PFOS: **(-) -0.025 (-0.043, -0.007)** 2) ln(P) PFOA: no association PFOS: **(-) -0.027 (-0.048, -0.007)** Among parous women, no association	Age, marital status, BMI, physical activity, history of hormone contraceptives, alcohol consumption, smoking status
Lewis 2015	Cross-sectional	Women in NHANES 2011-2012 from the US	824	12-80	Median by age groups 12-<20:1.5; 20-<40:1.5; 40-<60:1.6; 60-<80:2.6	Median by age groups 12-<20:3.8; 20-<40:4.2; 40-<60:4.9; 60-<80: 9.5	NA	Serum T, pmol/L	Percent change (95% CI) per doubling increase in PFAS	No association	Age, BMI,PIR, serum cotinine, race/ethnicity

									stratified by age groups		
Tsai 2015	Cross-sectional	Women recruited from China during 2006-2008	330	12-30	Median 3.6	Median 5.4	Including adolescent and young adult students	Serum SHBG, FSH, T, and E2 ln(pmol/L)	Predicted mean(SE) in PFAS categories (<median, median-p75, >p75-p90, >p90) stratified by age groups (12-17; 18-30)	Among adolescents, 1) SHBG PFOA: **(-) 3.5 (0.2), 3.5 (0.3), 3.4 (0.3), 3.0 (0.3)** PFOS: no association 2) FSH: no association 3) T PFOA: no association PFOS: **(-) 4.0 (0.2), 4.0 (0.2), 3.9 (0.2), 3.6 (0.4)** 4) E2: no association Among young adults, no association	Age, gender, BMI, high fat diet
Lopez-Espinosa 2016	Cross-sectional	Girls enrolled in the C8 Health Project during 2005-2006	1123	6-9	Median 35	Median 22	Excluding girls with menarche	Serum E2 and T, pmol/L	Percent change (95%CI) in the p75 vs. p25 of ln(PFAS)	PFOA: no association PFOS: **(-) -6.6% (-10.1%, -2.8%)**	Age, time of sampling
Zhou 2016	Cross-sectional	Girls enrolled in the Genetics and Biomarkers study for Childhood Asthma from China during 2009-2010	123	13-15	Median (IQR) 0.5 (0.4-1.2)	Median (IQR) 28.8 (14.8-42.6)	Including girls from seven public schools who had no personal or family history of asthma	Serum E2 and T, pmol/L	β (95%CI) per 1 ng/mL increase in PFAS	No association	Age, BMI, ETS exposure, parental education, regular exercise, and month of survey
McCoy 2017	Cross-sectional	Women undergoing IVF in the US during 2013-2014	36	Mean 34	Plasma, ng/g Mean±SD 2.4±0.3	Plasma, ng/g Median±SD 6.5±0.5	Including women at the Coastal Fertility Center in the Mount Pleasant, South Carolina	Plasma E2, pg/mL	Correlation coefficient (*P* value)	PFOA: no association PFOS: **(-) -0.47 (*P*<0.05)**	NA
Heffernan 2018	Case-control	Women with PCOS and age- and BMI-matched controls recruited from	59	20-45	GM (range) 2.4 (0.5-8.2)	GM (range) 3.5 (0.9-7.7)	Including women with BMI ≤35 and undergoing IVF; excluding those with immunological	Serum T, and SHBG, ln(pmol/L); FAI;	β (SE) per ln-unit increase in PFAS stratified by	Among PCOS cases, no association. Among controls, 1) ln(T) PFOA: **(+) 0.52 (0.15)**	Serum albumin

		the UK in 2015					disease, diabetes, renal insufficiency, infections, or inflammatory diseases.	Serum A4 and E2, pmol/L Luteal phase	cases and controls	PFOS: no association 2) SHBG: no association 3) ln(FAI) PFOA: no association PFOS: **(-) -0.61 (0.26)** 4) A4: no association 5) E2: no association	
Zhang 2018	Case-control	Women with overt POI and 120 healthy controls from China recruited during 2013-2016	240	20-40	Median (IQR) 11.1 (7.6-14.5)	Median (IQR) 8.4 (6.3-11.3)	Excluding women with chromosomal abnormalities, a history of radiotherapy or chemotherapy, ovarian surgery, thyroid-related diseases, or use of thyroid medications	Serum FSH, LH, PRL, T, and E2, ln(ng/mL) Early follicular phase	β (95%CI) per ln-unit increase in PFAS	Among POI cases, 1) ln(FSH) PFOA: no association PFOS: **(+) 0.3 (0.2-0.4)** 2) ln(LH): no association 3) ln(E2) PFOA: no association PFOS: **(-) -0.2(-0.4,-0.04)** 4)ln(PRL): PFOA: **(+) 0.2 (0.01-0.3)** PFOS: **(+) 0.2 (0.06-0.3)** 5)ln(T): no association Among controls, no association	Age, BMI, education, income, sleep quality, parity

Abbreviations: AMH, anti-Mullerian hormone; A4, androstenedione; BMI, body mass index; DHEA, dehydroepiandrosterone; ETS, environmental tobacco smoke; E2, estradiol; FAI, free androgen index, was calculated as 100×total T/SHBG; FSH, follicle-stimulating hormone; GM, geometric mean; IQR, interquartile range; NA, not available; NM, not measured; OR, odds ratio; P, progesterone; PCOS, polycystic ovarian syndrome; PFOA, perfluorooctanoic acid; PFOS, perfluorooctane sulfonic acid; POI, premature ovarian insufficiency; PRL, prolactin; p25, 75 and 90, 25^{th}, 75^{th} and 90^{th} percentiles; SHBG, sex hormone-binding globulin; T, testosterone; 95%CI, 95% confidence interval.

Table I. 3 Epidemiologic evidence on the associations of exposure to PFOA and PFOS with menarche.

1st author, Year	Study design	Population, location, and time period	Sample size	Age, year	PFOA, ng/mL	PFOS, ng/mL	Outcome	Measure of association	Results	Covariate adjusted
Christensen 2011	Case-control	Girls with age of menarche before 11.5 years and controls with age of menarche later than 11.5 years from the Avon Longitudinal Study of Parents and Children conducted during 1991-1992 in the UK	218 cases and 230 controls	8-13	Median (IQR) maternal exposure 3.7 (2.8-4.8)	Median (IQR) maternal exposure 19.8 (15.1-24.9)	Early menarche before age 11.5 years	OR (95%CI) by PFAS dichotomous categories	PFOA/PFOS: no association	Birth ord maternal delivery
Lopez-Espinosa 2011	Cross-sectional	Girls enrolled in the C8 Health Project from the Mid-Ohio Valley in the US during 2005-2006	2931	8-18	Median (IQR) 28.2 (11-58)	Median (IQR) 20.2 (14-27)	1) Being postmenarcheal 2) Delay in age of menarche, day	OR (95%CI) in the highest quartile vs. the lowest (the reference)	1) Being postmenarcheal PFOA: **(-) 0.57 (0.37-0.89)**. PFOS: **(-) 0.55 (0.35-0.87)**. 2) Delay in age of menarche PFOA: 130 PFOS: 138	Age
Kristensen 2013	Birth cohort	Female offspring enrolled in a Danish population-based cohort from 1988-1989 with follow-ups in 2008-2009	267	~20	Median (IQR) maternal exposure 3.6 (2.8-4.8)	Median (IQR) maternal exposure 21.1 (16.7-25.5)	Age of menarche, month	β (95%CI) in the highest tertile vs. the lowest (the reference)	PFOA: **(+) 5.3 (1.3-9.3)** PFOS: no association	Maternal during pr household income, daughter

Abbreviations: BMI, body mass index; IQR, interquartile range; OR, odds ratio; PFOA, perfluorooctanoic acid; PFOS, perfluorooctane sulfonic acid; 95%CI, 95% confidence interval.

Table I. 4 Epidemiologic evidence on the associations of exposure to PFOA and PFOS with menstrual cycle characteristics.

1st author, Year	Study design	Population, location, and time period	Sample size	Age, year	PFOA, ng/mL	PFOS, ng/mL	Outcome	Measure of association	Results	Covariate adjusted
Fei 2009	Cross-sectional	Women with planned pregnancy enrolled in the Danish National Birth Cohort during 1996-2002	1240	Mean 30.6	Median (IQR) 5.3 (4.0-7.0)	Median (IQR) 33.7 (26.6-43.5)	Self-reported irregular menses	Proportion of women in the lowest vs. the upper three quartiles	PFOA: 9.0% vs. 15.0% PFOS: 11.6% vs. 14.2%	None
Kristensen 2013	Birth cohort	Female offspring enrolled in a Danish population-based cohort from 1988-1989 with follow-ups in 2008-2009	267	~20	Median (IQR) prenatal exposure 3.6 (2.8-4.8)	Median (IQR) maternal exposure 21.1 (16.7-25.5)	1) Cycle length, day 2) Number of follicles per ovary	β (95% CI) per ln-unit increase stratified by OC use	PFOA/PFOS: no association	Maternal smoking during pregnancy, social class, BMI, smoking status.
Lyngsø 2014	Cross-sectional	Women with planned pregnancy enrolled in the Inuit-Endocrine Cohort during 2002-2004 from Greenland, Poland, and Ukraine	1623	19-49	Median (p10;90) 1.5 (0.7-3.1)	Median (p10;90) 8.0 (3.6-25.6)	1) Longer cycle with cycle length ≥32 days 2) Shorter cycle with cycle length ≤24 days 3) Irregular cycle with ≥7 days in difference between cycles	OR (95%CI) in the highest tertile vs. the lowest (the reference)	1) Longer cycle PFOA: **(+) 1.8 (1.0-3.3)**. PFOS: no association 2) Shorter cycle PFOA/PFOS: no association 3) Irregular cycle PFOA/PFOS: no association	Age at menarche, age at pregnancy, parity, BMI before pregnancy, smoking, and country
Lum 2017	Cross-sectional	Female attempting pregnancy enrolled in the Longitudinal Investigation of Fertility and Environment Study during 2005-2009 from the US	501	18-40	Median 3.2	Median 12.3	Relative difference in cycle length	AF (95%CI) in the highest tertile vs. the lowest (the reference)	PFOA: **(-) 0.98 (0.96-1.00)** PFOS: no association	Age, BMI, smoking status
Zhou 2017	Cross-sectional	Women who were attempting pregnancy	950	Median (IQR) 30	Median (IQR) 13.8	Median (IQR) 10.5	1) Longer periods with cycle length >35 days	OR (95%CI) in the highest	1) Longer periods PFOA: **(+) 2.0 (1.2-3.1)**. PFOS: no association	Age, BMI, income, age at

		recruited during 2013-2015 from China		(28-32)	(10.1-18.8)	(7.6-15.4)	2) Irregular periods with ≥7 days in difference between cycles 3) Menorrhagia as self-reported heavy or very heavy bleeding 4) Hypomenorrhea as self-reported light bleeding	quartile vs. the lowest (the reference)	2) Irregular periods PFOA: **(+) 2.0 (1.2-3.2)**. PFOS: no association 3) Menorrhagia PFOA: **(-) 0.2 (0.1-0.5)**. PFOS: **(-) 0.3 (0.1-0.6)**. 4) Hypomenorrhea PFOA/PFOS: no association	menarche, and parity

Abbreviations: AF, acceleration factor; BMI, body mass index; IQR, interquartile range; OR, odds ratio; PFOA, perfluorooctanoic acid; PFOS, perfluorooctane sulfonic acid; 95%CI, 95% confidence interval.

Table I. 5 Epidemiologic evidence on the associations of exposure to PFOA and PFOS with ovarian aging.

1st author, Year	Study design	Population, location, and time period	Sample size	Age, year	PFOA, ng/mL	PFOS, ng/mL	Outcome	Measure of association	Results	Covariate adjusted
Knox 2011	Cross-sectional	Women enrolled in the C8 Health Project from the Mid-Ohio Valley in the US during 2005-2006	25957	18-65	Median 23.6	Median 17.6	Natural menopause	OR (95%CI) in the highest quintile vs. the lowest (the reference) stratified by age groups (18-42 years, >42-51 years, and >51 years)	18-42 years PFOA/PFOS: no association >42-51 years PFOA: **(+) 1.4 (1.1-1.8)** PFOS: **(+) 1.4 (1.1-1.8)** >51 years PFOA: **(+)1.7 (1.3-2.3)** PFOS: **(+) 2.1 (1.6-2.8)**	Age, BMI, alcohol consumption, smoking status, exercise
Taylor 2014	Retrospective cohort	General adult women in NHANES 1999-2010 from the US	2732	20-65	Median 3.8	Median 14.0	1) Natural menopause 2) Hysterectomy	HR (95%CI) in the highest tertile vs. the lowest (the reference)	1) Natural menopause PFOA: **(+) 1.4 (1.1-1.8)** PFOS: no association 2) Hysterectomy PFOA: **(+) 2.8 (2.1-3.7)** PFOS: **(+) 2.6 (1.9-3.4)**	Age, race/ethnicity, education, smoking status, parity
Dhingra 2016	Prospective cohort	Premenopausal women enrolled in the C8 Health Project from the Mid-Ohio Valley in the US during 2005-2006 and followed up during 2008-2011	3334	≥40	P40, 60 17.8-33.6	NM	Natural menopause	HR (95% CI) in the highest quintile vs. the lowest (the reference) with hysterectomy censored or excluded	No association	Smoking status, education, BMI, parity
Zhang 2018	Case-control	Women with overt POI and healthy controls from China recruited during 2013-2016	240	20-40	Median (IQR) POI cases, 11.1 (7.6-14.5); Controls, 8.4 (6.3-11.3)	Median (IQR) POI cases, 8.2 (5.5-13.5); Controls, 6.0 (4.2-9.1)	POI as an elevated FSH level >25IU/L on two occasions >4 weeks apart and oligo/amenorrhea for ≥4 months	OR (95%CI) in the highest tertile vs. the lowest (the reference)	PFOA: **(+) 3.8 (1.9-7.5)** PFOS: **(+) 2.8 (1.5-5.4)**	Age, BMI, education, income, sleep quality, parity

Abbreviations: AF, acceleration factor; IQR, interquartile range; NM, not measured; OR, odds ratio; PFOA, perfluorooctanoic acid; PFOS, perfluorooctane sulfonic acid; POI, premature ovarian insufficiency; 95%CI, 95% confidence interval.

Table I. 6 Epidemiologic evidence on the associations of exposure to PFOA and PFOS with other chronic conditions.

1st author, Year	Study design	Population, location, and time period	Sample size	Age, year	PFOA, ng/mL	PFOS, ng/mL	Outcome	Measure of association	Results	Covariate adjusted
Barry 2013	Retrospective cohort	Female residents enrolled in the C8 Health Project and workers employed at DuPont from the US during 2005-2006 and followed up in 2008-2011	17360	Mean 53	Median (range) for residents 24.2 (0.25-4752), and for workers 112.7 (0.25-22412)	NM	Ovarian cancer	HR (95%CI) per ln-unit increase in PFAS	No association	Smoking, alcohol consumption, sex, education, birth year
Vieira 2013	Cross-sectional	Cancer patients living near the DuPont plant from the US during 1996-2005	25107	Median 67	Range 3.7-655 estimated based on geocoded address	NM	Ovarian cancer	OR (95%CI) in the highest category (110-655 ng/mL) vs. unexposed group (the reference)	No association	Age, race, sex, diagnosis year, insurance provider, smoking status
Wang 2019	Case-control	Infertile women diagnosed with PCOS and healthy controls from China in 2014	367	20-40	Median 5.1	Median 4.1	PCOS	OR (95%CI) in the highest tertile vs. the lowest (the reference)	No association	Age, BMI, household income, education, employment, age at menarche, menstrual volume

Abbreviations: NM, not measured; OR, odds ratio; PCOS, polycystic ovarian syndrome; PFOA, perfluorooctanoic acid; PFOS, perfluorooctane sulfonic acid; 95%CI, 95% confidence interval.

Table I. 7 Epidemiologic evidence on the effects of other PFAS compounds.

1st author, Year	Study design	Population, location, and time period	Sample size	Age, year	Other PFAS, ng/mL	Outcome	Measure of association	Results	Covariate adjusted
Menarche									
Christensen 2011	Case-control	Girls with age of menarche before 11.5 years and controls with age of menarche later than 11.5 years from the Avon Longitudinal Study of Parents and Children conducted during 1991-1992 in the UK	448	8-13	Median (IQR) maternal exposure PFOSA, 0.2 (0.2-0.3) EtFOSAA, 0.6 (0.4-0.9) MeFOSAA, 0.4 (0.3-0.8) PFHxS, 1.6 (1.2-2.2) PFNA, 0.6 (0.5-0.8)	Early menarche before age 11.5 years	OR (95%CI) by PFAS dichotomous categories at medians	No association	Birth order, maternal age at delivery
Menstrual cycle characteristics									
Zhou 2017	Cross-sectional	Women who were attempting pregnancy recruited during 2013-2015 from China	950	~30	Median (IQR) PFHxS, 0.7 (0.6-0.9) PFNA, 1.4 (1.0-1.9)	1) Longer cycle with cycle length >35 days 2) Irregular cycle with ≥7 days in difference between cycles 3) Menorrhagia as self-reported heavy or very heavy bleeding 4) Hypomenorrhea as self-reported light bleeding	OR (95%CI) in the highest quartile vs. the lowest (the reference)	1) Longer cycle PFHxS: **(+) 2.1 (1.3-3.5)** PFNA: **(+) 1.7 (1.0-2.7)** 2) Irregular cycle PFHxS: **(+) 2.1 (1.3-3.5)** PFNA: no association 3) Menorrhagia PFHxS: **(-) 0.3 (0.1-0.7)** PFNA: **(-) 0.4 (0.2-0.9)** 4) Hypomenorrhea PFHxS: **(+) 3.6 (1.5-8.6)** PFNA: no association	Age, BMI, income, age at menarche, and parity
Lum 2017	Cross-sectional	Female attempting pregnancy enrolled in the Longitudinal Investigation of Fertility and Environment Study during 2005-2009 from the US	501	18-40	Median (IQR) among women with normal cycle MeFOSAA, 0.3 (0.1-0.5) PFDeA, 0.4 (0.2-0.6) PFNA, 1.2 (0.8-1.7)	Relative difference in menstrual cycle length	AF (95%CI) in the highest tertile vs. the lowest (the reference)	No association	Age, BMI, smoking status
Ovarian aging									
Zhang 2018	Case-control	Women with overt POI and healthy controls from China	240	20-40	Median (IQR) in controls: PFHpA, 0.2 (0.1-0.3)	POI as an elevated FSH level >25IU/L on two occasions	OR (95%CI) in the highest tertile vs. the	PFHpA: no association PFNA: no association PFDeA: no association	Age, BMI, education,

		recruited during 2013-2016			PFNA, 1.8 (1.3-2.7) PFDeA, 1.7 (1.0-2.6) PFDoA, 0.17 (0.1-0.2) PFUnA, 1.3 (0.8-1.9) PFBS, 0.05 (0.04-0.1) PFHxS, 0.3 (0.2-0.4)	>4 weeks apart and oligo/amenorrhea for ≥4 months	lowest (the reference)	PFDoA: no association PFUnA: no association PFBS: no association PFHxS: **(+) 6.6 (3.2-13.7)**	income, sleep quality, parity
Taylor 2014	Retrospective cohort	General adult women in NHANES 1999-2010 from the US	2732	20-65	Median (IQR) in premenopausal women: PFHxS, 1.0 (0.6-1.8) PFNA, 0.9 (0.6-1.4)	1) Natural menopause 2) Hysterectomy	HR (95%CI) in the highest tertile vs. the lowest (the reference)	1) Natural menopause PFHxS: **(+) 1.7 (1.4-2.1)** PFNA: **(+) 1.5 (1.1-1.9)** 2) Hysterectomy PFHxS: **(+) 3.5 (2.7-4.5)** PFNA: **(+) 1.8 (1.3-2.4)**	Age, race/ethnicity, education, smoking status, parity
Sex steroid hormones									
Barrett 2015	Cross-sectional	Healthy women with natural cycling enrolled in the parent Energy Balance and Breast Cancer Aspects study from Norway during 2000-2002	178	25-35	Median (range) in nulliparous women: PFOSA, 0.2 (0.07-1.1) PFHxS, 1.1 (0.3-5.0) PFDeA, 0.2 (0.05-2.0) PFUnA, 0.4 (0.07-1.1) PFNA, 0.6 (0.2-2.9)	Saliva E2 and P, ln(pmol/L) Day -7 to -1 for E2 and day +2 to +10 for P	β (95%CI) stratified by parity	No association	Age, marital status, BMI, physical activity, history of hormone contraceptives, alcohol consumption, smoking status
Lewis 2015	Cross-sectional	Women in NHANES 2011-2012 from the US	824	12-80	Median by age groups 12-<20:PFHxS, 0.8; PFNA, 0.7; 20-<40:PFHxS, 0.7; PFNA, 0.7; 40-<60:PFHxS, 0.9; PFNA, 0.8; 60-<80:PFHxS, 1.5; PFNA, 1.1.	Serum T, pmol/L	Percent change (95% CI) per doubling increase in PFAS stratified by age groups	No association	Age, BMI,PIR, serum cotinine, race/ethnicity
Tsai 2015	Cross-sectional	Women recruited from China during 2006-2008	330	12-30	Median (IQR) PFUnA, 6.5 (1.5-13.4) P60, 90 PFNA, 1.6-6.9	Serum SHBG, FSH, T, and E2 ln(pmol/L)	Predicted mean(SE) in PFAS categories (<median, median-p75, >p75-p90, >p90)	Among adolescents, 1) SHBG: no association 2) FSH PFNA: no association PFUnA: only in girls 12-17 years, **(-) 1.6**	Age, gender, BMI, high fat diet

							stratified by age groups (12-17; 18-30)	**(0.3), 1.6 (0.2), 1.4 (0.2), 1.2 (0.2)** 3) T: no association 4) E2: no association	
Lopez-Espinosa 2016	Cross-sectional	Girls enrolled in the C8 Health Project during 2005-2006	1123	6-9	Median (IQR) PFHxS, 7.0 (3.8-13.8) PFNA, 1.7 (1.3-2.4)	Serum E2 and T, pmol/L	Percent change (95%CI) in the p75 vs. p25 of ln(PFAS)	No association	Age, time of sampling
Zhou 2016	Cross-sectional	Girls enrolled in the Genetics and Biomarkers study for Childhood Asthma from China during 2009-2010	123	13-15	Median (IQR) PFBS, 0.5 (0.4-0.5) PFHxS, 1.2 (0.5-3.0) PFHxA, 0.2 (0.1-0.3) PFNA, 0.9 (0.6-1.1) PFDeA, 1.0 (0.8-1.2) PFDoA, 3.1 (0.9-6.2) PFTeA, 4.5 (0.3-18.4)	Serum E2 and T, pmol/L	β (95%CI) per 1 ng/mL increase in PFAS	1) ln(T): Only for PFDoA, **(-) -0.012 (-0.023- -0.001)** 2) ln(E2): No association	Age, BMI, ETS exposure, parental education, regular exercise, and month of survey
McCoy 2017	Cross-sectional	Women undergoing IVF in the US during 2013-2014	36	Mean 34	Plasma, ng/g Mean±SD PFHxS, 2.2±0.4 PFNA, 0.8±0.1 PFDeA, 0.4±0.05 PFUnA, 0.3±0.03	Plasma E2, pg/mL	Correlation coefficient (*P* value)	No association	NA
Heffernan 2018	Case-control	Women with PCOS and age- and BMI-matched controls recruited from the UK in 2015	59	20-45	GM (range) PFHxS, 1.0 (0.2-10.2) PFNA, 0.6 (0.2-1.8)	Serum T, and SHBG, ln(pmol/L); FAI; Serum A4 and E2, pmol/L Luteal phase	β (SE) per ln-unit increase in PFAS stratified by cases and controls	Among PCOS cases, only for A4 and PFNA: **(+) 1.71 (0.65)** Among controls, only for ln(T), PFHxS: **(+) 0.50 (0.17)** PFNA: **(+) 0.46 (0.21)**	Serum albumin
Zhang 2018	Case-control	Women with overt POI and 120 healthy controls from China recruited during 2013-2016	240	20-40	Median (IQR) in controls PFHxS, 0.3 (0.2-0.4)	Serum FSH, LH, PRL, T, and E2, ln(ng/mL) Early follicular phase	β (95%CI) per ln-unit increase in PFAS	Among POI cases, 1) ln(FSH) **(+) 0.16 (0.04-0.28)** 2) ln(LH): no association 3) ln(E2) **(-) -0.19 (-0.37- -0.02)** 4)ln(PRL): no association 5)ln(T): no association Among controls, no association	Age, BMI, education, income, sleep quality, parity
Other chronic conditions									

Wang 2019	Case-control	Infertile women diagnosed with PCOS and healthy controls from China in 2014	367	20-40	Median (IQR) PFBS, 0.11 (0.1-0.12) PFHxS, 0.24 (0.17-0.3) PFHpA, 0.08 (0.05-0.1) PFNA, 0.5 (0.3-0.9) PFDeA, 0.5 (0.3-0.8) PFUnA, 0.4 (0.3-0.6) PFDoA, 0.24 (0.2-0.27)	PCOS	OR (95%CI) in the highest tertile vs. the lowest (the reference)	PFBS: no association PFHxS: no association PFHpA: no association PFNA: no association PFDeA: no association PFUnA: no association PFDoA: **(+) 3.0 (1.2-7.7)**	Age, BMI, household income, education, employment, age at menarche, menstrual volume

Abbreviations: AMH, anti-Mullerian hormone; A4, androstenedione; BMI, body mass index; DHEA, dehydroepiandrosterone; EtFOSAA, 2-(N-Ethylperlfuorooctane sulfonamide) acetic acid; E2, estradiol; FAI, free androgen index, was calculated as 100×total T/SHBG; FSH, follicle-stimulating hormone; GM, geometric mean; IQR, interquartile range; MeFOSAA, 2-(N-Methylperfluorooctane sulfonamido) acetic acid; NHANES, National Health And Nutrition Examination Survey; NM, not measured; OR, odds ratio; P, progesterone; PCOS, polycystic ovarian syndrome; PFBS, perfluorobutane sulfonic acid; PFDeA, perfluorodecanoic acid; PFDoA, perfluorododecanoic acid; PFHpA, perfluoroheptanoic acid; PFHxS, perfluorohexane sulfonic acid; PFNA, perfluorononanoic acid; PFOSA, perfluorooctane sulfonamide; PFUnA, perfluoroundecanoic acid; POI, premature ovarian insufficiency; PRL, prolactin; p60 and 90, 60th and 90th percentiles; SHBG, sex hormone-binding globulin; T, testosterone; 95%CI, 95% confidence interval.

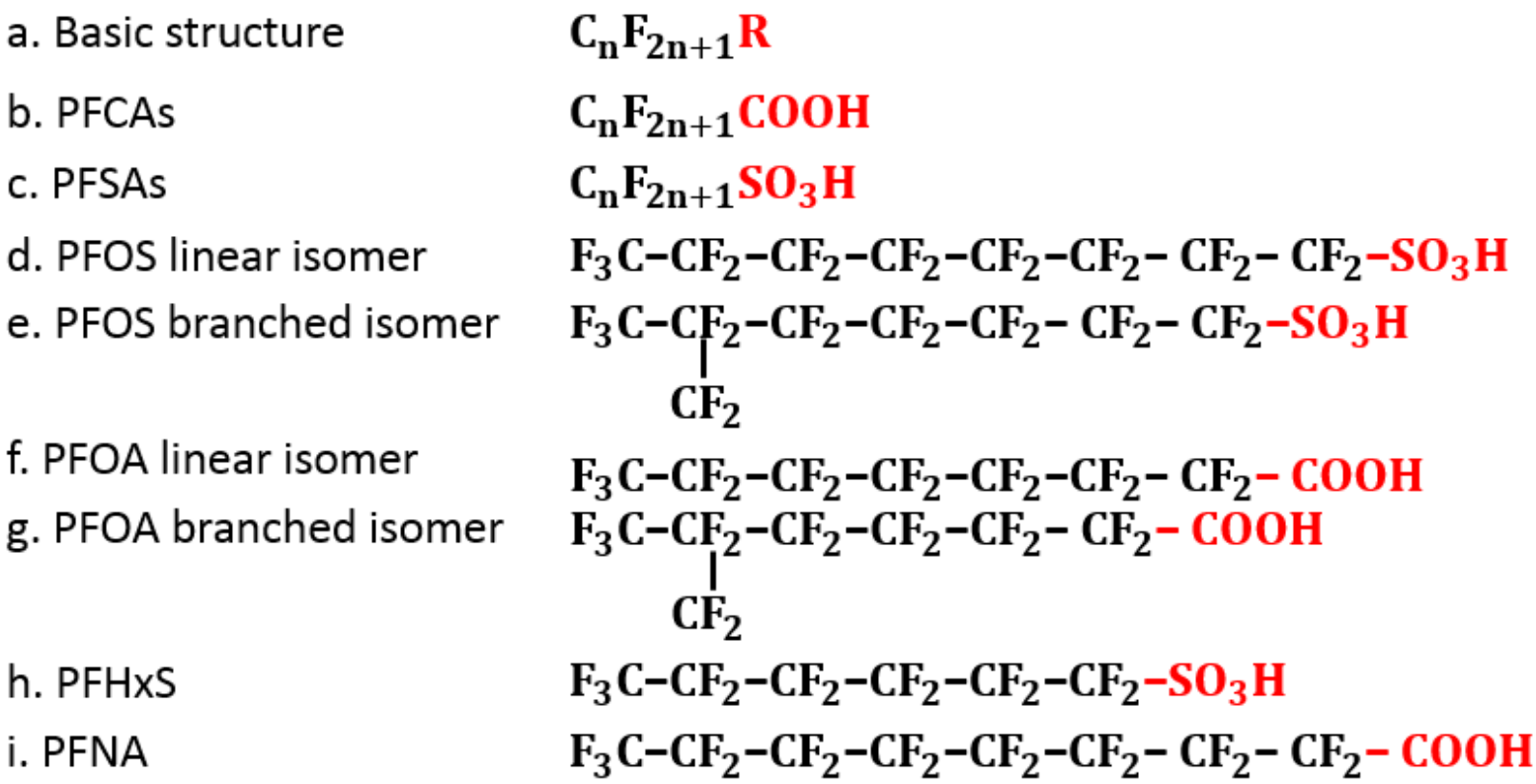

Figure I. 1 The chemical structures of perfluoroalkyl substances. a. The basic chemical structure of perfluoroalkyl substances, where "C_nF_{2n+1}" represents the length of the perfluoroalkyl chain and "R" defines the functional group. b. The general chemical structures of perfluoroalkyl carboxylic acids (PFCAs) with the functional group of *–COOH*. c. The general chemical structures of perfluoroalkane sulfonic acids (PFSAs) with the functional group of -SO_3H. d-i. The chemical structures of commonly detected perfluoroalkyl substances, including liner and branched perfluorooctane sulfonic acid (PFOS), linear and branched perfluorooctane carboxylic acid (PFOA), perfluohexane sulfonic acid (PFHxS), and perfluorononanoic acid (PFNA). Note that branched PFOA and PFOS isomers may have different rearrangements from the linear form, often mono-substituted, di-substituted, or cyclic structural isomers.

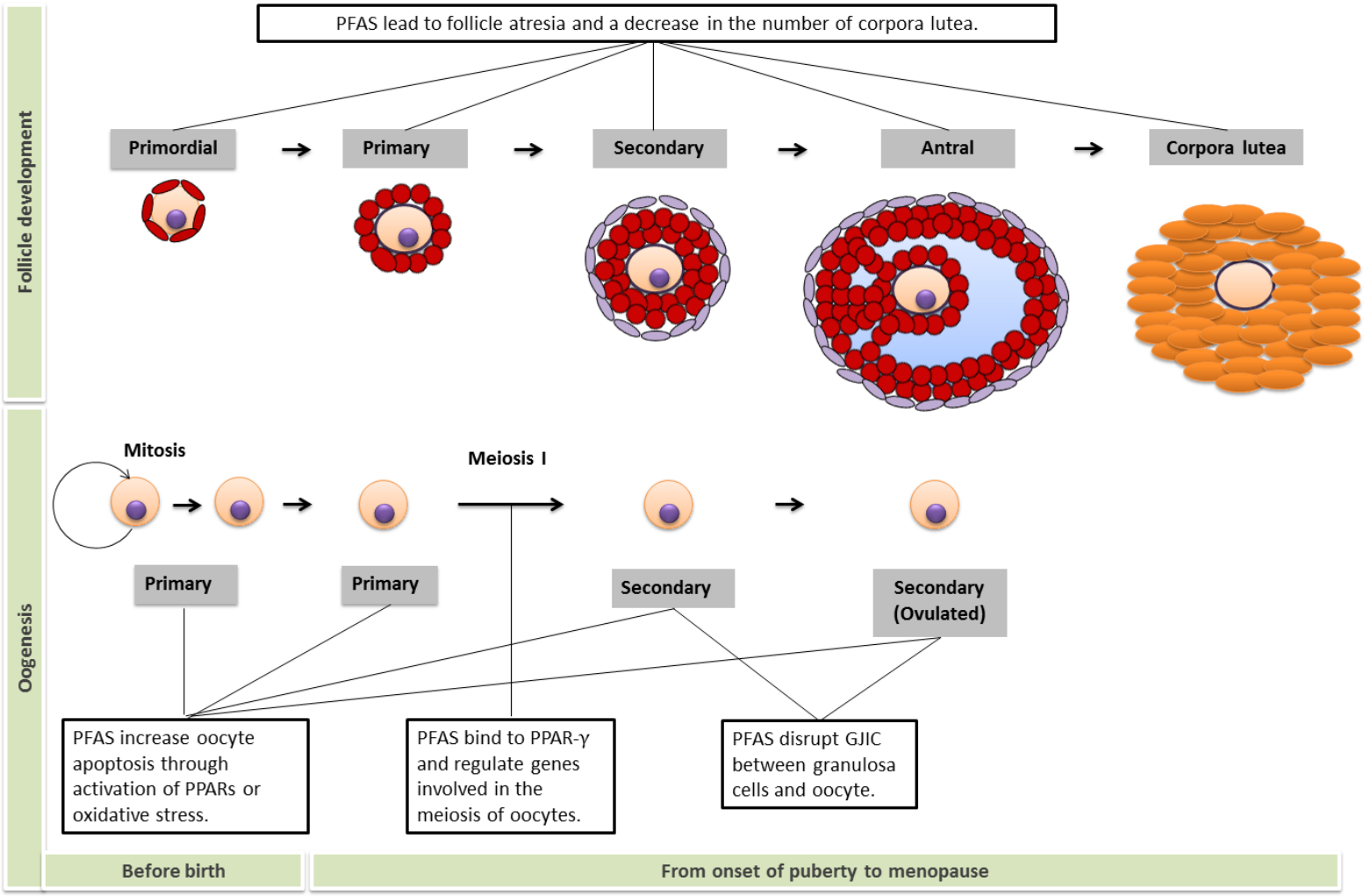

Figure I. 2 PFAS disrupt folliculogenesis. The upper part of the figure is about follicle development and the text box shows the effects of PFAS on the number of follicles at that stage of development. The part below displays the process of oogenesis and text boxes outline the major effects of PFAS at that stage of oocyte development and maturation. GJIC=gap junction intercellular communication; PPAR=peroxisome proliferator activated receptor.

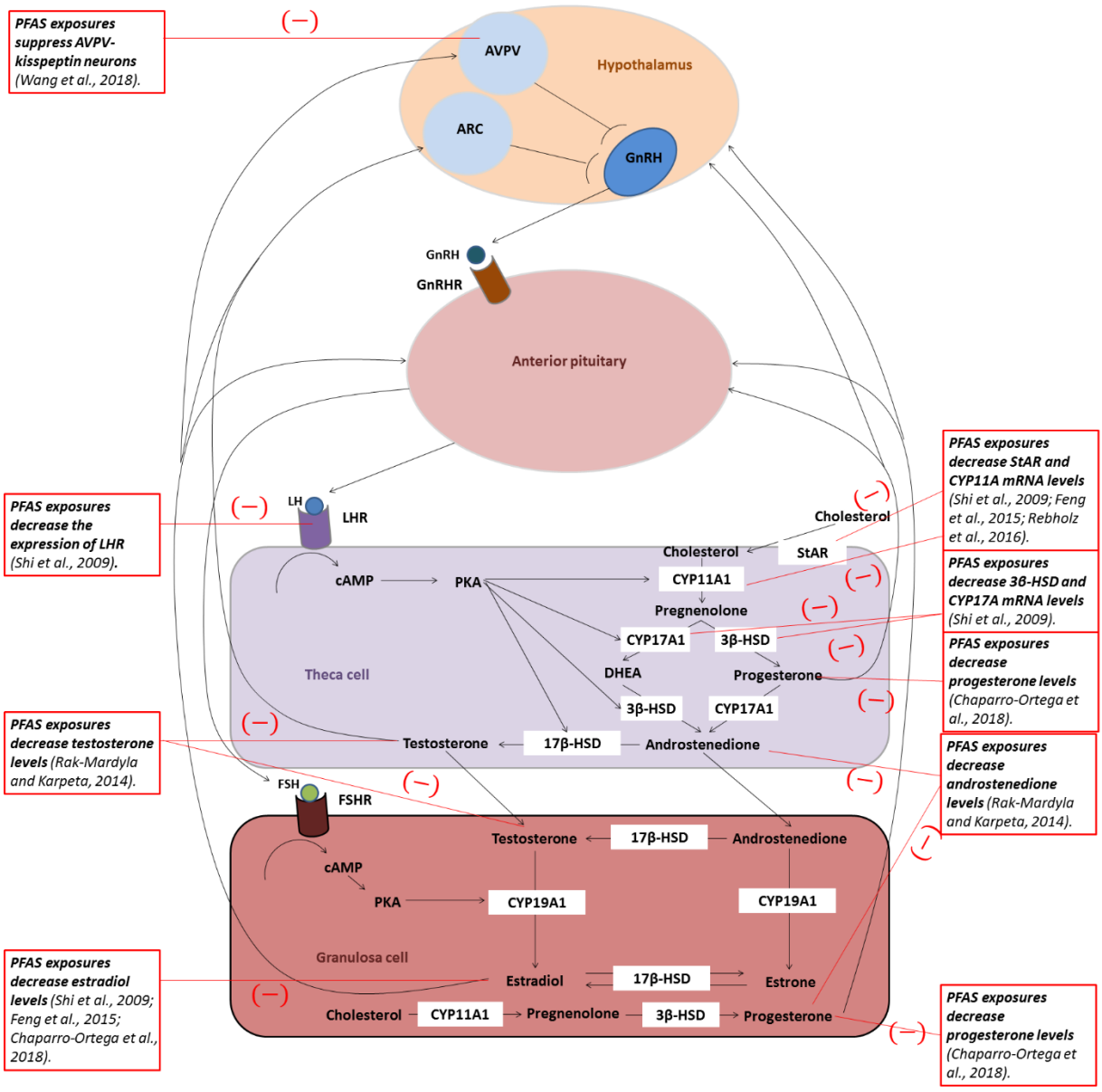

Figure I. 3 PFAS alter ovarian steroidogenesis. Ovarian steroidogenesis requires the cooperative interactions of the theca and granulosa cells within the follicles. This figure is a simplified overview of the two-cell ovarian steroidogenesis model, with black text boxes indicating PFAS targets from the experimental literature. ARC=arcuate nucleus; AVPV=anteroventral periventricular nucleus; cAMP=cyclic adenosine monophosphate; CYP11A1=cholesterol side chain cleavage enzyme; CYP17A1=17α-hydroxylase-17, 20-desmolase; CYP19A1=cytochrome P450 aromatase; ERα=estrogen receptor α; FSH=follicle-stimulating hormone; FSHR=follicle-stimulating hormone receptor; GnRH=gonadotropin-releasing hormone; GnRHR=gonadotropin-releasing hormone receptor; 3β-HSD=3β-hydroxysteroid dehydrogenase; 17β-HSD=17β-hydroxysteroid dehydrogenase; LH=luteinizing hormone; LHR=luteinizing hormone receptor; PKA=protein kinase A; StAR=steroid acute regulatory protein.

