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Associations between perfluorinated alkyl acids in blood and ovarian follicular fluid and ovarian function in women undergoing assisted reproductive treatment

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Abstract

Endocrine disrupting contaminants, in combination with other environmental variables, are associated with altered reproductive health. Assisted reproductive technology (ART) procedures offer valuable opportunities to explore the connections between environmental contaminants in the ovarian microenvironment and measures of fertility, including impaired responsiveness to gonadotropins. Here, we investigate an emerging class of environmental contaminants, the perfluorinated alkyl acids (PFAAs), to determine whether ovarian contaminant levels are associated with measures of ovarian responsiveness and fertility outcomes in a South Carolina population of women undergoing ART. Levels of PFAAs in plasma and follicular fluid samples collected from women undergoing ovarian stimulation were analyzed by liquid chromatography tandem mass spectrometry (LC-MS/MS). Six PFAAs were detected in both plasma and follicular fluid. PFAA concentrations in plasma correlate strongly to those detected in ovary and, with the exception of one compound, remain stable throughout ovarian stimulation. The concentration of PFHxS in follicular fluid inversely relates to baseline follicle counts. While no significant relationships were detected between ovarian response measures and PFAA concentrations, we

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identified a negative relationship between follicular fluid PFDA and PFuNA and blastocyst conversion rates. Our assessments indicate that plasma levels of PFAAs serve as a sound proxy of those in the ovarian compartment and that follicular fluid levels of specific PFAA compounds are inversely related to important clinical measures of reproductive health including baseline follicle count and post-fertilization success.

Keywords

PFAAs; EDCs; follicular fluid; ART

1. INTRODUCTION

Current estimates of human fecundity and fertility in the United States indicate that 10.9% (7.3 million) of married women between ages 15–44 display impaired fecundity and 6% (1.5 million) are classified as infertile [1, 2]. While it is difficult to evaluate whether these numbers have increased over recent decades, it is clear that the number of individuals seeking assisted reproductive technology (ART) therapy is on the rise [3]. Various reasons have been postulated to explain this trend including delayed age of first pregnancy, improved access to fertility clinics, and increased reporting of fertility problems. Additionally, exposure to environmental contaminants can significantly influence reproductive health, and exposures have been suggested as a likely contributor [4–7]. Here, we investigate an emerging class of environmental contaminants, the perfluorinated alkyl acids (PFAAs), to determine whether ovarian contaminant levels are associated with measures of overall ovarian health in a population of women undergoing fertility treatment.

The ovarian follicle represents the functional unit of the ovary, and follicular development is tightly regulated by both intra- and extra-ovarian factors [8]. Whereas pre-antral stages can occur independent of gonadotropin receptor activation and are characterized by growth and differentiation of the oocyte, the subsequent antral phases of follicular development are dependent upon pituitary derived gonadotropins (follicle stimulating hormone and luteinizing hormone, FSH and LH, respectively) and are characterized by differentiation and enlargement of the follicle [8]. Thus, the ability of the ovary to properly respond to gonadotropin stimulation is a fundamental aspect of its function. Current ART takes advantage of the regulatory mechanisms that control follicle growth to promote the development of multiple follicles. In this setting, controlled ovarian stimulation is conducted by pharmacologically silencing the hypothalamus-pituitary-ovary axis and subsequent stimulation with exogenous FSH, allowing for careful control and monitoring of the developing follicle pool. When optimal follicle count and size is achieved, ovulation is triggered and measures to promote fertilization (e.g., intrauterine insemination, harvesting of follicles subsequent *in vitro* fertilization) are performed [9]. Ovarian responsiveness to FSH treatment is not a standardized parameter across different fertility practices, but typically incorporates a combination of more common measures including peak 17β-estradiol (E2), peak follicle count, total FSH administered, and total oocytes retrieved. The etiology of variation in responsiveness to FSH is likely rooted in a complex of influences including age, body mass index (BMI), ovarian reserve, as well as genetic factors. There is, however, also

concern that exposures to environmental contaminants may also impair the mechanisms that regulate ovarian follicle development [10–12]. The treatment protocol and samples collected as part of standard ART procedures offer valuable opportunities to explore the connections between environmental contaminants in the ovarian microenvironment potentially providing insights into the underlying influences of impaired responsiveness to gonadotropins.

Here we focus on PFAAs, which exhibit surfactant properties that make them desirable components of various products including adhesives, water-repellant surfaces, lubricants, and aqueous film-forming foams finding use in packaging, as stain repellants on fabrics and as firefighting foams. Structurally, PFAAs consist of one or more carbon atoms where all of the hydrogen atoms have been replaced by fluorine atoms and an acid functional group [13]. The carbon-fluorine bond is the strongest known bond in organic chemistry hence PFAAs are stable and long-lived in the environment. PFAAs have also been shown to bioaccumulate and biomagnify in the ecosystem [14, 15]. In humans, PFAAs are poorly eliminated and exhibit half-lives up to 5 years [16]. Primary exposure routes include inhalation of air particles contaminated with PFAAs originating from numerous consumer products including non-stick cookware and water-resistant consumables as well as ingestion through food and water [17]. Some epidemiological data exists associating PFAAs and health outcomes including recent studies demonstrating an association between circulating PFAA levels and kidney dysfunction (increased uric acid, reduced glomerular filtration), prostate cancer risk, lipid metabolism, and sperm quality [18–21].

