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NHANES epidemiological analysis of associations between exposure to per- and polyfluoroalkyl substances (PFAS) and long-term amenorrhea in women of reproductive age

By

Yihan Fan
Degree to be awarded: MPH.

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By

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Bachelor of Medicine
Peking University
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A thesis submitted to the faculty of the
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Abstract

NHANES epidemiological analysis of associations between exposure to per- and polyfluoroalkyl substances (PFAS) and long-term amenorrhea in women of reproductive age

By Yihan Fan

Background: The per- and polyfluoroalkyl substances (PFAS) have been widely used in numerous consumer and industrial products. The high persistence of PFAS has led to extensive environmental contamination and bioaccumulation. Previous research revealed associations between exposure to PFAS and female reproductive disorders, but the underlying mechanisms remains elusive. Moreover, nearly all studies focused on perfluorooctanoic acid (PFOA) and perfluorooctane sulfonate (PFOS), the two long-chain legacy PFAS that have been gradually phased out, yet the reproductive impacts of other long-chain PFAS and short-chain alternatives are rarely studied.

Objectives: We aimed to use epidemiological approaches to investigate toxic effects of long- and short-chain PFAS on the female reproductive outcomes.

Methods: Using three consecutive 2-year cycles (2013-2018) of the National Health and Nutrition Examination Survey (NHANES) data, a cross-sectional analysis was performed to examine epidemiological associations between blood concentrations of PFAS and long-term amenorrhea in women of reproductive age, a proxy of ovarian dysfunction.

Results: The NHANES epidemiological analysis revealed that compared with menstruating women, women with long-term amenorrhea had significantly higher blood concentrations of several long-chain PFAS, including PFOA, PFOS, PFNA, PFHxS, and PFDeA. After the full adjustment for confounding factors, multiple logistic regression analysis revealed concentration-dependent associations between each of these long-chain PFAS and women's long-term amenorrhea.

Discussion: This study utilizes epidemiological approaches to establish that exposure to long-chain PFAS at environmentally relevant levels presents a significant risk to women's reproductive health. emphasizing an urgent need to reduce or eliminate exposure to PFAS to protect women's reproduction.

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Keywords: Per- and polyfluoroalkyl substances, long-chain PFAS, short-chain PFAS, alternative, NHANES

Table of Content

Introduction

The per- and polyfluoroalkyl substances (PFAS) are thousands of synthetic organic compounds that consist of a major carbon backbone and at least one fluoroalkyl moiety (C_nF_{2n+1}-) [1, 2]. PFAS that contain 8 or more carbons are defined as long-chain PFAS, including perfluorooctanoic acid (PFOA), perfluorooctane sulfonate (PFOS), and perfluorononanoic acid (PFNA). Short-chain PFAS containing 4-7 carbons, such as perfluorobutane sulfonic acid (PFBS) and GenX, are now increasingly manufactured and applied as alternatives [3, 4]. The strong C-F bonds of PFAS make render them of have low surface tension and thermally, chemically, and oxidatively resistant [5]. Since the 1960s, PFAS has been widely used in numerous consumer and industrial products, including textiles, food packaging, cookware coating, surfactants, personal care products, and aqueous firefighting foams [6-8]. PFAS are highly resistant to environmental degradation, which has led to extensive environmental contamination and bioaccumulation [9, 10] and earn the name “Forever Chemicals” [5, 11]. Due to their health threats to human and wildlife animals, there is a rising global environmental and public health concern [12].

Humans are exposed to PFAS primarily through drinking water, while it is also possible through contaminated foods, soil, contact with PFAS-containing products, and PFAS manufacturing process [13-17]. These exposures impact millions of residents in the US and also the larger population worldwide [18-20]. The strong C-F bonds make many PFAS rarely metabolize in human bodies after absorption, with long half-lives up to 8-9 years [21, 22]. Long-chain PFAS are detectable in > 90% of world populations [23-25], with blood

concentrations ranging from 1.5 to 220 μM [21, 23, 25-30].

Exposure to PFAS has been related to several adverse health outcomes in humans, such as liver toxicity [31, 32], cancers [33], immunotoxicity [34, 35], and metabolic disorders [36]. Growing epidemiological evidence revealed associations between PFAS and female reproductive dysfunctions related to the ovary, such as premature ovarian failure [37, 38], irregular menstrual cycles [39], polycystic ovarian syndrome (PCOS) [40-43], and infertility [44-46]. The ovary houses follicles at various stages to sustain female reproductive cycles and fertility. The early phase of follicle activation and development is largely gonadotropin independent, but the eventual maturation of a secondary or early antral follicle is governed by a pituitary gonadotropin follicle-stimulating hormone (FSH), and the ovulation of a fully mature preovulatory follicle is triggered by another pituitary gonadotropin, luteinizing hormone (LH) [47-50]. Experimental research has reported adverse impacts of long-chain PFAS on ovarian cyclicity [51-54], steroidogenesis [55-57], and oocyte maturation [58-61], but the underlying mechanism remains elusive. Moreover, nearly all studies focused on PFOA and PFOS, the two long-chain legacy PFAS that have been gradually phased out in the US and many other counties. Other long-chain PFAS such as PFNA and emerging short-chain PFAS such as GenX, however, may reach similar contamination levels [3, 4, 62], but few examined their impact on female ovarian functions and reproduction.

