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Perfluoroalkyl substances (PFASs) and ovarian hormone concentrations in naturally cycling women

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Abstract

Objective—To examine associations between environmental exposure to perfluoroalkyl substances (PFASs) and ovarian hormone concentrations in naturally cycling women.

Design—Estradiol and progesterone were measured in saliva samples collected daily for a single menstrual cycle and concentrations of PFASs (including perfluorooctane sulfonate [PFOS] and perfluorooctanoic acid [PFOA]) were measured in serum samples collected during the same cycle.

Setting—Tromsø, Norway.

Patients—178 healthy, naturally cycling women, ages 25–35.

Intervention—None.

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Main outcome measures(s)—Mean follicular estradiol (cycle days –7 to –1, where 0 is the day of ovulation); mean luteal progesterone (cycle days +2 to 10).

Results—Among nulliparous, but not parous women, PFOS concentrations were inversely associated with estradiol ($\beta=-0.025$, 95% CI: –0.043, –0.007) and progesterone ($\beta=-0.027$, 95% CI: –0.048, –0.007). Similar, but weaker results were observed for perfluorooctanesulfonic acid (PFOSA). No associations were observed between other PFASs (including PFOA) and ovarian steroid concentrations, nor were any associations noted in parous women.

Conclusions—Our results demonstrate that PFOS and PFOSA may be associated with decreased production of estradiol and progesterone in reproductive age women. These results suggest a possible mechanism by which PFASs affect women's health, and underscore the importance of parity in research on PFASs and women's reproductive health.

Keywords

Perfluoroalkyl substances; PFOS; estradiol; progesterone; endocrine disrupting chemicals; fecundity

Introduction

Female fecundity requires the intricate integration of multiple factors, including genetics, hormones from the hypothalamic-pituitary-ovarian axis, timely oocyte maturation and release, and endometrial proliferation. Just as this delicate balance may be upset by factors such as diet, physical activity, and stress, so may it be disrupted by exposure to the wide array of endocrine-disrupting chemicals that are ubiquitous in the modern environment (1). Among the chemicals of most concern are the perfluoroalkyl substances (PFASs), a class of oleophilic, hydrophobic chemicals that are widely used in the manufacture of consumer goods, including fabrics and upholstery, non-stick cookware, food packaging, and fire-fighting foams (2-5). In addition to their toxic (and potentially carcinogenic) effects, PFASs are highly resistant to breakdown and therefore tend to accumulate in the environment and in food chains (5-7). As a result, diet (particularly consumption of fish and dairy) (8-11) and drinking water (12-14) are believed to be leading sources of PFAS exposure in humans.

Most of the epidemiological research on PFASs has centered on perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA), the two PFASs which are typically present in the highest concentrations in humans (15, 16). The half-lives of PFOS and PFOA in the human body are estimated at approximately 4-5 years and 2-3 years, respectively (17-19) and in Western populations, PFASs are typically detectable in at least 98% of individuals sampled (15, 16, 20). Although much of the research on the health effects of PFAS exposure has focused on thyroid function, lipid profiles, and cancer risk (21-25), there has been increasing interest in possible effects on the female reproductive system. In animal models, PFOA exposure is associated with changes in ovarian function, timing of vaginal opening, and mammary tissue development (26-28). In humans, some studies have found associations between PFAS exposure and reproductive hormone-sensitive outcomes including pubertal timing (29, 30), fecundity (31, 32), and breast cancer (33), while others have not (34-36). Given the critical role that reproductive hormones play in all of these health outcomes, it is

important to determine the extent to which PFAS may disrupt ovarian hormone pathways in women.

Few studies have directly examined PFAS exposure in relation to gonadal steroids in humans. Maternal PFOA, but not PFOS, concentrations have been linked to hypogonadism in sons in adulthood (37). By contrast, no associations were found between maternal PFOS or PFOA concentrations and reproductive hormones in adult daughters, however most of the subjects were using hormonal contraception, thus it is unclear whether endogenous ovarian hormone production may have been altered (30). Several studies have examined adult PFAS exposure in relation to circulating reproductive hormone concentrations. Among occupationally exposed men, some studies have found positive associations between PFOA concentrations and gonadal steroid (estradiol and/or testosterone) concentrations (38, 39), while others have found no association after adjusting for other factors, such as body size (40, 41).

