Associations of Perfluoroalkyl Substances with Incident Natural Menopause: the Study of Women's Health Across the Nation

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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 perfluoroalkyl substances and incident natural menopause.

Design and Setting

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

Participants

1120 multi-racial/ethnic premenopausal women aged 45-56 years.

Methods

Serum concentrations of perfluoroalkyls were quantified by high performance liquid chromatography-isotope dilution-tandem mass spectrometry. Natural menopause was defined as the bleeding episode prior to at least 12 months of amenorrhea not due to surgery or hormone use. 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 equivalent to 2.0 years earlier median time to 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.

Keywords: per- and polyfluoroalkyl substances (PFAS), endocrine-disrupting chemicals, natural menopause, midlife women

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 (1,2). Earlier age at the final menstrual period (FMP) has been associated with an increased risk of overall mortality (3–5), cardiovascular disease (6,7), cardiovascular death (3,8,9), low bone mineral density (10) and osteoporosis (11), and other chronic conditions (12). Ovarian aging reflects the combined effects of genetic factors, socio-demographics, lifestyle and health characteristics (13–15). 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 (16–18).

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 (19,20), food packaging (21–23), outdoor apparel (24,25), and aqueous film-forming foams (26–29). 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 (30,31). 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 (32–34), likely through the activation of various transcriptional factors, such as peroxisome proliferator-activated receptors (PPARs) (35,36). 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 (37).

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 (38); 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 (39). 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 (39–42).

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 (44). Potential recall bias may have reduced the accuracy of reported age at natural menopause and presumably biased the study results toward the null (43). 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 perfluoroalkyl substances 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 chemical mixtures on natural menopause.

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 (45). A total of 3,302 premenopausal 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 articipants 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 Materials Figure S1** (46). 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 (47).

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 (48). 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 (49). The analytic methods and quality control procedures have been described elsewhere (48). 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 (50).

Time-varying lifestyle variables included annual self-reported active smoke exposure and physical activity. Self-reported smoking status was defined based on the questions asking about ever smoking, amount smoked and quit date (51). Women were classified as never smoked, former smoked only, or current smoking. Physical activity was assessed using an adaptation of the Kaiser Physical Activity Survey (52), 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 hazards (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. We applied the Cox PH models to generate adjusted survival curves and estimate median age at natural menopause. We calculated predicted survival probability of natural menopause (i.e., probability of not having natural menopause). Median age at natural menopause was defined as the time at which 50 percent had reached their natural menopause. 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 (53,54). 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 (55). 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 Materials Table S1**) (46). 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. The SWAN Data Coordinating Center 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 Material Table S2**) (46). Covariates were selected based on previous literature (14,15). 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 (56). 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. Furthermore, we excluded 29 women who reached their natural menopause since baseline to minimize the possibility of reverse causation bias. Lastly, it is possible that smokers enrolled in our study may be healthier than those who reached their natural menopause before baseline, especially those with premature menopause (before age 40) or early menopause (before age 45). We have conducted sensitivity analyses by removing smokers from our study sample.

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 Materials Table S3**) (46). 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 Materials Figure S2**) (46).

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 Material Figures S3-S7** (46). 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

274 women was 1.23 (95% CI: 1.06-1.44) and 1.33 (95% CI: 1.13-1.56) per doubling increase in
275 serum concentrations of n-PFOA and PFNA, respectively, after covariate adjustment. The
276 associations in Black or Asian women did not reach statistical significance. Neither did the
277 results for n-PFOS, Sm-PFOS and PFHxS (**Supplemental Materials Figure S8**) (46).

278 In sensitivity analyses, the pooled effect estimates from 10 imputations of age at FMP
279 were largely unchanged, while the 95% CIs became narrower (**Table 3**). However, the
280 significant associations between PFNA concentration and natural menopause disappeared. In the
281 competing risks analyses, 67 women (303 observations) who had hysterectomy and/or
282 oophorectomy were excluded from the analyses, but effect estimates remained similar
283 (**Supplemental Material Table S4**) (46). Exclusion of 29 women who reached natural
284 menopause in the six months since baseline did not change results (**Supplemental Material**
285 **Table S5**) (46), diminishing the likelihood that reverse causation bias drove the observed results.
286 The results were robust when restricting the study sample to never smokers (**Supplemental**
287 **Material Tables S6-S8**) (46).

