Maternal Exposure to perfluoroalkyl acids measured in whole blood and birth outcomes in offspring

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Abstract

Perfluoralkyl and polyfluoralkyl substances have been measured in plasma and serum of pregnant women as a measure of prenatal exposure. Increased concentrations of individual perfluoroalkyl acids (PFAAs), (typically perfluorooctanoic acid (PFOA) and perfluoroctane sulfonate (PFOS) have been reported to be associated with reductions in birth weight and other birth outcomes. We undertook a study of 14 PFAAs in whole blood (including PFOS, PFHxS, PFHpA, PFOA, PFNA, PFDA and PFUnDA) from 98 pregnant women in Western Australia from 2008 to 2011. Median concentrations (in µg/L) were: PFOS 1.99; PFHxS 0.33; PFOA 0.86; PFNA 0.30; PFDA 0.12 and PFUnDA 0.08. Infants born to women with the highest tertile of PFHxS exposure had an increased odds of being <95% of their optimal birth weight (OR 3.5, 95% CI 1.1-11.5). Conversely, maternal blood concentrations of PFUnDA were associated with non-significant increases in average birth weight (+102 g, 95% CI -41, 245) and significant increases in proportion of optimal birth weight (+4.7%, 95% CI 0.7, 8.8) per ln-unit change. This study has reported a range of PFAAs in the whole blood of pregnant women and suggests that PFHxS and PFUnDA may influence foetal growth and warrant further attention. Additional studies are required to identify the sources of PFAA exposure with a view to prevention, in addition to further studies investigating the long term health effects of these ubiquitous chemicals.

Key Words: PFAAs; whole blood; birth weight; maternal exposure; proportion of optimal birth weight; fetal growth

1. Introduction

Perfluoralkyl acids (PFAAs) have been widely used for many years as water repellants and protective coatings in industrial and domestic products, as a constituent in flooring materials, and in fire-fighting formulations, adhesives and electrical wire (ATSDR 2009; CDC 2009). Several types of PFAA, including perfluorooctanoic acid (PFOA) and perfluorooctane sulfonate (PFOS) have been phased out in a number of countries, nevertheless they persist in the environment and have been measured in a number of human exposure studies (Calafat et al., 2007; Kishi et al., 2015; Velez et al., 2015).

Concerns have been raised about the potential for health effects following PFAA exposure, and in particular, their role as endocrine disruptors and effects on foetal growth and development (Maisonet et al., 2012; Steenland et al., 2010; Velez et al., 2015). PFOA and PFOS (both containing eight carbons) are the most widely studied of the PFAAs, with fewer human exposure studies reporting concentrations of other PFAAs , despite evidence that exposure to longer chain compounds , such as the 9-carbon perfluorononanoic acid (PFNA) could be increasing (Kato et al., 2011). The exposure sources and health effects of exposure to other PFAAs are not as well understood.

PFAAs, especially PFOA and PFOS, have been measured in the blood of pregnant women as well as in breast milk, demonstrating the potential for prenatal and early life exposure (Gutzkow et al., 2012; Hamm et al., 2010; Kishi et al., 2015; Kubwabo et al., 2013; Maisonet et al., 2012). Concentrations of a number of PFAAs has been found to be correlated between maternal and cord blood, demonstrating the ability of these chemicals to cross the placenta (Gützkow et al.,

2012; Monroy et al., 2008). Inverse associations have been reported between maternal PFOS concentrations during pregnancy and birth weight (Maisonet et al., 2012; Washino et al., 2009), as well as increased risk of low birth weight (Stein et al., 2009). Similarly, decrements in birth weight, head circumference and ponderal index have been reported to be associated with cord blood PFOS concentrations (Apelberg et al., 2007; Chen et al., 2012). Maternal PFOA concentrations in pregnancy have been associated with decreased birth weight (Fei et al., 2007; Maisonet et al., 2012). Fewer studies have examined the potential for other PFAAs to have deleterious effects on foetal growth. Maternal perfluorohexanesulfonic acid (PFHxS) concentrations during pregnancy have been associated with reduced birth weight in girls (Maisonet et al., 2012), although other studies have reported no relationship (Arbuckle et al., 2013; Hamm et al. 2010).

The mechanisms by which prenatal PFAA exposure may influence foetal growth in humans are not well understood. However, alterations in lipid metabolism and thyroid hormone homeostasis have been observed in animal models (Seacat et al., 2003; Thibodeaux et al., 2003). PFAAs are structurally analogous to fatty acids and therefore it has been speculated that they may interfere in fatty acid metabolism (Hu et al., 2005), and consistent with this, an inverse relationship between PFOS exposure and polyunsaturated fatty acids in pregnant women has recently been reported (Kishi et al., 2015).

Typically, PFAAs are measured in blood plasma or serum samples (Knepper and Lange (Eds) 2012). The Australian Maternal Exposure to Toxic Substances (AMETS) study provided the opportunity to measure PFAAs in whole blood to investigate the presence and range of

concentrations in a sample of pregnant women. We were able to consider factors that may have influenced exposure to PFAAs in pregnant women, as well as the associations between maternal blood PFAA concentrations and birth outcomes.

2. Methods

Ninety eight whole blood samples were available for PFAA analysis. They were collected in the AMETS study, a cross-sectional study conducted between 2008 and 2011. This study recruited pregnant women aged greater than 18 years in Western Australia and the study design, recruitment methods and details of sample collection are described elsewhere (Reid et al., 2013). Whole blood samples were collected around 2 weeks prior to the due date of the participant during the third trimester of pregnancy (between June 2008 and April 2011). At this time a questionnaire regarding lifestyle and diet was also completed. Following the birth of their baby, participants were asked to complete a second questionnaire which was used to collect birth outcome data (Stasinska et al., 2014). Participants were requested to check the birth outcomes recorded for their child by the medical team at delivery when completing their questionnaire. This study received approval from the Edith Cowan University Human Research Ethics Committee. All participants provided written informed consent to participate.

2.1 Birth Outcome Variables

Birth outcomes included in the analysis were birth weight, birth length and head circumference.

