Assessing the relationship between perfluoroalkyl substances, thyroid hormones and binding proteins in pregnant women; a longitudinal mixed effects approach

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Abstract

The mechanisms involved in thyroid homeostasis are complex, and perfluoroalkyl substances (PFASs) have been indicated to interfere at several levels in this endocrine system. Disruption of the maternal thyroid homeostasis during early pregnancy is of particular concern, where subclinical changes in maternal thyroid hormones (THs) may affect embryonic and foetal development.

The present study investigated associations between THs, thyroid binding proteins (TH-BPs) and PFAS concentrations in pregnant women from Northern Norway.

Women participating in The Northern Norway Mother-and-Child contaminant Cohort Study (MISA) donated a blood sample at three visits related to their pregnancy and postpartum period (during the second trimester, 3 days and 6 weeks after delivery) in the period 2007-2009. Participants were assigned to quartiles according to PFAS concentrations during the second trimester and mixed effects linear models were used to investigate potential associations between PFASs and repeated measurements of THs, TH-BPs, thyroxin binding capacity and thyroid peroxidase antibodies (anti-TPO).

Women within the highest perfluorooctane sulfonate (PFOS) quartile had 24% higher mean concentrations of thyroid stimulating hormone (TSH) compared to the first quartile at all sampling points. Women within the highest quartiles of perfluorodecanoate (PFDA) had 4% lower mean concentrations of triiodothyronine (T3) and women within the highest quartile of perfluoroundecanoate (PFUnDA) had 3% lower mean concentrations of free triiodothyronine (FT3). Further, the difference in concentrations and the changes between three time points were the same for the PFAS quartiles. Thyroxin binding capacity was associated with all the THs and TH-BPs, and was selected as a holistic adjustment for individual changes in TH homeostasis during pregnancy. Finally, adjusting for maternal iodine status did not influence the model predictions.

Findings in the present study suggest modifications of TH homeostasis by PFASs in a background exposed maternal population. The variation in levels of THs between PFAS quartiles were within normal reference ranges and may not be of clinical significance in the pregnant woman. However, subtle individual changes in maternal THs may have significant consequences for foetal health.

Abbreviations:

Anti-TPO, Anti-thyroid peroxidase antibodies; HTP, Hypothalamic pituitary; LOD, Limit of detection; MISA, The Northern Norway Mother-and-Child contaminant Cohort Study; PFASs, Poly- and perfluoroalkyl substances; PFDA, Perfluorodecanoate; PFDoDA, Perfluorododecanoate; PFHpS, Perfluoroheptane sulfonate; PFHxS, Pefluorohexane sulfonate; PFNA, Perfluorononanoate; PFOA, Perfluorooctanoate; PFOS, Perfluorooctane sulfonate; PFUnDA, Perfluoroundecanoate; PLS, Partial least square; T3, Triiodothyronine; FT3, Free triiodothyronine; T4, Thyroxin; FT4, Free thyroxin; T-Uptake, Thyroxin binding capacity; TBG, Thyroid binding globulin; TH, Thyroid hormone; TH-BP, Thyroid hormone binding protein; TSH, Thyroid stimulating hormone; TTR, Transtyretin; UHPLC-MS/MS, Ultrahigh pressure liquid chromatography triple–quadrupole mass-spectrometry.

Keywords: Thyroid hormones; perfluoroalkyl substances; pregnant women; thyroxin binding capacity; endocrine disruption

1. Introduction

Thyroid hormones (THs) like thyroid stimulating hormone (TSH), thyroxin (T4) and triiodothyronine (T3), are involved in numerous physiological processes e.g. regulation of metabolism, bone remodelling, cardiac function and mental status in the adult. For the embryo and foetus, THs are crucial in all developmental stages. The onset of foetal thyroid function is at approximately 20 weeks gestation, and thus prior to this, maternal T4 is the sole source of TH to the developing foetal brain (Morreale De et al., 2004). In adults, THs are produced in the thyroid gland and transported to peripheral target tissues aided by thyroid hormone binding proteins (TH-BPs) e.g. thyroid binding globulin (TBG), transthyretin (TTR), and albumin. The thyroid function is regulated by negative feedback mechanisms, in which TSH stimulates the thyroid to synthesize T4 which is further converted to T3. TSH is in turn regulated by the hypothalamus as well as by the levels of circulating T3 and T4. In healthy individuals, serum levels of THs are maintained relatively stable with individuals having his or her specific set point (Feldt-Rasmussen et al., 1980).

During the first two trimesters of pregnancy, marked changes are seen in the maternal hypothalamic pituitary (HTP) thyroid axis to increase the availability of THs. In short, these changes lead to a two- to three-fold increase in TBG production and a subsequent decrease in levels of free thyroxin (FT4) and free triiodothyronine (FT3) followed by an increased production of T3 and T4. The increase in T3 and T4 is less than the increase in TBG, resulting in a decreased T4/TBG ratio, creating a state of relative hypothyroxinemia. Hence, these adaptions mimic hyperthyroidism, but thyroid function per se does not change during pregnancy. There is uncertainty regarding reference ranges for thyroid tests during pregnancy as pregnancy-induced changes in thyroid physiology affects laboratory interpretation and presently no universally accepted reference ranges exist (Fitzpatrick and Russell, 2010). Changes in individual TH levels throughout pregnancy varies by gestational age, number of foetuses and study population, but generally, the woman achieves a new steady state in HTP function at the end of 2nd trimester which is maintained until delivery. After delivery, the alterations in thyroid processes are gradually reversed over 4-6 weeks (Blackburn, 2013).

Endocrine systems like the thyroid are susceptible to disruption by naturally-occurring and man-made compounds, possibly by affecting the hormone homeostasis through carrier proteins and receptors. One group of potential endocrine disrupting chemicals are poly- and perfluoroalkyl substances (PFASs). PFASs are persistent substances that have been directly emitted to the environment, intentionally or as by-products, during their production and use (Prevedouros et al., 2006). Diet is currently suspected to be the major on-going exposure pathway of PFASs for humans (Fromme et al., 2009; Haug et al., 2011a; Vestergren and Cousins, 2009). In addition, these chemicals are passed to humans through air, house dust, drinking water and water based beverages (Eschauzier et al., 2013; Haug et al., 2011a; Haug et al., 2011b; Ullah et al., 2011).

