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Title Page

Association of perfluorinated chemical exposure in utero with maternal and infant thyroid hormone levels in the Sapporo cohort of Hokkaido Study on the Environment and Children's Health

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Key words

Perfluorooctanesulfonate, perfluorooctanoate, thyroid hormones, mother-infant pairs,
prenatal exposure

Abstract

Objectives: Perfluorooctanesulfonate (PFOS) and perfluorooctanoate (PFOA) have been widely used as industrial products, and are persistent organic pollutants due to their chemical stability. Previous studies suggested that PFOS and PFOA might disrupt thyroid hormone (TH) status. Although TH plays an important role in fetal growth during pregnancy, little attention has been paid to the relationships between maternal exposure to perfluorocarbons and TH statuses of mothers and fetuses. We investigated the effects of low levels of environmental PFOS and PFOA on thyroid function of mothers and infants.

Methods: Of the eligible subjects in a prospective cohort, 392 mother-infant pairs were selected. Concentration of maternal serum PFOS and PFOA was measured in samples taken during the second and third trimesters or within 1 week of delivery. Blood samples for measuring thyroid stimulating hormone (TSH) and free thyroxine (FT4) levels were obtained from mothers at early gestational stage (Median; 11.1 weeks), and from infants between 4 and 7 days of age, respectively.

Results: Median concentrations of PFOS and PFOA were 5.2 (95% confidence interval [CI]: 1.6-12.3) and 1.2 (95% CI: limitation of detection-3.4) ng/mL, respectively. Maternal PFOS levels were inversely correlated with maternal serum TSH and positively associated with infant serum TSH, whereas maternal PFOA showed no significant relationship with TSH or FT4 among mothers and infants.

Conclusions: These findings suggest that PFOS may independently affect the secretion and balances of maternal and infant TSH even at low levels of environmental exposure.

Introduction

Perfluorinated compounds (PFCs) are synthetic chemicals that have been manufactured since the 1950s. Perfluorooctanesulfonate (PFOS) and perfluorooctanoate (PFOA) are the most common chemicals among PFCs, and are quite stable because of the extremely strong bond between carbon and fluorine atoms. This characteristic has made them useful as surfactants, water repellants, and a variety of other industrial products for more than half a century. Human exposure to PFCs mainly occurs orally, via the intake of contaminated food, water, and dust [1]. Their presence in human blood has been reported in several countries [2-5]. Recently, potential adverse effects of PFCs on human health have been claimed, and PFOS was added to Annex B of the Stockholm Convention on Persistent Organic Pollutants in 2009 due to potential adverse human health and environmental effects. Although PFOS and PFOA are being voluntarily phased out by several industries and substituted by short chain products, they are still present in older products and in human serum due to their long half-lives (5.3 years for PFOS and 3.8 years for PFOA) [6]. Additionally, maternal PFOS and PFOA are transferred to fetuses across the placental barrier in humans [7, 8]. Therefore, there is significant concern over the adverse effects of in-utero exposure to PFOS and PFOA.

Epidemiological studies have reported that in-utero exposure to PFOS and PFOA had adverse effects on birth sizes and childhood neurodevelopment [9, 10]. We previously reported an inverse association between maternal PFOS serum levels and birth weight among female infants [11]. Moreover, we found inverse correlations between PFOS and essential fatty acid and triglyceride levels during pregnancy, at levels of PFOS and PFOA that were lower than those previously reported [12].

Some recent studies have suggested that PFCs may disrupt thyroid function. In rat

dams and their pups, PFOS exposure during pregnancy has been reported to reduce concentrations of the thyroid hormones triiodothyronine (T3) and thyroxine (T4) of dams and pups due to the competitive PFOS binding to protein and increased clearance of THs, without affecting the stimulation of thyroid stimulating hormone (TSH) [13, 14]. Furthermore, human cross-sectional studies in pregnant women have also revealed associations between higher levels of PFCs and increased maternal TSH levels, along with decreased T3 and T4 levels [15-17]. In addition, two prospective studies concluded that prenatal exposure to PFCs alters the balance of thyroid hormones in infants [18, 19]. However, their sample sizes were relatively small.

Thyroid hormones (THs) are very important for fetal growth in utero. Fetuses depend on their mothers' supply of THs in early gestation; hence, disruption of maternal TH homeostasis during early pregnancy might impair fetal development. Mild maternal hypothyroxinemia during pregnancy increases the risk for adverse effects on fetal neurodevelopment. However, there have been no reports describing the effects of PFCs on both maternal TH at early gestational stage and infant THs. Therefore, the aim of this study was to examine potential associations between levels of maternal PFOS and PFOA, and THs, TSH, and FT4 in mother-infant pairs.

Material and Methods

Study population and study design.

This study was based on data from the Hokkaido Study on the Environment and Children's Health, which involved mothers and their infants delivered at the Sapporo Toho Hospital in Sapporo, Hokkaido, Japan [20]. In brief, pregnant women between 23 to 35 weeks of gestation were recruited between 2002 and 2005. Of 1796 potentially

eligible pregnant women who visited the hospital for prenatal health care, 514 women (29%) agreed to participate in the study. Among 514 women, we excluded 10 who did not maintain participation to the end of the study (7 voluntarily withdrew, 2 had a miscarriage or stillbirth, and 1 relocated), as well as 7 who had twins. For the analysis of associations between maternal PFC levels and maternal and infant TH levels, the following subjects were excluded: those undergoing treatment for thyroid disease ($n = 14$), those of whom maternal and/or infants' TH data were not available ($n = 40$), and those with no data for serum PFOS and PFOA levels ($n = 51$). Ultimately, 392 mother-infant pairs were included in this study. The analysis of mothers regarding the associations between maternal PFOS and PFOA levels and maternal thyroid status during pregnancy were designed as the cross-sectional study, and the analysis of infants regarding the associations between maternal PFOS and PFOA levels and infants' thyroid status at birth were designed as the prospective study.

Data collection.