Ponderal Index was also calculated using the equation:

113 Birth weight (g)/ (birth length (cm))^3 * 100

114 Proportion of optimal birth weight (POBW), proportion of optimal birth length (POBL) and
115 proportion of optimal head circumference (POHC) were calculated using the previously reported
116 equations (Blair et al., 2005). These calculations incorporate adjustments for gestational age,
117 maternal height, parity, sex of the infant (and also maternal age for proportion of optimal head
118 circumference only) and provide an indication of the appropriateness of foetal growth for a given
119 infant. They were calculated based on a large cohort of Caucasian singleton births in Western
120 Australia and were found to account for 40.5% of the variation in birth weight, 32.2% of the
121 variation in birth length and 25.2% of the variation in head circumference (Blair et al., 2005).
122 Due to missing data in the variables that contributed to the calculations of proportion of optimal
123 birth outcomes it was only possible to calculate POBW for 83 infants, POBL for 89 infants,
124 POHC for 82 infants.

125 **2.2 Chemical Analysis of Samples**

126 Mass labeled internal standards (1 ng) were added to 0.2 mL whole blood. The 14 PFAAs that
127 were targeted in the analysis were then extracted with 1.5 mL 100% acetonitrile using
128 ultrasonication followed by vortex extraction, centrifugation and evaporation to 0.2 mL under a
129 gentle stream of nitrogen. Performance standards and 0.3 mL of 5 mM ammonium acetate in
130 water were added prior to analysis. The samples were analyzed by HPLC-MS/MS using an
131 API5500Q massspectrometer (AB/Sciex, Concord, Ontario, Canada) equipped with an
132 electrospray (TurboV) interface coupled to a Shimadzu Nexera HPLC system (Shimadzu Corp.,
133 Kyoto, Japan) . Details of chemicals and standard compounds used in the extraction and the
134 instrumental set-up are available in the Supplementary material (S1).

2.3 Quality assurance

The limit of detection (LOD) was calculated using an average of the lowest calibration point (n = 3) and 3*signal to noise (S/N). In the case of blank contamination the LOD was calculated using the average blank concentration (n=5) plus three times the standard deviation. The reproducibility of the method was calculated from multiple analyses of a pooled whole blood sample on different days (n = 6) using the internal standard method and mass labeled standards. More details are provided in the supplementary material.

2.4 Method performance

Method validation parameters are summarized in Table S1 in the supplementary material. Average recoveries were calculated for ^{13}C-labelled PFAAs and ranged between 77% and 88% in all samples, and LODs ranged between 0.03 and 0.06 ng/mL whole blood for all detected PFAAs. The reproducibility of PFAA analysis, measured as the relative standard deviation (RSD) of multiple analysis (n=6) of a pooled whole blood sample on different days, was below 17% for all quantified analytes.

2.5 Data Handling and Statistical Analysis

Questionnaire and birth outcome data were stored in a Microsoft Access database. Descriptive statistics were generated using IBM SPSS, version 22 (2012, Armonk, NY: IBM Corp.). Biological concentrations below the Method Detection Limit (MDL) were assigned a value of half the respective MDL. Variables that were skewed were normalized using natural logarithm transformation. Pearson correlations were performed to identify correlations between

concentrations of each individual PFAA and personal and lifestyle characteristics, as well as birth outcome variables. Only singleton births were included in the analysis with one participant who gave birth to twins excluded from the analysis of birth outcomes.

Maternal PFAA concentrations were divided into tertiles of exposure and Kruskal Wallis analysis was undertaken to determine whether differences exist in birth outcome by PFAA tertile. Multiple logistic regression was performed using POBW <95% and above 95% as the binary outcome as POBW is expressed as a percentage and the range is small. Multiple linear regression analyses were performed to examine the relationship between maternal PFAA concentrations in whole blood and birth outcomes, with a univariate and adjusted models generated. The adjusted models included variables known to influence birth outcomes including gestational age, maternal pre-pregnancy BMI, maternal weight gain during pregnancy (%), maternal height and the sex of infant.

3. Results

This sample of pregnant women had an average age of 31.8 years with 60% having their first child (Table 1). Nearly half women lived in metropolitan Perth and the remainder in regional areas. Most participants were well educated, with two thirds of participants having completed tertiary education. The majority of participants were from homes with household incomes of >$80,000, which exceeds the Australian median value (Table 1).

PFOS, PHxS, PFOA, PFNA and perfluorodecanoic acid (PFDa) were detected in most of the whole blood samples analysed (Table 2) while perfluoroundecanoic acid (PFUnDA) concentrations were below the limit of detection in 31% of samples. Perfluorobutanoic acid

(PFBA), perfluoropentanoic acid (PFPeA), perfluorohexanoic acid (PFHxA), perfluorododecanoic acid (PFDoDA), perfluorotridecanoic acid (PFTrDA), perfluorobutanesulfonate (PFBuS), and perfluorodecane sulfonate (PFDS) were below LOD in all samples. PFHpA was detected in only 3 of the samples and was excluded from subsequent statistical analyses (Table 2). Concentrations of all individual PFAAs were correlated with one another, with the strongest Pearson correlation coefficients between PFOA, PFNA and PFDA (Supplementary Data Table S3). Although mean concentrations of PFAAs decreased by year of sample collection, these differences were not significant (Supplementary Data Table S4).

3.1 Dietary and Lifestyle Factors associated with PFAA exposure

The women who were having their first child had higher blood concentrations of PFOS, PFHxS, PFOA, PFNA and PFUnDA than those who were not (Table 3). Pearson correlations were also performed to examine the relationships between PFAA concentrations and maternal characteristics (Supplementary Table S5). Whole blood concentrations of PFNA and PFDA were positively correlated with maternal age (Supplementary Table S5).

Maternal concentrations of PFHxS, PFOA and PFNA were higher in those who reported having a gross annual household income of <$80,000 ($z = 1.956$, $p = 0.05$; $z = 2.46$, $p = 0.015$ and $z = 2.29$, $p = 0.022$, respectively). There were no differences in PFAA concentrations by highest level of maternal education or place of residence (urban vs. rural) in this population.

Maternal PFUnDA concentrations were higher in those who reported ever eating canned fish during pregnancy (mean 0.093 vs 0.071 µg/L, Mann Whitney U 1335, $z = 3.14$, $p = 0.002$) and

also increased with a reported increase in the number of portions oily fish consumed per month (mean concentrations 0.071, 0.091, 0.095 and 0.106 µg/L for 0, 1-2, >2-4 and >4 portions per month, respectively, Kruskal Wallis p= 0.018).