Scientific and public concern regarding PFASs, are their potential to perturb maternal hormonal homeostasis and subsequently affect pregnancy outcome by increasing the risk of spontaneous abortions, placental disruptions, foetal distress, malformations, prematurity, decreased birth weight, and hypertension (Boas et al., 2012; Morreale De et al., 2000; Morreale De et al., 2004; Stahl et al., 2011). Disruption of the maternal thyroid homeostasis during early pregnancy is of particular concern, where subclinical changes in maternal THs may affect embryonic and foetal development (Boas et al., 2012). Compared to the wide population reference ranges for THs, the range of variation within each individual are narrower. Hence, subtle changes in the individual set point of thyroid homeostasis may have significant effects, especially if occurring during critical developmental periods (Feldt-Rasmussen et al., 1980).

T3 and T4 are the only biological molecules which are halogenated (iodine). Similarly, PFASs are halogenated (fluorine) with active sites that resembles those of T3 and T4 (Preau et al., 2014). When assessing effects of PFASs on TH homeostasis, the relevant mechanisms of disruption are; i) disturbance of the overall activity of the thyroid gland by interference with the TH receptors, ii) stimulation or inhibition of enzyme functions which mediates iodine uptake of the thyroid gland in the synthesis of T3 and T4, and iii) competitive displacement of THs on their binding proteins (Boas et al., 2012). Disruption of the thyroid function is often investigated in regards to hypothyroidism with the reporting of TSH concentrations. TSH levels can reflect mild thyroid functional impairment even when T4 and T3 concentrations are within normal ranges but hypothyroxinemia can still occur with normal TSH and T3 concentrations. Hence,

in the absence of assessment of the overall thyroid function; the clinical importance of individual TH levels is unclear (Braverman and Utiger, 1986). Therefore, the present study aims to investigate the overall thyroid function in relation to PFAS concentrations by investigating associations between all the THs (TSH, T3, T4, FT3, FT4), thyroxin binding capacity, anti-thyroid peroxidase antibodies (anti-TPO), thyroid hormone binding proteins (TH-BPs) (TBG, TTR and albumin) at three time points; 2nd trimester of pregnancy, 3 days and 6 weeks after delivery and PFAS concentrations in women from Northern Norway.

2. Materials and methods

2.1 Study participants and collection of blood samples

The selected subjects in the present study represent the 391 women who completed the Northern Norway Mother-and-Child Contaminant Cohort Study (MISA) which consists of 515 enrolled pregnant women, recruited from June 2007 to October 2009 (recruitment period; 867 days). All participants answered a detailed questionnaire about diet and lifestyle at enrolment, and donated a blood sample at three visits/time points related to their pregnancy (around gestational week 18, 3 days and 6 weeks after delivery). Detailed information about the study group characteristics, ethical approvals, the food frequency questionnaire (FFQ) and the blood collection procedures have been reported elsewhere (Hansen et al., 2010; Veyhe et al., 2012).

2.2 Chemical analyses

2.2.1 PFAS analyses

Blood samples donated at median gestational week 18 (ranging 10–34) were analysed for a variety of PFASs. A total of 26 PFASs were initially screened for in a sub-group of 50 serum samples. Compounds detected above the limit of detection (LOD) in more than 20% of the samples were further quantified in the remaining serum samples (N = 391). Detailed information about the compounds, sample preparation, extraction method, analytical method, reagents and instrumentation has been reported elsewhere (Berg et al., 2014; Hanssen et al., 2013). Briefly, PFASs were determined in serum samples using sonication-facilitated liquid–liquid extraction, activated ENVI-carb clean-up (Powley et al., 2005) and analysed by ultrahigh pressure liquid chromatography triple–quadrupole mass-spectrometry (UHPLC-MS/MS).

Quantification of the compounds was performed by the internal standard addition method with isotope-labeled PFASs (Hanssen et al., 2013). Further details regarding quality control have been reported elsewhere (Berg et al., 2014)

2.2.2 TH and TH-BP analyses

Determination of TH, TH-BP, thyroxin binding capacity and anti-TPO concentrations in non-fasting serum samples from three visits (second trimester, 3 days and 6 weeks after delivery) were performed by laboratory staff at the University Hospital of Northern Norway, Department of Laboratory Medicine. The analyses are routine analyses used in the clinic for diagnostic purposes except for T3, T4 and thyroxin binding capacity. Details on the different methods, instrumentation, analytical variation and reference ranges are provided in Table S1 in the Supplemental Material. The laboratory is certified according to ISO 151810 (Norwegian accreditation, 2014) and all reagents, calibrators and equipment were CE-approved. Quality controls are run at three different concentrations every day and additionally the laboratory participates in the LabQuality external quality assessment program (Labquality Finland, 2014).

2.3 Statistical analyses

Statistical analyses were performed using SPSS statistic software, version 22 (IBM SPSS Inc. Chicago, IL, USA). Statistical significance was defined as $p < 0.05$. All PFAS, TH and TH-BP results were log-transformed in the statistical analyses. For PFASs, only compounds with detection frequencies above 80% were evaluated in statistical models where concentrations below LODs were replaced by LOD/$\sqrt{2}$ (Anda et al., 2007). Partial least square (PLS) regressions were used for data reduction and screening for important variables. Mixed effects linear models were used to investigate potential associations between PFASs and three repeated measurement of THs, where the pregnant women were assigned to quartiles according to PFAS concentrations in 2nd trimester. Separate models were built for five dependent variables; TSH, T3, T4, FT3 and FT4. PFAS quartiles and TH-BPs were included as fixed factors and covariates, respectively. A quadratic development over time was included as fixed factor in all the models. The variance of the fixed factors and the distribution of significant covariates were

homogeneous across PFAS quartiles. Diagnostic plots of the residuals and potential influential points were evaluated.

