

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

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

20

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22 The financial supporters had no role in the study design, collection and analysis of data, data interpretation  
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25 **Conflict of interest**

26 None declared.

27

28 **ABSTRACT**

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

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

38 **Methods:** A total of 39 Danish women who underwent legal termination of pregnancy before gestational  
39 week 12 were included; 24 maternal blood samples were obtained together with 34 placenta tissue and  
40 108 fetal organs. PFASs and cotinine were assayed by liquid chromatography/ triple quadrupole mass  
41 spectrometry.

42 **Results:** In fetal organs perfluorooctanesulfonic acid (PFOS) 0.6 ng/g, perfluorooctanoic acid (PFOA) 0.2  
43 ng/g, perfluorononanoic acid (PFNA) 0.1 ng/g, perfluoroundecanoic acid (PFUnDa) 0.1 ng/g, and  
44 perfluorodecanoic acid (PFDA) 0.1 ng/g was detected. In fetal organs the mean concentrations of PFOS,  
45 PFOA, PFNA, and PFUnDA were reduced to 5–13% of the concentration found in maternal plasma; PFDA  
46 was reduced to 27%. A significant positive correlation was found between fetal age and fetal levels for all  
47 five PFASs evaluated. A significant positive correlation was also found between fetal age and fetal cotinine  
48 levels. A significant negative correlation was found between maternal BMI and maternal plasma PFNA and  
49 and PFUnDA concentrations. Smokers presented with 99 ng/g cotinine in plasma, 108 ng/g in placenta, and  
50 61 ng/g in fetal organs, non-smokers showed cotinine levels below 0.2 ng/g in all evaluated compartments.  
51 No correlation between maternal cotinine levels and PFASs levels were found.

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

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

58

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

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

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67

## 68 Introduction

69 Perfluoroalkyl substances (PFASs) are slowly degradable pollutants and belong to the group of water- and  
70 grease resistant fluorosurfactants used for many industrial and consumer applications, like outdoor clothes,  
71 non-stick cookware, food packaging, electronics, stain-resistant carpets and in fire-fighting foams (Key *et al.*,  
72 1997; Jensen and Leffers, 2008; De Solla *et al.*, 2012). The PFASs include perfluorooctanesulfonic acid  
73 (PFOS), perfluorooctanoic acid (PFOA), perfluorononanoic acid (PFNA), perfluorodecanoic acid (PFDA) and  
74 perfluoroundecanoic acid (PFUnDA), all of which suspected to have negative impact on fetal growth and  
75 development and may disturb the endocrine system ( de Cock *et al.*, 2014; Johnson *et al.*, 2014; Bach *et al.*,  
76 2015 ). Prenatal exposure to PFOS and PFOA has been associated with an increased risk of congenital  
77 cerebral palsy in Danish boys (Liew *et al.*, 2014) and maternal exposure to PFNA and PFDA has been  
78 associated with increased risk of pregnancy lost (Jensen *et al.*, 2015). In rodents, prenatal exposure to high  
79 doses of PFOS and PFOA reduced postnatal survival and birth weight, and disturbed lactation and growth of  
80 the pups (Lau *et al.*, 2004; Olsen *et al.*, 2009) and both pre and postnatal PFASs exposure have been  
81 associated with hypothyroidism and significant decreased T4 levels in pups (Yu *et al.*, 2009).

82 PFASs, and particularly PFOA and PFNA, have been shown to induce synthesis of the estrogen-responsive  
83 biomarker protein vitellogenin leading to an estrogen-like activity *in vivo* in rainbow trout (Benninghoff *et al.*,  
84 2011).

85

86 Notably, PFASs half-life in rats is as short as a few days (Kudo *et al.*, 2002), compared to 200 days in the  
87 cynomolgus monkey (Seacat *et al.*, 2002), and 2.5—4.5 years in humans (Olsen *et al.*, 2007; Zhang *et al.*,  
88 2013), suggesting a large difference in the elimination kinetics between species, and therefore animal  
89 models may only reflect the human situation to a limited extent.

90

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

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

109  
110 Maternal cigarette smoking, together with other lifestyle parameters, may impact maternal PFAS levels  
111 (Lauritzen *et al.*, 2016), why the present study included maternal smoking and other lifestyle factors in  
112 order to evaluate if maternal lifestyle affected maternal and fetal PFAS levels. Cotinine is the primary  
113 metabolite of nicotine and is a valid biomarker used to discriminate smokers from non-smokers (Benowitz  
114 *et al.*, 2003). The adverse effects that maternal smoking has on the unborn child is well-known and widely  
115 described (Mund *et al.*, 2013). Plasma cotinine concentrations in newborns have been reported to be  
116 approximately 60 ng/mL in children of heavy smokers, 30 ng/mL in children from moderate smokers, and 3  
117 ng/mL in children from non-smokers (Ivorra *et al.*, 2014). The actual concentrations in human fetuses have  
118 not previously been measured.