At enrollment, participants completed a self-administered questionnaire that included questions about relevant sociodemographic, medical, and behavioral information, as described previously [11, 21]. In brief, the questionnaire extracted information related to medical history, dietary habits, smoking status, alcohol intake, household income, and educational level. We also obtained information from medical records on maternal age, maternal height, maternal pre-pregnancy weight, parity, pregnancy complications, gestational age, infant gender, birth weight, and birth length. This study was conducted with written informed consent from all subjects and was approved by the ethics committees of Hokkaido University Graduate School of

Medicine (2002.10.28).

TH measurement.

Thyroid function and antithyroid antibody data of a mass screening test at the Sapporo City Institute of Public Health were obtained. The blood samples were collected from mothers between the 7th and 20th weeks of gestation, and from infants between 4 and 7 days old, respectively. TSH and FT4 levels were measured using enzyme-linked immunosorbent assay (TSH: Enzaplate N-TSH, Bayer Co., Tokyo, Japan; FT4: Enzaplate N-FT4, Bayer Co.). The FT4 values of all samples were detected. For 85 samples with TSH levels below the detection limit (0.50 $\mu\text{U/mL}$), we used a value of half the detection limit (0.25 $\mu\text{U/mL}$). Antimicrosome antibody (AMC) and antithyroglobulin antibody (ATG) were measured by indirect agglutination reactions (AMC: Serodia AMC, Fuji-Rebio, Tokyo, Japan; ATG: Serodia ATG, Fuji-Rebio). Values of AMC and ATG above 100-fold of baseline were classified as positive.

Blood sampling and measurement of PFOS and PFOA levels in maternal serum.

We used the data of PFOS and PFOA concentrations in maternal serum measured in our previous study. Detailed methods for the measurement of PFOS and PFOA have been described previously [11, 21]. Forty milliliters of blood were collected from the maternal peripheral vein between 24 and 41 weeks of gestational age or within 5 days after delivery, and were stored at -80°C until analysis. PFOS values of all samples were determined; for samples with PFOA levels below the detection limit (0.50 ng/mL), we used a value of half the detection limit (0.25 ng/mL).

Data Analysis.

The Mann-Whitney and Spearman correlation tests were conducted to examine associations between levels of PFOS and PFOA in maternal serum and characteristics of the subjects. Associations of maternal serum PFOS and PFOA levels with TSH and FT4 levels of mothers and infants were analyzed using Spearman's correlation test. We also performed multiple linear regression analysis; the PFOS and PFOA values as well as the TSH and FT4 values were converted to a log₁₀ scale because they did not fall into a normal distribution. In addition, the relationships between quartile PFOS concentrations in maternal serum and TSH levels of mothers and infants with a p-value of < 0.10 in adjusted models shown in multiple linear regression analysis were assessed to clarify the dose-response relationship between maternal serum PFCs and THs; PFOS and PFOA levels were divided into 4 quartiles, and TSH and FT4 levels were converted to a log₁₀ scale. The least square means of TSH and FT4 were calculated and back-transformed, and the p-value for their trends determined. The first quartile was also compared to the second, third, and fourth quartiles of PFOS and PFOA, and p-values were calculated using the Hsu-Dunnett's test. Analyses were conducted fully adjusting for confounding variables that were found to be significantly associated with PFOS, PFOA, TSH, and FT4 levels in our results (those with p < 0.05 in Table 3 and Supplemental Table 1) and in previous literatures. For maternal analyses, values were adjusted for maternal age at delivery (years), body mass index (BMI) before pregnancy (kg/m²), parity (0/≥1), educational level (≤12/≥13 years), positive for ATG and/or AMC (yes/no), intake of seaweed more than once a week (yes/no), blood sampling period for PFOS and PFOA (before/after delivery), and the gestational week at which blood sampling for TSH and FT4 was performed (continuous variable). For infant analysis, we

selected maternal confounding factors (maternal age at delivery [years], parity [0/ $\geq$ 1], positivity for ATG and/or AMC [yes/no], blood sampling period for PFOS and PFOA [before/after delivery], and log10 values of TSH and FT4 [continuous variable]), and infant factors (gestational weeks for birth [continuous variable], low birth weight < 2500 g [yes/no], and born via caesarean section [yes/no],).

All statistical analyses were performed using JMP Pro 12 (SAS Institute Inc., NC, USA). Results were considered statistically significant if the *p*-value was below 0.05.

Results

Table 1 shows the comparison of maternal characteristics and the concentrations of PFOS, PFOA, TSH, and FT4 by infant sex. Among 392 mothers of total infants, the median concentrations of PFOS and PFOA were 5.2 (95% confidence interval [CI]: 1.6-12.3) and 1.2 (95% CI: limit of detection [LOD]-3.4) ng/mL, respectively. TSH levels from 307 (78.3%) maternal blood samples were above the LOD. The median levels of maternal TSH and FT4 levels were 1.0 μ U/mL (95% CI: LOD-4.1 μ U/mL) and 0.99 ng/mL (95% CI: 0.62-1.67 ng/mL), respectively. There was no significant difference of between maternal levels of PFOS, PFOA, TSH, and FT4, and other maternal characteristics between mothers of male infants and female infants. As for infants in Table 2, the values of TSH above the LOD were detected from 373 (95.2 %) of infant's blood samples. The median levels of infants' TSH and FT4 levels were 2.2 μ U/mL (95% CI: LOD-7.9 μ U/mL) and 2.03 ng/mL (95% CI: 1.39-2.89 ng/mL), respectively. The percentage of female infants with low birth weight (< 2500 g) was slightly higher than that of male infants, however, it was not significant. The correlations between maternal TSH and infants' FT4 were found to be significant

inversely (Spearman's $\rho = -0.104$, $p\text{-value} = 0.040$), while maternal TSH did not correlated with infants' TSH. The maternal serum PFOS and PFOA concentrations in relation to various maternal characteristics are shown in Table 3. PFOS and PFOA levels were significantly higher among women who were primiparous and whose blood samples were obtained before delivery. Maternal age at delivery was negatively associated with PFOS levels among mothers of total infants and female infants. Negative correlation between maternal age at delivery and PFOA level was found only among mothers of female infants. As for mothers of male infants, PFOA level of mothers who smoked during pregnancy was significantly lower than that of non-smoking mothers, and inverse correlation was found between PFOA level and blood sampling period of TSH, FT4 (weeks of pregnancy), while those difference were not found among mothers of total and female infants.