Both epidemiological and *in vitro* studies suggest that PFAA compounds might influence ovarian cell signaling and measures of overall reproductive health [22]. In human populations, elevated concentrations of perfluorooctanoate (PFOA) and perfluorooctane sulfonate (PFOS) are associated with moderate to severe endometriosis as well as polycystic ovarian syndrome [23, 24]. Approaches that incorporate transient transfection assays have linked PFAA exposure to activation of mouse and human peroxisome proliferator activated receptor alpha (PPARα) [25, 26]. PPARs are ligand dependent nuclear receptors with roles in many physiological processes including inflammation, energy homeostasis, glucose metabolism and cellular proliferation and differentiation. The observed interaction between PFAAs and receptors of the PPAR family is intriguing given the documented role of PPAR receptor activation in the regulation of folliculogenesis and steroidogenesis and offers rationale for inclusion of the PFAA survey conducted here [27, 28]. To our knowledge, only two studies have directly assessed the relationship between PFAAs and ovarian health, specifically responsiveness to gonadotropin stimulation through fertility treatment. The first did not present details regarding individual PFAA concentrations [29]. A more recent study involved assessments in a population of Belgium women seeking ART and focused on examining post-oocyte retrieval outcomes such as fertilization and cleavage rates and identified a direct relationship between PFAA concentrations and embryo quality [30].

The development of the ovarian follicle is a tightly regulated process dependent on a critical balance of hormones and growth factors. Given the observations linking PFAAs to disrupted ovarian signaling, we set out to address four primary questions (1) Which PFAAs are measurable in the ovarian follicular fluid?, (2) Are plasma PFAA levels predictive of levels measured in follicular fluid?, (3) Do the concentrations of PFAAs fluctuate over the course

of ovarian stimulation with FSH?, and (4) Is there relationship between measures of ovarian responsiveness or fertilization success and PFAA body burden? The results will provide information regarding abundance and distribution of these compounds in a population of women seeking ART. Also important is that the analysis framework described here can be applied to study the influence of other compounds of interest on ovarian function and responsiveness in the setting of ART.

2. METHODS

2.1 Patients

This study was approved by the Medical University of South Carolina institutional review board (IRB# Pro00015729). Informed consent was obtained from all subjects before inclusion in the study. A total of 50 subjects undergoing *in vitro* fertilization at the Coastal Fertility Center in Mount Pleasant, South Carolina, USA, were enrolled between May 2013 and August 2014. Of the enrolled subjects, we collected follicular fluid from 36 subjects. No exclusions were applied. All subjects underwent pelvic ultrasound examination to determine baseline antral follicle counts. The initial dosage of gonadotropin was determined based on baseline antral follicle counts, baseline FSH levels, and patient age. Ultrasound tracking and hormone measures were carried out at appropriate intervals and human chorionic gonadotropin (hCG) was administered to trigger final oocyte maturation. Ultrasound guided oocyte retrieval was carried out approximately 36 hours post hCG trigger and all visible follicles larger than 10 mm in diameter were aspirated.

2.2 Sampling Protocol

Blood and follicular fluid were collected from enrolled subjects. A baseline blood and urine sample was collected prior to the gonadotropin stimulation phase. Additional blood samples were collected throughout the stimulation phase. At the time of oocyte retrieval, ovarian follicular fluid was collected from follicles >10mm in diameter. The total volume of follicular fluid from each patient was pooled and collected directly into glass containers, transferred to 50 mL conical centrifuge tubes (Thermo Fisher Scientific, Grand Island, NY, USA) and immediately processed by centrifugation at 600 RCF for 10 min to pellet associated follicular cells. The cleared supernatant was removed and stored in glass vials at −40 °C until further analysis. Whole blood was centrifuged at 1500 RCF for 20 min at room temperature and the cleared plasma supernatant was stored at −40 °C until further analysis.

2.3 Calculating ovarian responsiveness index and measures of fertilization success

For each patient, data regarding patient age, body mass index (BMI), basal and peak E_2 levels (pg/mL), amount of gonadotropin applied, number of oocytes retrieved (>10 mm), and other related fertility measures were collected. From this information, we calculated three measures of ovarian responsiveness: ΔE2 (pg/mL), Δ antral follicle account and oocytes at retrieval. The ΔE2 was calculated by determining the proportional increase of E_2 over the course of FSH stimulation. The Δ antral follicle count was calculated by determining proportional change in antral follicle count over the course of FSH stimulation (as determined by ultrasound examination). The oocytes at retrieval measure reflects the total number of oocytes retrieved. Additionally, fertilization rate, blastocyst conversion rate,

and pregnancy outcomes were collected for each subject and incorporated into the analysis. Fertilization rate was determined 17–20 hours post insemination by calculating the number of zygotes with two pronuclei (2pn) / total number of mature oocytes retrieved. Blastocyst development was defined as embryos with blastocoel development and expansion of at least grade 2 using the Gardner scale on day 5 or 6 of development [31]. Blastocyst conversion is defined as the number of blastocysts generated by day 6 of development / the number of embryos cultured past day 3 of development. Whether the *in vitro* fertilization procedure resulted in the birth of one or more babies was recorded and categorized as pregnancy outcome.

2.4 Measurement of perfluorinated alkyl acids

The National Institute of Standards and Technology (NIST) Standard Reference Material (SRM) 1958 organic contaminants in fortified human serum was employed as a control material during PFAA analysis. The SRM was reconstituted with deionized water based on the certificate of analysis (www.nist.gov/srm/).

