LEVELS OF PERFLUORINATED ALKYLATED SUBSTANCES (PFAs) IN BREAST MILK, MATERNAL AND CORD SERUM OF FRENCH WOMEN AND THEIR NEWBORNS: RESULTS OF THE CONTREPERF RESEARCH PROGRAM

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Introduction

Consumers from industrialized countries are exposed every day with several chemicals of anthropogenic origin. Among others, perfluoroalkylated substances (PFAS) are used for their hydrophobic and lipophobic characteristics in a broad range of applications[1]. The CONTREPERF project (2010-2013), funded by the French National Agency of Research (ANR), aims at producing knowledge regarding human exposure to PFAS and related toxicological impact. One task of the project is dedicated to the exposure assessment of fetus and breast fed newborn as population sub-groups particularly sensitive to endocrine disruption. To this end, 5 Perfluoroalkyl Sulfonates (PFBS, PFHxS, PFHpS, PFOS, PFDS), 11 Perfluoroalkyl Carboxylic Acids (PFBA, PFPA, PFHxA, PFHpA, PFOA, PFNA, PFDA, PFUnA, PFDoA, PFTrDA, PFTeDA), perfluorooctanesulfonamide (PFOSA) and perfluorooctanesulfinate (PFOSi) were quantified in cord and maternal serum as well as breast milk samples collected from *ca.* 100 French volunteer mothers and their newborns. The present work aims at presenting the data obtained for internal exposure levels. Related human risk assessment, but also genotoxicity and metabolomic disruptions induced at hepatic level, as well as nuclear receptor binding and transactivation assays, are also under the scope of this project and will be presented elsewhere.

Material and Methods

Samples. Samples were collected by the Centre Hospitalier Universitaire de Toulouse from 102 volunteer women aged from 20 to 46 years old and planned for Caesarean delivery between June 2010 and January 2013. Breast milk from 61 women collected 4-5 days post-partum and serum from 100 women and their 106 corresponding cords (incl. 6 twin pairs) were also analyzed. The protocol was approved by an ethical committee, in accordance with French regulations.

Sample preparation. The analytical strategy used for isolating and measuring PFAS from milk samples, validated and accredited according to the ISO 17025 standard, has been described elsewhere[2]. Briefly, a protein precipitation step was performed by adding acetone to 5 mL breast milk or potassium hydroxide 0.1 M and acetic acid to 1 mL serum. A two stage Solid Phase Extraction was then applied, using Oasis® HLB and carbon graphitized (Envicarb®) cartridges, respectively. Final extracts were reconstituted in 200 µL of a fluorometholone solution as external standard in a methanol/water mixture (30:70, v/v). Mass-labeled PFAS surrogates (^{13}C and/or ^{18}O) were added prior to extraction as internal standards used for quantification according to the isotope dilution method.

LS-MS/MS measurements. The system used included a 1200 series HPLC pump (Agilent, Palo Alto, CA, USA) fitted to a reverse phase column Gemini C18 (3 µm, 50 × 2.0 mm) equipped with a guard column (3 µm, 10 × 2.0 mm) (Phenomenex, Torrance, CA, USA). The mobile phase consisted of a gradient of methanol and ammonium acetate 20 mM. The HPLC system was interfaced with a triple quadrupole tandem mass spectrometer (Agilent 6410, Palo Alto, CA, USA) fitted with an electrospray ion source operating in the negative ion mode. Acquisition was performed in the selected reaction monitoring mode.

Results and Discussion

Maternal and Cord Serum

Six target compounds (PFDS, PFOSi, PFHxA, PFOSA, PFPA and PFBA) were not detected at LOQs ranging from 0.05 to 0.38 ng/mL. Three other compounds (PFBS, PFDoA, PFTeDA) presented detection rates lower than 8% at LOQs ranging from 0.05 to 0.20 ng/mL. These 9 compounds were consequently excluded from further data analyses and interpretation. Two compounds (PFHpA and PFTrDA) presented detection rates around 10% at LOQs ranging from 0.05 to 0.20 ng/mL. Data for other compounds (7 PFAS presenting detection rates ranging from 25 to 100%) are presented in Table 1. As shown in Figure 1, the main contributors to the PFAS internal exposure are PFOS, followed by PFOA, then PFHxS and PFNA. It should be pointed out that the relative contribution of PFOS *versus* PFOA is significantly lower in cord than in maternal serum.

