Concentration of perfluorinated compounds and cotinine in human fetal organs, placenta, and maternal plasma

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Running title: PFASs in maternal plasma, placenta, and fetuses

Financial interest declaration

The financial supporters had no role in the study design, collection and analysis of data, data interpretation or in writing the report. The corresponding author had full access to all data presented in the study and had the final responsibility for the decision to submit this paper for publication.

Conflict of interest

None declared.

ABSTRACT

Background: Perfluoroalkyl substances (PFASs) have been frequently used for many years in industrial and consumer products. Maternal cigarette smoking may be associated with maternal PFAS levels. Further, prenatal exposure to PFASs is suggested impact on human fetal development and may have long-term adverse health effects later in life. Fetal exposure has previously been estimated from umbilical cord plasma, but the actual concentration in fetal organs has never been measured.

Objectives: Concentrations of 5 PFASs and cotinine – the primary metabolite of nicotine – was measured in human fetuses, placentas, and maternal plasma to evaluate to what extend these compounds were transferred from mother to fetus, and to see if PFAS concentration was associated with maternal cigarette smoking.

Methods: A total of 39 Danish women who underwent legal termination of pregnancy before gestational week 12 were included; 24 maternal blood samples were obtained together with 34 placenta tissue and 108 fetal organs. PFASs and cotinine were assayed by liquid chromatography/ triple quadrupole mass spectrometry.

Results: In fetal organs perfluorooctanesulfonic acid (PFOS) 0.6 ng/g, perfluorooctanoic acid (PFOA) 0.2 ng/g, perfluorononanoic acid (PFNA) 0.1 ng/g, perfluoroundecanoic acid (PFUnDa) 0.1 ng/g, and perfluorodecanoic acid (PFDA) 0.1 ng/g was detected. In fetal organs the mean concentrations of PFOS, PFOA, PFNA, and PFUnDA were reduced to 5—13% of the concentration found in maternal plasma; PFDA was reduced to 27%. A significant positive correlation was found between fetal age and fetal levels for all five PFASs evaluated. A significant positive correlation was also found between fetal age and fetal cotinine levels. A significant negative correlation was found between maternal BMI and maternal plasma PFNA and and PFUnDA concentrations. Smokers presented with 99 ng/g cotinine in plasma, 108 ng/g in placenta, and 61 ng/g in fetal organs, non-smokers showed cotinine levels below 0.2 ng/g in all evaluated compartments. No correlation between maternal cotinine levels and PFASs levels were found.

Conclusions: PFASs were transferred from mother to fetus, however with a markedly differently efficacy. The concentrations of PFOS, PFOA, PFNA, and PFUnDA in fetal organs were 7—20 times lower than maternal levels, whereas PFDA was four times lower. Furthermore, a significant correlation between fetal age and all evaluated PFASs was found. The health-compromising levels of these substances in fetal life are unknown.

KEYWORDS: Prenatal exposure, perfluorinated compounds, cigarette smoke, maternal plasma, placenta

ABBREVIATIONS: BMI, body mass index; EDTA, ethylenediamine tetraacetic acid; IS, internal standard; LOD, limit of detection; pc, post conception; PCR, polymerase chain reaction; PFAS, perfluoroalkyl substance;

PFASs, perfluoroalkyl substances; PFDA, perfluorodecanoic acid; PFNA, perfluorononanoic acid; PFOA, perfluorooctanoate; PFOS, perfluorooctanesulfonate; PFUnDA, perfluoroundecanoic acid; TDI, tolerable daily intake.

ACKNOWLEDGEMENTS /FUNDING:

We thank The Research Pools of Rigshospitalet and EU interregional project ReproUnion for founding this study.