The objective of this study was to integrate epidemiological approaches to investigate the ovarian disrupting effects of PFAS. Long-chain PFAS have been shown to exhibit longer half-lives after absorption and stronger PPARs protein binding affinity than short-chain PFAS [63-65]. We performed a cross-sectional analysis to examine epidemiological associations between blood concentrations of PFAS and long-term amenorrhea, a marker of ovarian dysfunctions, in

women of reproductive age using three consecutive 2-year cycles of the National Health and Nutrition Examination Survey (NHANES) in 2013-2018.

Materials and Methods

Study population

All recruited subjects and associated measurements in this study were obtained from NHANES, a national-wide survey that is conducted by the National Center for Health Statistics (NCHS) of the US Centers for Disease Control and Prevention (CDC) to assess the health and nutritional status of adults and children in the US and track changes over time conducted by the National Center for Health Statistics (NCHS) of the US Centers for Disease Control and Prevention (CDC). NHANES consists of multi-year, stratified, and clustered four-stage samples, and all data are released in 2-year cycles. All details of the NHANES background, design, operation, data access and download, weighting, and data analysis are available at the NHANES website [66]. Although NHANES began to measure blood concentrations of PFAS in the cycle of 1999-2000 [67], herein, we used three consecutive 2-year cycles of 2013-2014, 2015-2016, and 2017-2018, in which both women's menstrual cycles and blood concentrations of PFAS were measured using the same methodologies. For instance, NHANES measured total PFOS or PFOA before 2013 and began to measure the linear and branched isoforms of PFOS and PFOA beginning in 2013. All data including women's menstrual cycles, blood concentrations of PFAS, and sociodemographic and physical measurements were downloaded from the CDC's website and processed following NHANES guidelines [66, 68].

Measurements of blood (serum) concentrations of PFAS in NHANES participants and PFAS inclusion strategy

For the cross-sectional epidemiological analysis, seven long-chain PFAS, including perfluorooctanoic acid (PFOA), perfluorooctane sulfonic acid (PFOS), perfluoro decanoic acid (PFDeA), perfluoro hexane sulfonic acid (PFHxS), 2- (N-methyl-perfluoroctane sulfonamido) acetic acid (Me-PFOSA-AcOH), perfluoro nonanoic acid (PFNA), and perfluoroundecanoic acid (PFUA) were included because the blood concentrations of these seven PFAS were available in all three selected NHANES cycles. With respect to short chain PFAS, such as perfluoroheptanoic acid (PFHpA), perfluorobutane sulfonic acid (PFBS), and GenX, the blood concentrations in the majority of NHANES participants were below the lower limit of detection (LLOD) [69]. Thus, we did not include short-chain PFAS for the epidemiological analysis. The quantification of blood serum concentrations of PFAS was performed using the online solid phase extraction coupled with the high-performance liquid chromatography-turboionspray ionization-tandem mass spectrometry (online SPE-HPLC-TIS-MS/MS). The LLOD of each PFAS was 0.10 ng/mL, and the PFAS concentrations below the LLOD were imputed by dividing the LLOD by the square root of two (LLOD/sqrt2), with the final imputed PFAS concentrations reported as 0.07 ng/mL. The detailed laboratory procedures of PFAS measurements are available at the CDC NHANES website of https://wwwn.cdc.gov/Nchs/Nhanes/2013-2014/PFAS_H.htm. Different from other PFAS, the blood concentration of PFOA was calculated as the sum of branched PFOA isomers (Sb-PFOA) and the linear isomers (n-PFOA), and the blood concentration of PFOS for each participant was calculated as the sum of the branched PFOS isomers (perfluoromethylheptane sulfonate isomers, Sm-PFOS) and the linear isomers (n-PFOS) [70]. For the calculation of either PFOA or PFOS, if the concentration of one isomer was below the LLOD, the PFOA or PFOS concentration was the sum of detected value of one isomer and imputed 0.07 ng/mL; if the

concentrations of both isomers were below the LLOD, the concentration of PFOA or PFOS was the sum of two imputed 0.07 ng/mL, which gave 0.14 ng/mL.