In women, even less is known. In a small study of lactating women, serum levels of another PFAS, perfluorooctanesulfonic acid (PFOSA), were positively correlated with estradiol concentrations in serum and breast milk (42). Among the female participants in the C8 Health Project, who were inadvertently exposed to high levels of PFOA through contaminated drinking water for decades, PFOS (but not PFOA) concentrations were inversely associated with serum estradiol in peri-menopausal and menopausal women (43). In cycling women (ages 18-42), similar, but non-significant, negative associations were observed between PFOS and estradiol. However, serum samples, from which estradiol was assayed, were collected without regard to menstrual cycle phase and parity (potentially an important confounder or effect modifier) was not considered. Interestingly, despite their supranormal PFOA exposure, circulating PFOS concentrations in the C8 cohort were similar to those measured in the general U.S. population, (15, 44), suggesting that environmental levels of PFOS may affect estradiol production in women.

The objective of the current analysis was to further investigate the possibility that PFAS exposure may affect ovarian steroid production in naturally cycling, reproductive age women. To that end, we evaluated the association between circulating serum PFAS concentrations and ovarian hormones (estradiol and progesterone), as measured daily in saliva samples collected across an entire menstrual cycle.

Methods

Study population and overview

From 2000-2002, women were recruited into the parent Energy Balance and Breast Cancer Aspects (EBBA-I) study, based in Tromsø, Norway. Eligible women were between the ages of 25-35 with self-described regular menstrual cycles (22-38 days) and no use of hormonal contraceptives within the past six months. In addition, women who had been pregnant or had breast-fed within the previous six months were excluded from the study, as were women with known histories of infertility, gynecological disorders, or chronic illness (e.g. type II diabetes or hypothyroidism). Primary participation in the study lasted for the duration of a single menstrual cycle, during which subjects collected daily saliva samples and participated

in several visits and physical examinations. The study population, design, and methods have been described in detail elsewhere (45). The study was approved by human subjects review boards at all relevant institutions and the Norwegian Data Inspectorate and all subjects signed informed consent prior to participating in study activities.

Baseline examination and questionnaires

At baseline, subjects completed extensive questionnaires (both by self-report and interview) including items on demographics, reproductive history, lifestyle, diet and exercise, and health. The questionnaires included specific items on age, age at menarche, reproductive history, breast-feeding history, smoking status, physical activity, and history of hormonal contraception use (*e.g.* oral contraceptives, injections, and intrauterine devices that included hormones). Women completed an extensive series of questions on their leisure time activities over the last year and based on that, their physical activity level was quantified on a scale of 1-4 (1= sedentary or low activity, 2=moderate activities at least 4 hours per week, 3=hard activities to keep fit for at least 4 hours per week; 4=hard training or exercise for competition several times per week) (46). For the current analyses, we dichotomized physical activity into low or moderate (1, 2, or 3 on the scale) versus high (4 on the scale). Subjects underwent a physical examination, during which height and weight were measured by a trained research nurse. Body mass index (BMI) was subsequently calculated as weight (kg)/ height (m²). In addition, during the baseline visit, a fasting blood sample was collected and the resulting serum was stored at -70° C. Subjects were provided with saliva collection supplies and taught how to properly collect samples during their participation.

Salivary steroid collection and analysis

Estimation of free ovarian steroid concentrations in saliva is reliable and well-validated, and concentrations are typically well correlated with those measured in serum (47-50). Subjects collected waking saliva samples at home every day for the duration of a single menstrual cycle following published protocols (48). Using a reverse numbering system whereby day 1 represents the start of the next menstrual cycle, we selected saliva samples from days -5 to -24 for estradiol assay and samples from day -1 to -14 for progesterone assay. Free estradiol and progesterone concentrations were measured at the Reproductive Ecology Laboratory at Harvard University using I-125 based radioimmunoassay (RIA) kits (Diagnostic Systems Laboratories, Webster, TX, USA), with minor modifications described elsewhere (45). The sensitivity of the estradiol assay was 4 pmol/L, average intra-assay variability was 9%, and interassay variability ranged from 23% for low pools to 13% for high pools. For the progesterone assay, the sensitivity was 13 pmol/L, average intra-assay variability was 10%, and the interassay variability was 19% and 12% for the low and high pools, respectively. In general, this level of precision of measurement is typical for salivary steroid assays. Considered as a percentage, the coefficient of variation for the low estradiol pool was relatively high due to the very low concentration of hormone in those samples; however the absolute variation was still quite low (51).