3. Results:

3.1 Population characteristics and PFAS concentrations

The median age was 32 and the majority of the participants were nulliparous or primiparous (parity varied from 0-4), for the MISA study population. Further details on demographic characteristics are briefly presented in the Supplemental Material, Table S2 and described in detail elsewhere (Veyhe et al., 2012). Seven PFASs; pefluorohexane sulfonate (PFHxS), perfluoroheptane sulfonate (PFHpS), perfluorooctane sulfonate (PFOS), perfluorooctanoate (PFOA), perfluorononanoate (PFNA), perfluorodecanoate (PFDA) and perfluoroundecanoate (PFUnDA) were detected in more than 80% of blood samples and were included in the statistical analyses. PFOS (median of 8.03 ng/mL) was the dominating compound followed by PFOA (1.53 ng/mL), PFNA (0.56 ng/mL), PFHxS (0.44 ng/mL), PFUnDA (0.26 ng/mL), PFDA (0.23 ng/mL) and PFHpS (0.10 ng/mL). Spearman correlation coefficients showed high to moderate correlations between PFOS and PFOA (r=0.65), PFHxS, (r=0.63), PFHpS (r=0.68), PFNA (r=0.60), PFDA (r=0.57) and PFUnDA (r=0.45). The correlation between PFDA and PFUnDA was higher than between PFOS and any of these two compounds. PFAS concentrations and their predictors are described in detail in a previous publication (Berg et al., 2014).

3.2 Concentrations of THs and TH-BPs

Concentrations and specific study group reference ranges of THs, TH-BPs and thyroxin binding capacity are provided in Table 1. Sixteen women were excluded from the study due to self-reported thyroid related disease and/or use of medications. Further, 22, 15 and 15 women had thyroid peroxidase antibodies above 34 IU/L at visit 1, 2 and 3 respectively, and were categorized as anti-TPO positive according to the reference range applied by the manufacturer. The anti-TPO positive women were included in all analyses, tables and figures as they did not alter the variance in TH concentrations compared to anti-TPO negative women. The specific

study group reference range (2.5–97.5th percentile) for the THs were within the normal reference ranges for the respective hormones.

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Table 1

Maternal concentrations[a] of THs, TH-BPs, thyroxin binding capacity and anti-TPO, and study group spesific reference ranges at three repeated measurments

	Visit 1: 2nd trimester, N= 375				Visit 2: 3 days postpartum, N=372				Visit 3: 6 weeks postpartum, N= 374			
Compound	**Median (Range)**	**AM**	**SD**	**Reference range[b]**	**Median (Range)**	**AM**	**SD**	**Reference range[b]**	**Median (Range)**	**AM**	**SD**	**Reference range[b]**
TSH (mlU/L)	1.55 (0.06-10.2)	1.76	1.04	0.44-4.48	2.37 (0.15-9.51)	2.56	1.20	0.98-5.39	1.39 (0.06-6.54)	1.55	0.80	0.47-3.38
T3 (nmol/L)	2.71 (1.47-4.75)	2.75	0.46	1.97-3.73	2.75 (1.32-4.66)	2.80	0.50	1.81-3.88	1.70 (1.15-2.53)	1.72	0.22	1.29-2.22
T4 (nmol/L)	145 (92.00-215)	146	21.1	111-190	144 (77.0-232)	145	26.3	97.1-204	97.0 (63.0-153)	98.5	14.3	72-130
FT3 (pmol/L)	4.59 (2.99-7.08)	4.62	0.53	3.66-5.79	4.50 (2.72-6.77)	4.52	0.57	3.50-5.73	4.63 (3.14-6.48)	4.66	0.45	3.80-5.77
FT4 (pmol/L)	13.0 (9.00-20.0)	13.4	1.62	10.0-17.0	13.0 (8.00-19.0)	13.0	1.87	10.0-17.0	14.0 (10.0-25.0)	14.3	1.74	12.0-18.0
TBG (mg/L)	36.7 (23.2-69.6)	37.2	6.74	26.2-53.3	37.0 (19.9-56.7)	37.3	6.35	25.2-51.4	17.9 (11.7-39.1)	18.4	3.44	12.9-26.54
TTR (g/L)	0.19 (0.09-0.27)	0.19	0.03	0.15-0.25	0.19 (0.09-0.42)	0.19	0.04	0.11-0.29	0.22 (0.13-0.38)	0.22	0.03	0.16-0.29
Albumin (g/L)	40.0 (33.9-47.4)	40.2	2.42	36.0-46.0	34.9 (24.3-46.4)	34.8	3.15	28.0-40.0	46.5 (40.9-53.6)	46.6	2.27	42.0-51.0
Thyroxin binding capacity (TBI)[c]	1.26 (0.84-1.50)	1.26	0.09	1.07-1.43	1.29 (0.20-1.50)	1.28	0.10	1.10-1.42	1.01 (0.50-1.18)	1.00	0.07	0.81-1.11
	Positive > 34 IU/L	**%**			**Positive > 34 IU/L**	**%**			**Positive > 34 IU/L**	**%**		
Anti-TPO (IU/mL)	22	6			15	4			15	4		

[a]Anti-TPO positive women are included in medians. The same 15 women were anti-TPO positive at all three visits

[b]Defined as the 2.5 percetile (lower range) and 97.5 percentile (upper range) for this population

240 [c]Thyroxin binding index, the measure unit for thyroxin binding capacity

3.3 THs and associations to PFAS concentrations

After adjusting for significant covariates such as parity, age, thyroxin binding capacity and BMI, and regardless of including anti-TPO positive women or not, TSH was positively associated with PFOS; T3 was negatively associated with PFDA; and FT3 was negatively associated with PFUnDA (Table 2). The significant covariates were included in the respective models (Table 2), but many more were evaluated (e.g. iodine sufficiency and gestational week) and are listed in Table S2 in the Supplemental material. Thyroxin binding capacity was significantly associated with all the thyroid hormones and the individual binding proteins, and was selected as a holistic adjustment for individual changes in thyroid hormone homeostasis during pregnancy and postpartum periods. Several PFASs (Results not presented) were inversely associated with T4 and FT4 after adjusting for age and BMI, but after including thyroxin binding capacity the associations were no longer significant. Further, adjusting for estimated daily dietary intakes of iodine (µg/day) and concentrations of iodine in urine (µg/L for 212 participants), did not influence the model predictions (results not presented) when grouped into iodine sufficient, mildly deficient and deficient, according to guidelines from the World Health Organisation (World Health Organization et al., 2014).