119  
120 The present study is, to our knowledge, the first to measure the actual concentrations of PFASs and  
121 cotinine in human first trimester fetuses. These findings provide (i) important new knowledge on the  
122 perfusion of PFASs and cotinine from mother over the early placenta barrier to fetal circulation and organs,  
123 and (ii) evaluate if fetal age is associated with fetal PFASs levels indicating a fetal accumulation over time  
124 and (III) whether maternal cigarette smoking affects PFASs accumulation in mother and fetus.

## 125 **Materials and Methods**

### 126 *2.1. Participating women*

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

## 133 2.2. Human fetal tissues and maternal blood samples

134 Legal abortions were performed at the Department of Obstetrics and Gynaecology, University Hospital  
135 Skejby, Denmark and at Department of Obstetrics and Gynaecology, Regional Hospital Randers, Denmark,  
136 in collaboration with the Laboratory of Reproductive Biology, Rigshospitalet, Denmark. All fetuses were  
137 morphologically normal. Within one hour after the surgical procedure the fetal organs and placenta tissue  
138 were isolated, washed in sterile saline snap frozen on dry ice, and stored at  $-80^{\circ}\text{C}$  until analysis. Fetal  
139 organs and placenta tissue processed for freezing more than one hour after collection were not included in  
140 the present study. Fetal age was measured by crown-rump lengths via ultrasound in connection with the  
141 surgical procedure. Gestational age was converted to age post conception by subtracting two weeks.  
142 Maternal blood samples were obtained in connection with anaesthesia prior to surgery, collected in  
143 ethylenediamine tetraacetic acid (EDTA) tubes, and kept on ice until centrifugation ( $4.000g$  for 15 min) to  
144 isolate plasma. Plasma was aliquoted to  $200\ \mu\text{L}$  microinserts in 1.5 mL vials (Skandinaviska GenTec, Västra  
145 Frölunda, Sweden) and stored at  $-20^{\circ}\text{C}$  until analysis.

146

## 147 2.3. Tissue processing

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

154

## 155 2.4. Analysis of PFASs and cotinine

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

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

175

## 176 *2.6. Statistical methods*

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

185

## 186 *2.7. Limitations*

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

196

## 197 **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  $\pm$ SEM, 52  $\pm$ 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 ( $\pm$ SEM)  
208 in smokers were 99.3 ng/g  $\pm$  26.9, range: 6.2—326.1 ng/g, and in non-smokers 0.2 ng/g  $\pm$  0.1; range: 0—1.1  
209 ng/g (Table 2). Cotinine concentration in smoke-exposed placentas was 107.8 ng/g  $\pm$ 22.3; 3.8—374.7 (mean  
210  $\pm$ SEM; range) and in non-exposed placentas 0.4 ng/g  $\pm$ 0.1; 0.0—1.8 (mean  $\pm$ SEM; range) (Table 2). Cotinine  
211 levels in smoke-exposed and non-exposed fetal organs were 61.1 ng/g  $\pm$ 9.2; 1.1—336 (mean  $\pm$ 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,



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

231  
232 Maternal plasma, placenta tissue and fetal organs were available for each of 21 cases. PFASs in placenta  
233 and fetal organs were greatly reduced compared to maternal plasma. The relative concentrations of PFOS,  
234 PFOA, PFNA, and PFUnDA in placentas were 11–15% of the concentration found in maternal plasma, and  
235 were further reduced to 5–13% in fetal organs (Table 3). PFDA was detected in relatively higher  
236 concentration in placenta (43%) and fetal organs (27%) compared the other PFASs (Table 3). PFOS in  
237 maternal plasma was significantly higher compared to the other PFASs measured. Interestingly, PFOS was  
238 detected in the lowest relative concentration in fetal organs (5%) (Table 3).