288 ***Mixture effects of PFAS on incident natural menopause***

289 Participants were classified into clusters based on their overall PFAS concentrations
290 profiles using the k-means method (**Supplemental Material Figure S9)** (46). Women were
291 classified into four clusters based on serum PFAS concentrations, including “low”, “low-
292 medium”, “medium-high”, and “high” overall concentration patterns. Women in the “low”
293 concentration group had the lowest overall concentrations of PFAS, while those classified into
294 the “high” group exhibited the highest concentrations. After adjusting for confounding, the HRs
295 for natural menopause comparing the “high”, “medium-high”, “low-medium” concentration

296 groups with the low concentration group were 1.63 (95% CI: 1.08-2.45), 1.31 (95% CI: 0.94-
297 1.83), and 1.30 (95% CI: 0.97-1.74), respectively (**Supplemental Material Table S9**) (46).
298 Participants in the high concentration group had an earlier onset of natural menopause compared
299 to those in other groups (**Figure 2**). The predicted median age at natural menopause in the low
300 concentration group was 52.8 years compared to 51.8 years, 52.0 years and 50.8 years for low-
301 medium, medium-high, and high concentration groups, respectively.

302 **DISCUSSION**

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

310 ***Comparison with previous epidemiologic studies***

311 To date, evidence on the influence of PFAS exposure on the timing of menopause and
312 ovarian aging has been limited and inconsistent, and has been primarily generated from cross-
313 sectional studies that could not establish causal relationships (38,39). Knox et al. found that
314 higher concentrations of PFOA and PFOS were associated with earlier menopausal age in a
315 cross-sectional study of women aged 18-65 years from the C8 Health Project (38). This study
316 collected data from highly exposed communities and workers in six public water districts
317 contaminated with PFOA from the DuPont Washington Works Plant near Parkersburg (57).

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 (39). 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 (43). However, reverse causation could not be ruled out as women appeared to have higher PFAS concentrations after menopause (39–42).

Serum concentrations of PFAS appear to be higher in males than in females across all age groups (58). Wong et al. (59) found that menstruation could explain the PFOS elimination half-life difference between men and women. The differences by sex narrows with age, suggesting that PFAS may reaccumulate after cessation of menstrual bleeding in postmenopausal women (47,60–62). Given that 90% to 99% of PFAS in the blood are bound to serum albumin (63,64), menstrual bleeding could be an important elimination pathway in women. It is still unclear whether PFAS exposure is the cause of earlier natural menopause or the cessation of menstruation leads to increased serum concentrations of PFAS. Therefore, previously observed associations identified in cross-sectional or retrospective designs (38,39,43) 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 timing of natural menopause are not in concordance with Dhingra et al. (43), the only other published study to our knowledge that has explored the associations between PFOA exposure and incident

menopause. Dhingra et al. (43) included 8,759 women aged ≥40 years in 2005-2006 who were exposed to high levels of PFOA from a chemical facility in the Mid-Ohio Valley, West Virginia (the C8 Health Project). Women were recruited in 2005-2006 and interviewed in 2008-2011 to ascertain the timing of menopause. They found no significant association between PFOA exposure (using either estimated cumulative exposure during 1951 and 2011, or measured serum concentrations in 2005-2006) and incident natural menopause. They observed that women exhibited considerable digit preference and tended to round off their age at menopause to 40, 45 or 55, suggesting that their results may suffer from the reduced accuracy of recalled ages and are presumably biased towards the null. The availability of a standardized questionnaire administered prospectively at regular, approximately annual, intervals 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 (65). People may be normally exposed to multiple PFAS through drinking water, food intake, or use of consumer products (66,67). 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 (68), 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

364 environmental exposure in general is sometimes expected to be higher in racial minority groups
365 and socioeconomically disadvantaged neighborhoods, the concentration patterns tended to
366 depend on the PFAS. Serum concentrations of n-PFOA were found to be higher in White women
367 and PFNA concentrations were relatively higher in White and Chinese women, whereas serum
368 concentrations of n-PFOS and Sm-PFOS were higher in Black women. This is consistent with
369 previous findings (47,69–71). White women with higher n-PFOA and PFNA tended to have
370 earlier natural menopause.

371 PFOA is used as a surfactant and emulsifier in compounds used to coat a variety of food
372 packaging materials, including microwave popcorn bags (37,72,73) and is essential in
373 manufacture of the fluoropolymer polytetrafluoroethylene (PTFE) used in non-stick coatings and
374 waterproof fabrics (74). Uses of PFOS included inks, varnishes, waxes, fire-fighting foams, and
375 coating formulations (75). Use of consumer products may have contributed to more exposure to
376 PFOA, while the most dominant source of PFOS exposure might have been intake of
377 contaminated drinking water (76). Although production and use of some PFAS, including PFOA
378 and PFOS, in the USA is on the decline, environmental exposures to many of these pervasive
379 chemicals continue with associated potential hazards to human reproductive health.