Chapter II. Longitudinal Trends in Perfluoroalkyl and Polyfluoroalkyl Substances among Midlife Women from 1999 to 2011: the Study of Women's Health Across the Nation

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Abstract

Background: Limited information exists regarding longitudinal trends in midlife women's exposure to per- and polyfluoroalkyl substances (PFAS). Further, little is known about how patterns of exposure differ by race/ethnicity and reproductive characteristics including parity and menopause.

Objective: We aimed to examine temporal variations in serum PFAS concentrations among midlife women from the Study of Women's Health Across the Nation.

Methods: Serum concentrations of 11 PFAS compounds were measured in 75 White, Black and Chinese women with blood samples collected in 1999-2000, 2002-2003, 2005-2006, and 2009-2011. Rates of changes in PFAS concentrations were calculated assuming a first-order elimination model. Associations between PFAS concentrations and race/ethnicity, menstruation and parity were evaluated with linear mixed models, adjusting for age, body mass index and study site.

Results: Serum concentrations of linear-chain perfluorooctanoic acid (n-PFOA), linear- and branched-chain perfluorooctane sulfonic acid (n-PFOS and Sm-PFOS) decreased significantly (-6.0%, 95% CI: -8.3%, -3.6% per year for n-PFOA; -14.8%, 95% CI: -17.3%, -12.3% per year for n-PFOS; -16.9%, 95% CI: -19.1%, -14.6% per year for Sm-PFOS); whereas perfluorononanoic acid (PFNA) increased (16.0%, 95% CI: 10.6%, 21.6% per year). Detection rates of perfluorodecanoic acid (PFDeA) and perfluoroundecanoic acid (PFUA) doubled. Temporal trends varied significantly by race/ethnicity. Chinese women tended to have consistently higher PFNA concentrations at each follow-up visit, compared with White and Black women. Serum PFHxS concentrations significantly decreased in White and Black women, but not in Chinese.

Menstruating women consistently had lower concentrations. Parity was associated with lower concentrations at baseline but the differences between nulliparous and parous women became smaller over time.

Conclusions: Our results suggest longitudinal declines in serum concentrations of legacy PFAS and increases in serum concentrations of emerging compounds from 1999 to 2011 in midlife women. Temporal trends in PFAS concentrations are not uniform across race/ethnicity and parity groups.

Keywords: Perfluoroalkyl and polyfluoroalkyl substances; Biomonitoring; Midlife women; Racial/ethnic disparities; Menstruation; Parity.

1. Introduction

Per- and polyfluoroalkyl substances (PFAS) are anthropogenic compounds that have been widely used since the discovery of polytetrafuoroethylene (commonly known as Teflon) (Kotthoff *et al.*, 2015; Prevedouros *et al.*, 2006). Due to the strong electronegativity and small atomic size of fluorine, the perfluoroalkyl moiety imparts unique water- and oil-repellency, and thermal and chemical stability to these compounds, compared to their hydrocarbon counterparts. Many consumer products contain specific members of this family of chemicals, such as nonstick cookware, weatherproof clothing, surface protectants, carpets, greaseproof food packaging, aqueous film-forming foams, and etc. (Begley *et al.*, 2005; Butenhoff *et al.*, 2006; Kantiani *et al.*, 2010; Kissa, 2011; Trudel *et al.*, 2008).

PFAS are of particular concern as these compounds have been linked to hepatocellular damage (Darrow *et al.*, 2016), chronic kidney disease (Shankar *et al.*, 2011) and metabolic disorders (Liu *et al.*, 2018; Sun *et al.*, 2018), and have also been identified as potential reproductive toxicants (Jensen and Leffers, 2008). Perfluorooctanoic acid (PFOA) and perfluorooctane sulfonic acid (PFOS) (so-called C8 compounds) have been the most extensively produced and studied chemicals. Epidemiological studies have shown associations between exposure to elevated concentrations of PFOA and PFOS with menstrual cycle irregularity (Lyngsø *et al.*, 2014; Zhou *et al.*, 2017), premature ovarian insufficiency and accelerated ovarian aging (Knox et al., 2011; Taylor et al., 2014; Zhang et al., 2018), steroidogenic defects (Barrett *et al.*, 2015), and infertility (Bach *et al.*, 2016). Given the toxicity, persistence and bioaccumulation of PFAS, government and regulatory bodies in some parts of the world have been working towards agreements and regulations to limit the production of PFOA and PFOS since 2000 (Significant New Use Rule Final Rule and Supplemental Proposed Rule, 2002;

Stockholm Convention, 2016; US EPA, 2000, 2016). At the same time, several other PFAS have increased steadily in the general population (Calafat *et al.*, 2007). Therefore, the quantitation of multiple PFAS in human serum is important to adequately assess human exposure and associated health risks.

Previous human biomonitoring studies primarily focused on repeated cross-sectional data and have shown declines in PFOS, PFOA and their precursors (Calafat *et al.*, 2006, 2007; Kato, Wong, *et al.*, 2011; Liu *et al.*, 2015; Olsen *et al.*, 2012). To date, few published studies have reported longitudinal trends in the United States (Kato *et al.*, 2014; Wu *et al.*, 2015). These longitudinal studies (Kato *et al.*, 2014; Wu *et al.*, 2015) included only two recorded time points within a short time frame (~6-12 months) among pregnant women in Ohio and children and adults in California, respectively. Given that many PFAS are slowly eliminated, e.g., half-lives can exceed 2 years, it is important to have multiple measurements over a relatively long follow-up period to adequately describe within-person changes.

Understanding the health effects of common exposures is challenging as exposures may vary by participant characteristics such as race/ethnicity and geography. Serum concentrations also cannot easily be related to probable ongoing background exposures in midlife women, since factors such as menstruation and parity may not properly be accounted for in female elimination rates. To improve our understanding of exposure to PFAS in midlife women, we describe the longitudinal changes in PFAS concentrations during the menopausal transition and evaluate whether time trends differed by reproductive aging (i.e. menstruation status), parity, or race/ethnicity. The present study was based on four repeated measurements of serum PFAS concentrations collected 1999 through 2011 in a cohort of 75 multiethnic midlife women aged 45-56 years at baseline (1999-2000) from the United States.

2. Methods

2.1 Study populations. Participants were drawn from the Study of Women's Health Across the Nation (SWAN), a multicenter, multi-ethnic, community-based cohort of midlife women. Detailed study designs were described elsewhere (Sowers *et al.*, 2000). Briefly, 3,302 women were recruited at baseline during 1996-1997 from 7 study sites in the United States (Boston, MA; Chicago, IL; southeast Michigan, MI; Los Angeles, CA; Newark, NJ; Oakland, CA; Pittsburgh, PA). Each site recruited White women and women from a specified minority group (black in Boston, Chicago, southeast Michigan, and Pittsburgh; Chinese in Oakland; Japanese in Los Angeles; Hispanic in Newark). Baseline eligibility criteria for enrollment into the longitudinal cohort included the following: aged 42 to 52 years, having an intact uterus and at least one ovary, not currently using exogenous hormones affecting ovarian functions, having a menstrual period in the previous 3 months, and self-identified with a site's designated racial/ethnic group.

The SWAN Multi-Pollutant Study (MPS) was initiated in 2016 to examine multiple environmental chemical exposures, including PFAS, polychlorinated biphenyls (PCBs), organochlorine pesticides, polybrominated diphenyl ethers (PBDEs), metals, phenols, phthalates, and organophosphate pesticide among midlife women. A schematic diagram of the SWAN MPS sampling procedure is shown in **Supplemental Table II.1**. Repository samples available from the third follow-up visit (V03, 1999-2000) were used for environmental exposure assessments. Of 2,694 women enrolled at V03, we excluded women from Chicago (n=368) and Newark (n=278) because urine samples were not available in these two sites. We additionally excluded 648 women with insufficient serum or urine samples at V03 or insufficient urine samples at V06 (for the assessment of non-persistent phenols and phthalates), yielding the sample size of 1400. Details of the study design are described elsewhere (Park *et al.*, 2019).

We also designed a pilot project at the SWAN V03 (1999-2000), V06 (2002-2003), V09 (2005-2006) and V12 (2009-2011) to examine temporal trends in a panel of persistent organic pollutants, including PCBs, PBDEs, and PFAS. Of 1,400 participants with serum samples available at V03, we picked three study sites (Boston, MA in the East; southeast Michigan in the Midwest; Oakland, CA in the West), to capture temporal variations in different race/ethnic groups and geographical locations with limited resources. Because the menopausal transition and related body composition changes may impact chemical distributions of persistent organic pollutants, we then conducted random sampling to get a subsample of women (n=75) with 4 follow-up visits at V03, V06, V09 and V12 (n=300 observations in total) stratified on changes in waist circumferences and race/ethnicity. The sampling procedure of this pilot project is given in **Supplemental Figure II.2**.

Characteristics among 1,400 participants at V03 were compared to those included in the temporal trend study. Women who were followed until V12 had a higher level of education ($P<0.05$, **Supplemental Table II.2**). No significant differences were found for other socio-demographic factors ($P>0.05$, **Supplemental Table II.2**) or for PFAS serum concentrations at the baseline ($P>0.05$, **Supplemental Table II.3**). In addition, waist circumference changes did not impact the temporal trends of PFAS concentrations (data not shown).

2.2 PFAS measurements. We obtained serum samples from 75 women at SWAN V03 (1999/2000), V06 (2002/2003), V09 (2005/2006), and V12 (2009/2011). Serum samples were sent to the Division of Laboratory Sciences, National Center for Environmental Health, Centers for Disease Control and Prevention.

Perfluoroalkyl acids (PFAAs) are some of the most simple PFAS molecules, which are essentially non-degradable. The PFAAs contain two major groups, perfluoroalkyl carboxylic acids (PFCAs) and perfluoroalkane sulfonic acids (PFSAs). PFCAs included PFOA, perfluorononanoic acid (PFNA), perfluorodecanoic acid (PFDeA), perfluoroundecanoic acid (PFUA), and perfluorododecanoic acid (PFDoA). PFSAs included perfluorohexane sulfonic acid (PFHxS), and PFOS. PFOA and PFOS may be present as mixtures of linear- (n-) and branched- (sm-) chain isomers depending on the manufacturing process used. A linear isomer is composed of carbon atoms bonded to only one or two carbons, which form a straight backbone. A branched isomer consists of at least one carbon atom bonded to more than two carbon atoms. Note that 2-(N-ethyl-perfluorooctane sulfonamide) acetate (EtFOSAA) and 2-(N-methyl-perfluorooctane sulfonamide) acetate (MeFOSAA) are perfluoroalkane sulfonamide acetic acids (FASAAs) which belong to polyfluorinated compounds and act as intermediate environmental transformation products of PFOS. Chemical names and formulas of PFAS analyzed in this study are shown in **Supplemental Table II.1**.

We measured PFHxS, n-PFOS, sum of Sm-PFOS, EtFOSAA, MeFOSAA, n-PFOA, sum of Sb-PFOA, PFNA, PFDeA, PFUA, and PFDoA in 1 mL of serum, using an online solid phase extraction-high performance liquid chromatography-isotope dilution-tandem mass spectrometry (on-line SPE-HPLC-MS/MS) method (Kato, Basden, *et al.*, 2011) that allows for selective analyses of serum. The limits of detection (LODs) were determined during method validation by running 5 repeated measurements of low-level standards spiked onto calf serum (Taylor, 1981) and then calculating the standard deviation of the instrument response. The limit of detection was then defined as three times the standard deviation. The LODs were 0.1 ng/mL for all PFAS analytes.

In parallel with sample analyses, the following quality control (QC) procedures were conducted: (a) standard reference materials (SRMs) and spike and surrogate recoveries were tested periodically; (b) linearity and drift checks were performed with each sample batch; (c) internal standards consisting of deuterated standards are used on each sample; (d) duplicates were analyzed in each batch; (e) method detection limits (MDLs) for each target compound were determined for each matrix; and (f) blanks (instrumental, field and laboratory) were run with each sample batch. Each sample run contained 9 calibration standards, two low-concentration QCs (QCL), two high-concentration QCs (QCH), three serum blanks, and two reagent blanks. All solvents and other materials contacting samples are proved to be clean, as confirmed using blanks. The coefficient of variation was 5.9-12.1% for the low QC pools; and 5.9-10.6% for the high QC pools.

2.3 Covariates. Time-independent covariates included race/ethnicity, study site, and baseline age, measured body mass index, and parity. Parity, which represents the sum of the number of live births and stillbirths, was classified into nulliparous or parous. Time-varying variables included menstruation status. Menstruation was determined from self-administered questionnaires. At each visit women were asked: "Did you have any menstrual bleeding since your last study visit?" All these variables were fully observed in the study sample.

2.4 Comparison with NHANES data. We used the NHANES 1999-2010 data to compare temporal trends in PFAS between our study and the contemporary US representative population. Only women with the same age range at each cycle matched to our longitudinal follow-up visits were included in the analyses. Thus, participants included in the comparison were 91 women aged 45-56 years from NHANES 1999-2000, 119 women aged 48-59 years from NHANES 2003-2004, 124 women aged 51-62 years from NHANES 2005-2006, and 232 women aged 55-

68 years from NHANES 2009-2010 (a total of 566). Survey-weighted medians and interquartile ranges were computed at each cycle.

2.5 Statistical analysis. The concentrations of 11 PFAS were described using geometric mean (GM), geometric standard deviation (GSD), median, interquartile range (IQR), 95th and 99th percentiles, and range. For measurements below the LODs, the values were substituted with LOD/$\sqrt{2}$. An intra-class correlation coefficient (ICC) was calculated to assess reliability of serum PFAS measurements using the following formula $\mathrm{ICC} = {\sigma_B{}^2}\big/{(\sigma_B{}^2 + \sigma_W{}^2)}$, where $\sigma_B{}^2$ is the between-subject variation and $\sigma_W{}^2$ is the within-subject variance. An ICC is very useful for analyzing continuous measures. A high ICC indicates that differences in PFAS serum concentration between subjects have greater variability than that within subjects over the study period (Enderlein, 2007).

PFAS chemicals that were detected in at least 70% of the samples were included in the trend analysis. Under the assumption of a first-order elimination model, halving time ($T_{1/2}$) for PFAS were calculated by ${\ln(2)}\big/{\beta}$, where β was the fixed effect coefficient of time; $(e^{\beta} - 1) \times 100\%$ was expressed as the excretion constant rate. Repeated measure analysis of variance (ANOVA) tests were conducted to compare serum PFAS concentrations by participant characteristics.

In the adjusted analyses, linear mixed models (LMMs) were fitted with random intercepts to calculate effect estimates and standard errors (SEs) for assessment of time-varying PFAS serum concentrations by participant characteristics. PFAS concentrations were modeled as a function of follow-up visits with baseline visit as the reference, to capture non-linear trends. A

natural logarithmic transformation was applied to serum PFAS concentrations to approximate a normal distribution. Both crude analyses and analyses with adjustment for age at baseline, race/ethnicity, study site (Santoro *et al.*, 2011), BMI at baseline, parity, and menstrual bleeding were conducted. To explore temporal variations by covariates, interactions terms between time and these covariates were included in the regressions of SWAN follow-up visits. Likelihood ratio tests were used to compare models with and without interaction terms to determine whether time trajectories differed by these factors.

Given the limited sample size we used the most parsimonious adjusted model as shown below,

$$\begin{aligned} PFAS_{ij} = {} & \beta_0 + \beta_1 V06_i + \beta_2 V09_i + \beta_3 V12_i + \beta_4 Menstruating_{ij} + \beta_5 White_i + \beta_6 Chinese_i \\ & + \beta_7 Parous_i + \beta_8 V06_i \times White_i + \beta_9 V09_i \times White_i + \beta_{10} V12_i \times White_i \\ & + \beta_{11} V06_i \times Chinese_i + \beta_{12} V09_i \times Chinese_i + \beta_{13} V12_i \times Chinese_i \\ & + \beta_{14} V06_i \times Parous_i + \beta_{15} V09_i \times Parous_i + \beta_{16} V12_i \times Parous_i \\ & + \beta Covariates_i + b_{0j} + \varepsilon_{ij}, \end{aligned}$$

where $PFAS_{ij}$ is serum concentrations for the ith subject with the jth observation; $X \times Y$ is the interaction term between X and Y; $V06$ is SWAN visit 06 during $2002 - 2003$, $V09$ is SWAN visit 09 during $2005 - 2006$, and $V12$ is SWAN visit 12 during $2009 - 2011$; Covariates include age at baseline, BMI at baseline, and study site.

Statistical analyses were conducted by R 3.4.4 (R Core Team (2018). R Foundation for Statistical Computing, Vienna, Austria) and SAS, version 9.4 (SAS Institute, Inc., Cary, North Carolina).

3. Results

3.1 Participant characteristics. Characteristics of 75 women at SWAN V03 (1999/2000), V06 (2002/2003), V09 (2005/2006) and V12 (2009/2011) are described in **Table II.1**. The average age of participants was 49 years (range = 45 to 56 years) at V03, the baseline period for this analysis, in 1999-2000. The median (IQR) BMI was 26.1 (22.7-32.0) kg/m^2 at baseline and did not change much during the follow-up visits. All 75 women lived in the same place during the follow-up visits. Among participants, 25.3% resided in southeast Michigan, 30.7% in Boston, and 44.0% in Oakland; 49.3% were White, 25.3% were Black and 25.3% were Chinese. 89.3% of participants experienced menstrual bleeding since their last visit at V03 (on average, approximately 12 months after V02) decreasing to 4.0% at V12. Sixteen percent were nulliparous at V03 and no one became pregnant or breastfed during the follow-up visits.

3.2 Longitudinal trends of serum PFAS concentrations from 1999 to 2011. Distributions of serum PFAS concentrations across follow-up visits are displayed for selected compounds with detection rates >70% in **Figure II.1** and presented for all the PFAS analytes in **Supplemental Table II.4**. Over 70% of the serum samples had detectable concentrations above LODs at each time point for n-PFOA, n-PFOS, Sm-PFOS, PFHxS, and PFNA. Substantial decreases were observed in median concentrations of n-PFOA (3.30 to 2.60 ng/mL; P=0.001), n-PFOS (17.00 to 7.50 ng/mL; P<.0001), and Sm-PFOS (6.20 to 2.50 ng/mL; P<.0001) during 1999 and 2011. Over 98% of the samples had detectable EtFOSAA and MeFOSAA at V03 while the detectable percentages decreased to 1.33% and 50.67%, respectively, during the follow-up visits. PFHxS serum concentrations did not change significantly during 1999 and 2011 (1.50 to 1.20 ng/mL; P=0.05).

In contrast to other PFAS, PFNA showed increase steeply (0.50 to 1.30 ng/mL), comparing V03 (1999/2000) to V12 (2009/2011) (*P*<.0001). PFDeA and PFUA concentrations increased during 1999-2011 with a detection rate of 89.3% and 66.7% at V12, respectively, from 42.7% and 36% at V03. Fewer than 20% of the samples had detectable levels of Sb-PFOA and PFDoA. Intraclass correlation coefficients ranged 0.16 to 0.48, indicating low similarity and highly variable values from the same subject over time.

On average, serum concentrations of n-PFOA, n-PFOS, Sm-PFOS and PFHxS were estimated to be decreased by 6.0% (95% CI: -8.3%, -3.6%), 14.8% (95% CI: -17.3%, -12.3%), 16.9% (95% CI: -19.1%, -14.6%) and 6.2% (95% CI: -9.1%, -3.2%) per year, respectively, as shown in **Table II.2**. The halving time was estimated to be 11.2 (95% CI: 8.0-19.0) years for n-PFOA, and 4.3 (95% CI: 3.6-5.3), 3.7 (95% CI: 3.3-4.4) and 10.8 (95% CI: 7.2-21.5) years and for n-PFOS, Sm-PFOS and PFHxS, respectively. On the contrary, PFNA increased by 16.0% (95% CI: 10.6%, 21.6%) during follow-up visits with a doubling time of 4.7 (95% CI: 3.5-6.9) years. Adjustment for age at baseline, race and study site and menstruation did not account for temporal variations of PFAS serum concentrations, except for PFNA, of which the time effects became insignificant (*P*=0.12).

3.3 Comparison with NHANES data. Comparisons of the present study with the contemporary NHANES data of general U.S. women with the same age range are displayed in **Supplemental Figure II.3**. Both showed serum concentrations of PFOA, PFOS, PFHxS, and PFNA with detection rates>70%. SWAN participants had lower median concentrations over time, but the spread of IQRs in SWAN and the NHANES datasets generally overlapped. Both had decreasing trends of PFOA, PFOS and PFHxS, and increasing trends of PFNA. Detection rates of PFDeA and PFUA increased in both SWAN and NHANES data but decreased for EtFOSAA (data not

shown). Neither showed detectable concentrations of PFDoA.

3.4 Determinants of temporal changes in PFAS concentrations. Unadjusted median (IQR) log-transformed serum concentrations of n-PFOA, n-PFOS, Sm-PFOS, PFHxS and PFNA by race/ethnicity, menstruation status and parity over time are displayed in **Figures II.2-II.4**. In the unadjusted analyses, temporal trends differed significantly by race/ethnicity for n-PFOA (P=0.007), n-PFOS (P=0.02), and PFHxS (P=0.04) but not for Sm-PFOS (P=0.13) and PFNA (P=0.19). Menstruating women had lower PFAS concentrations and the differences remain almost the same over time during the follow-up visits (P=0.31 for n-PFOA, P=0.29 for n-PFOS, P=0.80 for Sm-PFOS, P=0.36 for PFHxS, and P=0.07 for PFNA). Nulliparous women had higher serum PFAS concentrations at baseline but the time trajectories of n-PFOA and n-PFOS had changed significantly during the follow-up visits (P=0.03 for n-PFOA, P=0.03 for n-PFOS). In contrast, the trajectories of other PFAS did not differ by parity (P=0.19 for Sm-PFOS, P=0.99 for PFHxS, and P=0.28 for PFNA).

Figure II.5 depicts the adjusted trends of n-PFOA, n-PFOS, Sm-PFOS, PFHxS and PFNA across the four visits stratified by race/ethnicity, menstrual bleeding and parity, controlling for age at baseline, study site, and BMI at baseline (see effect estimates and standard errors from mixed regression models in **Supplemental Table II.5**).

Race/ethnicity was an independent predictor of PFAS concentrations and their trends over time. For n-PFOA, women had significantly lower concentrations from baseline to V12, but trends differed significantly by race/ethnicity (P for interaction=0.001). White women had the highest exposures to n-PFOA at baseline; however, time mitigated the racial/ethnic differences. Chinese women showed a much slower decline in from V03 to V12. For n-PFOS, all three

racial/ethnic groups had significantly lower concentrations at V12 compared to V03, but White women had a more rapid decline from baseline to V12, compared with that of Chinese and Black (*P* for interaction=0.0007). Temporal trends of Sm-PFOS and PFHxS also differed significantly by race/ethnicity (*P* for interaction=0.03 and 0.008, respectively), with racial/ethnic differences decreased over time. However, PFNA serum concentrations did not change significantly over time across racial/ethnic groups (*P* for interaction=0.13) but Chinese women showed consistently higher concentrations compared to other racial/ethnic groups (*P*=0.03 at baseline for race/ethnicity).

Compared to women without menstrual bleeding since the last visit, menstruating women had 16.4% (95% CI: -26.7%, -4.7%), 18.3% (95% CI: -31.9%, -1.9%), 13.4% (95% CI: -22.8%, -2.9%) lower serum concentrations of n-PFOA, PFNA, and Sm-PFOS, respectively, during follow-up visits. n-PFOS or PFHxS were not associated with menstruation status (*P*=0.14 and 0.15, respectively). Interaction terms between time and menstrual bleeding were not significant.

In addition, serum concentrations of n-PFOA, n-PFOS and Sm-PFOS also varied significantly across parity. Concentrations decreased by -40.5% (95% CI: -58.5%, -14.6%) for n-PFOA, -47.7% (95% CI: -65.6%, -20.7%) for n-PFOS, and -45.5% (95% CI: -64.0%, -17.4%) for Sm-PFOS, comparing parous to nulliparous women at baseline; whereas the differences became significantly smaller for n-PFOA and n-PFOS (*P* for interaction=0.02 for n-PFOA, and 0.008 for n-PFOS) over time, but did not change for Sm-PFOS (*P* for interaction=0.21). No significant differences by parity status were observed for PFHxS (*P*=0.50) and PFNA (*P*=0.31) at baseline, or during follow-up visits (*P* for interaction= 0.95 for PFHxS, and 0.47 for PFNA). Further adjustment for education, employment status, and difficulty paying for basics did not eliminate the observed differences by race/ethnicity, menstruation, or parity (data not shown).

4. Discussion

This study updates the existing knowledge on human exposure to PFAS. It provides valuable data on midlife women's exposure to PFAS as they transition through menopause and temporal trends of PFAS serum concentrations, which have rarely been reported before. This study also provides new evidences on the contribution of race/ethnicity, menstruation, and parity to the temporal variations of PFAS concentrations.

4.1 Longitudinal trends of serum PFAS concentrations from 1999 to 2011. Overall, serum concentrations of legacy compounds, including n-PFOA, n-PFOS, Sm-PFOS, EtFOSAA and MeFOSAA peaked at baseline. In contrast, increasing trends were observed for PFNA, PFUA, and PFDeA from 1999 to 2011.

Along with a recent study summarizing PFAS data in NHANES (Calafat *et al.*, 2007), our findings indicated effectiveness of deliberate efforts to reduce the production of PFOA, PFOS and its precursors in the United States. Unlike the majority of legacy PFAS, the current study and previous studies have suggested increases in serum concentrations of other long-chain PFAS, including PFNA (Calafat *et al.*, 2006, 2007; Kato, Wong, *et al.*, 2011; Spliethoff *et al.*, 2008), PFUA (Calafat *et al.*, 2006, 2007; Kato, Wong, *et al.*, 2011; Spliethoff *et al.*, 2008), and PFDeA (Calafat *et al.*, 2006, 2007; Kato, Wong, *et al.*, 2011; Spliethoff *et al.*, 2008).These increasing trends indicate an ongoing exposure. For example, PFNA was found to be present in several commonly used consumer products, e.g. paper-based food contact materials and textiles (Kotthoff *et al.*, 2015). In addition, PFNA and PFUA are believed to be manufactured through a transformation of fluorotelomer olefins (Buck *et al.*, 2011), which could be formed by telomere-derived PFAS precursors. It is also possible that the observed increase in PFNA concentrations

was related to internal metabolisms (e.g. cessation of menstruation) in midlife women. However, the routes of exposure and control mechanisms for these compounds remain obscure, as the main exposure pathway for PFCAs varies according to exposure scenarios (Gebbink *et al.*, 2015).

No significant changes were observed for serum PFHxS concentrations among the midlife women. However, PFHxS was the second most abundant PFSAs next to PFOS during 2003 and 2011. Previous research indicates that higher prevalence of PFHxS could be associated with increased use of stovetop Teflon cookware (Hu *et al.*, 2018), preheated packaged/microwavable foods (Hu *et al.*, 2018; Wu *et al.*, 2015), as well as indoor dust (Wu *et al.*, 2015) and lower vacuuming frequency (Siebenaler *et al.*, 2017).

4.2 Differential changes in PFAS concentrations by race/ethnicity from 1999-2011.

Race/ethnicity has previously been correlated with PFAS exposures (Boronow *et al.*, 2019; Calafat *et al.*, 2007; Park *et al.*, 2019). Although serum concentrations of n-PFOA, n-PFOS, and Sm-PFOS have declined over all, to our knowledge no longitudinal study to date had assessed whether temporal changes in serum PFAS concentrations differ by race/ethnicity. The present study addresses this gap. Our findings suggest that temporal trends in PFAS exposure are not uniform across racial/ethnic groups, and subpopulations with higher initial PFAS exposures often experienced the greater change over the study period. For example, we observed a more rapid decline in n-PFOA and n-PFOS concentrations among White women, who had higher baseline concentrations compared with other racial/ethnic groups, possibly reflecting differences in consumer product use. A scenario-based risk assessment study (Trudel *et al.*, 2008) reported that in female adults, the most dominant source of PFOA exposure was likely from consumer products including impregnation spray, treated carpets in homes, and coated food contact materials, while a large proportion of PFOS exposure was through intake of contaminated food.

Substantial declines in serum concentrations of n-PFOA and n-PFOS among White women might result from changes in product preferences and food consumption. Conversely, White women had a slight increase in PFHxS serum concentrations in 2009-2011. Another recent study of middle-aged women also found that White women with higher serum concentrations of PFHxS compared with Black, likely attributable to exposure from dental floss with Oral-B Glide (Boronow *et al.*, 2019).

Unlike other racial/ethnic groups, Chinese women had increasing exposures to PFHxS since 2002, and consistently higher serum concentrations of PFNA during the follow-up visits. However, little is known about potential sources of exposure in Chinese populations. Socioeconomic characteristics, lifestyle factors or genetics may account for the observed disparities. Compared to White women, both Black and Chinese had lower education attainment, less physical activity; Black women had more difficulty paying for basics; Chinese women had more fish intake (**Supplemental Table II.6**). Although biomonitoring studies are useful for documenting population exposures to environmental chemicals, they are limited in their ability to identify the contribution of specific sources to personal exposure. Nonetheless, our understanding of sources of PFAS exposure remains incomplete, however, these findings prompt follow-up in future studies.

4.3 PFAS concentrations by parity. Parity was a significant determinant of PFAS serum concentrations, especially with parous women having lower concentrations of n-PFOA, n-PFOS and Sm-PFOS than nulliparous women. This is the first study examining longitudinal trends of PFAS serum concentrations by parity in midlife women. Previous studies found that nulliparous women had higher PFAS maternal concentrations (Berg et al., 2014; Brantsæter et al., 2013; Fei et al., 2007; Jensen et al., 2015; Lewin et al., 2017; Ode et al., 2013). PFAS can be transferred to

infants or to the placenta through the umbilical cord (Beesoon et al., 2011; Hanssen et al., 2010; Kato et al., 2014). Blood loss during delivery might also decrease maternal body burden of PFAS (Lorber et al., 2015). After birth, PFAS may gradually re-accumulate in women, and as a result the differences at baseline narrowed over time (Fei et al., 2009). On the other hand, given the longer half-life of long-chain PFAS (e.g. PFNA) they might not be easily excreted during delivery.

4.4 PFAS concentrations by menstruation status. This study strengthens the evidence that PFAS concentrations are influenced by menstruation status. Menstruating women tended to have lower serum concentrations compared to those without. The results are consistent with a previous pharmacokinetics modeling, in which Wong et al. found that approximately 30% of the PFOS elimination half-life difference between females and males (Wong et al., 2014). Decreased serum concentrations have been shown in premenopausal versus postmenopausal women (Dhingra et al., 2017; Taylor et al., 2014) and, analogously, in men undergoing venesections for medical treatment (Lorber et al., 2015). Given that approximately 90% to 99% of these compounds in the blood are bound to serum albumin (Han et al., 2003; Ylinen and Auriola, 1990), blood loss through menstruation may be an important elimination pathway.

Kudo et al. examined the role of sex hormones and transport proteins on the renal clearance and concluded that: in ovariectomized female rats, 1) estradiol could facilitate transporting PFOA across the membranes of kidney tubules into the glomerular filtrate; 2) treatments with testosterone reduced the clearance of PFOA (Kudo et al., 2002). This conforms to humans. Zhang et al. study reported that the rate of renal elimination was 0.024 mL/day/kg among women >50 years while 0.043 mL/day/kg among women ≤50 years (Zhang et al., 2013). We cannot rule out the possibility of other unknown elimination routes that might elucidate the

change of PFAS serum concentrations among midlife women during menopausal transition.

4.5 Strengths and limitations. Our study is limited by its small sample size. We do not have sufficient power to examine the changes over time stratified by participant characteristics. Instead, we relied on the tests of statistical interactions between time and covariates. We also oversampled Chinese women to better capture racial/ethnic differences. In addition, we did not have information on important sources of exposure including food contact materials, microwavable or packaged food consumption, or use of carpet treatment procedures because no such information was available in this cohort. We were also unable to fully assess the impact of genetics and lifestyle factors which may account for racial/ethnic disparities. Future study with a larger sample size would provide a clearer picture of the complex relationships between race/ethnicity in PFAS exposure. Lastly, our study participants were 75 midlife women from three geographic locations, so results might not be generalizable to people living in other areas. The study sample is not representative of the base population of the SWAN Multipollutant Study. Women who have completed all 4 visits and provided serum samples were eligible. Also, by design, only women from Oakland, South Michigan, and Boston were included in selection.

A major strength of this study was the opportunity to include persons from different geographical locations in the United States, and Chinese, whose exposures have not been characterized. The repeated measurements also allow for examination of longitudinal intra-individual changes in PFAS serum concentrations. As the ICCs for repeated measurements were relatively low, indicating that one single serum measurement may not be enough to provide a reliable biomarker of PFAS exposures.

5. Conclusions

In summary, our results depict longitudinal declines in legacy PFAS (i.e. PFOA and PFOS), as well as their branched isomers and precursors MeFOSAA and EtFOSAA among midlife women living in the US during 1999-2011. The findings are consistent with reduced environmental exposures since 2000-2002. We also identified differential patterns of exposure by race/ethnicity, which can provide useful information for developing hypotheses about possible sources of exposure that, especially for PFHxS and PFNA, are poorly understood. Additional analyses should be performed nationwide to examine whether similar racial/ethnic disparities exist across different regions of the country and which compounds (e.g. behaviors, consumer products) are the primary determinants of this risk disparity.

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Table II. 1 Characteristics of participants in the Study of Women's Health Across the Nation (SWAN) 1999-2011.

SWAN visit	V03	V06	V09	V12
Year of sample collection	1999-2000	2002-2003	2005-2006	2009-2011
No. of participants	75	75	75	75
Age at interview[1], years	49.4 (47.1-51.2)	52.5 (50.1-54.2)	55.4 (53.2-57.3)	59.6 (57.8-62.1)
Body mass index [1], kg/m^2	26.1 (22.7-32.0)			
Study site				
Southeast Michigan	19 (25.3%)			
Boston, MA	23 (30.7%)			
Oakland, CA	33 (44.0%)			
Race/ethnicity				
Black	19 (25.3%)			
White	37 (49.3%)			
Chinese	19 (25.3%)			
Menstrual bleeding since last visit	67 (89.3%)	46 (61.3%)	23 (30.7%)	3 (4.0%)
Parity				
Nulliparous	12 (16.0%)			
Parous	63 (84.0%)			

[1]Median (interquartile range).

Table II. 2 Halving or doubling time for serum PFAS1 concentrations among 75 women (300 observations in total) in SWAN 1999-2011.

	n-PFOA	n-PFOS	Sm-PFOS	PFHxS	PFNA
Unadjusted					
Percent change per year	-6.0%	-14.8%	-16.9%	-6.2%	16.0%
95% CI	-8.3%, -3.6%	-17.3%, -12.3%	-19.1%, -14.6%	-9.1%, -3.2%	10.6%, 21.6%
p-value	<.0001	<.0001	<.0001	<.0001	<.0001
Halving or doubling time, year	11.2	4.3	3.7	10.8	-4.7
95% CI	8.0, 19.0	3.6, 5.3	3.3, 4.4	7.2, 21.5	-6.9, -3.5
Adjusted[2]					
Percent change per year	-6.9%	-12.0%	-14.7%	-6.3%	4.7%
95% CI	-10.0%, -3.7%	-15.4%, -8.4%	-17.7%, -11.6%	-10.2%, -2.1%	-1.2%, 10.8%
p-value	<.0001	<.0001	<.0001	0.004	0.12
Halving or doubling time, year	9.6	5.4	4.4	10.7	-15.3
95% CI	6.6, 18.3	4.1, 7.9	3.6, 5.6	6.4, 32.9	-58.4, 6.7

[1]PFAS with serum concentrations above limit of detection more than 60% were included in the analyses.
[2]Adjusting for age at baseline, body mass index at baseline, race/ethnicity, study site, menstrual bleeding, and parity.

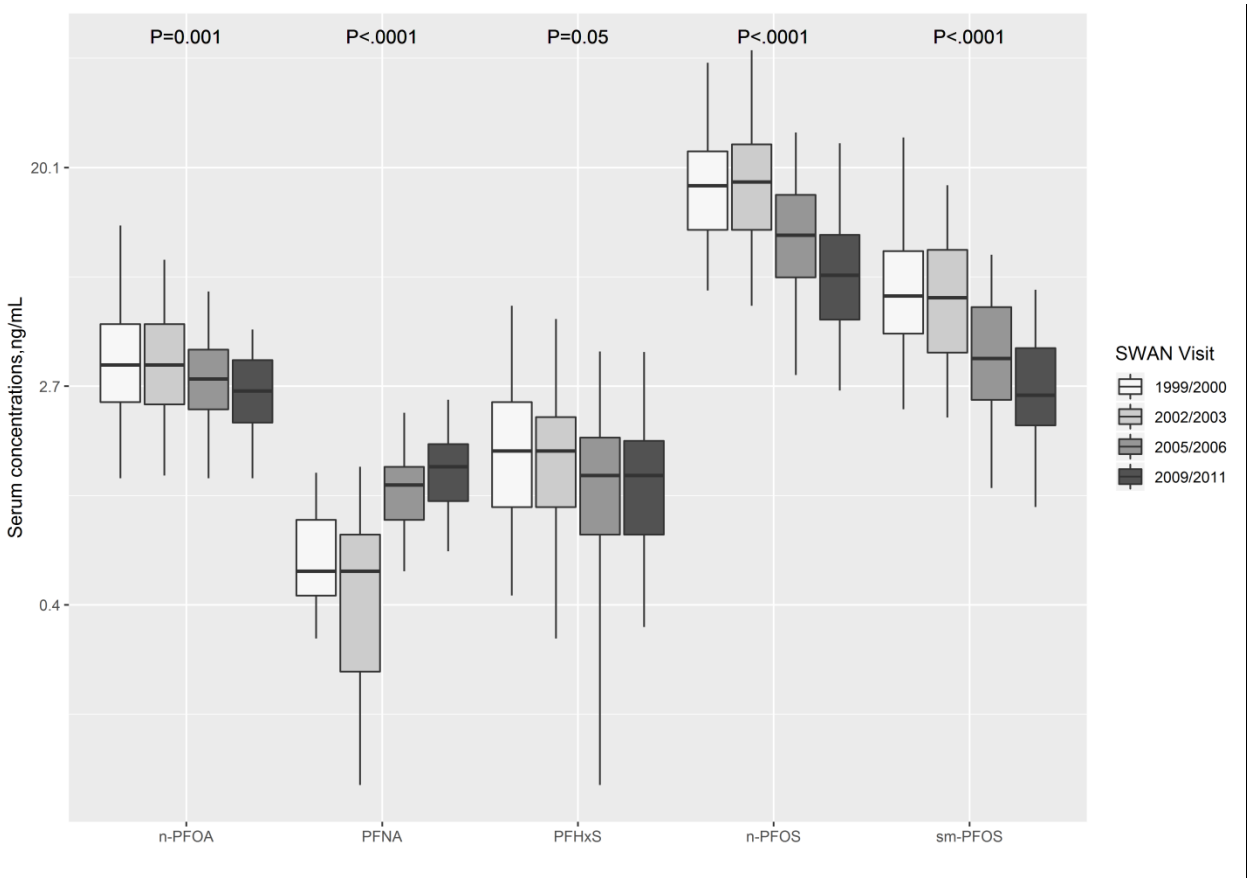


Figure II. 1 Concentrations of selected PFAS with detection rates >70% analyzed in repeated serum samples of women (n=75) across the United States for four SWAN visits. Boxes represent the 25th-75th percentiles, horizontal lines represent the median, and whiskers indicate 5th and 95th percentiles, respectively. Note that a log scale is used for Y axis. The limits of detection were 0.1 ng/mL for all PFAS analytes. Abbreviations: n-PFOA, linear-chain perfluorooctanoic acid; PFNA, perfluorononanoic acid; PFHxS, perfluorohexane sulfonic acid; n-PFOS, linear-chain perfluorooctane sulfonic acid; Sm-PFOS, sum of branched-chain perfluorooctane sulfonic acid.