The results of the correlations and the multiple linear regression analyses between maternal PFOS/PFOA concentrations and maternal TSH/FT4 are shown in Table 4. Maternal PFOS was significantly correlated with decreased maternal TSH levels. After adjustment for covariates, PFOS levels were inversely associated with TSH levels in mothers of total, male and female infants. There was no significant association between PFOS and FT4. PFOA showed no significant association with either TSH or FT4.

Table 5 shows the results of the correlations and multiple linear regression analyses between maternal PFOS/PFOA concentrations and infant TSH/FT4. There is no significant correlation between maternal PFCs and infants' THs. Among total infants, we observed a significantly positive association between maternal PFOS and infant TSH levels in the adjusted model. After stratifying by gender, we also observed a positive association of PFOS with TSH levels in both male and female infants. No

significant association was found between PFOS and infant FT4. PFOA showed no significant association with infant TSH or FT4.

The relationships between quartile PFOS concentrations ($1.5 \leq \text{Quartile 1} \leq 3.5$ ng/mL, $3.5 < \text{Quartile 2} \leq 5.2$ ng/mL, $5.2 < \text{Quartile 3} \leq 7.0$ ng/mL, $7.0 < \text{Quartile 4} \leq 16.2$ ng/mL) in maternal serum and TSH levels of mothers and infants are shown in Fig. 1. Maternal TSH showed a significantly decreasing trend in relation to quartile PFOS. Infant TSH showed an increasing trend in relation to the PFOS quartile in total infants, although this was not significant after stratifying by sex.

Discussion

The fetal period is a particularly vulnerable stage during which many critical neurodevelopmental events occur. THs are known to be crucial for fetal growth [22]. Our results suggest that PFOS exposure at common environmental levels during pregnancy is likely to decrease maternal TSH and increase infant TSH. Such subclinical hyper- or hypothyroidism [23] might lead to the imbalance of FT4, an important hormone for fetal neurodevelopment.

PFOS and PFOA levels in this study were lower compared to other areas of Japan and Korea [24]. Moreover, mothers who had experienced delivery before the participation to this study had significantly lower levels of PFOS and PFOA, which is consistent with other study [25]. According to previous reports, PFOS and PFOA levels of pregnant women are lower than non-pregnant women, indicating the placental transfer to fetus [26, 27].

Three previous studies in Norway, Taiwan, and Canada have reported positive associations between serum PFOS levels in pregnant women and their thyroid hormone

levels [15-17]. Our data, which shows an inverse association between maternal PFOS concentration and maternal TSH, is inconsistent with these studies. There are some difficulties in comparisons due to the difference of the timing of blood collection for thyroid hormone measurement between previous studies and ours. First, circulating thyroid hormone levels during pregnancy greatly change according to the gestational week. In previous studies, maternal blood was obtained after the second trimester (between 15–18 gestational weeks for Webster et al. in Canada, in the second trimester and 3 days to 6 weeks post-delivery for Berg et al. in Norway, and in the third trimester for Wang et al. in Taiwan), while our study measured the levels during the first trimester (median, 11.1 weeks of gestation). Fetal thyroid glands begin to secrete hormones after the second trimester of gestation [28, 29]. During early pregnancy, fetuses rely on maternal THs, and the disruption of maternal TH homeostasis can affect both maternal and fetal health. Deficiency or imbalance of maternal THs likely disrupts normal neurological development of fetuses. Therefore, our study clarified the effect of maternal THs on fetuses in early gestational stages. Additionally, reproductive hormone levels also drastically change at each stage of pregnancy. The level of human chorionic gonadotropin (hCG) increases rapidly until 10 weeks of gestation, and decreases soon. Then, estrogen and progesterone gradually increase until delivery. One previous report showed that estrogen indirectly increased the thyroxine binding globulin [30], which suggests the hormonal interaction. Therefore, the differences of the blood sampling timing may lead the inconsistent results between ours and previous studies due to the different hormonal balances. Second, the concentrations of PFOS in serum were different in previous studies compared to ours. Two of the previous studies reported higher PFOS concentrations in serum (Berg et al., 8.03 ng/mL; Wang et al., 12.73

ng/mL) compared to our study (5.3 ng/mL), while Webster et al. reported a lower concentration (4.8 ng/mL) [15-17]. Although whether PFOS and PFOA are metabolized in humans is still unclear, our results suggest an association between human thyroid homeostasis and exposure to background environmental levels of PFOS.

There are two previous studies regarding the effect of PFCs on infant THs. Kim et al. investigated the association of PFCs in maternal blood sera and cord blood with infant TH in cord blood among participants in South Korea (n=44) [18]. They reported negative correlation between maternal PFOS and fetal total T3, and between maternal perfluorotridecanoic acid (PFTrDA) and total T3 and T4 in fetuses; moreover, maternal PFOA positively correlated with fetal TSH. A Dutch study reported that a high level of PFOA in cord blood was associated with increased total T4 levels in infant girls as measured during heel prick blood draws (n=83) [19]. We found a significant positive association between maternal PFOS levels and infants' TSH in a larger sample size than those of previous studies. Our result is partially in line with Kim et al. that PFOS in maternal serum was associated with increased TSH, though their result was not significant. There are some discrepancies between our results and previous studies. We measured free T4 levels, which we assumed would be a more reliable indicator of hyper- or hypothyroidism, because FT4 is not affected by the concentration of binding proteins, while two previous studies used the data of total T4. Our results of elevated TSH without FT4 change indicate that prenatal PFOS exposure may cause subclinical hypothyroidism in fetuses. In addition, we added maternal TH levels as the confounders in the multiple regression analysis, considering the maternal TH effects on infants' TH. The correlations between maternal TSH and infants' FT4 were found to be significant inversely (Spearman's $\rho = -0.104$, p-value = 0.040) in this study, which is consistent

