Perfluorinated alkyl acids in the serum and follicular fluid of UK women with and without polycystic ovarian syndrome undergoing fertility treatment and associations with hormonal and metabolic parameters

Heffernan AL[1,2*], Cunningham TK,[3,4*] Drage DS,[1,5] Aylward LL,[1,6] Thompson K,[1] Vijayasarathy S,[1] Mueller JF[1], Atkin SL,[7] Sathyapalan T[3#]

1. Queensland Alliance for Environmental Health Sciences, The University of Queensland, Australia
2. The Florey Institute of Neuroscience and Mental Health, Melbourne, Australia
3. Centre for Cardiovascular and Metabolic Research, Hull York Medical School, University of Hull
4. The Hull IVF Unit, Women's and Childrens' Hospital, Hull Royal Infirmary, Hull, UK;
5. School of Geography, Earth and Environmental Sciences, University of Birmingham, UK
6. Summit Toxicology, LLP, USA
7. Weill Cornell Medicine Qatar, Education City, Qatar Foundation, Doha, Qatar
8. The Hull IVF Unit, Womens and Childrens Hospital, Hull Royal Infirmary, Hull, UK

*Joint first authors

Corresponding Author
T Sathyapalan
Michael White Diabetes Centre, Hull Royal Infirmary, Hull, UK, HU3 2RW
Tel +441482675312
Fax +441482675370
Email thozhukat.sathyapalan@hyms.ac.uk

Abstract

Women undergoing treatment for infertility could be a sensitive subpopulation for endocrine effects of exposure to perfluorinated alkyl acids (PFAAs), persistent organic pollutants with potential endocrine activity. Women with polycystic ovarian syndrome (PCOS, n=30) and age- and BMI-matched controls (n=29) were recruited from a UK fertility clinic in 2015. Paired serum and follicular fluid samples were collected and analysed for 13 PFAAs. Sex steroid and thyroid hormones, metabolic markers, and serum biochemical parameters were measured and assessed for associations with serum PFAAs. Four PFAAs were detected in all serum and follicular fluid samples and concentrations in the two matrices were highly correlated ($R^2>0.95$): perfluorooctane sulfonate (PFOS), perfluorooctanoic acid (PFOA), perfluorohexane sulfonate (PFHxS), and perfluorononanoic acid (PFNA). Serum PFOS was positively associated with age ($p<0.05$) and was higher in PCOS cases than controls (geometric mean 3.9 vs. 3.1 ng/mL, $p<0.05$) and in women with irregular vs. regular menstrual cycles ($p<0.05$). When adjusted for PCOS case status and serum albumin, serum testosterone was positively associated with PFOA, and sex hormone binding globulin was positively associated with PFOS ($p<0.05$); no other associations between sex steroid or thyroid hormones and PFAA concentrations were observed. Fasting glucose was significantly positively associated with PFOA, adjusted for age, PCOS status, and serum albumin ($p<0.05$). Serum insulin and HbA1c were positively associated with BMI ($p<0.01$), but not with PFAAs. Serum PFAA concentrations can be used as surrogates for follicular fluid concentrations due to the high correlations observed. Limited associations between serum PFAAs and sex steroid hormones and fasting glucose were observed. Associations were modified by serum albumin, which can influence serum PFAA concentrations, and these interrelationships should be considered in assessing endocrine associations for PFAAs.

Key words: polycystic ovary syndrome; IVF; PFAA, endocrine disrupting chemicals; perfluorinated alkyl acids; PFOS; PFNA

Introduction

Perfluorinated alkyl acids (PFAAs; perfluorinated chemicals (PFCs)) consist of a fluorinated hydrophobic alkyl chain with a hydrophilic end group, and are used widely as surfactants in household and industrial applications such as textile treatments, food packaging, and as aqueous film-forming foams. The dominant exposure pathway for humans is diet, particularly meat and fish, and via breast milk for infants (Gebbink et al., 2015; Haug et al., 2010; Kärrman et al., 2007). PFAAs are persistent and bioaccumulative. Elimination of PFAAs depends on chain length and they sequester particularly in the liver and kidney. They are non-covalently bound to protein in serum, particularly serum albumin (Andersen et al., 2008; Bischel et al., 2010). Serum elimination half-life is approximately 3.8 and 5.4 years for perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA), respectively (Li et al., 2017b; Olsen et al., 2007). PFAAs can cross the placenta (Kim et al., 2011), and have been associated with adverse effects on fertility, birth outcomes, and early development in humans (Bach et al., 2015; Goudarzi et al., 2016; Lyngso et al., 2014; Olsen et al., 2009). PFOS and PFOA have intrinsic estrogenic activity and anti-estrogenic effects *in vitro* (Henry and Fair, 2013), and PFOS is capable of modulating steroidogenesis (Kraugerud et al., 2011). *In vivo*, PFAAs were associated with increased breast cancer risk in Inuit women, perhaps related to their estrogenic effects (Bonefeld-Jorgensen et al., 2011), while other studies have shown increased serum PFAAs associated with an earlier menopause, and with PFOS being inversely associated with estradiol levels (Knox et al., 2011). Because PFAAs are eliminated via both menstruation and renal elimination, it may be difficult to assess and interpret relationships between serum PFAA concentrations and outcomes such as birth weight, which can be affected by glomerular filtration rates, or timing of menopause, which can influence PFAA levels due to decreased elimination of PFAAs post-menopause (Ruark et al., 2017; Verner et al., 2015).