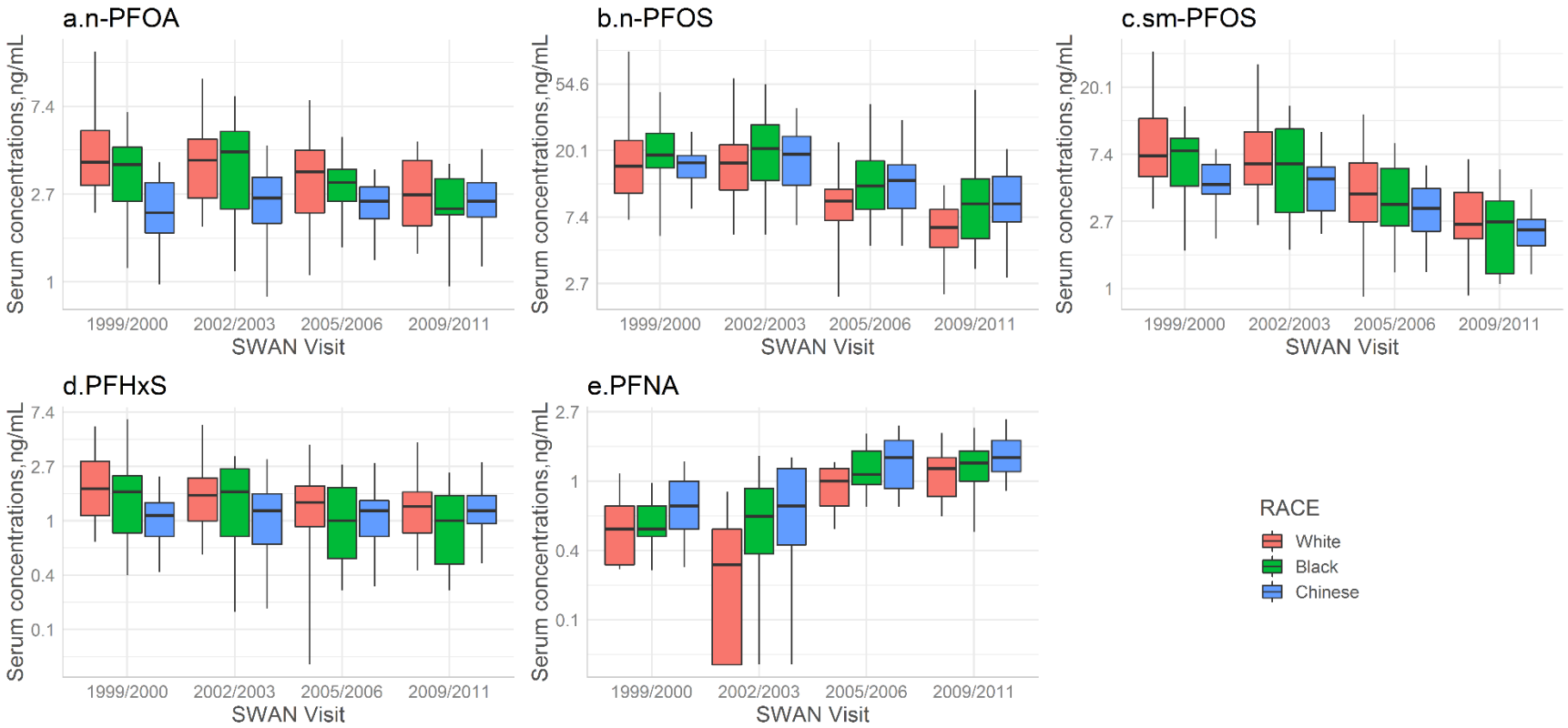


Figure II. 2 Serum concentrations of selected PFAS with detection rates >70% by race/ethnicity in women (n=75) across the United States for four SWAN visits. Boxes represent the 25th-75th percentiles, horizontal lines represent the median, and whiskers indicate 5th and 95th percentiles, respectively. Repeated measure analysis of variance tests was conducted to compare temporal variations of PFAS concentrations by racial/ethnic groups: ***P*=0.007 for n-PFOA; *P*=0.02 for n-PFOS; *P*=0.13 for Sm-PFOS; *P*=0.04 for PFHxS; and *P*=0.19 for PFNA**. Note that a log scale is used for Y axis. The limits of detection were 0.1 ng/mL for all PFAS analytes.
Abbreviations: n-PFOA, linear-chain perfluorooctanoic acid; PFNA, perfluorononanoic acid; PFHxS, perfluorohexane sulfonic acid; n-PFOS, linear-chain perfluorooctane sulfonic acid; Sm-PFOS, sum of branched-chain perfluorooctane sulfonic acid.

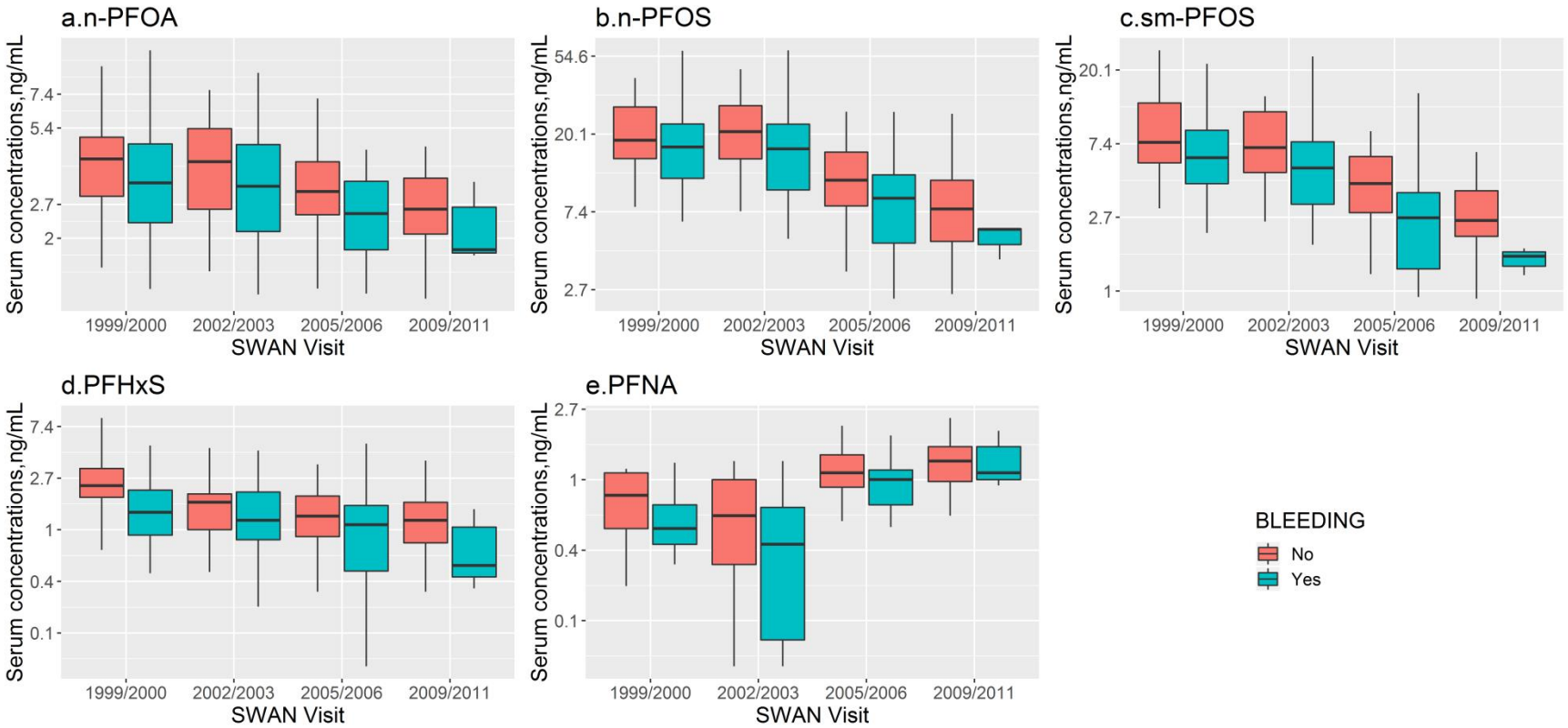


Figure II. 3 Serum concentrations of selected PFAS with detection rates >70% by menstruation status (i.e. whether had menstrual bleeding since last visit) in women (n=75) across the United States for four SWAN visits. Boxes represent the 25th-75th percentiles, horizontal lines represent the median, and whiskers indicate 5th and 95th percentiles, respectively. Repeated measure analysis of variance tests was conducted to compare temporal variations of PFAS concentrations by menstruation status: ***P*=0.31 for n-PFOA; *P*=0.29 for n-PFOS; *P*=0.80 for Sm-PFOS; *P*=0.36 for PFHxS; *P*=0.07 for PFNA**. Note that a log scale is used for Y axis. The limits of detection were 0.1 ng/mL for all PFAS analytes. Abbreviations: n-PFOA, linear-chain perfluorooctanoic acid; PFNA, perfluorononanoic acid; PFHxS, perfluorohexane sulfonic acid; n-PFOS, linear-chain perfluorooctane sulfonic acid; Sm-PFOS, sum of branched-chain perfluorooctane sulfonic acid.

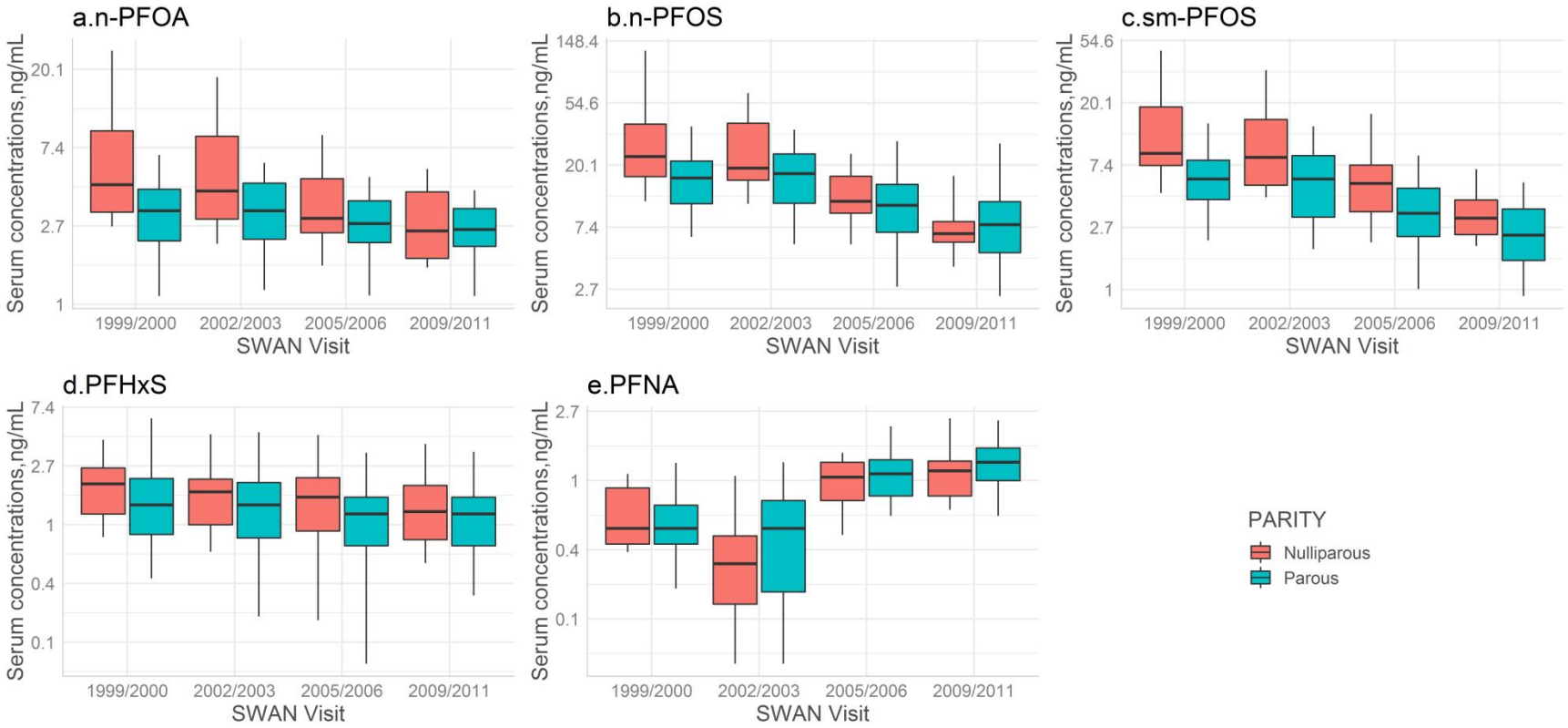


Figure II. 4 Serum concentrations of selected PFAS with detection rates >70% by parity status (nulliparous or parous) in women (n=75) across the United States for four SWAN visits. Boxes represent the 25th-75th percentiles, horizontal lines represent the median, and whiskers indicate 5th and 95th percentiles, respectively. Repeated measure analysis of variance tests was conducted to compare temporal variations of PFAS concentrations by parity group: ***P*=0.03 for n-PFOA; *P*=0.03 for n-PFOS; *P*=0.19 for Sm-PFOS; *P*=0.99 for PFHxS; *P*=0.28 for PFNA**. Note that a log scale is used for Y axis. The limits of detection were 0.1 ng/mL for all PFAS analytes. Abbreviations: n-PFOA, linear-chain perfluorooctanoic acid; PFNA, perfluorononanoic acid; PFHxS, perfluorohexane sulfonic acid; n-PFOS, linear-chain perfluorooctane sulfonic acid; Sm-PFOS, sum of branched-chain perfluorooctane sulfonic acid.

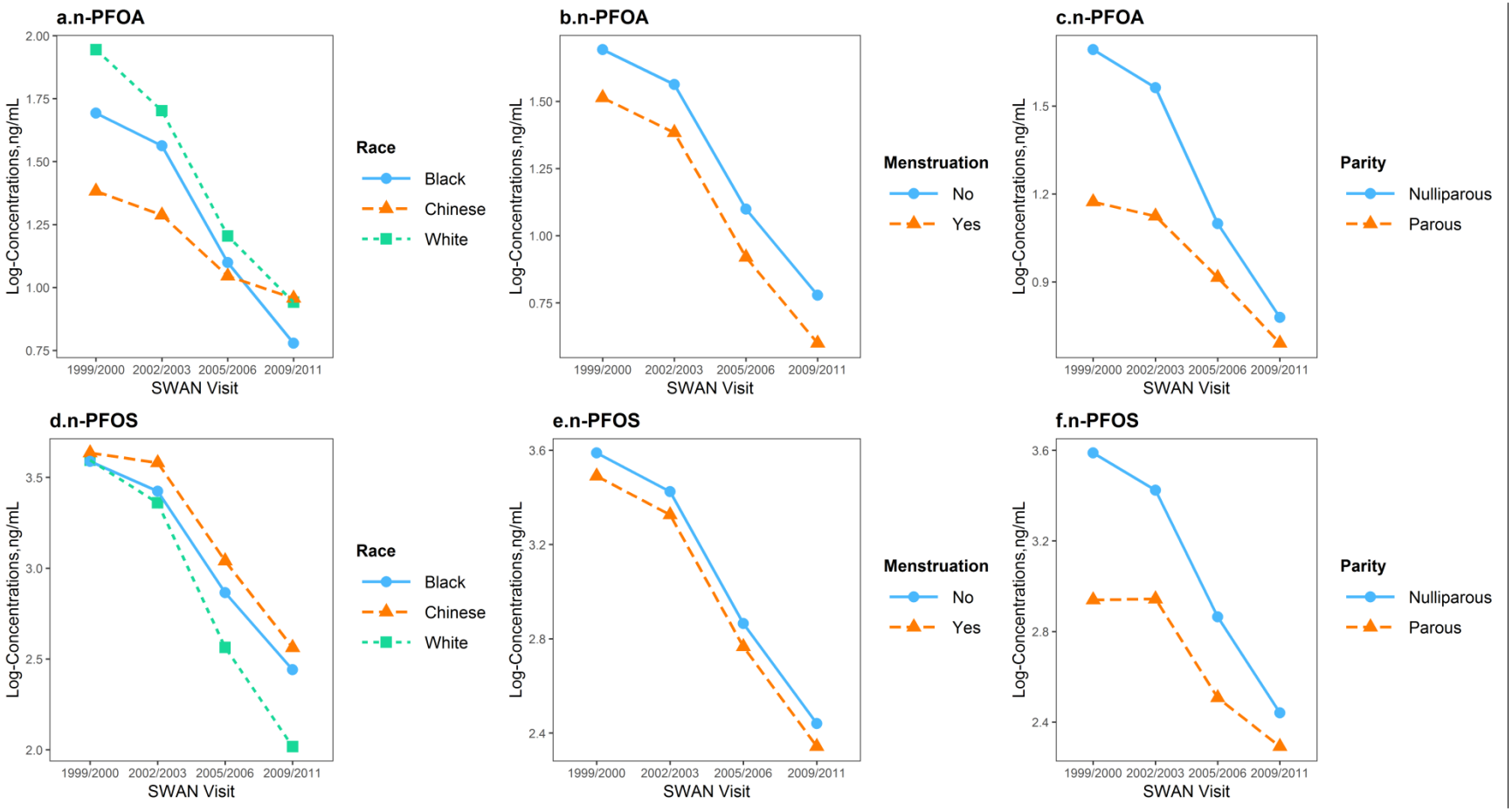


Figure II. 5 Predicted temporal trends of log-transformed n-PFOA, n-PFOS, Sm-PFOS, PFHxS and PFNA serum concentrations at SWAN V03 (1999/2000), V06 (2002/2003), V09 (2005/2006) and V12 (2009/2011), stratified by race/ethnicity, menstruation status and parity. The models were adjusted for age at baseline, study site and body mass index at baseline, based on fixed effects estimated from mixed regression models. Abbreviations: n-PFOA, linear-chain perfluorooctanoic acid; PFNA, perfluorononanoic acid; PFHxS, perfluorohexane sulfonic acid; n-PFOS, linear-chain perfluorooctane sulfonic acid; Sm-PFOS, sum of branched-chain perfluorooctane sulfonic acid.

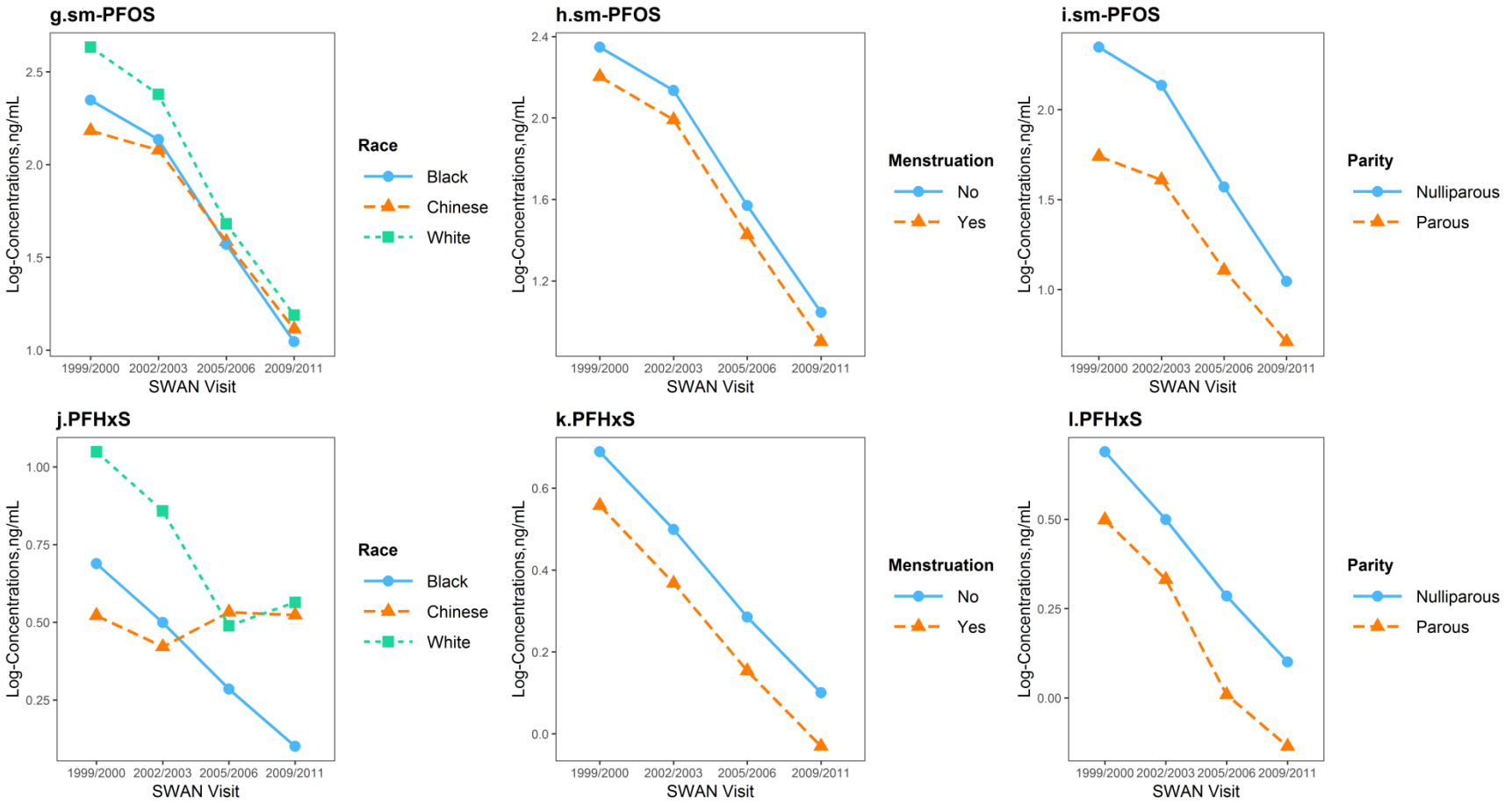


Figure II.5 Continued Predicted temporal trends of log-transformed n-PFOA, n-PFOS, Sm-PFOS, PFHxS and PFNA serum concentrations at SWAN V03 (1999/2000), V06 (2002/2003), V09 (2005/2006) and V12 (2009/2011), stratified by race/ethnicity, menstruation status and parity. The models were adjusted for age at baseline, study site and body mass index at baseline, based on fixed effects estimated from mixed regression models. Abbreviations: n-PFOA, linear-chain perfluorooctanoic acid; PFNA, perfluorononanoic acid; PFHxS, perfluorohexane sulfonic acid; n-PFOS, linear-chain perfluorooctane sulfonic acid; Sm-PFOS, sum of branched-chain perfluorooctane sulfonic acid.

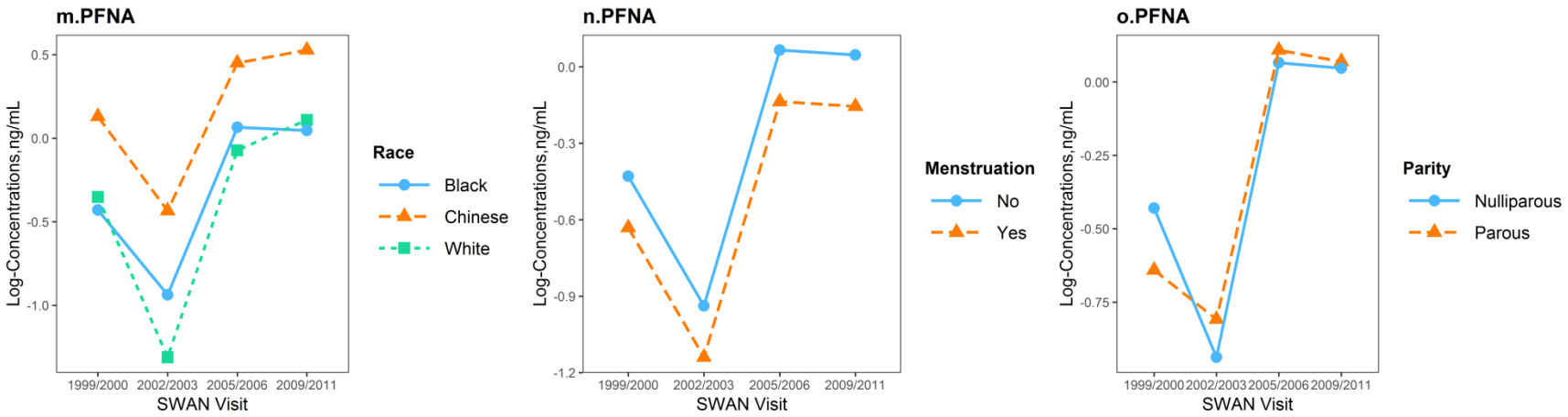


Figure II.5 Continued Predicted temporal trends of log-transformed n-PFOA, n-PFOS, Sm-PFOS, PFHxS and PFNA serum concentrations at SWAN V03 (1999/2000), V06 (2002/2003), V09 (2005/2006) and V12 (2009/2011), stratified by race/ethnicity, menstruation status and parity. The models were adjusted for age at baseline, study site and body mass index at baseline, based on fixed effects estimated from mixed regression models. Abbreviations: n-PFOA, linear-chain perfluorooctanoic acid; PFNA, perfluorononanoic acid; PFHxS, perfluorohexane sulfonic acid; n-PFOS, linear-chain perfluorooctane sulfonic acid; Sm-PFOS, sum of branched-chain perfluorooctane sulfonic acid.

Supplemental Table II. 1 Perfluoroalkyl and polyfluoroalkyl substances (PFAS) analyzed in the serum samples.

Analyte (Long Name)	Analyte	Formula
2-(N-ethyl-perfluorooctane sulfonamide) acetate	ET-PFOSA-ACOH	$C_{12}H_8F_{17}NO_4S$
2-(N-methyl-perfluorooctane sulfonamide) acetate	ME-PFOSA-ACOH2	$C_{11}H_6F_{17}NO_4S$
n-perfluorooctanoate	n-PFOA	$C_8HF_{15}O_2$
branched perfluorooctanoates[1]	Sb-PFOA	$C_8HF_{15}O_2$
perfluorohexane sulfonate	PFHxS	$C_6HF_{13}O_3S$
perfluorododecanoate	PFDOA	$C_{12}HF_{23}O_2$
perfluoroundecanoate	PFUA	$C_{11}HF_{21}O_2$
perfluorodecanoate	PFDeA	$C_{10}HF_{19}O_2$
branched perfluorooctane sulfonate[2]	Sm-PFOS	$C_8HF_{17}O_3S$
n-perfluorooctane sulfonate	n-PFOS	$C_8HF_{17}O_3S$
perfluorononanoate	PFNA	$C_9HF_{17}O_2$

[1]Sb-PFOA include perfluoro-3-methylheptanoic acid, perfluoro-4-methyheptanoic acid, perfluoro-5-methyheptanoic acid, perfluoro-6-methyheptanoic acid, perfluoro-4,4-dimethylhexanoic acid, perfluoro-5,5-dimethylhexanoic acid, perfluoro-3,5-dimethylhexanoic acid, and perfluoro-4,5-dimethylhexanoic acid.

[2]Sm-PFOS include perfluoro-3-methylheptane sulfonate, perfluoro-4-methylheptane sulfonate, perfluoro-5-methylheptane sulfonate, and perfluoro-6-methylheptane sulfonate.

Supplemental Table II. 2 Comparisons of characteristics between study participants at SWAN V03 (1999/2000) (n=1,400) and those in the PFAS temporal variation sub-study at V03 (n=75).

	Participants (n=1,400)	**Substudy**	
		Unweighted (n=75)	**Weighted[a] (n=345)**
Socio-demographic characteristics	**Mean (SD) or N (%)**	**Mean (SD) or N (%)**	**Mean (95% CI) or % (95% CI)**
Age at interview, years	49.5 (2.6)	49.4 (2.4)	49.4 (48.8-50.0)
Education			
High school or less	252 (18.1%)	9 (12.0%)	9.0% (2.0%-16.0%)
Some college	448 (32.2%)	20 (26.7%)	28.8% (17.1%-40.5%)
College degree or higher	693 (49.7%)	46 (61.3%)	62.2% (50.0%-74.4%)
Employed	1,213 (86.6%)	70 (93.3%)	91.7% (84.3%-99.1%)
Difficulty paying for basics (V00)			
Very difficult	89 (6.5%)	5 (6.9%)	7.8% (0.7%-14.9%)
Somewhat difficult	347 (25.2%)	16 (22.2%)	20.9% (10.5%-31.2%)
Not at all difficult	942 (68.3%)	51 (70.8%)	71.3% (59.9%-82.7%)
Study sites			
Michigan	257 (18.4%)	19 (25.3%)	25.0% (14.2%-35.7%)
Boston	233 (16.6%)	23 (30.7%)	29.0% (17.5%-40.5%)
Oakland	309 (22.1%)	33 (44.0%)	46.0% (33.1%-58.8%)
Los Angeles	366 (26.1%)	NA	NA
Pittsburgh	235 (16.8%)	NA	NA
Race/ethnicity			
Black	308 (22.0%)	19 (25.3%)	24.9 (14.0%-35.8%)
White	708 (50.6%)	37 (49.3%)	47.0% (34.2%-59.7%)
Japanese	207 (14.8%)	NA	NA
Chinese	177 (12.6%)	19 (25.3%)	28.1% (16.2%-40.0%)
Physical activity[b]	7.8 (1.7)	7.7 (2.0)	7.9 (7.5-8.4)
Biomarkers	**Mean (SD) or %**	**Mean (SD) or %**	**Mean (95% CI) or % (95% CI)**
Body mass index, kg/m^2	27.9 (7.3)	28.0 (7.1)	27.6 (26.0-29.2)

Percent body fat, % (V06)	37.3 (7.9)	37.0 (7.7)	36.9 (34.9-38.8)
Total body water, kg (V06)	33.6 (5.5)	33.7 (5.0)	34.0 (32.6-35.4)
Estradiol, pg/mL	65.8 (81.4)	64.8 (78.6)	69.2 (48.7-89.7)
Daily dietary intake (V00)	**Mean (SD) or %**	**Mean (SD) or %**	**Mean (95% CI) or % (95% CI)**
Protein, g	70.1 (26.8)	71.1 (29.2)	70.9 (63.4-78.3)
Fiber, g	12.9 (6.2)	13.0 (6.2)	13.4 (11.7-15.1)
Total calorie, kcal	1816.8 (695.1)	1841.5 (760.3)	1851.5 (1643.4-2059.6)
Tuna			
Never	414 (29.9%)	23 (31.1%)	31.4% (19.5%-43.3%)
2 times per month	356 (25.7%)	18 (24.3%)	23.1% (12.5%-33.6%)
4 times per month	324 (23.4%)	14 (18.9%)	20.2% (9.8%-30.6%)
12 times per month	167 (12.1%)	9 (12.2%)	13.4% (4.4%-22.4%)
23 times per month	78 (5.6%)	6 (8.1%)	8.3% (1.6%-15.0%)
Once per day	38 (2.8%)	4 (5.4%)	3.6% (0-7.8%)
Twice per day	6 (0.4%)	NA	NA
30 times per week	NA	NA	NA
Shellfish			
Never	568 (41.1%)	32 (43.2%)	42.1% (29.4%-54.8%)
2 times per month	409 (29.6%)	21 (28.4%)	25.8% (14.7%-36.9%)
4 times per month	263 (19.0%)	11 (14.9%)	16.8% (6.9%-26.7%)
12 times per month	88 (6.4%)	7 (9.5%)	10.9% (2.7%-19.0%)
23 times per month	35 (2.5%)	1 (1.4%)	2.2% (0-6.5%)
Once per day	16 (1.2%)	2 (2.7%)	2.3% (0-6.2%)
Twice per day	2 (0.1%)	NA	NA
30 times per week	1 (0.1%)	NA	NA
Other fish			
Never	365 (26.4%)	20 (27.0%)	29.6% (17.9%-41.4%)
2 times per month	315 (22.8%)	12 (16.2%)	14.4% (6.3%-22.4%)
4 times per month	332 (24.0%)	17 (23.0%)	24.2% (13.0%-35.5%)
12 times per month	236 (17.1%)	14 (18.9%)	16.6% (7.2%-26.1%)
23 times per month	96 (6.9%)	9 (12.2%)	11.3% (3.4%-19.2%)
Once per day	29 (2.1%)	2 (2.7%)	3.9% (0-9.2%)
Twice per day	4 (0.3%)	NA	NA
30 times per week	6 (0.4%)	NA	NA

Fried fish or fish sandwich			
Never	715 (51.7%)	48 (64.9%)	62.1% (49.8%-74.5%)
2 times per month	259 (18.7%)	10 (13.5%)	16.5% (6.6%-26.4%)
4 times per month	195 (14.1%)	8 (10.8%)	9.5% (2.5%-16.5%)
12 times per month	138 (10.0%)	5 (6.8%)	8.5% (1.0%-16.0%)
23 times per month	60 (4.3%)	2 (2.7%)	1.9% (0-4.7%)
Once per day	14 (1.0%)	1 (1.4%)	1.5% (0-4.5%)
Twice per day	1 (0.1%)	NA	NA
30 times per week	1 (0.1%)	NA	NA
Menstruation	**%**	**%**	**% (95% CI)**
Menstrual bleeding since last visit	1,206 (86.1%)	67 (89.3%)	88.6% (80.2%-96.9%)

[a] Descriptive statistics were calculated after taking into account sampling weights from stratified random sampling. We created a population of 345 women which were the same as the sampling frame at the selection of study subjects into the pilot project.

[b] Physical activity was assessed with a modified version of the Kaiser Physical Activity Survey (KPAS) as per Ainsworth et al. 2000 at visit 03. Adapted from the Baecke physical activity questionnaire (Baecke et al. 1982), the KPAS assesses activity levels during the previous 12 months in 3 distinct domains: active living (e.g. frequency of television viewing (reverse coded), active transportation such as walking to work); household/caregiving (e.g. housework, childcare); and sports/exercise (e.g. participation in recreational activity). Domain-specific activity indices were calculated from mostly ordinal Likert scale categorical responses, with higher scores indicating greater activity in that specific domain (range: 1-5).

Supplemental Table II. 3 Comparisons of serum PFAS concentrations between study participants at SWAN V03 (1999/2000) (n=1,400) and those in the PFAS temporal variation sub-study at V03 (n=75).

Serum concentrations, ng/mL	Participants (n=1,400)		Substudy (n=75)	
	Percent detected	GM (GSD)	Percent detected	GM (GSD)
Linear PFOA	99.9%	4.08 (1.82)	100%	3.46 (1.90)
Branched PFOA	18.3%	0.11 (2.46)	17.3%	0.10 (2.38)
Linear PFOS	100%	17.88 (1.80)	100%	17.21 (1.90)
Branched PFOS	99.9%	7.18 (2.02)	100%	6.55 (2.05)
ET-PFOSA-ACOH	99.0%	1.24 (2.53)	98.7%	1.21 (2.91)
ME-PFOSA-ACOH2	99.6%	1.45 (2.04)	100%	1.45 (1.99)
PFHxS	99.6%	1.58 (2.23)	98.7%	1.47 (2.30)
PFDeA	41.1%	0.13 (2.21)	42.7%	0.13 (2.11)
PFUA	32.0%	0.12 (2.26)	36.0%	0.12 (2.07)
PFDoA	3.8%	<LOD	4.0%	<LOD
PFNA	97.1%	0.55 (1.82)	97.3%	0.54 (1.80)

[a] Descriptive statistics were calculated after taking into account sampling weights from stratified random sampling. We created a population of 345 women which were the same as the sampling frame at the selection of study subjects into the pilot project.

[b] Values below level of detection (LOD) were replaced by LOD/$\sqrt{2}$.

Supplemental Table II. 4 Summary of PFAS serum concentrations (ng/mL) measured in SWAN PFAS temporal trend sub-study.

