Identifying risk factors for levels of per- and polyfluoroalkyl substances (PFAS) in the placenta in a high-risk pregnancy cohort in North Carolina

Jacqueline Bangma[1], Lauren A Eaves[1], Kirsi Oldenburg[1], Jessica L Reiner[2], Tracy Manuck[3,4], and Rebecca C Fry[1,4,5]

SUPPLEMENTAL INFORMATION

Number of pages: 17

Number of files: 1

Number of tables: 9

Number of figures:1

File S1. File of calculated RL for each PFAS in each extracted placenta sample (See supplemental Excel file).

Figure S1. Correlogram demonstrating the associations between each of PFAS levels measured in the placenta for PFOS, PFHxS, PFHpS, and PFUnA. Blue circles indicate a positive correlation while red circles indicated a negative correlation. Intensity of color and size of the circle correspond to the magnitude of the association, in terms of absolute value of Spearman correlation coefficient. Blank indicates a non-significant association.

Table S1. Spearman correlation coefficients and associated p-values (in parentheses) of the correlations between PFAS measured in the placenta for PFOS, PFHxS, PFHpS, and PFUnA (ng/g).

Table S2. Comparisons of medians of PFAS levels (ng/g) by selected demographic variables. Tests of difference in medians evaluated by Wilcoxon test or Kruskal-Wallis test depending on if comparing two groups or over two groups, respectively, and p value of test listed.

Table S3. Comparisons of medians, IQR, maximum and minimum values of PFAS levels (ng/g) by hypertensive disorders of pregnancy (preeclampsia or gestational hypertension). p-value of test of difference in medians is listed.

Table S4. Comparisons of medians, IQR, maximum and minimum values of PFAS levels (ng/g) by fetal growth by the categories small for gestational age (SGA), appropriate for gestational age (AGA), and large for gestational age (LGA). p-value of test of difference in medians is listed.

Table S5A. Beta values, standard error and associated p-values of crude linear regressions of PFAS level (ng/g) onto gestational age. Beta values represent predicted change in ng/g of PFAS with each additional 1 week of gestational age.

Table S5B. Beta values, standard error and associated p-values of adjusted, multivariate linear regressions of PFAS level (ng/g) onto gestational age. Beta values represent predicted change in ng/g of PFAS with each additional 1 week of gestational age, adjusted for maternal age, maternal smoking during pregnancy and maternal race.

Table S6. Crude (unadjusted) binomial regression models for relationship between gestational age and PFAS detection above RL for the seven PFAS detected below RL in approximately 50% or more of samples. Beta estimate represent the change in probability of PFAS being detected over the LOD with each 1 week increase in gestational age.

Table S7. Beta values, standard error and associated p-values of crude linear regressions of PFAS level (ng/g) onto delivery date. Beta values represent predicted change in ng/g of PFAS with each subsequent year of delivery (range 2015-2017). 2018 delivery dates (n=2) were removed from the analysis due to low sample size.

Table S8. NIST Standard Reference Material (SRM) 1947 certificate of analysis values compared to values obtained from methods employed in this study.

MATERIALS AND METHODS

2.2 Placenta PFAS quantification

Chemicals

Standard mixtures PFAC-MXC, HFPO-DA, and 6:2FTS from Wellington labs were combined to create calibration solutions. Combined, the solution contained a total of 22 PFAS as follows: (1) perfluorobutyric acid (PFBA), (2) per- fluoropentanoic acid (PFPeA), (3) perfluorohexanoic acid (PFHxA), (4) perfluoroheptanoic acid (PFHpA), (5) Perfluorooctanic acid (PFOA), (6) perfluorononanoic acid (PFNA), (7) perfluorodecanoic acid (PFDA), (8) perfluoroundecanoic acid (PFUdA), (9) perfluorododecanoic acid (PFDoA), (10) perfluorotridecanoic acid (PFTrDA), (11) perfluorotetradecanoic acid (PFTeDA), (12) Perfluorohexadecanoic acid (PFHxDA), (13) perfluorobutanesulfonic acid (PFBS), (14) perfluoropentanesulfonic acid (PFPeS), (15) perfluorohexanesulfonic acid (PFHxS), (16) Perfluoroheptanesulfonic acid (PFHpS), (17) perfluorooctanesulfonic acid (PFOS), (18) perfluorononanesulfonic acid (PFNS), (19) perfluorodecanesulfonic acid (PFDS), (20) perfluorododecancesulfonic acid (PFDoS), (21) 1H, 1H, 2H, 2H-perfluorooctane sulfonic acid (6:2 FTS), and (22) 2,3,3,3-Tetrafluoro-2-(1,1,2,2,3,3,3-heptafluoropropoxy)propanoic acid (HFPO-DA also known as GenX).