Polycystic ovary syndrome (PCOS) is one of the most common endocrine disorders and affects 6-20% of reproductive-aged women (Bozdag et al., 2016; March et al., 2010; Teede et al., 2013; Yildiz et al., 2012) with clinical manifestations of irregular menstruation, hyperandrogenism and/or polycystic ovaries (Bozdag et al., 2016; PCOS Consensus Workshop, 2004). PCOS is associated with infertility, hirsutism, and acne (Ehrmann, 2005; Norman et al., 2007). Thus, PCOS patients may be a sensitive subpopulation for compounds that alter endocrine outcomes. The previously reported association of PFAAs with menstrual irregularity and infertility (Lyngso et al., 2014; Velez et al., 2015) may have been influenced by the inclusion of women with PCOS in the studies. The aim of the study was to examine correlation of serum and follicular fluid measures of PFAAs, and to explore the associations of PFAAs with hormonal parameters in women with and without PCOS, and undergoing fertility treatment.

Materials and Methods

This prospective cohort study was performed within the Hull IVF Unit, UK following approval by The Yorkshire and The Humber NRES ethical committee, UK (approval number 02/03/043). The PCOS subjects were recruited sequentially in 2015, using the revised 2003 criteria from the Rotterdam ESHRE/ASRM sponsored PCOS consensus workshop group, indicating PCOS to be present if any 2 out of 3 criteria were met: menstrual disturbance (oligo or amenorrhoea), clinical and/or biochemical signs of androgenism or polycystic ovaries on ultrasound (PCOS Consensus Workshop, 2004). Inclusion criteria were age 20-45 years, BMI ≤35 and undergoing *in vitro* fertilisation. Patients with known immunological disease, diabetes, renal or liver insufficiency, acute or chronic infections, or inflammatory diseases were excluded from the study. No comparative study on which to base formal power calculations was available; therefore, power and sample size for pilot studies has been reviewed (Birkett and Day, 1994) that concluded that a minimum of 20 degrees-of-freedom was required to estimate effect size and variability. Hence, we planned to recruit 25 patients per group with an additional 5 patients allowing for drop-outs and covariate adjustment. A total of 59 women were recruited into the study, 30 PCOS cases and 29 control subjects matched for age and weight.

Sample Collection

The subjects fasted from midnight and had a fasting blood sample taken on day 21 of the luteal phase of the cycle before commencing their IVF treatment. Fasting venous blood samples were collected, separated by centrifugation at 3500 x g for 15 min at 4°C, and the aliquots stored at -80°oC within 1 hour of collection. Plasma glucose was measured using a Synchron LX20 analyzer (Beckman-Coulter), and serum insulin was assayed using a competitive chemiluminescent immunoassay performed using the DPC Immulite 2000 analyzer (Euro/DPC, Llanberis, UK). C reactive protein (CRP) was measured enzymatically using a Synchron LX20 analyzer (Beckman-Coulter, UK). Estradiol and all thyroid assays were performed on an Abbott Architect i4000 immunoassay analyser (Abbott Diagnostics Division, UK). Serum testosterone and androstenedione were measured by liquid chromatography tandem mass spectrometry (LC/MS/MS; Acquity UPLC-Quattro Premier XE-MS, Waters, Manchester, UK). Sex hormone binding globulin (SHBG) was measured by an immunometric assay with fluorescence detection (DPC Immulite 2000 analyzer; upper limit 2.0 nmol/l) . Glycosylated hemoglobin A1c (HbA1c) measurements were made using ion-exchange chromatography.