	SWAN V03	SWAN V06	SWAN V09	SWAN V12	ICC
Year of sample collection	1999-2000	20002-2003	2005-2006	2009-2011	
No. of participants	75	75	75	75	
Serum PFAS concentrations, ng/mL					
Total PFOA					0.47
GM (GSD)	3.63 (1.89)	3.36 (1.92)	2.88 (1.74)	2.60 (1.56)	
Median (IQR)	3.57 (2.37, 5.07)	3.37 (2.37, 4.97)	2.97 (2.27, 3.87)	2.67 (1.97, 3.57)	
95th percentile	14.00	9.97	6.67	4.77	
99th percentile	35.87	26.77	11.27	7.07	
Min, max	0.77-35.87	0.37-26.77	0.47-11.27	0.57-7.07	
Linear PFOA					0.18
Percent detected	100%	100%	100%	100%	
GM (GSD)	3.46 (1.90)	3.27 (1.96)	2.80 (1.78)	2.52 (1.59)	
Median (IQR)	3.30 (2.30, 4.80)	3.30 (2.30, 4.90)	2.90 (2.20, 3.80)	2.60 (1.90, 3.50)	
95th percentile	12.30	9.90	6.60	4.70	
99th percentile	35.80	26.70	11.20	7.00	
Min, max	0.7-35.8	0.3-26.7	0.4-11.2	0.5-7.0	
Branched PFOA					NA[b]
Percent detected	17.33%	0%	0%	0%	
GM (GSD)	0.10 (2.38)	<LOD	<LOD	<LOD	
Median (IQR)	<LOD	<LOD	<LOD	<LOD	
95th percentile	1.00	<LOD	<LOD	<LOD	
99th percentile	1.70	<LOD	<LOD	<LOD	
Min, max	<LOD-1.7	<LOD	<LOD	<LOD	
Total PFOS					0.46
GM (GSD)	24.04 (1.91)	23.37 (1.89)	13.81 (1.95)	10.18 (1.93)	
Median (IQR)	22.80 (16.90, 34.70)	23.70 (16.10, 33.20)	15.30 (9.90, 21.10)	10.40 (7.20, 14.90)	
95th percentile	96.20	78.40	43.30	32.90	
99th percentile	177.80	103.60	53.70	57.00	
Min, max	6.1-177.8	6.1-103.6	2.7-53.7	1.3-57.0	
Linear PFOS					0.33
Percent detected	100%	100%	100%	100%	
GM (GSD)	17.21 (1.90)	17.17 (1.88)	10.03 (1.97)	7.46 (2.03)	
Median (IQR)	17.00 (11.30, 23.40)	17.60 (11.20, 25.30)	10.80 (7.30, 15.70)	7.50 (4.90, 11.00)	
95th percentile	63.50	58.80	29.80	29.40	
99th percentile	141.50	67.30	48.00	53.50	
Min, max	4.3-141.5	5.1-67.3	1.0-48.0	0.8-53.5	
Branched PFOS					0.25

Percent detected	100%	100%	100%	100%	
GM (GSD)	6.55 (2.05)	5.95 (2.00)	3.55 (2.00)	2.50 (1.79)	
Median (IQR)	6.20 (4.30, 9.40)	6.10 (3.50, 9.50)	3.50 (2.40, 5.90)	2.50 (1.80, 3.90)	
95th percentile	32.50	23.70	9.60	6.70	
99th percentile	63.00	36.30	21.50	7.80	
Min, max	1.2-63.0	1.0-36.3	0.8-21.5	0.5-7.8	
EtFOSAA					NA[b]
Percent detected	98.67%	65.33%	2.67%	1.33%	
GM (GSD)	1.21 (2.91)	0.23 (2.83)	0.07 (1.23)	0.07 (1.28)	
Median (IQR)	1.10 (0.60, 2.40)	0.30 (<LOD, 0.50)	<LOD	<LOD	
95th percentile	7.70	1.70	0.07	0.07	
99th percentile	112.50	3.70	0.30	0.60	
Min, max	<LOD-112.5	<LOD-3.7	<LOD-0.3	<LOD-0.6	
MeFOSAA					0.48
Percent detected	100%	62.67%	80%	50.67%	
GM (GSD)	1.42 (1.99)	0.29 (3.58)	0.30 (2.43)	0.18 (2.64)	
Median (IQR)	1.50 (0.80, 2.30)	0.30 (<LOD, 0.80)	0.30 (0.20, 0.60)	0.20 (<LOD, 0.40)	
95th percentile	5.00	1.90	1.10	0.80	
99th percentile	6.60	8.40	1.60	1.10	
Min, max	0.3-6.6	<LOD-8.4	<LOD-1.6	<LOD-1.1	
PFHxS					0.40
Percent detected	98.67%	98.67%	93.33%	97.33%	
GM (GSD)	1.47 (2.30)	1.31 (2.39)	1.01 (2.69)	1.07 (2.25)	
Median (IQR)	1.50 (0.90, 2.50)	1.50 (0.90, 2.10)	1.20 (<LOD, 1.70)	1.20 (<LOD, 1.70)	
95th percentile	6.30	5.30	3.80	4.20	
99th percentile	11.20	8.90	5.70	5.10	
Min, max	<LOD-11.2	<LOD-8.9	<LOD-5.7	<LOD-5.1	
PFDEA					0.36
Percent detected	42.67%	32%	89.33%	89.33%	
GM (GSD)	0.13 (2.11)	0.13 (2.59)	0.33 (1.99)	0.37 (2.12)	
Median (IQR)	<LOD (<LOD, 0.30)	<LOD (<LOD, 0.30)	0.40 (0.30, 0.50)	0.40 (0.30, 0.60)	
95th percentile	0.40	0.70	0.80	1.20	
99th percentile	0.70	8.40	2.00	2.30	
Min, max	<LOD-0.7	<LOD-8.4	<LOD-2.0	<LOD-2.3	
PFUA					0.44
Percent detected	36%	26.67%	46.67%	66.67%	
GM (GSD)	0.12 (2.07)	0.11 (2.22)	0.15 (2.42)	0.22 (2.43)	
Median (IQR)	<LOD (<LOD, 0.30)	<LOD (<LOD, 0.20)	<LOD (<LOD, 0.30)	0.30 (<LOD, 0.50)	

95th percentile	0.50	0.60	0.70	0.80	
99th percentile	0.50	1.10	1.00	1.00	
Min, max	<LOD-0.5	<LOD-1.1	<LOD-1.0	<LOD-1.0	
PFDOA					NA[b]
Percent detected	4%	2.67%	0%	1.33%	
GM (GSD)	<LOD	<LOD	<LOD	<LOD	
Median (IQR)	<LOD	<LOD	<LOD	<LOD	
95th percentile	<LOD	<LOD	<LOD	<LOD	
99th percentile	0.20	0.20	<LOD	0.20	
Min, max	<LOD-0.2	<LOD-0.2	<LOD	<LOD-0.2	
PFNA					0.16
Percent detected	97.33%	77.33%	100%	100%	
GM (GSD)	0.54 (1.80)	0.36 (2.80)	1.06 (1.51)	1.22 (1.55)	
Median (IQR)	0.50 (0.40, 0.80)	0.50 (0.20, 0.70)	1.10 (0.80, 1.30)	1.30 (0.90, 1.60)	
95th percentile	1.30	1.30	2.20	2.40	
99th percentile	1.60	2.40	2.70	3.50	
Min, max	<LOD-1.6	<LOD-2.4	0.4-2.7	0.3-3.5	

Abbreviations: GM, geometric mean; GSD, geometric standard deviation; IQR, interquartile range.

P value estimated using Kruskal-Wallis test to assess temporal variations of serum PFAS concentrations.

[a] Values below level of detection (LOD) were replaced by LOD/$\sqrt{2}$.

[b] ICC cannot be estimated because serum congener concentrations can barely be detected in at least one of the follow-up visits.

Supplemental Table II. 5 Effect estimates (standard errors) from linear mixed regressions on log (serum PFAS1 concentrations) among 75 women with 300 observations in SWAN 1999-2011.

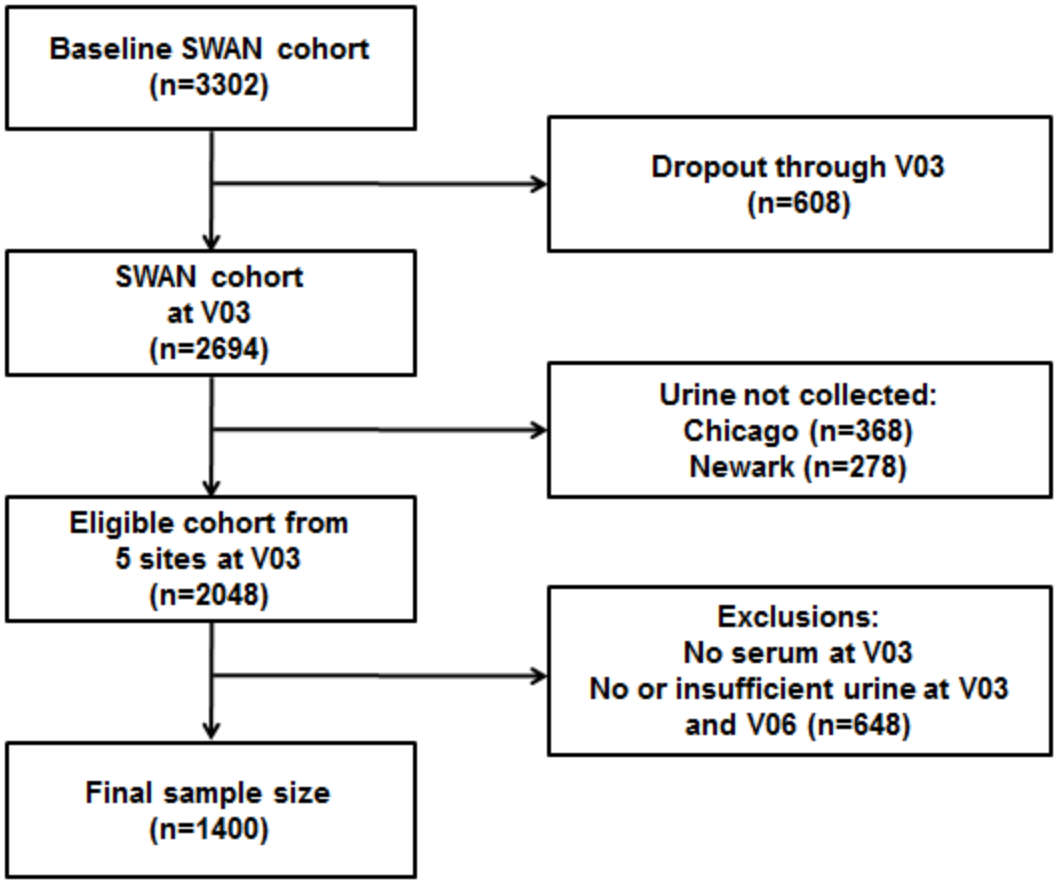
Predictor	n-PFOA	n-PFOS	Sm-PFOS	PFHxS	PFNA
Intercept	1.70 (0.24)***	3.63 (0.28)***	2.34 (0.28)***	0.78 (0.38)*	-0.43 (0.28)
Age at baseline[2]	0.02 (0.02)	0.05 (0.03)	0.04 (0.03)	0.06 (0.04)	0.03 (0.02)
BMI at baseline[3]	0.002 (0.009)	-0.003 (0.01)	0.01 (0.01)	-0.02 (0.01)	0.007 (0.009)
Period					
1999/2000	Ref	Ref	Ref	Ref	Ref
2002/2003	-0.13 (0.18)	-0.16 (0.18)	-0.21 (0.16)	-0.19 (0.24)	-0.51 (0.27)
2005/2006	-0.59 (0.19)**	-0.72 (0.18)***	-0.78 (0.16)***	-0.40 (0.25)	0.49 (0.27)
2009/2011	-0.91 (0.19)***	-1.15 (0.19)***	-1.30 (0.16)***	-0.59 (0.25)*	0.48 (0.28)
Menstruation					
No	Ref	Ref	Ref	Ref	Ref
Yes	-0.18 (0.07)**	-0.10 (0.07)	-0.15 (0.06)*	-0.13 (0.09)	-0.20 (0.09)*
Race/ethnicity					
Black	Ref	Ref	Ref	Ref	Ref
Chinese	-0.31 (0.24)	0.05 (0.29)	-0.16 (0.19)	-0.17 (0.39)	0.56 (0.26)*
White	0.25 (0.18)	0.006 (0.21)	0.29 (0.21)	0.36 (0.28)	0.08 (0.20)
Parity					
Nulliparous	Ref	Ref	Ref	Ref	Ref
Parous	-0.52 (0.18)**	-0.65 (0.21)**	-0.61 (0.21)**	-0.19 (0.28)	-0.21 (0.20)
Site					
Michigan	Ref	Ref	Ref	Ref	Ref
Boston	0.26 (0.15)	-0.10 (0.18)	0.16 (0.19)	-0.12 (0.24)	0.17 (0.14)
Davis	0.05 (0.19)	-0.21 (0.23)	0.05 (0.23)	-0.28 (0.31)	-0.15 (0.18)
Period × Race/ethnicity					
2002/2003 × Chinese	0.03 (0.16)	0.11 (0.15)	0.11 (0.14)	0.09 (0.21)	-0.05 (0.23)
2005/2006 × Chinese	0.26 (0.16)	0.13 (0.15)	0.18 (0.14)	0.41 (0.21)*	-0.17 (0.23)
2009/2011 × Chinese	0.49 (0.16)**	0.08 (0.15)	0.23 (0.14)	0.59 (0.21)**	-0.08 (0.23)
2002/2003 × White	-0.11 (0.14)	-0.07 (0.14)	-0.04 (0.12)	-0.0003 (0.19)	-0.45 (0.20)*
2005/2006 × White	-0.15 (0.14)	-0.31 (0.14)*	-0.17 (0.12)	-0.16 (0.18)	-0.22 (0.20)
2009/2011 × White	-0.09 (0.14)	-0.43 (0.14)**	-0.14 (0.12)	0.10 (0.19)	-0.02 (0.20)

Period × Parity					
2002/2003 × Parous	0.08 (0.16)	0.17 (0.15)	0.08 (0.14)	0.02 (0.21)	0.34 (0.23)
2005/2006 × Parous	0.34 (0.16)*	0.29 (0.15)	0.14 (0.14)	-0.09 (0.21)	0.25 (0.23)
2009/2011 × Parous	0.43 (0.16)**	0.50 (0.15)**	0.27 (0.14)*	-0.05 (0.21)	0.23 (0.23)

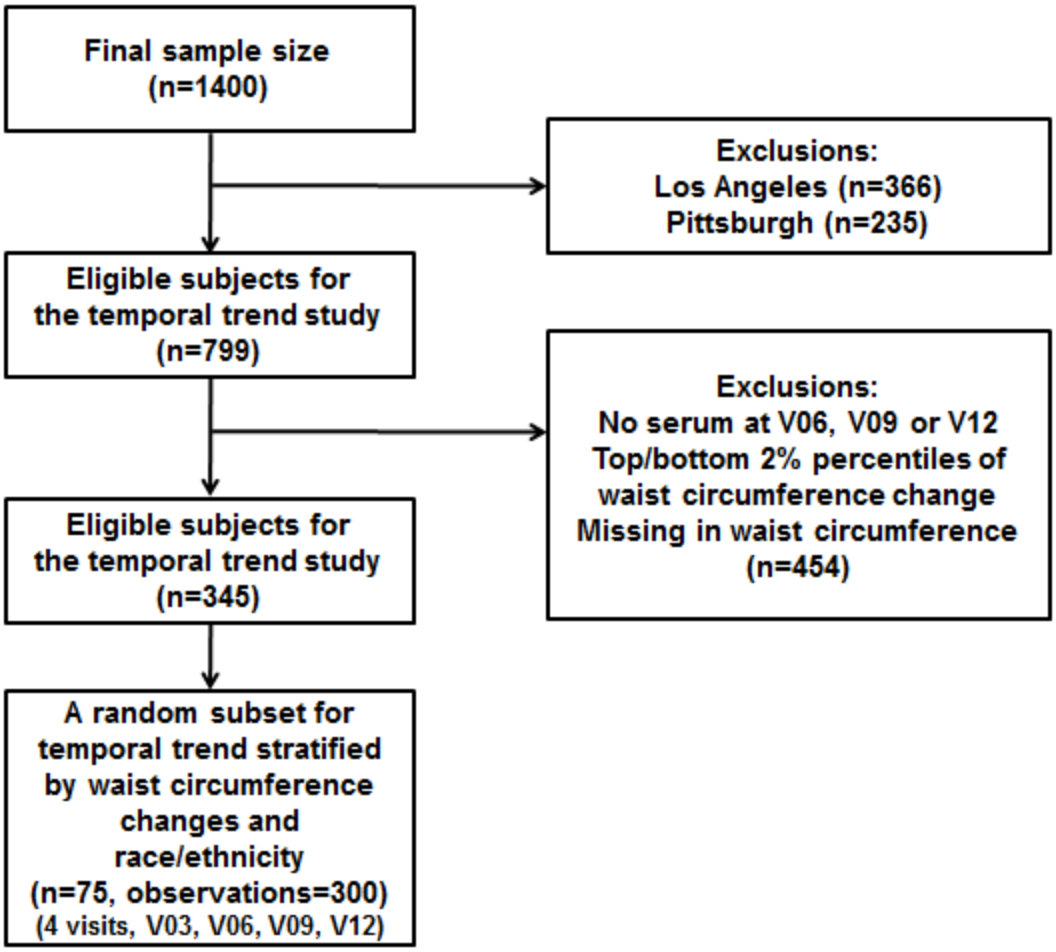
[1]PFAS with serum concentrations above limit of detection more than 70% were included in the analyses.
[2]Age at baseline was centered at 50 years.
[3]BMI at baseline was centered at 25 kg/m^2.
*P<0.05, **P<0.01, ***P<0.001.

Supplemental Table II. 6 Baseline characteristics of study participants at SWAN V03 (1999/2000) by race/ethnicity.

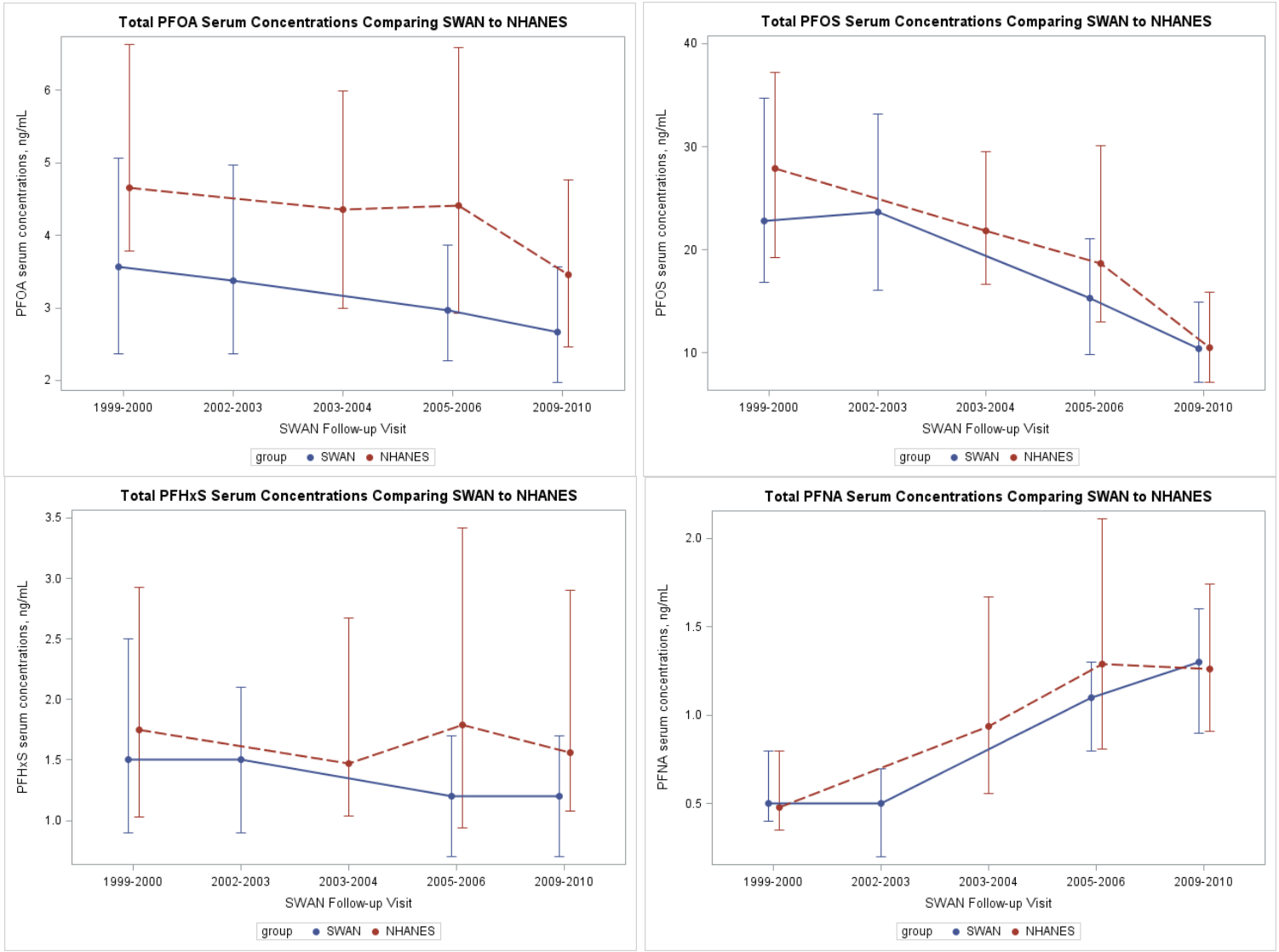
	Total (n=75)	**White (n=37)**	**Black (n=19)**	**Chinese (n=19)**
	Mean (SD) or N (%)	**Mean (SD) or N (%)**	**Mean (SD) or N (%)**	**Mean (SD) or N (%)**
Age at interview, years	49.4 (2.4)	48.8 (2.4)	50.1 (2.5)	49.8 (2.1)
Education				
High school or less	9 (12.0%)	2 (5.4%)	2 (10.5%)	5 (26.3%)
Some college	20 (26.7%)	9 (24.3%)	9 (47.4%)	2 (10.5%)
College degree or higher	46 (61.3%)	26 (70.3%)	8 (42.1%)	12 (63.2%)
Employed	70 (93.3%)	36 (97.3%)	17 (89.5%)	17 (89.5%)
Difficulty paying for basics				
Very difficult	5 (7.0%)	2 (5.7%)	3 (16.7%)	0
Somewhat difficult	16 (22.2%)	9 (25.7%)	4 (22.2%)	3 (15.8%)
Not at all difficult	51 (70.8%)	24 (68.6%)	11 (61.1%)	16 (84.2%)
Study sites				
Michigan	19 (25.3%)	8 (21.6%)	11 (57.9%)	0
Boston	23 (30.7%)	15 (40.5%)	8 (42.1%)	0
Oakland	33 (44.0%)	14 (37.8%)	0	19 (100%)
Physical activity	7.7 (2.0)	8.2 (1.8)	7.2 (2.4)	7.4 (1.7)
Body mass index, kg/m^2	28.0 (7.1)	27.2 (5.8)	33.2 (9.1)	24.3 (4.1)
Menstrual bleeding since last visit	67 (89.3%)	33 (89.2%)	17 (89.5%)	17 (89.5%)
Daily dietary intake				
Protein, g	71.1 (29.2)	67.6 (25.4)	83.1 (37.5)	66.0 (24.7)
Fiber, g	13.0 (6.2)	12.7 (5.7)	13.1 (6.5)	13.7 (7.1)
Total calorie, kcal	1841 (760)	1722 (624)	2223 (1024)	1691 (583)
Fish				
<1 per week	23 (31.1%)	15 (41.7%)	6 (31.6%)	2 (10.5%)
1-2 per week	24 (32.4%)	12 (33.3%)	3 (15.8%)	9 (47.4%)
>2 per week	27 (36.5%)	9 (25.0%)	10 (52.6%)	8 (42.1%)



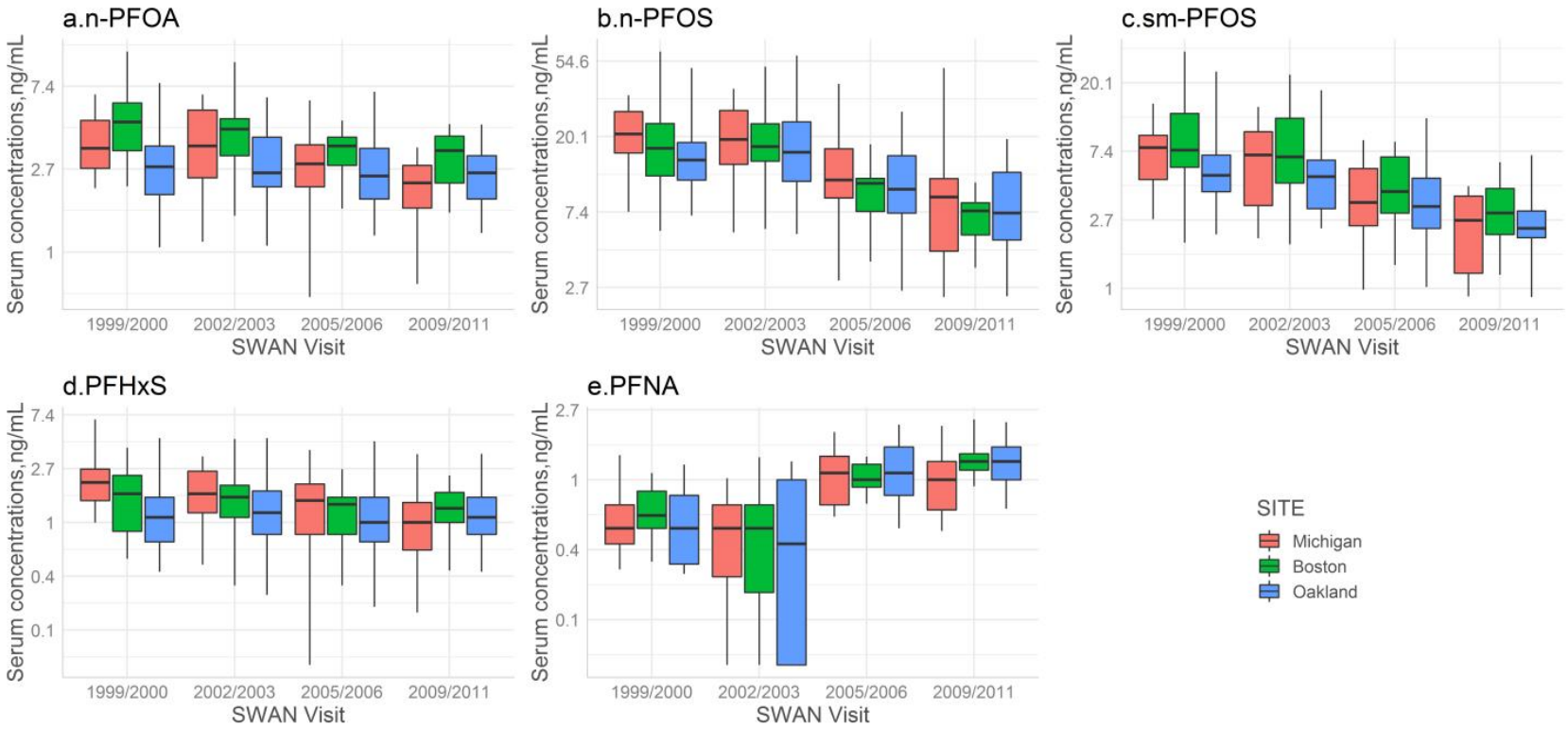
Supplemental Figure II. 1 The study designs of the Study of Women's Health Across the Nation Multi-Pollutant Study (SWAN MPS).



Supplemental Figure II. 2 The study designs of the pilot project to examine temporal variations over time.



Supplemental Figure II. 3 Median (interquartile range) of serum PFAS concentrations (ng/mL) of measured in SWAN among women (n=75) aged 45-56 years at V03(1999-2000), V06 (2002-2003), V09 (2005-2006), and V12 (2009-2010);and in NHANES 1999-2000 (n=91) among women aged 45-56 years, 2003-2004 (n=119) among those aged 48-59 years, 2005-2006 (n=124) among those aged 51-62 years, and 2009-2010 (n=232) among those aged 55-68 years.



Supplemental Figure II. 4 Serum concentrations of selected PFAS with detection rates >70% by study site in women (n=75) across the United States for four SWAN visits. Boxes represent the 25th-75th percentiles, horizontal lines represent the median, and whiskers indicate 5th and 95th percentiles, respectively. Note that a log scale is used for the Y axis. The limits of detection were 0.1 ng/mL for all PFAS analytes. Abbreviations: n-PFOA, linear-chain perfluorooctanoic acid; PFNA, perfluorononanoic acid; PFHxS, perfluorohexane sulfonic acid; n-PFOS, linear-chain perfluorooctane sulfonic acid; Sm-PFOS, sum of branched-chain perfluorooctane sulfonic acid.

Chapter III. Associations between Perfluoroalkyl Substances and Incident Natural Menopause: the Study of Women's Health Across the Nation 1999-2017

Abstract

Context

Previous epidemiologic studies of per- and polyfluoroalkyl substances (PFAS) and menopausal timing conducted in cross-sectional settings were limited by reverse causation because PFAS serum concentrations increase after menopause.

Objectives

To investigate associations between PFAS serum concentrations and incident natural menopause.

Design and Setting

A prospective cohort of midlife women, the Study of Women's Health Across the Nation, from 1999 to 2017.

Participants

1120 multi-racial/ethnic premenopausal women (White, Black, Chinese and Japanese) aged 45-56 years in 1999-2000.

Methods

Serum PFAS concentrations were quantified by online solid phase extraction-high performance liquid chromatography-isotope dilution-tandem mass spectrometry. The final menstrual period was determined during the annual follow-up visits. Cox proportional hazards models were used to calculate hazard ratios (HRs) and 95% confidence intervals (CIs).

Results

Participants contributed 5466 person-years of follow-up, and 578 had incident natural menopause. Compared to the lowest tertile, women at the highest tertile of baseline serum concentrations had adjusted HR for natural menopause of 1.26 (95%CI: 1.02-1.57) for n-perfluorooctane sulfonic acid (n-PFOS) (P_{trend}=0.03), 1.27 (95%CI: 1.01-1.59) for branched-PFOS (P_{trend}=0.03), and 1.31 (95%CI: 1.04-1.65) for n-perfluorooctanoic acid (P_{trend}=0.01). Women were classified into four clusters based on their overall PFAS concentrations as mixtures: low, low-medium, medium-high, and high. Compared to the low cluster, the high cluster had a HR of 1.63 (95% CI: 1.08-2.45), which is interpreted as 2.0 years earlier experience of natural menopause.

Conclusion

This study suggests that select PFAS serum concentrations are associated with earlier natural menopause, a risk factor for adverse health outcomes in later life.

1. Introduction

Menopause marks the cessation of ovarian function, and its timing has physiologic impacts beyond the reproductive system, affecting the overall health of midlife women (Snowdon *et al.*, 1989; Wise *et al.*, 1996). Earlier age at the final menstrual period (FMP) has been associated with an increased risk of overall mortality (Jacobsen *et al.*, 2003; Mondul *et al.*, 2005; Ossewaarde *et al.*, 2005), cardiovascular disease (Atsma *et al.*, 2006; Hu *et al.*, 1999), cardiovascular death (de Kleijn *et al.*, 2002; Mondul *et al.*, 2005; van der Schouw *et al.*, 1996), low bone mineral density (Parazzini *et al.*, 1996) and osteoporosis (Kritz-Silverstein and Barrett-Connor, 1993), and other chronic conditions (Shuster *et al.*, 2010). Ovarian aging reflects the combined effects of genetic factors, socio-demographics, lifestyle and health characteristics (de Bruin *et al.*, 2001; Gold *et al.*, 2001, 2013). Although the etiology of premature menopause (before age 40 years) and early menopause (before age 45 years) is not fully understood, accumulating evidence has suggested that certain environmental exposures may play an important role in the acceleration of ovarian aging (Diamanti-Kandarakis *et al.*, 2009; Grindler *et al.*, 2015; Vabre *et al.*, 2017).

Per- and polyfluoroalkyl substances (PFAS) are a family of anthropogenic environmentally persistent chemicals, some of which also persist in the human body, that have been widely used in many industrial and consumer products, such as non-stick cookware (Bradley *et al.*, 2007; Ewan Sinclair *et al.*, 2007), food packaging (Begley *et al.*, 2005; Schaider *et al.*, 2017; Trier *et al.*, 2011), outdoor apparel (Hill *et al.*, 2017; Lee *et al.*, 2017), and aqueous film-forming foams (Butenhoff *et al.*, 2006; Kantiani *et al.*, 2010; Kissa, 2011; Trudel *et al.*, 2008a). These compounds, especially the most studied perfluorooctanoic acid (PFOA) and perfluorooctane sulfonic acid (PFOS), have been identified as plausible endocrine disruptors

with the potential to cause reproductive disturbances (Jensen and Leffers, 2008; Kar *et al.*, 2017). The potential for reproductive impact is supported by findings from animal toxicology studies with effects on female reproduction, including altered ovarian function, histopathological changes in the reproductive tract and ovarian cell steroidogenesis (Chaparro-Ortega *et al.*, 2018a; Dixon *et al.*, 2012; Zhao *et al.*, 2012), likely through the activation of various transcriptional factors, such as peroxisome proliferator-activated receptors (PPARs) (Andersen *et al.*, 2008; White *et al.*, 2011). However, extrapolations of findings from animal studies to the potential effects of PFAS on human ovarian health are clearly limited, given the species-specific toxicokinetics, metabolism and tissue distributions of PFAS (Lau *et al.*, 2007).

Although three human studies have examined the associations of natural menopause with PFOS, PFOA, perfluorononanoic acid (PFNA) and perfluorohexane sulfonic acid (PFHxS), the results have been inconsistent. A cross-sectional study of mid-Ohio Valley residents found that earlier age at natural menopause was associated with higher concentrations of PFOA and PFOS (Knox *et al.*, 2011); whereas using data from the National Health and Nutrition Examination Survey (NHANES), Taylor et al. observed a significant relationship of earlier natural menopause with PFHxS but not with PFOA, PFOS, PFNA (Taylor *et al.*, 2014). These studies also raised concerns about reverse causation, in that it is unclear whether PFAS exposure contributed to earlier menopause, or cessation of PFAS excretion via cessation of menstruation led to increased serum concentrations of PFAS in women (Dhingra *et al.*, 2017; Konkel, 2014; Ruark *et al.*, 2017; Taylor *et al.*, 2014).

A retrospective cohort study reported no association between PFOA exposure and natural menopause [43]. That study relied on recalled information on age at menopause that had occurred on average >10 years prior to the interview. It is difficult to ascertain the precise timing

of FMP without longitudinal observations of menstrual cycles (Santoro and Johnson, 2019). Potential recall bias may have reduced the accuracy of reported age at natural menopause and presumably biased the study results toward the null (Dhingra *et al.*, 2016). Annual interviews can determine relatively accurate estimates of FMP, and a prospective cohort design with a large, diverse population can provide insights regarding causality that can be more generalizable.

We, therefore, examined the associations between PFAS exposures and incidence of natural menopause in the multi-racial/ethnic sample of women who were premenopausal at baseline from a prospective cohort, i.e., the Study of Women's Health Across the Nation (SWAN). Women were followed every year from 1999-2010 and every other year from 2011-2017. We also assessed whether the relationship differed by racial/ethnic groups and evaluated the combined effects of PFAS mixtures on natural menopause.

2. Materials and methods

Study design

The SWAN cohort, a multi-racial/ethnic, longitudinal study, was designed to characterize physiological and psychosocial changes that occur during the menopausal transition to observe their effects on subsequent risk factors for age-related chronic diseases, as previously described (Sowers *et al.*, 2000). A total of 3,302 premenopaal women aged 42-52 years at baseline were recruited from seven study sites, including Boston, MA; Chicago, IL; Detroit, MI; Los Angeles, CA; Newark, NJ; Oakland, CA; Pittsburgh, PA. Eligible participants had to have an intact uterus, at least one menstrual period in the prior three months, and not have taken hormone medications within the prior three months. Participants self-identified as non-Hispanic White women or one designated minority group, including Black, Chinese, Hispanic and Japanese in a proportion for

each site. Data and specimens were collected every year from 1999-2010 and every other year from 2011-2017. The institutional review board at each participating site approved the study protocol, and all participants provided written, signed informed consent.

The SWAN Multi-Pollutant Study (MPS) was initiated in 2016, using the SWAN follow-up visit 03 (V03, 1999-2000) as the baseline to examine the potential health effects of multiple environmental chemicals, including PFAS, polychlorinated biphenyls, organochloride pesticides, polybrominated diphenyl ethers, metals, phenols, phthalates, and organophosphate pesticide among midlife women. The study design of the SWAN MPS is shown in **Supplemental Figure III.1**. We used repository serum and urine samples collected at SWAN V03, considered the MPS baseline for environmental exposure assessments. Of 2,694 women enrolled at SWAN V03, we did not include women from Chicago (n=368) and Newark (n=278), because urine samples were not available at these two sites. An additional 648 women were excluded due to insufficient volumes of serum or urine samples. Of the remaining1,400 participants with serum samples available at the SWAN-MPS baseline, we excluded 232 women who had already reached natural menopause and 48 women who had had a hysterectomy and/or oophorectomy at the MPS baseline, resulting in a final analytic sample of 1120 women with 6586 observations and 5466 person-years of follow-up through 2017. Additional details of the study design are described elsewhere (Park *et al.*, 2019).

Ascertainment of natural menopause incidence

The age at the natural FMP was determined from annual interviews indicating 12 months of amenorrhea since the last menstrual period for no other causes (including hysterectomy, bilateral oophorectomy or hormone therapy, HT). If a participant was reliably observed to have

had a menstrual bleed followed by at least 12 consecutive months that were both HT-free and bleed-free, her FMP was ascertained. If a woman missed at least three consecutive visits prior to the first post-menopause visit, the FMP date was set to missing.

Measurements of PFAS serum concentrations

Baseline MPS serum samples were sent to the Division of Laboratory Sciences, National Center for Environmental Health, Centers for Disease Control and Prevention (CDC). The CDC laboratory's involvement did not constitute engagement in human-subjects research. Serum samples from subsequent SWAN visits were not analyzed because serum concentrations of the target analytes are relatively stable over time (Ding *et al.*, 2019). We measured perfluorohexane sulfonic acid (PFHxS), n-PFOS, sum of branched isomers of PFOS (Sm-PFOS), n-PFOA, sum of branched PFOA (Sb-PFOA), perfluorononanoic acid (PFNA), perfluorodecanoic acid (PFDA), perfluoroundecanoic acid (PFUnDA), and perfluorododecanoic acid (PFDoA) in 0.1 mL of serum, using an online solid phase extraction-high performance liquid chromatography-isotope dilution-tandem mass spectrometry method (Kato, Basden, *et al.*, 2011). The analytic methods and quality control procedures have been described elsewhere (Ding *et al.*, 2019). The coefficient of variation of low- and high-quality controls ranged from 6% to 12%. The limit of detection (LOD) was 0.1 ng/mL for all the analytes. Concentrations below the LODs were substituted with LOD/$\sqrt{2}$.

Assessments of covariates

Annual visits included an in-person interview, self-administered questionnaires, and measurements of weight and height. All questionnaires were translated into Spanish, Cantonese and Japanese and back-translated; translation discrepancies were resolved by two translators.

Socio-demographic variables included race/ethnicity, study site, and educational attainment from the screening questionnaire. Race/ethnicity was classified into self-identified Black, Chinese, Japanese, or White. We categorized education as high school or less, some college, or college degree or higher. Baseline time-invariant health-related variables included prior oral contraceptive and other exogenous hormone use, and body mass index (BMI) at baseline. We did not consider time-varying BMI in case of over-adjustment bias because PFAS might contribute to weight gain (Liu *et al.*, 2018).

Time-varying lifestyle variables included annual self-reported active smoke exposure and physical activity. Self-reported smoking status was classified as never smoked, former smoked only, or current smoking (Ferris, 1978). Physical activity was assessed using an adaptation of the Kaiser Physical Activity Survey (Sternfeld *et al.*, 1999), which consists of 38 questions with primarily Likert-scale responses about physical activity in various domains, including sports/exercise, household/caregiving, and daily routine (defined as walking or biking for transportation and hours of television watching, which are reverse-coded). Domain-specific indices were derived by averaging the ordinal responses to questions in each domain, resulting in values from 1 to 5. Thus, the total physical activity score ranged from 3 to 15 with 15 indicating the highest level of activity.