with one previous report that showed the inverse relationship between maternal TSH and neonatal total T4 [31]. Although maternal TSH is not transferred to fetus, the associations of PFOS levels with decreased TSH among mothers might disrupt infants' hormone levels. Further study is needed to clarify the association between disrupted TH by PFOS exposure in maternal serum and infants' TH levels. It is noted that our previous study reported the inverse association between maternal PFOS and birth weight among female infants [11]. In the current study, there was a significant inverse correlation between birth weight and TSH ($r = -0.155$). Decreased birth weight by PFOS exposure might lead to affect TSH level of infants, however, our results showed the increased TSH by PFOS exposure. Further study is needed to investigate the intermediation of known relationships by PFC exposure. Since the quartile analysis showed the significant p for trend (p for trend < 0.05) in total infants and the tendency (p for trend < 0.10) in female infants for the increasing TSH, our results indicate the PFOS dose-response toxicity for TSH even at low exposure level. On the contrary, male infants' TSH showed highest levels at 2nd quartiles of PFOS and slightly decreased at 3rd and 4th quartiles. That suggests no dose-response relationships among male infants. Although the reason for sex differences is not understood, one explanation for the sex difference is the faster elimination time of PFCs in female compared to male in animal studies [32, 33].

We found that the PFOA level had no effect on TSH or FT4, implying that PFOS is more influential than PFOA on human THs. One study observed the negative association of PFOA levels with free T4 and positive association with T3 among male adults exposed to PFOA occupationally [34]. Additionally, two studies regarding the effects of in-utero exposure on infants' THs reported the significant associations of

PFOA with TSH or FT4 [18, 19]. Our results were inconsistent with those previous studies. Concentrations of PFOA in cord blood were reported to be much closer to concentrations in maternal blood compared to PFOS [8, 35]. This suggests that PFOA transfers more easily across the placental barrier than PFOS. However, Olsen et al. reported that the half-life of elimination of PFOS was longer than that of PFOA [6]. One explanation for our results is that PFOS may have stronger effects than PFOA on THs in this study by remaining in maternal serum at a higher concentration due to its longer elimination time.

There are some inconsistencies between human and animal studies, such as higher doses of PFCs used in animal studies as well as physiologic differences. In rats and mice, PFOS has been reported to reduce concentrations of T3 and T4 without affecting stimulation of TSH [13, 14]. Although our results differed in that PFOS had no effect on FT4 in either maternal or infant serum, findings in rodents are difficult to extrapolate to humans because the high level of exposure to PFOS and PFOA in rodents is probably not comparable to the chronic low level exposures examined in our study. Moreover, PFOS may remain in the human body much longer than in other animals. A serum elimination half-life of 7.5 days has been reported for rats [36], whereas the half-life is 200 days in monkeys [37] and approximately 5.4 years in humans [6]. In animal experiments, repeated doses maintain the high serum levels even with fast elimination, while in human serum levels also remain low but stable due to long half-lives. Another discrepancy is the role of transthyretin (TTR). Although PFOS has been reported to competitively bind to TTR [38], only 10% of circulating T4 binds to TTR in humans, while the percentage in rats is 75%.

A major strength of the present study is its prospective design, which allowed

us to estimate the effects of prenatal PFC exposure on fetal thyroid functions using prenatal and perinatal blood samples. However, there are several limitations in this study. The detection of PFOS and PFOA was also limited by the amount of blood obtained from mothers. Compared to the excluded women in original cohort population (122 mothers), the median levels of maternal PFOS and PFOA did not significantly differ between the analyzed participants (PFOS: 5.2 ng/mL; PFOA: 1.2 ng/mL) and the excluded participants (PFOS: 5.0 ng/mL, PFOA: 1.5 ng/mL). However, the infants who were included in our analyses had a higher gestational age and a heavier birth weight (data not shown), which indicates a possible selection bias. Among the 392 mother-infant pairs, blood samples were obtained from 106 mothers at a later time post-delivery due to anemia, and these post-delivery blood samples exhibited significantly lower PFOS and PFOA (before delivery: PFOS = 5.2 and PFOA = 1.4 ng/mL; post-delivery: PFOS = 3.5 ng/mL and PFOA = 1.3 ng/mL). Although there is no report regarding PFOS and PFOA half-lives among pregnant women, PFOS and PFOA were reported to have long half-lives in adult human serum (5.4 years and 3.8 years, respectively). We believed that the post-delivery samples accurately reflected these women's PFC levels during their pregnancy. In addition, PFC concentrations during pregnancy are reported to decrease across trimesters [26]. We found the significant inverse correlation between PFOS and PFOA levels and gestational weeks of blood sampling for the measurement of PFOS and PFOA during pregnancy (PFOS; $r = -0.224$, PFOA; $r = -0.177$). We also analyzed using continuous variables of gestational weeks for PFOS and PFOA measurement, and the significance and direction of the results were almost same as dichotomous variables. Therefore, we retained those mothers whose blood samples were obtained after delivery to increase this study's

statistical power, and also included the blood sampling period as a potential confounder in the adjusted models. It is noted that we also included 40 mothers with AMC and/or ATG positive in this study. They were not under medical treatments at the recruitment, however, TSH levels of 40 mothers with AMC and/or ATG positive (Median; 1.5 $\mu\text{U/mL}$) were significantly higher than those of 352 mothers with AMC and/or ATG negative (Median; 0.9 $\mu\text{U/mL}$), and FT4 levels of 40 mothers with AMC and/or ATG positive (Median; 0.91 ng/mL) were significantly lower than those of 352 mothers with AMC and/or ATG negative (Median; 1.00 ng/mL), respectively. We did the analysis excluding 40 women with AMC/ATC positive, and the results were almost same as Table 4 and 5 in the significance and directions. Thus, we included these 40 women in this study, adding the presence of AMC and/or ATG into one of confounders. Lastly, we did not have data for hCG levels. TSH levels in early pregnancy stages are suppressed because of the elevation of hCG [39, 40]. Maternal TH levels were measured at 11.1 weeks of gestation in our study, when hCG is usually very high. However, all TH data were obtained before the 20th weeks of gestation to ensure that there was an actual relationship between PFOS and TSH.