Analysis for PFAAs

Samples were analysed for 13 PFAAs including PFOS, PFOA, perfluorohexane sulfonate (PFHxS), perfluorononanoic acid (PFNA), and perfluorodecanoic acid (PFDA) (Table 1). 200 µL serum or follicular fluid was transferred to a 2 mL Eppendorf tube, followed by addition of the internal standards. Proteins were precipitated with acetonitrile, centrifuged, filtered (2 µm GHP membrane; Pall, East Hills, NY, USA), and concentrated under gentle stream of nitrogen. Samples were reconstituted in 5mM ammonium acetate in water prior to analysis via high performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS) using a Nexera HPLC (Shimadzu Corp., Kyoto, Japan) coupled to API5500 QTRAP mass spectrometer (Sciex, Melbourne, Australia) with electrospray ionization (ESI) interface operating in negative mode. Chromatographic separation of the analytes was achieved with a Gemini C_{18} column (50x2.0 mm, 4 µm; Phenomenex, Torrance, CA), maintained at 45 °C, with a flow rate of 0.3 mL/min and injection volume of 5 µL. Mobile phases consisted of methanol:water (1:99, v/v) (A), and methanol:water (95:5, v/v) (B), with 5mM ammonium acetate in both phases. An isolator column (Phenomenex) was included inline directly after the mobile phase mixing chamber to delay the elution of solvent-derived background PFAA contamination. Data acquisition and processing was carried out using Analyst® TF 1.6 and MultiQuantTM software (Sciex). Further details of reagents, standards, and mass spectrometry settings are provided in the Supplementary Material.

Statistics.

Descriptive data are presented as mean ± SD for continuous data and n (%) for categorical data. T-tests were used to compare means where appropriate. A p-value of <0.05 was considered to indicate statistical significance except for exploratory Pearson correlation coefficient evaluations for regression modelling development (p<0.1).

Measured serum PFAA, hormone concentrations, and metabolic markers were assessed for normality and ln-transformed where appropriate. Estimated glomerular filtration rate (eGFR) was calculated using the Modification of Diet in Renal Disease (MDRD) study method (Levey et al., 1999). Insulin resistance (IR) was calculated from basal glucose and insulin concentration using the homeostasis model assessment (HOMA) ((Insulin x glucose)/22.5)(Matthews et al., 1985). Free androgen index (FAI) was calculated as 100 times the ratio of serum testosterone and SHBG concentrations. A sum PFAA (∑PFAA) variable by calculated by adding the molar concentrations of the four frequently detected PFAA compounds (i.e. sum of PFOS, PFOA, PFHxS, PFNA).

Pairwise Pearson correlation coefficients and significance were examined as an initial step in assessing potential associations and identifying potential covariates. Multivariable linear regression was used to assess predictors for PFAA concentrations and potential associations between measured hormone concentrations or metabolic endpoints and serum PFAA concentrations. Tobit regressions were used to examine oestradiol concentrations due to left censoring of the data (concentrations below 75 pmol/L were not quantified). Statistical analyses were conducted using Stata (IC 12.1, Stata Corp., College Station, TX).

Results

Cases and controls were similar in age, BMI, and age at menarche (Table 2). Measured hormone concentrations were not available for all cases and controls (see Table 2). PCOS cases more frequently had irregular menstrual cycles (87% vs. 14%, $p<0.001$). PCOS cases were more likely to be taking metformin (47% vs. 0%), and had significantly lower average fasting glucose concentrations than controls (4.4 nmol/L vs. 4.9 nmol/L, $p<0.01$), though HbA1c did not differ to controls.

PCOS cases had higher androgen levels with significantly elevated FAI and androstenedione compared to controls, though testosterone and oestradiol did not differ. PCOS cases also had lower eGFR on average than controls (88.3 vs. 97.4 mL/min/ 1.73m^{-2}, $p<0.05$). Serum insulin, HOMA-IR and CRP did not differ between PCOS and controls.

Detection frequencies and descriptive statistics for serum and follicular fluid PFAA concentrations are presented in Table 1. Four PFAAs were detected in all serum and follicular fluid samples: PFOS, PFOA, PFHxS, and PFNA, and all were significantly correlated with one another. Detection frequencies for PFDA were 76%; and <50% for PFPeA, PFBS, PFHpA and PFUnDA, (49, 7, 17 and 36%, respectively). In general, PFOS was present at the highest concentrations, followed by PFOA, PFHxS, and PFNA. Geometric mean serum concentrations of PFOS were significantly greater in PCOS cases than controls (Table 1, Figure 1). Other serum PFAAs were not significantly different between PCOS and controls, and geometric mean follicular fluid concentrations were not different between groups (Table 2), due to greater variation in measured PFAS concentrations. For the four frequently detected PFAAs, concentrations in follicular fluid were highly correlated with serum concentrations ($R^2 > 0.95$ for all four, Figure 2). The mean ratios of follicular fluid to serum concentrations were 0.59, 0.78, 0.86, and 0.77 for PFOS, PFOA, PFHxS, and PFNA, respectively (Table 1).

Patients with irregular menstrual cycles (from both PCOS and control groups; n=30, GM: 4.16 ng/mL) had significantly higher PFOS concentrations than those with regular cycles (n=29, GM 3.25 ng/mL) ($p=0.011$, 2-tailed t-test; Figure 3), but the PFOS concentration was not associated with the degree of irregularity in PCOS patients (not shown). No associations between other PFAA concentrations and menstrual cycle regularity were observed. No associations between parity (nulliparous versus primiparous) and PFAA concentrations were observed (data not shown).