Statistical analyses

Bivariate statistics were calculated for participant characteristics at baseline and PFAS serum concentrations stratified by racial/ethnic groups. Chi-square or Fisher's exact statistics were computed for categorical variables; and analysis of variance (ANOVA) or Kruskal-Wallis tests were used for continuous variables. We censored a participant's data when she reported

initiating HT if no subsequent HT-free bleeding occurred, at the date of hysterectomy or bilateral oophorectomy, or at the last menstrual period at the end of data collection if it occurred before 12 months of amenorrhea, on the date of death or on the date of the participants' last follow-up visits. Of the 1120 participants, 578 had an observed date at the natural FMP. The remaining 542 were censored for one of the following reasons: hysterectomy and/or oophorectomy before having ≥12 months of amenorrhea (n=69); had an unknown FMP date because of HT use (n=451); or end of data collection before ≥12 months of amenorrhea (n=22).

Hazard ratios (HRs) and 95% confidence intervals (CIs) of natural menopause incidence were estimated by Cox proportional hazard (PH) regression. We used time since baseline as the time scale. Serum PFAS concentrations were also categorized into tertiles. HRs and 95% CIs were calculated comparing the medium and the highest tertiles of PFAS concentrations to the lowest tertiles (the reference group). To assess the linear trend of the associations between PFAS exposures and incident natural menopause, tertiles of PFAS concentrations were used as continuous variables in the regression models. We also tested the log-linear relationships using log-transformed PFAS concentrations (log-transformed with base 2). In this case, HRs and 95% CIs were interpreted as effects of a two-fold increase in PFAS serum concentrations. Covariates considered in multivariate adjustments included baseline age (continuous), race/ethnicity (White, Black, Chinese, Japanese), educational attainment (high school or less, some college, college graduate, or post-college), time-varying parity status (nulliparous, or parous), time-varying smoking status (never, former, or current smoker), time-varying physical activity, prior HT use at baseline, and baseline BMI. To examine effect modifications by race/ethnicity, we used statistical interaction terms between PFAS exposure and race/ethnicity. Chinese and Japanese were combined because of the small sample sizes of these groups.

People are exposed to multiple and often inter-correlated chemicals. Efforts to study chemical mixtures in isolation can thus result in underestimated environmental effects (Wang, Mukherjee, and Park, 2019; Wang, Mukherjee, *et al.*, 2018). To identify subgroups corresponding to distinct MPS baseline PFAS concentration profiles, a nonparametric portioning method, k-means clustering, was used to find an optimal number of clusters and assign membership to each cluster for each participant (Jain, 2010). The k-means clustering was conducted using PROC FASTCLUS procedures. All PFAS serum concentrations were log-transformed and standardized to z scores to make the distributions normal and comparable before the k-means analysis. The number of clusters was chosen based on cubic clustering criterion, pseudo F statistic (i.e., the ratio of between-cluster variance to within-cluster variance), r-squared statistics, and interpretability. Participant characteristics differed significantly by k-means clusters (**Supplemental Table III.1**). It is possible that there is uncertainty in classifying women based on their overall concentration patterns. Therefore, we utilized inverse probability treatment weighting method to account for confounding due to differences in distributions of these characteristics among clusters (96). HRs of natural menopause incidence were estimated for women with different clusters. We estimated conservative 95% CIs based on the robust variance estimator. All the analyses were performed using SAS, version 9.4 (SAS Institute, Inc., Cary, North Carolina).

Sensitivity analyses

HT use or loss to follow up masked the actual FMP date. We therefore conducted multiple imputations with chain equations for missing FMP age using a comprehensive list of covariates related to timing of menopause (see the list of covariates in the **Supplemental Table III.2**). The imputations were conducted using IVEware. Because we could not impute FMP age

perfectly, we used ten sets of imputations to account for uncertainty, and the pooled results were computed using PROC MIANALYZE.

Hysterectomy and/or oophorectomy was a competing risk in our analyses. Previous studies have suggested that exposures to PFOS, PFOA, and PFNA were associated with increased risk of endometriosis (Campbell *et al.*, 2016). Many women undergo a hysterectomy to help alleviate intolerable symptoms of endometriosis. Because such surgery would mask the age at which a woman would have become menopausal in the absence of surgery, the competing risk may preclude women from participation due to no longer being at risk of reaching the natural FMP. To examine the potential impact of this competing risk on our results, we excluded women who had hysterectomy in the sensitivity analyses. Lastly, we excluded 29 women who reached their natural menopause since baseline to minimize the possibility of reverse causation bias.

3. Results

Study participants

The median (interquartile range, IQR) age of the 1120 premenopausal women was 48.9 (47.0-50.8) years with a range of 45-56 years at baseline (1999-2000) (**Table 1)**. Most women had at least some college education. Educational attainment differed significantly by race/ethnicity, with Black women more likely to receive a high school education or less (p<0.0001) compared to other racial/ethnic groups. Less than 40% of the women had ever smoked; a higher proportion of Black women were current smokers compared to the other racial/ethnic groups (p<0.0001). Physical activity also differed significantly by race/ethnicity, with White women having higher activity scores (p<0.0001). BMI at baseline was significantly higher among Black women and was the lowest in Chinese and Japanese women (p<0.0001).

Chinese and Japanese women were more likely to be nulliparous (p<0.0001) and to report prior use of HT at baseline (p=0.0005).

PFOS and PFOA were the PFAS detected at the highest concentrations (**Supplemental Table III.3**). The median (interquartile range, IQR) serum concentration was 17.1 (12.2-24.5) ng/mL for n-PFOS, 7.2 (4.6-10.8) ng/mL for Sm-PFOS, and 4.0 (2.8-5.7) ng/mL for n-PFOA, 1.5 (0.9-2.3) ng/mL for PFHxS, 0.6 (0.4-0.8) ng/mL for PFNA. PFUnDA, PFDoA, PFDA, and Sb-PFOA were detected in fewer than 40% of baseline samples, and thus they were not considered further in these analyses. Significant racial/ethnic differences were observed in serum PFAS concentrations: White women had the highest concentrations of n-PFOA; Black women had the highest concentrations of n-PFOS, and Sm-PFOS; Chinese and Japanese women had the lowest PFHxS concentrations; White, Chinese and Japanese women had a higher detection rate of PFNA, and significantly higher median concentrations compared to Black women. PFAS were positively correlated amongst each other with Spearman ρs ranging from 0.35-0.82 (**Supplemental Figure III.2**).

Associations between PFAS and incident natural menopause

n-PFOS, Sm-PFOS, n-PFOA and PFNA were associated with earlier age at natural FMP (**Table 2**). After multivariate adjustment for age at baseline, race/ethnicity, study site, education, parity, BMI at baseline, and time-varying physical activity and smoking status, and prior hormone use at baseline, comparing the highest to the lowest tertiles, the HR for natural menopause was 1.26 (95% CI: 1.02-1.57) for n-PFOS (p_{trend} =0.03), 1.27 (95% CI: 1.01-1.59) for Sm-PFOS (p_{trend}=0.03), and 1.31 (95% CI: 1.04-1.65) for n-PFOA (p_{trend} =0.01). The relationship between PFNA and incident natural menopause was not linear but log linear. The HR of natural

menopause was 1.12 (95% CI: 1.01-1.24) per doubling increase in PFNA serum concentrations. No significant association with age of menopause was found for PFHxS in either trend (p_{trend}=0.24) or log-linear analyses (p=0.15). Adjusted survival curves by tertiles of PFAS concentrations are presented in **Supplemental Figures III.3-III.7**. The predicted age at natural menopause in women with tertile 1, tertile 2, and tertile 3 of serum concentrations was: 52.6 years, 52.3 years and 51.6 years for n-PFOS; 52.6 years, 51.9 years and 51.7 years for Sm-PFOS; 52.7 years, 51.9 years and 51.6 years for n-PFOA; 52.7 years, 51.8 years and 51.8 years for PFNA; and 52.4 years, 51.9 years and 51.8 years for PFHxS.

When we examined interaction terms between PFAS concentrations and race/ethnicity, significant associations with incidence of natural menopause were observed for PFNA and n-PFOA among White women but not in other racial/ethnic groups (**Figure 1**). The HR for White women was 1.23 (95% CI: 1.06-1.44) and 1.33 (95% CI: 1.13-1.56) per doubling increase in serum concentrations of n-PFOA and PFNA, respectively, after covariate adjustment. The associations in Black or Asian women did not reach statistical significance. Neither did the results for n-PFOS, Sm-PFOS and PFHxS (**Supplemental Figure III.8**).

In sensitivity analyses, the pooled effect estimates from 10 imputations of age at FMP were largely unchanged, while the 95% CIs became narrower (**Table 3**). However, the significant associations between PFNA concentration and natural menopause disappeared. In the competing risks analyses, 67 women (303 observations) who had hysterectomy and/or oophorectomy were excluded from the analyses, but effect estimates remained similar (**Supplemental Table III.4**). Exclusion of 29 women who reached natural menopause in the six months since baseline did not change results (**Supplemental Table III.5**), diminishing the likelihood that reverse causation bias drove the observed results.

Mixture effects of PFAS on incident natural menopause

Participants were classified into clusters based on their overall PFAS concentrations profiles using the k-means method (**Supplemental Figure III.9)**. Women were classified into four clusters based on serum PFAS concentrations, including "low", "low-medium", "medium-high", and "high" overall concentration patterns. Women in the "low" concentration group had the lowest overall concentrations of PFAS, while those classified into the "high" group exhibited the highest concentrations. After adjusting for confounding, women in the high concentration group were 1.63 (95% CI: 1.08-2.45) times more likely to reach natural menopause earlier, 1.31 (95% CI: 0.94-1.83) for medium-high group and 1.30 (95% CI: 0.97-1.74) for low-medium group, compared to those in the low concentration group (**Supplemental Table III.6**). Participants in the high concentration group had an earlier onset of natural menopause compared to those in other groups (**Figure 2**). The predicted median age at natural menopause in the low concentration group was 52.8 years compared to 51.8 years, 52.0 years and 50.8 years for low-medium, medium-high, and high concentration groups, respectively.

4. Discussion

In this 17-year prospective cohort of 1120 women with 5466 person-years of observation in annual follow-up visits, we found that higher baseline serum concentrations of n-PFOS, Sm-PFOS, n-PFOA, and PFNA were significantly associated with an earlier age at natural FMP. PFHxS concentrations were not associated with incidence of natural menopause. The analysis of mixtures also suggested that the combined PFAS mixtures were associated with earlier onset of natural menopause. These results suggest that PFAS may play an important role in ovarian aging, perhaps through its endocrine disruptive actions.

Comparison with previous epidemiologic studies

To date, evidence on the influence of PFAS exposure on the timing of menopause and ovarian aging has been limited and inconsistent, and has been primarily generated from cross-sectional studies that could not establish causal relationships (Knox *et al.*, 2011; Taylor *et al.*, 2014). Knox et al. found that higher concentrations of PFOA and PFOS were associated with earlier menopausal age in a cross-sectional study of women aged 18-65 years from the C8 Health Project (Knox *et al.*, 2011). This study collected data from highly exposed communities and workers in six public water districts contaminated with PFOA from the DuPont Washington Works Plant near Parkersburg (Frisbee *et al.*, 2009). Taylor et al. reported significant relationships between higher PFHxS concentrations and earlier menopause, but not for PFOA, PFOS and PFNA among the U.S. general women aged 20-65 years from NHANES (Taylor *et al.*, 2014). Using estimated retrospective year-specific serum concentrations for 1951-2011 and PFOA concentrations measured in 2005-2006, no association was observed between earlier age at menopause with PFOA exposure in either retrospective or prospective cohort of C8 Science Panel (Dhingra *et al.*, 2016). However, reverse causation could not be ruled out as women appeared to have higher PFAS concentrations after menopause (Dhingra *et al.*, 2017; Konkel, 2014; Ruark *et al.*, 2017; Taylor *et al.*, 2014).

Given that approximately 90% to 99% of PFAS in the blood are bound to serum albumin (Han *et al.*, 2003; Ylinen and Auriola, 1990), menstrual bleeding could be an important elimination pathway in women. Wong et al.(Wong *et al.*, 2014) found that menstruation could explain the PFOS elimination half-life difference between men and women. Therefore, previously observed associations identified in cross-sectional or retrospective designs (Dhingra *et al.*, 2016; Knox *et al.*, 2011; Taylor *et al.*, 2014) could result from the impact of reproductive

aging on serum PFAS concentrations, rather than their adverse effects on ovarian reserve.

To our knowledge, the current investigation is among the first of studies to evaluate the associations of exposures to various PFAS with the occurrence of natural menopause in a prospective cohort of multi-racial/ethnic midlife women. Our findings of PFOA and natural menopause are not in concordance with Dhingra et al. (Dhingra *et al.*, 2016), the only other published study to our knowledge that has explored the associations between PFOA exposure and incident menopause. Adult women drawn from the C8 Health Project in 2005-2006 were interviewed in 2008-2011 to ascertain the timing of menopause, as part of the work of C8 Science Panel (Dhingra *et al.*, 2016). The different results obtained by the present study and Dhingra et al. (Dhingra *et al.*, 2016) might be attributed to different sampling time intervals, sources and duration of exposures, as well as different demographics of the study participants. The availability of standardized and regular (approximately annual) visits to confirm menopausal status and ascertain age at the natural FMP is a major strength of SWAN and may account for the observed differences in the findings.

No previous research of which we are aware has explored the mixture effects of PFAS on ovarian aging. PFAS are ubiquitous and environmentally persistent (Olsen *et al.*, 2007). People may be normally exposed to multiple PFAS through drinking water, food intake, or use of consumer products (Domingo and Nadal, 2017, 2019). Understanding concentration patterns of multiple PFAS is an important first step before examining the association between PFAS mixtures and incident natural menopause. Results of mixture analyses showed a larger joint effect on ovarian aging compared with single PFAS. Along with our recent study of profiles of urinary concentrations of metal mixtures among midlife women (Wang, Mukherjee, Batterman, *et al.*, 2019), the results of this study suggested that k-means clustering is a useful tool to identify

clusters in the population.

This is also the first study of which we are aware to explore effect modification by race/ethnicity on the associations between PFAS exposure and natural menopause. Although environmental exposure in general is sometimes expected to be higher in racial minority groups and socioeconomically disadvantaged neighborhoods, the concentration patterns tended to depend on the PFAS. Serum concentrations of n-PFOA were found to be higher in White women and PFNA concentrations were relatively higher in White and Chinese women, whereas serum concentrations of n-PFOS and Sm-PFOS were higher in Black women. This is consistent with previous findings (Calafat, Kuklenyik, *et al.*, 2007; Calafat, Wong, *et al.*, 2007; Jain, 2014; Park *et al.*, 2019). White women with higher n-PFOA and PFNA tended to have earlier natural menopause.

PFOA is used as a surfactant and emulsifier in compounds used to coat a variety of food packaging materials, including microwave popcorn bags (Lau *et al.*, 2007; Lindstrom *et al.*, 2011; State Water Resources Control Board, 2010) and is essential in manufacture of the fluoropolymer polytetrafluoroethylene (PTFE) used in non-stick coatings and waterproof fabrics (EFSA, 2016). Uses of PFOS included inks, varnishes, waxes, fire-fighting foams, and coating formulations (Paul *et al.*, 2009). Use of consumer products may have contributed to more exposure to PFOA, while the most dominant source of PFOS exposure might have been intake of contaminated drinking water (Trudel *et al.*, 2008b). Although production and use of some PFAS, including PFOA and PFOS, in the USA is on the decline, environmental exposures to many of these pervasive chemicals continue with associated potential hazards to human reproductive health.

Our study results showed no difference in the effects of n-PFOS and Sm-PFOS by racial/ethnic groups, possibly because of the exclusion of women with premature (before the age of 40 years) or early menopause (before age 45 years), or censoring of Black women who had surgical menopause had higher concentrations of n-PFOS and Sm-PFOS. Asian women with similar PFNA concentrations as White women did not reach their natural menopause earlier. Previous studies have shown increases in PFNA concentrations since 2000 (Calafat *et al.*, 2006; Calafat, Kuklenyik, *et al.*, 2007; Calafat, Wong, *et al.*, 2007; Kato, Wong, *et al.*, 2011; Spliethoff *et al.*, 2008). Future studies with more recent PFNA measurements are needed to confirm our findings and better understand exposure trends.

Biological evidence

PFAS exposures have been associated with diminished ovarian reserve (i.e., the number of ovarian follicles and oocytes) (Bellingham *et al.*, 2009; Chen *et al.*, 2017; Domínguez *et al.*, 2016; Du *et al.*, 2019; Feng *et al.*, 2015, 2017; Hallberg *et al.*, 2019; López-Arellano *et al.*, 2019). The mechanisms of PFAS-induced effects have widely been thought to occur through a peroxisome proliferator-activated receptor (PPAR) mechanism (Andersen *et al.*, 2008; Elcombe *et al.*, 2010; White *et al.*, 2011). PPARs are expressed in the female hypothalamic-pituitary-gonadal axis, and they act on critical processes for ovarian function. For example, PPARs may inhibit transactivation of the estrogen receptor (ER) through competition for estrogen response element (ERE) binding (Keller *et al.*, 1995), down-regulate aromatase expression via nuclear factor-κB (NF-κB) pathway (Fan *et al.*, 2005), and affect enzymatic activity in steroidogenesis (Rak-Mardyła and Karpeta, 2014; Toda *et al.*, 2003).

Accumulating evidence from experimental research suggests that PFAS can directly

interfere with steroidogenic enzyme activities (Chaparro-Ortega *et al.*, 2018b; Shi *et al.*, 2009a; Wang, Bai, *et al.*, 2018). Recently, it was also reported that PFNA and PFOA are weak xenoestrogens, inducing ERα-dependent transcriptional activation *in vitro* and *in vivo* (Benninghoff *et al.*, 2011). As potential endocrine disruptors, PFAS might also suppress the effects of 17β-estradiol (E2) on estrogen-responsive gene expression (Henry and Fair, 2013; Shi *et al.*, 2009b), reduce E2 production and alter the expression of major steroidogenic genes and regulator steroidogenic factors 1 (SF-1) (Du *et al.*, 2013). Disruption of ER signaling pathways may contribute to adverse health effects, such as reproductive failure and acceleration of ovarian aging, thus supporting the notion that women may be particularly vulnerable to reproductive toxicity of PFAS. In addition, experimental studies suggest that PFOA may lead to minimal but significant histopathologic changes in the uterus, vagina, and cervix (Dixon *et al.*, 2012).

Strengths and limitations

The primary strengths of this study included direct measurements of PFAS serum concentrations prior to menopause, prospectively determination of FMP date, and a large cohort of community-based midlife women from four racial/ethnic groups followed for up to 17 years. The reproductive toxicity of PFAS has not been previously characterized among Chinese and Japanese women, to our knowledge. The prospective design also minimized the possibility of reverse causation. Standard annual follow-up visits instead of one-time questionnaire provided reliable estimates of date of FMP. We also consider multiple factors simultaneously in the Cox PH model, censoring at initiation of HT use or at hysterectomy or oophorectomy, thus providing HRs for natural menopause for the independent relations of all exposure factors examined.

Several limitations should be considered as well. First, enrollment at age 45-56 years was

limited to menstruating women, thus women with earlier menopause were excluded. This left-truncation resulted in an overestimation of median age at FMP (Cain *et al.*, 2011). Women who experienced menopause before baseline, especially those with premature menopause (before age 40 years) or early menopause (before age 45 years) were not included in the cohort, which could bias our effect estimates towards the null. Second, more than 40% of the cohort was censored at the initiation of HT, before the participants were classified as post-menopausal. This could have resulted in an underestimation of the age at FMP because these women had higher education levels, which has been associated with later age at menopause. To minimize potential bias, we imputed their FMP age based on covariates related to the timing of menopause. Imputing age at menopause increased sample size and broadened generalizability to women with HT use and thus might have reduced bias. Finally, hysterectomy could be a competing risk of natural menopause. Hysterectomy can be undertaken for medical conditions (such as endometriosis or uterine fibroids, cancer or menorrhagia). We did not have data on the date of onset of these conditions and hence were unable to examine directly the potential effects of PFAS on cause-specific subsets of menopause (either surgically or naturally occurring).

5. Conclusions

Our findings suggest that exposure to select PFAS was associated with earlier natural menopause. Women with highest tertiles of n-PFOS serum concentrations tended to have 1.0 years earlier median time to natural menopause, and 0.9 years and 1.1 years earlier for Sm-PFOS and n-PFOA, respectively, compared to those in the lowest tertiles. High overall PFAS concentration patterns might contribute to 2.0 years earlier median time to natural menopause, compared to the low group. These estimates were roughly equivalent to or even larger than an effect estimate of 1.1 years comparing current smokers vs. never smokers in our sample. Due to

PFAS widespread use and environmental persistence, their potential adverse effects remain a public health concern.

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Table III. 1 Baseline (1999-2000) characteristics of multi-racial/ethnic midlife women by racial/ethnic groups in the Study of Women's Health Across the Nation (n=1120).

Baseline characteristic	Total (n=1120)	White (n=577)	Black (n=235)	Chinese (n=142)	Japanese (n=166)	*p* value[a]
	Median (IQR) or n (%)	Median (IQR) or n (%)	Median (IQR) or n (%)	Median (IQR) or n (%)	Median (IQR) or n (%)	
Age, years	48.9 (47.0-50.8)	48.7 (47.0-50.8)	48.7 (46.8-50.7)	49.3 (47.3-50.7)	49.2 (47.4-50.9)	0.23
Study site						NA
Southeast MI	202 (18.0%)	90 (15.6%)	112 (47.7%)	0	0	
Boston, MA	182 (16.3%)	118 (20.4%)	64 (27.2%)	0	0	
Oakland, CA	242 (21.6%)	100 (17.3%)	0	142 (100%)	0	
Los Angeles, CA	299 (26.7%)	133 (23.1%)	0	0	166 (100%)	
Pittsburgh, PA	195 (23.4%)	136 (23.6%)	59 (25.1%)	0	0	
Educational attainment						<0.0001
≤High school	197 (17.7%)	69 (12.0%)	65 (28.0%)	35 (24.7%)	28 (16.9%)	
Some college	350 (31.4%)	174 (30.3%)	90 (38.8%)	28 (19.7%)	58 (34.9%)	
College	271 (24.3%)	137 (23.9%)	41 (17.7%)	43 (30.3%)	50 (30.1%)	
Post-college	296 (26.6%)	194 (33.8%)	36 (15.5%)	36 (25.3%)	30 (18.1%)	
Parity						<0.0001
Nulliparous	215 (19.2%)	146 (25.3%)	21 (8.9%)	21 (14.8%)	27 (16.3%)	
Parous	905 (80.8%)	431 (74.7%)	214 (91.1%)	121 (85.2%)	139 (83.7%)	
Prior hormone use	248 (22.1%)	151 (26.2%)	54 (23.0%)	21 (14.8%)	22 (13.3%)	0.0005
Smoking status						<0.0001
Never smoker	720 (64.4%)	343 (59.5%)	134 (57.3%)	134 (94.4%)	109 (65.7%)	
Former smoker	291 (26.0%)	187 (32.5%)	55 (23.5%)	7 (4.9%)	42 (25.3%)	
Current smoker	107 (9.6%)	46 (8.0%)	45 (19.2%)	1 (0.7%)	15 (9.0%)	
Physical activity score	7.9 (6.6-9.0)	8.1 (6.9-9.3)	7.3 (6.4-8.6)	7.2 (6.0-8.5)	7.8 (6.7-8.9)	<0.0001
Body mass index, kg/m^2	26.1 (22.7-31.5)	26.5 (22.9-31.7)	31.4 (26.5-37.9)	23.0 (20.9-25.0)	23.3 (21.5-26.2)	<0.0001

IQR, inter-quartile range. NA, not available.

[a] Chi-square tests or Fisher's exact tests were used for categorical variables; analysis of variance tests or Kruskal-Wallis tests were conducted for continuous variables. The significance level was set at 0.05.

Table III. 2 Hazard ratios (HR) (95% confidence intervals, 95% CI) for incident natural menopause with tertile changes and per doubling increase in serum concentrations of n-PFOS, Sm-PFOS, n-PFOA, PFNA, and PFHxS.

PFAS	Tertile of PFAS concentrations			*p* value for trend[c]	Per doubling increase HR (95%CI)	*p* value[c]
	Tertile 1 HR (95%CI)	Tertile 2 HR (95%CI)	Tertile 3 HR (95%CI)			
n-PFOS						
Median (IQR), ng/mL	10.4 (8.1-12.2)	16.9 (15.6-18.7)	28.3 (24.2-37.8)			
no. cases/person-years	183/1861	192/1883	203/1880			
Model 1[a]	Ref	1.04 (0.85-1.27)	1.19 (0.97-1.47)	0.09	1.06 (0.96-1.18)	0.26
Model 2[b]	Ref	1.06 (0.86-1.31)	1.26 (1.02-1.57)	0.03	1.11 (0.99-1.23)	0.06
Sm-PFOS						
Median (IQR), ng/mL	3.8 (2.9-4.5)	7.1 (6.2-8.0)	13.0 (10.7-16.8)			
no. cases/person-years	195/1842	194/1923	189/1858			
Model 1[a]	Ref	1.03 (0.84-1.27)	1.12 (0.90-1.39)	0.30	1.04 (0.95-1.14)	0.37
Model 2[b]	Ref	1.11 (0.90-1.37)	1.27 (1.01-1.59)	0.03	1.08 (0.99-1.19)	0.09
n-PFOA						
Median (IQR), ng/mL	2.3 (1.8-2.8)	4.0 (3.5-4.5)	6.6 (5.6-8.6)			
no. cases/person-years	183/1818	195/1936	200/1870			
Model 1[a]	Ref	1.15 (0.92-1.42)	1.29 (1.03-1.61)	0.02	1.06 (0.95-1.19)	0.27
Model 2[b]	Ref	1.12 (0.90-1.40)	1.31 (1.04-1.65)	0.01	1.11 (0.99-1.24)	0.07
PFNA						
Median (IQR), ng/mL	0.3 (0.3-0.4)	0.5 (0.5-0.6)	0.9 (0.7-1.0)			
no. cases/person-years	168/1930	181/1679	229/2015			
Model 1[a]	Ref	1.18 (0.95-1.46)	1.21 (0.99-1.49)	0.07	1.13 (1.02-1.25)	0.02
Model 2[b]	Ref	1.18 (0.95-1.47)	1.20 (0.97-1.49)	0.10	1.12 (1.01-1.24)	0.04
PFHxS						
Median (IQR), ng/mL	0.8 (0.6-1.0)	1.5 (1.3-1.6)	3.0 (2.3-4.5)			
no. cases/person-years	203/1957	168/1728	207/1939			
Model 1[a]	Ref	0.92 (0.75-1.13)	1.15 (0.94-1.41)	0.19	1.04 (0.97-1.13)	0.27
Model 2[b]	Ref	1.05 (0.84-1.30)	1.11 (0.90-1.37)	0.33	1.03 (0.95-1.11)	0.50

[a] Model 1 was adjusted for age at baseline, race/ethnicity, and study site.

[b] Model 2 was additionally adjusted for education, parity, BMI at baseline, physical activity, smoking status, and prior hormone use at baseline.

[c] The significance level was set at 0.05.

Table III. 3 Pooled hazard ratios (HR) (95% confidence intervals, 95% CI) for incident natural menopause with tertile changes and per doubling increase in serum concentrations of n-PFOS, Sm-PFOS, n-PFOA, PFNA, and PFHxS with 10 imputations.

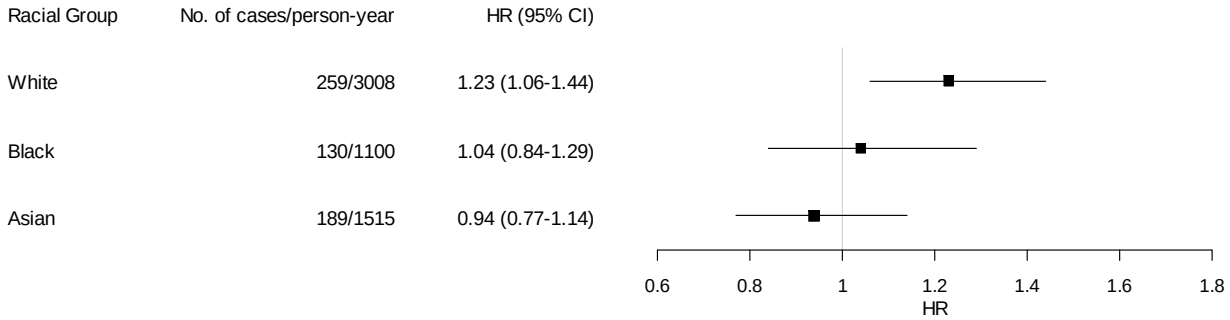
PFAS	Tertile of PFAS concentrations			P value for trend[c]	Per doubling increase HR (95%CI)	P value[c]
	Tertile 1 HR (95%CI)	Tertile 2 HR (95%CI)	Tertile 3 HR (95%CI)			
n-PFOS						
Median (IQR), ng/mL	10.4 (8.1-12.2)	16.9 (15.6-18.7)	28.3 (24.2-37.8)			
no. cases/person-years[a]	315/1487	322/1499	344/1483			
Model 1[b]	Ref	0.98 (0.84-1.16)	1.23 (1.05-1.46)	0.01	1.10 (1.01-1.20)	0.02
Model 2[c]	Ref	0.99 (0.84-1.17)	1.26 (1.06-1.49)	0.01	1.11 (1.02-1.21)	0.02
Sm-PFOS						
Median (IQR), ng/mL	3.8 (2.9-4.6)	7.2 (6.2-8.1)	13.1 (10.9-17.2)			
no. cases/person-years[a]	320/1496	331/1510	330/1463			
Model 1[b]	Ref	1.01 (0.86-1.19)	1.20 (1.01-1.43)	0.04	1.09 (1.01-1.17)	0.02
Model 2[c]	Ref	1.02 (0.86-1.20)	1.25 (1.04-1.50)	0.01	1.11 (1.03-1.20)	0.009
n-PFOA						
Median (IQR), ng/mL	2.3 (1.8-2.8)	4.0 (3.5-4.5)	6.6 (5.6-8.6)			
no. cases/person-years[a]	313/1448	334/1553	334/1468			
Model 1[b]	Ref	1.11 (0.94-1.30)	1.15 (0.98-1.35)	0.06	1.10 (1.01-1.20)	0.03
Model 2[c]	Ref	1.14 (0.96-1.35)	1.23 (1.03-1.47)	0.02	1.10 (1.01-1.21)	0.02
PFNA						
Median (IQR), ng/mL	0.3 (0.3-0.4)	0.5 (0.5-0.6)	0.9 (0.7-1.0)			
no. cases/person-years[a]	331/1522	295/1362	374/1585			
Model 1[b]	Ref	1.00 (0.85-1.19)	1.14 (0.96-1.34)	0.12	1.07 (0.99-1.16)	0.10
Model 2[c]	Ref	0.98 (0.82-1.18)	1.11 (0.94-1.33)	0.20	1.05 (0.97-1.14)	0.23
PFHxS						
Median (IQR), ng/mL	0.8 (0.6-1.0)	1.5 (1.3-1.6)	3.0 (2.3-4.5)			
no. cases/person-years[a]	337/1592	299/1324	344/1553			
Model 1[b]	Ref	1.08 (0.90-1.28)	1.13 (0.97-1.35)	0.10	1.05 (0.99-1.12)	0.09
Model 2[c]	Ref	1.02 (0.86-1.23)	1.11 (0.94-1.31)	0.24	1.05 (0.98-1.12)	0.15

[a] Averaged no. cases and person-years from 10 imputations. [b] Model 1 was adjusted for age at baseline, race/ethnicity, and study site.

[b] Model 2 was additionally adjusted for education, parity, BMI at baseline, physical activity, smoking status, and prior hormone use at baseline.

[c] The significance level was set at 0.05.

a) Exposure to **n-PFOA** and incidence of natural menopause by racial groups



b) Exposure to **PFNA** and incidence of natural menopause by racial groups

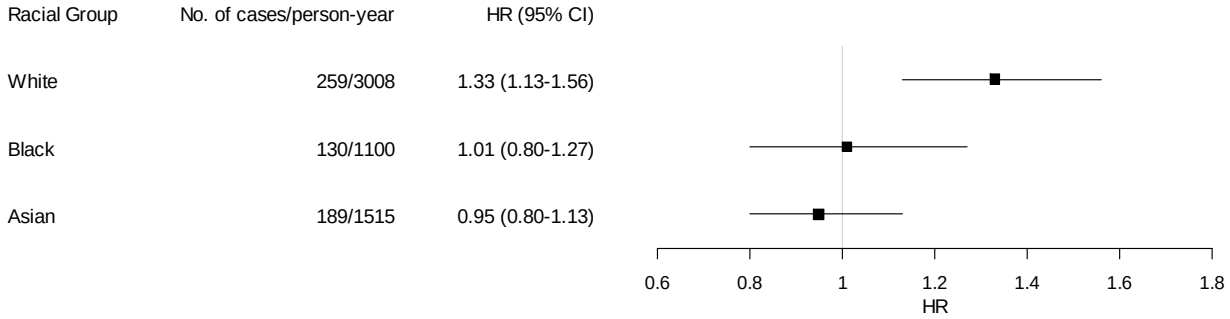


Figure III. 1 Adjusted hazard ratios (HR) (95% confidence intervals, 95% CI) for incident natural menopause with per doubling increase in serum concentrations of n-PFOA and PFNA. Models were adjusted for age at baseline, study site, education, parity, BMI at baseline, physical activity, smoking status, and prior hormone use at baseline. *P* values for the interaction terms with race/ethnicity are 0.08 for n-PFOA and 0.01 for PFNA.

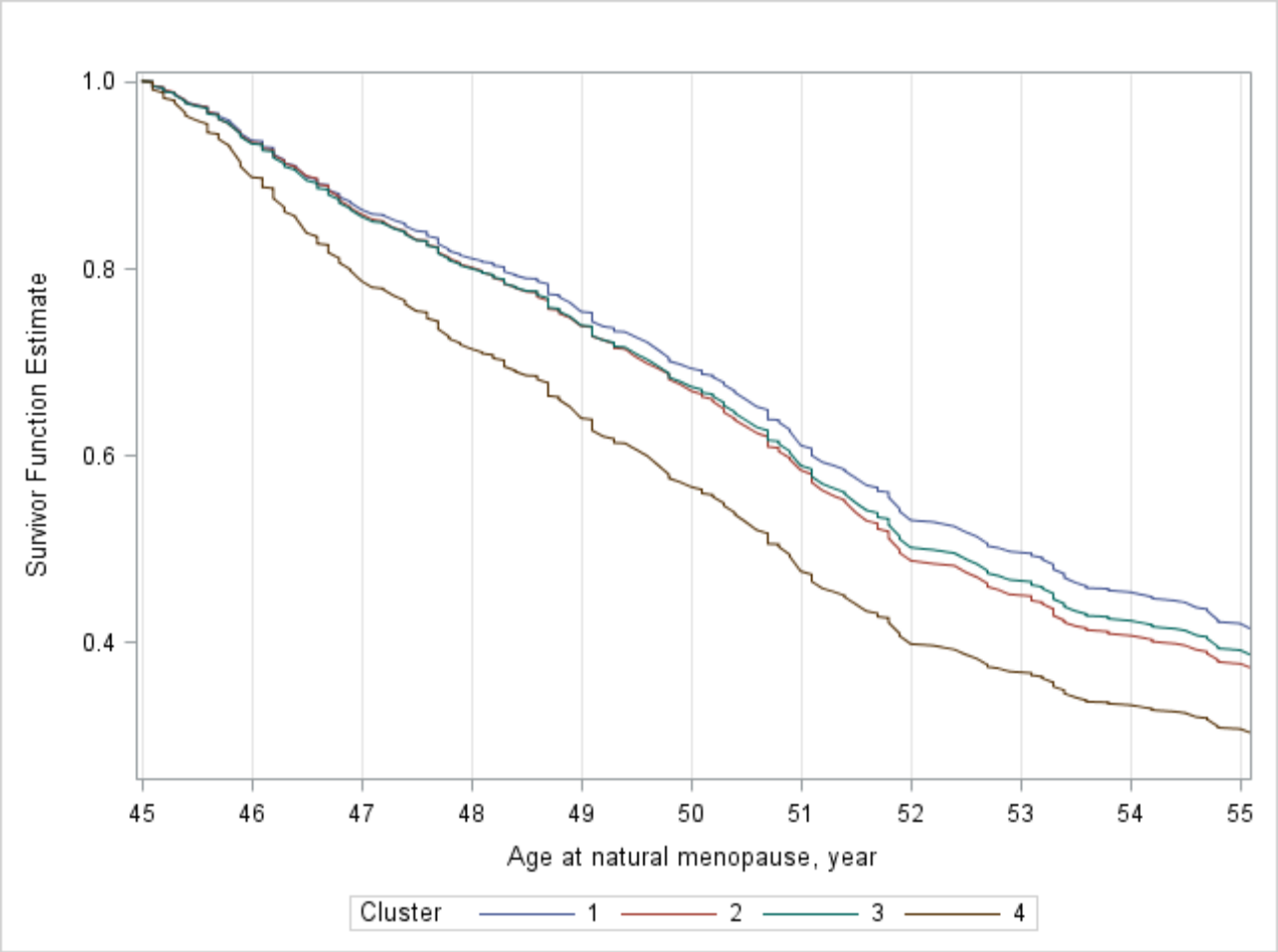


Figure III. 2 Adjusted survival curves for natural menopause by participant clusters. The model was adjusted for age at baseline, race/ethnicity, study site, education, parity, BMI at baseline, physical activity, smoking status, and prior hormone use at baseline. The hazards ratio for low-medium, medium-high, and high groups were 1.30 (95% CI: 0.97-1.74), 1.31 (95% CI: 0.94-1.83), and 1.63 (95% CI: 1.08-2.45), respectively, compared to the low group. The predicted median age at natural menopause for women with low overall PFAS concentration profile was 52.8 years, and 51.8 years, 52.0 years and 50.8 years for those with low-medium, medium-high, and high overall concentration patterns, respectively.

Supplemental Table III. 1 Baseline participant characteristics overall and by K-means clusters.