In conclusion, our study revealed an inverse association between maternal PFOS concentration and maternal TSH during early gestation, and a positive association between maternal PFOS concentration and infant TSH at background-level PFOS exposures. Our group previously reported a decreasing trend for PFOS and PFOA levels, and an increasing trend for PFC levels with longer carbon chains (e.g., perfluorononanoic acid and perfluorodecanoic acid), in the plasma of pregnant women between 2003 and 2011 [41]. Therefore, future studies are required to verify the effects of PFCs with longer carbon chains on fetal thyroid functions. Moreover, multi-exposure

analysis are required because our study reported that most concentrations of individual chemicals, such as polychlorinated biphenyls, PFOS, PFOA and mercury, were correlated due to the similar exposure sources [42]. Additionally, these studies should also investigate the long-term effect of altered hormone levels on fetal neurodevelopment.

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This study was conducted with written informed consent from all subjects and was approved by the ethics committees of Hokkaido University Graduate School of Medicine.

Disclosures

The authors declare that they have no conflicts of interest.

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Table 1. Comparisons of maternal characteristics and the concentrations of PFOS, PFOA, TSH, and FT4 by infant sex.

Characteristics of mothers		Mothers of Total infants	Mothers of male infants	Mothers of female infants	p-value ^a
		(n=392)	(n=180)	(n=212)	
		Median ± SD	Median ± SD	Median ± SD	
		or n (%)	or n (%)	or n (%)	
Age (years) at delivery		31.1 ± 7.1	31.4 ± 7.2	30.8 ± 7.1	0.373
BMI (kg/m ²) before pregnancy		21.3 ± 3.3	21.2 ± 3.2	21.3 ± 3.3	0.678
Parity (times)	0	176 (44.9)	85 (47.2)	91 (42.9)	0.384
	≥1	212 (54.1)	93 (51.6)	119 (56.1)	
	≤12	178 (45.4)	83 (46.1)	95 (44.8)	
Educational level (years)	≥13	214 (54.6)	97 (53.9)	117 (55.2)	0.797
	<5	269 (68.6)	121 (67.2)	148 (69.8)	
Annual income (millions of yens)	≥5	120 (30.6)	57 (31.7)	63 (29.7)	0.645
	Yes	67 (17.1)	28 (15.6)	39 (18.4)	
Smoking during pregnancy	No	325 (82.9)	152 (84.4)	173 (81.6)	0.457
Fish intake (g/day)		37.2 ± 33.9	37.0 ± 30.1	37.4 ± 36.9	0.924
AMC and/or ATG positive	Yes	40 (10.2)	19 (10.6)	21 (9.9)	0.832
	No	352 (89.8)	161 (89.4)	191 (90.1)	

Blood sampling period for PFOS and PFOA	Before delivery	286 (73.0)	133 (73.9)	153 (72.2)	0.703
	After delivery	106 (27.0)	47 (26.1)	59 (27.8)	
Blood sampling period of TSH, FT4 (weeks of pregnancy)		11.1 ± 2.6	11.2 ± 2.9	11.1 ± 2.3	0.740
PFOS (ng/mL) ^b		5.2 (1.6-12.3)	5.2 (1.6-12.2)	5.3 (1.6-12.6)	0.985
PFOA (ng/mL) ^b		1.2 (LOD-3.4)	1.3 (LOD-3.6)	1.2 (LOD-3.4)	0.834
TSH (μU/mL) ^b		1.0 (LOD-4.1)	1.0 (LOD-4.3)	1.0 (LOD-4.0)	0.835
FT4 (ng/mL) ^b		0.99 (0.62-1.67)	0.98 (0.58-1.84)	1.00 (0.72-1.67)	0.269

^a comparing the differences between mothers of male infants and mothers of female infant. P-values were calculated by the t-test and the chi-square test. ^b Median (95% confidence interval). ATC: antimicrosome antibody; ATG: antithyroglobulin antibody; BMI: body mass index; FT4: free thyroxine; LOD: limit of detection; PFOA: perfluorooctanoate; PFOS: perfluorooctanesulfonate; SD: standard deviation; TSH: thyroid stimulating hormone.

Table 2. Comparisons of infants' characteristics and the concentrations of TSH and FT4 by infant sex.

		Total infants (n=392)	Male infants (n=180)	Female infants (n=212)	
Characteristics of infants		Median $\pm$ SD	Median $\pm$ SD	Median $\pm$ SD	p-value ^a
		or n (%)	or n (%)	or n (%)	
Gestational age at birth (weeks)		38.9 $\pm$ 1.5	38.9 $\pm$ 1.3	38.9 $\pm$ 1.6	0.984
Birth weight (g)		3055.3 $\pm$ 401.0	3075.6 $\pm$ 380.9	3038.0 $\pm$ 417.5	0.355
Low birth weight (< 2500g)	Yes	25 (6.4)	10 (5.6)	15 (7.1)	0.539
Caesarean section	Yes	71 (19.6)	35 (19.4)	42 (19.8)	0.955
Blood sampling (day after birth) of TSH and FT4		4.5 $\pm$ 0.9	4.5 $\pm$ 1.0	4.5 $\pm$ 0.9	0.691
TSH (μ U/mL) ^b		2.2 (LOD-7.9)	2.1 (LOD-7.0)	2.4 (LOD-8.5)	0.353
FT4 (ng/mL) ^b		2.03 (1.39-2.89)	2.03 (1.39-2.74)	2.04 (1.38-2.93)	0.646

^a comparing the differences between mothers of male infants and mothers of female infant. P-values were calculated by the t-test and the chi-square test. ^b Median (95% confidence interval). ATC: antimicrosome antibody; ATG: antithyroglobulin antibody; BMI: body mass index; FT4: free thyroxine; LOD: limit of detection; PFOA: perfluorooctanoate; PFOS: perfluorooctanesulfonate; SD: standard deviation; TSH: thyroid stimulating hormone.