380 Our study results showed no difference in the effects of n-PFOS and Sm-PFOS by
381 racial/ethnic groups, possibly because of the exclusion of women with premature (before the age
382 of 40 years) or early menopause (before age 45 years), or greater censoring of Black women who
383 had surgical menopause before age 45. However, caution should be taken in interpreting the
384 findings because of the modest sample sizes in those racial/ethnic groups. Asian women with
385 similar PFNA concentrations as White women did not reach their natural menopause earlier.
386 Previous studies have shown increases in PFNA concentrations since 2000 (69,70,77–79). Future

studies with more recent PFNA measurements are needed to confirm our findings and better understand exposure trends. It is also important to explore the role of genetic background and changes in lifestyle factors (80–83).

Biological evidence

PFAS exposures have been associated with diminished ovarian reserve (i.e., the number of ovarian follicles and oocytes) (84–91). The mechanisms of PFAS-induced effects have widely been thought to occur through a peroxisome proliferator-activated receptor (PPAR) mechanism (35,36,92). 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 (93), down-regulate aromatase expression via nuclear factor-κB (NF-κB) pathway (94), and affect enzymatic activity in steroidogenesis (95,96).

Accumulating evidence from experimental research suggests that PFAS can directly interfere with steroidogenic enzyme activities (97–99). Recently, it was also reported that PFNA and PFOA are weak xenoestrogens, inducing ERα-dependent transcriptional activation *in vitro* and *in vivo* (100). As potential endocrine disruptors, PFAS might also suppress the effects of 17β-estradiol (E2) on estrogen-responsive gene expression (101,102), reduce E2 production and alter the expression of major steroidogenic genes and regulator steroidogenic factors 1 (SF-1) (103). 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 (32).

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 (104). 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. However, the effect estimates remained similar when restricting our study sample to never smokers. 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).

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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FIGURE LEGENDS

Figure 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. a) Exposure to n-PFOA and incidence of natural menopause by racial/ethnic groups; b) Exposure to PFNA and incidence of natural menopause by racial/ethnic groups. 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 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.

TABLES

Table 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

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

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

Table 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.

852 **Table 3 Pooled** hazard ratios (HR) (95% confidence intervals, 95% CI) for incident natural menopause with tertile changes and per
853 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

854 [a] Averaged no. cases and person-years from 10 imputations. [b] Model 1 was adjusted for age at baseline, race/ethnicity, and study site.
855 [b] Model 2 was additionally adjusted for education, parity, BMI at baseline, physical activity, smoking status, and prior hormone use at baseline.
856 [c] The significance level was set at 0.05.

Figure 1a

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

Racial Group	No. of cases/person-year	HR (95% CI)
White	259/3008	1.23 (1.06-1.44)
Black	130/1100	1.04 (0.84-1.29)
Asian	189/1515	0.94 (0.77-1.14)

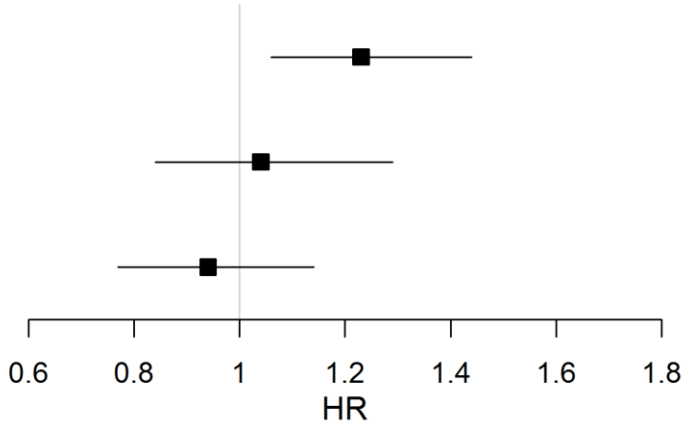


Figure 1b

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

Racial Group	No. of cases/person-year	HR (95% CI)
White	259/3008	1.33 (1.13-1.56)
Black	130/1100	1.01 (0.80-1.27)
Asian	189/1515	0.95 (0.80-1.13)