We then examined the daily estradiol concentrations to determine the day of the greatest mid-cycle drop in estradiol (day -18 to -12, in the reverse numbering system) following methods described elsewhere (51). All cycles were aligned on this mid-cycle drop day ("day

0”), which provides a good estimate of the day of ovulation. From the resulting, aligned hormone profiles, we calculated two hormone indices: (1) mean follicular estradiol (days –7 to –1); and (2) mean luteal progesterone (days +2 to +10). Due to the intensive schedule of sample collection, some subjects were missing one or more saliva samples. We therefore imputed the missing data using the following algorithms: (1) missing values at the beginning or end of the interval were assigned the neighboring value in the interval; and (2) values missing between two observed values were assigned the geometric mean of the neighboring two values.

PFAS analysis

The serum samples collected at baseline were shipped from the University of Tromsø, Norway to the Norwegian Institute of Public Health in Oslo where concentrations of ten PFASs were determined using high-performance liquid chromatography/tandem mass spectrometry. The methods have been thoroughly described elsewhere (52). In brief, 150 µL of serum was transferred to a centrifugation tube and internal standards and methanol were then added. The samples were centrifuged, the supernatant transferred to a glass autosampler vial, and 500 µL of 0.1 M formic acid was added. 400 µL of extract was injected into a column switching liquid chromatography system coupled to a triple quadrupole mass spectrometer. Calibration solutions were prepared in serum from newborn calves, which has been proven to be an acceptable surrogate matrix for human serum in a thorough method validation (52). Concentrations for most of the congeners (including our primary exposures, PFOS and PFOA) were above the limit of detection (LOD) in 99-100 percent of subjects (Table 2). However, for several congeners including perfluoroheptanoate (PFHpA), perfluoroheptane sulfonate (PFHpS), and perfluorododecanoate (PFDoDA), less than 30% of subjects had detectable levels, therefore these PFASs were excluded from further analysis. For the remaining PFASs, any missing values were assigned as the LOD divided by the square root of two (53).

For quality assurance and control, procedure blanks as well as in-house quality controls were analyzed along with the samples. The between-batch coefficients of variation were between 5-10% for PFOA, PFOS, perfluorononanoate (PFNA), and perfluoroundecanoate (PFUnDA), but higher for the other congeners (20, 25, and 41% for perfluorohexane sulfonate [PFHxS], PFOSA, and perfluorodecanoate [PFDA], respectively). The procedure blanks did not contain any of the PFASs above the LOD.

Statistical Analysis

Due to the preponderance of literature on the reproductive toxicity of PFOS and PFOA and the relative moiety on the other PFASs, an *a priori* decision was made to focus the primary analyses on PFOS and PFOA. Secondly, we then considered an additional five, less well-characterized PFASs that were present in measurable levels in at least 70% of subjects: PFNA, PFDA, PFUnDA, PFHxS, and PFOSA. Salivary hormone indices were non-normal and were thus log-transformed for all analyses. We conducted univariate analyses (mean, standard deviation, minimum, median, maximum, and frequencies, when relevant) to examine descriptive statistics for all relevant variables for the full cohort as well as stratified by parity. We selected a set of covariates of interest for inclusion in full models based on the

existing literature: age, BMI, history of use of oral contraceptives (ever/never), alcohol consumption (any/none), smoking (any/none), marital status (married/single), and physical activity level (low/high). We visually examined the relationships between our variables of interest in a series of bivariate analyses and calculated Pearson correlations.