Introduction

Perfluoroalkyl substances (PFASs) are slowly degradable pollutants and belong to the group of water- and grease resistant fluorosurfactants used for many industrial and consumer applications, like outdoor clothes, non-stick cookware, food packaging, electronics, stain-resistant carpets and in fire-fighting foams (Key *et al.*, 1997; Jensen and Leffers, 2008; De Solla *et al.*, 2012). The PFASs include perfluorooctanesulfonic acid (PFOS), perfluorooctanoic acid (PFOA), perfluorononanoic acid (PFNA), perfluorodecanoic acid (PFDA) and perfluoroundecanoic acid (PFUnDA), all of which suspected to have negative impact on fetal growth and development and may disturb the endocrine system (de Cock *et al.*, 2014; Johnson *et al.*, 2014; Bach *et al.*, 2015). Prenatal exposure to PFOS and PFOA has been associated with an increased risk of congenital cerebral palsy in Danish boys (Liew *et al.*, 2014) and maternal exposure to PFNA and PFDA has been associated with increased risk of pregnancy lost (Jensen *et al.*, 2015). In rodents, prenatal exposure to high doses of PFOS and PFOA reduced postnatal survival and birth weight, and disturbed lactation and growth of the pups (Lau *et al.*, 2004; Olsen *et al.*, 2009) and both pre and postnatal PFASs exposure have been associated with hypothyroidism and significant decreased T4 levels in pups (Yu *et al.*, 2009).

PFASs, and particularly PFOA and PFNA, have been shown to induce synthesis of the estrogen-responsive biomarker protein vitellogenin leading to an estrogen-like activity *in vivo* in rainbow trout (Benninghoff *et al.*, 2011).

Notably, PFASs half-life in rats is as short as a few days (Kudo *et al.*, 2002), compared to 200 days in the cynomolgus monkey (Seacat *et al.*, 2002), and 2.5—4.5 years in humans (Olsen *et al.*, 2007; Zhang *et al.*, 2013), suggesting a large difference in the elimination kinetics between species, and therefore animal models may only reflect the human situation to a limited extent.

Human fetal exposure has been estimated from levels measured in maternal circulation and umbilical cord blood in newborns. Prenatal exposure to PFOA has been associated with decreased birth weight in a dose dependent manner (Johnson *et al.*, 2014; Lauritzen *et al.*, 2017). An association between PFOS exposure and birth weight has also been suggested, but reported results are conflicting (Bach *et al.*, 2015) whereas prenatal PFOS and PFOA exposure has been suggested to negatively affect thyroid function. In new-born boys, T4 levels decreased with increasing prenatal exposure to PFOS and PFOA. Surprisingly, the same study found the opposite effect in girls where the T4 level increased with increasing prenatal exposure to PFOA (de Cock *et al.*, 2014). In human breast cancer cells, PFOA are suggested to be cytotoxic and to exert an estrogen effect, though an anti-estrogen effect was found when cells were co-exposed to estradiol (Henry and Fair, 2013). Information on the actual concentration of PFASs in human tissues is limited, but a few

reports do exist (Olsen *et al.*, 2003a; Maestri *et al.*, 2006; Kärrman *et al.*, 2010; Pérez *et al.*, 2013). Lung tissue is suggested to accumulate the highest concentration of PFASs in general, though PFOS and PFOA tend to accumulate with highest prevalence in liver and bone structures, respectively (Pérez *et al.*, 2013). In liver, PFOS was detected in higher concentrations than PFOA (Maestri *et al.*, 2006; Kärrman *et al.*, 2010; Pérez *et al.*, 2013). In human liver, PFOA has been detected in higher levels than PFNA and PFDA in one report (Pérez *et al.*, 2013) and in similar levels in another (Kärrman *et al.*, 2010). PFASs were found in all human tissues (Pérez *et al.*, 2013). The tissue concentrations in human fetal organs are currently not available.