Sample Preparation

All internal standards (IS) were purchased from Cambridge Isotope Laboratories (Andover, MA), RTI International (Research Triangle Park, NC), and Wellington Laboratories (Guelph, Ontario), in order to create an IS mixture that was comprised of a total of 15 isotopically labeled PFAS. The mixture is as follows: [13C4]PFBA,

[13C5]PFPeA, [13C5]PFHxA, [13C4]PFHpA, [13C8]PFOA, [13C9]PFNA, [13C6]PFDA, [13C7]PFUdA, [13C]PFDoA, [13C2]PFTeDA, [13C3]PFBS, [13C3]PFHxS, [13C8]PFOS, [13C2]6:2FTS, and [13C3]HFPO-DA.

For each blank, calibrant, and NIST Standard Reference Material (SRM) 1947 Lake Michigan Fish Tissue (used as control), approximately 1.0 g of sample was gravimetrically added to a 50 mL Falcon tube. A minimum of three blanks were included in the method. Each blank subsisted of approximately 1.0 g of de-ionized water. For the placental tissues, after trimming to approximately 1.0 g, excess blood was removed using methanol-rinsed foil. The sample was then re-weighed in the 50 mL falcon tube and the final weight recorded prior to extraction. The internal standard solution (approximately 200 μL) was then added to each placenta, blank, and calibrant. Samples were vortexed and left to equilibrate for 1.5 h. A solution of 0.01 mol/L KOH (2.5 mL) in methanol was added, and the samples were sonicated for 30 min. The samples were then centrifuged for 5 min at 2500 rpm. The supernatant was removed and transferred to a clean glass culture tube. The addition of 0.01 mol/L KOH methanol and sonication was repeated. The combined supernatant was then evaporated under nitrogen at 35° C, resulting in a final volume of 2 mL using the TurboVap LV (Biotage, Uppsala, Sweden).

Each sample was further cleaned by being loaded into Supelco Supelclean ENVI-Carb SPE columns (3 mL, 250 mg, 120 to 400 mesh; Bellefonte, PA) and eluted with 2.5 ml methanol twice. The SPE cleaned fractions were evaporated at 48 KPa and 35 °C to 1 mL and the extracts were transferred to autosampler vials for analysis. Individual autosampler vials were analyzed using a liquid chromatograph coupled with a tandem mass spectrometer (LC-MS/MS). Three aliquots of SRM (1 mL each) were analyzed

alongside samples and used as controls for this investigation. Values on the Certificate of Analysis were compared to PFAS measurements to assess the accuracy of the method.

LC-MS/MS data acquisition

Samples were analyzed using an Agilent 1100 High Performance Liquid Chromatography 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. Samples (5 µL) were injected onto a Kinetex 2.6 µ PFP analytical column, (50 mm x 3 mm). The solvent gradient flow started at 25% 20 mmol ammonium acetate (volume fraction) in methanol and 75% 20 mmol ammonium acetate (volume fraction) in water (flow rate 150 µL/min). Gradient flow increased to 28.5% methanol by 4.20 min, and further increased to 55% methanol by 15 min which held for 5 min. Methanol continued to increase to 62.5 % by 22 min and then a slow ramp to 87.5% methanol occurred by 52 min. The flow was then increased to 100 % methanol at 55 min and held for 5 min. Following, the flow decreased to 25% methanol by 60.5 min and held until 70 min. Two multiple reaction monitoring (MRM) transitions for each PFAS were monitored to ensure no interferences. One MRM was employed for quantitation and the other transition was used for confirmation.[1]

Quantification

Each PFAS was quantitated using a linear equation of the calibration curve when compared to internal standards of similar compounds. Using an established gravimetric method, the reporting limit (RL) is calculated in two ways: (1) using the lowest

detectable calibrant divided by the mass of extracted sample or (2) taking the average amount of compound detected in the blanks divided by the mass of extracted sample. To be conservative, from the two methods of calculating the RL, the RL that was the highest for that PFAS and sample was designated as the respective RL for PFAS in that sample (**Supplemental File S1**).[2,3] The concentrations determined include isomers within applicable PFAS. PFAS were included in further statistical analysis if approximately 50% or more of the samples were detected above the RL.[4] Values on the Certificate of Analysis for SRM 1947 were compared to PFAS measurements to assess the accuracy of the method (**Supplemental Table S8**).