Calibration solutions were comprised of two solutions produced by the NIST Reference Materials (RMs): RM 8446 Perfluorinated Carboxylic Acids and Perfluorooctane Sulfonamide in Methanol and RM 8447 Perfluorinated Sulfonic Acids in Methanol. Together, the solution contained 15 PFAAs as follows: perfluorobutyric acid (PFBA), perfluoropentanoic acid (PFPeA), perfluorohexanoic acid (PFHxA), perfluoroheptanoic acid (PFHpA), PFOA, perfluorononanoic acid (PFNA), perfluorodecanoic acid (PFDA), perfluoroundecanoic acid (PFUnA), perfluorododecanoic acid (PFDoA), perfluorotridecanoic acid (PFTriA), perfluorotetradecanoic acid (PFTA), perfluorobutanesulfonic acid (PFBS), perfluorohexanesulfonic acid (PFHxS), PFOS, and perfluorooctanesulfonamide (PFOSA). Internal standards (IS) were purchased from Cambridge Isotope Laboratories (Andover, MA), RTI International (Research Triangle Park, NC), and Wellington Laboratories (Guelph, Ontario) to generate an internal standard (IS) mixture containing eleven isotopically labeled PFAAs, and they were as follows: $[^{13}C_4]$PFBA, $[^{13}C_2]$PFHxA, $[^{13}C_8]$PFOA, $[^{13}C_9]$PFNA, $[^{13}C_9]$PFDA, $[^{13}C_2]$PFUnA, $[^{13}C_2]$PFDoA, $[^{18}O_2]$PFBS, $[^{18}O_2]$PFHxS, $[^{13}C_4]$PFOS, and $[^{18}O_2]$PFOSA.

The isolation and purification methods are outlined in detail by Reiner and colleagues [32]. Purified extracts from 1 mL of follicular fluid were analyzed for PFAAs by liquid chromatography tandem mass spectrometry (LC-MS/MS) using an Agilent 1100 LC system (HPLC; Santa Clara, CA) coupled to an Applied Biosystems API 4000 triple quadrupole mass spectrometer (Applied Biosystems, Foster City, CA) with electrospray ionization in negative mode. A total of 5 μL of resulting sample extract was injected onto an Agilent Zorbax Eclipse Plus C18 analytical column (2.1 mm x 150 mm x 5 μm). The following solvent gradient flow was employed: 50% 20 mmol/L ammonium acetate in methanol, 50% 20 mmol/L ammonium acetate in water (at a flow rate of 250 μL/min). By 20 min, the methanol increased to 75% methanol and held for 5 min, followed by an increase to 95% methanol by 35 min with a subsequent return to 50% methanol at 40 min. Two multiple reaction monitoring (MRM) transitions for each PFAA were monitored to ensure no interferences with PFAA measurement, one MRM was employed for quantitation and

the other one was used for confirmation [32]. All plasma and follicular fluid samples were processed alongside quality control material NIST SRM 1958, a follicular fluid quality control sample, and blanks to determine the accuracy and precision of the method. The PFAA concentrations of SRM 1958 processed during our extraction met established values reported on the Certificate of Analysis (CoA). Repeated measurements of the follicular fluid quality control material showed good agreement (RSD < 15 %). The PFAAs were considered to be above the limit of detection (LOD) if the mass of an analyte in the sample was greater than the mean plus three standard deviations of all blanks.

2.5 Statistics

All statistical analyses were performed with SPSS Statistics version 22 (IBM, Armonk, NY, USA) and GraphPad Prism version 6.01 (GraphPad Software, La Jolla, CA, USA). PFAA concentrations were log transformed prior to analysis. Measures were analyzed using a ROUT test to identify outliers, Q=0.5. For each individual PFAA, a T-test was used to detect differences between plasma and follicular fluid concentrations. To determine whether plasma PFAA concentration related to follicular fluid PFAA concentration, Pearson correlation analyses were used. For assessments aimed at determining if concentrations of PFAAs changed over the course of ovarian stimulation, a repeated measures ANOVA was utilized. To identify relationships among ovarian response and contaminant burden, a partial correlation analysis was conducted with a correction for age built into the model. A correction for BMI was not applied in this context because unlike age, a direct association between BMI and ovarian responsiveness was not detected. For statistical analysis of PFAA measurement data, PFAAs measured below the LOD were set equal to half the LOD prior to running the statistical tests [33]. Statistical significance was set at $p<0.05$ for all tests.

3. RESULTS

3.1 Description of Subjects

Of the 50 subjects enrolled, we collected both baseline and stimulatory phase plasma samples, along with follicular fluid samples, for 26 individual women participating in the standard *in vitro* fertilization treatment protocol. Baseline and stimulatory phase samples were collected (i.e. plasma samples) from an additional 8 patients but corresponding follicular fluid samples at time of egg retrieval were not collected. These samples were used when addressing questions regarding fluctuations of PFAAs over the course of ovarian stimulation and ovarian responsiveness. Descriptive data including average age (33.7 ± 4.5 years) and average BMI (24.8 ± 4.2 kg/m^2) were determined for the study cohort and accounted for in subsequent statistical analyses.

3.2 PFAA burden in ovarian follicular fluid and plasma

Of the panel of fifteen PFAAs measured, six were detected above the LOD in all patient follicular fluid samples, with the exception of one patient that had PFDA and PFUnA concentrations below the LOD (PFDA >0.3 ng/g, PFUnA > 0.2). In order of abundance, these include: PFOS, PFOA, PFNA, PFHxS, PFDA, and PFUnA (Figure 1A). Correlation analysis revealed a high degree of association among the individual PFAA levels, indicating

that exposures to the compounds are linked either by source or perhaps by behavior (Table 1).