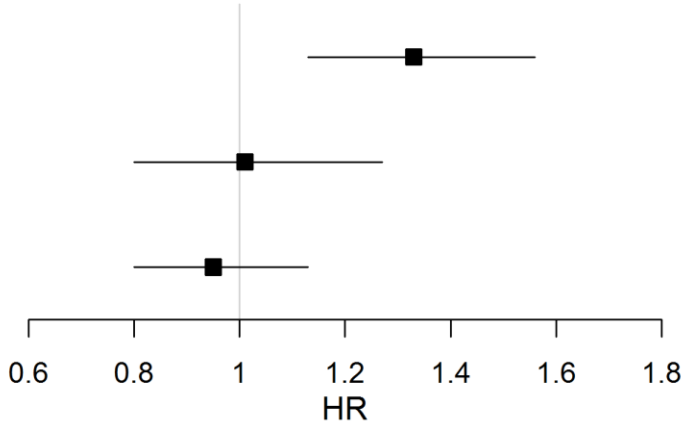
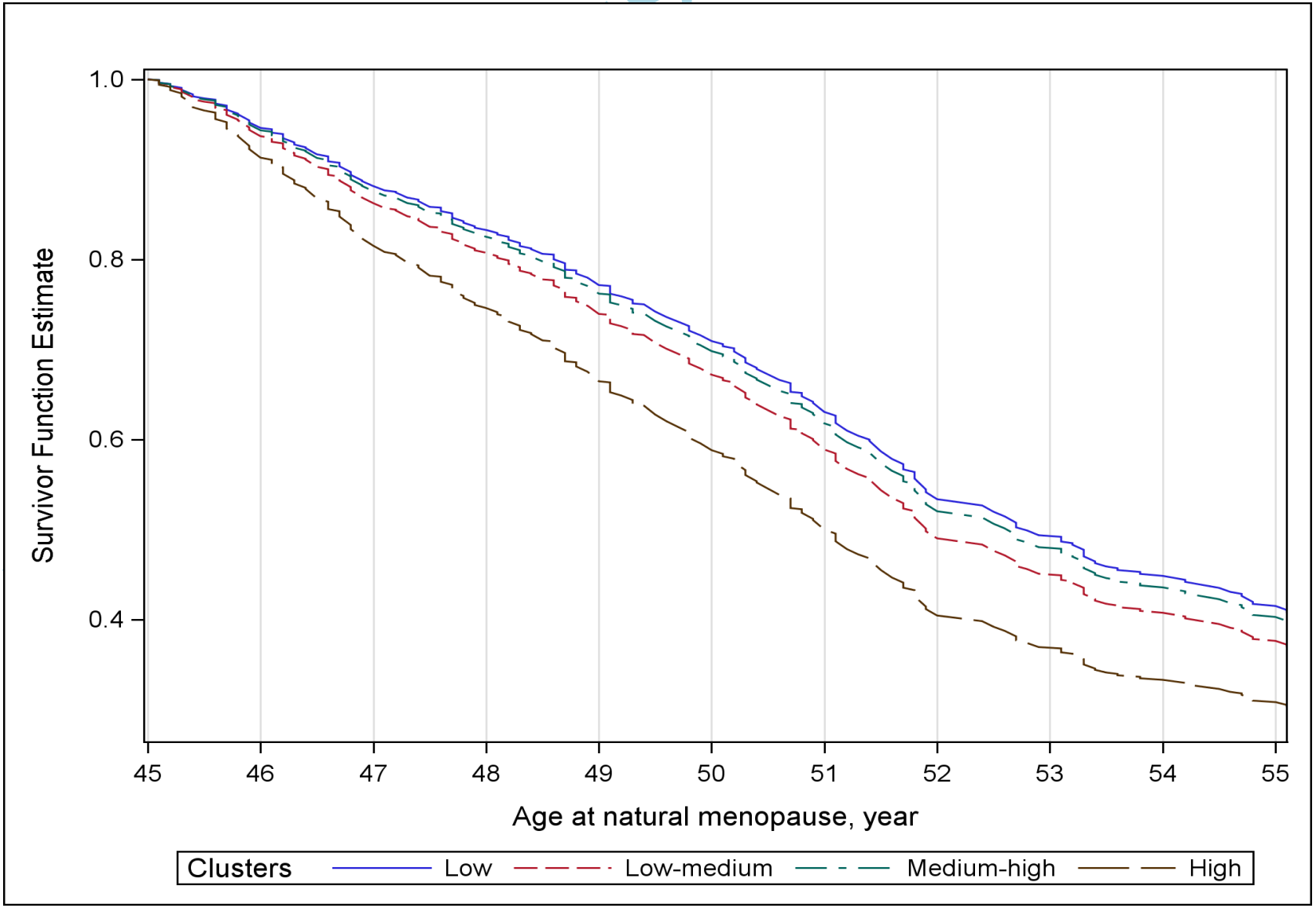


Figure 2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Survivor Function Estimate
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Age at natural menopause, year
Clusters
Low
Low-medium
Medium-high
High