Table 1: Concentration levels (ng/mL) measured for the 7 most frequently detected PFAS compounds in 100 maternal and 100 cord serum samples. Mean values were considered for twins (n=2x6); LB: lower bound; UB: upper bound.

	PFHxS		PFHpS		PFOS		PFOA		PFNA		PFDA		PFUnA	
	Mat.	Cord	Mat.	Cord	Mat.	Cord	Mat.	Cord	Mat.	Cord	Mat.	Cord	Mat.	Cord
Detected	99%	93%	50%	25%	100%	99%	100%	100%	98%	91%	93%	46%	79%	35%
Min LOQ	0.10	0.09	0.05	0.05	-	0.10	-	-	0.08	0.08	0.05	0.04	0.05	0.05
Max LOQ	0.10	0.10	0.24	0.19	-	0.10	-	-	0.17	0.18	0.18	0.18	0.23	0.23
Min.	<LOQ	<LOQ	<LOQ	<LOQ	0.32	<LOQ	0.15	0.15	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
LB Med.	0.62	0.34	0.03	0.00	3.07	1.12	1.05	0.86	0.43	0.22	0.20	0.00	0.13	0.00
UB Med.	0.62	0.34	0.19	0.13	3.07	1.12	1.05	0.86	0.43	0.22	0.20	0.09	0.16	0.08
Max.	31.0	16.0	0.81	0.37	24.5	8.04	7.31	7.06	3.29	2.25	1.99	0.60	2.60	0.74

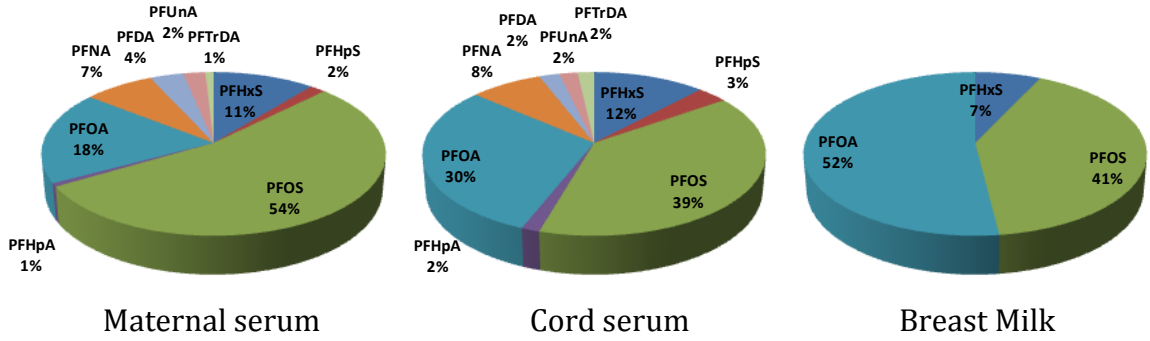

Figure 1: Median PFAS contamination profiles observed in maternal and cord serum samples (medium bound).

PFOS, PFOA, PFHxS and PFNA were found to contribute together to around 90% of the total PFAS contamination both in maternal and cord serum. Distribution frequencies observed for these 4 compounds are typically Log-normals (Figure 2). Individual patterns for these 4 major compounds exhibit important inter-individual variability. Thus, although PFOS is usually the main contributor (Figure 1), the pattern observed for the 3 pairs presenting the highest cumulated concentrations is dominated by PFHxS (Figure 2).