Baseline characteristic	**Total (n=1120)**	**Low (n=143)**	**Low-medium (n=414)**	**Medium-high (n=406)**	**High (n=157)**	**P value[a]**
	Median (IQR) or n (%)	Median (IQR) or n (%)	Median (IQR) or n (%)	Median (IQR) or n (%)	Median (IQR) or n (%)	
Age, years	48.9 (47.0-50.8)	49.1 (47.0-50.7)	49.2 (47.2-51.1)	48.6 (46.9-50.4)	48.4 (46.9-50.8)	0.04
Race/ethnicity						<.0001
White	577 (51.5%)	60 (42.0%)	199 (48.1%)	226 (55.7%)	92 (58.6%)	
Black	235 (21.0%)	30 (21.0%)	63 (15.2%)	88 (21.7%)	54 (34.4%)	
Chinese	142 (12.7%)	36 (25.1%)	73 (17.6%)	33 (8.1%)	0 (0.0%)	
Japanese	166 (14.8%)	17 (11.9%)	79 (19.1%)	59 (14.5%)	11 (7.0%)	
Study site						<.0001
Southeast MI	202 (18.0%)	17 (11.9%)	67 (16.2%)	70 (17.2%)	48 (30.6%)	
Boston, MA	182 (16.3%)	23 (16.1%)	62 (14.9%)	78 (19.2%)	19 (12.1%)	
Oakland, CA	242 (21.6%)	51 (35.7%)	115 (27.8%)	61 (15.0%)	15 (9.6%)	
Los Angeles, CA	299 (26.7%)	34 (23.8%)	115 (27.8%)	122 (30.1%)	28 (17.8%)	
Pittsburgh, PA	195 (23.4%)	18 (12.6%)	55 (13.3%)	75 (18.5%)	47 (29.9%)	
Educational attainment						0.002
≤High school	197 (17.7%)	28 (19.6%)	73 (17.7%)	61 (15.1%)	35 (22.4%)	
Some college	350 (31.4%)	43 (30.0%)	110 (26.8%)	144 (35.6%)	53 (34.0%)	
College	271 (24.3%)	48 (33.6%)	109 (26.5%)	86 (21.3%)	28 (18.0%)	
Post-college	296 (26.6%)	24 (16.8%)	119 (29.0%)	113 (28.0%)	40 (25.6%)	
Parity						0.02
Nulliparous	215 (19.2%)	16 (11.2%)	76 (18.4%)	92 (22.7%)	31 (19.8%)	
Parous	905 (80.8%)	127 (88.8%)	338 (81.6%)	314 (77.3%)	126 (80.2%)	
Prior hormone use	248 (22.1%)	33 (23.1%)	87 (21.0%)	83 (20.4%)	45 (28.7%)	
Smoking status						0.001
Never smoker	720 (64.4%)	99 (69.7%)	291 (70.3%)	246 (60.7%)	84 (53.5%)	
Former smoker	291 (26.0%)	34 (23.9%)	96 (23.2%)	109 (26.9%)	52 (33.1%)	
Current smoker	107 (9.6%)	9 (6.3%)	27 (6.5%)	50 (12.4%)	21 (13.4%)	
Physical activity score	7.9 (6.6-9.0)	7.5 (6.3-8.8)	7.9 (6.5-9.1)	7.9 (6.8-9.0)	7.7 (6.6-8.6)	0.11
Body mass index, kg/m^2	26.1 (22.7-31.5)	24.6 (22.4-30.2)	25.6 (22.3-30.1)	26.4 (22.7-31.8)	29.4 (24.9-35.1)	<.0001

[a] The significance level was set at 0.05.

Supplemental Table III. 2 Covariates included in the imputation model for FMP age.

Covariates related to timing of menopause
Race/ethnicity
Study site
Frequency of vasomotor symptoms
Bleeding pattern
Estradiol
Follicle-stimulating hormone
Day of menstrual cycle corresponding to blood draw
Age
History of oral contraceptive use
History of exogenous hormone use other than oral contraceptives
Number of live births
Diabetes
Cardiovascular disease diagnosis
Smoking status
Alcohol consumption
Body mass index
Total physical activity without work
Education
Financial strain
Self-reported health
Marital status
Employment status
Months of amenorrhea reported

Supplemental Table III. 3 Median (inter-quartile range, IQR) serum concentrations of n-PFOS, Sm-PFOS, PFHxS, PFDoA, PFDeA, PFNA, n-PFOA, and Sb-PFOA by racial/ethnic groups at the MPS baseline (1999-2000).

PFAS serum concentrations, ng/mL	Total (n=1120)		Caucasian (n=577)		African American (n=235)		Chinese (n=142)		Japanese (n=166)		P value[a]
	%>LOD	Median (IQR)	%>LOD	Median (IQR)	%>LOD	Median (IQR)	%>LOD	Median (IQR)	%>LOD	Median (IQR)	
n-PFOS	100%	17.1 (12.2-24.5)	100%	16.5 (12.0-23.5)	100%	21.7 (15.3-32.2)	100%	15.6 (10.7-20.1)	100%	15.6 (11.2-20.8)	<.0001
Sm-PFOS	99.9%	7.2 (4.6-10.8)	100%	7.6 (5.2-11.6)	100%	8.1 (5.2-12.5)	99.3%	4.4 (3.0-6.1)	100%	6.9 (4.1-9.3)	<.0001
PFHxS	99.6%	1.5 (0.9-2.3)	99.8%	1.6 (1.1-2.8)	99.6%	1.6 (1.0-2.6)	99.3%	1.0 (0.6-1.5)	99.4%	1.2 (0.8-1.6)	<.0001
PFUA	30.0%	<LOD (<LOD-0.2)	17.9%	<LOD	33.6%	<LOD (<LOD-0.2)	50.7%	0.15 (<LOD-0.4)	49.4%	<LOD (<LOD-0.5)	NA
PFDoA	4.0%	<LOD	2.6%	<LOD	8.5%	<LOD	0.7%	<LOD	5.4%	<LOD	NA
PFDeA	39.3%	<LOD (<LOD-0.3)	29.8%	<LOD (<LOD-0.2)	47.2%	<LOD (<LOD-0.3)	52.1%	0.15 (<LOD-0.3)	50.0%	<LOD (<LOD-0.3)	NA
PFNA	97.0%	0.6 (0.4-0.8)	97.4%	0.5 (0.4-0.7)	94.5%	0.6 (0.5-0.8)	96.5%	0.6 (0.4-0.8)	99.4%	0.6 (0.5-0.8)	<.0001
n-PFOA	99.9%	4.0 (2.8-5.7)	100%	4.5 (3.3-6.1)	99.6%	4.0 (2.8-5.6)	100%	2.2 (1.6-3.0)	100%	4.0 (3.0-5.2)	<.0001
Sb-PFOA	18.2%	<LOD	21.3%	<LOD	17.4%	<LOD	12.7%	<LOD	13.3%	<LOD	NA

NA, not available.

[a] Kruskal-Wallis tests were used to examine racial/ethnic differences in median concentrations of PFAS with detection rate >70%.

Supplemental Table III. 4 Hazard ratio (HR) (95% confidence interval, 95% CI) of n-PFOS, Sm-PFOS, n-PFOA, PFNA, and PFHxS serum concentrations on incidence of natural menopause with surgical menopause excluded instead of censored (n=1051).

PFAS	Tertile of PFAS concentrations			P value for trend	Per doubling increase HR (95%CI)	P value
	Tertile 1 HR (95%CI)	Tertile 2 HR (95%CI)	Tertile 3 HR (95%CI)			
n-PFOS						
Median (Range), ng/mL	10.4 (8.1-12.2)	16.9 (15.6-18.7)	28.3 (24.2-37.8)			
no. cases/person-years	183/1741	192/1719	203/1832			
Model 1[a]	Ref	1.08 (0.88-1.32)	1.15 (0.93-1.41)	0.19	1.05 (0.95-1.16)	0.34
Model 2[b]	Ref	1.15 (0.93-1.42)	1.22 (0.98-1.51)	0.07	1.10 (0.99-1.22)	0.07
Sm-PFOS						
Median (Range), ng/mL	3.8 (2.9-4.6)	7.2 (6.2-8.1)	13.1 (10.9-17.2)			
no. cases/person-years	195/1704	194/1782	189/1807			
Model 1 (Unadjusted)[a]	Ref	1.03 (0.84-1.26)	1.05 (0.85-1.31)	0.63	1.03 (0.94-1.12)	0.52
Model 2 (Adjusted)[b]	Ref	1.11 (0.90-1.37)	1.18 (0.95-1.48)	0.14	1.07 (0.97-1.17)	0.16
n-PFOA						
Median (Range), ng/mL	2.3 (1.8-2.8)	4.0 (3.5-4.5)	6.6 (5.6-8.6)			
no. cases/person-years	183/1686	195/1804	200/1802			
Model 1 (Unadjusted)[a]	Ref	1.14 (0.92-1.41)	1.24 (0.99-1.55)	0.06	1.04 (0.93-1.16)	0.50
Model 2 (Adjusted)[b]	Ref	1.12 (0.90-1.39)	1.26 (1.01-1.58)	0.04	1.08 (0.97-1.21)	0.16
PFNA						
Median (Range), ng/mL	0.3 (0.3-0.4)	0.5 (0.5-0.6)	0.9 (0.7-1.0)			
no. cases/person-years	168/1778	181/1572	229/1942			
Model 1 (Unadjusted)[a]	Ref	1.14 (0.92-1.41)	1.16 (0.94-1.42)	0.18	1.10 (0.99-1.22)	0.06
Model 2 (Adjusted)[b]	Ref	1.14 (0.91-1.42)	1.14 (0.92-1.41)	0.25	1.09 (0.98-1.21)	0.11
PFHxS						
Median (Range), ng/mL	0.8 (0.6-1.0)	1.5 (1.3-1.6)	3.0 (2.3-4.5)			
no. cases/person-years	203/1820	168/1610	207/1862			
Model 1 (Unadjusted)[a]	Ref	0.92 (0.74-1.13)	1.10 (0.90-1.35)	0.36	1.03 (0.95-1.11)	0.48
Model 2 (Adjusted)[b]	Ref	1.06 (0.85-1.31)	1.06 (0.86-1.31)	0.59	1.01 (0.94-1.10)	0.74

[a] Model 1 was adjusted for age at baseline, race/ethnicity, and study site.

[b] Model 2 was additionally adjusted for education, parity, BMI at baseline, physical activity, smoking status, and prior hormone use at baseline.

Supplemental Table III. 5 Hazard ratio (HR) (95% confidence interval, 95% CI) of n-PFOS, Sm-PFOS, n-PFOA, PFNA, and PFHxS serum concentrations on incidence of natural menopause after excluding women who reached natural menopause in 6 months since baseline (n=1091).

PFAS	Tertile of PFAS concentrations			P value for trend	Per doubling increase HR (95%CI)	P value
	Tertile 1 HR (95%CI)	Tertile 2 HR (95%CI)	Tertile 3 HR (95%CI)			
n-PFOS						
Median (Range), ng/mL	10.4 (8.1-12.2)	16.9 (15.6-18.7)	28.3 (24.2-37.8)			
no. cases/person-years	170/1857	185/1880	194/1878			
Model 1[a]	Ref	1.08 (0.88-1.33)	1.23 (0.99-1.52)	0.06	107 (0.96-1.18)	0.24
Model 2[b]	Ref	1.10 (0.89-1.37)	1.30 (1.04-1.62)	0.02	1.11 (0.99-1.24)	0.06
Sm-PFOS						
Median (Range), ng/mL	3.8 (2.9-4.6)	7.2 (6.2-8.1)	13.1 (10.9-17.2)			
no. cases/person-years	183/1839	185/1920	181/1856			
Model 1 (Unadjusted)[a]	Ref	1.05 (0.85-1.29)	1.14 (0.91-1.42)	0.26	1.04 (0.95-1.14)	0.35
Model 2 (Adjusted)[b]	Ref	1.11 (0.89-1.38)	1.28 (1.02-1.61)	0.03	1.08 (0.99-1.19)	0.10
n-PFOA						
Median (Range), ng/mL	2.3 (1.8-2.8)	4.0 (3.5-4.5)	6.6 (5.6-8.6)			
no. cases/person-years	173/1815	184/1933	192/1868			
Model 1 (Unadjusted)[a]	Ref	1.13 (0.90-1.41)	1.29 (1.03-1.62)	0.03	1.06 (0.95-1.19)	0.30
Model 2 (Adjusted)[b]	Ref	1.11 (0.88-1.39)	1.31 (1.04-1.66)	0.02	1.10 (0.98-1.24)	0.10
PFNA						
Median (Range), ng/mL	0.3 (0.3-0.4)	0.5 (0.5-0.6)	0.9 (0.7-1.0)			
no. cases/person-years	163/1928	167/1675	219/2012			
Model 1 (Unadjusted)[a]	Ref	1.13 (0.90-1.40)	1.20 (0.97-1.48)	0.09	1.12 (1.01-1.24)	0.03
Model 2 (Adjusted)[b]	Ref	1.13 (0.90-1.41)	1.18 (0.95-1.47)	0.14	1.10 (0.99-1.23)	0.08
PFHxS						
Median (Range), ng/mL	0.8 (0.6-1.0)	1.5 (1.3-1.6)	3.0 (2.3-4.5)			
no. cases/person-years	193/1954	158/1725	198/1936			
Model 1 (Unadjusted)[a]	Ref	0.90 (0.73-1.12)	1.15 (0.93-1.41)	0.21	1.05 (0.97-1.13)	0.25
Model 2 (Adjusted)[b]	Ref	1.02 (0.82-1.27)	1.09 (0.88-1.35)	0.44	1.02 (0.94-1.11)	0.56

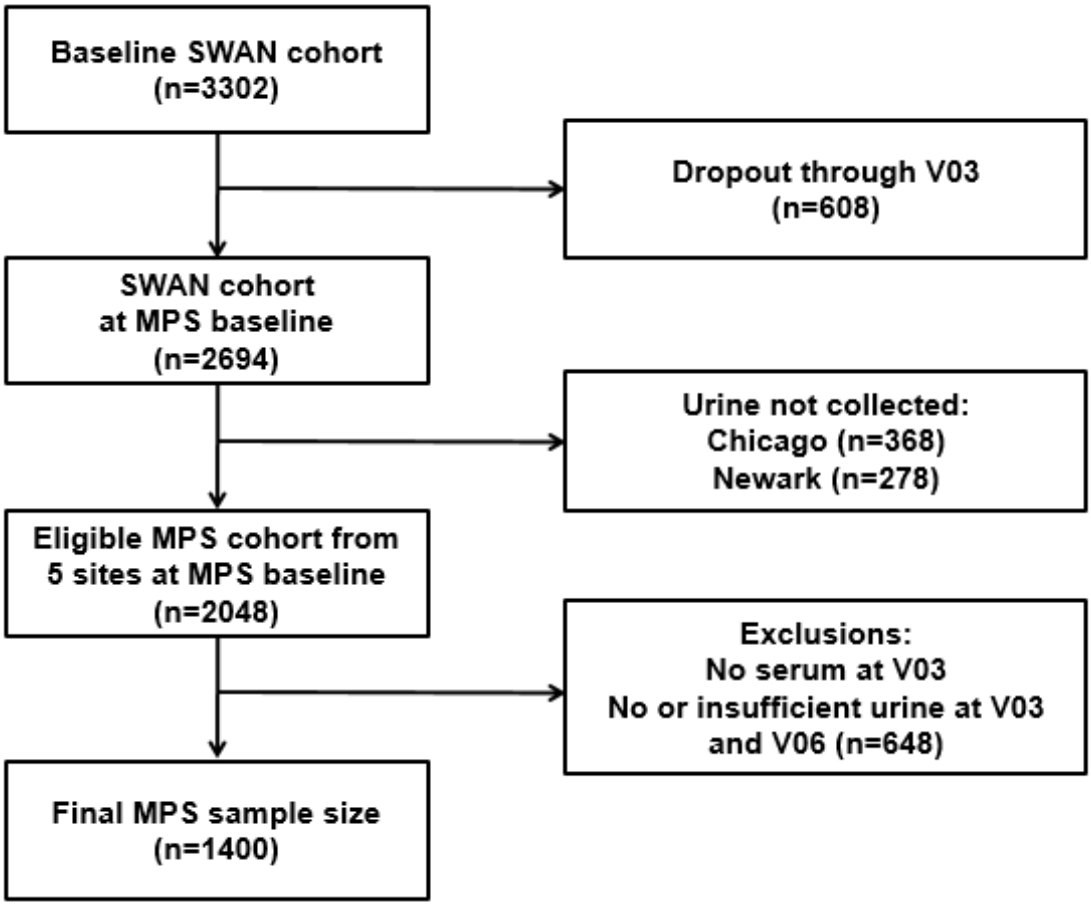
[a] Model 1 was adjusted for age at baseline, race/ethnicity, and study site.

[b] Model 2 was additionally adjusted for education, parity, BMI at baseline, physical activity, smoking status, and prior hormone use at baseline.

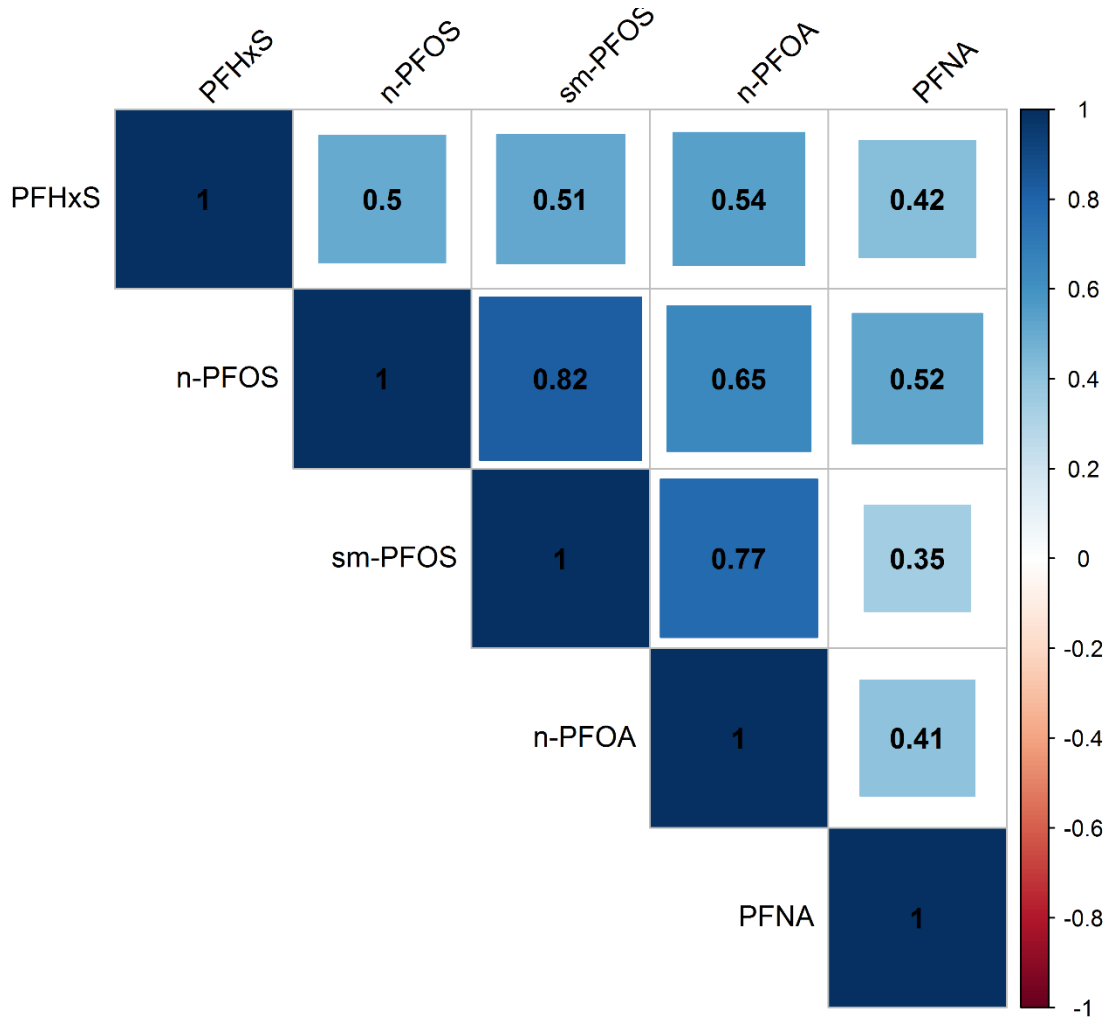
Supplemental Table III. 6 Hazard ratio (HR) (95% confidence interval, 95% CI) of natural menopause incidence with by PFAS clusters.

PFAS cluster	Population clusters			
	Cluster 1 HR (95%CI)	**Cluster 2 HR (95%CI)**	**Cluster 3 HR (95%CI)**	**Cluster 4 HR (95%CI)**
no. cases/person-years	68/765	220/2010	209/2122	81/727
Model 1[a]	Ref	1.31 (0.98-1.75)	1.33 (0.96-1.85)	1.60 (1.07-2.39)
Model 2[b]	Ref	1.30 (0.97-1.68)	1.31 (0.94-1.83)	1.63 (1.08-2.45)

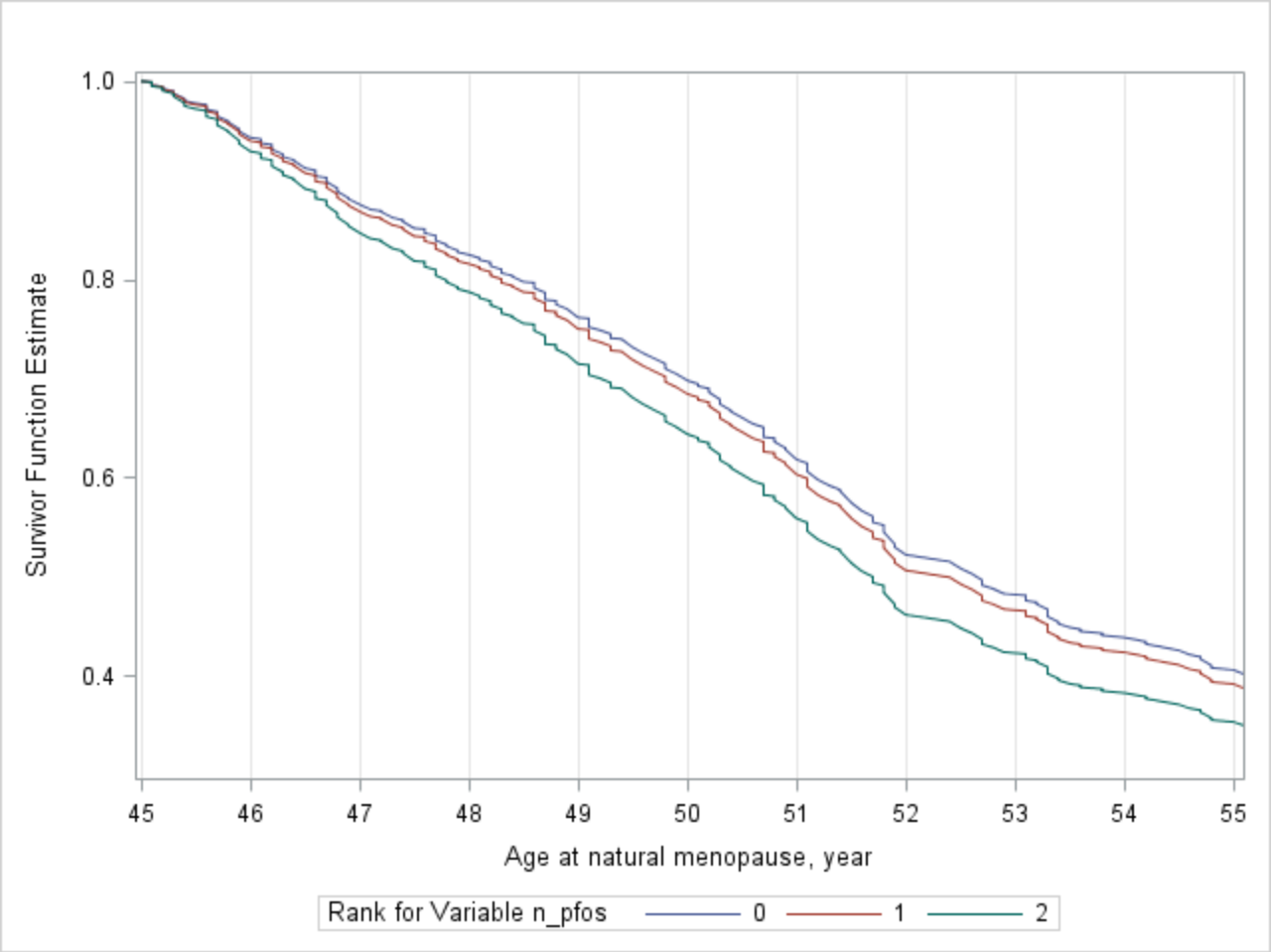
[a] Model 1 was adjusted for age at baseline, race/ethnicity, and study site.
[b] Model 2 was additionally adjusted for education, parity, BMI at baseline, physical activity, smoking status, and prior hormone use at baseline.



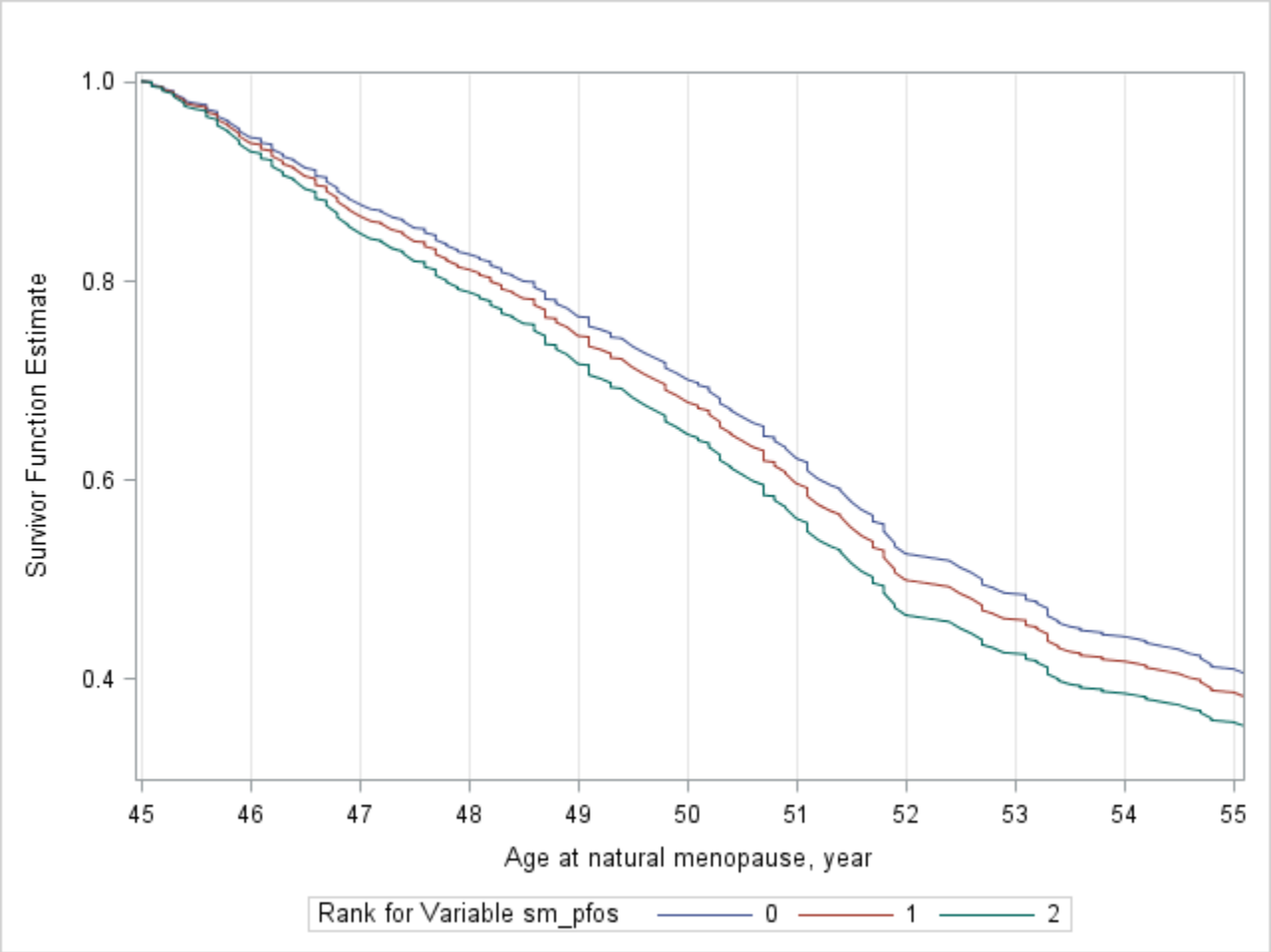
Supplemental Figure III. 1 The study designs of the Study of Women's Health Across the Nation (SWAN) Multi-Pollutant Study (MPS).



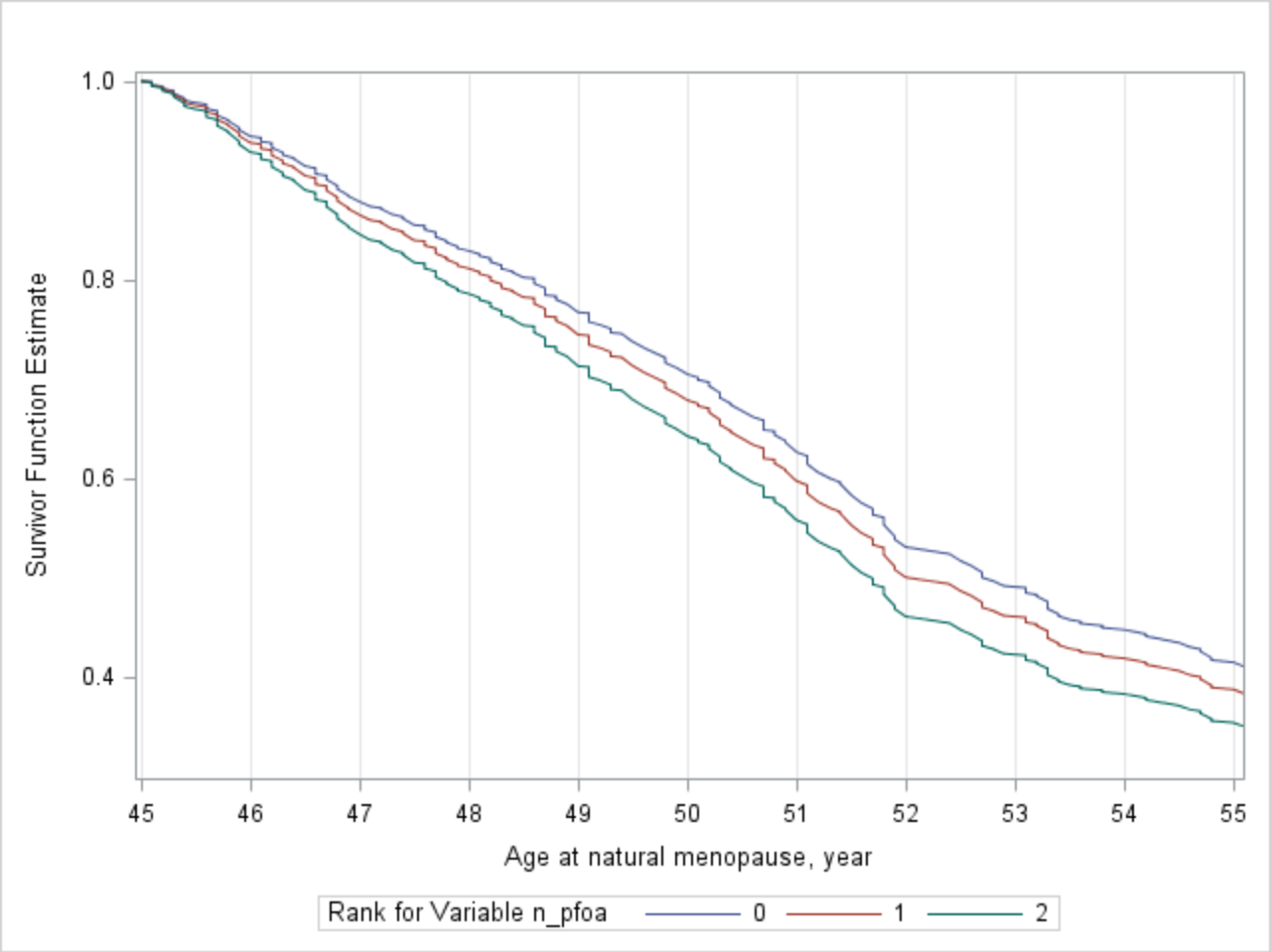
Supplemental Figure III. 2 Spearman correlation matrix of PFAS biomarkers in SWAN 1999-2000.



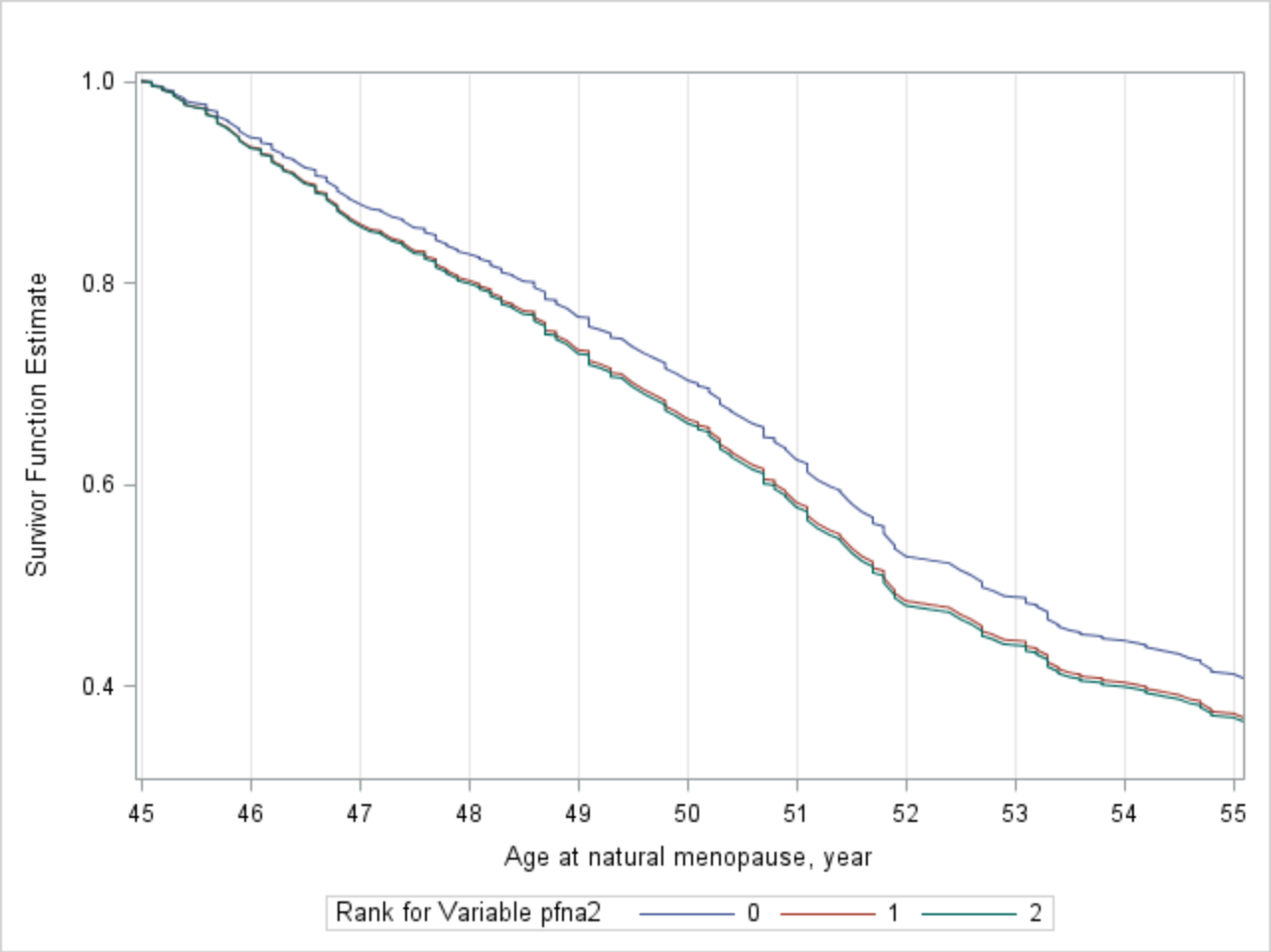
Supplemental Figure III. 3 Adjusted survival curves of natural menopause by tertiles of n-PFOS serum concentrations. The model was adjusted for age at baseline, race/ethnicity, study site, education, parity, BMI at baseline, physical activity, smoking status, and prior hormone use at baseline. The hazards ratio of tertile 2 and tertile 3 was 1.06 (0.86-1.31) and 1.26 (1.02-1.57), compared to tertile 1 (ptrend=0.03). The predicted median age at natural menopause was for tertile 1 was 52.6 years, and 52.3 years, and 51.6 years for tertiles 2 and 3, respectively.



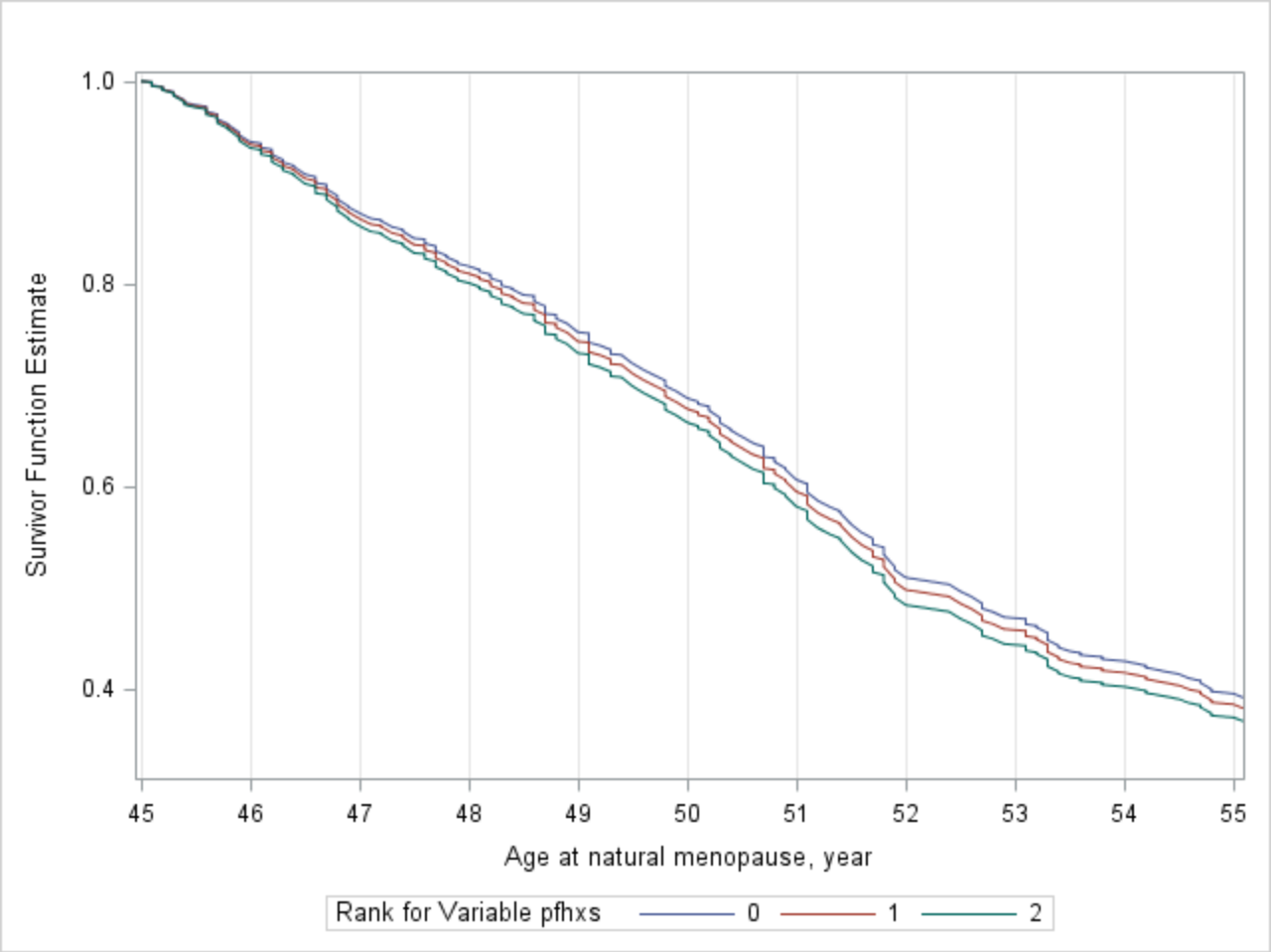
Supplemental Figure III. 4 Adjusted survival curves of natural menopause by tertiles of Sm-PFOS serum concentrations. The model was adjusted for age at baseline, race/ethnicity, study site, education, parity, BMI at baseline, physical activity, smoking status, and prior hormone use at baseline. The hazards ratio of tertile 2 and tertile 3 was 1.11 (0.90-1.37) and 1.27 (1.01-1.59), compared to tertile 1 (ptrend=0.03). The predicted median age at natural menopause for tertile 1 was 52.6 years, and 51.9 years, and 51.7 years for tertiles 2 and 3, respectively.