Women in the highest PFOS quartile had higher mean concentration of TSH at all three time point compared to women in the first quartile (Figure 1 A and B). Similar, women in the highest PFDA and PFUnDA quartiles had lower T3 and FT3 concentrations, respectively (Figure 1 C, D and E, F). Further, the proportion of women with a depleted supply of T4/FT4 and T3/FT3 (subclinical hypothyroidism), characterized by elevated TSH concentrations (>3.6 mlU/L) but with normal FT4 and FT3 concentrations (Fitzpatrick and Russell, 2010), increased for each PFOS quartile (Q1: n=12, Q2: n=16, Q3: n=24, Q4: n=30). Women in the extreme quartiles of PFHxS and PFOA had higher concentrations of TSH compared to the lowest quartiles, but when including PFOS concentration as a covariate, the associations were no longer significant (results not presented). PFDA weas negatively associated with FT3, but not after adjusting for PFUnDA (results not presented). Finally, the difference as well as the relative change in thyroid hormone concentrations over time was constant between PFAS quartiles (parallel growth curves, Figure 1 B, D and E).

Table 2

Mixed-effects model estimated mean[a] differences in thyroid hormone concentrations over time between PFAS quartiles

Fixed factor		**Model[b]**		
		Model 1[d]: TSH mlU/L		
PFOS (ng/mL)[c]		Ŷ	95 % Confidence Interval	*p*
Quartile 1: 0.3-5.7	N=94	Reference		
Quartile 2: 5.8-8.0	N=90	0.18	0.06, 0.31	0.11
Quartile 3: 8.1-11.0	N=95	0.26	0.13, 0.40	0.03
Quartile 4: 11.1-35.9	N=96	0.35	0.21, 0.50	0.00
		Model 2[f]: T3 nmol/L		
PFDA (ng/mL)[e]		Ŷ	95 % Confidence Interval	*p*
Quartile 1: 0.05-0.17	N=93	Reference		
Quartile 2: 0.17-0.23	N=94	-0.04	-0.08, 0.04	0.46
Quartile 3: 0.23-0.31	N=94	-0.05	-0.08, 0.00	0.52
Quartile 4: 0.31-2.34	N=94	-0.1	-0.14,-0.06	0.03
		Model 3[f]: FT3 pmol/L		
PFUnDA (ng/mL)[g]		Ŷ	95 % Confidence Interval	*p*
Quartile 1: LOD-0.15	N=92	Reference		
Quartile 2: 0.16-0.25	N=94	-0.08	-0.15, -0.00	0.14
Quartile 3: 0.26-0-37	N=94	-0.09	-0.16, -0.01	0.23
Quartile 4: 0.4-0.96	N=95	-0.18	-0.25, -0.12	0.00

[a]Mean differences in TH concentrations (Ŷ) are backtransformed from log-estimates of fixed effect variables

[b]Models are based on three measurements of THs per subject and included a subject-specific random intercept

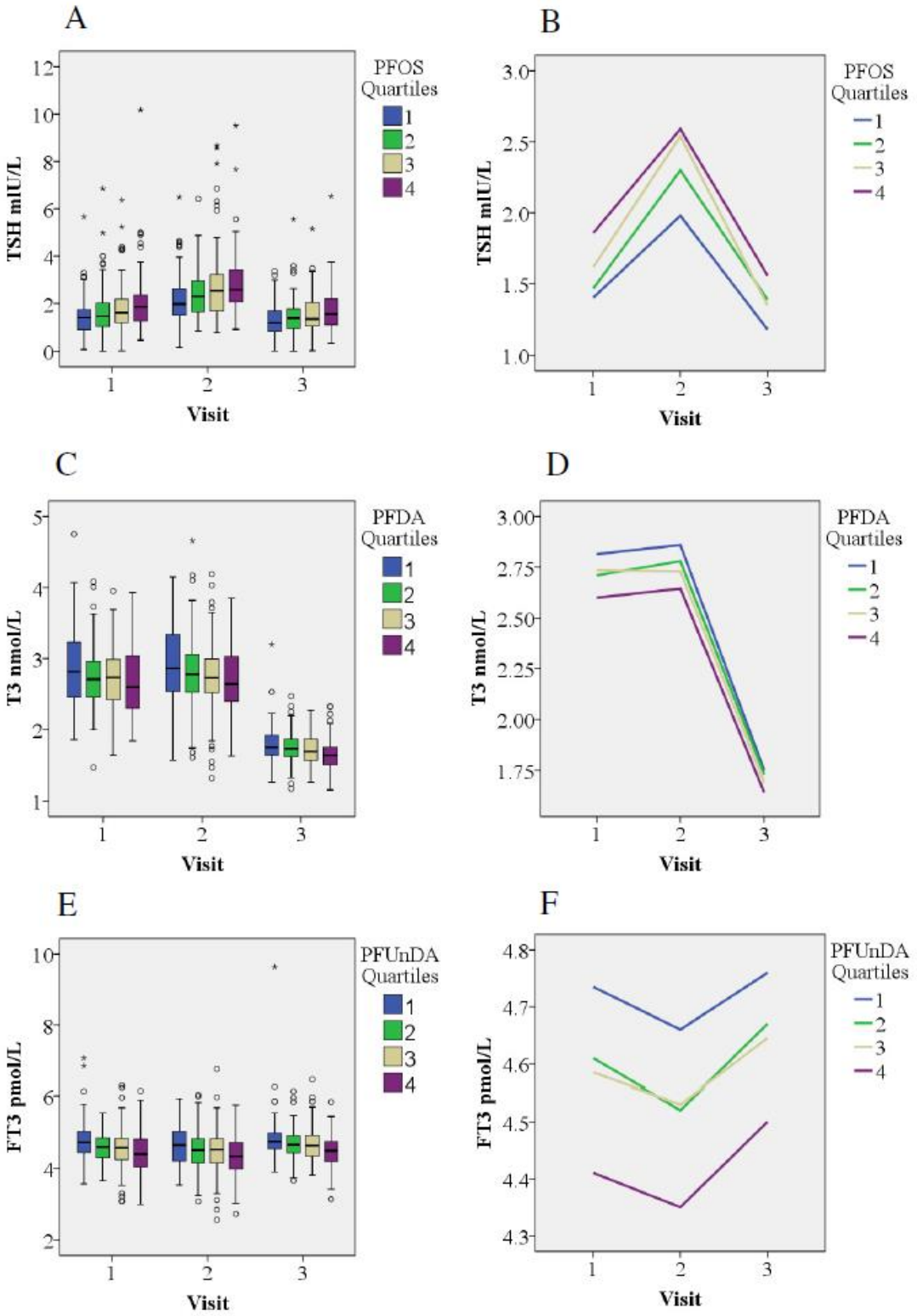
[c]Coefficients express change for TSH concentrations across PFOS quartiles, with quartile 1 as the reference group

[d]Parity and thyroxin binding capacity were included as covariates (fixed effects variables) in the model

[e]Coefficients express change for T3 concentrations across PFDA quartiles, with quartile 1 as the reference group

[f]Age, BMI and thyroxin binding capacity were included as covariates (fixed effects variables) in the model

[g]Coefficients express change for FT3 concentrations across PFUnDA quartiles, with quartile 1 as the reference group



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278 Figure 1. TSH, T3 and FT3 concentrations for the PFAS quartiles at three sampling points,
279 presented as boxplot (A, C and E) and as median concentrations (B, D and F).