Relationships between the different PFAS levels in a same biological matrix were further investigated. Determination coefficients (R^2) higher than 0.80 were observed in cord serum for PFHxS *versus* PFHpS (R^2=0.82, n=25) and for PFOA *versus* PFNA (R^2=0.82, n=91). No other significant correlation was found, most compounds showing independent occurrences. Relationships between cord and maternal serum were also investigated: correlations observed for the 4 major detected compounds are showed in Figure 3. Interestingly, the observed determination coefficients were always >0.83, excepted for PFTrDA (R^2=0.45, but n=8 only).

Mean ratios between cord and maternal levels were 0.30 (PFDA, n=46), 0.38 (PFOS, n=99), 0.39 (PFUnA, n=35), 0.41 (PFHpS, n=25), 0.53 (PFNA, n=91), 0.57 (PFHxS, n=93), 0.78 (PFOA, n=100), 1.15 (PFTrDA, n=8) and 1.35 (PFHpA, n=7), respectively. These proportions are in line with the litterature[3]. These values strongly suggest the existence of congener specific transplacental transfer rates. However, our results demonstrate that Human fetus is exposed to a range of various PFAS.

Additional analyses of blood sediments were achieved for 13 maternal/cord sample pairs, including the most contaminated ones. Only PFOS, PFOA and PFHxS had quantification levels suited for further interpretation. Assuming hematocrits at 0.344 for women in their third trimester of pregnancy[4] and at 0.472 for cord blood at cesarean delivery[5], about 10% of the PFAS present in blood is contained in the cellular fraction, which may represents a source of slight underestimation of exposure determined on the basis of plasma or serum as is usually the case. Besides this practical implication, such finding also raises the question of the nature of interactions between PFAS and blood components.

Compared to the literature overview from 10 sample sets from various regions[3], median values observed for PFOS, PFOA and PFHxS in the present study stand among the lowest ones, prolonging a secular tendency already observed and reported since 2002 (with the closing of a major production plant) to lower body burden levels in the general population. This tendency is corroborated by significant decrease observed when compared with 11 cord serum samples previously obtained from the same clinical and sampling protocol, for women recruited in 2006-07. Median medium bound values observed in these older samples for PFOS, PFOA, and PFNA, where 0.38, 0.49 and 0.63 fold lower, respectively, compared to more recently collected samples. For PFHxS this difference was not significant.

Breast milk

Twelve target compounds (PFBS, PFHpS, PFDS, PFOSi, PFBA, PFPA, PFDA, PFTeDA, PFHxA, PFHpA, PFDoA and PFTrDA) were not detected at LOQs ranging from 0.01 to 0.20 ng/mL. Two other compounds (PFNA and PFUnA) presented detection rates lower than 4% at LOQs ranging from 0.01 to 0.10 ng/mL. All these 14 compounds were excluded from further interpretations. Data for PFOS, PFOA, and PFHxS are presented in Table 2. As shown in Figure 1, the main contributors to the total PFAS contamination level are PFOS and PFOA, PFHxS being detected in only 20% of samples.

Table 2: Concentration levels (ng/mL) measured in 61 breast milk samples for the 3 most frequently detected compounds. Mean values were considered for twins (n=2x6); LB: lower bound; UB: upper bound.

	PFHxS	PFOS	PFOA
Detected	12 (20%)	50 (82%)	47 (77%)
Min LOQ	0.009	0.010	0.010
Max LOQ	0.028	0.032	0.033
Min.	<LOQ	<LOQ	<LOQ
LB Med.	0.000	0.026	0.033
UB Med.	0.009	0.028	0.033
Max.	0.217	0.376	0.308