RESULTS

Comparison to National Vital Statistics Birth Data 2016 and 2017

In order to assess the representativeness of the present study cohort to pregnant women in the general US population, we compared demographic distributions in this cohort to National Vital Statistics Birth Data Report for 2017 (which includes data on 2016 as well).[5] In 2016, the total percentage of women reporting smoking in pregnancy was 7.2%, in 2017, it was 6.9%. In this cohort 7.4% of women reported smoking, indicating that the distribution of smokers was similar to the US population. The mean age of mother at first birth in the total population was 26.8 in 2017 and 26.6 in 2016, in our study the mean age in 31.0, indicating that our cohort is slightly older than average, however the age of mother at first birth is rising.[5] As in the present study population, in the general population, the largest percentage of births are paid by either private insurance (49.1%, 2017) or Medicaid (43%, 2017). In our study, private insurance and Medicaid were payment options for 53.9% and 38.5%, respectively, which is not

substantially different than the general population. The current study population is 37.% White non-Hispanic, 26.8% White, Hispanic, 33% non-Hispanic Black and 2.7% listed their race as "Other." The racial demographics of women who gave birth in 2017 vary slightly from this distribution with 51.68% of birth being to White mothers, 14.54% to non-Hispanic Black mothers, 23.3% to Hispanic mothers. The largest difference, as expected for a cohort of high risk of spontaneous preterm birth women, is in the gestational age at delivery, with extremely preterm births comprising 36.4%, later preterm births 9.9% and term 53.7% of the current cohort. Nationally, however, 90.07% of births in 2017 were term births. In sum, while this cohort is high-risk for spontaneous preterm birth and thus differ from the general population with regard to gestational age at delivery, for a number of key demographics (racial demographics, maternal age, insurance type and percentage of smokers) are not substantially different from pregnant women in the general U.S population.

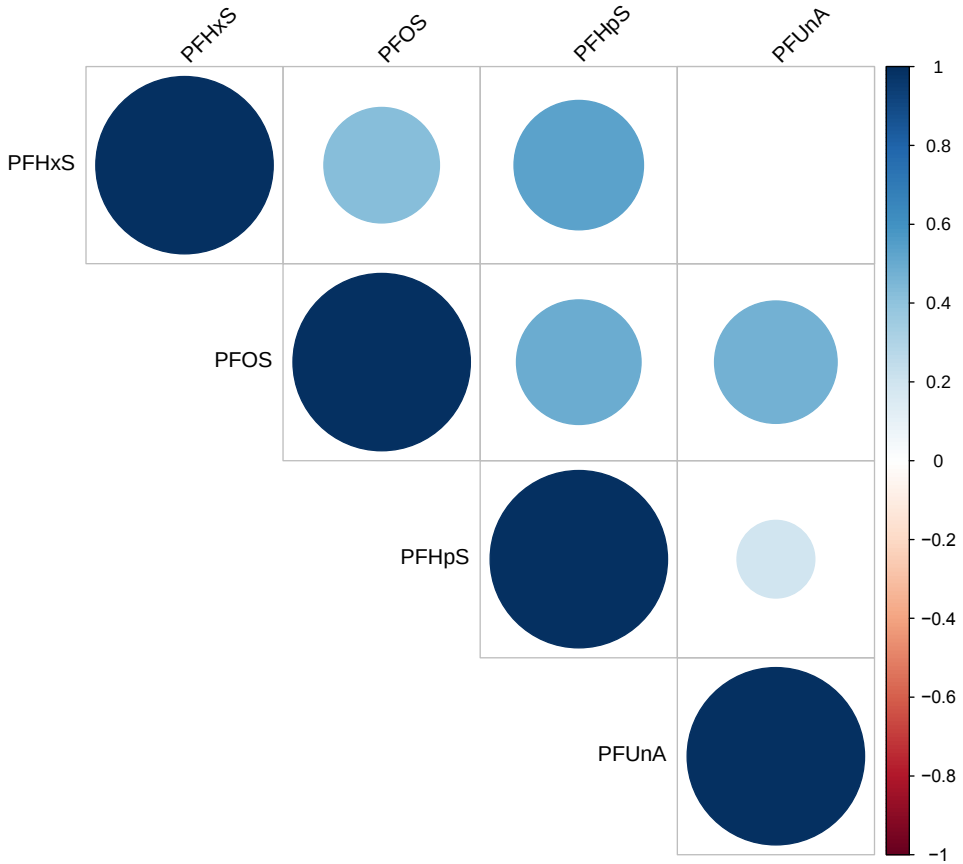

Figure S1. Correlogram demonstrating the associations between each of the PFAS levels measured in the placenta for PFOS, PFHxS, PFHpS, and PFUnA. Blue circles indicate a positive correlation while red circles indicated a negative correlation. Intensity of color and size of the circle correspond to the magnitude of the association, in terms of absolute value of Spearman correlation coefficient. Blank indicates a non-significant association.