Maternal cigarette smoking, together with other lifestyle parameters, may impact maternal PFAS levels (Lauritzen *et al.*, 2016), why the present study included maternal smoking and other lifestyle factors in order to evaluate if maternal lifestyle affected maternal and fetal PFAS levels. Cotinine is the primary metabolite of nicotine and is a valid biomarker used to discriminate smokers from non-smokers (Benowitz *et al.*, 2003). The adverse effects that maternal smoking has on the unborn child is well-known and widely described (Mund *et al.*, 2013). Plasma cotinine concentrations in newborns have been reported to be approximately 60 ng/mL in children of heavy smokers, 30 ng/mL in children from moderate smokers, and 3 ng/mL in children from non-smokers (Ivorra *et al.*, 2014). The actual concentrations in human fetuses have not previously been measured.

The present study is, to our knowledge, the first to measure the actual concentrations of PFASs and cotinine in human first trimester fetuses. These findings provide (i) important new knowledge on the perfusion of PFASs and cotinine from mother over the early placenta barrier to fetal circulation and organs, and (ii) evaluate if fetal age is associated with fetal PFASs levels indicating a fetal accumulation over time and (III) whether maternal cigarette smoking affects PFASs accumulation in mother and fetus.

Materials and Methods

2.1. Participating women

The participants were healthy women aged 18-46 years (mean ±SEM, 26.4 ±1.1), who had decided to terminate pregnancy for other reasons than fetal abnormality. Exclusion criteria: age under 18 years, chronic diseases, dependency on an interpreter. The project was approved by the Research Ethics Committees of the Regional Capital (H-KF 01 258206); all participants received oral and written information and gave their informed consent. The participants answered a detailed questionnaire concerning lifestyle habits during pregnancy, including smoking and drinking habits.

2.2. Human fetal tissues and maternal blood samples

Legal abortions were performed at the Department of Obstetrics and Gynaecology, University Hospital Skejby, Denmark and at Department of Obstetrics and Gynaecology, Regional Hospital Randers, Denmark, in collaboration with the Laboratory of Reproductive Biology, Rigshospitalet, Denmark. All fetuses were morphologically normal. Within one hour after the surgical procedure the fetal organs and placenta tissue were isolated, washed in sterile saline snap frozen on dry ice, and stored at –80 °C until analysis. Fetal organs and placenta tissue processed for freezing more than one hour after collection were not included in the present study. Fetal age was measured by crown-rump lengths via ultrasound in connection with the surgical procedure. Gestational age was converted to age post conception by subtracting two weeks.

Maternal blood samples were obtained in connection with anaesthesia prior to surgery, collected in ethylenediamine tetraacetic acid (EDTA) tubes, and kept on ice until centrifugation (4.000*g* for 15 min) to isolate plasma. Plasma was aliquoted to 200 µl microinserts in 1.5 mL vials (Skandinaviska GenTec, Västra Frölunda, Sweeden) and stored at -20°C until analysis.

2.3. Tissue processing

Fetal- and placenta samples were homogenized in 70% acetonitril solution containing isotopically labelled cotinine and PFASs as internal standards (IS) with three parts solution and one part tissue. Homogenization was performed using a TissueLyser (Qiagen, Copenhagen, Denmark) with a 0.5 mm. stainless steel bead for one min. at 15 Hz, thereafter shaken at room temperature (RT) for 30 min. followed by 1600 g. centrifugation. The supernatant was transferred to 100 µl inserts fitted for 1.5 mL vials. The samples were transported at –20°C to the Division of Occupational and Environmental Medicine for further analysis.

2.4. Analysis of PFASs and cotinine

The analyses of PFOS, PFOA, PFNA, PFUnDa, PFDA and cotinine in the plasma, placenta and fetal tissues samples were performed by LC/MS/MS (QTRAP 5500; AB Sciex, Foster City, CA, USA coupled to a liquid chromatography system (UFLCXR, Shimadzu Corporation, Kyoto, Japan) according to procedures set out by Lindh et al. (Lindh *et al.*, 2012). Plasma samples for calibration standards were obtained from healthy volunteers at the laboratory in Lund. Plasma was also used as a proxy matrix for the tissue samples. The levels were quantified and samples with low amounts of all compounds were selected for the calibration standards. Calibration standards were prepared by adding a standard solution containing all analyzed compounds. Concentrations were determined by peak area ratios between the analytes and the IS. The levels of all compounds in the pooled serum used for preparation of standards were quantified in each batch, and the calibration standards were corrected for the concentration found in this sample. Also, all