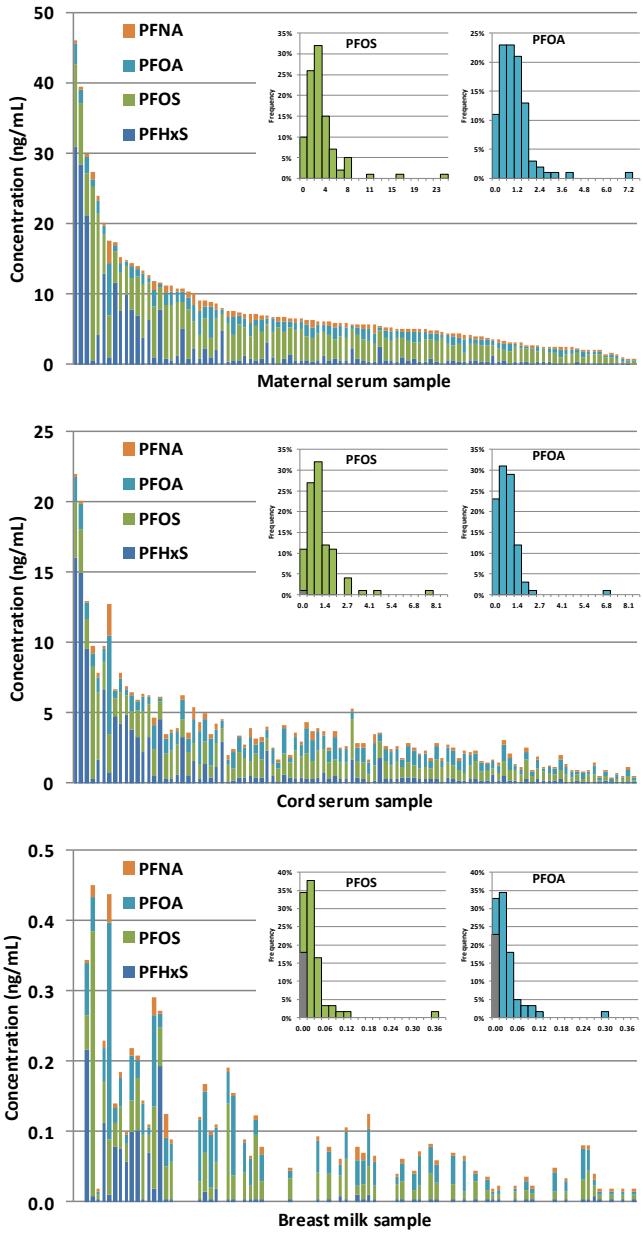

Figure 2: Cumulated medium bound concentrations levels (ng/mL) determined for the 4 main PFAS detected in maternal serum (n=100, decreasing order), cord serum and breast milk (identical order), and associated distribution frequencies of PFOS and PFOA. Mean values were considered for twins; Grey: <LOQ.