We then fit two sets of multivariable linear regression models to examine associations between our study outcomes (log-transformed follicular estradiol and log-transformed luteal progesterone) and PFAS concentrations, adjusting for the covariates specified above. In the first set of models, parity (nulliparous/parous) was included as a covariate. In the second set of models, we included a PFAS*parity interaction term. This permits separate slopes for parous and nulliparous women without losing power by stratifying the sample and allowed us to determine whether those slopes differed significantly. Finally, we fit a final set of models limited to parous women in which we also included time since last birth and duration of last breast-feeding (in months) as potential predictors of follicular estradiol and luteal progesterone. Regression assumptions (including linearity, homoscedasticity, and normality) were checked for all models and we investigated any outliers or influential points. All analyses were conducted with R 3.1.1 (R Foundation for Statistical Computing, Vienna, Austria). The p-values presented are two-tailed with an alpha-level of 0.05.

Results

Two hundred and seven women participated in the EBBA-I study and ultimately, 178 women were included in the current analyses. Twenty-two women were excluded either due to lack of a discernable estradiol drop day or excessive missing progesterone or estradiol values. One subject was missing data on history of hormonal contraceptive use and PFAS concentrations were not measured in six additional subjects due to lack of serum samples. There were no significant differences in PFAS concentrations between subjects included in the current analyses and women whose cycles could not be aligned (not shown).

Subjects included in the current analysis were 31 years old on average and 63% percent were married (Table 1). Approximately half (49%) of subjects were parous. Most (79%) were non-smokers, and a majority (94%) reported at least some alcohol use. In bivariate analyses, parous women were slightly older than nulliparous women, with a slightly higher BMI. Parous women were more likely to smoke and less likely to engage in intense physical activity.

Of the PFASs, PFOS concentrations were highest, followed by PFOA (Supplemental Table 1). Correlations among the PFASs were positive and ranged in magnitude from 0.03 (for PFOSA and PFDA) to 0.84 (for PFDA and PNFA) (Supplemental Table 2). Follicular estradiol and luteal progesterone were also moderately, positively correlated ($r=0.53$). In bivariate analyses, the direction of association between estradiol and PFAS concentrations was inverse for all PFASs, whereas directionality differed by congener for progesterone analyses (Supplemental Table 2).

In our primary models, when parity was considered as a covariate, PFOS concentrations were inversely related to estradiol ($\beta=-0.013$, 95% CI: -0.026 , -0.001) (Table 2 and Figure

1), but there was no association with progesterone ($\beta=-0.011$, 95% CI: -0.025 , 0.003) (Table 2, Figure 2). When we fit a second set of models with an interaction term (PFOS*parity) to allow separate slopes for parous and nulliparous women, we found that associations between PFOS concentrations and ovarian hormones were limited to nulliparous women. Among nulliparous, but not parous, women, PFOS concentrations were associated with both reduced estradiol ($\beta=-0.025$, 95% CI: -0.043 , -0.007) and progesterone ($\beta=-0.027$, 95% CI: -0.048 , -0.007) concentrations. No associations between PFOA and ovarian steroid concentrations were found regardless of whether parity was considered as a covariate or an effect modifier.

Our secondary models considered the other PFASs measured. When parity was considered a covariate, a positive (but not statistically significant) association between PFDA concentrations and luteal progesterone ($\beta=0.472$, 95% CI: -0.043 , 0.987) was found, but no other associations with ovarian hormones were observed. When we fit the second set of models including PFAS*parity interaction terms, in nulliparous women, PFOSA concentrations were associated with somewhat lower estradiol ($\beta=-0.645$, 95% CI: -1.320 , 0.030) and progesterone ($\beta=-0.662$, 95% CI: -1.432 , 0.108). No other associations were observed between PFAS and ovarian hormones concentrations. Across most models, BMI and activity levels were positively associated with estradiol levels. Similarly, across models, parity and BMI were often negatively associated with progesterone, while being married was associated with higher progesterone levels (not shown).

In our models limited to parous women only, all PFASs were inversely (but non-significantly) related to estradiol concentrations (Supplemental Table 3). With the exception of PFHxS, all PFAS were positively (but not significantly) related to progesterone concentrations. Time since last birth was a positive predictor of estradiol and progesterone concentrations in models including PFOS (not shown). Duration of last breast-feeding was inversely associated with estradiol, but positively associated with progesterone in models including PFOS. Time since last birth and duration of last breast-feeding were not predictive of hormone concentrations in any of the models including other PFASs.