Overall, follicular fluid levels of PFAAs are similar to those detected in plasma. Of the six compounds detected, only PFUnA concentrations differed between the two matrices with plasma levels being approximately 1.5 times higher than those detected in follicular fluid (unpaired T-Test, p=0.01) (Figure 1, Table 2).

3.3 PFAA concentrations in plasma are highly correlated to those detected in follicular fluid

For total and individual PFAA compounds, significant positive correlations between plasma and follicular fluid concentrations are detected (Figure 1, B–H). However, the strength of these relationships varies among the individual compounds (range r=0.81 – 0.99).

3.4 PFAA plasma concentrations remain stable over the course of ovarian stimulation

To evaluate whether the average plasma levels of PFAA compounds vary significantly over the course of ovarian stimulation, we measured compounds in plasma samples collected from women prior to stimulation with FSH and at three subsequent time points during the stimulation protocol, reflecting an average of 17 days total (min. 12 days, max. 26 days). Results indicate that levels generally remain unchanged (Figure 2, Supplementary Table 1), with the exception of PFOA, which decreased over the course of stimulation (repeated measures ANOVA, p=0.001, F=7.4).

3.5 Connections between ovarian responsiveness and plasma PFAAs

To assess the potential influence of PFAAs on ovarian health, we first considered whether detected plasma and follicular fluid PFAAs relate to baseline follicle counts, E_2 levels, and patient age (Figure 3, Table 3). A non-significant relationship was determined for total PFAA burden and baseline measures of E_2 and follicle counts (Figure 3). However, the follicular fluid levels of one particular PFAA, PFHxS, negatively correlate to baseline follicle counts (Pearson correlation, r= −0.43, Table 3). We did not detect an association between patient age and PFAA body burden (Table 3).

Subsequently, we assessed whether PFAAs relate to ovarian response to exogenous gonadotropins. Three different, but related, measures of ovarian responsiveness were considered: (1) ΔE_2, (2) Δ antral follicle count, and (3) oocytes retrieved. The range of responsiveness observed in our sample set is displayed in Figure 4. The oocytes retrieved measure of responsiveness negatively correlates with age (Pearson correlation, r= −0.47, p = 0.007). To control for this relationship, an age correction was applied when examining the link between PFAA burden and ovarian responsiveness. The analysis did not reveal any significant relationships linking ovarian responsiveness to total or individual PFAA concentrations in plasma or follicular fluid (Figure 4, Table 3).

3.6 Fertility outcome measures and PFAAs

When considering the relationship between PFAA body burden and fertilization measures we found that both PFDA and PFuNA levels in follicular fluid are inversely related to

blastocyst conversion rate (r=−0.52 and r=−0.60, respectively, Table 3). A relationship was not detected between PFAA levels and fertilization rates or pregnancy outcomes (Table 3).

4. DISCUSSION

Previously, we demonstrated that wildlife exposed to environmental endocrine disrupting contaminants display reduced ovarian responsiveness upon exogenous stimulation with gonadotropins [34]. In the context of human fertility, women also display a wide range of ovarian responsiveness (Figures 4A, C and E). We hypothesized that certain contaminants present in the ovarian microenvironment influence the ability of the ovary to properly respond to stimulatory signals, such as gonadotropins. This study represents one of the first efforts to quantify multiple PFAAs in the plasma and ovarian follicular fluid of women and subsequently connect those levels to measures of ovarian health in response to gonadotropin stimulation. In addition, it examines the relationship between PFAAs and fertilization outcomes and establishes a valuable framework for future investigations into the environmental determinants of ovarian sensitivity to gonadotropin stimulation.

Six PFAAs were quantified in plasma and ovarian follicular fluid samples, providing evidence that these persistent environmental compounds accumulate in the ovary at levels similar to those detected in the plasma. In previous assessments aimed at quantifying different classes of environmental contaminants (mostly organochlorines, OCs), a two-fold or greater difference was reported between the two tissues, with levels being greater in the plasma relative to the ovary [4]. This stands in contrast to our findings and those reported previously for PFAA compounds, which were found to be approximately equal in both compartments. A likely explanation for this lies in the distinct chemical properties that characterize the different contaminant groups. While OC pollutants are lipophilic and stored in adipose tissue, PFFAs display lipophobic properties and are frequently detected in human plasma samples [13]. PFAAs circulate bound to carrier proteins, which unlike lipid bound OCs, and easily transported across the blood follicle barrier [35, 36]. Therefore, it is likely that the observed near equal abundance of PFAAs in circulation and in follicular fluid may be due to the chemical properties that facilitate transport of these compounds into the follicle from the local bloodstream.

The two most abundant PFAAs detected in our samples (both plasma and follicular fluid) were PFOS and PFOA, which is similar to recent NHANES reports examining PFAA burden in human samples [37]. Our results show that circulating PFAAs are highly predictive of PFAA burden in the ovarian follicular fluid, indicating that plasma sampling can serve as an appropriate proxy for ovarian exposure levels. The time course results indicate that PFAAs remain fairly stable over the course of ovarian stimulation (Figure 2). This finding further supports the use of plasma measurements of PFAAs as an indicator of exposures in the ovarian compartment. If a high degree of variation had been observed, then predictions linking plasma PFAA levels to ovarian health would be challenging. We did note a decrease in the measurable levels of one PFAA compound, PFOA, over the stimulatory course. Due to the long half-life of PFAAs, it is doubtful that this observation is linked to changes in lifestyle (exposure) during the period of ovarian stimulation, but instead might reflect physiological changes associated with a change in circulating hormones (i.e. increased E_2).