Subject inclusion

Figure 1 illustrates the process of subject recruitment in our study. In NHANES 2013-2018, the weighting of PFOA and PFOS is different from the other five selected PFAS. We thus analyzed the epidemiological associations of PFOA and PFOS separately with the same methodology. With respect to PFDeA, PFHxS, Me-PFOSA-AcOH, PFNA, and PFUA ('n1' in Figure 1), there were 6642 subjects, including 3030 males and 3327 females in three selected NHANES cycles. Although all subjects over 12 years had the laboratory measurements of PFAS, because NHANES collected female reproductive health data only from participants who were 20 years and older, we only included reproductive aged women (20-45 years). After the exclusion of males (n = 3212), females under 20 years (n = 611), and females over 45 years (n = 1609),1210 women of reproductive age. A total of 862 women of reproductive age were eventually included after further excluding women who had missing data on long-term amenorrhea (n=153), underwent hysterectomy (n=40), and had missing data on blood PFAS concentrations (n=74), household income (n=75), BMI (n=5), and smoking history (n=1). With respect to PFOA and PFOS ('n2' in Figure 1), the subject inclusion method was the same, and the process of inclusion and exclusion details leading to the eventual inclusion of 831 subjects were summarized in Figure 1.

Definition of women with menstruation and long-term amenorrhea

Women with long-term amenorrhea were defined as those who answered "No" to the question of "Have you had at least one menstrual period in the past 12 months? (Please do not include bleedings caused by medical conditions, hormone therapy, or surgeries.)," and answered "Other"

or “Don’t know” to the question of “What is the reason that you have not had a period in the past 12 months?”. Menstruating women were defined as women who answered “Yes” to the first question. Women who answered who answered “Pregnancy,” “Breast feeding,” and “Menopause/Change of life” to the question of “What is the reason that you have not had a period in the past 12 months?” were excluded from this study.

Covariates selection

Demographic variables including race/ethnicity, education, and Poverty Income Ratio were included as covariates. Age, marital status, health insurance coverage, smoking status, and BMI have all been shown to influence women's reproductive health and fertility management and are therefore also included as covariates. The CDC defines BMI as underweight (<18.5), healthy weight (18.5 to <25), overweight (25 to <30), and obesity (30 or more) [71]. The use of hormonal contraceptives is also included, as women are often prescribed hormones to regulate menstruation or prevent menstruation and unwanted pregnancy.

Data and statistical analysis

Both sampling weights and sample design variables were used following the NHANES recommendation, because the NHANES sampling is a clustered design and incorporates differential selection probabilities. The statistical software SAS v9.4 (SAS Institute, Cary, NC) was used for all NHANES analyses, incorporating sampling weights and non-responses while adjusting for cluster (PSUs) and strata of the complex sample design in NHANES [72, 73]. Weights were calculated using NHANES subsample weights according to NHANES protocols and documentation [74].

The statistical difference between various covariates and descriptive outcomes was analyzed using the chi-square test for categorical variables and t-test for continuous variables. Since the blood concentrations of all five PFAS had skewed distributions based on the normality test, logarithmic conversion of blood PFAS concentrations was used for the multiple logistic regression analysis. Multiple logistic regression analysis was performed to assess the independent association between blood concentrations of each PFAS and women's long-term amenorrhea with the full adjustment for all covariates and four others unexamined PFAS. Adjusted odds ratios (OR) and their corresponding 95% confidence intervals (CI) were presented. We first used the blood concentrations of each PFAS as continuous variables to examine its associations with women's long-term amenorrhea. In addition, we also categorized the blood concentrations of each PFAS in eligible women into four quartiles and used the lowest quartile as a reference to perform multiple regression analysis and calculate OR for quartiles 2 - 4.

Results

We first used NHANES 2013-2018 to investigate epidemiological associations between blood concentrations of seven major long-chain PFAS and long-term amenorrhea in reproductive aged women. Figure. 1 illustrates participant inclusion and exclusion criteria. For the analysis of PFDeA, PFHxS, Me-PFOSA-AcOH, PFNA, and PFUA (n1), 862 reproductive aged women were included with 828 of them having normal menstruation and 34 having long-term amenorrhea. For the analysis of PFOA and PFOS (n2), 831 women were included with 799 of them having normal menstruation and 32 with long-term amenorrhea. Menstruating women and women with long-term amenorrhea had similar distributions of all covariates, including age, education, marriage, health insurance, income, BMI, smoking history, and the use of

hormonal contraception (all $p > 0.05$), except those women with long-term amenorrhea tended to be Non-Hispanic White ($p<0.05$, Table 1 and Supplemental Table 1)

The medians and log transformed means of the blood concentrations of 7 PFAS are summarized in Table 2. Compared with menstruating women, women with long-term amenorrhea had comparable median concentrations of PFDeA, PFHxS, Me-PFOSA-AcOH, PFNA, and PFOA ($p>0.05$), borderline higher median of PFOS ($p=0.0769$), and significantly higher median of PFUA ($p=0.0227$). Regarding log transformed means of PFAS in women with long-term amenorrhea, PFNA was borderline higher ($p=0.0825$) and PFHxS, PFOA, and PFOS were significantly higher than menstruating women ($p<0.05$). There was no significant difference for Me-PFOSA-AcOH and PFUA ($p>0.05$).