Table S1: Spearman correlation coefficients and associated p-values (in parentheses) of the correlations between PFAS measured in the placenta for PFOS, PFHxS, PFHpS, and PFUnA (ng/g).

PFAS	PFHxS	PFOS	PFHpS	PFUnA
PFHxS	1	0.425 (1.097×10^{-6})	0.532 (2.907×10^{-10})	0.037 (0.688)
PFOS	0.425 (1.097×10^{-6})	1	0.493 (7.990×10^{-9})	0.477 (2.786×10^{-8})
PFHpS	0.532 (2.907×10^{-10})	0.493 (7.990×10^{-9})	1	0.192 (0.034)
PFUnA	0.037 (0.688)	0.477 (2.786×10^{-8})	0.192 (0.034)	1

Table S2: Comparisons of medians of PFAS levels (ng/g) by selected demographic variables. Tests of difference in medians evaluated by Wilcoxon test or Kruskal-Wallis test depending on if comparing two groups or over two groups, respectively, and p value of test listed.

	N	PFHxS					PFOS					PFHpS					PFUnA				
		Median	IQR	P-value	Min	Max	Median	IQR	P-value	Min	Max	Median	IQR	P-value	Min	Max	Median	IQR	P-value	Min	Max
	122	0.067	0.115		0.017	0.446	0.480	0.525		0.001	4.87	0.009	0.010		0.004	0.063	0.027	0.032		0.002	0.240
Race & Hispanic Ethnicity				0.16					0.16					0.37					**<0.01**		
White non-Hispanic	42	0.117	0.130		0.017	0.446	0.454	0.500		0.006	1.42	0.010	0.012		0.004	0.028	0.019	0.020		0.016	0.100
White Hispanic	30	0.069	0.102		0.017	0.427	0.626	0.552		0.129	4.87	0.009	0.008		0.005	0.063	0.047	0.056		0.018	0.240
Non-Hispanic Black	37	0.052	0.079		0.017	0.362	0.432	0.407		0.001	1.71	0.006	0.010		0.004	0.025	0.04	0.042		0.017	0.217
Other	3	0.047	0.165		0.043	0.208	0.552	0.386		0.495	0.881	0.013	0.0030		0.011	0.014	0.081	0.111		0.045	0.156
Maternal Smoking				**0.03**					0.12					0.28					0.90		
Yes	9	0.124	0.116		0.040	0.377	0.569	0.503		0.164	1.91	0.010	0.002		0.004	0.043	0.039	0.032		0.017	0.217
No	112	0.063	0.181		0.017	0.446	0.459	0.502		0.001	4.87	0.009	0.010		0.004	0.063	0.027	0.031		0.002	0.240
Missing	1																				
Child's Gender				0.89					0.66					0.32					0.81		
Female	54	0.068	0.117		0.017	0.446	0.476	0.515		0.004	4.87	0.001	0.011		0.004	0.063	0.020	0.003		0.016	0.177
Male	67	0.067	0.116		0.017	0.377	0.490	0.546		0.0005	1.91	0.009	0.008		0.004	0.055	0.037	0.003		0.002	0.240
Missing	1																				
Maternal pre-pregnancy BMI				0.35					0.25					0.60					0.80		
<18.5	6	0.006	0.089		0.018	0.213	0.627	0.668		0.166	0.978	0.015	0.014		0.004	0.028	0.024	0.025		0.017	0.156
18.5-<25	24	0.050	0.078		0.0165	0.256	0.403	0.404		0.006	1.82	0.005	0.007		0.004	0.055	0.020	0.054		0.017	0.240
25-<30	30	0.075	0.139		0.017	0.363	0.506	0.602		0.001	1.18	0.010	0.009		0.004	0.027	0.038	0.028		0.017	0.217
30+	62	0.073	0.105		0.017	0.446	0.495	0.495		0.004	4.87	0.010	0.011		0.004	0.063	0.020	0.032		0.002	0.177
Missing	0																				
Maternal age				0.82					0.35					***0.09***					0.21		
<20	1	0.064	0		0.064	0.064	0.166	0		0.166	0.166	0.004	0		0.004	0.004	0.017	0		0.017	0.017
20-<35	77	0.067	0.102		0.017	0.446	0.484	0.486		0.001	1.91	0.009	0.009		0.004	0.043	0.034	0.028		0.002	0.134
35+	44	0.068	0.116		0.017	0.427	0.485	0.569		0.004	4.87	0.011	0.011		0.004	0.063	0.022	0.057		0.016	0.240