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4. Discussion:

4.1 Associations between TH and PFAS concentrations

The observed associations in our study suggest modifications of the thyroid homeostasis by PFASs in a background exposed maternal population. The results demonstrate higher TSH concentrations with higher PFOS concentrations in pregnant women. Women within the highest PFOS quartile had 24% higher mean TSH concentrations compared to the first quartile at all sampling points. These observations are in accordance with those in another pregnant population in Norway (Wang et al., 2013), where PFOS concentrations were positively associated to TSH concentrations. Furthermore, the proportion of woman being classified with subclinical hypothyroidism at visit 1 (2^{nd} trimester) were higher with increasing PFOS concentrations in our study. Higher PFHxS and PFOA concentrations were associated to higher TSH concentrations, although not significantly when adjusting for PFOS concentrations. In comparison, PFHxS was positively associated to TSH levels but not to PFOS in a pregnant population from the Taiwan Maternal and Infant Cohort Study (Wang et al., 2014). The results indicate that PFOS can interfere with the production and elimination of T3 and T4, where elevated levels of TSH in women with high concentrations of PFOS could be the adjusted homeostasis state due to a reduction in T3 and T4. TSHs ability to maintain equilibrium in the TH homeostasis, may further explain that the variance in levels of T3 and T4 between PFOS quartiles were within the normal reference ranges in our study. In accordance with investigations carried out in rats, a single dose of PFOS caused a reduction in T3 and T4 levels (Chang et al., 2008).

In the present study PFDA concentrations were inversely associated to T3, and the women within the highest quartiles had 4% lower concentrations compared to women in the first quartile at all sampling points. Similar, PFUnDA concentrations were inversely associated to FT3 where women within the highest quartile had 3% lower concentrations. To the best of our knowledge, inverse associations between longer chained PFASs and maternal serum T3 and FT3 levels have not been reported previously, whereas Wang et al. (2014) reported a significant association between PFDA and cord blood T3 levels. Presently, there are no reports on the mechanisms by which PFDA and PFUnDA might modify thyroid hormones in humans or animals, but in a study conducted on a rat pituitary tumour cell line expressing intracellular thyroid receptor (TR), PFDA and PFUnDA significantly decreased the T3-induced cell

proliferation (Long et al., 2013). The authors therefore believe that PFDA and PFUnDA might compete with T3 binding to TR. Considering the increasing temporal trends of longer chained PFASs in humans (Nost et al., 2014) their endocrine disrupting potencies are a growing concern and studies on thyroid disrupting effects of these longer chained PFASs are warranted.

PFASs have been indicated to interfere at several levels of the thyroid homeostasis. We observed that PFASs were associated to individual TH set points but not to the relative change in TH levels across sampling period. The quartile differences in TH levels were consistent at all the time points with no interaction between time and quartiles, thus PFASs exert its effect in a constant matter. Further, we did not observe any associations between thyroxin binding capacity and PFOS, as was reported in the C8 health project (Knox et al., 2011) suggesting competitive displacement of T4 by PFOS on TH-BPs. This could be explained by elevated TH-BP levels in pregnancy and a subsequent excess in thyroxin binding sites, which likely renders it difficult to detect any displacement of T4 by PFASs on TH-BPs in this study.

Studies performed on pregnant women present conflicting results in regards to thyroid disrupting potencies of PFASs (Chan et al., 2011; Wang et al., 2013; Wang et al., 2014; Webster et al., 2014), whereas similar studies in animals demonstrate consistent physiological effects including disruption of thyroid hormone homeostasis (Fuentes et al., 2006; Lau et al., 2003; Thibodeaux et al., 2003). Different exposure histories in different populations may influence the associations between PFASs and THs as well as variation in lifestyle predictors such as parity, breastfeeding, birth year and study period that affect PFAS concentrations (Berg et al., 2014). Hence, measurement of PFASs at one time point may not reflect the historic exposure to PFASs and potential early effects on thyroid function. Further, the human exposure scenario with lifelong exposure to a mixture of chemicals in low doses, the large physiological variation in TH levels between individuals and complicated pathways, render human effect studies more difficult to perform. Finally, TSH, T3 and T4 are tightly regulated within a given individual, where the expected inter-individual variations may camouflage differences associated with exposure.

4.2 Study design and strengths

To the best of our knowledge, this is the first study investigating the effect of PFASs on ten thyroid hormone parameters, and we have also included three repeated measurements. Due to the complex thyroid system, assessment of potential thyroid impairment cannot be interpreted from individual TH levels only. Furthermore, T3 and T4 levels per se are not adequate hormone indicators in pregnant women (Glinoer and Spencer, 2010) due to the alterations in TH levels, blood composition and volume. To accompany this complexity we have adjusted for the increase in thyroxin binding capacity (reflects elevated levels of all the TH-BPs) as a proxy for the pregnancy related alterations in blood THs and TH-BPs in statistical models. We have also reported concentrations of FT3 and FT4 in addition to T3 and T4, as the evaluation of these are generally preferred in pregnant women due to that the increase in TH-BPs may mask an actual decrease in levels of T4 and T3. We have reported TSH levels as well because TSH reflects the thyroid status more directly, and can reveal abnormalities in T3 and T4 levels despite apparently normal levels of these THs (Glinoer and Spencer, 2010). Still, in a situation with a low supply of T4, normal T3 levels might prevent an increase in circulating TSH and consequently, hypothyroxinemia will not be detected if only TSH is measured (Braverman and Utiger, 1986). Further, as the thyroid homeostasis is dynamic and a single measurement may not adequately characterize the maternal thyroid function, we have reported repeated measurements of THs.