3.2 Maternal PFAA concentrations and birth outcomes

Concentrations of PFAAs in blood were categorized into tertiles. Both mean birth weight and POBW declined with increasing maternal blood concentrations of PFOS, PFHxS, and PFOA concentrations, with the trend most evident in female infants (Table 4). Conversely birth weight and POBW increased with increasing tertiles of PFDA and PFUnDA concentrations, again with the trend most evident in females (Table 4). No statistically significant differences in birth weight or POBW were seen by tertile of PFAA concentration. Maternal concentrations of PFUnDA were significantly positively associated with proportion of optimal birth weight by Pearson correlation (r=0.258, p= 0.019) (Supplementary Table S4).

By logistic regression it was identified that babies born to mothers with the highest tertile of blood PFHxS concentration had an increased odds of being <95% of their calculated optimal birth weight (OR 3.5, 95% CI 1.1-11.5) (Table 5).

In both univariate and adjusted multiple linear regression models a non-significant negative association was observed between maternal concentrations of PFOS, PFOA and PFHxS and infant birth weight. In the adjusted model, a ln-unit increase in maternal blood concentration (equivalent to a ~2.7 fold increase) was associated with a decrease in mean birth weight of 69 g (95% CI -231, 94), 48 g (-203, 108) and 103 g (-221, 15) for PFOS, PFOA and PFHxS,

respectively. When Proportion of Optimal Birth Weight was used as the outcome measure, the negative association between PFOS and PFOA concentrations was attenuated, however a non-significant negative association remained for PFHxS with a ln-unit increase in maternal blood concentrations associated with a 2% decrease in the Proportion of Optimal Birth weight (95% CI -5.4, 1.3). PFOS, PFOA and PFHxS were all associated with decreased head circumference, POHC and ponderal index in univariate and adjusted models, but these relationships were not significant in this study population. Maternal PFUnDA concentrations were associated with increased birth weight, birth length, POBW and POBL, although only the relationship between PFUnDA and POBW was statistically significant with an average 5.3% increase in POBW (95% CI 1.2, 9.3) associated with a ln-unit increase in maternal concentrations.

An adjusted combined pollutant model was generated to reflect the fact that exposure to individual PFAAs is not occurring in isolation and that simultaneous exposure to chemicals with potentially opposing effects is occurring. In the adjusted combined pollutant model the change in average birth weight per change in ln-unit PFHxS and PFUnDA concentrations was more pronounced than in the models for the respectively single analytes (Table 7). The model, which included gestational age, maternal height, prepregnancy BMI, weight gain during pregnancy (%) and sex of infant, PFHxS and PFUnDA explained 22% of the variability in birth weight, noting the large confidence intervals. When proportion of optimal birth weight was used in place of birth weight as the outcome variable in the combined pollutant model, the estimated change in outcome per ln-unit increase in PFAA concentration was again more pronounced than in the single pollutant models, although only PFUnDA was significant in the final combined model (Table 7).Due to the proportion of participants with PFUnDA concentrations below the limit of

detection the combined pollutant regression model was also re-run with only participants with detectable PFUnDA concentrations included. Removal of participants with PFUnDA below the limit of detection strengthened the relationships between PFUnDA and PFHxS and POBW in the model.

4. Discussion

The concentrations of PFAAs measured in whole blood of pregnant women were low in this study, with concentrations considerably lower than those reported in the plasma and sera (Kannan et al., 2004) and whole blood (Kärrman et al., 2006) of adult women. Few studies have measured PFAA concentrations in whole blood, however where this has occurred multiplication of the resulting concentrations by 2 to account for the dilution of whole blood, has been reported as an acceptable means of obtaining an estimate of serum concentrations (Kannan et al., 2004). However other studies have suggested that given the differing ratios of individual PFAAs between whole blood and plasma, whole blood may actually be the most appropriate matrix in which to assess PFAA exposure (Kärrman et al., 2006). When the concentrations of PFOS and PFOA in maternal blood samples in this study were compared specifically with those of pregnant women reported elsewhere, concentrations in this study were lower than those reported by Inoue et al. 2004 (Japan), Fei et al., 2007 (Denmark) and Maisonet et al., 2012 (United Kingdom) but comparable with those in Washino et al., 2009 and Kishi 2015 (both conducted in Japan). Maisonet et al., (2012) also reported blood concentrations of PFHxS in pregnant women that were higher than those in this study. Toms et al., 2014 reported that PFOS and PFOA in serum have decreased in Australia from concentrations measured in the year 2002/2003. However few studies have reported concentrations of PFHxS, PFNA, PFDA and PFUnDA in pregnant women so it is difficult to make comparisons.

In this study most of the PFAAs measured were significantly higher in primiparous women. Other researchers have also observed the same effect with maternal plasma concentrations of PFOS and PFOA (Fei et al., 2007; Kishi et al., 2015), however few studies have investigated the relationship between parity and blood concentrations of other PFAAs. Given the small sample size in this study it was not possible to distinguish between the effects of parity and previous breast feeding on PFAA concentrations in this study, as of the women who had given birth previously 74% reported having breast fed. Diet is an important source of PFAA exposure, with seafood intake known to be a major contributor to serum concentrations (Haug et al., 2010). Consistent with this we found increasing blood concentrations of PFUnDA in pregnant women with increasing reported portions of oily fish consumption and also in those who consumed canned fish. Despite the relatively homogenous nature of the study population, in terms of the high average level of education and high household annual incomes (≥$80,000) in the participants, differences in maternal concentrations of several PFAAs by household income were observed, with higher concentrations of PFHxS, PFOA and PFNA in those with a reported household income <$80,000.

Despite these low PFAA concentrations and relatively small sample size, associations were observed between increased PFHxS and decreased birth weight and increased PFUnDA and both increased birth weight and POBW in regression models, noting the wide confidence intervals. Whilst negative associations were observed between PFOS and PFOA and birth weight, head circumference, POBW, POHC and ponderal index, these were not statistically significant in this study. There are several potential reasons why significant negative association between PFOS

and PFOA and birth outcomes were not detected in this study, including the small sample size of this study and also the low concentrations of these PFAAs measured in this population compared with international studies. In addition it is important to note that the quantification of total PFOS and PFOA in blood samples includes both linear and branched isomers of these analytes (Berger et al. 2011; Beeson et al., 2011). The differences in the proportions of these isomers in individuals, the partitioning between mother and foetus and the potential for differing physiological responses, are not well understood and this may contribute to the discrepancies in the strength of the associations between PFOS and PFOA and birth outcomes reported in studies.