Hormonal fluctuations associated with the menstrual cycle have been linked to observed differences in the pharmacokinetics and pharmacodymamics of certain pharmaceuticals [38, 39]. In addition, PFAA dosing studies in rodents have revealed sex differences in clearance rates. Relative to female rats, males display a reduced excretion rate of PFOA. Treatment of castrated male rats with E_2 results in an increased excretion of PFOA, similar to that observed in females [40].Therefore, it seems plausible that the changes associated with exogenous FSH treatment may directly impact clearance of circulating PFAAs and contribute to the reduction observed in PFOA over the course of ovarian stimulation.

We tested the hypothesis that ovarian health is correlated with PFAA body burden in women seeking ART. We found that baseline follicle count is inversely related to plasma PFHxS concentrations, flagging this particular PFAA as a potential compound of interest in the context of ovarian pathology. The identified relationship between PFHxS and baseline fertility measures is intriguing given the documented ability of PFAAs to bind PPARs *in vitro* and recognized roles for PPAR signaling in proper ovarian follicle maturation and ovulation [26]. Gene expression analysis in rodents reveals that fluctuations in ovarian PPAR transcription characterize different stages of follicle development. Transcript abundance of PPARγ, for example, declines dramatically as follicles transition into the late antral stage and prepare for ovulation through activation of human chorionic gonadotropin (HCG) signaling cascades [28]. Therefore, it seems plausible that persistent activation of ovarian PPARs, through binding to PFAA compounds, could impede follicle maturation and responsiveness to ovulation triggers contributing to impaired fertility. Support linking environmental contaminant exposure to impaired ovarian responsiveness via PPAR mediated signaling comes from *in vitro* studies demonstrating the ability of monoethylhexyl phthalate (MEHP) to activate PPARs and subsequently lead to reduced aromatase mRNA and protein along with a corresponding reduction in E_2 synthesis, an action that could ultimately lead to impaired follicle maturation and ovulation [41]. Given the correlative nature and small sample size of these analyses, a causal relationship is certainly not demonstrated with our current study design. Future studies employing larger samples sizes are still needed to test the robustness of the relationship between PFAA exposure and ovarian responsiveness to FSH. Further, experimental approaches capable of establishing causality will be required to determine the potential molecular mechanisms by which PFAAs might influence ovarian health.

In addition to ovarian responsiveness, we also assessed whether PFAA body burden relates to ART outcome measures and found that increased follicular fluid PFOA and PFuNA levels are associated with lower rates of blastocyst conversion, but not fertilization or pregnancy outcomes. In human IVF, there is a trend toward blastocyst stage transfer and cryopreservation [42]. Culturing embryos to the blastocyst stage allows for the *in vitro* selection of embryos with the highest implantation potential through morphokinetic assessment [43]. Blastocyst conversion is thus an important measure of IVF cycle quality. Interestingly, a previous report observed a positive relationship between PFAA levels and fertilization rates as well as higher top quality embryo rates [30]. However, this study incorporated a principle component analysis in which potential negative impacts of organochlorine endocrine disrupting contaminants (e.g., *p,p'*-DDE) on these endpoints were corrected for [44]. The current analyses did not measure or include corrections for this class

of contaminants, and thus, observed negative correlations between blastocyst conversion rate and PFOA and PFuNA levels could be confounded by effects of co-occurring compounds to which individuals are exposed.

The current study as well as the Petro, et al., 2014 report are based on relatively small sample sizes and future studies examining the relationship of these compounds to clinical measures of fertility more broadly across the population are warranted. Further, mechanistic studies, aimed at identifying causal roles of PFAAs in affecting early embryonic development, are needed to more fully understand the impact of these exposures on reproduction.