In the present study, PFHxS, PFDA and PFUnDA were inversely associated to FT4 and T4. However, when adjusting for the levels of TH-BPs by including thyroxin binding capacity, associations were no longer observed. Consequently, had we not corrected for the influence of TH-BPs on TH levels, we would have reported significant associations between several PFASs and FT4/T4. This might explain why our observations are not in accordance to the most recent publication on PFASs and THs in a pregnant population (Wang et al., 2014), who reported PFUnDA to be significant inversely associated with FT4 and T4. That study did not report on adjustments for gestational week or elevated levels of TH-BPs. Further, due to differences in PFAS concentrations and sampling points during pregnancy, the results from the two studies may not be comparable. Hence, inconsistencies in analytes and covariates investigated may account for some of the discrepancies in observed relationships between PFASs and THs in different studies which complicate the conclusion on definite relations.

4.3 Clinical relevance

Concentrations of all the THs varied within normal reference ranges and as the thyroid system is tightly regulated, small changes in TH concentrations will likely be adjusted for through the negative mechanisms which naturally secure TH equilibrium. Consequently, the indicated PFAS induced changes in TH concentrations, may not have caused clinical effects in the mother, thus the physiological importance of the observations is not established. Several studies have described ranges for thyroid hormone levels during pregnancy but have demonstrated variation by gestational age, number of foetuses, population studied, laboratory, and testing method (Fitzpatrick and Russell, 2010). In the present study, increasing levels of TSH according to increased PFOS concentrations may indicate low individual levels of T4 in these women. The specific reference ranges for T4 and FT4 in this study group were within the mid to high end of the normal reference ranges (Table 1 and Table S1), while the specific reference ranges for PFOS quartile four (results not presented) were within the lower end. Considering that levels of THs in pregnant women should be 40-100% higher than in non-pregnant (Blackburn, 2013), the subsequent distribution should have been towards the higher end of the reference range for the respective T4 and FT4 levels to be "normal". Hence, the clinical relevance of individual levels might be masked in non-pregnant population reference ranges.

Independent of the mode of action of PFASs on the maternal thyroid function and the subsequent clinical relevance, disruption of maternal TH homeostasis in any degree would only increase the difficulties encountered by the newborn in meeting postnatal hormone requirements (Morreale De et al., 2000). The foetus relies on maternal THs throughout gestation and a normal supply of maternal T4 has an important protective role also after midgestation. This is underlined by reports of poor developmental outcome e.g. impaired mental development and growth in babies faced with a premature interruption of the maternal supply of THs (Morreale De et al., 2000). Although the indicated PFAS induced changes in TH concentrations were within the reference ranges in the present study, small changes in THs may affect foetal development, especially if occurring during critical periods. Therefore, concerns have been raised regarding the effect of mild maternal thyroid hormone deficiency on foetal neurodevelopment. Decreases in childhood intellectual performance can occur even when a pregnant woman's hypothyroidism is subclinical (mild and asymptomatic) where marginally low T4 levels in the pregnant woman cause reduction in cognitive functions of the offspring (Berbel et al., 2009; Haddow et al., 1999; Pop et al., 2003).. Hence, subtle changes in THs may have significant consequences for foetal health and consistent evidence confirms that disrupted

maternal thyroid homeostasis negatively affects newborn development (Morreale De et al., 2000).

4.4 Thyroid function and iodine

The two principal causes of maternal hypothyroidism are iodine deficiency and exposure to xenobiotic thyroid disruptors. The negative health effects during pregnancy of thyroid hormones that have been associated to PFAS exposures, can resemble those related to iodine deficiency (eg. decreased maternal FT4 and increased maternal TSH, increased risk of prematurity, spontaneous abortion, and neurodevelopmental impairment) (Morreale De et al., 2000; Morreale De et al., 2004; Stahl et al., 2011). Hence, the importance of assessing iodine status when investigating associations between thyroid disruptors and THs is obvious. In the present study, maternal iodine status did not influence the observed associations between PFASs and THs and was not included as a covariate in the final models. Still, iodine status may affect the variance in TH concentrations, as the degree of iodine sufficiency or deficiency affects individual TH set points and changes in concentrations throughout the pregnancy (Blackburn, 2013; Morreale De et al., 2004). For example, iodine deficient women may be more susceptible to TH disruption. This could not be observed in our cohort as the variation in iodine status was low. This is also important for the general population and emphasizes that iodine status may interact with PFAS effects on thyroid homeostasis. Still, the majority of studies are not considering iodine status in regards to thyroid disruptors.

4.5 Mixture effects

Although the statistical analyses investigate the relationship between one or several dependent and independent variables at the same time, contaminants do not occur isolated in the human blood circulation. This means that the actual causality between physiological processes and the impairment by contaminants is complicated by the complex correlation of exposures. Also, there might be interactions and dose dependencies that we are not able to capture. This is evident in the observation of PFOA, PFHxS and PFDA being significantly associated to THs in individual models, and where the associations were no longer significant when adjusting for dominant components such as PFOS. Due to the strong correlation between the contaminants and their joint explanation of the outcome, it was not possible to isolate the variance in THs

explained by PFOA, PFHxS or PFDA alone. A possible solution to this is to summarize contaminants based on similarities e.g. chemical properties, mode of action and emission patterns, and report mixture effects, but this is beyond the scope of this publication. For further progression in the research on contaminants and human health effects, it is important to assess multiple exposure scenarios. Therefore, in subsequent studies we aim to include several contaminants and thyroid related outcomes in the children, to assess associations of thyroid function with combined exposures.

5. Conclusions

Women with the highest concentrations of PFOS, PFDA and PFUnDA had consistently higher TSH and lower T3 and FT3 concentrations, respectively, at all sampling points compared to women with the lowest concentrations. The difference in concentrations and the changes between three time points were the same for the PFAS quartiles. Despite significant associations between PFASs and THs, TH levels were within normal reference ranges and may not be of clinical significance in the pregnant woman. However, the foetus relies on maternal THs throughout gestation and a normal supply of maternal T4 has an important protective role also after midgestation, hence, subtle individual changes in thyroid hormones may have significant consequences for foetal health.

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Reference List

Anda EE, Nieboer E, Dudarev AA, Sandanger TM, and Odland JO. Intra- and intercompartmental associations between levels of organochlorines in maternal plasma, cord plasma and breast

494 milk, and lead and cadmium in whole blood, for indigenous peoples of Chukotka,
495 Russia.Journal of environmental monitoring 8-6-2007;9:884-93.