In the previous studies that have stratified their analysis on the basis of infant sex, stronger associations between prenatal PFOS exposure and reduced foetal growth were observed for girls (Kishi et al., 2015; Washino et al., 2009). In this study, although by descriptive analyses the change in birth weight and POBW by tertile of PFAA concentrations appeared more pronounced in female infants, the small sample size precluded a detailed analysis stratified by sex of infant.

The potential for these results to have impacts on children later in life need to be considered. Maisonet et al. (2012) reported decreases in birth weight with higher prenatal PFAA concentrations, but at 20 months an increase in weight compared with those with lower PFAA exposure, perhaps as a result of accelerated growth to compensate for foetal growth restriction. Furthermore prenatal exposure to PFOA has been related to increased risk of obesity in women at the age of 20 years (Halldorsson et al., 2012) suggesting that the impacts of prenatal exposure to PFAAs may have longer term consequences. Prenatal exposure to PFOA has also been found to be associated with delayed age of menarche in girls (Kristensen et al., 2013; Lopez-Espinosa

et al., 2011) and impaired semen quality and reproductive hormone levels in adult men (Vested et al., 2013).

PFAA concentrations in maternal blood decrease towards the end of pregnancy (Kishi et al., 2015), therefore the concentrations reported here (collected late in the 3rd trimester) may not be representative of the concentrations earlier in pregnancy, during critical developmental time points. Even at low maternal PFAA concentrations, associations were seen between increased PFHxS in maternal blood and decreased foetal growth and also conversely between increased PFUnDA concentrations and increased foetal growth. PFUnDA concentrations were below the limit of detection in 31% of samples and at very low concentrations for the rest of the study population, which means that the results of this study need to be interpreted with caution. When the analysis was restricted to only participants with detectable PFUnDA concentrations, PFUnDA remained associated with increased POBW in the combined pollutant model, suggesting that this relationship is valid. Some PFAAs have been demonstrated to have the potential to result in impacts on lipid metabolism in animal models (Thibodeaux et a., 2003; Seacat et al., 2003) and this is likely to be a mechanism contributing to the relationships between PFOA and PFOS and foetal growth reported in some studies. However the mechanism by which PFHxS and PFUnDA could have opposing impacts on foetal growth remain to be determined. Indeed the relationship between a longer chain PFAAs and increased foetal growth needs confirmation in other cohorts.

This study was limited by the small sample size, which reduced the power of the study and also by the measurement of PFAA concentrations in whole blood, which reduced the ability to

compare these results with previous studies. In addition, it was not possible to address the potential confounding by glomerular filtration rates, which are altered during pregnancy and may influence both the blood concentrations of PFAAs and also foetal growth (Verner et al., 2015). Failure to adjust for kidney function may lead to an overestimation of the relationship between PFAA exposure and reduced foetal growth, with physiologically based pharmacokinetic modeling suggesting that confounding of this nature may have more of an impact in studies with samples collected late in pregnancy (Verner et al., 2015), as was the case in our study. However, one of the strengths of this study is the reporting of a range of PFAAs in maternal blood, many of which have rarely been measured in pregnant women and could represent emerging exposures of concern, as some studies suggest the exposure to longer chain PFAAs may be increasing (Kato et al., 2011).In addition this study incorporated a wider variety of birth outcomes than is usually employed in studies of this type. The calculation of POBW, POBL and POHC as an attempt to assess the appropriateness of foetal growth for an individual, rather than relying on birth weight alone represents another strength of this work.

5. Conclusions

Despite the low concentrations of PFAAs measured in maternal whole blood, relationships were observed between concentrations and birth weight. Given the ubiquitous nature of exposure to low levels of these compounds and the potential long term impacts associated with inappropriate foetal growth, this study adds further evidence to support the notion that PFAAs represent a public health concern and suggests that exposure to PFAAs warrants further study. Specifically,

363 the potential for maternal exposure to PFHxS and PFUnDA to affect birth outcomes needs to be
364 confirmed in a larger study and future studies need to control for glomerular filtration rates.

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Table 1 Demographic, maternal and infant characteristics of study population (n=98, unless otherwise specified)

Maternal Characteristics	Mean ± SD or n (%)
Age (years)	31.8 ± 4.6
range	19 – 44
First Pregnancy	n=57 (60.0%)
Breastfed Other children	n= 28 (28.6%)*
Mean Reported pre-pregnancy weight for population (Kg)	69.4 ± 13.5
Pregnancy BMI	
<18.5	n =5 (5.3%)
18.5 – 24.9	n=51 (54.3%)
25.0 – 29.9	n=18 (19.1%)
≥30	n=20 (21.3%)
Region of residence	
Perth Metropolitan	n=46 (46.9%)
Great Southern	n=7 (7.1%)
South West	n=19 (19.4%)

Goldfields Esperance	n=15 (15.3%)
Other	n=11 (11.2%)
Urban or rural residence	
Urban	n=66 (72.5%)
Rural	n=25 (27.5%)
Education Level	
Secondary Education	n=5 (5.4%)
Trade/Diploma	n=25 (26.9%)
Tertiary	n=63 (67.7%)
Gross Annual Household Income	
<80,000AUD	n=25 (27.5%)
>80,000AUD	n=66 (72.5%)
Average reported Oily Fish consumption (portions /month)	
0	n=36 (44.4%)
1-2	n= 20 (24.6%)
>2 – 4	n=12 (14.8%)
>4	n=13 (16%)
Infant characteristics	
Sex	

Female Male	n=56 (59.6%) n=38 (40.4%)
Gestational age (weeks) Range	39.7 ± 1.1 35 - 42
Birth weight (g)	3561 ± 467
Length (cm)	51.0 ± 2.3
Head circumference (cm)	35.0 ± 1.6
Proportion of Optimal Birth weight (POBW) (%)	101 ± 12
Proportion of Optimal Birth Length (POBL) (%)	100 ± 4
Proportion of Optimal Head Circumference (POHC) (%)	100 ± 4

*74% of women who had been pregnant previously also reported having breast fed.