values were corrected for the chemical blank. The limit of detection (LOD) was determined as the concentration corresponding to three times the standard deviation of the ratio of the peak at the same retention time as the analyzed compounds, and the corresponding IS determined in the chemical blank samples. Tissue extraction time was tested for 5, 15, 30, 45 min. It was found that after 30 min. no additional chemicals were extracted from the tissue, and a 30 min. extraction time was subsequently used for all samples included in the present study. To make sure the study set-up was not contaminated with other chemicals, blank samples (IS solution) were included for all batches of tissue prepared and treated exactly the same way as the actual samples (see section 2.3.). No chemical contamination detected. Further quality-assurance analysis was not made due to limited fetal material.

2.6. Statistical methods

All statistical analyses were performed using GraphPad Prism 6.07 program (GraphPad Software, Inc., CA, USA) and RStudio program (RStudio software, Boston, Massachusetts, USA). Significance level was defined as a probability lower that 0.05 (p=0.05). An unpaired non-parametric *t*-test was used to compare PFA levels in plasma from smoking versus non-smoking women. Spearman's rank test was performed to test for correlation between PFASs and cotinine, PFASs and fetal age, PFASs and lifestyle parameters. Additionally a linear regression model was used to test the potential correlations between the PFAS levels and lifestyle parameters, maternal and fetal ages. A pairwise correlation model was used to test if the lifestyle parameters correlated.

2.7. Limitations

A direct measurement of PFAS levels in fetal blood cannot be performed as it is not possible to obtain blood samples from fetuses at these early developmental stages. Fetal organs were used instead and their PFASs content compared to levels present in maternal circulation. Previously, PFOS have been measured in serum and liver tissue and a mean liver to serum ratio was reported to 1.3 to 1 in adults (34—70 years) (Olsen *et al.*, 2003b). However, PFOS has been found in the higher concentration in liver compared to other organs (Pérez *et al.*, 2013). Taken together, these finding suggest similar concentrations in serum and tissues and it is unlikely that the comparison between maternal plasma (mL) and fetal organs (g) significantly affects the results. The number of fetal organs obtained from the same fetus was limited why a potential organ specific accumulation cannot be evaluated.

Results

198 Thirty-nine women were included in this study and a total of 34 placenta samples, 108 fetal organs and 24
199 blood samples were obtained from the participants (for detailed distribution see Fig. 1). The 108 organs
200 were obtained from a total of 36 fetuses aged 37—68 days pc (mean ±SEM, 52 ±1.3)(Fig. 1).
201
202 *3.1. Cotinine concentrations in plasma, placenta, and fetal organs*
203 A plasma cut-off value of 3 ng/mL was used to discriminate between smokers and non-smokers (Benowitz
204 *et al.*, 2009). Three participants reported themselves as non-smokers but presented with cotinine levels
205 above the cut-off value. They were grouped as smokers and their questionnaire excluded from analysis.
206 Women who smoked had significantly higher levels cotinine levels in maternal plasma, placenta, and fetal
207 organs compared to non-smokers ($p<0.001$, $p<0.0001$, $p<0.0001$, respectively). Mean plasma levels (±SEM)
208 in smokers were 99.3 ng/g ± 26.9, range: 6.2—326.1 ng/g, and in non-smokers 0.2 ng/g ± 0.1; range: 0—1.1
209 ng/g (Table 2). Cotinine concentration in smoke-exposed placentas was 107.8 ng/g ±22.3; 3.8—374.7 (mean
210 ±SEM; range) and in non-exposed placentas 0.4 ng/g ±0.1; 0.0—1.8 (mean ±SEM; range) (Table 2). Cotinine
211 levels in smoke-exposed and non-exposed fetal organs were 61.1 ng/g ±9.2; 1.1—336 (mean ±SEM; range)
212 ($p<0.0001$) and 0.4 ng/g (range: 0—2.6), respectively (Table 2). Cotinine was detected in all different fetal
213 organs exposed to cigarette smoke (Fig. 2). Cotinine was hardly detectable in the placentas and fetal organs
214 of non-smokers (Fig. 2). In the group who smoked, a significant positive correlation was found between
215 fetal age and the fetal to maternal cotinine ratio ($p=0.0168$) (Fig 3.). There were no association between the
216 number of cigarettes smoked and the cotinine concentration in fetal tissue ($p>0.1$) (data not shown).