In summary, the experimental design and analysis approach presented here provides a framework for investigating the relationships between ovarian contaminant burden and function. Our assessments indicate that plasma levels of PFAAs are fairly stable and highly predictive of those present in the ovarian compartment follicular fluid. While future studies incorporating an increased number of patient samples as well as unbiased, untargeted analytical approaches to profile the suite of contaminants that accumulate in the follicular fluid will certainly provide insight and targets for future investigations, additional mechanistic studies are also needed to better understand the downstream effects of PFAA exposure in the ovary. An *in vitro* system incorporating ovarian granulosa cells cultured in the presence or absence of PFAAs, and stimulated with FSH could be used to identify potential genomic or transcriptional level PFAA responsive targets.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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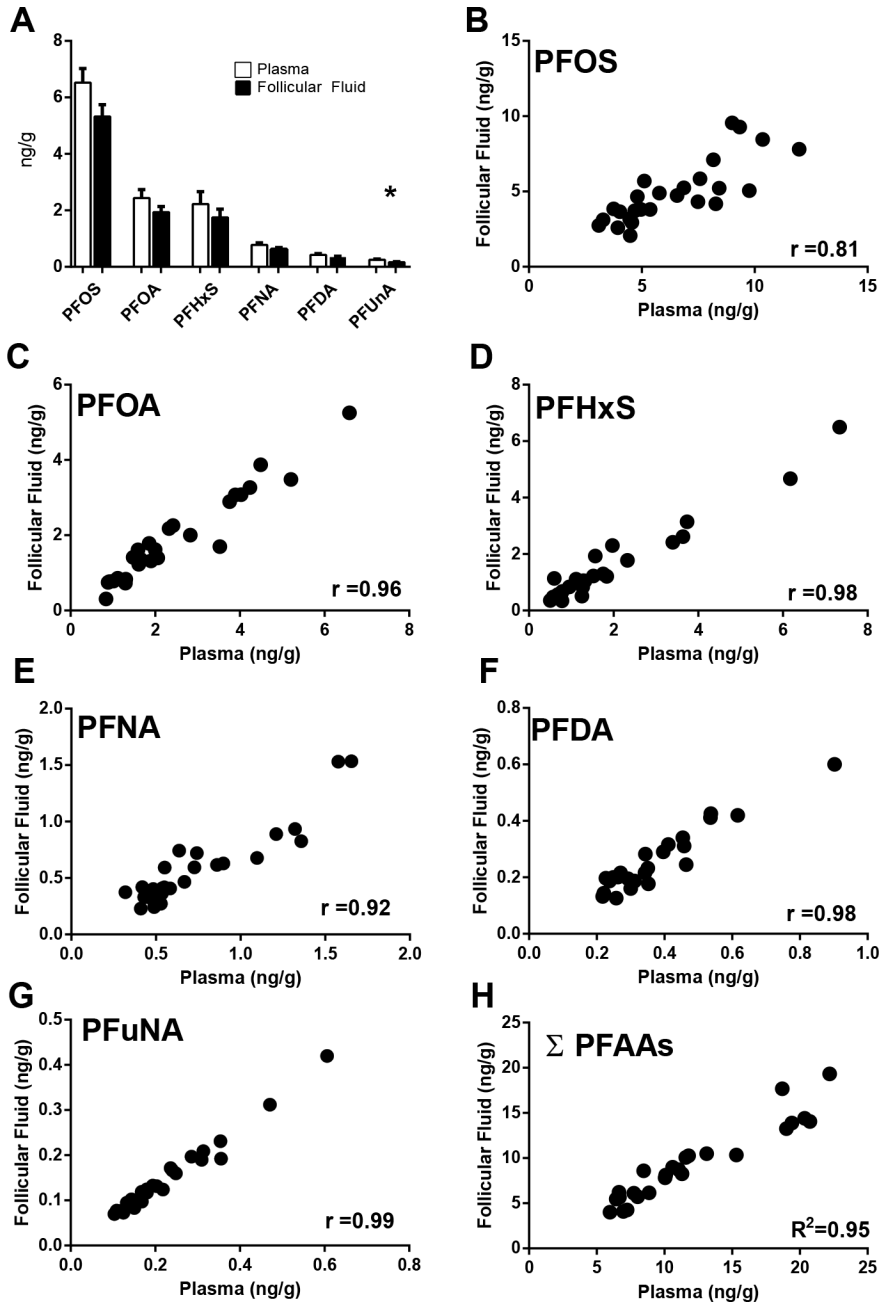

Figure 1.

Measurable PFAA compounds in plasma and follicular fluid. Mean concentrations (A) and tissue level correlations (B-H) for each individual compound are displayed. The asterisk (*) in panel A denotes a statistically significant difference was determined by T-test ($p<0.05$). The Pearson correlation coefficients are displayed in panels B-H, $p<0.05$. Error bars displayed in panel A reflect SEM.