Missing data:

Maternal characteristics:

Age n=5, Maternal height, weight, BMI and weight gain, n=4, first pregnancy n=3, breast fed previously n=5, household income n=7, place of residence n=7, highest level of education n=5.

Birth Outcomes:

Sex of infant n=4, gestational age n=9, birth weight and length, n=4, head circumference n=5, ponderal index n= 4, POBW, n= 15, POBL n = 9, POHC n=16.

Table 2 Mean, median and range of whole blood PFAA concentrations (µg/L) (n=98).

PFAA Type	Mean ± SD	Median	Min-Max	% Below LOD*
PFOS	2.32 ± 1.42	1.99	0.45-8.1	0
PFHxS	0.47 ± 0.44	0.33	0.06-3.3	0
PFHpA	<0.06 ± 0.02	<0.06	<0.06-0.12	97
PFOA	1.00 ± 0.60	0.86	0.21-3.1	0
PFNA	0.30 ± 0.18	0.30	0.05-1.3	0
PFDA	0.15 ± 0.08	0.12	<0.05-0.39	4
PFUnDA	0.09 ± 0.06	0.08	<0.06-0.36	31

*LOD Limit of Detection

Table 3. Median and range of PFAA concentrations (µg/L) by parity

Primiparous	PFOS	PFOA	PFHxS	PFNA	PFDA	PFUnDA
Yes (n=57)						
Median	2.2	1.1	0.41	0.28	0.14	0.08
range	0.55-8.1	0.22-3.1	0.06-1.3	0.05-1.3	<0.05-0.39	<0.06-0.34
No (n=38)						
Median	1.6	0.62	0.28	0.21	0.12	0.06
Range	0.45-7.3	0.21-1.6	0.07-3.3	0.08-0.49	<0.05-0.28	<0.06-0.18
Mann Whitney U	686	570	769	766	853	755
z	-3.02	-3.90	-2.39	-2.41	-1.75	-2.53
p value	**0.003**	**<0.001**	**0.017**	**0.016**	0.080	**0.011**

Table 4: Mean Birth weight (g) and Proportion of Optimal Birth weight (%) by tertile of maternal PFAA concentration µg/L*

		Birth weight (g)						Proportion of Optimal Birth weight (%)					
		Total		Male		Female		Total		Male		Female	
	µg/L	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
PFOS	<1.60	3606	±431	3613	±348	3599	±508	101	±11	99	±8	104	±13
	≥1.60 - <2.53	3591	±452	3685	±406	3530	±479	102	±13	101	±12	103	±14
	≥2.53	3481	±522	3577	±551	3433	±515	101	±12	106	±12	98	±12
PFOA	<0.63	3621	±465	3595	±376	3645	±547	102	±12	99	±9	104	±13
	≥0.63 – <1.16	3586	±500	3754	±447	3509	±513	102	±14	104	±16	101	±14
	≥ 1.16	3474	±436	3570	±454	3406	±421	101	±10	103	±8	99	±12
PFHxS	<0.271	3670	±425	3673	±287	3668	±533	103	±11	101	±8	106	±13
	≥0.271 - <0.490	3579	±477	3587	±548	3575	±449	103	±12	102	±12	103	±12
	≥0.490	3431	±481	3610	±454	3319	±474	98	±13	101	±12	96	±13

PFNA	<0.21	3562	±439	3594	±366	3537	±497	101	±11	98	±9	103	±13
	≥0.21- <0.33	3567	±428	3714	±382	3486	±439	101	±12	104	±12	99	±12
	≥0.33	3553	±543	3593	±513	3524	±576	102	±13	103	±12	102	±15
PFDA	<0.105	3528	±451	3671	±432	3419	±446	101	±11	101	±12	101	±11
	≥0.105 -<0.155	3502	±499	3458	±381	3521	±551	100	±13	99	±9	100	±15
	≥0.155	3650	±458	3705	±425	3598	±495	104	±12	103	±10	104	±13
PFUnDA	<0.060	3530	±476	3589	±408	3459	±555	99	±12	98	±9	100	±16
	≥0.060- <0.098	3510	±441	3823	±321	3374	±421	100	±12	105	±11	98	±11
	≥0.098	3647	±489	3512	±483	3726	±488	106	±11	103	±11	107	±11

*Differences between tertiles analysed by Kruskal Wallis tests, no statistically significant differences identified.

Table 5 Logistic regression showing odds of infant being <95% POBW by tertile of maternal PFHxS concentration

	µg/L	Exp (B)	95% CI for Exp (B)	
			Lower	Upper
PFHxS Tertile 1	<0.271	1.000		
PFHxS Tertile 2	≥0.271 - <0.490	1.065	0.308	3.683
PFHxS Tertile 3	≥0.490	3.500	1.066	11.495

Table 6 Estimated change in birth outcome with change in maternal PFAA concentrations equal to one ln-unit