One observation with the highest concentration of PFOA and PFDA was an influential observation in several models. Sensitivity analyses were conducted excluding this individual and several extreme outliers. The overall conclusions were unchanged when these subjects were excluded (not shown).

Discussion

This is the first study to examine PFAS exposure in relation to ovarian hormones in naturally cycling women across the entire menstrual cycle. We found that among nulliparous women, follicular estradiol and luteal progesterone concentrations were inversely associated with PFOS and PFOSA concentrations. These associations were not evident in parous women, and were attenuated when parity was considered as a confounder, rather than an effect modifier. These findings concur with those reported by Knox *et al.* (2011), who noted inverse associations between serum estradiol levels and PFOS concentrations, but no relationships with PFOA. We extend that work to further suggest that progesterone

production may be impacted by PFOS as well, and that PFOSA may be another important congener to consider in relation to ovarian function.

Understanding how ovarian hormone production is impacted by PFASs (and other environmental chemicals) is of primary importance given the role that estradiol and progesterone play in women's reproductive health and disease. Estradiol stimulates follicular development and oocyte maturation, helps to regulate hormone production in the hypothalamus and pituitary, and promotes proliferation of endometrial tissue to support a pregnancy following conception (54-56). Endogenous estradiol concentrations are positively associated with odds of conception, successful *in vitro* fertilization (IVF), and ovulation induction (51, 57-60). Progesterone production is also needed for ovulation, and more importantly, promotes the final differentiation of the endometrial lining and stimulates its secretory functions during the luteal phase to prepare the uterus for implantation (61). Beyond fertility, ovarian hormones are important in the etiology of breast and other reproductive tract cancers (62-65), osteoporosis (66, 67), and cardiovascular disease (68, 69). They play important roles in sexual function (70), immune function (71, 72), cognition (73), and memory (74) as well. Therefore, to the extent that PFASs impact circulating ovarian hormone levels, they may have profound effects on women's health and well-being. Researchers have begun to examine PFASs in relation to some of these outcomes, including endometriosis (75) and breast cancer (33, 76), however further study is clearly needed, as is more research on the underlying mechanisms.

In vitro, PFOA (and to a lesser extent, PFOS) shows xenoestrogenic properties, inducing proliferation of estrogen-dependent cells and expression of estrogen-sensitive biomarkers (77-80). Both PFOS and PFOA stimulate estrogen receptor (ER) transactivity moreover, and increase ER- α expression (77, 81). At least one study has noted PFOS-induced upregulation of progesterone production *in vitro* as well (82). In rodent models, the action of PFASs on ovarian hormone pathways is less clear-cut. Depending on the particular study methods and context, PFASs may increase estrogen and progesterone concentrations (80, 83) or suppress hormone activity (26). Thus there is some discrepancy between animal models and *in vitro* experiments as compared to the current findings. However the relevance of the rodent models for understanding human effects is questionable, given that rodents' clearance of PFOS and PFOA occurs within hours or days, compared to years in humans (84). Non-human primates may more closely approximate human PFAS metabolism, and in cynomolgus monkeys, low dose exposure to PFOS was associated with reduced estradiol concentrations in both sexes (85), whereas high dose administration of C8 (PFOA's ammonium salt) did not elicit any changes in testicular hormone production, even after long-term exposure (86). These findings are consistent with our current work that PFOS, but not PFOA, is associated with lower ovarian hormone concentrations in reproductive age women.

There have been a number of recent studies on PFASs' possible effects on female fecundity, and our results further speak to the importance of carefully considering parity in such analyses. Unlike other persistent organic pollutants, PFASs accumulate in serum and organs rather than fat tissue (87-90). Therefore parturition, during which fetus, placenta, and other tissues are expelled, is believed to be the major route of PFAS clearance in reproductive age women (91). Indeed, parity is the strongest determinant of PFAS concentrations in women,