496 Berbel P, Mestre JL, Santamaria A, Palazon I, Franco A, Graells M, Gonzalez-Torga A, and de
497 Escobar GM. Delayed neurobehavioral development in children born to pregnant women with
498 mild hypothyroxinemia during the first month of gestation: the importance of early iodine
499 supplementation.Thyroid 2009;19:511-9.

500 Berg V, Nost TH, Huber S, Rylander C, Hansen S, Veyhe AS, Fuskevag OM, Odland JO, and
501 Sandanger TM. Maternal serum concentrations of per- and polyfluoroalkyl substances and
502 their predictors in years with reduced production and use.Environ.Int 6-5-2014;69C:58-66.

503 Blackburn, S. T.Pituitary, Adrenal, and Thyroid function.Elsevier 2013;Fourth:627-652.

504 Boas M, Feldt-Rasmussen U, and Main KM. Thyroid effects of endocrine disrupting
505 chemicals.Mol.Cell Endocrinol. 22-5-2012;355:240-8.

506 Braverman LE and Utiger RD.The thyroid: A fundemental and clinical text.9.Lippincott;1986

507 Chan E, Burstyn I, Cherry N, Bamforth F, and Martin JW. Perfluorinated acids and hypothyroxinemia
508 in pregnant women.Environ.Res. 2011;111:559-64.

509 Chang SC, Thibodeaux JR, Eastvold ML, Ehresman DJ, Bjork JA, Froehlich JW, Lau C, Singh RJ,
510 Wallace KB, and Butenhoff JL. Thyroid hormone status and pituitary function in adult rats
511 given oral doses of perfluorooctanesulfonate (PFOS).Toxicology. 20-1-2008;243:330-9.

512 Eschauzier C, Hoppe M, Schlummer M, and de VP. Presence and sources of anthropogenic
513 perfluoroalkyl acids in high-consumption tap-water based beverages.Chemosphere
514 2013;90:36-41.

515 Feldt-Rasmussen U, Hyltoft PP, Blaabjerg O, and Horder M. Long-term variability in serum
516 thyroglobulin and thyroid related hormones in healthy subjects.Acta Endocrinol.(Copenh)
517 1980;95:328-34.

518 Fitzpatrick DL and Russell MA. Diagnosis and management of thyroid disease in
519 pregnancy.Obstet.Gynecol.Clin.North Am. 2010;37:173-93.

520 Fromme H, Tittlemier SA, Volkel W, Wilhelm M, and Twardella D. Perfluorinated compounds--
521 exposure assessment for the general population in Western countries.Int.J.Hyg.Environ.Health
522 2009;212:239-70.

523 Fuentes S, Colomina MT, Rodriguez J, Vicens P, and Domingo JL. Interactions in developmental
524 toxicology: concurrent exposure to perfluorooctane sulfonate (PFOS) and stress in pregnant
525 mice.Toxicol.Lett. 20-6-2006;164:81-9.

526 Glinoer D and Spencer CA. Serum TSH determinations in pregnancy: how, when and
527 why?Nat.Rev.Endocrinol. 2010;6:526-9.

528 Haddow JE, Palomaki GE, Allan WC, Williams JR, Knight GJ, Gagnon J, O'Heir CE, Mitchell ML,
529 Hermos RJ, Waisbren SE, Faix JD, and Klein RZ. Maternal thyroid deficiency during
530 pregnancy and subsequent neuropsychological development of the child.N.Engl.J.Med. 19-8-
531 1999;341:549-55.

532 Hansen S, Nieboer E, Odland JO, Wilsgaard T, Veyhe AS, and Sandanger TM. Levels of
533 organochlorines and lipids across pregnancy, delivery and postpartum periods in women from
534 Northern Norway.J.Environ.Monit. 2010;12:2128-37.

Hanssen L, Dudarev AA, Huber S, Odland JO, Nieboer E, and Sandanger TM. Partition of perfluoroalkyl substances (PFASs) in whole blood and plasma, assessed in maternal and umbilical cord samples from inhabitants of arctic Russia and Uzbekistan.Sci.Total Environ. 1-3-2013;447:430-7.

Haug LS, Huber S, Becher G, and Thomsen C. Characterisation of human exposure pathways to perfluorinated compounds--comparing exposure estimates with biomarkers of exposure.Environ.Int. 2011a;37:687-93.

Haug LS, Huber S, Schlabach M, Becher G, and Thomsen C. Investigation on per- and polyfluorinated compounds in paired samples of house dust and indoor air from Norwegian homes.Environ.Sci.Technol. 1-10-2011b;45:7991-8.

Knox SS, Jackson T, Frisbee SJ, Javins B, and Ducatman AM. Perfluorocarbon exposure, gender and thyroid function in the C8 Health Project.J.Toxicol.Sci. 2011;36:403-10.

Labquality Finland. External quality assessment for medical laboratories;2014. Available from: http://www.labquality.fi/eqa-eqas/eqa-eqas-program-scheme/external-quality-assessment/.

Lau C, Thibodeaux JR, Hanson RG, Rogers JM, Grey BE, Stanton ME, Butenhoff JL, and Stevenson LA. Exposure to perfluorooctane sulfonate during pregnancy in rat and mouse. II: postnatal evaluation.Toxicol.Sci. 2003;74:382-92.

Long M, Ghisari M, and Bonefeld-Jorgensen EC. Effects of perfluoroalkyl acids on the function of the thyroid hormone and the aryl hydrocarbon receptor.Environ.Sci.Pollut.Res.Int. 2013;20:8045-56.

Morreale De EG, Obregon MJ, and Escobar del RF. Is neuropsychological development related to maternal hypothyroidism or to maternal hypothyroxinemia?J Clin.Endocrinol.Metab 2000;85:3975-87.

Morreale De EG, Obregon MJ, and Escobar del RF. Role of thyroid hormone during early brain development.Eur.J Endocrinol. 2004;151 Suppl 3:U25-U37.

Norwegian accreditation. Norwegian accreditation;2014. Available from: http://www.akkreditert.no/en/hva-er-akkreditering/hva-vi-akkredtierer/laboratorier/.