Model	**PFOS** β (95% CI)	**PFOA** β (95% CI)	**PFHxS** β (95% CI)	**PFNA** β (95% CI)	**PFDA** β (95% CI)	**PFUnDA** β (95% CI)
Birth weight (g)						
Univariate	-58 (-225, 109)	-59 (-216, 98)	-111 (-230, 7)	28 (-155, 211)	54 (-116, 224)	100 (-46, 246)
Adjusted [a]	-69 (-231, 94)	-48 (-203, 108)	-103 (-221, 15)	14 (-169, 196)	4 (-161, 170)	102 (-41, 245)
Birth length (cm)						
Univariate	-0.33 (-1.1, 0.47)	-0.17 (-0.93, 0.59)	-0.36 (-0.94, 0.22)	0.08 (-0.80, 0.97)	0.45 (-0.37, 1.23)	0.31 (-0.40, 1.01)
Adjusted [a]	-0.22 (-1.0, 0.57)	0.06 (-0.70, 0.81)	-0.20 (-0.78, 0.38)	0.20 (-0.68, 1.09)	0.36 (-0.44, 1.15)	0.32 (-0.37, 1.02)
Head circumference (cm)						
Univariate	-0.34 (-0.90, 0.22)	-0.28 (-0.81, 0.24)	-0.24 (-0.64, 0.16)	-0.05 (-0.66, 0.56)	0.04 (-0.53, 0.61)	-0.35 (-0.84, 0.14)
Adjusted [a]	-0.39 (-0.98, 0.20)	-0.40 (-0.96, 0.16)	-0.31 (-0.74, 0.12)	-0.14 (-0.80, 0.52)	-0.07 (-0.67, 0.53)	-0.29 (-0.81, 0.24)
Ponderal Index (g/cm^3 x 100)						
Univariate	-0.004 (-0.11, 0.10)	-0.03 (-0.13, 0.07)	-0.03 (-0.11, 0.04)	-0.003 (-0.12, 0.11)	-0.04 (-0.15, 0.07)	0.02 (-0.08, 0.11)
Adjusted [a]	-0.03 (-0.14, 0.08)	-0.06 (-0.16, 0.05)	-0.05 (-0.13, 0.03)	-0.03 (-0.16, 0.09)	-0.06 (-0.18, 0.05)	0.01 (-0.09, 0.11)
POBW (%)						
Univariate	-0.18 (-4.7, 4.3)	0.44 (-3.8, 4.7)	-2.1 (-5.4, 1.3)	2.2 (-2.8, 7.6)	1.9 (-2.7, 6.5)	4.8 (0.8, 8.7)*
Adjusted [b]	0.48 (-4.2, 5.2)	0.83 (-3.6, 5.3)	-1.7 (-5.2, 1.8)	2.1 (-3.3, 7.4)	1.9 (-2.9, 6.7)	5.3 (1.2, 9.3)*
POBL (%)						
Univariate	-0.37 (1-1.9, 1.1)	0.08 (-1.4, 1.5)	-0.58 (-1.7, 0.5)	0.39 (-1.3, 2.1)	0.82 (-0.7, 2.4)	0.99 (-0.41, 2.3)
Adjusted [b]	-0.12 (-1.4, 1.7)	0.42 (-1.0, 1.9)	-0.12 (-1.28, 1.0)	0.50 (-1.3, 2.2)	0.85 (-0.7, 2.4)	1.1 (-0.22, 2.5)
POHC (%)						
Univariate	-0.47 (-2.1, 1.2)	-0.54 (-2.1, 1.0)	-0.39 (-1.6, 0.8)	0.06 (-1.8, 1.9)	0.11 (-1.6, 1.8)	-0.56 (-2.0, 0.93)

Adjusted [c]	-0.60 (-2.3, 1.1)	-0.66 (-2.3, 1.0)	-0.53 (-1.8, 0.8)	-0.06 (-2.0, 1.9)	0.08 (-1.7, 1.8)	-0.47 (-2.0, 1.1)

Change in end point per ln-unit presented, equivalent to a 2.7 fold increase in PFAA concentrations. *Statistically significant (p <0.05)

a. Models adjusted for gestational age, maternal height, prepregnancy BMI, weight gain during pregnancy (%) and sex of infant.
b. Models adjusted for weight gain during pregnancy (%), maternal age and annual household income (<$80,000 vs ≥$80,000). POBW and POBL are calculated using gestational age, sex of infant, parity, maternal height.
c. Models adjusted for weight gain during pregnancy (%) and annual household income (<$80,000 vs ≥$80,000). POHC is calculated using gestational age, sex of infant, parity, maternal height and maternal age.

Table 7 Combined adjusted multiple linear regression models showing estimated change in birth outcome with a one ln-unit change in concentrations of PFHxS and PFUnDa

			Beta	95% CI		p	Adj R^2
	Model			Lower	Upper		
Birth Weight (g)[a]	1 (n=89)	PFHxS	-99.5	-221	22	0.107	0.22
		PFUnDA	164	19.4	308	0.027	
Proportion of Optimal Birth weight (%)[b]	1 (n=83)	PFHxS	-2.6	-6.0	0.8	0.134	0.06
		PFUnDA	5.9	1.8	10.0	0.006	
	2 (n =57)	PFHxS	-4.3	-8.6	0.07	0.054	0.11
		PFUnDA	8.0	0.5	15.5	0.037	

Change in end point per ln-unit presented, equivalent to a 2.7 fold increase in PFAA concentrations.

a. Model adjusted for gestational age, maternal height, prepregnancy BMI, weight gain during pregnancy (%), maternal age, annual household income (<$80,000 vs. ≥$80,000) and sex of infant. n=89 due to missing values for gestational age.

b. Models adjusted for weight gain during pregnancy (%), maternal age and annual household income (<$80,000 vs. ≥$80,000). Model 1: Final model included 83 infants due to missing data in variables used to calculate POBW (gestational age, parity, maternal; height and sex of infant). Model 2: As for model 1 but restricted to participants with PFUnDA concentrations >LOD (n=57).

Supplementary material

Chemical Analysis: Chemicals and Extraction

Ammonium acetate (>97%) was purchased from Chem-Supply (Gillman, SA, Australia), and methanol (99.8%, LiChrosolv®) and acetonitrile (99.9%, LiChrosolv®) from Merck (Darmstadt, Germany). All water used was laboratory produced ultra pure water. A native standard mix containing native PFAAs (perfluorobutanoic acid (PFBA), perfluoropentanoic acid (PFPeA), perfluorohexanoic acid (PFHxA), perfluoroheptanoic acid (PFHpA), perfluorooctanoic acid (PFOA), perfluorononanoic acid (PFNA), perfluorodecanoic acid (PFDA), perfluoroundecanoic acid (PFUnDA), perfluorododecanoic acid (PFDoDA), perfluorotridecanoic acid (PFTrDA), perfluorobutanesulfonate (PFBuS), perfluorohexane sulfonate (PFHxS), perfluorooctane sulfonate (PFOS), Perfluorodecane sulfonate (PFDS) and a mass labelled internal standard mix ($^{13}C_4$-PFBA, $^{13}C_2$-PFHxA, $^{13}C_4$-PFOA, $^{13}C_5$-PFNA, $^{13}C_2$-PFDA, $^{13}C_2$-PFUnDA, $^{13}C_2$-PFDoDA, $^{18}O_2$-PFHxS, and $^{13}C_4$-PFOS) were from Wellington Laboratories (Guelph, Ontario, Canada). An aliquot of 200 µL whole blood was transferred to a 2 mL Eppendorf tube using a Gilson's MICROMAN® pipette followed by addition of the internal standards. Acetonitrile was used to precipitate the proteins and the extraction was facilitated by ultrasonication and vortex mixing. After centrifugation, the supernatant was filtrated into a LC vial through a 2 µm GHP membrane (Pall, East Hills, NY, USA) and reduced to 200 µL using nitrogen, after which 300 µL 5 mM ammonium acetate in water and the performance standards $^{13}C_8$-PFOS and $^{13}C_8$-PFOA were added (Wellington Laboratories).