217 Maternal plasma, placenta and fetal organs from the same women were available in 21 cases. From each
218 woman, the mean cotinine concentration (ng/g) in fetal organs was calculated and the overall mean
219 presented (Table 3). The percentage was calculated as concentrations in fetal organs and placenta
220 respectively in relation to the corresponding maternal plasma (100%) (Table 3).

221 *3.2. PFASs concentrations in maternal plasma, placenta, and fetal organs*
222 Significant positive correlations between fetal age and the fetal to maternal ratio of all five PFASs were
223 found: PFOS $p=0.0008$, 95% CI [0.33—0.86]; PFOA $p=0.0246$, 95% CI [0.06—0.77], PFNA $p=0.0106$, 95% CI
224 [0.13—0.80]; PFUnDA $p=0.0197$, 95% CI [0.08—0.77]; PFDA $p=0.0390$, 95% CI [0.01—0.75] (Fig. 3). Further,
225 significantly linear correlations were found for PFOS ($p=0.0011$), PFNA ($p=0.0243$), and PFUnDA ($p=0.0467$)
226 (Fig. 3).
227 The concentration of PFASs in maternal plasma, placenta, and fetal organs was presented in Table 2. The
228 PFAS levels in plasma from women who smoked were higher compared to women who did not smoke,

though not at a significant level for any of the PFASs. All five measured PFASs were present in the evaluated fetal organs. No association between maternal plasma cotinine levels and plasma PFASs was found ($p>0.1$).

Maternal plasma, placenta tissue and fetal organs were available for each of 21 cases. PFASs in placenta and fetal organs were greatly reduced compared to maternal plasma. The relative concentrations of PFOS, PFOA, PFNA, and PFUnDA in placentas were 11—15% of the concentration found in maternal plasma, and were further reduced to 5—13% in fetal organs (Table 3). PFDA was detected in relatively higher concentration in placenta (43%) and fetal organs (27%) compared the other PFASs (Table 3). PFOS in maternal plasma was significantly higher compared to the other PFASs measured. Interestingly, PFOS was detected in the lowest relative concentration in fetal organs (5%) (Table 3).

3.3. Correlation between lifestyle and PFAS levels

Maternal characteristic and life-style habits were presented in Table 1. The concentrations of PFNA and PFUnDA showed a significant negative association with BMI ($p=0.0391$; $p=0.0085$, respectively) (Fig. 4), while there was no significant association between BMI and fetal PFASs levels ($p>0.1$) and between PFASs and maternal cigarette smoking, maternal age, or rual/urban residence ($P>0.1$).

Further, a positive correlation (over 50%) was found between first-hand smoking and second-hand smoking ($r=0.65$, $p<0.0001$), second-hand smoke smoking also correlated positively with fathers smoking habits ($r=0.50$, $p=0.004$) (Supp. Table 1). Alcohol consumption correlated positively with soft drink consumption ($r=0.57$, $p=0.0002$) (Supp. Table 1). Use of over the counter medication correlated positively with use of prescription medicine ($r=0.56$, $p=0.0003$) (Supp. Table 1). There was no significant correlation between any other pairs of measured parameters (Supp. Table 1).