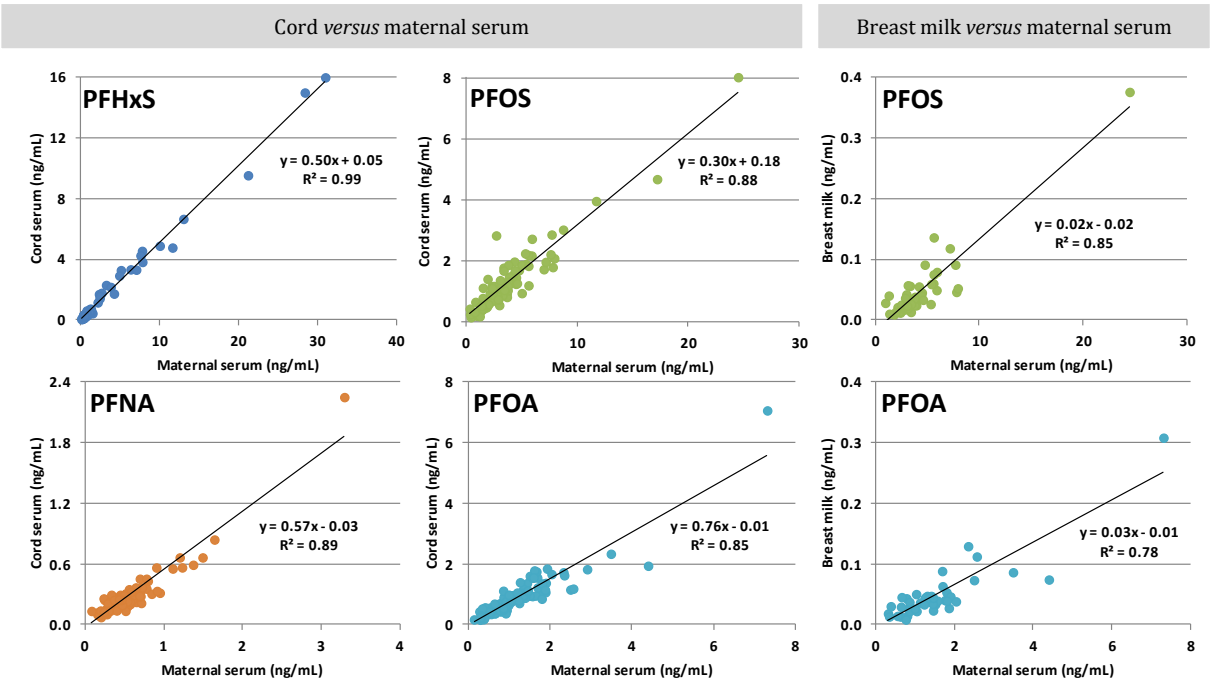

Figure 3: Concentration levels (ng/mL) determined in cord *versus* maternal serum for PFHxS (n=93), PFOS (n=99), PFOA (n=100) and PFNA (n=91), and in breast milk *versus* maternal serum levels for PFOS (n=45) and PFOA (n=48).

The relative contribution of PFOS/PFOA was found to be significantly lower in breast milk than in maternal serum, so that PFOA exceeds PFOS in breast milk. Distribution frequencies observed for these 2 compounds were classically found to be Log-normals (Figure 2). Similarly to what had been observed in serum, PFAS contamination patterns in breast milk displayed significant inter-individual variability. PFOS and PFOA were usually the main contributors (Figure 1). However, PFHxS was also a significant, if not major, contributor in certain serum profiles (Figure 2). As expected, no correlation was found between the main detected compounds.
Correlations for PFOS and PFOA between breast milk and maternal serum are showed in Figure 3. Concentration ratios were 0.011 (PFOS, n=48), 0.012 (PFHxS, n=11) and 0.033 (PFOA, n=45). Again, PFOA appeared more likely to cross the placental barrier and was also more extensively excreted in breast milk than PFOS. Compared to the literature overview from 25 breast milk sample sets from various regions[6], median values observed for PFOS, PFOA and PFHxS in the present study stand among the lowest ones. This could confirm a global decreasing exposure to the targeted substances. The tendency is corroborated with significant decreases observed for PFOS and PFOA when compared with 29 breast milk samples previously obtained from the same sampling protocol, for women recruited in 2005-06, with medium bound mean folds at 0.49 ($p=2.10^{-6}$, non parametric Mann-Whitney test) and at 0.61 ($p=3.10^{-3}$), respectively.

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References
1. Fromme H, Tittlemier SA, Volkel W, Wilhelm M, Twardella D (2009). *Int. J. Hyg. Environ. Health* 212:239.
2. Kadar H, Veyrand B, Barbarossa A, Legrand A, Boquien CY, Antignac JP (2011). *Chemosphere* 85:473-480.
3. Reproduction et environnement (2011). Page 535-597 of 716. Collective expertise, INSERM, in French.
4. Fontaine P, Deruelle P, Fermon C, Vambergue A (2009). *Diabetes & Metabolisms* 35(S1):70.
5. Zanardo V, Gabrieli C, de Luca F, …, Scambia G, Straface G (2013). *J Matern Fetal Neonatal Med.* In press.
6. Antignac JP, Veyrand B, Kadar H, Oleko A, Le Bizec B, Vandentorren S (2013). *Chemosphere* 91:802-808.