We examined pair-wise Pearson correlations for the ln-transformed concentrations of the frequently detected PFAAs and age, BMI, serum albumin, and ln-transformed eGFR (Supplementary Information, Tables S1 to S3). No correlations with BMI were observed for any of the PFAAs. PFOS and PFNA were negatively correlated with eGFR and positively correlated with serum albumin. Based on the correlation matrix, we examined predictors for each PFAA concentration in multivariable regressions with dependent variables of age, ln-transformed eGFR, serum albumin, and PCOS status. PFOS was significantly positively associated with age (approximately 0.1 ng/ml increase per year of age) and status as a PCOS case vs. control. PFNA was negatively associated with eGFR. No other statistically significant predictors for PFAA concentrations were identified.

Associations between metabolic endpoints (fasting glucose, serum insulin, HbA1C, and HOMA-IR) and serum PFAAs were assessed considering potential confounders. In pairwise correlations, fasting glucose was positively correlated with age and negatively correlated with serum albumin and status as a PCOS case; these variables were retained in multivariable regressions examining potential associations between fasting glucose and ln-transformed PFAA concentrations. Significant positive associations were observed between fasting glucose and ln-transformed PFOA (β=0.18, 95% CI 0.01,0.36, p=0.035; i.e. 1.2 ng/mL increase in PFOA for every 1nmol/L increase in glucose) and ln-transformed $\sum PFAA$ (β=0.18, 95% CI 0.00,0.37, p=0.05; i.e. 1.2 µmol/L increase in ∑PFAA for every 1nmol/L increase in glucose). Serum insulin, HbA1c, and HOMA-IR were all significantly positively associated with BMI, but no significant associations with any of the PFAAs were observed. These results were not affected by Inclusion of metformin in use in the analysis or stratification by metformin use.

We examined associations between ln-transformed concentrations of each of the four frequently detected PFAAs and steroid hormone concentrations, SHBG concentrations, and FAI, adjusting for status as a PCOS case vs. control, as well as for serum albumin concentrations (Table 3). $\sum PFAA$ was assessed in association with hormone concentrations. Serum testosterone concentrations were positively associated with ln-transformed PFOA and with $\sum PFAA$, though not with the degree of testosterone elevation. SHBG concentrations were significantly positively associated with PFOS and with $\sum PFAA$. No other significant associations between measured steroid hormone levels and serum PFAA concentrations were observed in adjusted models (Table 3).

No significant associations between any of the frequently detected PFAAs and TSH, fT3, or fT4 were observed. Both fT3 and fT4 were significantly negatively associated with serum albumin, consistent with non-specific binding of total T3 and T4 to serum protein (not shown).

Discussion

The potential effects of PFAAs on reproductive and thyroid hormones and potential influences on reproductive health are of interest given previous reports linking PFAAs to alterations in endocrine activity and function(Bach et al., 2016; Coperchini et al., 2017). The profile of PCOS also includes alterations in reproductive hormones; thus, this population might represent a sensitive subpopulation for chemical exposures that also influence hormone concentrations.

The four frequently detected PFAAs were correlated with each other in this study, consistent with previous reports (Calafat et al., 2007; Ye et al., 2017). Concentrations in follicular fluid of each PFAA were strongly associated with the corresponding serum concentrations, with average ratios ranging from approximately .59 to .86 for the four frequently detected PFAAs (Table 1). These ratios are similar to those reported by McCoy et al. (2017) (McCoy et al., 2017) for PFOA, PFHxs, and PFNA, but somewhat lower than the value reported for PFOS (0.59 in the current study vs. 0.82 in McCoy et al. 2017). The lower concentrations in follicular fluid relative to serum might reflect a lower total protein concentration in follicular fluid relative to serum (Leroy et al., 2004), which is pertinent because PFAAs are known to be protein bound (Andersen et al., 2008; Bischel et al., 2010). The high correlations between PFAA concentrations in follicular fluid and serum suggest that measures of PFAAs in serum likely are good surrogates for examining potential dose-effect relationships on the ovary.

The assessment of associations between PFAA levels and sex steroid hormones resulted in limited findings of a positive association between testosterone and PFOA and the molar sum of PFAAs after adjusting for serum albumin concentrations and PCOS case status; no significant findings remained after adjustment for the other hormone-PFAA combinations (Table 3). When the data were restricted to PCOS cases alone there was no linear relationship of PFOS to increased testosterone. This may be due to a combination of the small sample size (n= 27 PCOS cases with measured testosterone) and the high degree of variation in testosterone levels in the PCOS cases. In a recent systematic review, Bach et al. (2016) reported that associations between reproductive hormone levels and PFAA exposures was mixed (Bach et al., 2016).