Supplemental Figure III. 5 Adjusted survival curves of natural menopause by tertiles of n-PFOA serum concentrations. The model was adjusted for age at baseline, race/ethnicity, study site, education, parity, BMI at baseline, physical activity, smoking status, and prior hormone use at baseline. The hazards ratio of tertile 2 and tertile 3 was 1.12 (0.90-1.40) and 1.31 (1.04-1.65), compared to tertile 1 (p_{trend}=0.01). The predicted median age at natural menopause for tertile 1 was 52.7 years, and 51.9 years, and 51.6 years for tertiles 2 and 3, respectively.



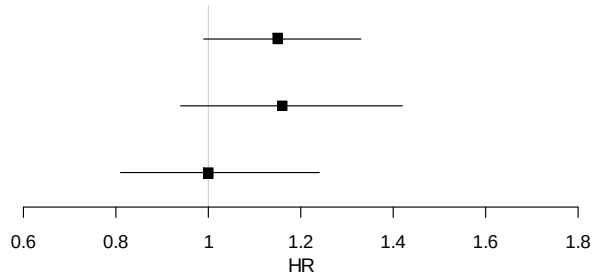
Supplemental Figure III. 6 Adjusted survival curves of natural menopause by tertiles of PFNA serum concentrations. The model was adjusted for age at baseline, race/ethnicity, study site, education, parity, BMI at baseline, physical activity, smoking status, and prior hormone use at baseline. The hazards ratio of tertile 2 and tertile 3 was 1.18 (0.95-1.47) and 1.20 (0.97-1.49), compared to tertile 1 (p_{trend}=0.10). The predicted median age at natural menopause for tertile 1 was 52.7 years, and 51.8 years, and 51.8 years for tertiles 2 and 3, respectively.



Supplemental Figure III. 7 Adjusted survival curves of natural menopause by tertiles of PFHxS serum concentrations. The model was adjusted for age at baseline, race/ethnicity, study site, education, parity, BMI at baseline, physical activity, smoking status, and prior hormone use at baseline. The hazards ratio of tertile 2 and tertile 3 was 1.05 (0.84-1.30) and 1.11 (0.90-1.37), compared to tertile 1 (p_{trend}=0.33). The predicted median age at natural menopause for tertile 1 was 52.4 years, and 51.9 years, and 51.8 years for tertiles 2 and 3, respectively.

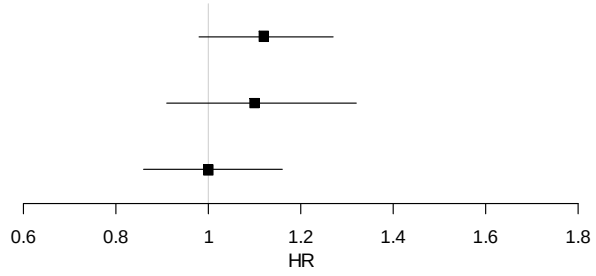
a) Exposure to **n-PFOS** and incidence of natural menopause by racial groups

Racial Group	No. of cases/person-year	HR (95% CI)
White	259/3008	1.15 (0.99-1.33)
Black	130/1100	1.16 (0.94-1.42)
Asian	189/1515	1.00 (0.81-1.24)

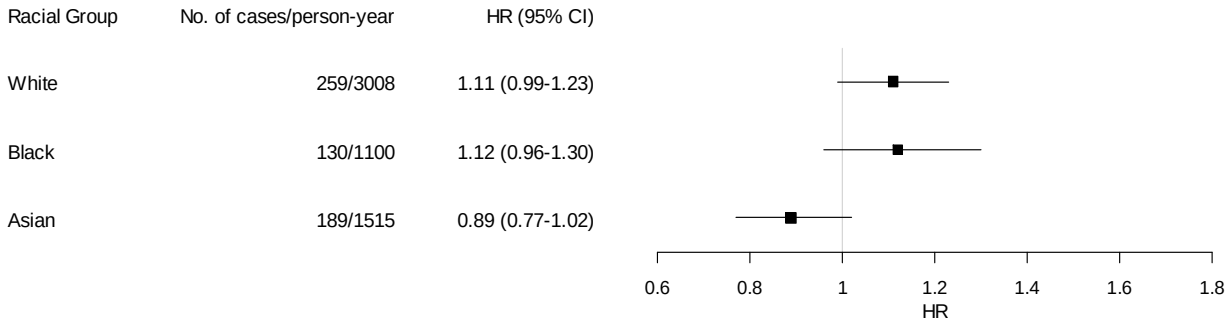


b) Exposure to **Sm-PFOS** and incidence of natural menopause by racial groups

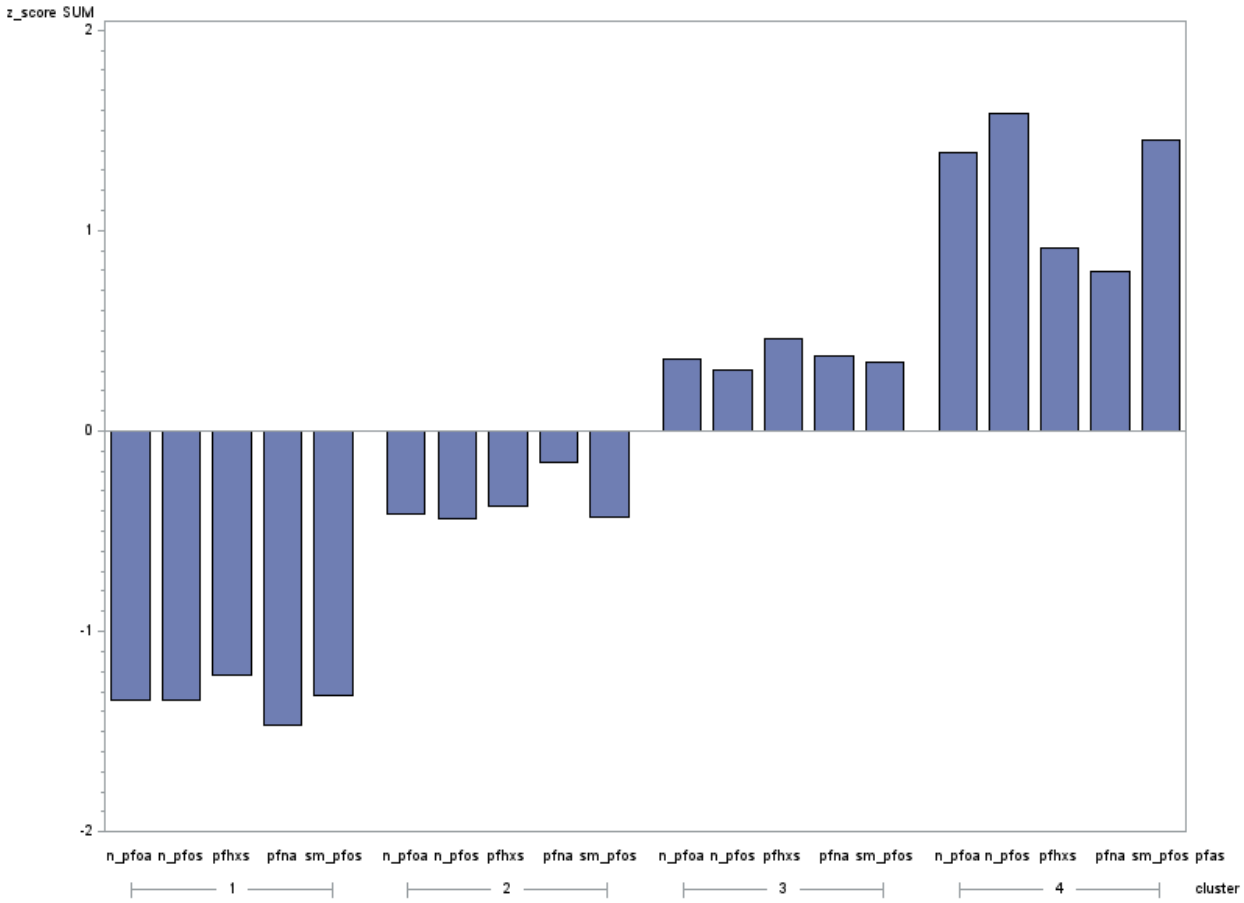
Racial Group	No. of cases/person-year	HR (95% CI)
White	259/3008	1.12 (0.98-1.27)
Black	130/1100	1.10 (0.91-1.32)
Asian	189/1515	1.00 (0.86-1.16)



c) Exposure to **PFHxS** and incidence of natural menopause by racial groups



Supplemental Figure III. 8 Adjusted hazard ratio (HR) (95% confidence interval, 95% CI) of natural menopause incidence with per doubling increase in serum concentrations of n-PFOS, Sm-PFOS and PFHxS. Models were adjusted for age at baseline, study site, education, parity, BMI at baseline, physical activity, smoking status, and prior hormone use at baseline. P values for the interaction terms with race/ethnicity are 0.54 for n-PFOS, 0.52 for Sm-PFOS, and 0.03 for PFHxS.



Supplemental Figure III. 9 Cluster means of the 5 standardized log-transformed serum PFAS concentrations using k-means clustering. Y-axis (cluster means) represents the mean standardized z-scores of log2-transformed PFAS concentrations. Cluster 1 (n=143): "low" overall PFAS exposure pattern; clusters 2 (n=414): "moderate low" overall PFAS exopsure pattern; cluster 3 (n=406): "moderate high" overall PFAS exposure pattern; cluster 4 (n=157): "high" overall PFAS exposure pattern.

Chapter IV. The Mediating Role of Follicle-Stimulating Hormone on the Relationships between Exposure to Perfluoroalkyl Substances and Incident Natural Menopause

Abstract

Exposure to perfluoroalkyl substances (PFAS) has been associated with earlier natural menopause, possibly through depletion of ovarian reserve and disturbance of hormone homeostasis. We aimed to investigate and quantify the degree to which follicle-stimulating hormone (FSH) could mediate the associations between PFAS exposure and natural menopause among 1120 premenopausal women aged 45-56 years in 1999-2000 from the Study of Women's Health Across the Nation (SWAN). Serum concentrations of linear- and branched-chain perfluorooctane sulfonic acid (n-PFOS and Sm-PFOS), linear-chain perfluorooctanoic acid (n-PFOA), and perfluorononanoic acid (PFNA) were measured in 1999-2000 and included in the analyses because their detection rates were larger than 70%. Accelerated failure time models were utilized to evaluate time to incident natural menopause. 578 women reached natural menopause, with a median survival time of 6.5 (95% CI: 6.1, 6.8) years. The proportion of the effect mediated through FSH was 8.5% (95% CI: -11.7%, 24.0%) for n-PFOS, 13.2% (95% CI: 0.0%, 24.5%) for Sm-PFOS, 26.9% (95% CI: 15.6%, 38.4%) for n-PFOA, and 21.7% (6.8%, 37.0%) for PFNA. No significant associations were observed for perfluorohexane sulfonic acid (PFHxS). PFAS are associated with an earlier age at natural menopause. The effect of PFAS on natural menopause may be partially explained by variation in FSH concentrations.

Keywords: perfluoroalkyl substances, endocrine-disrupting chemicals, natural menopause, follicle-stimulating hormone, mediation, midlife women

1. Introduction

Menopause is ascertained after 12 months of amenorrhea and represents the depletion of ovarian reserve and the near complete cessation of estrogen secretion. Earlier age at the final menstrual period (FMP) has been associated with an increased risk of overall mortality (Jacobsen *et al.*, 2003; Mondul *et al.*, 2005; Ossewaarde *et al.*, 2005), cardiovascular disease (Atsma *et al.*, 2006; Hu *et al.*, 1999) and cardiovascular death (de Kleijn *et al.*, 2002; Mondul *et al.*, 2005; van der Schouw *et al.*, 1996), low bone mineral density (Parazzini *et al.*, 1996) and osteoporosis (Kritz-Silverstein and Barrett-Connor, 1993), and other chronic conditions (Shuster *et al.*, 2010). Evidence also supports its clinical importance in quality of life (i.e. vasomotor symptoms, sleep disturbance, and depressive symptoms) (Bromberger *et al.*, 2010; Dennerstein *et al.*, 2000; Divakaran *et al.*, 2001; Maki *et al.*, 2010; Woods and Mitchell, 2010). Women with higher serum concentrations of perfluoroalkyl substances (PFAS) had an earlier onset of natural menopause (Ning Ding *et al.*, 2019).

PFAS are anthropogenic chemicals that have been widely used in consumer and industrial products such as non-stick cookware (Teflon) (Bradley *et al.*, 2007; Ewan Sinclair *et al.*, 2007); food packaging materials (Begley *et al.*, 2005; Schaider *et al.*, 2017; Trier *et al.*, 2011); stain- and water-resistant coating for clothing, furniture, and carpets (Scotchgard and Gore-Tex) (Hill *et al.*, 2017; Lee *et al.*, 2017); and aqueous fire-fighting foam (Butenhoff *et al.*, 2006; Kantiani *et al.*, 2010; Kissa, 2011; Trudel *et al.*, 2008). A recent study found that drinking water supplies for at least 6 million U.S. residents exceed the U.S. Environmental Protection Agency's lifetime health advisory of 70 parts per trillion for two commonly detected compounds, perfluorooctanoic acid (PFOA) and perfluorooctane sulfonic acid (PFOS), separately or

combined (Hu *et al.*, 2016). Once consumed, these chemicals can build up in the human body and persist for years (Ding *et al.*, 2019).

These compounds, especially PFOA and PFOS, have been identified as plausible endocrine disrupting chemicals with the potential to accelerate ovarian aging (Jensen and Leffers, 2008; Kar *et al.*, 2017). Animal studies have reported effects on female reproduction including altered ovarian function, histopathologic changes in the reproductive tract and ovarian cell steroidogenesis (Chaparro-Ortega *et al.*, 2018; Dixon *et al.*, 2012; Zhao *et al.*, 2012), likely through the activation of various transcriptional factors such as peroxisome proliferator-activated receptors (PPARs) (Andersen *et al.*, 2008; White *et al.*, 2011). Several hormones in the hypothalamic-pituitary-ovarian axis are markers of ovarian aging, including follicle-stimulating hormone (FSH) (Strauss and Barbieri, 2013). FSH levels increase progressively as the final menstrual period is approached (Randolph *et al.*, 2004, 2011; Sowers *et al.*, 2008), and thus is considered a biomarker of reproductive aging from active reproduction, through the stages of the menopausal transition (MT) to menopause (Harlow *et al.*, 2012; Soules *et al.*, 2001). Our recent finding based on data from the Study of Women's Health Across the Nation (SWAN) has revealed that higher serum concentrations of PFAS were associated with increased levels of FSH in midlife women during the menopausal transition (Sung Kyun Park).

Understanding the mediation mechanisms between PFAS exposure and natural menopause can provide evidence of associations, identify risk factors for ovarian aging, and inform precision health studies that seek to target specific biological pathways for interventions. However, it remains unknown whether and to what extent the association between PFAS and natural menopause is mediated by FSH. The goal of the present study was to examine and quantify the mediating role of FSH in the association between PFAS exposure and incident

natural menopause incidence using data from the SWAN during 1999 and 2017. Specifically, we used a causal mediation approach to decompose natural direct effect (NDE) and natural indirect effect (NIE) in the survival setting (VanderWeele, 2011).

2. Methods

Study population

Data for this study are from the participants of SWAN, a multi-site, multi-ethnic, longitudinal cohort of community-based group of midlife women, designed to characterize physiological and psychosocial changes that occur during the menopausal transition and observed their effects on subsequent risk factors for chronic disease (Sowers *et al.*, 2000). Between 1996 and 1997, 3320 women were enrolled in the cohort study. Eligibility criteria for entry into the longitudinal cohort included age 42-52 years, an intact uterus and at least one ovary, at least one menstrual period and no use of hormone medications within the three months before screening, , the ability to speak English or other designated languages including Spanish, Cantonese or Japanese, and self-identification as a member of one of the five eligible racial/ethnic groups. Each study site recruited White women and women with one of the pre-specified race/ethnicities. Black women were enrolled in Pittsburgh, Boston, Chicago and Detroit, Japanese women in Los Angeles, Chinese women in Oakland, and Hispanic women in Newark. Data and specimens were collected in annual follow-up visits from 1996/97 through 2016/17. The institutional review board at each participating site approved the study protocol.

The SWAN Multi-Pollutant Study was initiated to examine the associations of multiple environmental pollutants including PFAS, polychlorinated biphenyls, organochlorine pesticides, polybrominated diphenyl ethers, metals, phenols, phthalates, and organophosphate pesticide

among midlife women (Park *et al.*, 2019). We used repository samples available from the third follow-up visit (Visit 3 in 1999-2000, the MPS baseline) for environmental exposure assessment. The MPS included only the four study sites: Boston, Oakland, Los Angeles, and Pittsburgh. Therefore, only White, Black, Chinese and Japanese women were included in the sample. Of 1400 participants with serum samples available at baseline, we excluded 232 women who had already reached natural menopause and 48 women who had had a hysterectomy and/or oophorectomy prior to Visit 3, resulting in a final sample size of 1120 premenopausal women eligible for this study.

PFAS measurement

Serum PFAS concentrations were assessed at the Division of Laboratory Sciences, National Center for Environmental Health, Centers for Disease Control and Prevention. Serum samples were analyzed using an online solid phase extraction-high performance liquid chromatography-isotope dilution-tandem mass spectrometry (online SPE-HPLC-MS/MS) method (Kato *et al.*, 2011). The analytic methods and quality control procedures have been described in detail (Ding *et al.*, 2019). We measured 9 PFAS chemicals, including perfluorohexane sulfonic acid (PFHxS), linear-chain PFOS (n-PFOS), sum of branched-chain PFOS (Sm-PFOS), linear-chain PFOA (n-PFOA), sum of branched-chain PFOA (Sb-PFOA), perfluorononanoic acid (PFNA), perfluorodecanoic acid (PFDeA), perfluoroundecanoic acid (PFUA), and perfluorododecanoic acid (PFDoA). The limit of detection (LOD) was 0.1 ng/mL for all analytes. Measurements below the LODs were assigned LOD/$\sqrt{2}$. The coefficient of variation (CV) was 6-12% for quality controls. PFHxS, n-PFOS, Sm-PFOS, n-PFOA and PFNA with detection rates >70% were included in the statistical analyses.

FSH assessment

Collection of biological specimens was scheduled for each participant before 10:00 A.M. on day 2-5 of a spontaneous menstrual cycle occurring within 60 days of recruitment at the baseline visit and annually thereafter. If a follicular phase sample could not be obtained, a random fasting sample was taken within 90 days of the anniversary of the baseline visit. Serum FSH measurements were conducted with a two-site chemiluminometric immunoassay using an ACS-180 automated analyzer (Bayer Diagnostics Corp., Norwood, MA). The LOD was 1.1 mIU/mL. FSH was detected in all serum samples at the MPS baseline. The inter-and intra-assay CV were 12% and 6%, respectively. We used serum concentrations of FSH at Visit 3 (the MPS baseline) in 1999-2000 because there is currently no well-established method to handle multiple, correlated, time-varying mediators in the context of censored survival outcomes.

Natural menopause incidence

The ascertainment of natural menopause was based on self-reported bleeding patterns during the annual follow-up visits. Natural menopause was defined as 12 months of amenorrhea since the last menstrual period not because of hysterectomy, bilateral oophorectomy, or hormone therapy (HT). If a woman missed at least three consecutive visits prior to the first post-menopause visit, the FMP date was set to missing. Of the 1120 premenopausal women, 578 had an observed date at the natural FMP during the follow-up visits through 2016/17, and 542 were censored for one of the following reasons: hysterectomy and/or oophorectomy before having ≥12 months of amenorrhea (n=69); unknown FMP date because of HT use (n=451); or end of data collection before ≥12 months of amenorrhea (n=22).

Covariates

Data on study site, race/ethnicity, and level of education and parity (the number of live births) were collected at the SWAN enrollment interview. Race/ethnicity was classified into self-identified Black, Chinese, Japanese, or White. We categorized educational attainment as high school or less, some college, or college degree or higher. Information on prior hormone use was also collected at baseline. Other covariates were obtained at Visit 3. Age was calculated based on date of birth and date of visit and centered at 50 years. Weight and height were measured using a stadiometer and calibrated scales, respectively. Body mass index (BMI) was calculated as weight (kg)/height^2 (m^2) at baseline. Self-reported smoking status was classified as never smokers, former smokers only, or current smokers based on seven smoking questions adapted from the American Thoracic Society standard questions (Ferris, 1978). Physical activity was assessed using an adaptation of the Kaiser Physical Activity Survey (Sternfeld *et al.*, 1999), which consists of 38 questions with primarily Likert-scale responses about physical activity in various domains, including sports/exercise, household/caregiving, and daily routine (defined as walking or biking for transportation and hours of television watching, which are reverse-coded). Domain-specific indices were derived by averaging the ordinal responses to questions in each domain, resulting in values from 1 to 5. Thus, total physical activity score ranged from 3 to 15 with 15 indicating the highest level of activity.

Statistical analyses

Univariate statistics were calculated for baseline participant characteristics and PFAS serum concentrations by racial/ethnic groups. Chi-square or Fisher's exact statistics were computed for categorical variables; and analysis of variance (ANOVA) or Kruskal-Wallis tests were used for continuous variables. We censored a participant's data if she reported initiating HT if no subsequent HT-free bleeding occurred at the date of hysterectomy or bilateral

oophorectomy, or at the last menstrual period at the end of data collection if it occurred before 12 months of amenorrhea, or because of ending data collection.

The primary analytic objective was to estimate the extent to which FSH mediated associations between PFAS exposure and natural menopause incidence, while adjusting for baseline confounders (age, race/ethnicity, study site, education, parity, BMI, physical activity, smoking status, and prior hormone use) of the PFAS-natural menopause and FSH-natural menopause associations. Time to natural menopause was modeled using accelerated failure time (AFT) models with a Weibull distribution. AFT models were chosen because they outperformed Cox proportional hazards regression models in causal mediation analysis setting with non-rare outcomes (>10%) (Gelfand *et al.*, 2016; VanderWeele, 2011). The Weibull distribution was selected by comparing Akaike information criterion (AIC) values.

The outcome AFT model initially took the following general form:

$$\log(T) = \theta_0 + \theta_1 PFAS + \theta_2 FSH + \theta_3 PFAS \times FSH + \theta_4 Covariates + \sigma\varepsilon,$$

where *PFAS* corresponds to log-transformed (base 2) serum PFAS concentrations, *FSH* is log-transformed serum FSH concentrations at Visit 3, $PFAS \times FSH$ is the interaction between PFAS and FSH, $Covariates$ are baseline confounders, σ describes the Weibull distribution scale and shape parameters, and ε symbolizes the errors which is independently and identically distributed. After evaluation of the interaction term, there was no significant exposure-mediator interaction, thus the model was reduced to

$$\log(T) = \theta_0 + \theta_1 PFAS + \theta_2 FSH + \theta_3 Covariates + \sigma\varepsilon. \quad (1)$$

$\exp(\theta_1)$ is interpreted as relative survival (i.e. ratio of time to natural menopause) per doubling

increase in PFAS concentrations. If exp (θ_1) equals to 1, then there is null association between PFAS and natural menopause; if exp (θ_1) < 1, PFAS exposure is associated with earlier time to natural menopause; and if exp (θ_1) > 1, PFAS exposure is associated with postponed onset of natural menopause. We then fit a linear regression model for the mediator, FSH,

$$E(FSH|PFAS, Covariates) = \beta_0 + \beta_1 PFAS + \beta_2 Covariates + \varepsilon. \quad (2)$$

Within the causal framework, we investigated the natural direct effect (NDE) and natural indirect effect (NIE) with survival data, as illustrated in **Figure 1**. Considering the mean time to natural menopause (*T*), the NDE refers to differences in the mean event time in association with a defined change in PFAS exposure (*A*) from the exposed level (*a)* to the reference level (*a*),* while holding the mediator level (*M*) for each participant that it would have been with *A* set at the reference value *a** if levels of confounders had otherwise remained unchanged. The NIE represents difference in mean time to natural menopause if *A* was held at *a* and if *M* for each participant changed to the levels that would have seen if that subject had been assigned to *a**. So then the total effects are decomposed into NDE and NIE:

$E(T_a)/E(T_{a*}) = \left[E\left(T_{aM_{a*}}\right)/E\left(T_{a*M_{a*}}\right)\right] \times \left[E\left(T_{aM_a}\right)/E\left(T_{aM_{a*}}\right)\right]$ (VanderWeele, 2011).

If model (1) holds for the outcome and model (2) holds for the mediator, these models yield the NDE and NIE of PFAS exposure on natural menopause as follows:

$$\text{NDE} = \exp(\theta_1);$$

and,

$$\text{NIE} = \exp[(\theta_2\beta_1)(a - a^*)],$$

for changes in PFAS exposure from a to a^* on a counterfactual setting. The statistical significance was determined using 95% confidence intervals (95% CIs) that were calculated using bootstrap method with 1000 re-samplings.

Assumptions and sensitivity analyses

The mediation analysis described above assumes that the measured covariates control for confounding of: (1) the exposure-outcome association; (2) the mediator-outcome association; (3) the exposure-mediator relationship; and (4) that none of the mediator-outcome confounders are influenced by the exposure. For assumptions (1)-(4), which cannot be assessed directly, sensitivity analyses were conducted to estimate the potential bias in NDE and NIE estimates due to unmeasured confounding (Ding and Vanderweele, 2016; Vanderweele, 2010; VanderWeele, 2013). Assuming a binary unknown confounder (e.g. having healthy lifestyle; exposure to other endocrine-disrupting chemicals), we used the bias formula to correct for unmeasured confounding (VanderWeele, 2013),

$$Bias(NDE) = \frac{1 + (\gamma - 1)\pi_a}{1 + (\gamma - 1)\pi_{a*}} \; and \; Bias(NIE) = \frac{1 + (\gamma - 1)\pi_{a*}}{1 + (\gamma - 1)\pi_a},$$

where γ is the effect of the hypothesized unadjusted confounder, U, on natural menopause; π_a is the prevalence of U among women with the 75th percentile of PFAS concentrations given the baseline covariates; π_{a*} is the prevalence of U among women with the 25th percentile of PFAS concentrations given the baseline covariates.

We conducted sensitivity analyses by testing interaction terms in the regression models. In this case, the controlled direct effect (CDE), NDE and NIE are defined as follows:

$$\text{CDE} = \exp\left[(\theta_1 + \theta_3 m)(a - a^*)\right];$$

$$\text{NDE} = \exp\left[\theta_1 + \theta_3(\beta_0 + \beta_1 a^* + \beta_2 Covariates + \theta_2\sigma^2)(a - a^*) + 0.5{\theta_3}^2\sigma^2(a^2 - a^{*2})\right];$$

and,

$$\text{NIE} = \exp[(\theta_2\beta_1 + \theta_3\beta_1 a)(a - a^*)],$$

where PFAS exposure levels changed from a to a^* (per doubling increase in serum PFAS concentrations) in a counterfactual setting. Without an interaction term between PFAS and FSH, CDE should equal NDE.

To ensure the temporality between the exposure and mediator, we conducted further analyses using FSH levels measured at SWAN Visit 4 in 2000-2001. We excluded 101 women who reached their FMP or were censored by Visit 4 and an additional 53 women with missing values of FSH levels, after which 966 women available for the sensitivity analyses. All the analyses were performed using SAS, version 9.4 (SAS Institute, Inc., Cary, North Carolina).

3. Results

The analytic sample consists of 1120 premenopausal women with median age of 48.9 (interquartile range, IQR: 47.0, 50.8) years at baseline. Baseline characteristics are displayed in **Table III.1**. A total of 577 (51.5%) were White, 235 (21.0%) were Black, 142 (12.7%) were Chinese, and 166 (14.8%) were Japanese women. More than half had received college or post-college education and had never smoked. Most women had given birth to at least one child and 22.1% had a history of prior hormone use. The median physical activity score was 7.9 (IQR: 6.6, 9.0), indicating moderate physical activity. The median BMI was 26.1 (IQR: 22.7, 31.5) kg/m^2.

Adjusted percent changes in serum FSH concentrations per doubling increase in serum PFAS concentrations are presented in **Table III.2**. Exposure to Sm-PFOS and n-PFOA were

associated significantly with serum FSH concentrations. Participants had a 9.4% (95% CI:1.5%, 18.0%) increment in baseline FSH concentrations per doubling increase in serum concentrations of Sm-PFOS. Additionally, women had FSH concentrations increased by 15.0% (95% CI: 4.9%, 26.0%) per doubling increase in n-PFOA concentrations. No association was found for n-PFOS (percent change=4.1%, 95% CI: -4.8%, 13.7%), PFNA (percent change=4.9%, 95% CI: -3.6%, 14.0%), and PFHxS (percent change=3.7%, 95% CI: -2.9%, 10.6%).

Population median time to natural menopause was 6.5 (95% CI: 6.1, 6.8) years. As expected, women with higher FSH concentrations at Visit 3 tended to have earlier onset of natural menopause. After adjusting for age, race/ethnicity, study site, educational attainment, BMI, parity, physical activity score, smoking status and prior hormone use, women had 17% (95% CI: 12%, 22%) earlier time to natural menopause per doubling increase in serum FSH concentrations

Table III.3 shows the results of causal mediation analysis decomposing total effects into NDE and NIE. For the total effects, the adjusted relative survival of natural menopause was 0.82 (95% CI: 0.69, 0.96) per doubling increase in serum n-PFOS concentrations, 0.84 (95% CI: 0.69, 1.00) for Sm-PFOS, 0.79 (95% CI: 0.66, 0.93) for n-PFOA, 0.84 (95% CI: 0.71, 0.97) for PFNA, and 0.90 (95% CI: 0.76, 1.05) for PFHxS. When FSH was included as a mediator, 26.9% (95% CI: 15.6%, 38.4%) of the total effects of n-PFOA on natural menopause was attributable to indirect effects through FSH. 21.7% (95% CI: 6.8%, 37.0%) and 13.2% (95% CI: 0.0%, 24.5%) of the total effects of PFNA and Sm-PFOS was attributable to indirect effects, respectively. No causal mediation effects were observed for n-PFOS (percent mediated=8.5%, 95% CI: -11.7%, 24.0%) and PFHxS (percent mediated=18.3%, 95% CI: -13.0%, 40.8%).

Details of the assumption testing results are provided in the **Supplemental Tables IV.1-IV.4**. Assuming that an unmeasured confounder (e.g. healthy lifestyle) decreases the risk of earlier natural menopause, and that it is more prevalent among women with lower n-PFOA or Sm-PFOS concentrations, the observed NDE seems to be overestimated and the NIE seems to be underestimated. For example, if there is an unmeasured confounder with a relative survival of 1.2 on natural menopause and a larger prevalence in women with higher n-PFOA concentrations (10% vs. 25% in the 75^{th} and 25^{th} percentiles of n-PFOA concentrations), the estimated NDE would have a relative survival of 0.96 and the estimated NIE would have a relative survival of 0.94 (**Supplemental Tables IV.1-IV.2**). Therefore, the estimated percent of mediation would be 59% larger than the observed 26.9%. The sensitivity analyses also confirmed that there was no interaction between PFAS exposure and FSH (**Supplemental Table IV.5**). The mediating role of FSH on the associations between n-PFOA and natural menopause remained when using FSH at SWAN Visit 4 instead of FSH at Visit 3 as the mediator (percent mediated=36.4%, 95% CI: 1.0%, 54.8%), as shown in **Supplemental Table IV.6**.

If the unknown confounder (e.g. other endocrine-disrupting chemicals) increases the risk of earlier natural menopause, and it is more prevalent among women with higher n-PFOA or Sm-PFOS concentrations, the NDE would be overestimated, then the NIE would be underestimated. In these scenarios, the degree of mediation of exposure to n-PFOA and Sm-PFOS by FSH would be underestimated without adjustment. Otherwise, the observed natural direct effect underestimates the true direct effect, whereas the observed natural indirect effect overestimates the true indirect effect.

4. Discussion

In this population-based cohort study, we found that the effects of exposure to n-PFOA

and Sm-PFOS on shortening time to incident natural menopause is partially explained through increasing serum concentrations of FSH. Although PFAS exposure has been linked to earlier menopause and higher FSH concentrations, the potential mediating role of FSH in the association between PFAS and menopause has not been previously examined. This study is the first, to our knowledge, that supports a potential mechanistic pathway for the associations between PFAS exposure and reproductive outcomes in human populations using a causal mediation approach.

As proposed by Baron and Kenny in 1986, mediation requires strong relationships (1) between the exposure and the mediating variable, and (2) between the mediating variable and the outcome of interest (Baron and Kenny, 1986). Previous studies have documented associations between PFAS exposure and circulating levels of FSH in the human body (43,56). Exposure to PFOS and PFHxS has been associated with increased serum concentrations of FSH in patients with premature ovarian insufficiency (Zhang *et al.*, 2018). This longitudinal study using SWAN data also detected positive relationships between n-PFOA exposure and increases in FSH concentrations among midlife women (Sung Kyun Park). Experimental studies have confirmed the endocrine-disrupting role of PFAS, possibly through an activation of peroxisome proliferator-activated receptors (PPARs) (White *et al.*, 2011).

The second criterion, an association between FSH and natural menopause, is well established. FSH is a glycoprotein hormone that is critical for ovarian folliculogenesis. FSH acts through FSH receptors (FSHR) located on the membrane of granulosa cells to facilitate antral follicle development and, in combination with luteinizing hormone, stimulate preovulatory follicle growth and form a corpus luteum after ovulation (Hillier, 2001; McGee *et al.*, 2015). FSH serum concentrations begin increasing about 7 years before the FMP; and the rates of

changes accelerate approximately 2 years before the FMP; and FSH concentrations stabilize approximately 2 years after the FMP (Randolph *et al.*, 2011). FSH is also a candidate biomarker assay to estimate ovarian reserve as a reflection of decreased negative feedback from a diminishing cohort of follicles, with elevated FSH levels commonly used to confirm the onset of menopause (Roudebush *et al.*, 2008).

In the present study, exposure to PFAS was related to shorter time to natural menopause in midlife women. In experiments *in vitro* and in *in vivo* animal models, inverse associations were reported for PFAS exposure with diminished ovarian reserve (i.e. the number of ovarian follicles and oocytes) (Bellingham *et al.*, 2009; Chen *et al.*, 2017; Domínguez *et al.*, 2016; Du *et al.*, 2019; Feng *et al.*, 2015, 2017; Hallberg *et al.*, 2019; López-Arellano *et al.*, 2019) and reduced steroidogenic enzyme activities (Chaparro-Ortega *et al.*, 2018; Shi *et al.*, 2009; Wang *et al.*, 2018). We found that FSH was a statistically significant mediator for the associations of exposure to n-PFOA and Sm-PFOS with natural menopause incidence, with the proportion mediated by FSH of 26.8% and 23.8%, respectively. Other possible mechanisms for the effect of PFAS on menopause timing include interruption of gap junction intercellular communication between oocytes and granulosa cells (Domínguez *et al.*, 2016; López-Arellano *et al.*, 2019), oxidative stress (Wielsøe *et al.*, 2015), and/or distribution of thyroid hormone homeostasis (Chang *et al.*, 2008; Lau *et al.*, 2003; Thibodeaux *et al.*, 2003).

This study has several strengths. First, the prospective design minimized the possibility of reverse causation. Second, standard annual follow-up visits instead of one-time questionnaire provided reliable estimates of date of FMP. Furthermore, the availability of serum concentrations of FSH at Visit 3 (the MPS baseline) allowed assessment of the mediating role of FSH on the association between PFAS exposure and incident natural menopause.

There are limitations that should be acknowledged. First, the age range for the cohort was restricted to 45-56 years at the MPS baseline. This left-truncation resulted in an overestimation of median age at FMP (Cain *et al.*, 2011). Women who experienced menopause before baseline, especially those with premature menopause (before age 40) or early menopause (before age 45) were not included in the MPS cohort, which could bias our effect estimates towards the null. Second, more than 40% of the cohort was censored at the initiation of HT before the participant was classified as post-menopausal. Although we considered many confounding variables in the analyses, residual or unmeasured confounding might still bias our effect estimates. Given the expected direction of residual confounding (i.e. unmeasured confounders like healthy lifestyle would likely decrease the risk of earlier natural menopause, while other endocrine-disrupting chemicals may shorten the time to natural menopause), our sensitivity analyses suggest that the observed indirect effects may have underestimated the true mediated effect while the observed direct effects were overestimated. Finally, the indirect effects through changing FSH concentrations assessed at Visit 3 alone ignore the mediating process and therefore underestimate the proportion mediated (Vansteelandt *et al.*, 2019).

In conclusion, this study is the first to investigate and quantify the degree to which FSH is an explanatory factor for the shortened time to natural menopause in midlife women with higher exposure to PFAS. Although mediation analysis still does not concretely establish a causal pathway, our findings provide evidence that PFAS exposure may accelerate ovarian aging through endocrinologic mechanisms associated with changing serum concentrations of FSH. Any potential mechanism underlying the relationships between PFAS exposure and natural menopause is likely to involve an interplay of hormones, beyond the action of single hormone levels. Future replication of our findings should consider other hormones in the analysis.

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Table IV. 1 Baseline characteristics among 1120 premenopausal women in the Study of Women's Health Across the Nation.

Baseline characteristic	**Total (n=1120)**
	Median (IQR) or n (%)
Age at baseline, years	48.9 (47.0-50.8)
Race/ethnicity	
White	577 (51.5%)
Black	235 (21.0%)
Chinese	142 (12.7%)
Japanese	166 (14.8%)
Study site	
Southeast MI	202 (18.0%)
Boston, MA	182 (16.3%)
Oakland, CA	242 (21.6%)
Los Angeles, CA	299 (26.7%)
Pittsburgh, PA	195 (23.4%)
Educational attainment	
≤High school	197 (17.7%)
Some college	350 (31.4%)
College	271 (24.3%)
Post-college	296 (26.6%)
Parity	
Nulliparous	215 (19.2%)
Parous	905 (80.8%)
Prior hormone use	248 (22.1%)
Smoking status	
Never smoker	720 (64.4%)
Former smoker	291 (26.0%)
Current smoker	107 (9.6%)
Physical activity score	7.9 (6.6-9.0)
Body mass index, kg/m^2	26.1 (22.7-31.5)
Follicle-stimulating hormone, mIU/mL	21.5 (12.7-41.6)

Table IV. 2 Adjusted[a] percent changes (95% confidence interval, 95% CI) in serum concentrations of follicle-stimulating hormone (FSH) per doubling increase in serum PFAS concentrations.