Table 3. Relationships between maternal factors and PFOS and PFOA concentrations in maternal serum.

Characteristics of mothers		Mothers of Total infants (n=392)		Mothers of male infants (n=180)		Mothers of female infants (n=212)	
		PFOS	PFOA	PFOS	PFOA	PFOS	PFOA
Age (years) at delivery ^a		r = -0.170 **	r = -0.071	r = -0.115	r = 0.026	r = -0.209 **	r = -0.152 *
BMI (kg/m ²) before pregnancy ^a		r = -0.065	r = -0.067	r = -0.0046	r = 0.022	r = -0.083	r = -0.146 *
Parity (times) ^{b,c}	0	6.0 (1.8–13.4) **	1.5 (0.4–4.3) **	6.1 (1.8-13.6) **	1.5 (0.6-4.7) **	5.7 (1.6-13.9) **	1.5 (0.3-4.2) **
	≥1	4.8 (1.5–11.1)	0.9 (0.3–2.7)	4.9 (1.4-10.2)	0.9 (0.3-2.6)	4.7 (1.6-11.3)	0.9 (0.3-2.9)
Educational level (years) ^b	≤12	5.0 (1.7–12.2)	1.2 (0.3–3.7)	4.9 (1.4-12.1)	1.2 (0.3-4.2)	5.1 (1.7-12.9)	1.2 (0.3-3.7)
	≥13	5.5 (1.6–12.7)	1.3 (0.3–3.4)	6.1 (1.6-13.3)	1.3 (0.3-3.7)	5.4 (1.5-12.7)	1.3 (0.3-2.8)
Annual income (millions of yens) ^b	<5	5.2 (1.6–12.3)	1.2 (0.3–3.7)	5.2 (1.5-12.4)	1.2 (0.3-4.3)	5.1 (1.6-12.4)	1.2 (0.3-3.6)
	≥5	5.5 (1.6–13.1)	1.3 (0.3–2.6)	5.6 (2.0-13.0)	1.3 (0.3-2.6)	5.5 (1.6-13.2)	1.3 (0.3-3.0)
Smoking during pregnancy ^b	Yes	4.8 (1.5–12.5)	1.1 (0.3–3.1)	4.5 (1.5-12.1)	1.0 (0.3-2.4) *	5.2 (1.5-13.3)	1.1 (0.3-3.2)
	No	5.3 (1.6–12.4)	1.3 (0.3–3.6)	5.4 (1.6-12.5)	1.3 (0.3-3.8)	5.3 (1.6-12.6)	1.3 (0.3-3.5)
Fish intake (g/day) ^a		r = 0.000	r = -0.052	r = -0.006	r = -0.026	r = 0.028	r = -0.078
AMC and/or ATG positive ^b	Yes	5.8 (1.4–13.3)	1.4 (0.3–3.8)	4.2 (1.4-12.1)	1.5 (0.6-2.4)	5.8 (2.5-13.3)	1.1 (0.3-3.8)
	No	5.2 (1.6–12.3)	1.2 (0.3–3.4)	5.2 (1.8-12.4)	1.2 (0.3-3.7)	5.2 (1.6-12.4)	1.2 (0.3-3.3)

Blood sampling period for PFOS and PFOA ^b	Before	5.7 (1.8–12.6) **	1.3 (0.3–3.7) **	5.6 (2.0–12.6) **	1.4 (0.3–4.1) *	5.8 (1.7–12.8) **	1.3 (0.3–3.5) **
	delivery						
	After	3.8 (1.4–11.2)	1.0 (0.3–2.5)	4.0 (1.4–10.4)	1.0 (0.3–2.6)	3.4 (1.4–13.1)	1.0 (0.3–2.6)
	delivery						
Blood sampling period of TSH, FT4 (weeks of pregnancy) ^a		r = 0.023	r = −0.077	r = −0.016	r = −0.152 *	r = 0.053	r = −0.011

^a P-values were calculated by the Spearman correlation test. ^b Median (95% confidence interval) (ng/mL). P-values were calculated by the Mann-Whitney U test.

* $p < 0.05$, ** $p < 0.01$. ATC: antimicrosome antibody; ATG: antithyroglobulin antibody; BMI: body mass index; FT4: free thyroxine; LOD: limit of detection;

PFOA: perfluorooctanoate; PFOS: perfluorooctanesulfonate; TSH: thyroid stimulating hormone.

Table 4. Spearman's correlation test and multiple linear regression analyses of maternal TSH and FT4 by maternal PFOS and PFOA levels.

	PFOS						PFOA					
	Correlation ^a		Crude ^b		Adjusted ^{b,c}		Correlation ^a		Crude ^b		Adjusted ^{b,c}	
	ρ	p-value	β	p-value	β	p-value	ρ	p-value	β	p-value	β	p-value
All mothers (n = 392)												
TSH	-0.179	<0.001	-0.187	<0.001	-0.214	<0.001	0.042	0.412	0.056	0.272	0.039	0.478
FT4	0.083	0.100	0.069	0.176	0.061	0.236	0.023	0.651	0.023	0.646	0.004	0.946
Mothers of Male infants (n = 180)												
TSH	-0.194	0.009	-0.219	0.003	-0.246	0.002	-0.034	0.652	0.004	0.959	-0.067	0.435
FT4	0.118	0.114	0.113	0.130	0.093	0.208	0.058	0.437	0.023	0.755	0.023	0.762
Mothers of Female infants (n = 212)												
TSH	-0.164	0.017	-0.163	0.018	-0.210	0.005	0.108	0.119	0.101	0.142	0.114	0.124
FT4	0.056	0.422	0.032	0.648	0.030	0.684	-0.017	0.810	0.024	0.727	0.006	0.936

^a Spearman's correlation test.

^b Concentrations of PFOS, PFOA, TSH, and FT4 are log10 transformed.

^c Adjusted for maternal age at delivery, BMI, parity, educational level, thyroid antibody, intake of seaweed, blood sampling period before/after delivery for PFOS and PFOA, and gestational week at which blood sampling was obtained for TSH and FT4. FT4: free thyroxine; PFOA: perfluorooctanoate PFOS: perfluorooctanesulfonate; TSH: thyroid stimulating hormone.