239  
240 *3.3. Correlation between lifestyle and PFAS levels*

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

245  
246 Further, a positive correlation (over 50%) was found between first-hand smoking and second-hand smoking  
247 ( $r=0.65$ ,  $p<0.0001$ ), second-hand smoke smoking also correlated positively with fathers smoking habits  
248 ( $r=0.50$ ,  $p=0.004$ ) (Supp. Table 1). Alcohol consumption correlated positively with soft drink consumption  
249 ( $r=0.57$ ,  $p=0.0002$ ) (Supp. Table 1). Use of over the counter medication correlated positively with use of  
250 prescription medicine ( $r=0.56$ ,  $p=0.0003$ ) (Supp. Table 1). There was no significant correlation between any  
251 other pairs of measured parameters (Supp. Table 1).

252  
253 **4. Discussion**

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

262 *4.1. Fetal age correlated positively with fetal concentrations of all five PFASs*

263 Fetal age correlated positively with fetal concentrations of all five PFASs evaluated, suggesting that these  
264 compounds accumulate in the fetal tissues and may continue to increase during pregnancy. It has been  
265 shown that phthalates and PFOS accumulate in the human amniotic fluid during the second trimester at  
266 almost 10% per gestational week (Jensen *et al.*, 2012), supporting the present findings. Given that the  
267 development of keratinized epidermal skin is first seen in human fetuses from week 22 pc (Hardman *et al.*,  
268 1999), the fetal skin will prior to this age be permeable and chemicals from the amniotic fluid may be  
269 absorbed by this route. Hence in early pregnancy the fetus may be exposed to PFASs from both placental  
270 blood and from the amniotic fluid.

271

272 *4.2. PFASs in human fetal organs in relation to maternal levels*

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

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

289 Previously, fetal concentrations of PFOS, PFOA, and PFNA have been estimated from the concentrations  
290 measured in the umbilical cord blood (Monroy *et al.*, 2008; de Cock *et al.*, 2014; Manzano-Salgado *et al.*,  
291 2015). Our results help qualify estimations of fetal exposure by providing actual measured levels. The  
292 present study detected actual fetal concentration of PFASs to be 3–12 times lower than the previous  
293 estimated from umbilical cords, which may either reflect that fetal PFAS levels increase during gestation

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

#### 296 4.3. PFAS levels in maternal plasma

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

310 We found a negative correlation between maternal BMI and the levels of PFNA and PFUnDA in maternal  
311 plasma, though the correlation disappeared when compared to fetal levels, suggesting that maternal BMI  
312 does not affect the PFNA and PFUnDA levels in the fetus. Maternal cigarette smoking was associated with  
313 slightly higher plasma concentration of all five PFASs, but not to a significant level. Conflicting results of the  
314 association between maternal cigarette smoking and maternal PFASs levels has been reported (Cho *et al.*,  
315 2015; Lauritzen *et al.*, 2016, 2017) and the effect of maternal cigarette smoke may be questionable.  
316 Nevertheless, this association was not reflected in placenta and fetal tissues, suggesting that smoking did  
317 not affect the levels of PFASs transferred to the fetus. The included women were recruited from both rural  
318 and urban areas, and no association was found between where the women were resident and her plasma  
319 levels of PFASs, suggesting that exposure to pollutants from urban living does not impact PFAS levels in  
320 pregnant women in Denmark.

#### 321 4.4. Health compromising PFASs levels

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

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

337

#### 338 4.5. PFASs and lifestyle

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

348

#### 349 4.6. Cotinine concentration in maternal plasma, placenta, and fetal organs

350 Among smokers, cotinine was detected in significantly higher concentrations in fetal organs compared to  
351 PFASs. Cotinine accumulated in placenta and 106% of the concentration found in maternal plasma was  
352 detected in placenta. The concentrations in smoke-exposed fetal tissues were reduced to 52% of the  
353 concentration found in maternal plasma, indicating that cotinine crosses the placenta relatively easy and  
354 reaches the fetal circulation. The mean concentration of cotinine in smoke-exposed fetuses was 61.1 ng/g,  
355 which was very close to the concentration previously measured in serum from newborns of heavy smokers  
356 (59.1 ng/mL) (Ivorra *et al.*, 2014). This may indicate that even though cotinine reaches the fetus in high  
357 concentrations, its clearance is rapid (half-life: 16 hours, total clearance: 48 hours (Benowitz and Jacob,  
358 1994)) compared to PFASs ( $\approx$ 4 years). Where PFAS tends to accumulate in the fetus, cotinine levels may

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

363

## 364 **5. Conclusions**

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

## 375 **Ethical approval**

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

## 379 **Authors’ roles**

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

389

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519

520 **Figure Legends:**

521

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 PUnDA 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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