and in a Norwegian pregnancy cohort, parous women had 46% lower PFOS and 70% lower PFOA than nulliparous women (92). After birth, PFASs may gradually re-accumulate in the woman, and as a result, time since last pregnancy predicts PFAS concentrations (31, 35). The most highly fecund women may therefore have the lowest PFAS concentrations, simply because of more frequent elimination of PFASs through parturition and breast-feeding. For this reason, the possibility of reverse causality has complicated attempts to determine whether PFAS may interfere with fecundity, as marked by time to pregnancy (31, 35, 36, 93, 94). In the current study, parity was a potential confounder given that many PFASs are lower in parous women (92) and ovarian hormones may be as well (95-97). At the same time, because concentrations of PFASs are so dependent on parity and breast-feeding history, the relationship between PFASs and ovarian hormones may differ in parous and nulliparous women, with the latter providing a “cleaner” population in which to study the possible effects of PFASs on the ovary. Indeed, in our study, PFOS (and possibly PFOSA) concentrations were associated with lower progesterone and estradiol concentrations in nulliparous, but not parous, women. Even after adjusting for time since last birth and duration of last breast-feeding, we did not observe relationships between PFAS and ovarian steroid concentrations in parous women. We propose that nulliparous women are a more suitable population for studies of the effects of PFASs on ovarian function and that more work is needed to understand the complex relationships in parous women.

There is some possibility of reverse causation in the current study. If women with higher ovarian hormone levels tend to have a more proliferative endometrial lining (98) and by extension, heavier, more regular menstrual bleeding, then their lower levels of PFOS and PFOSA may be attributable to greater clearance in menstrual blood (91, 99, 100). Indeed, in NHANES, levels of multiple PFASs were higher in post-menopausal women than premenopausal, and highest in women with hysterectomies (43, 101), suggesting that menstrual bleeding may be a major route of clearance of PFASs. Similarly, in a European cohort, PFOA and PFOS concentrations were higher in women reporting irregular or long (PFOA only) cycles, possibly due to reduced menstrual clearance (102). We expect that if our results were attributable to differences in menstrual clearance of PFAS, levels of all congeners measured would likely be inversely associated with ovarian hormone concentrations, which they were not. Nevertheless, we cannot completely rule out this possibility and it may be important to further consider menstrual clearance of PFASs in future work in cycling women.

The median PFOA and PFOS concentrations in this study are similar to those reported in several other cohort studies including the contemporaneous Norwegian Mother and Child Cohort Study (PFOA: 2 ng/mL; PFOS: 14 ng/mL) (35). PFOS concentrations were also similar to those documented in the C8 cohort (median 15.0 ng/mL), in which a relationship between PFOS and estradiol was previously noted in women (43). Levels in some other populations studied, notably several Danish cohorts (31, 36), have much been higher, in the range of 5-6 ng/mL and 35-36 ng/mL for PFOA and PFOS, respectively (31, 36). Although some of the differences in PFAS concentrations may be due to slight temporal differences in sample collection given that bioburden is generally declining (15, 103), most of the variation is likely to be due to geographical and lifestyle differences. It is unknown, at this point, whether PFASs affect health outcomes in a linear fashion or whether there may be non-

linear, low-dose effects, as have been suggested for other endocrine disrupting chemicals (104).

We observed inverse associations between ovarian hormones and PFOSA. In contrast to the sizeable literature on the possible effects on PFOA and PFOS on reproductive health, very little is known about PFOSA. PFOSA was used in consumer products to repel grease and water and is also a metabolite of other parent fluorochemicals, including the *N*-alkylated sulphonamides and *N*-methyl sulphonamidoethanol. It can be further metabolized to PFOS in the body (105) and therefore it is not surprising that the two congeners were correlated ($r=0.57$) in this study. Although *in vitro* research has suggested that PFOSA may be among the most toxic PFASs (106), there is little human data on health outcomes associated with PFOSA. One recent, prospective time to pregnancy study found a 18-21% reduction in fecundability for every standard deviation increase in women's log-transformed PFOSA concentrations (107), however PFOSA was only detectable in 10% of samples and a similar study reported no associations between PFOSA and women's fecundability (36). Another small study of lactating women reported that serum PFOSA was positively correlated with estradiol concentrations in both breast milk ($r=0.44$) and maternal serum (0.53) in unadjusted models; few additional study details have been published (42). Finally, in a case-control study of breast cancer patients, women in the highest quintile of PFOSA concentrations during pregnancy had increased odds of breast cancer 10-15 years later, adjusting for age and other covariates (76). Because PFOS and PFOSA are fairly highly correlated, it is possible that the associations with ovarian hormones found here are driven by one of the two congeners. Nevertheless, our finding highlights the possibility that other PFASs, beyond PFOA and PFOS, may be of reproductive health concern. This is further highlighted by the fact that progesterone was positively, albeit non-significantly, associated with PFDA and PFUnDA concentrations in parous women in our study. The health effects of "newer" PFASs is also an important question given that even as PFOA and PFOS bioburden may be declining, concentrations of other PFASs, such as PFNA and PFHxS may be rising (108, 109).