Table 5. Spearman's correlation test and multiple linear regression analyses of infant TSH and FT4 by maternal PFOS and PFOA levels.

	PFOS						PFOA					
	Correlation ^a		Crude ^b		Adjusted ^{b,c}		Correlation ^a		Crude ^b		Adjusted ^{b,c}	
	ρ	p-value	β	p-value	β	p-value	ρ	p-value	β	p-value	β	p-value
All infants (n = 392)												
TSH	0.061	0.229	0.080	0.112	0.177	0.001	-0.077	0.127	-0.066	0.190	-0.014	0.801
FT4	0.015	0.774	0.003	0.961	-0.043	0.452	-0.012	0.815	0.014	0.786	0.003	0.960
Male infants (n = 180)												
TSH	0.057	0.444	0.084	0.264	0.205	0.014	-0.146	0.051	-0.125	0.095	-0.095	0.277
FT4	0.012	0.877	-0.040	0.598	-0.037	0.670	-0.031	0.678	0.033	0.658	0.023	0.795
Female infants (n = 212)												
TSH	0.071	0.301	0.081	0.241	0.173	0.021	-0.017	0.808	-0.013	0.847	0.054	0.465
FT4	0.022	0.753	0.032	0.642	-0.044	0.567	0.001	0.988	-0.001	0.990	-0.017	0.819

^a Spearman's correlation test.

^b Concentrations of PFOS, PFOA, TSH, and FT4 are log10 transformed.

^c Adjusted for maternal factors (maternal age at delivery, parity, thyroid antibody, blood sampling period before/after delivery for PFOS and PFOA, and values of TSH and FT4); and infant factors (gestational weeks at birth, low birth weight < 2500g, and mode of delivery). FT4: free thyroxine; PFOA: perfluorooctanoate PFOS: perfluorooctanesulfonate; TSH: thyroid stimulating hormone.

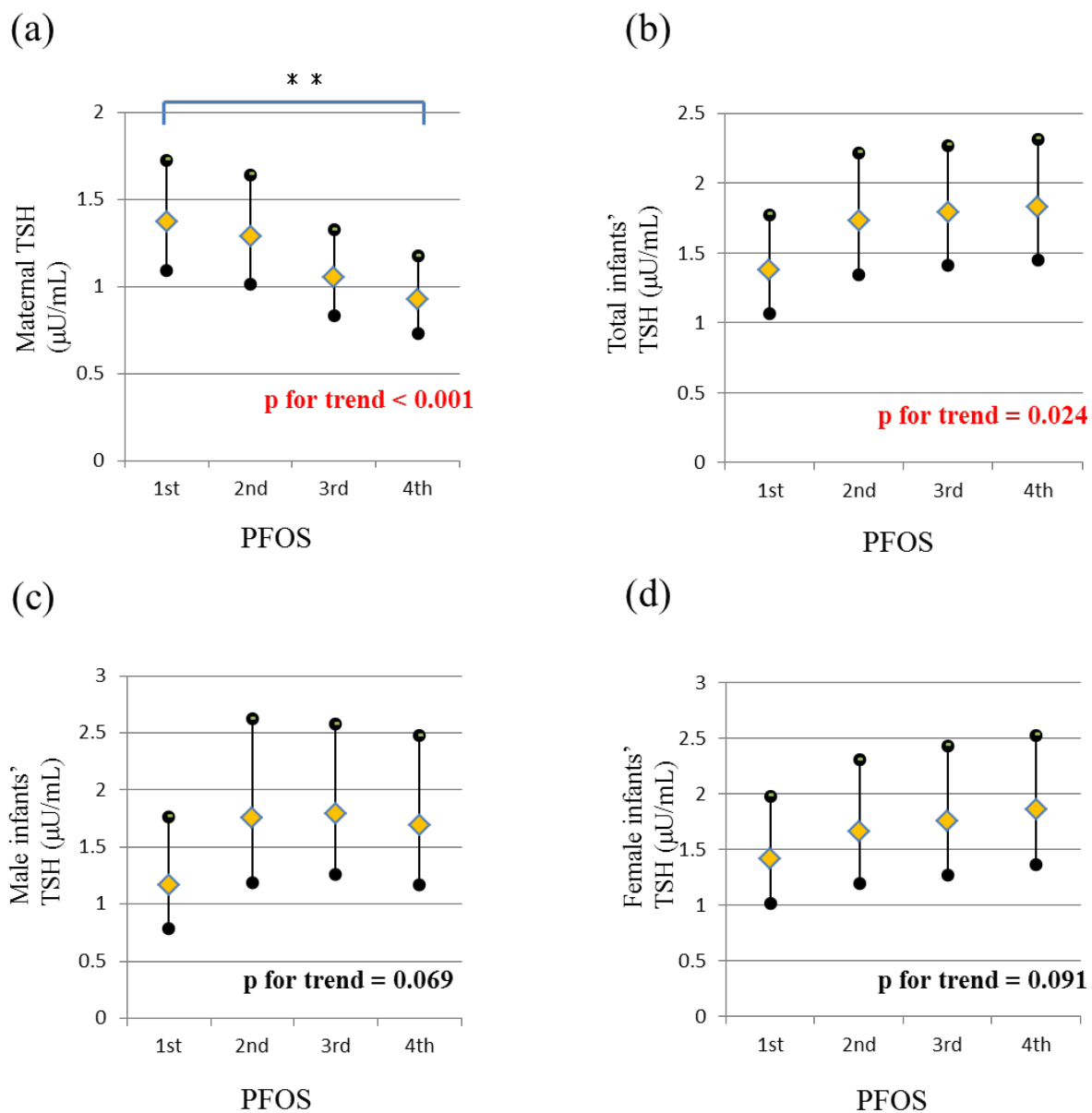


Figure 1.