Missing	0																		
Maternal medical insurance				0.19				0.60				0.11				0.29			
Private insurance	56	0.105	0.129		0.017	0.363	0.501	0.529		0.001	1.82	0.010	0.011		0.004	0.028	0.037	0.034		0.016	0.156
Medicare/Medicaid	40	0.051	0.100		0.017	0.446	0.362	0.486		0.006	1.91	0.005	0.009		0.004	0.043	0.020	0.025		0.002	0.240
Self-pay	8	0.067	0.026		0.024	0.241	0.649	0.504		0.192	1.26	0.010	0.008		0.005	0.019	0.047	0.051		0.018	0.094
Missing	18																				
Maternal Education				0.43				0.40				0.65				0.70			
Less than high school	13	0.071	0.058		0.018	0.142	0.608	0.794		0.141	1.72	0.009	0.065		0.005	0.018	0.025	0.035		0.017	0.240
Completed high school	42	0.074	0.155		0.017	0.446	0.556	0.581		0.006	4.87	0.010	0.014		0.004	0.063	0.020	0.028		0.016	0.217
More than high school	34	0.090	0.092		0.017	0.363	0.432	0.553		0.0005	1.18	0.011	0.010		0.004	0.025	0.020	0.029		0.0170	0.156
Declined/missing	33																				
Marital status				0.73				1.00				0.24				0.59			
Married	81	0.072	0.119		0.017	0.446	0.475	0.557		0.001	4.87	0.009	0.009		0.004	0.063	0.036	0.034		0.016	0.240
Not married	33	0.065	0.073		0.017	0.266	0.490	0.469		0.006	1.71	0.010	0.011		0.004	0.025	0.020	0.024		0.002	0.217
Missing	8																				
Delivered Preterm				0.65				0.57				0.16				0.31			
Extremely preterm (<34 weeks)	45	0.053	0.101		0.017	0.361	0.490	0.385		0.006	1.71	0.005	0.007		0.004	0.028	0.036	0.030		0.002	0.240
Preterm (34-<37 weeks)	12	0.085	0.098		0.017	0.332	0.538	0.465		0.151	1.11	0.012	0.011		0.004	0.025	0.020	0.022		0.016	0.052
Term (37+weeks)	65	0.073	0.104		0.017	0.446	0.432	0.563		0.001	4.87	0.010	0.011		0.004	0.063	0.020	0.036		0.017	0.217

P-values obtained from Wilcoxon test for sociodemographic variables of two groups (child's gender, maternal smoking and marital status) and from Kruskal-Wallis test for all other sociodemographic variables, which have more than two groups.
Values below the RL have been imputed as half the RL.

Table S3. Comparisons of medians, IQR, maximum and minimum values of PFAS levels (ng/g) by hypertensive disorders of pregnancy (preeclampsia or gestational hypertension). p-value of test of difference in medians is listed.

		PFHxS					PFOS					PFHpS					PFUnA				
	N	**Median**	**IQR**	**Min**	**Max**	**p-value**	**Median**	**IQR**	**Min**	**Max**	**p-value**	**Median**	**IQR**	**Min**	**Max**	**p-value**	**Median**	**IQR**	**Min**	**Max**	**p-value**
	122	0.067	0.115	0.017	0.446		0.480	0.525	0.001	4.87		0.009	0.010	0.004	0.063		0.027	0.032	0.002	0.240	
Hypertensive disorders of pregnancy[1]						0.47					0.11					0.22					***0.09***
No	108	0.066	0.114	0.017	0.446		0.459	0.497	0.001	4.87		0.009	0.001	0.004	0.063		0.020	0.030	0.002	0.240	
Yes	14	0.112	0.118	0.017	0.377		0.765	0.583	0.151	1.91		0.011	0.015	0.005	0.043		0.044	0.037	0.018	0.217	

P-values obtained from Wilcoxon test.
Values below the RL have been imputed as half the RL.
[1]Defined as either gestational hypertension or preeclampsia

Table S4. Comparisons of medians, IQR, maximum and minimum values of PFAS levels (ng/g) by fetal growth by the categories small for gestational age (SGA), appropriate for gestational age (AGA), and large for gestational age (LGA). p-value of test of difference in medians is listed.