TSH, free T3, and free T4 were not associated with any of the frequently detected PFAAs or the sum of these PFAAs in this study. We did observe a negative correlation between serum albumin and free T4 and free T3. Previous studies have reported mixed results regarding associations between PFAAs and thyroid hormones. Crawford et al. (2017) in a study of women without infertility found no associations between TSH and PFAAs, and reported a positive association between free T4 and PFNA (Crawford et al., 2017). Lin et al. (2013) found a similar relationship between free T4 and PFNA in adolescents and young adults from the NHANES survey (Lin et al., 2013). Chan et al. 2011 found no associations between serum PFAA concentrations and hypothyroxinemia in 974 pregnant women (Chan et al., 2011). Lewis et al. (2015) found a positive association between free T4 and serum concentrations of all four PFAAs considered here in women of reproductive age in the NHANES 2011-2012 survey, but other thyroid hormone concentrations were not associated with PFAAs in women of reproductive age (Lewis et al., 2015). Overall, studies have reported a mixed pattern of associations between PFAAs and thyroid hormone concentrations (de Cock et al., 2014; Jain, 2013; Ji et al., 2012; Kato et al., 2016; Li et al., 2017a; Shah-Kulkarni et al., 2016; Tsai et al., 2017).

PCOS cases had higher geometric mean concentrations of PFOS than controls, but concentrations of other PFAAs were similar between cases and controls. Vagi et al. (2014) found elevated PFOS and PFOA concentrations in another study of PCOS cases relative to controls (Vagi et al., 2014). The current study also found an association of PFOS concentrations with menstrual irregularity, similar to one previous study (Lyngso et al., 2014). Menstruation may be an important elimination pathway for PFAAs, and has been hypothesized to be responsible for lower PFAA concentrations in females compared to males (Lorber et al., 2015; Wong et al., 2014) and in post-menopausal women (Lorber et al., 2015). In PCOS women who may suffer from oligomenorrhea or amenorrhea, and thus menstruate less frequently than controls, the same exposure dose could result in higher serum PFAAs concentrations (Vagi et al., 2014). However, for the PCOS women, there was no significant difference in PFOS concentration between cases with cycles greater than or less than 40 days. Similarly, in this dataset, PFAAs other than PFOS were not significantly associated with menstrual cycle regularity.

In addition, we found that PCOS cases had significantly lower eGFR than controls. Evidence from the literature suggestd this may be due to inflammation and reflected in a higher CRP (Gozukara et al., 2015); however, in our study, CRP did not differ between PCOS and controls. . Renal elimination is another pathway for elimination of PFAA compounds (Han et al., 2012; Verner et al., 2015). Thus, lower eGFR for PCOS cases may result in higher serum PFAA concentrations for the same external exposure level, which was observed for PFOS, and potentially compounded by menstrual irregularity, thereby reducing PFAA elimination. Previous cross-sectional studies considered the possibility that PFAAs may negatively impact renal function, resulting in decreased eGFR (Kataria et al., 2015; Watkins et al., 2013). In this dataset, PFOS and PFNA were inversely correlated with eGFR ($p<0.05$); PFOA and PFHxS were not significantly correlated with eGFR. Thus, decreased eGFR in PCOS cases may result in some increase in serum concentrations of selected PFAA compounds in these cases compared to controls, but this relationship may be compound-specific.

Recent temporal studies report decreasing serum concentrations of PFOS and PFOA, and a corresponding increase of alternative PFAAs used as replacement chemicals, likely due to action from manufacturers and legislators to phase out PFOS and PFOA (Land et al., 2015; US EPA, 2015). In comparison with the biomonitoring literature, PFOS concentrations in this UK cohort were lower than most previously reported pregnancy cohorts globally (reviewed in (Miralles-Marco and Harrad, 2015)), whereas PFOA and PFHxS were higher (Table S4).

The strengths of the study lie in the age and BMI matched population, with measurement of a range of hormone and metabolic markers. In addition, PCOS patients may represent a sensitive subpopulation for effects of endocrine active substances. The study was limited by the small sample size and by missing data for some hormone measurements. A relatively large number of statistical evaluations were conducted (approximately 12 outcome measures by four PFAAs and the molar sum of the four PFAAs, plus assessments of predictors for PFAA concentrations), suggesting that some findings might be expected to be observed by chance.