Table 3 summarizes epidemiological associations between blood concentrations of PFAS and women's long-term amenorrhea. Multiple logistic regression analysis using continuous log transformed data revealed that as the blood concentrations of PFDeA, PFHxS, PFNA, PFOA, and PFOS increased, women were more likely to experience long-term amenorrhea. When PFAS concentrations were divided into 4 quartiles, the categorical multiple logistic regression analysis showed that the blood concentrations of PFDeA, PFHxS, PFNA, PFOA, and PFOS in quartile 4 were significantly associated with the risk of women's long-term amenorrhea. Moreover, the odds of quartiles 2 to 4 of all these five PFAS exhibited a concentration-dependent manner. With respect to the other two PFAS, Me-PFOSA-AcOH and PFUA, both the continuous and categorical multiple logistic regression analyses showed insignificant associations. Taken together, these epidemiological results are in line with our experimental findings, indicating that long-chain PFAS may exhibit ovarian disrupting effects and increase the odds of long-term amenorrhea in reproductive aged women.

Discussion

Approximately 10-15% of reproductive aged women experience reproductive disorders and infertility, with ovarian disorders as the leading cause [75-77]. The etiology of these ovarian dysfunctions is not fully understood but has been related to exposure to environmental EDCs (endocrine disrupting chemicals) [78], including PFAS [79]. Here, we conducted a cross-sectional analysis using NHANES 2013-2018 and revealed that the blood concentrations of long-chain PFAS concentration-dependently increased the risk of long-term amenorrhea in women of reproductive age, a proxy of ovarian dysfunction.

PFOA and PFOS have been classified as Persistent Organic Pollutants (POPs) by the Stockholm Convention, an international environmental treaty, in 2017 [80], and both were designated as hazardous substances by the US EPA (Environmental Protection Agency) [81]. Together with several major global PFAS manufactures, EPA targeted to eliminate the production and emission of PFOA and PFOS by 95% by the 2010s [82, 83]. Lately, EPA proposed to establish legally enforceable Maximum Contaminant Levels (MCLs) of six PFAS (PFOA, PFOS, PFNA, PFHxS, PFBS, and GenX) in drinking water through the program of National Primary Drinking Water Regulation (NPDWR). In line with these efforts, a decline in the blood circulating levels of PFAS, particularly PFOA/S has been observed in populations in the US and other countries [26, 84]. In NHANES participants, including both males and females, the geometric mean of PFOA decreased from 5.22 ng/mL in 1999-2000 to 1.42 ng/mL in 2017-2018, the mean of PFOS decreased from 30.3 ng/mL in 2009-2010 to 4.25 ng/mL 2017-2018, and the mean of PFNA increased from 0.55 ng/mL in 1999-2000 to 1.26 ng/mL in 2009-2010 and then decreased to 0.41 ng/mL in 2017-2018 [85, 86]. However, due to the high persistence and long half-lives of long chain PFAS, exposure to PFAS remains prevalent. Our

NHANES analysis showed that PFOA and PFOS were detectable in nearly all participants (>99%) and PFNA were detectable in 93.74% of participants. Moreover, their blood levels were concentration-dependently associated with increased odds of long-term amenorrhea in women of reproductive age, suggesting that the relatively low exposure levels of long-chain PFAS remain a concern on women's reproductive health.

Previous epidemiological studies reported associations between long-chain PFAS and women's menstrual health. A study from Fei et al included 1240 pregnant women from the Danish National Birth Cohort and found that higher blood levels of PFOA/S were correlated with an increased risk of irregular periods before pregnancy, with odds of 1.66 and 1.22, respectively [45]. Another cross-sectional study enrolling 1623 pregnant women from Greenland, Poland, and Ukraine measured women's blood concentrations of PFOA/S and reported that higher blood concentrations of PFOA/S were correlated with women's historical longer or irregular menstrual cycles, with odds of 1.8 and 1.7, respectively [87]. Zhou and colleagues studied the blood concentrations of 10 PFAS and their associations with 950 pre-pregnant women's menstrual cycles. It was also found that women with higher levels of PFOA, PFOS, PFNA, and PFHxS were more likely to experience irregular menstrual cycles, with odds of 1.52, 1.29, 1.50, and 1.80, respectively [88]. Our results using NHAENS participants concur with these epidemiological findings. It is worth noting that the odds between the blood levels of long-chain PFAS and women's impaired menstrual health are higher in our study (Zhao paper vs our study: PFOA = 0.09 vs 1.05, PFOS = 0.09 vs 2.72, and PFNA = 0.02 vs 0.34 ng/mL). This discrepancy might be caused by the distinct health status of examined subjects. For example, the first two aforementioned studies recruited pregnant women and focused on their historical menstrual cycles, indicating that all included subjects were able to become pregnant although the time to pregnancy (TPP) might be > 12 months and/or they may receive infertility