			PFHxS					PFOS					PFHpS					PFUnA				
		N	Median	IQR	Min	Max	p-value	Median	IQR	Min	Max	p-value	Median	IQR	Min	Max	p-value	Median	IQR	Min	Max	p-value
		122	0.067	0.115	0.017	0.446		0.480	0.525	0.001	4.87		0.009	0.0010	0.004	0.063		0.027	0.032	0.002	0.240	
Fetal growth							0.98					0.82					0.87					0.37
	SGA	9	0.072	0.142	0.017	0.446		0.381	0.525	0.242	1.82		0.009	0.006	0.005	0.025		0.042	0.052	0.002	0.112	
	AGA	92	0.067	0.111	0.017	0.427		0.480	0.501	0.0005	4.87		0.01	0.011	0.004	0.063		0.020	0.028	0.016	0.240	
	LGA	12	0.060	0.081	0.018	0.363		0.589	0.433	0.006	1.26		0.009	0.014	0.005	0.025		0.044	0.036	0.018	0.094	

P-values obtained from Kruskal-Wallis test.
Values below the RL have been imputed as half the RL.

Table S5A. Beta values, standard error and associated p-values of crude linear regressions of PFAS level (ng/g) onto gestational age. Beta values represent predicted change in ng/g of PFAS with each additional 1 week of gestational age.

	Beta value	**Standard error**	**P-value**
PFOS	0.000	0.009	0.98
PFHxS	0.002	0.002	0.18
PFUnA	0.000	0.001	0.94
PFHpS	0.002	0.000	0.20

Table S5B. Beta values, standard error and associated p-values of adjusted, multivariate linear regressions of PFAS level (ng/g) onto gestational age. Beta values represent predicted change in ng/g of PFAS with each additional 1 week of gestational age, adjusted for maternal age, maternal smoking during pregnancy and maternal race.

	Beta value	**Standard error**	**P-value**
PFOS	-0.005	0.009	0.56
PFHxS	0.002	0.002	0.22
PFUnA	-0.001	0.001	0.36
PFHpS	0.000	0.000	0.40

Table S6: Crude (unadjusted) binomial regression models for relationship between gestational age and PFAS detection above RL for the seven PFAS detected below RL in approximately 50% or more of samples (ie. 0=below RL, 1=detected above RL). Beta value represents the change in probability of PFAS being detected over the LOD with each 1 week increase in gestational age.

	Beta value	**Standard error**	**p-value**
PFOA	0.015	0.007	0.04
PFNA	0.006	0.007	0.36
PFDA	0.004	0.008	0.66
PFTriA	-0.013	0.008	0.10
PFPeS	0.007	0.008	0.38
PFTA	-0.007	0.004	0.11
PFHxA	-0.002	0.002	0.32

Table S7: Beta values, standard error and associated p-values of crude linear regressions of PFAS level (ng/g) onto delivery date. Beta values represent predicted change in ng/g of PFAS with each subsequent year of delivery (range 2015-2017). 2018 delivery dates (n=2) were removed from the analysis due to low sample size.

	Beta value	**Standard error**	**P-value**
PFOS	-0.044	0.067	0.51
PFHxS	-0.024	0.011	0.04
PFUnA	-0.001	0.001	0.36
PFHpS	0.007	0.005	0.14

Table S8: NIST Standard Reference Material (SRM) 1947 certificate of analysis values compared to values obtained from methods employed in this study. Not provided (NP)

Measured			**Certificate of Analysis**		
PFAS	**Measured Mean (ng/g)**	**Measured 95% CI (lower bound- upper bound)**	**Reported Mean (ng/g)**	**Uncertainty**	**Reported 95% CI (lower bound- upper bound)**
PFOS	5.53	5.14-5.92	5.90	0.39	5.51-6.29
PFNA	0.217	0.17-0.26	0.2	NP	NP
PFDA	0.202	0.12-0.28	0.26	NP	NP
PFUnA	0.263	0.23-0.29	0.28	NP	NP
PFTriA	0.172	0.17-0.18	0.2	NP	NP

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