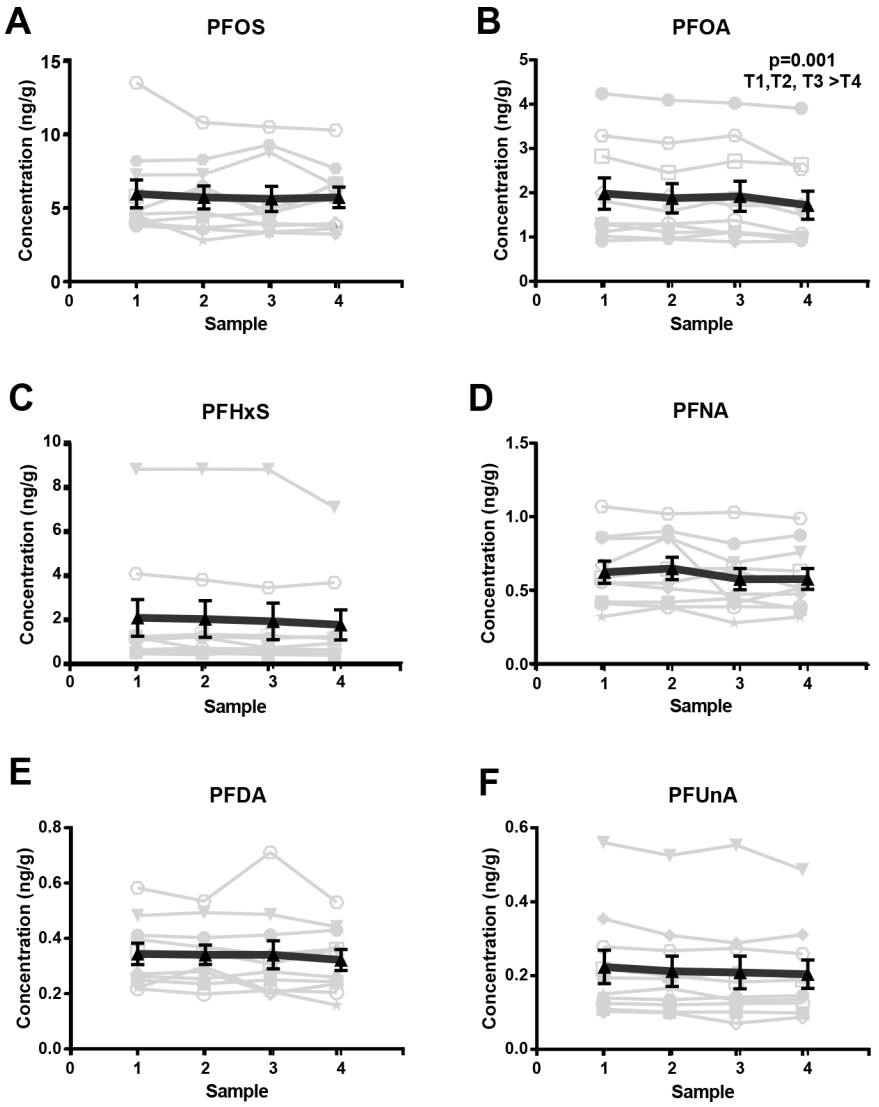

Figure 2.

Plasma PFAA concentrations (ng/g) measured over the study time period: (A) PFOS, (B) PFOA, (C) PFHxS, (D) PFNA, (E) PFDA, and (F) PFUnA. Plots depict the detectable levels of individual plasma PFAAs collected from women (n=10) prior to and throughout the course of ovarian stimulation. Individual (light grey) and average (n=10, black) values are displayed. Error bars reflect S.E.M.

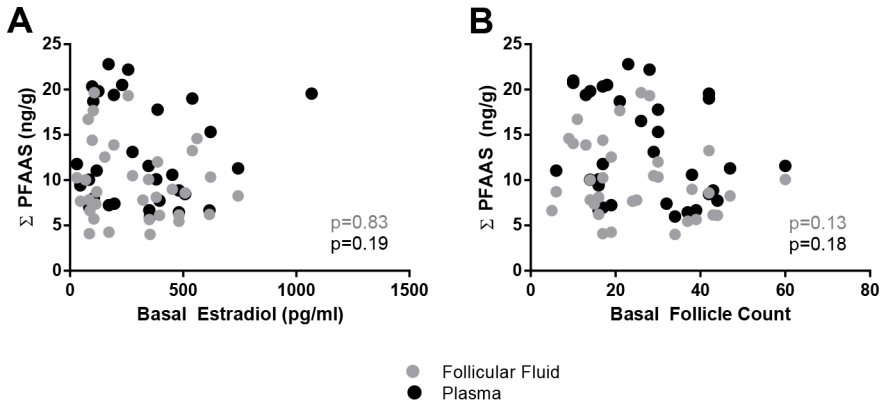

Figure 3.

PFAA concentrations (ng/g) related to basal E_2 and follicle count measurements. Plasma (black circles) and follicular fluid (gray circles) are depicted along with significance values (p) obtained from partial correlation analysis.