treatments [45, 87]. However, all participants in our study were non-pregnant at the time of the NHANES survey. While the study from Zhou et al. surveyed non-pregnant women, but women who were failed to conceive after 1 year of trying and who received medical assistance to conceive were excluded [88]. In contrast, NHANES participants in our study with similar conditions were not excluded. The difference on subject recruitment indicates that women with high levels of PFAS and abnormal menstrual cycles were more likely to be included in our study, which may result in higher odds. Another potential reason of the odds discrepancy is that NHANES only collected information on the presence or absence of menstruation for the past 12 months [89], but those three referred studies examined more specific characteristics such as the regularity, length, and amount of menstruation [45, 87, 88]. We classified women who had no menstruation for the past 12 months as having long-term amenorrhea. While these women self-reported not having menopause, it is possible that some of them may already have suspected early menopause or POF, which could indicate more severe reproduction impairment and result in higher odds.

Compared with women without recent menstrual bleeding and menopausal women, menstruating women have been shown to have lower blood PFAS concentrations [90-92], indicating that menstruation might be a crucial pathway of PFAS elimination. Our NHANES analysis reveal that women with long-term amenorrhea had significantly higher blood concentrations of several long-chain PFAS, including PFDeA, PFHxS, PFNA, PFOA, and PFOS. However, these epidemiological associations do not allow us to distinguish whether the more often menstrual bleeding leads to lower blood levels of PFAS or the higher blood levels of PFAS cause amenorrhea.

Conclusion

In conclusion, we utilize epidemiological approaches to demonstrate the ovarian disrupting effects of PFAS. PFNA, a long-chain PFAS that can have similar environmental contamination levels to the legacy PFOA/S, acts as a PPARγ agonist in follicular granulosa cells to interfere with gonadotropin-dependent follicle maturation, ovulation, and hormone secretion. The widespread contamination, ovarian accumulation, and long half-lives of PFAS pose a significant risk to women's reproductive health, including imbalanced ovarian hormone secretion, anovulation, irregular menstrual cycles, and infertility. It is an urgent need to reduce or eliminate exposure to PFAS to safeguard women's reproductive health and fertility.

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Appendices

Table

Table 1: Characteristics of NHANES reproductive aged women for investigating associations between blood PFAS concentrations and long-term amenorrhea.

Characteristics	Total Sample N (%)	Menstruating[1] N (%)	Amenorrhea[2] N (%)	*p*-value[3]
Total subjects	862	828 (95.0)	34 (5.0)	
Age, *mean* ± *SE* (years)	31.8 ± 0.36	31.7 ± 0.35	32.5 ± 1.58	0.6904
Race/Ethnicity				0.0043**
Hispanic	208 (18.1)	203 (17.6)	5 (8.8)	
Non-Hispanic White	309 (58.9)	289 (54.9)	20 (79.1)	
Non-Hispanic Black	195 (12.5)	188 (12.1)	7 (9.8)	
Other Race Including Multi-Racial	150 (10.5)	148 (10.4)	2 (2.3)	
Education Level				0.2388
Less than High School	130 (10.2)	125 (9.7)	5 (9.8)	
High School	158 (17.7)	149 (16.3)	9 (28.7)	
More than High School	574 (72.1)	554 (69.0)	20 (61.5)	
Marital Status				0.3841
Married / Living with Partner	493 (60.3)	471 (59.7)	22 (71.4)	
Divorced / Widowed / Separated	101 (10.4)	100 (10.7)	1 (3.3)	
Never Married	268 (29.3)	257 (29.6)	11 (25.2)	
Covered by Health Insurance				0.5707
Yes	672 (82.6)	643 (82.8)	29 (77.9)	
No	190 (17.4)	185 (17.2)	5 (22.1)	
Poverty Income Ratio, *mean* ± *SE*.	3.57 ± 0.06	3.38 ± 0.07	3.86 ± 0.25	
<1	234 (19.5)	229 (20.0)	5 (9.1)	
1-1.99	223 (22.8)	211 (22.4)	12 (30.1)	
2-2.99	125 (15.3)	118 (14.9)	7 (23.0)	0.2166
3-3.99	93 (12.2)	88 (11.6)	5 (22.4)	
4-4.99	65 (10.3)	63 (10.4)	2 (7.9)	
>=5	122 (20.0)	119 (20.6)	3 (7.5)	
Body Mass Index (kg/m2)**				0.5903
Underweight (<18.5)	22 (2.3)	21 (2.3)	1 (1.7)	
Normal Weight (18.5-24.9)	279 (34.1)	266 (34.2)	13 (30.9)	
Overweight (25.0-29.9)	202 (23.1)	194 (22.6)	8 (32.3)	
Obesity (>30)	359 (40.5)	347 (40.8)	12 (35.1)	
Ever Smoked				0.5794
Yes	239(30.0)	231 (30.3)	8 (24.1)	
No	623 (70.0)	597 (69.7)	26 (75.9)	
Use hormonal contraception				0.6152
Yes	598 (76.6)	574 (76.4)	24 (80.3)	
No	264 (23.4)	254 (23.6)	10 (19.7)	