Our study has several notable strengths. Rather than relying on hormones measured in a single serum sample, we collected daily saliva samples over an entire menstrual cycle, which allowed us to ascertain the day of ovulation for each woman and calculate hormone levels during specific periods of the cycle corresponding to maximum steroid production. This is important given that estradiol and progesterone production vary quite dramatically across the cycle. Importantly, salivary hormone levels reflect only the free, biologically relevant, fraction, whereas in previous, related work, it is not clear whether the hormone values reported represent free or total concentrations (43). In addition, PFASs and ovarian hormones were analyzed in samples collected during the same menstrual cycle, which is ideal, even if PFAS concentrations are expected to be stable over long time periods (17-19, 91, 110). Finally, because our study was relatively small, compared to some previous work, we were able to collect extensive, reliable data on covariates related to ovarian steroid concentrations including reproductive history and physical activity.

At the same time, we note several limitations of our study. First, for logistic reasons, it only spans a single menstrual cycle, whereas ideally we would study hormone profiles over

several cycles to account for intra-subject variation. In addition, women with highly irregular cycles or a history of fertility problems were not eligible to participate in the parent EBBA-I study, thus we may have inadvertently excluded the very women most impacted by PFAS exposure. We did not observe any differences in PFAS concentrations between the women included in this analysis and the EBBA-I subjects whose cycles lacked a discernable day of ovulation, however it is possible that effects might be observed in women with more dramatically suppressed ovarian function. Because of the intensive biospecimen collection protocols, our sample size was necessarily small, and thus our analyses may have been underpowered to detect associations in some cases, particularly when we examined parous women separately. With a larger sample size, we may have been able to discern whether patterns in parous women were similar to those in nulliparous women after adjusting for time since last birth and duration of last breast-feeding. Finally, we fit a large number of models, and although our main analyses focused on PFOS and PFOA, it remains possible that the associations found were spurious.

In summary, we have found further evidence that PFOS and PFOSA are associated with decreased production of the ovarian steroids estradiol and progesterone in reproductive age women. These results help to establish a possible mechanism by which PFASs may act to affect women's health, and as a result, may inform future research on PFASs in relation to hormone-sensitive women's reproductive health outcomes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Capsule

Among 178 naturally cycling women, concentrations of perfluorooctane sulfonate (PFOS) and perfluorooctanesulfonic acid (PFOSA) were inversely associated with salivary estradiol and progesterone concentrations. Associations were only evident in nulliparous women.

Figure 1. (Log) mean follicular (day -7 to -1) estradiol concentrations (pmol/L) in relation to PFOS concentrations, by parity (parous/nulliparous) (n=178). Lines show slopes by parity.

Figure 2.
(Log) mean luteal (day +2 to +10) progesterone concentrations (pmol/L) in relation in relation to PFOS concentrations (parous/nulliparous) (n=178). Lines show slopes by parity.

Table 1

Descriptive statistics for EBBA-I cohort by parity (n=178).