4. Discussion

These data demonstrate that PFASs and cotinine are transferred from mother to fetus during the first trimester of pregnancy and that fetal PFASs levels increase with fetal age, suggesting that these substances may accumulate in the fetus during gestation. All five evaluated PFASs were significantly higher in maternal circulation as compared to fetal levels. Further a negative correlation between maternal BMI and plasma concentrations of PFNA and PFUnDA were found supporting previous reports (Lauritzen *et al.*, 2016). Collectively, these data provide information on the exact PFAS concentrations present in fetal organs, placenta, and maternal plasma during first trimester of pregnancy together with the transfer rate from mother to fetus.

4.1. Fetal age correlated positively with fetal concentrations of all five PFASs

Fetal age correlated positively with fetal concentrations of all five PFASs evaluated, suggesting that these compounds accumulate in the fetal tissues and may continue to increase during pregnancy. It has been shown that phthalates and PFOS accumulate in the human amniotic fluid during the second trimester at almost 10% per gestational week (Jensen *et al.*, 2012), supporting the present findings. Given that the development of keratinized epidermal skin is first seen in human fetuses from week 22 pc (Hardman *et al.*, 1999), the fetal skin will prior to this age be permeable and chemicals from the amniotic fluid may be absorbed by this route. Hence in early pregnancy the fetus may be exposed to PFASs from both placental blood and from the amniotic fluid.

4.2. PFASs in human fetal organs in relation to maternal levels

The five PFASs were detected in all the different fetal organs evaluated indicating that fetuses are systemically exposed to these compounds. Although the majority of the organogenesis are completed around weeks six to eight (Zhou *et al.*, 2012), the organs are still at an early immature stage, and an organ-specific accumulation is not expected at this early age. In adults, PFOA and PFOS accumulate in liver and bone structures (Pérez *et al.*, 2013), further all the five PFASs evaluated in the present study have been detected in adult liver tissue (Kärrman *et al.*, 2010) suggesting that the tissue accumulation the present study find in fetal life continues in adulthood.

The concentrations of PFOS, PFOA, PFNA, and PFUnDA in placenta were reduced to 11—15% of the concentrations found in the maternal circulation. These levels were further attenuated across the placenta to the fetal organs, to a level of 5—13% of the concentration found in maternal plasmas, indicating that the fetal exposure to these compounds was 7—20 times lower than the maternal levels. Of the PFASs examined, PFDA were found in the lowest concentrations in maternal plasma, but showed the relative highest concentration in both placenta (43%) and fetal tissues (27%) as compared to maternal plasma. Although the assay of measurement is close to the detection limit, these values are within the standard curve and are considered valid. These data suggest that PFASs accumulate in fetal tissue with different efficiency. The differences in in fetal uptake may be due to different placental clearance or fetal age.

Previously, fetal concentrations of PFOS, PFOA, and PFNA have been estimated from the concentrations measured in the umbilical cord blood (Monroy *et al.*, 2008; de Cock *et al.*, 2014; Manzano-Salgado *et al.*, 2015). Our results help qualify estimations of fetal exposure by providing actual measured levels. The present study detected actual fetal concentration of PFASs to be 3—12 times lower than the previous estimated from umbilical cords, which may either reflect that fetal PFAS levels increase during gestation

(the present study find a significantly positive correlation between PFASs and fetal age), or indicate that the actual fetal exposure may be less than previously anticipated.

4.3. PFAS levels in maternal plasma

In maternal plasma, PFOS were present in the highest concentration of all PFASs measured followed by PFOA. The lowest concentrations were found in PFNA, PFUnDa, and PFDA, respectively. These findings were reflected in the levels measured in the fetal organs, where PFOS and PFOA also were preset in highest concentrations followed by PFNA, PFUnDa, and PFDA. The maternal PFAS levels support previous measurements from pregnant women (Monroy *et al.*, 2008; Okada *et al.*, 2013, 2014; Cho *et al.*, 2015; Manzano-Salgado *et al.*, 2015; Papadopoulou *et al.*, 2015; Callan *et al.*, 2016; Wang *et al.*, 2016) except for PFUnDA, which has been detected both in higher and lower concentrations (Okada *et al.*, 2013; Callan *et al.*, 2016; Wang *et al.*, 2016). Literature is not conclusive with regards to the concentration of PFOS and PFOA in plasma. In a study of 1,400 women, plasma levels of PFOS and PFOA were 4 and 8 times higher than the present study (Fei *et al.*, 2007) whereas Hannsen and colleagues reported 5 and 1.5 times the concentrations of the present study (Hanssen *et al.*, 2010). These differences may be explained by variations in local exposure or by the year in which the samples were taken; during the last decade changing levels of PFASs have been observed (Glynn *et al.*, 2012; Olsen *et al.*, 2012).