We found high correlations between PFAAs in follicular fluid and serum, supporting the use of serum as a relevant matrix for biomonitoring for PFAAs for assessment of potential ovarian responses. Concentrations of PFOS, but not other PFAAs, were higher in the PCOS cases than in controls in this study. We found evidence of a positive associations between PFOA concentrations and summed PFAA concentrations and testosterone, and between PFOS and summed PFAAs and SHBG concentrations in the PCOS cases and controls in this study. The relationships to summed PFAAs appear to be largely driven by the contributions from the individual significant predictors; that is, the relationships to summed PFAAs are not stronger in magnitude or significance than the relationships to PFOA or PFOS. Fasting glucose was positively associated with PFOA concentrations and with summed PFAAs, but not other PFAAs. Again, the relationship with summed PFAAs appears to be largely due to the specific association with PFOA.

We identified a number of factors that should be considered in the evaluation of PFAA concentrations and potential associations with reproductive outcomes, steroid hormone concentrations, and related endpoints. Characteristics such as menstrual irregularity, parity, eGFR, and serum albumin levels may influence serum PFAA concentrations due to elimination mechanisms or physical/chemical properties of these compounds, and some of these characteristics may also be altered in populations under study for reproductive outcomes. In addition, serum albumin concentrations can influence both measured serum hormone concentrations and serum concentrations of PFAAs. Non-covalent binding of sex steroid hormones to serum protein is recognized as a factor affecting transport and metabolism of these hormones (Egloff et al., 1981; Pardridge, 1986), and we observed positive associations between serum albumin concentrations and the measured levels of the sex steroid hormones. Similarly, T3, and T4 are protein bound in serum (Koulouri et al., 2013). We observed a negative correlation between free T3 and free T4 and albumin in this dataset (Table S2), consistent with this protein binding. PFAAs are also protein-bound in serum. These interrelationships suggest that characteristics that influence PFAA elimination or serum concentrations, and which may also be associated with outcome variables, be carefully considered as covariates in future assessments of associations between hormone concentrations, reproductive outcomes, and serum PFAAs.

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Tables and Figures

Table 1. Summary of PFAA serum concentrations in PCOS case-control study (ng/mL)

	Detection frequency, n (%)		Geometric Mean		Range		Average ratio	LOR	
	Serum	FF	Serum	FF	Serum	FF	FF:Serum	Serum	FF
PFOS	59	58	3.46	2.00	0.93-7.71	0.60-4.29	0.59	0.5	0.2
PFOA	59	58	2.39	1.82	0.5-8.16	0.43-6.64	0.78	0.1	0.1
PFHxS	59	58	1.04	0.88	0.2-10.2	0.1-9.07	0.86	0.05	0.1
PFNA	59	58	0.57	0.41	0.2-1.79	0.1-1.43	0.77	0.2	0.1
PFDA	45 (76)	14 (24)	0.31	-	<LOR-	<LOR-	-	0.2	0.2
PFPeA	29 (49)	0	-	-	<LOR-	-	-	0.5	0.3
PFUnDA	21 (36)	0	-	-	<LOR-	-	-	0.2	0.7
PFHpA	10 (17)	9 (15)	-	-	<LOR-	<LOR-	-	0.1	0.1
PFBS	4 (6.8)	12 (21)	-	-	<LOR-	<LOR-	-	0.2	0.2
PFBA	0	0	-	-	-	-	-	0.5	0.2
PFHxA	0	0	-	-	-	-	-	0.5	0.1
PFDS	0	0	-	-	-	-	-	0.5	0.1
PFDoDA	0	0	-	-	-	-	-	0.5	0.4

FF, follicular fluid; LOR, limit of reporting; PFOS, perfluorooctane sulfonate; PFOA, perfluorooctanoic acid; PFHxS, perfluorohexane sulfonate; PFNA, perfluorononanoic acid; PFDA, perfluorodecanoic acid; PFPeA, perfluoropentanoic acid; PFUnDA, perfluoroundecanoic acid; PFHpA, perfluoroheptanoic acid; PFBS, perfluorobutanesulfonate; PFBA, perfluorobutanoic acid; PFHxA, perfluorohexanoic acid; PFDS, Perfluorodecane sulfonate; PFDoDA, perfluorododecanoic acid

Table 2. Demographics, hormone and biochemistry endpoints, and serum and follicular PFAA concentrations for PCOS patients and controls.