	All women (n=178)	Nulliparous (n=90)	Parous (n=88)	p-value ^I
<i>Demographic factors and covariates</i>			Mean (SD)	
Age (years)	30.7 (3.0)	29.2 (2.8)	32.2 (2.5)	<0.0001
BMI (kg/m ²)	24.4 (3.8)	23.8 (3.7)	24.9 (3.8)	0.04
Age at menarche	13.1 (1.4)	13.2 (1.4)	13.0 (1.4)	0.33
		N (%)		
Married (or living as married)	112 (62.9)	41 (45.6)	71 (80.7)	<0.0001
History of hormonal contraceptive use (ever) ²	149 (83.7)	70 (77.8)	79 (89.8)	0.05
Current smoker (any)	38 (21.3)	14 (15.6)	24 (27.3)	0.08
Alcohol use (any)	168 (94.4)	84 (93.3)	84 (95.5)	0.77 ²
Physical activity level (high)	41 (23.0)	24 (26.7)	17 (19.3)	0.32
<i>Reproductive hormone concentrations</i>			Mean (SD)	
Mean progesterone (days +2 to +10; pmol/L)	153.9 (80.8)	164.3 (86.0)	143.4 (74.0)	0.89
Mean follicular estradiol (days −7 to −1; pmol/L)	20.0 (10.9)	20.1 (10.7)	19.8 (11.1)	0.08
<i>Maternal characteristics</i>			Mean (SD)	
Parity			1.9 (0.9)	
Time since last birth (years)			4.6 (3.0)	
Months breast-feeding last child			10.4 (5.3)	

^IBased on t-tests and chi-square tests comparing nulliparous and parous women.²Chi-squared approximation assumption may not hold.

Table 2

Regression coefficients (with 95% confidence intervals) of models predicting log-transformed salivary ovarian hormone concentrations (estradiol and progesterone) from serum PFAS concentrations (n=178).

Model 1: No interaction ^a		Model 2: Parity*PFAS interaction ^b		
Total sample		Nulliparous	Parous	p-value, interaction
β (95% CI)		β (95% CI)	β (95% CI)	
<i>Follicular estradiol (day -7 to -1)</i>				
PFOS	-0.013 (-0.026, -0.001)	-0.025 (-0.043, -0.007)	-0.003 (-0.020, 0.014)	0.09
PFOA	-0.017 (-0.073, 0.038)	-0.0001 (-0.070, 0.070)	-0.048 (-0.141, 0.045)	0.42
PFNA	0.021 (-0.282, 0.323)	0.174 (-0.180, 0.529)	-0.367 (-0.927, 0.193)	0.11
PFDA	0.101 (-0.354, 0.557)	0.258 (-0.221, 0.818)	-0.504 (-1.404, 0.396)	0.13
PFUnDA	-0.171 (-0.529, 0.188)	-0.055 (-0.636, 0.526)	-0.237 (-0.682, 0.208)	0.62
PFHxS	-0.015 (-0.051, 0.020)	-0.040 (-0.187, 0.107)	-0.014 (-0.050, 0.022)	0.73
PFOSA	-0.271 (-0.686, 0.144)	-0.645 (-1.320, 0.030)	-0.045 (-0.570, 0.479)	0.17
<i>Luteal progesterone (day +2 to +10)</i>				
PFOS	-0.011 (-0.025, 0.003)	-0.027 (-0.048, -0.007)	0.003 (-0.016, 0.023)	0.04
PFOA	-0.003 (-0.066, 0.061)	0.001 (-0.078, 0.081)	-0.012 (-0.117, 0.096)	0.86
PFNA	0.254 (-0.089, 0.597)	0.179 (-0.226, 0.584)	0.443 (-0.196, 1.082)	0.49
PFDA	0.472 (-0.043, 0.987)	0.330 (-0.260, 0.920)	0.908 (-0.114, 1.929)	0.33
PFUnDA	0.212 (-0.197, 0.621)	-0.152 (-0.811, 0.507)	0.422 (-0.083, 0.926)	0.17
PFHxS	-0.003 (-0.043, 0.037)	0.014 (-0.154, 0.182)	-0.004 (-0.046, 0.038)	0.84
PFOSA	-0.107 (-0.583, 0.368)	-0.662 (-1.432, 0.108)	0.227 (-0.371, 0.825)	0.07

^a Adjusted for age, marital status, parity (parous/nulliparous), BMI, physical activity, history of hormonal contraceptive use, alcohol use, and smoking. Assumes a common slope for parous and nulliparous women.

^b In addition to adjusting for covariates listed above, also includes interaction terms allowing for parity-specific slopes, as shown.