Values for continuous variables are mean +/- the Standard Error of the Mean.
Values for categorical variables are N (unweighted sample counts) and % (weighted sample percentages to account for NHANES survey design).
If the percentages do not equal 100% it is due to rounding.
1. 'Menstruating' if answered "Yes" to the question "Have you had at least one menstrual period in the past 12 months? (Please do not include bleedings caused by medical conditions, hormone therapy, or surgeries.)"
2. 'Long-term amenorrhea' answered "no" to the question "Have you had at least one menstrual period in the past 12 months? (Please do not include bleedings caused by medical conditions, hormone therapy, or surgeries.)" and answered "Other" or "Don't know" to the question "What is the reason that you have not had a period in the past 12 months?"
3. *p*-value for categorical variables comes from a chi-squared test, which determines if there is a significant difference between demographics in long-term amenorrhea vs. menstruating women. *p*-values for continuous variables comes from a t-test to determine if there is a significant difference between the means of long-term amenorrhea vs. menstruating. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

Table 2: Medians and log transformed means of blood PFAS concentrations in menstruating women and women with long-term amenorrhea.

PFAS	Total Sample	Menstruating[1]	Amenorrhea[2]	*p*-value[3]
PFDeA				
Median, IQR (ng / mL)	0.10 (0.07, 0.19)	0.10 (0.07, 0.18)	0.16 (0.08, 0.26)	0.9186
Log Transformed, mean, SE	-1.90 ± 0.04	-1.91 ± 0.04	-1.70 ± 0.09	0.0892
PFHxS				
Median, IQR (ng / mL)	0.58 (0.36, 1.00)	0.57 (0.35, 0.99)	0.79 (0.53, 1.27)	0.2281
Log Transformed, m*ean, SE*	-0.40 ± 0.04	-0.42 ± 0.04	-0.01 ± 0.08	0.0228*
Me-PFOSA-AcOH				
Median, IQR (ng / mL)	0.07 (0.07, 0.09)	0.07 (0.07, 0.09)	0.07 (0.07, 0.09)	0.5779
Log Transformed, m*ean, SE*	-2.27 ± 0.04	-2.27 ± 0.04	-2.25 ± 0.08	0.8962
PFNA				
Median, IQR (ng / mL)	0.34(0.19, 0.56)	0.33 (0.18, 0.56)	0.41 (0.24, 0.63)	0.3995
Log Transformed, m*ean, SE*	-1.00 ± 0.04	-1.01 ± 0.05	-0.78 ± 0.11	0.0825
PFUA				
Median, IQR (ng / mL)	0.07 (0.07, 0.10)	0.07 (0.07, 0.10)	0.07 (0.07, 0.10)	0.0227 *
Log Transformed, m*ean, SE*	-2.25 ± 0.03	-2.24 ± 0.03	-2.31 ± 0.06	0.5054
PFOA				
Median, IQR (ng / mL)	1.05 (0.66, 1.63)	1.04 (0.66, 1.61)	1.31 (0.86, 1.95)	0.1823
Log Transformed, mean, SE	0.09 ± 0.03	0.08 ± 0.03	0.34 ± 0.08	0.0357*
PFOS				
Median, IQR (ng / mL)	2.72 (1.77, 4.16)	2.70 (1.74,4.02)	4.46 (2.55, 5.35)	0.0769
Log Transformed, m*ean, SE*	0.99 ± 0.04	0.98 ± 0.04	1.34 ± 0.13	0.0172*

The distribution of blood PFAS concentrations was skewed. Therefore, we presented the median and IQR (25th and 75th percentile) and log transformed means of blood PFAS levels. These results are weighted to account for NHANES survey design.

1. 'Menstruating' if answered "Yes" to the question "Have you had at least one menstrual period in the past 12 months? (Please do not include bleedings caused by medical conditions, hormone therapy, or surgeries.)"
2. 'Long-term amenorrhea' answered "no" to the question "Have you had at least one menstrual period in the past 12 months? (Please do not include bleedings caused by medical conditions, hormone therapy, or surgeries.)" and answered "Other" or "Don't know" to the question "What is the reason that you have not had a period in the past 12 months?"
3. *p*-values represent a t-test to determine if there is a significant difference between the means of long-term amenorrhea vs. menstruating. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

Table 3: Associations between blood concentrations of PFAS and women's long-term amenorrhea