	Control (n=28) Mean ± SD	PCOS (n=31) Mean ± SD
Age (years)	32.9 ± 4.6	30.7 ± 4.6
Body mass index (kg/m^2)	25.6 ± 3.7	25.9 ± 3.8
Menarche (years)	13.1 ± 1.8	12.8 ± 1.3
Irregular menstrual cycle (%)	14%	87%***
Nulliparous (%)	97%	83%
Insulin (µIU/ml)	7.9 ± 4.1	7.9 ± 4.6
Fasting glucose (nmol/L)	4.9 ± 0.4	4.4 ± 0.8**
HbA1C (mmol/mol)	30.9 ± 6.5 (n=27)	31.8 ± 3.0 (n=28)
HOMA-IR	1.8 ± 1.0	1.9 ± 1.6
Metformin use (%)	0%	47%***
SHBG (nmol/L)	104.2 ± 80.3 (n=28)	71.7 ± 62.2 (n=28)
Testosterone (nmol/L)	0.85 ± 0.56 (n=27)	1.04 ± 0.37 (n=27)
Free androgen index (FAI)	1.44 ± 1.47 (n=27)	3.32 ± 4.08 (n=27)*
Estradiol (pmol/L)	398.0 ± 423.4 (n=27)	259.1 ± 276.0 (n=26)
Androstenedione (nmol/L)	2.7 ± 1.4 (n=23)	4.0 ± 1.5 (n=24)**
TSH (mU/L)	2.3 ± 1.0 (n=27)	2.0 (0.8) (n=28)
Free T3 (pmol/L)	4.8 ± 0.7 (n=26)	4.8 ± 0.7 (n=24)
Free T4 (pmol/L)	11.2 ± 1.3 (n=26)	11.4 ± 2.2 (n=24)
eGFR (mL/min 1.73m^{-2})	97.4 ± 18.9 (n=28)	88.3 ± 10.1 (n=28)*
C Reactive Protein (mg L^{-1})	2.34 ± 2.34 (n=27)	2.77 ± 2.57 (n=28)
Albumin (g/L)	40.1 ± 2.9 (n=28)	40.6 ± 3.1 (n=28)
Serum[a]		
PFOS, (ng/mL)	3.1 (2.6-3.6)	3.9 (3.4-4.4)*
PFOA, (ng/mL)	2.4 (1.9-2.9)	2.4 (2.0-2.9)
PFHxS, (ng/mL)	0.9 (0.8-1.2)	1.1 (0.9-1.4)
PFNA, (ng/mL)	0.5 (0.4-0.6)	0.6 (0.5-0.7)
Follicular fluid[a]		
PFOS, (ng/mL)	1.8 (1.6-2.1)	2.2 (1.9-2.5)

PFOA, (ng/mL)	1.9 (1.6-2.3)	1.7 (1.4-2.1)
PFHxS, (ng/mL)	0.8 (0.6-1.0)	0.9 (0.7-1.2)
PFNA, (ng/mL)	0.4 (0.3-0.5)	0.4 (0.3-0.5)

*p<0.05, **p<0.01, ***p<0.001; [a] Geometric mean (95% CI)

Table 3: Associations of measured steroid hormone concentrations and related endpoints with PFAA concentrations, adjusted for serum albumin and PCOS case status. Bolded associations are significant at $p<0.05$.

	β (SE) *p value*				
	ln(Testosterone nmol/L)	ln(SHBG, nmol/L)	ln(FAI, unitless)	Androstenedione nmol/L	Oestradiol pmol/L
ln(PFOS, ng/ml)	0.21 (0.18)	**0.76 (0.30)**	-0.55 (0.32)	0.57 (0.61)	-22.63 (115.46)
	0.254	***0.015***	*0.090*	*0.356*	*0.845*
ln(PFOA, ng/ml)	**0.34 (0.14)**	0.36 (0.24)	-0.04 (0.31)	0.96 (0.52)	95.78 (150.78)
	0.021	*0.137*	*0.900*	*0.071*	*0.528*
ln(PFHxS, ng/ml)	0.25 (0.14)	0.29 (0.17)	-0.04 (0.25)	0.68 (0.44)	86.94 (122.72)
	0.083	*0.087*	*0.864*	*0.131*	*0.482*

ln(PFNA, ng/ml)	0.29 (0.17) *0.093*	0.38 (0.24) *0.124*	-0.09 (0.34) *0.795*	0.97 (0.57) *0.094*	85.15 (157.92) *0.592*
ln($\sum PFAA$, umol/L)	**0.41 (0.18)** ***0.024***	**0.61 (0.28)** ***0.035***	-0.21 (0.36) *0.572*	1.11 (0.63) *0.084*	88.04 (163.56) *0.593*

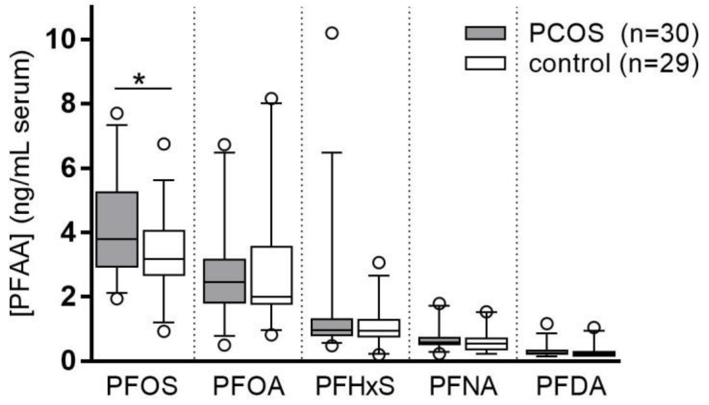

Figure 1. Plot of serum concentration (ng/mL, n=59) of PFAAs with detection frequencies >50%. Box indicates interquartile range, whiskers indicate 5th% and 95th %, horizontal line indicates median. Significantly different geometric means ($p<0.05$) denoted by asterisk.