We found a negative correlation between maternal BMI and the levels of PFNA and PFUnDA in maternal plasma, though the correlation disappeared when compared to fetal levels, suggesting that maternal BMI does not affect the PFNA and PFUnDA levels in the fetus. Maternal cigarette smoking was associated with slightly higher plasma concentration of all five PFASs, but not to a significant level. Conflicting results of the association between maternal cigarette smoking and maternal PFASs levels has been reported (Cho *et al.*, 2015; Lauritzen *et al.*, 2016, 2017) and the effect of maternal cigarette smoke may be questionable. Nevertheless, this association was not reflected in placenta and fetal tissues, suggesting that smoking did not affect the levels of PFASs transferred to the fetus. The included women were recruited from both rural and urban areas, and no association was found between where the women were resident and her plasma levels of PFASs, suggesting that exposure to pollutants from urban living does not impact PFAS levels in pregnant women in Denmark.

4.4. Health compromising PFASs levels

Dietary intake has been suggested as the primary source of PFASs exposure (Domingo, 2012; Lauritzen *et al.*, 2016) with the largest contribution coming from meat, animal fat, and snacks (Halldorsson *et al.*, 2008). The EFSA CONTAM panel has established a tolerable daily intake (TDI) for PFOS and PFOA of 150 ng/kg/day and 15 µg/kg/day, respectively, based on the lowest non-observed adverse effect level identified in animal

exposure studies. The estimated intake of PFOS and PFOA in the present study was 2.7 and 2.1 respectively, which is well below advised TDI. In the Swedish adult population the mean dietary PFAS exposure has been estimated to 0.6—8.5 ng/kg/day (Domingo, 2012), which is also well below the estimated health compromising levels and suggests a minimal health risk at these levels in Scandinavian adults. PFOS and PFOA plasma levels in Swedish women were 10.2 ng/mL and 2.9 ng/mL, respectively (Axmon *et al.*, 2014), which is slightly higher than the maternal plasma levels in the present study. This can be interpreted as neither the Swedish nor the present Danish plasma levels being health compromising in adult women, given that uptake from other sources than diet is insignificant. However, the health compromising levels of PFASs during fetal live is to our knowledge not defined. In pregnant women, plasma PFOA levels above 3.9 ng/mL was significantly associated with reduced birth weight (Fei *et al.*, 2007), suggesting that health compromising levels may be lower in fetal life compared to adulthood.

4.5. PFASs and lifestyle

We did not find a correlation between the evaluated self-reported lifestyle parameters except between smoking, second-hand smoke and fathers smoking habits, indicating that smokers are more likely to be surrounded by other smokers, and may therefore expose their fetus to more cigarette smoke than their personal cigarette consumption indicates. For all other aspects the lifestyles of the participants were similar. The variation in PFAS concentrations between fetuses could not be explained by the monitored lifestyle parameters: smoking habits, coffee-, tea-, soft drink, soft drink light -consumption, or exercise. Fetal age was found to significantly correlate with PFAS levels in fetal organs and may be the most likely explanation for the variation between fetuses. Differences in placental clearance may also impact on the fetal PFAS levels.