	PFAS	Total N (%)	Full sample (Amenorrhea[1] vs Menstruating[2] n=957)		
			Menstruating[2]	Amenorrhea[1]	*Full Adj* OR (95% CI)
PFDeA	Log mean (SD)	-1.89 (0.79)	-1.89 (0.79)	-1.68 (0.67)	2.16 (1.38 3.37) *
	Quartiles, n (%)				
	Q1 (0-0.07)	268 (31.09)	263 (30.51)	5 (0.58)	Ref
	Q2 (0.08-0.1)	180 (20.88)	173 (20.07)	7 (0.81)	1.20 (0.34, 4.19)
	Q3 (0.11-0.2)	223 (25.87)	212 (24.59)	11 (1.28)	2.15 (0.86, 5.38)
	Q4 (0.21–14.8)	191 (22.16)	180 (20.88)	11 (1.28)	7.08 (2.12, 23.58) *
PFHxS	Log mean (SD)	-0.49 (0.86)	-0.51 (0.86)	-0.08 (0.79)	1.84 (1.15, 2.97) *
	Quartiles, n (%)				
	Q1 (0-0.3)	198（22.97）	196 (22.74)	2 (0.23)	Ref
	Q2 (0.31-0.5)	199（23.09）	193 (22.39)	6 (0.70)	3.13 (0.43, 22.99)
	Q3 (0.51-0.9)	228（26.45）	220 (25.52)	8 (0.93)	4.74 (0.66, 34.00)
	Q4 (0.91-21.3)	237（27.49）	219 (25.41)	18 (2.09)	10.22 (1.43, 73.32) *
Me-PFOSA-AcOH	Log mean (SD)	-2.30 (0.65)	-2.31 (0.64)	-2.17 (0.71)	0.96 (0.62, 1.49)
	Quartiles, n (%)				
	Q1 (0-0.07)	553 (64.15)	536 (62.18)	17 (1.97)	Ref
	Q2 (0.08-0.1)	138 (16.01)	130 (15.08)	8 (0.93)	2.11 (0.68, 6.49)
	Q3 (0.11-0.2)	81 (9.40)	77 (8.93)	4 (0.46)	1.30 (0.20, 8.33)
	Q4 (0.21-4.1)	90 (10.44)	85 (9.86)	5 (0.58)	0.94 (0.35, 2.58)
PFNA	Log mean (SD)	-0.97 (0.79)	-0.98 (0.79)	-0.73 (0.69)	2.13 (1.35, 3.35) *
	Quartiles, n (%)				
	Q1 (0-0.2)	224 (25.99)	219 (25.41)	5 (0.58)	Ref
	Q2 (0.21-0.4)	267 (30.97)	255 (29.58)	12 (1.39)	1.78 (0.44, 7.21)
	Q3 (0.41-0.6)	166 (19.26)	158 (18.33)	8 (0.93)	2.49 (0.78, 7.98)
	Q4 (0.61 -6)	205 (23.78)	196(22.74)	9 (1.04)	4.20 (1.27, 13.83) *
PFUA	Log mean (SD)	-2.20 (0.70)	-2.20 (0.71)	-2.24 (0.52)	0.79 (0.34, 1.76)
	Quartiles, n (%)				
	Q1 (0-0.07)	473 (54.87)	455 (52.78)	18 (2.09)	Ref
	Q2 (0.08-0.1)	157 (18.21)	152 (17.63)	5 (0.58)	0.98 (0.35, 2.76)
	Q3 (0.11-0.2)	120 (13.92)	111 (12.88)	9 (1.04)	1.67 (0.61, 4.52)
	Q4 (0.21 -5.8)	112 (12.99)	110 (12.76)	2 (0.23)	0.17 (0.01, 6.16)
PFOA	Log mean (SD)	0.03 (0.68)	0.02 (0.68)	0.31 (0.63)	2.46 (1.38, 4.40) *
	Quartiles, n (%)				
	Q1 (0.14-0.67)	240 (28.88)	236 (28.40)	4 (0.48)	Ref
	Q2 (0.7-0.97)	171 (20.58)	164 (19.74)	7 (0.84)	2.43 (0.57, 10.31)
	Q3 (1.07-1.50)	202 (24.31)	195 (23.47)	7 (0.84)	3.52 (0.86, 14.34)
	Q4 (1.57–52.87)	218 (26.23)	204 (24.55)	14 (1.68)	7.42 (2.00, 27.54) *
PFOS	Log mean (SD)	0.99 (0.79)	0.97 (079)	1.34 (0.74)	2.47 (1.31, 4.68) *
	Quartiles, n (%)				
	Q1 (0.14-1.7)	212 (25.51）	208 (25.03)	4 (0.48)	Ref
	Q2 (1.8-2.7)	205（24.67）	199 (23.95)	6 (0.72)	1.34 (0.17, 10.45)
	Q3 (2.8-4.1)	198（23.83）	192 (23.10)	6 (0.72)	1.38 (0.37, 5.18)
	Q4 (4.2-84.3)	216（25.99）	200 (24.07)	16 (1.93)	5.68 (1.48, 21.75) *

*Statistically significant and corresponding *p*-value <0.05
Values are unweighted sample counts and percentages.