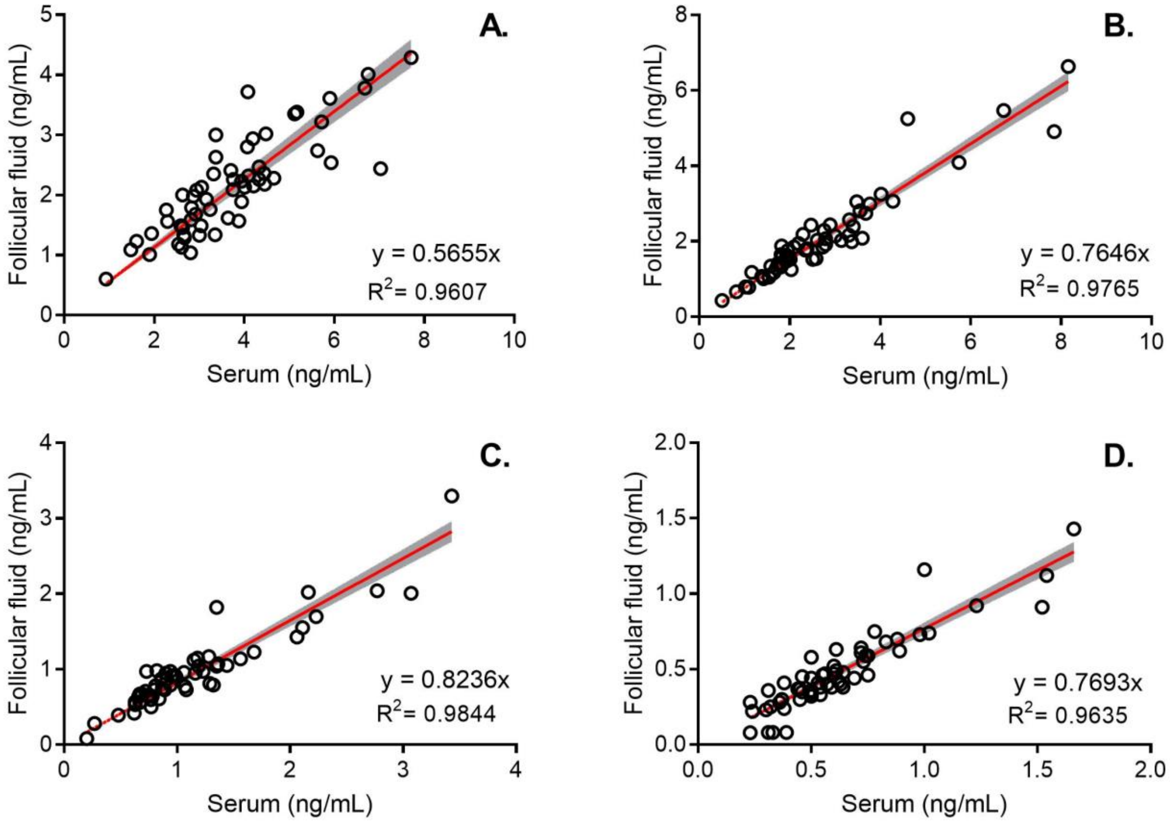

Figure 2. Correlation of PFOS (A), PFOA (B), PFHxS (C) and PFNA (D) in serum and follicular fluid. Shaded area represents 95% confidence interval of regression line forced through the origin (red); R^2 of weighted linear regression shown in bottom right corner.

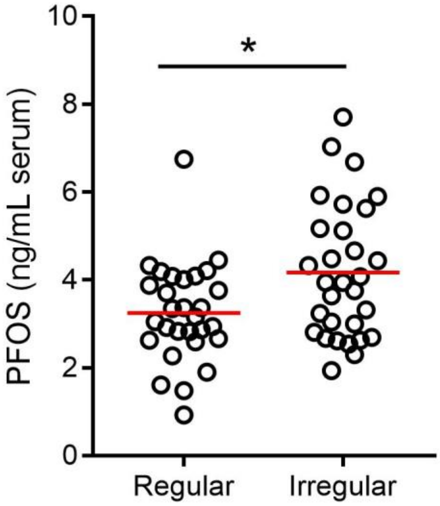

Figure 3: Perfluorooctane sulfonate (PFOS) concentration in women with regular versus irregular menstrual cycles ($p<0.05$ for difference of means). Horizontal line represents the geometric mean.