LC/ESI-MS/MS Analysis. Chromatographic separation of the analytes was carried out using a Nexera HPLC system (Shimadzu Corp., Kyoto, Japan) and a 4 micron 50x2.0mm Phenomenex C18 Gemini column (Phenomenex, Torrance, CA). The injection volume was 5

µL. The column unit was held at 45 °C and the flow rate was 0.3 mL/min. An isolator column (Phenomenex) was placed directly after the mobile phase mixing chamber to delay the elution of solvent derived background. Mobile phases A and B were, respectively, 1% methanol in water and 5% water in methanol with 5mM ammonium acetate in both phases. The initial gradient (25% B) was held for 0.10 min. A gradient ramp followed over 9 min to 100% B, which was held for 2 min, then returned to the initial composition in 0.10 min, followed by equilibrium for 4 min. The HPLC was coupled to API5500Q mass spectrometer (AB/Sciex, Concord, Ontario, Canada) with electrospray ionization (ESI) interface working in negative ionization mode, and the MS was operated in MRM acquisition mode. The data acquisition and processing was carried out using Analyst® TF 1.6 and MultiQuantTM software (AB Sciex).

Quality Assurance and Quality control (QA/QC). Method validation parameters are summarized in table S1. A calibration range between 0.1-50 ng/mL whole blood was used to establish linearity and was performed with non-extracted standards dissolved in 40% methanol in aqueous 5 mM ammonium acetate (0.5mL final volume). The signal to noise ratio (S/N) was calculated in samples with levels close to the LOD and samples with a S/N <3 were reported as non-detects. Quantification was performed using the internal standard method. PFHxS, PFOS, PFHxA, PFOA, PFNA, PFDA, PFUnDA and PFDoDA were quantified against labelled analogues. PFBuS was calculated against $^{18}O_2$-PFHxS, PFPeA and PFHpA against $^{13}C_2$-PFHxA, PFDS against $^{13}C_4$-PFOS and PFTrDA against $^{13}C_2$-PFDoDA. Ultra pure water was used as a procedural blank and was prepared for each batch of approximately 20 samples and extracted in the same way as the real samples. Reproducibility was calculated as the relative standard deviation, RSD, of analyses of a pooled whole blood sample extracted on different days (n=6) and was found to be between 7% and 17% for all quantified analytes. The percent

recovery (%R) of the ^{13}C-labelled internal standards was measured in the sample extracts using the formula: $\%R = ((A_{is}/A_{ps})_{sample}/(A_{is}/A_{ps})_{standard})*100$, where A_{is} is the area of the labelled internal standard and A_{ps} is the area of the performance standard. For the evaluation of matrix effects we used a pooled whole blood sample in spiking experiments (the same sample that was used to evaluate method reproducibility). Matrix effects of 15-20% is generally thought of as acceptable. The whole blood was spiked at three different levels: 0.5 ng/mL, 5 ng/mL and 20 ng/mL in triplicate samples. Due to that the sample already contained all PFAAs assessed in this study (except PFHpA) at levels above 0.05 ng/mL (≥10% of the spiking level) matrix effects were difficult to evaluate at the lowest spiking level for most of the PFAAs (Table S2).

Table S1. Summary of method validation parameters. Values are given in ng/mL whole blood.

QA/QC parameters	PFBA	PFPeA	PFHxA	PFBS	PFHXS	PFOS[1]	PFDS	PFHpA	PFOA	PFNA	PFDA	PFUnDA	PFDoDA	PFTrDA
MRM transitions[2]	213> **169**	263> **219**	313>**269**/ 119	299> **80**/99	399> **80**/99, 119	499>8 0/**99**	599> **80**/99	363> **319**/169	413>**369** /169	463>**419** /169	513>**469** /269	563>**519** /269	613>**569**/ 169	663>**619**/ 169
MRM transitions ^{13C}-labelled PFAAs	217> 172	N/A	315>270	N/A	403> 103	503>8 0	N/A	N/A	417>372 421>372	468>423	515>470	565>520	615>570	N/A
Linearity, r^2	0.999	0.999	0.999	0.999	0.999	0.999	0.999	0.999	0.999	0.999	0.999	0.999	0.999	0.999
LOD[3]	0.15	0.11	0.05	0.02	0.03[4]	0.03	0.03	0.03	0.05	0.05	0.05	0.06	0.05	0.06
Reproducibility (n=6), RSD (%)	<LOD	<LOD	<LOD	<LOD	9	9	<LOD	<LOD	11	7	14	17	<LOD	<LOD
Average recovery rates of ^{13}C-labelled PFAAs (%) (min, max)	NC	N/A	82 (60, 100)	N/A	82 (57-98)	80 (59-92)	N/A	N/A	88 (62-109)	77 (55-90)	84 (62-99)	88 (64, 102)	NC	N/A

N/A = Not available

NC = Not calculated

[1] Total PFOS (the sum of all structural isomers) was calculated against linear PFOS.

[2] Transition used for quantification is shown in bold.

[3] Limit of detection calculated from an average of the lowest calibration point (n=3) and 3*signal to noise (S/N).

[4] Limit of detection calculated from the average blank concentration (n=5) plus three times the standard deviation.

Table S2. Recovery results of matrix spike experiments of a pooled human whole blood sample (sample 0) at three different concentration levels.