Figure legend

Fig 1. Least square means of maternal and infant TSH, according to maternal serum PFOS concentration quartiles ($1.5 \leq \text{Quartile 1} \leq 3.5$ ng/mL, $3.5 < \text{Quartile 2} \leq 5.2$ ng/mL, $5.2 < \text{Quartile 3} \leq 7.0$ ng/mL, $7.0 < \text{Quartile 4} \leq 16.2$ ng/mL). (a) maternal TSH according to PFOS, (b) total infant TSH according to PFOS, (c) male infant TSH according to PFOS, (d) female infant TSH according to PFOS. Results are based on multiple linear regression models that were adjusted for the following factors: for (a), maternal age at delivery (years), BMI before pregnancy (kg/m^2), parity ($0/\geq 1$), educational level ($\leq 12/\geq 13$ years), positivity for ATG and/or AMC (yes/no), intake of seaweed more than once a week (yes/no), blood sampling period for PFOS and PFOA (before/after delivery), and gestational week of blood sampling for TSH and FT4 (continuous variable); and for (b), (c), and (d), maternal factors (maternal age at delivery [years], parity [$0/\geq 1$], positivity for ATG and/or AMC [yes/no], blood sampling period for PFOS and PFOA [before/after delivery], log10 values of TSH and FT4 [continuous variable]) and infant factors (gestational weeks at birth [continuous variable], low birth weight [yes/no], and born via cesarean section [yes/no]). $**p < 0.01$ compared to the first quartile, as calculated using the Hsu-Dunnett's method. LSMs are indicated in rhombus and the black dots depict the upper and lower 95% CI. ATC: antimicrosome antibody; ATG: antithyroglobulin antibody; BMI: body mass index; FT4: free thyroxine; PFOA: perfluorooctanoate PFOS: perfluorooctanesulfonate; TSH: thyroid stimulating hormone.

Supplemental Table 1. Characteristics of mothers and infants in relation to maternal and infants' TSH, and FT4.

Characteristics of mothers		Median $\pm$ SD or n (%)	TSH ^c (μ U/mL)	FT4 ^c (ng/mL)
Age (years) at delivery ^a		31.1 $\pm$ 7.1	r = 0.075	r = -0.063
BMI (kg/m ²) before pregnancy ^a		21.3 $\pm$ 3.3	r = 0.066	r = -0.112 *
Parity (times) ^b	0	176 (44.9)	1.0 (LOD-4.0)	0.99 (0.71-1.59)
	≥ 1	212 (54.1)	0.9 (LOD-4.4)	0.98 (0.6-1.77)
Educational level (years) ^b	≤ 12	178 (45.4)	1.0 (LOD-4.0)	0.98 (0.60-1.65) *
	≥ 13	214 (54.6)	0.9 (LOD-4.3)	1.00 (0.64-1.73)
Annual income (millions of yens) ^b	<5	269 (68.6)	1.0 (LOD-4.0)	0.99 (0.62-1.76)
	≥ 5	120 (30.6)	1.0 (LOD-4.3)	0.99 (0.61-1.67)
Smoking during pregnancy ^b	Yes	67 (17.1)	1.0 (LOD-4.2)	0.94 (0.55-1.66)
	No	325 (82.9)	1.0 (LOD-4.3)	1.00 (0.66-1.68)
Fish intake (g/day) ^a		37.2 $\pm$ 33.9	r = -0.016	r = -0.017
AMC and/or ATG positive ^b	Yes	40 (10.2)	1.5 (LOD-5.3) *	0.91 (0.53-1.49) *
	No	352 (89.8)	0.9 (LOD-3.9)	1.00 (0.65-1.69)
Intake of seaweed more than once a week ^b	Yes	350 (89.3)	0.9 (LOD-4.3)	0.99 (0.63-1.70) *
	No	42 (10.7)	1.4 (LOD-3.4)	0.91 (0.55-1.58)

Intake of iodine including supplements/eggs more than once a week ^b	Yes	14 (3.6)	1.2 (LOD-5.8)	0.98 (0.71-1.22)
	No	288 (73.5)	1.1 (LOD-4.2)	0.97 (0.60-1.68)
Gargling with iodine more than once a month ^b	Yes	50 (12.8)	1.0 (LOD-4.3)	1.00 (0.66-2.26)
	No	341 (87.0)	1.0 (LOD-4.1)	0.98 (0.61-1.67)
Blood sampling period for PFOS and PFOA ^b	Before delivery	286 (73.0)	1.0 (LOD-3.5)	1.00 (0.67-1.61)
	After delivery	106 (27.0)	1.0 (LOD-4.7)	0.95 (0.60-2.05)
Blood sampling period of TSH, FT4 (weeks of pregnancy) ^a		11.1 ± 2.6	r = 0.060	r = -0.203 **
Characteristics of infants		Median ± SD or n (%)	TSH (μU/mL)	FT4 (ng/mL)
Gestational age at birth (weeks) ^a		38.9 ± 1.5	r = -0.058	r = 0.092
Caesarean section ^b	Yes	71 (19.6)	2.0 (LOD-5.9) *	1.98 (1.30-2.69)
	No	306 (78.1)	2.2 (LOD-8.5)	2.03 (1.39-2.89)
Birth weight (g) ^b	Male	3075.6 ± 380.9	r = -0.072	r = 0.010
	Female	3038.0 ± 417.5	r = -0.155 *	r = 0.038
Low birth weight (< 2500g) ^b	Yes	25 (6.4)	1.7 (LOD-5.0) *	1.84 (0.85-2.67)
	No	367 (93.6)	2.3 (LOD-8.36)	2.03 (1.40-2.91)
Blood sampling (day after birth) of TSH and FT4 ^a		4.5 ± 0.9 ^a	r = -0.013	r = -0.046

^a Spearman correlation test. ^b Median (95% confidence interval). P-values were calculated by the Mann-Whitney U test. **p* < 0.05, ***p* < 0.01. ATC: antimicrosome antibody; ATG: antithyroglobulin antibody; BMI: body mass index; FT4: free thyroxine; LOD: limit of detection; PFOA: perfluorooctanoate; PFOS:

perfluorooctanesulfonate; TSH: thyroid stimulating hormone.