The model was only adjusted for the other PFAS when analyzing PFOA and PFOS
1 'Menstruating' if answered "Yes" to the question "Have you had at least one menstrual period in the past 12 months? (Please do not include bleedings caused by medical conditions, hormone therapy, or surgeries.)"
2 'Long-term amenorrhea' answered "no" to the question "Have you had at least one menstrual period in the past 12 months? (Please do not include bleedings caused by medical conditions, hormone therapy, or surgeries.)" and answered "Other" or "Don't know" to the question "What is the reason that you have not had a period in the past 12 months?"

Supplementary table 1: Characteristics of NHANES reproductive aged women for investigating associations between blood PFOA and PFOS concentrations and long-term amenorrhea.

Characteristics	Total Sample N (%)	Menstruating[1] N (%)	Amenorrhea[2] N (%)	*p*-value[3]
Total Subjects	831	799 (95.0)	32 (5.0)	
Age, *mean* ± *SE* (years)	31.7 ± 0.36	31.6 ±0.35	32.9 ±1.60	0.5313
Race/Ethnicity				0.0028**
Hispanic	203 (18.1)	198 (18.6)	5 (8.7)	
Non-Hispanic White	297 (58.4)	278 (54.4)	19 (80.4)	
Non-Hispanic Black	186 (12.7)	180 (12.3)	6 (8.4)	
Other Race Including Multi-Racial	145 (10.7)	143 (10.6)	2 (2.4)	
Education Level				0.2402
Less than High School	125 (10.2)	120 (10.2)	5 (10.4)	
High School	155 (18.0)	146 (17.4)	9 (29.8)	
More than High School	551 (71.7)	533 (72.4)	18 (59.8)	
Marital Status				0.4061
Married / Living with Partner	474 (60.1)	453 (59.5)	21 (72.4)	
Divorced / Widowed / Separated	95 (9.9)	94 (10.2)	1 (3.7)	
Never Married	262 (30.0)	254 (30.3)	10 (23.8)	
Covered by Health Insurance				0.4728
Yes	650 (82.5)	623 (82.8)	27 (76.2)	
No	181 (17.5)	176 (17.2)	5 (23.8)	
Poverty Income Ratio, *mean* ± *SE*.	3.57 ± 0.06	3.38 ± 0.08	3.90 ± 0.24	
<1	224 (19.3)	229 (20.0)	5 (9.6)	
1-1.99	214 (22.8)	211 (22.4)	10 (28.0)	
2-2.99	120 (15.1)	118 (14.9)	7 (23.8)	0.2306
3-3.99	92 (12.5)	88 (11.6)	5 (22.9)	
4-4.99	64 (10.4)	63 (10.4)	2 (8.1)	
>=5	117 (19.9)	119 (20.6)	3 (7.6)	
Body Mass Index (kg/m2)**				n/a
Underweight (<18.5)	21 (2.2)	21 (2.4)	0 (.)	
Normal Weight (18.5-24.9)	268 (33.9)	256 (34.1)	12 (31.5)	
Overweight (25.0-29.9)	193 (23.2)	185 (22.7)	8 (32.7)	
Obesity (>30)	349 (40.6)	337 (40.8)	12 (35.9)	
Ever Smoked				0.6980
Yes	230(30.0)	222 (30.2)	8 (25.7)	
No	601 (70.0)	577 (69.8)	24 (74.3)	
Use hormonal contraception				0.5946
Yes	578 (76.4)	555 (76.4)	23 (80.8)	
No	253 (23.6)	244 (23.6)	9 (19.2)	

Values for continuous variables are mean +/- the Standard Error of the Mean.
Values for categorical variables are N (unweighted sample counts) and % (weighted sample percentages to account for NHANES survey design).
If the percentages do not equal 100% it is due to rounding.
1. 'Menstruating' if answered "Yes" to the question "Have you had at least one menstrual period in the past 12 months? (Please do not include bleedings caused by medical conditions, hormone therapy, or surgeries.)"
2. 'Long-term amenorrhea' answered "no" to the question "Have you had at least one menstrual period in the past 12 months? (Please do not include bleedings caused by medical conditions, hormone therapy, or surgeries.)" and answered "Other" or "Don't know" to the question "What is the reason that you have not had a period in the past 12 months?"
3. *p*-value for categorical variables comes from a chi-squared test, which determines if there is a significant difference between

demographics in long-term amenorrhea vs. menstruating women. *p*-values for continuous variables comes from a t-test to determine if there is a significant difference between the means of long-term amenorrhea vs. menstruating. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

Figure legends

Figure 1: Schematic diagram depicting the participant inclusion process from NHANES 2013–2018. n1 indicates the analysis of PFDeA, PFHxS, Me-PFOSA-AcOH, PFNA, and PFUA; and n2 indicates the analysis of PFOA and POFS. We included women aged 20-45. Women under 20 were not included because of the NHANES sampling design. Women over 45 were not included because our study assessed only women of reproductive age. All inclusions and exclusions were made using the DOMAIN statement in the SAS software, according to NHANES guidelines.