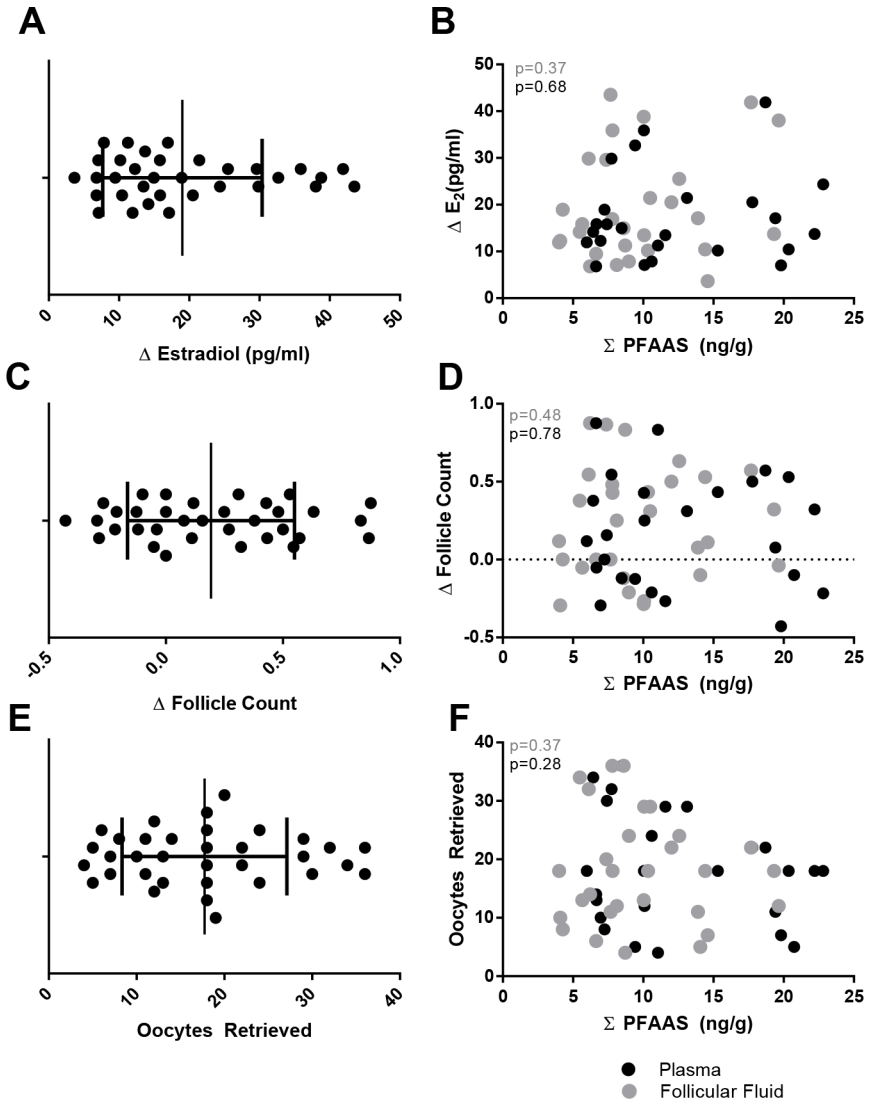

Figure 4.

Ovarian response measures related to total PFAA concentrations (ng/g). The range of observed ovarian responsiveness for three different response measures is displayed in panels A, C, and E. The relationship between ovarian responsiveness and total PFAA concentrations (ng/g) are depicted in panels B, D, and F. Plasma (black circles) and follicular fluid (gray circles) are depicted along with significance values (p) obtained from partial correlation analysis.

Table 1.

Intra-PFAA correlations values (r) in plasma and follicular fluid.

Follicular Fluid	**lnPFOA**	**lnPFNA**	**lnPFDA**	**lnPFUnA**	**lnPFHxS**	**lnPFOS**
PFOA	-	**0.741** **	**0.648** **	0.326	**0.363** *	**0.524** **
PFNA		-	**0.850** **	**0.438** *	**0.411** *	**0.770** **
PFDA			-	**0.586** **	0.276	**0.714** **
PFUnA				-	0.336	**0.383** *
PFHxS					-	**0.451** *
PFOS						-
Plasma	lnPFOA	lnPFNA	lnPFDA	lnPFUnA	lnPFHxS	lnPFOS
PFOA	-	**0.814** **	**0.617** **	0.289	0.173	**0.540** **
PFNA		-	**0.809** **	**0522** **	**0.421** *	**0613** **
PFDA			-	**0.699** **	0.324	**0.506** **
PFUnA				-	**0.571** **	0.363
PFHxS					-	**0534** **
PFOS						-

* significant at the 0.05 level (2-tailed).

** significant at the 0.01 level (2-tailed).

Table 2.

Detected concentrations (ng/g) of individual and total PFAAs in plasma and follicular fluid (FF).

	Plasma (ng/g; n=26)	FF (ng/g; n=31)	Plasma/ FF (n=26)	p value
PFOS	6.52 ± 0.50	5.33 ± 0.42	1.22	0.07
PFOA	2.44 ± 0.30	1.94 ± 0.20	1.26	0.16
PFHxS	2.23 ± 0.44	1.75 ± 0.30	1.27	0.36
PFNA	0.78 ± 0.08	0.63 ± 0.06	1.24	0.12
PFDA	0.42 ± 0.05	0.33 ± 0.04	1.27	0.19
PFUnA	0.25 ± 0.03	0.17 ± 0.02	1.47	0.01*
ΣPFAAs	12.6 ± 1.1	10.1 ± 0.8	1.25	0.06

Table 3.

Correlation values (r) between PFAA levels (ng/g) and clinical measurements

Follicular Fluid	**lnPFHxS**	**lnPFOA**	**lnPFOS**	**lnPFNA**	**lnPFDA**	**lnPFUnA**	**lnΣPFAAs**
Estradiol (pg/ml)	−0.34	−0.11	0.07	0.05	0.08	0.18	−0.04
Follicle Count	**−0.43**	−0.25	−0.16	−0.23	−0.25	−0.29	−0.29
Δ E2	−0.09	0.02	−0.19	−0.20	−0.17	−0.36	−0.18
Δ Follicle #	0.19	0.07	0.09	0.15	0.30	0.40	0.14
Ooocytes Retrieved	−0.23	−0.16	−0.07	−0.18	−0.13	−0.09	−0.17
Fertilization (%)	−0.07	0.04	0.15	−0.12	−0.06	−0.18	0.06
Blast Conversion	0.00	−0.24	−0.13	−0.33	**−0.52**	**−0.60**	−0.23
Age	−0.08	−0.04	−0.25	−0.20	−0.24	−0.37	−0.18

Plasma	lnPFHxS	lnPFOA	lnPFOS	lnPFNA	lnPFDA	lnPFUnA	lnΣPFAAs
Estradiol (pg/ml)	−0.18	0.07	**−0.47**	−0.16	−0.25	−0.35	−0.27
Follicle Count	−0.32	−0.06	−0.38	−0.20	−0.22	−0.27	−0.27
Δ E2	−0.18	−0.07	−0.06	−0.13	0.07	−0.27	−0.18
Δ Follicle #	0.06	0.22	−0.08	0.14	0.18	0.25	0.06
Ooocytes Retrieved	−0.29	−0.13	−0.13	−0.24	−0.08	−0.15	−0.22
Fertilization (%)	0.01	0.20	−0.05	0.19	0.14	0.03	0.08
Blast Conversion	−0.10	−0.17	−0.11	−0.21	−0.29	**−0.54**	−0.23
Age	−0.27	−0.21	−0.15	−0.12	−0.07	−0.29	−0.22

Bolded values are significant at the 0.05 level (2-tailed).