Nost TH, Vestergren R, Berg V, Nieboer E, Odland JO, and Sandanger TM. Repeated measurements of per- and polyfluoroalkyl substances (PFASs) from 1979 to 2007 in males from Northern Norway: assessing time trends, compound correlations and relations to age/birth cohort.Environ.Int. 2014;67:43-53.

Pop VJ, Brouwers EP, Vader HL, Vulsma T, van Baar AL, and de Vijlder JJ. Maternal hypothyroxinaemia during early pregnancy and subsequent child development: a 3-year follow-up study.Clin.Endocrinol.(Oxf) 2003;59:282-8.

Powley CR, George SW, Ryan TW, and Buck RC. Matrix effect-free analytical methods for determination of perfluorinated carboxylic acids in environmental matrixes.Anal.Chem. 1-10-2005;77:6353-8.

Preau L, Fini JB, Morvan-Dubois G, and Demeneix B. Thyroid hormone signaling during early neurogenesis and its significance as a vulnerable window for endocrine disruption.Biochim.Biophys.Acta 27-6-2014;

Prevedouros K, Cousins IT, Buck RC, and Korzeniowski SH. Sources, fate and transport of perfluorocarboxylates.Environ.Sci.Technol. 1-1-2006;40:32-44.

Stahl T, Mattern D, and Bruun H. Toxicology of perfluorinated compounds.Environmental Sciences Europe 2011;23:1-52.

Thibodeaux JR, Hanson RG, Rogers JM, Grey BE, Barbee BD, Richards JH, Butenhoff JL, Stevenson LA, and Lau C. Exposure to perfluorooctane sulfonate during pregnancy in rat and mouse. I: maternal and prenatal evaluations.Toxicol.Sci. 2003;74:369-81.

Ullah S, Alsberg T, and Berger U. Simultaneous determination of perfluoroalkyl phosphonates, carboxylates, and sulfonates in drinking water.J Chromatogr.A 16-9-2011;1218:6388-95.

Vestergren R and Cousins IT. Tracking the pathways of human exposure to perfluorocarboxylates.Environ.Sci.Technol. 1-8-2009;43:5565-75.

Veyhe AS, Hansen S, Sandanger TM, Nieboer E, and Odland JO. The Northern Norway mother-and-child contaminant cohort study: implementation, population characteristics and summary of dietary findings.Int J Circumpolar Health 2012;71:18644.

Wang Y, Rogan WJ, Chen PC, Lien GW, Chen HY, Tseng YC, Longnecker MP, and Wang SL. Association between maternal serum perfluoroalkyl substances during pregnancy and maternal and cord thyroid hormones: Taiwan maternal and infant cohort study.Environ.Health.Perspect. 2014;122:529-34.

Wang Y, Starling AP, Haug LS, Eggesbo M, Becher G, Thomsen C, Travlos G, King D, Hoppin JA, Rogan WJ, and Longnecker MP. Association between perfluoroalkyl substances and thyroid stimulating hormone among pregnant women: a cross-sectional study.Environ.Health 2013;12:76.

Webster GM, Venners SA, Mattman A, and Martin JW. Associations between Perfluoroalkyl acids (PFASs) and maternal thyroid hormones in early pregnancy: a population-based cohort study.Environ.Res. 2014;133:338-47.

World Health Organization, UNICEF, ICCIDD. Assessment of iodine deficiency disorders and monitoring their elimination;2014. Available from: http://www.who.int/nutrition/publications/micronutrients/iodine_deficiency/9789241595827/en/.

606 **SUPPLEMENTAL MATERIAL**

607

608 **Assessing the relationship between perfluoroalkyl substances, thyroid hormones and**
609 **binding proteins in pregnant women; a longitudinal mixed effects approach**

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Contents:

Table S1

Analytical methods, instrumentation, analytical variation and reference ranges

Analysis	Method/Instrument	Analytical variation (%)[a]	Reference range[b]
TSH[c]	ECLIA/Cobas 8000, e602	3.2	0.20-4.30 mlU/L
T3	ECLIA/Cobas 8000, e602	1.9	1.3-3.1 nmol/L
T4	ECLIA/Cobas 8000, e602	4.2	66-181 nmol/L
FT3[c]	ECLIA/Cobas 8000, e602	5.1	2.8-7.1 pmol/L
FT4[c]	ECLIA/Cobas 8000, e602	3.6	9-22 pmol/L
Anti-TPO[c]	ECLIA/Cobas 8000, e602	9.9	< 34 IU/ml
T-Uptake	ECLIA/Cobas 8000, e602	4.8	0.8-1.3 TBI
TBG	Chemiluminescence enzyme IA/IMMULITE 2000	7.5	47-45 mg/L
TTR[c]	Immuneturbidimetry/Cobas 8000, c702	2.6	0.15-0.29 g/L
Albumin[c]	Colometric assay (bromcresol green)/Cobas 8000, c702	1.9	39.7-49.4 g/L

[a]The analytical variation is calculated from all samples run in the diagnostic routine in the year 2013, except for T3, T4, TTR and Thyroxin binding capasity (T-Uptake) where the analytical variation was calculated during the verification of the analyses for the present study

[b]Reference ranges (2.5-97.5th percentiles) are those recommended by the manufacturer (Roche) representing a healthy nonpregnant population, except for TBG which is for pregnant women (third trimester)

647 [c]The analysis is accreditated according to ISO standard 15189

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Table S2

Covariates evaluated in mixed effects models

Predictor	Median	Range
Age	32	18-43
Children/Parity	1	0-4
Gestational week at visit 1	18	10-34
Sampling time visit 2 (Days after delivery)	3	1-13
Sampling time visit 3 (Weeks after delivery)	7	3-24
Prepregnancy BMI	23	18-44
BMI at visit 1	25	18-43
BMI at visit 2	27	18-45
BMI at visit 3	24	17-40
Education: Years in school	16	8-20
Thyroxin binding capacity (T-uptake)	Table S1	-
TBG	Table S1	-
TTR	Table S1	-
Albumin	Table S1	-
Blood sampling season	Month of the year	-
Iodine sufficiency[ab]	Yes/No	-
Alchohol during pregnancy	Yes/No	-
Smoking	Yes/no	-

[a]Participants were categorized as iodine sufficient according to a prepregnancy dietary intake of iodine above 150 µg/d (manuscript in preparation by Hansen et al.)

[b]Participants were categorized as iodine sufficient according to concentrations of iodine in urine above 150 µg/L (manuscript in preparation by Hansen et al.)

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