	FSH, mIU/mL	
	Percent change	**95% CI**
n-PFOS	4.1%	-4.8%, 13.7%
Sm-PFOS	9.4%	1.5%, 18.0%
n-PFOA	15.0%	4.9%, 26.0%
PFNA	4.9%	-3.6%, 14.0%
PFHxS	3.7%	-2.9%, 10.6%

[a] Models were adjusted for baseline covariates including age, race/ethnicity, study site, education, body mass index, parity, physical activity, smoking status, and prior hormone use.
Abbreviations: n-PFOS, linear-chain perfluorooctane sulfonic acid; Sm-PFOS, branched-chain perfluorooctane sulfonic acid; n-PFOA, linear-chain perfluorooctanoic acid; PFNA, perfluorononanoic acid; PFHxS, perfluorohexane sulfonic acid; 95% CI, 95% confidence interval.

Table IV. 3 Natural direct effects of PFAS exposure on natural menopause, natural indirect effects of PFAS exposure on natural menopause through altering follicle-stimulating hormone (FSH) levels, total effects of PFAS exposure on natural menopause and percent mediated.[a,b,c]

	Natural direct effects		**Natural indirect effect**		**Total effect**		**Percent mediated**	
	Relative survival[d]	**95% CI**	**Relative survival**[d]	**95% CI**	**Relative survival**[d]	**95% CI**	**Percent**	**95% CI**
n-PFOS	0.84	0.71, 0.97	0.98	0.94, 1.02	0.82	0.69, 0.96	8.5%	-11.7%, 24.0%
Sm-PFOS	0.86	0.72, 1.02	0.97	0.94, 1.00	0.84	0.69, 1.00	13.2%	0.0%, 24.5%
n-PFOA	0.85	0.72, 0.98	0.93	0.89, 0.97	0.79	0.66, 0.93	26.9%	15.6%, 38.4%
PFNA	0.88	0.74, 1.01	0.96	0.92, 0.99	0.84	0.71, 0.97	21.7%	6.8%, 37.0%
PFHxS	0.92	0.79, 1.07	0.98	0.94, 1.01	0.90	0.76, 1.05	18.3%	-13.0%, 40.8%

[a] Causal Mediation analysis was based on (1) AFT for the outcome model with time to natural menopause as the dependent variable and both exposure (PFAS) and mediator (FSH) as independent variables with adjustment for confounders; (2) linear regression for the mediator model with FSH as the dependent variable and PFAS as independent variable with adjustment for confounders.
[b] Models were adjusted for age at baseline, race/ethnicity, study site, education, BMI at baseline, parity, physical activity, smoking status and prior hormone use at baseline.
[c] Serum concentrations of PFAS were log-transformed with base 2. The effects of PFAS exposure were interpreted in relative survival and related 95% CI with a doubling increase in PFAS concentrations.
[d] Relative survival less than 1 means that PFAS exposure is associated with earlier onset of natural menopause.
Abbreviations: n-PFOS, linear-chain perfluorooctane sulfonic acid; Sm-PFOS, branched-chain perfluorooctane sulfonic acid; n-PFOA, linear-chain perfluorooctanoic acid; PFNA, perfluorononanoic acid; PFHxS, perfluorohexane sulfonic acid.; 95% confidence interval, 95% CI.

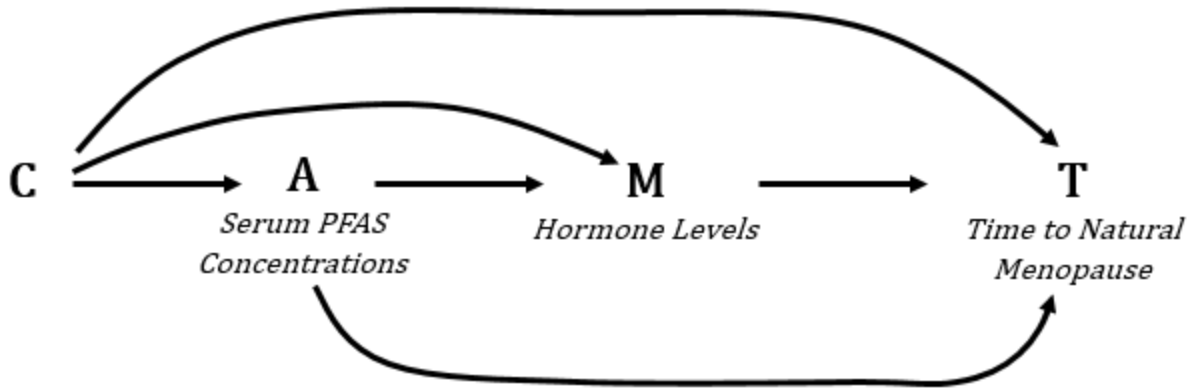

Figure IV. 1 Conceptual model for mediation analysis in the context of the present study.*C* represents baseline confounders, including age at baseline, race/ethnicity, study site, education, BMI at baseline, parity at baseline, physical activity without work at baseline, smoking status at baseline, prior oral contraceptive use at baseline; *A* is serum concentrations of PFAS at baseline; *M* is follicle-stimulating hormone at baseline; *T* represents time to natural menopause. Abbreviations: PFAS, perfluoroalkyl substances.

Supplemental Table IV. 1 Natural direct effects of PFAS exposure on natural menopause, natural indirect effects of PFAS exposure on natural menopause through altering follicle-stimulating hormone (FSH) levels, total effects of PFAS exposure on natural menopause, and percent mediated. [a,b,c,d]

	Controlled direct effects		Natural direct effects		Natural indirect effect		Total effect		Percent mediated	
	Relative survival[e]	95% CI	Relative survival[e]	95% CI	Relative survival[e]	95% CI	Relative survival[e]	95% CI	Percent	95% CI
n-PFOS	0.92	0.84, 1.00	0.92	0.84, 1.00	0.99	0.97, 1.01	0.91	0.83, 0.99	7.5%	-15.3%, 25.4%
Sm-PFOS	0.95	0.88, 1.02	0.95	0.88, 1.02	0.98	0.97, 1.00	0.93	0.86, 1.00	24.4%	6.0%, 39.0%
n-PFOA	0.93	0.85, 1.03	0.92	0.84, 1.01	0.97	0.95, 0.99	0.90	0.82, 0.98	23.9%	10.0%, 36.6%
PFNA	0.92	0.85, 1.00	0.93	0.85, 1.02	0.99	0.98, 1.01	0.92	0.84, 1.02	4.5%	-16.2%, 23.4%
PFHxS	0.98	0.92, 1.04	0.98	0.92, 1.04	0.99	0.98, 1.01	0.97	0.91, 1.03	23.1%	-42.5%, 49.2%

[a] Causal Mediation analysis was based on (1) AFT for the outcome model with time to natural menopause as the dependent variable and both exposure (PFAS) and mediator (FSH) as independent variables with adjustment for confounders; (2) linear regression for the mediator model with FSH as the dependent variable and PFAS as independent variable with adjustment for confounders.
[b] Models were adjusted for age at baseline, race/ethnicity, study site, education, BMI at baseline, parity, physical activity, smoking status and prior hormone use at baseline.
[c] Interaction terms between PFAS exposure and serum FSH levels were considered in the analyses. Controlled direct effects were computed when keeping FSH at its geometric mean level (23.8 mIU/mL).
[d] Serum concentrations of PFAS were log-transformed with base 2. The effects of PFAS exposure were interpreted in relative survival and related 95% CI with a doubling increase in PFAS concentrations.
[e] Relative survival less than 1 means that PFAS exposure is associated with earlier onset of natural menopause.
Abbreviations: n-PFOS, linear-chain perfluorooctane sulfonic acid; Sm-PFOS, branched-chain perfluorooctane sulfonic acid; n-PFOA, linear-chain perfluorooctanoic acid; PFNA, perfluorononanoic acid; PFHxS, perfluorohexane sulfonic acid.; 95% confidence interval, 95% CI.

Supplemental Table IV. 2 Natural direct effects of PFAS exposure on natural menopause, natural indirect effects of PFAS exposure on natural menopause through altering follicle-stimulating hormone (FSH) levels, total effects of PFAS exposure on natural menopause and percent mediated (N=966). [a,b,c,d]

	Natural direct effects		**Natural indirect effect**		**Total effect**		**Percent mediated**	
	Relative survival[e]	**95% CI**	**Relative survival**[e]	**95% CI**	**Relative survival**[e]	**95% CI**	**Percent**	**95% CI**
n-PFOS	0.96	0.89, 1.03	0.99	0.98, 1.01	0.96	0.88-1.02	10.9%	-53.8%, 36.9%
Sm-PFOS	0.98	0.91, 1.04	0.99	0.97, 1.00	0.97	0.90-1.03	35.1%	-11.8%, 55.7%
n-PFOA	0.97	0.90, 1.05	0.98	0.97, 1.00	0.96	0.88-1.03	36.4%	1.0%, 54.8%
PFNA	0.94	0.88, 1.00	1.00	0.98, 1.02	0.94	0.88-1.01	0%	-35.2%, 19.6%
PFHxS	0.99	0.94, 1.04	1.01	0.99, 1.02	1.00	0.94-1.05	-118.1%	-201.2%, 43.8%

[a] Causal Mediation analysis was based on (1) AFT for the outcome model with time to natural menopause as the dependent variable and both exposure (PFAS) and mediator (FSH) as independent variables with adjustment for confounders; (2) linear regression for the mediator model with FSH as the dependent variable and PFAS as independent variable with adjustment for confounders.
[b] Models were adjusted for age at baseline, race/ethnicity, study site, education, BMI at baseline, parity, physical activity, smoking status and prior hormone use at baseline.
[c] Serum concentrations of FSH measured at SWAN V04 were used in the analyses.
[d] Serum concentrations of PFAS were log-transformed with base 2. The effects of PFAS exposure were interpreted in relative survival and related 95% CI with a doubling increase in PFAS concentrations.
[e] Relative survival less than 1 means that PFAS exposure is associated with earlier onset of natural menopause.
Abbreviations: n-PFOS, linear-chain perfluorooctane sulfonic acid; Sm-PFOS, branched-chain perfluorooctane sulfonic acid; n-PFOA, linear-chain perfluorooctanoic acid; PFNA, perfluorononanoic acid; PFHxS, perfluorohexane sulfonic acid.; 95% confidence interval, 95% CI.

Supplemental Table IV. 3 Bias-corrected natural direct effect of n-PFOA exposure on natural menopause incidence for varying prevalence of unmeasured confounder (U) among women with the 25th and 75th percentiles of n-PFOA serum concentrations, with relative survival of U on natural menopause=0.8 or 1.2.

Relative survival=0.8								
	Prevalence of U among the 75th percentile							
Prevalence of U among the 25th percentile		1%	10%	25%	50%	75%	90%	99%
	1%	0.93	0.95	0.97	1.03	1.09	1.13	1.16
	10%	0.91	0.93	0.96	1.01	1.07	1.11	1.14
	25%	0.88	0.90	0.93	0.98	1.04	1.08	1.10
	50%	0.84	0.85	0.88	0.93	0.98	1.02	1.04
	75%	0.79	0.81	0.83	0.88	0.93	0.96	0.99
	90%	0.76	0.78	0.80	0.85	0.90	0.93	0.95
	99%	0.75	0.76	0.78	0.83	0.88	0.91	0.93
Relative survival=1.2								
	Prevalence of U among the 75th percentile							
Prevalence of U among the 25th percentile		1%	10%	25%	50%	75%	90%	99%
	1%	0.93	0.91	0.89	0.85	0.81	0.79	0.78
	10%	0.95	0.93	0.90	0.86	0.82	0.80	0.79
	25%	0.97	0.96	0.93	0.89	0.85	0.83	0.81
	50%	1.02	1.00	0.97	0.93	0.89	0.87	0.85
	75%	1.07	1.05	1.02	0.97	0.93	0.91	0.89
	90%	1.09	1.08	1.04	1.00	0.95	0.93	0.92
	99%	1.11	1.09	1.06	1.01	0.97	0.94	0.93

[a] The 25th and 75th percentiles of serum n-PFOA concentrations were 2.8 ng/mL and 5.7 ng/mL. The interquartile change was approximately a doubling increase in n-PFOA serum concentrations, so it was used in the sensitivity analyses.

[b] $\text{Percent mediated} = {NDE \times (NIE - 1)}\Big/{(NDE \times NIE - 1)} \times 100\%.$

Abbreviations: NDE, natural direct effect; NIE, natural indirect effect; n-PFOA, linear-chain perfluorooctanoic acid.

Supplemental Table IV. 4 Bias-corrected natural indirect effect of n-PFOA exposure on natural menopause incidence for varying prevalence of unmeasured confounder (U) among women with the 25th and 75th percentiles of n-PFOA serum concentrations, with relative survival of U on natural menopause=0.8 or 1.2.

Relative survival=0.8								
	Prevalence of U among the 75th percentile							
Prevalence of U among the 25th percentile		1%	10%	25%	50%	75%	90%	99%
	1%	0.97	0.95	0.92	0.87	0.83	0.80	0.78
	10%	0.99	0.97	0.94	0.89	0.84	0.81	0.79
	25%	1.02	1.00	0.97	0.92	0.87	0.84	0.82
	50%	1.07	1.06	1.02	0.97	0.92	0.88	0.86
	75%	1.14	1.12	1.08	1.03	0.97	0.94	0.91
	90%	1.18	1.16	1.12	1.06	1.00	0.97	0.95
	99%	1.21	1.18	1.15	1.09	1.03	0.99	0.97
Relative survival=1.2								
	Prevalence of U among the 75th percentile							
Prevalence of U among the 25th percentile		1%	10%	25%	50%	75%	90%	99%
	1%	0.97	0.99	1.02	1.06	1.11	1.14	1.16
	10%	0.95	0.97	1.00	1.05	1.09	1.12	1.14
	25%	0.92	0.94	0.97	1.02	1.06	1.09	1.11
	50%	0.88	0.90	0.92	0.97	1.01	1.04	1.06
	75%	0.84	0.86	0.88	0.93	0.97	0.99	1.01
	90%	0.82	0.84	0.86	0.90	0.94	0.97	0.98
	99%	0.81	0.82	0.85	0.89	0.93	0.95	0.97

[a] The 25th and 75th percentiles of serum n-PFOA concentrations were 2.8 ng/mL and 5.7 ng/mL. The interquartile change was approximately a doubling increase in n-PFOA serum concentrations, so it was used in the sensitivity analyses.

[b] Percent mediated $= {NDE \times (NIE - 1)}\Big/{(NDE \times NIE - 1)} \times 100\%$.

Abbreviations: NDE, natural direct effect; NIE, natural indirect effect; n-PFOA, linear-chain perfluorooctanoic acid.

Supplemental Table IV. 5 Bias-corrected natural direct effect of Sm-PFOS exposure on natural menopause incidence for varying prevalence of unmeasured confounder (U) among women with the 25th and 75th percentiles of Sm-PFOS serum concentrations, with relative survival of U on natural menopause=0.8 or 1.2.

Relative survival=0.8								
	Prevalence of U among the 75th percentile							
Prevalence of U among the 25th percentile		1%	10%	25%	50%	75%	90%	99%
	1%	0.94	0.96	0.99	1.04	1.10	1.14	1.17
	10%	0.92	0.94	0.97	1.02	1.08	1.12	1.15
	25%	0.89	0.91	0.94	0.99	1.05	1.09	1.11
	50%	0.85	0.86	0.89	0.94	0.99	1.03	1.05
	75%	0.80	0.81	0.84	0.89	0.94	0.97	1.00
	90%	0.77	0.79	0.81	0.86	0.91	0.94	0.96
	99%	0.75	0.77	0.79	0.84	0.89	0.92	0.94
Relative survival=1.2								
	Prevalence of U among the 75th percentile							
Prevalence of U among the 25th percentile		1%	10%	25%	50%	75%	90%	99%
	1%	0.94	0.92	0.90	0.86	0.82	0.80	0.79
	10%	0.96	0.94	0.91	0.87	0.83	0.81	0.80
	25%	0.98	0.97	0.94	0.90	0.86	0.84	0.82
	50%	1.03	1.01	0.98	0.94	0.90	0.88	0.86
	75%	1.08	1.06	1.03	0.98	0.94	0.92	0.90
	90%	1.11	1.09	1.06	1.01	0.96	0.94	0.92
	99%	1.12	1.10	1.07	1.02	0.98	0.95	0.94

[a] The 25th and 75th percentiles of serum Sm-PFOS concentrations were 4.6 ng/mL and 10.8 ng/mL. The interquartile change was approximately a doubling increase in Sm-PFOS serum concentrations, so it was used in the sensitivity analyses.

[b] Percent mediated $= {NDE \times (NIE - 1)}\Big/{(NDE \times NIE - 1)} \times 100\%$.

Abbreviations: NDE, natural direct effect; NIE, natural indirect effect; Sm-PFOS, branched-chain perfluorooctane sulfonic acid.

Supplemental Table IV. 6 Bias-corrected natural indirect effect of Sm-PFOS exposure on natural menopause incidence for varying prevalence of unmeasured confounder (U) among women with the 25th and 75th percentiles of Sm-PFOS serum concentrations, with relative survival of U on natural menopause=0.8 or 1.2.

Relative survival=0.8								
	Prevalence of U among the 75th percentile							
Prevalence of U among the 25th percentile		1%	10%	25%	50%	75%	90%	99%
	1%	0.98	0.96	0.93	0.88	0.83	0.80	0.79
	10%	1.00	0.98	0.95	0.90	0.85	0.82	0.80
	25%	1.03	1.01	0.98	0.93	0.88	0.84	0.83
	50%	1.09	1.07	1.03	0.98	0.92	0.89	0.87
	75%	1.15	1.13	1.09	1.04	0.98	0.94	0.92
	90%	1.19	1.17	1.13	1.07	1.01	0.98	0.96
	99%	1.22	1.20	1.16	1.10	1.04	1.00	0.98
Relative survival=1.2								
	Prevalence of U among the 75th percentile							
Prevalence of U among the 25th percentile		1%	10%	25%	50%	75%	90%	99%
	1%	0.98	1.00	1.03	1.08	1.12	1.15	1.17
	10%	0.96	0.98	1.01	1.06	1.10	1.13	1.15
	25%	0.94	0.95	0.98	1.03	1.07	1.10	1.12
	50%	0.89	0.91	0.93	0.98	1.02	1.05	1.07
	75%	0.85	0.87	0.89	0.94	0.98	1.01	1.02
	90%	0.83	0.85	0.87	0.91	0.95	0.98	0.99
	99%	0.82	0.83	0.86	0.90	0.94	0.96	0.98

[a] The 25th and 75th percentiles of serum Sm-PFOS concentrations were 4.6 ng/mL and 10.8 ng/mL. The interquartile change was approximately a doubling increase in Sm-PFOS serum concentrations, so it was used in the sensitivity analyses.

[b] $\text{Percent mediated} = {NDE \times (NIE - 1)}\Big/{(NDE \times NIE - 1)} \times 100\%.$

Abbreviations: NDE, natural direct effect; NIE, natural indirect effect; Sm-PFOS, branched-chain perfluorooctane sulfonic acid.

Chapter V. Conclusions

Perfluoroalkyl and polyfluoroalkyl substances (PFAS) are ubiquitous environmental toxicants that are widely used in consumer products and industry, such as non-stick cookware (Teflon) (Bradley *et al.*, 2007); food packaging materials (Schaider *et al.*, 2017); cosmetics (Danish EPA, 2018) and personal care products (Boronow *et al.*, 2019); and fire-fighting foam (Kissa, 2011). PFAS have received unprecedented attention recently due to nationwide drinking water contamination that impacts up to 110 million residents in the United States (EWG *et al.*, 2019). Due to the widespread use of PFAS and their persistence in the environment, several PFAS such as perfluorooctane sulfonic acid (PFOS), perfluorooctanoic acid (PFOA), perfluorohexane sulfonic acid (PFHxS), and perfluorononanoic acid (PFNA) are detectable in >98% of serum samples of the U.S. general population (ATSDR, 2018). Upon exposure, PFAS have the potential to impact the process of reproductive aging in women. However, few human studies have examined associations between these compounds and the timing of natural menopause. This dissertation fills a critical research gap in the current literature by advancing our understanding of potential reproductive and endocrinological mechanisms by which PFAS may impact ovarian aging during the menopausal transition.

1. Summary of findings

Aim 1

To improve our understanding of exposure to per- and polyfluoroalkyl substances (PFAS) in midlife women, we describe longitudinal changes in PFAS serum concentrations during the menopausal transition and evaluated whether time trends differed by participant characteristics. This chapter was based on four repeated measurements of blood serum samples collected 1999 through 2011 in a cohort of 75 multiethnic midlife women (including White,

Black and Chinese) 45-56 years of age at baseline from three study sites (i.e. Boston, MA; southeast MI, and Oakland, CA) in the United States. We measured 11 PFAS compounds, including linear-and branched-chain isomers of perfluorooctanoic acid (n-PFOA and Sb-PFOA, respectively), linear- and branched-chain isomers of perfluorooctane sulfonic acid (n-PFOS and Sm-PFOS, respectively), 2-(N-ethyl-perfluorooctane sulfonamide) acetate (EtFOSAA), 2-(N-methyl-perfluorooctane sulfonamide) acetate (MeFOSAA), perfluorohexane sulfonic acid (PFHxS), perfluorononanoic acid (PFNA), perfluorodecanoic acid (PFDeA), perfluoroundecanoic acid (PFUA), and perfluorododecanoic acid (PFDoA).

Serum concentrations of n-PFOA, n-PFOS and Sm-PFOS decreased significantly (P=0.001 for n-PFOA; P<.0001 for n-PFOS and Sm-PFOS) from 1999 to 2011. Detection rates of PFOS precursor compounds EtFOSAA and MeFOSAA also decreased. In contrast, serum PFHxS concentrations remained unchanged (P=0.05). An alternative compound, PFNA serum concentrations increased significantly (P<.0001). Detection rates of other replacement compounds, including PFDeA and PFUA doubled.

After adjusting for age at baseline, study site, body mass index at baseline, parity and menstruation status, temporal trends of PFAS exposures were not uniform across racial/ethnic groups (P=0.001 for n-PFOA, P=0.0007 for n-PFOS, P=0.03 for Sm-PFOS, P=0.008 for PFHxS). Compared to other racial/ethnic groups, Chinese women showed a much slower decline in serum concentrations of n-PFOA, n-PFOS and Sm-PFOS, and little difference in PFHxS concentrations over time. Although serum concentrations of PFNA did not change significantly by race/ethnicity (P=0.13), Chinese women had consistently higher concentrations compared to others (P=0.03 at baseline for racial/ethnic differences). Menstrual bleeding was negatively associated with serum PFAS concentrations at baseline whereas time trends did not differ by

menstruation status. Nulliparity was positively correlated with serum PFAS at the MPS baseline while the differences between nulliparity and parity narrowed over time.

This chapter updates the existing knowledge on human exposure to PFAS. It provides valuable data on midlife women's exposure to PFAS as they transitioned through menopause in the 1990s and first decade of the 2000s and temporal trends of PFAS serum concentrations, which have rarely been reported before. This chapter also provides new evidence on the contribution of race/ethnicity, menstruation and parity status to the longitudinal changes of PFAS serum concentrations. The main strengths of this analysis include:

- We examined 11 PFAS in the population of multiethnic midlife women.
- The repeated measurements during 1999 and 2011 also allow for an examination of longitudinal intra-individual changes in PFAS serum concentrations.
- The wide geographical and racial/ethnic coverage of the SWAN participants enabled us to compare differences in biomarkers levels across multiple groups and increased the generalizability of our findings.

Aim 2

In this chapter, we examined whether exposure to PFAS was associated with incident natural menopause. While the association between PFAS and natural menopause have been reported previously (Dhingra *et al.*, 2016; Knox *et al.*, 2011; Taylor *et al.*, 2014), the results of those studies have been inconsistent. These studies also raised concerns about reverse causation in that it is unclear whether PFAS exposure contributed to earlier menopause, or cessation of PFAS excretion via cessation of menstruation led to increased serum concentrations of PFAS in women.

We, therefore, utilized a prospective cohort of 1,120 multiethnic women (including White, Black, Chinese and Japanese) who were premenopausal at baseline from the Study of Women's Health Across the Nation with approximately annual clinic visits during 1999 and 2017. Concentrations of PFHxS, linear-chain PFOS (n-PFOS), branched-chain PFOS (Sm-PFOS), linear-chain PFOA (n-PFOA), and PFNA were measured in blood serum samples collected at baseline (1999-2000). We evaluated the associations between baseline serum concentrations of PFAS and incidence of natural menopause and assessed whether the relationships differed by racial/ethnic groups. We next identified subgroups exposed to different overall patterns of PFAS using k-means clustering method and evaluated the combined effects of PFAS mixtures on natural menopause.

In this 17-year prospective cohort of 1,120 women with 5,466 person-years of observations in annual follow-up visits, we found that higher baseline serum concentrations of n-PFOS, Sm-PFOS, and n-PFOA were significantly associated with earlier onset of natural menopause. After adjusting for age at baseline, race/ethnicity, study site, education, parity, baseline body mass index, time-varying physical activity, time-varying smoking status and prior hormone use at baseline, women with the highest tertile of baseline serum concentrations had a hazards ratio of natural menopause of 1.26 (95% CI: 1.02-1.57) for n-PFOS (*Ptrend*=0.03), 1.27 (95% CI: 1.01-1.59) for Sm-PFOS (*Ptrend*=0.03), and 1.31 (95% CI: 1.04-1.65) for n-PFOA (*Ptrend*=0.01). When examining interactions between PFAS exposure and race/ethnicity, significant associations with incident natural menopause were observed for PFNA and n-PFOA in White women but not in other racial/ethnic groups.

In the mixture analysis, we identified four overall exposure patterns of PFAS, i.e., "low", "low-medium", "medium-high", and "high" exposure groups. Women with high overall PFAS

exposure had significantly earlier natural menopause (HR=1.66, 95% CI: 1.17-2.36), compared to those with low overall exposure profiles. High overall PFAS exposure patterns might lead to 1.8 years earlier median time to natural menopause.

As age at the final menstrual period reflects a woman's overall health, it has become clinically more important to investigate the role of potential endocrine-disrupting chemicals and timing of natural menopause. This chapter updates the existing knowledge on the endocrine-disrupting roles of PFAS. It is the first prospective cohort, to our knowledge, to evaluate the associations of exposure to various PFAS with the occurrence of natural menopause in multiethnic midlife women. Additionally, no previous research of which we are aware has explored the mixture effects of PFAS on timing of ovarian aging.

This chapter includes direct measurements of PFAS exposure prior to menopause, prospectively determination of date of the final menstrual period, and a large cohort of community-based midlife women from four racial/ethnic groups followed for up to 17 years. The main advantages of this chapter include:

- The prospective design minimized the possibility of reverse causation which cannot be ruled out in the previous studies as women appear to have higher PFAS concentrations after menopause.
- Standard approximately annual follow-up visits instead of one-time recall provided more reliable estimates of the date of the final menstrual period.
- The wide geographical and racial/ethnic coverage of the SWAN participants enabled us to compare differences in biomarkers levels across multiple groups and increased the generalizability of our findings.

Aim 3

Our previous studies have shown that PFAS exposure was associated with earlier onset of natural menopause (Ning Ding *et al.*, 2019). In a separate analysis, higher serum concentrations of PFAS were associated with increased levels of follicle-stimulating hormone (FSH) in midlife women during the menopausal transition . In this chapter, we conducted causal mediation analysis of the relationships between PFAS exposure and incident natural menopause by altering FSH levels. We utilized the same prospective cohort of 1,120 multiethnic women (including White, Black, Chinese and Japanese) who were premenopausal at baseline from the Study of Women's Health Across the Nation. Concentrations of PFHxS, linear-chain PFOS (n-PFOS), branched-chain PFOS (Sm-PFOS), linear-chain PFOA (n-PFOA), and PFNA were measured in blood serum samples collected at baseline (1999-2000). Serum concentrations of FSH were also assessed at baseline. Time to natural menopause was modeled using accelerated failure time (AFT) models with a Weibull distribution. AFT models were chosen because they outperformed Cox proportional hazards regression models in causal mediation analysis with non-rare outcomes (Gelfand *et al.*, 2016; VanderWeele, 2011). Baseline covariates included age, race/ethnicity, study site, educational attainment, BMI, parity, physical activity score, smoking status and prior hormone use.

With FSH as being the mediator, 26.8% (95% CI: 10.3%, 39.3%) of the total effects of n-PFOA on natural menopause is attributable to indirect effects through the mediator, and 23.8% (95% CI: 3.8%, 38.3%) of the total effects of Sm-PFOS is attributable to indirect effects. No causal mediation effects were observed for n-PFOS (percent mediated=8.1%, 95% CI: -15.8%, 22.6%), PFNA (percent mediated=8.9%, 95% CI: -8.5%, 22.0%), and PFHxS (percent mediated=20.3%, 95% CI: -24.8%, 42.0%). We conducted comprehensive sensitivity analyses to

test the assumptions of no unmeasured confounding. Assuming an unknown confounder, either protective against earlier menopause (e.g. healthy lifestyle) or other hazardous endocrine-disrupting chemicals, it is possible that the observed percent of mediation are underestimated.

Use of mediation analysis in environmental epidemiology is surprisingly limited although many studies in environmental epidemiology measure markers of intermediate biological changes (e.g. hormone levels) in addition to examining exposure and health outcomes. This chapter updates existing knowledge regarding the endocrine-disrupting roles of PFAS. It is the first study, to our knowledge, to evaluate the mediating role of hormones on the associations between PFAS exposure and reproductive outcomes, specifically age at menopause.

2. Public health implications

Biomonitoring studies at the population level are important to alert researchers about geographical or time trends and provide a picture of the amount of PFAS actually accumulated in the blood for a specific period of time. In particular, time trends in population-level biomonitoring data provide evidence of whether policy change are effective and can contribute to lowering population-wide exposure levels. For example, the phase-out of leaded gasoline in 1970s has contributed to a tremendous reduction in blood lead levels in the U.S. general population. Similarly, the voluntary phase-outs of PFOA and PFOS by industries and regulatory bodies since 2000 in the United States (USEPA, 2002; 3M, 2000), have resulted in population-wide reductions in exposure to legacy compounds, which was confirmed in this dissertation. However, serum concentrations of PFHxS remained unchanged and PFNA exposure increased, suggesting that emerging compounds need to be restricted in industrial applications and consumer products.

PFAS are widespread drinking water contaminants because they are mobile in groundwater, as well as persistent and bioaccumulative in the environment (Post et al. 2017). There are currently no federal drinking water standards in the U.S. despite widespread drinking water contamination and ubiquitous population-wide exposure. Instead, the U.S. EPA released a non-enforceable lifetime health advisory for PFOA and PFOS at 70 parts per trillion (ppt), separately or combined, in 2016 (U.S. EPA 2016). Without an enforceable standard, public water systems are not required to routinely test for PFOA and PFOS or to treat water exceeding the health standards. In the absence of federal standards, seven U.S. states have adopted or proposed their own health-based drinking water guideline levels (ITRC 2017), ranging from 13 to 1000 ppt. Several states have established guideline levels below EPA's health advisory, suggesting that EPA's approach may not be sufficiently protective.

In addition, there are no health reference guidelines for other compounds. In 2019, the Michigan Department of Environment, Great Lakes, and Energy (EGLE) has proposed Maximum Contaminant Levels (MCLs) for seven types of PFAS including PFNA at 6 ppt, PFOA at 8 ppt, PFHxA at 400000 ppt, PFOS at 16 ppt, PFHxS at 51 ppt, PFBS at 420 ppt, and GenX at 370 ppt (EGLE 2019). Discrepancies in PFAS drinking water guidelines between the U.S. EPA and the states that adopted stricter guidelines suggest the remaining scientific uncertainty and assumptions that underlie these decisions. More research on risk assessment for PFAS in different exposure scenarios through drinking water consumption is urgently needed to set PFAS drinking water guidelines.

Most previous studies were limited to one race/ethnic group and conducted within high-exposed communities (e.g. the C8 Health Project conducted in residents from the Mid-Ohio Valley area). They also focused primarily on children and women of childbearing age. My

dissertation fills research gaps on race/ethnic disparities and provides data from midlife women that have been understudied. Midlife women could be identified as susceptible populations for public health interventions, because serum PFAS concentrations may reaccumulate years after pregnancy and cessation of menstrual bleeding. This dissertation also recommends that future toxicokinetic studies account for menstruation and parity as important routes of elimination.

With the aging population in the U.S., the number of individuals aged 65 years and older is projected to more than double in the upcoming decades, rising from 40.2 million in 2010 to an estimated 88.5 million by 2050 (Richardson *et al.*, 2014). Women will comprise the majority of the aging population, as nearly half of them are in the age range during which women typically experience perimenopause, the menopausal transition, or have entered the postmenopausal phase of their lives (Ortman *et al.*, 2014). Few studies have collected data on midlife women and focused on menopausal transition and reproductive outcomes in women's later life. The SWAN study design and its longitudinal nature allowed for the study of long-term reproductive effects from endocrine-disrupting chemicals (EDCs) and their mixtures.

Age at the final menstrual period may be marker for not only reproductive aging but also for general health and somatic aging. Menopause could be the first step of a causal pathway that, because of hormonal changes, results in organ dysfunction. Many undesirable side effects result from the menopausal transition and reproductive senescence. Common symptoms during this period include vasomotor hot flushes, sweating, vaginal dryness, vaginal atrophy, dyspareunia, urinary incontinence, irritability, depression, and insomnia. More serious side effects can result from a prolonged estrogen deficiency and premature menopause, including a significant loss of bone mineral density and an increased risk of serious cardiovascular diseases, such as atherosclerosis and myocardial infarction. The timing of reproductive aging has become an

important public health concern because with advancing life expectancy a full one-third of women's lives are spent postmenopause.

Using the SWAN data, this dissertation suggests that exposure to PFAS may lead to earlier onset of menopause in midlife women, a risk factor for chronic disease and mortality. Women who had high overall concentration profiles of PFAS exposure reached their natural menopause 2.0 years earlier compared to those in the low group. It has been reported that various factors such as educational attainment, occupation, use of oral contraceptives, age at menarche, obesity, smoking, and alcohol consumption (Gold *et al.*, 2013; Morris *et al.* 2012). Among these factors, smoking has been identified as one of the most significant cause of accelerating the onset of natural menopause. In our sample, current smokers tended to reach their natural menopause 1.1 years earlier compared to women who have never smoked before. Thus, similar to cigarette smoking, PFAS may also be an important risk factor for earlier onset of menopause.

PFAS compounds are found in the blood of most people and are all potentially modifiable. Given the endocrine-disrupting role of these chemicals, it is important to set up early control of PFAS exposure in women, especially those living in a city with a PFAS-contaminated public water supply. The medical community should be aware of potential risk posed by PFAS and other EDCs to maintenance of reproductive function. Health practitioners should also be prepared for questions about these chemicals because it is a topic of concern to patients who may present with these endocrine-disrupting chemicals. In addition, altering consumer product behaviors is one way for individuals to lower their personal exposure to these harmful chemicals. Consumer products known to contain PFAS based on product testing include dental floss, non-stick cookware, food packaging materials, sofa and furniture treated with PFAS prior to sale, ski and floor waxes, and thread seal tape (Boronow *et al.*, 2019; Kotthoff *et al.*, 2015; Herzke *et al.*

2012; Guo *et al.*, 2009). From a precautionary point of view, it makes sense to avoid the use of PFAS-containing consumer products.

Furthermore, our findings suggest that PFAS exposure may accelerate ovarian aging through endocrinologic mechanisms related to variations in FSH levels. It revealed an underlying mechanism of how PFAS exposure disrupted ovarian function and led to earlier onset of natural menopause. The estimation of the mediating role of FSH could inform future precision health studies that seek to target specific biological pathways for interventions. Overall, this dissertation contributed in providing implications for policy makers and health professionals to help them with the improvement of public's health, especially among women in their later life stage.

3. Future directions

In combination with this dissertation's research findings and study limitations, several additional research questions and improvements for future studies are proposed as follows:

(1) Measurement of emerging PFAS compounds: The phase out of PFOA and PFOS has led to an increasing usage of alternative compounds (Ateia *et al.*, 2019). For example, GenX chemicals are used to make high-performance fluoropolymers and non-stick coatings without the use of PFOA; and PFBS is a replacement chemical for PFOS (USEPA, 2018). However, there is inadequate evidence of general toxicity as well as ovarian toxicity of such alternative compounds. Future studies should fill these gaps with regard to sources and pathway of exposure and toxic effects of emerging PFAS on reproductive outcomes and their related chronic conditions.

(2) Expanded measurements of biomarkers: In regard to Aim 3, FSH could partially explain the associations between FPAS exposure and natural menopause. Besides the effect on FSH,

other possible mechanisms include oxidative stress (Wielsøe *et al.*, 2015) and/or distribution of thyroid hormone homeostasis (Chang *et al.*, 2008; Lau *et al.*, 2003; Thibodeaux *et al.*, 2003). It would be informative to measure thyroid hormone levels and biomarkers of oxidative stress (e.g. Gamma-glutamyl transferase). By doing so, we can better capture the pathways that might lead to earlier onset of natural menopause and understand the biological mechanisms through which PFAS alter reproductive function

(3) Analysis of EDC mixtures: Previous studies have linked phthalates, polychlorinated biphenyls (PCBs), polybrominated diphenyl esters (PBDEs), and other EDCs to impaired ovarian function (Craig and Ziv-Gal, 2018; Grindler *et al.*, 2015; Harley *et al.*, 2019). It is increasingly recognized that environmental endocrine disruption is most often not due to the effect of a single compound, but rather due to co-exposure to mixtures of chemicals at low concentrations (Alyea and Watson, 2009; Braun *et al.*, 2016; Wang *et al.*, 2018, 2019). Thus, future research should examine the effects of exposure to a mixture of persistent and non-persistent EDCs on ovarian health.

(4) Linking natural menopause to health conditions: This dissertation estimates the associations between PFAS exposure and incident natural menopause. The toxicity of PFAS on ovarian function is not fully understood. Future studies should explore the associations of PFAS exposure with vasomotor symptoms (e.g. hot flashes, night sweats), duration of menopausal transition, and hormone trajectories. The physiology of reproductive aging likely represents an interplay between multiple central and peripheral physiologic systems. Overall, a major follow-up question that arises from this dissertation is "what is the health consequences to midlife women?" An important next step is to link data on environmental exposures, age at natural menopause, and chronic diseases (e.g. cardiovascular disease, osteoporosis, and cognitive

decline). By doing so, we can better inform policy makers and health practitioners about population-level burden of environmental exposures and costs to society.

4. Overall conclusion

In conclusion, this dissertation fills a critical research gap in the current literature on endocrine disruption and reports evidence that exposure to PFAS is associated with earlier onset of natural menopause, possibly though altering FSH levels and depleting the ovarian reserve. By analyzing the temporal variations of PFAS serum concentrations and their determinants, this dissertation also identifies midlife women as potential susceptible subpopulations. Recent biomonitoring data, health effects data, and additional information to be solicited from the public will inform the development of public health strategies and safety regulation for a broader class of PFAS in the future.

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