4.6. Cotinine concentration in maternal plasma, placenta, and fetal organs

Among smokers, cotinine was detected in significantly higher concentrations in fetal organs compared to PFASs. Cotinine accumulated in placenta and 106% of the concentration found in maternal plasma was detected in placenta. The concentrations in smoke-exposed fetal tissues were reduced to 52% of the concentration found in maternal plasma, indicating that cotinine crosses the placenta relatively easy and reaches the fetal circulation. The mean concentration of cotinine in smoke-exposed fetuses was 61.1 ng/g, which was very close to the concentration previously measured in serum from newborns of heavy smokers (59.1 ng/mL) (Ivorra *et al.*, 2014). This may indicate that even though cotinine reaches the fetus in high concentrations, its clearance is rapid (half-life: 16 hours, total clearance: 48 hours (Benowitz and Jacob, 1994)) compared to PFASs (≈4 years). Where PFAS tends to accumulate in the fetus, cotinine levels may

rather reflect the actual maternal level, which diffuses to the fetus. The highest cotinine concentration was detected in fetal liver, which was close to the same concentration found in the placenta, indicating that cotinine may accumulate in the liver. The lowest cotinine concentration was found in the ribs, likely reflecting that cotinine reaches more perfused organs easier than bone structures.

5. Conclusions

In conclusion, the present study provides actual measurements on PFASs and cotinine concentrations in human fetuses, together with comparisons to placenta and maternal plasma samples. Fetal age was positively correlated with all evaluated PFASs, suggesting a fetal accumulation over time. In maternal plasma PFOS levels were more than 4 times higher than PFOA, which were twice as high as PFDA, PFUnDa and PFDA. The PFOS, PFOA, PFNA, and PNUnDA levels were reduced in placenta to 11—15% of the concentration found in maternal plasma, and further reduced to 5—13%. In contrast PFDA, which was present in the lowest concentrations of the evaluated PFASs in maternal plasma, was detected in the relatively highest concentration in the fetus (27%), suggesting that the placenta retains PFASs with different efficiency. These data suggest that fetal PFAS levels increase with fetal age, though the health compromising level of these substances in fetal life is unknown.

Ethical approval

'The Scientific Ethical Committee for the Capital Region' [KF (01) 258206] and [KF (01) 170/99] has given their approval for this study. All participants gave informed consent before taking part and have given written consent to their data can be included in publications.

Authors' roles

L.S.M. was responsible for writing the paper, collected the human fetuses and placenta samples, prepared samples for chemical analysis, did the statistics, and interpreted data. B.A.G.J. and C.H.L. was responsible for the design of the chemical analysis and interpreted data. R.O. did the scanning during the evacuation procedure. A.L. assisted collecting fetal material and placenta samples. E.E. was responsible for the surgical procedure of terminating pregnancies and consulted the participating women in prior to the operation for completion of questionnaires and obtained the blood samples. T.W.K interpreted data, assisted in the statistical correlation analysis, and assisted in writing the paper. C.Y.A. interpreted data, assisted in writing the paper and was responsible for the study design. All authors approved the last version of the manuscript.

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520 **Figure Legends:**

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522 **Figure 1**. Study population, plasma and placenta samples, and fetuses included.

523 **Figure 2.** Concentrations of cotinine and PFASs in maternal plasma (A) and in placenta and different fetal
524 organs (B). Error bars represent min. and max. values. Maternal plasma cotinine (n=14); Cotinine NS: non-
525 smokers; S: Smokers; CT: Connective tissue.*Cotinine concentrations in fetuses exposed to maternal
526 cigarette smoke (n=21).

527 **Figure 3.** Mean concentration of PFOS and PFNA per fetus in relation to fetal age in days post conception
528 (pc). Solid lines are unadjusted regression lines, dotted lines are error bars. A significant positive correlation
529 between fetal age and PFOS and PFNA were found.

530 **Figure 4.** Concentration of PFNA and PFUnDA in maternal plasma in relation to maternal body mass index
531 (BMI). Solid lines are unadjusted regression lines, dotted lines are error bars. A significant positive
532 correlation between fetal age and PFOS and PFNA were found.

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