Sample ID	Spiked level (ng/mL)	PFHpA	PFOA	PFNA	PFDA	PFUnDA	PFHxS	PFOS
		Concentration (ng/mL)						
0	-	0.05	1.1	0.38	0.22	0.08	0.33	2.2
		Recovery (%)						
1	0.5	107	299	168	140	114	171	416
2	0.5	116	310	184	153	121	184	431

3	0.5	112	278	162	143	116	168	393
4	5	105	111	108	107	111	109	136
5	5	108	117	107	107	111	110	130
6	5	112	110	117	107	110	100	131
7	20	106	97	106	104	106	94	108
8	20	113	100	111	109	108	107	108
9	20	106	101	110	101	101	104	108

Table S3: Correlations (Pearsons) between ln transformed concentrations of individual PFAAs

	PFOS	PFHxS	PFOA	PFNA	PFDA
PFOS					
PFHxS	.747**				
PFOA	.748**	.712**			
PFNA	.690**	.506**	.838**		
PFDA	.660**	.443**	.791**	.871**	
PFUnDA	.506**	.204*	.442**	.516**	.535**

Supplementary Table S4: Mean PFAA concentrations in maternal blood (in µg/L) by year of sample collection (n=98)

Year of Sample Collection		**PFOS**	**PFHxS**	**PFOA**	**PFNA**	**PFDA**	**PFUnDA**
2008	**N**	3	3	3	3	3	3
	Mean	3.09	0.43	1.86	0.61	0.27	0.11
	SD	1.46	0.28	1.15	0.33	0.14	0.02
	Minimum	1.79	0.23	0.76	0.24	0.12	0.08
	Maximum	4.67	0.75	3.06	0.89	0.39	0.12
2009	**N**	13	13	13	13	13	13
	Mean	2.83	0.43	1.09	0.31	0.16	0.10
	SD	2.03	0.30	0.65	0.16	0.08	0.05
	Minimum	0.55	0.10	0.22	0.05	0.03	0.03
	Maximum	8.05	1.00	2.38	0.61	0.28	0.21
2010	**N**	59	59	59	59	59	59
	Mean	2.38	0.52	1.02	0.31	0.15	0.09
	SD	1.41	0.52	0.59	0.19	0.08	0.07
	Minimum	0.67	0.07	0.24	0.08	0.03	0.03
	Maximum	7.31	3.29	2.64	1.31	0.38	0.36
2011	**N**	23	23	23	23	23	23
	Mean	1.79	0.38	0.78	0.24	0.12	0.06
	SD	0.84	0.26	0.43	0.09	0.06	0.03
	Minimum	0.45	0.06	0.21	0.08	0.03	0.03
	Maximum	3.75	1.21	1.84	0.51	0.28	0.13
Kruskal Wallis	***p***	.193	.870	.129	.103	.100	.161

Table S5: Pearson correlation coefficients for the relationship between maternal characteristics, parity and birth outcomes for each PFAA (ln transformed)

	PFOS	PFHxS	PFOA	PFNA	PFDA	PFUnDA
Age (years)	.071	-.018	.095	**.211***	**.205***	.057
Maternal weight prior to pregnancy (Kg)	.161	.032	.142	**.206***	.187	-.066
Maternal Weight Gain during pregnancy (Kg)	-.136	-.174	-.151	-.127	-.038	.188
Maternal Weight Gain (%)	-.185	-.168	-.196	-.175	-.104	.158
Pre-pregnancy BMI	.123	-.014	.096	.172	.152	-.042
Gestational Age	-.016	-.110	-.107	-.057	.009	.040
Birth Weight (grams)	-.072	-.191	-.078	.032	.065	.141
Birth length (cm)	-.085	-.128	-.047	.020	.113	.089
Head Circumference (cm)	-.127	-.122	-.111	-.017	.014	-.146
Ponderal Index (g/cm^3 x100)	-.007	-.091	-.058	-.006	-.078	.037

Proportion of Optimal Birth Weight (%)	-.009	-.135	.023	.096	.090	**.258***
Proportion of Optimal Birth Length (%)	-.052	-.110	.012	.050	.113	.147
Proportional Optimal Head circumference (%)	-.064	-.071	-.077	.007	.014	-.083

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

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

Supplementary Data Table S6: Comparison between participants in this study and full AMETS study population

	PFAA Analysis (n=98)	AMETS study[a] (n=173)	p value (mann whitney or χ^2)
Maternal Characteristics	Mean ± SD or n (%)	Mean ± SD or %	
Age (years)	31.8 ± 4.6	31.9 ± 4.2	0.603
range	19 – 44	19 - 44	
First Pregnancy	n=57 (60.0%)	52.4%	**0.031**
Breastfed Other children	n= 28 (28.6%)*	39.4%	**0.007**
Mean Reported pre-pregnancy weight for population (Kg)	69.4 ± 13.5	68.2 ± 13.9	0.089
Pregnancy BMI			
<18.5	n =5 (5.3%)	4.2%	0.129
18.5 – 24.9	n=51 (54.3%)	61.2%	
25.0 – 29.9	n=18 (19.1%)	18.2%	
≥30	n=20 (21.3%)	16.4%	
Region of residence			

Perth Metropolitan	n=46 (46.9%)	49.1%	0.527
Great Southern	n=7 (7.1%)	4.8%	
South West	n=19 (19.4%)	21.8%	
Goldfields Esperance	n=15 (15.3%)	15.2%	
Other	n=11 (11.2%)	9.1%	
Urban or rural residence			
Urban	n=66 (72.5%)	70.4%	0.297
Rural	n=25 (27.5%)	29.6	
Education Level			
Secondary Education	n=5 (5.4%)	7.3%	0.393
Trade/Diploma	n=25 (26.9%)	23%	
Tertiary	n=63 (67.7%)	69.7%	
Gross Annual Household Income			
<80,000AUD	n=25 (27.5%)	29.6%	0.603
>80,000AUD	n=66 (72.5%)	70.4%	
Average reported Oily Fish consumption			
(portions /month) 0	n=36 (44.4%)	38.4%	0.323
1-2	n= 20 (24.6%)	28.1%	
>2 – 4	n=12 (14.8%)	17.1%	

>4	n=13 (16%)	16.4%	
Infant characteristics Sex Female Male	 n=56 (59.6%) n=38 (40.4%)	 54.2% 45.8%	 0.120
Gestational age (weeks) Range	39.7 ± 1.1 35 - 42	39.8 ± 1.1 35 - 42	0.113
Birth weight (g)	3561 ± 467	3534 ± 448	0.651
Length (cm)	51.0 ± 2.3	50.8 ± 2.5	0.443
Head circumference (cm)	35.0 ± 1.6	35.0 ± 1.5	0.672
Proportion of Optimal Birth weight (POBW) (%)	101 ± 12	99 ± 11	**0.019**
Proportion of Optimal Birth Length (POBL) (%)	100 ± 4	100 ± 4	0.069
Proportion of Optimal Head Circumference (POHC) (%)	100 ± 4	100 ± 4	0.203

a. Callan et al., 2013.