Table 6 Linear regression analyses for infant psychomotor development (BSID-II, PDI) in relation to maternal antenatal depression (EPDS) and confounding variables

Antenatal EPDS	Crude[a] adjusted $R^2 = -0.01$, $F = 0.01$, $p = 0.927$			Model 1[b] adjusted $R^2 = 0.09$, $F = 2.93$, $p = 0.005$			Model 2[c] adjusted $R^2 = 0.13$, $F = 3.34$, $p = 0.001$			Model 3[d] adjusted $R^2 = 0.14$, $F = 3.09$, $p = 0.001$		
	β	95 % CI	p	β	95 % CI	p	β	95 % CI	p	β	95 % CI	p
Antenatal EPDS	−0.01	[−0.00, 0.00]	0.927	0.03	[−0.00, 0.00]	0.709	0.05	[−0.00, 0.00]	0.533	0.04	[−0.00, 0.00]	0.659
Infant factors												
Sex				−0.09	[−0.03, 0.01]	0.284	−0.10	[−0.03, 0.00]	0.219	−0.10	[−0.03, 0.01]	0.217
IUGR				−0.02	[−0.04, 0.03]	0.769	0.01	[−0.03, 0.01]	0.924	−0.00	[−0.03, 0.03]	0.957
Gestational age				0.28	[0.00, 0.00]	0.003	0.25	[0.00, 0.03]	0.006	0.23	[0.00, 0.00]	0.012
Birth weight				0.01	[0.00, 0.00]	0.928	0.04	[0.00, 0.00]	0.752	0.03	[0.00, 0.00]	0.832
Length				0.03	[−0.01, 0.01]	0.795	0.06	[−0.01, 0.01]	0.609	0.07	[−0.01, 0.01]	0.557
Head circumference				−0.04	[−0.01, 0.01]	0.695	−0.09	[−0.01, 0.00]	0.385	−0.07	[−0.01, 0.01]	0.477
Age at 6-month assessment				0.25	[0.00, 0.00]	0.002	0.24	[0.00, 0.00]	0.003	0.24	[0.00, 0.00]	0.002
Childcare factor												
Avoidance of restriction and punishment							0.20	[0.01, 0.07]	0.010	0.23	[0.02, 0.08]	0.004
Maternal factor												
Caffeine intake during pregnancy							−0.09	[0.00, 0.00]	0.254	−0.09	[0.00, 0.00]	0.262
Paternal factors												
Age										−0.14	[−0.00, 0.00]	0.082
Education level										−0.03	[−0.20, 0.01]	0.662

[a] $n = 154$ in linear regression analyses

[b] Model 1: Adjusted for infant factors (sex, IUGR, gestational age, birth weight, length, head circumference and age at 6-month assessment)

[c] Model 2: Adjusted as in Model 1 as well as for childcare factor (avoidance of restriction and punishment) and maternal factor (caffeine intake during pregnancy)

[d] Model 3: Adjusted as in Model 2 as well as for paternal factors (age and education level)

Table 7 Gestational age and IUGR in relation to maternal antenatal depression (EPDS) and confounding variables[a]

	Gestational age[b]			IUGR[c]		
	β	95 % CI	p	OR	95 % CI	p
Crude	Adjusted $R^2 = 0.03$, $F = 5.07$, $p = 0.026$			Nagelkerke $R^2 = 0.003$, $\chi^2 = 0.16$, $p = 0.686$		
Antenatal EPDS	−0.18	[−0.92, −0.06]	0.026	0.96	[0.78, 1.18]	0.697
Adjusted[d]	Adjusted $R^2 = 0.12$, $F = 2.19$, $p = 0.006$			Nagelkerke $R^2 = 0.27$, $\chi^2 = 18.24$, $p = 0.571$		
Antenatal EPDS	−0.25	[−1.20, −0.17]	0.010	0.81	[0.59, 1.11]	0.199
Confounding variables						
Stressful life events before pregnancy	−0.18	[−5.91, −0.30]	0.030	2.75	[0.61, 12.322]	0.186
Planned pregnancy	0.20	[0.26, 6.43]	0.034	0.80	[0.15, 4.38]	0.795
Infant sex; female	0.17	[0.02, 5.57]	0.048	0.43	[0.08, 2.38]	0.333
First born	−0.23	[−6.84, −1.11]	0.007	1.14	[0.23, 5.77]	0.875

[a] Factors shown in this table were found to be statistically significant in relation to gestational age or IUGR

[b] Multiple linear regression analyses

[c] Logistic regression analyses; $n = 154$

[d] Adjusted for maternal factors (age, education level, household income, worked during pregnancy, smoked during pregnancy, caffeine intake during pregnancy, alcohol intake during pregnancy, stressful life events before pregnancy, past depressive symptoms, worrying, obsessiveness, planned pregnancy, wanted pregnancy), paternal factors (age and education level) and infant factors (sex and parity)

($\beta = -0.18$, 95 % CI [−0.92, −0.06], $p = 0.026$; goodness of fit: adjusted $R^2 = 0.026$, $F = 5.07$, $p = 0.026$) bit not to IUGR (OR = 0.96, 95 % CI [0.78, 1.19], $p = 0.697$; goodness of fit: Nagelkerke $R^2 = 0.003$, $\chi^2 = 0.16$, $p = 0.686$). This trend did not change even when the confounders were adjusted. In the adjusted model analysing the association between antenatal EPDS and gestational age, the regression coefficient of antenatal EPDS was the highest of all variables ($\beta = -0.25$, 95 % CI [−1.20, −0.17], $p = 0.010$; goodness of fit: adjusted $R^2 = 0.123$, $F = 2.19$, $p < 0.000$).

Discussion

Summary of study findings

In this study, we evaluated the hypothesis that maternal depression during pregnancy has an adverse relationship with infant development using improved adjustments for confounding variables. Although a trend of association between maternal antenatal depression and infant development was found in the univariable analysis, this correlation was lost in the multivariable analyses. However, the results of the regression analyses highlighted the fact that depression during pregnancy was significantly related to shorter gestational age and that shorter gestational age was significantly related to developmental delay in infant cognitive function. Therefore, gestational age can be considered to be an important confounder in the association between maternal antenatal depression and infant mental development. This is the first study to investigate the relationship between maternal depression during pregnancy and infant development with a proper control for gestational age, and the results thus provide new insights into the seemingly inconsistent results from previous studies.

Prevalence of maternal depression and scoring of infant development

The prevalence of maternal depression during pregnancy, defined using a cut-off of 8/9 on the EPDS, was 5.8 % in our study, which is relatively low compared with rates reported from Europe and the USA. Previous studies evaluating maternal depression during the second or third trimester reported prevalence levels of 7.0 % in the USA [6], 13.9 % in England [28], and 17.4 % in Sweden [29] using the EPDS, and 8.7 % in Hong Kong using the Beck Depression Scale [30]. However, the prevalence of depression during pregnancy based on the DSM-III-R Major Depressive Episode in Japan was reported to be 5.6 % [31]. In addition, according to a meta-analysis of perinatal depression in developed countries [32], the prevalence of major and minor depression during pregnancy ranges from 6.5 to 12.9 % (with minor depression ranging from 1.0 to 12.9 %); maternal depression at 1–2 months after delivery was estimated to be 10–15 %. The prevalence of antenatal and postnatal maternal depression in our study was within those ranges, thereby supporting the credibility of our results.

We used the BSID-II score to evaluate infant development in our study. The median MDI and PDI scores were 90 and 88, respectively, and both are lower than standardised scores (mean score 100). Since both cultural and language

differences exist between Japan and the USA, the BSID-II must be used with care in Japan. However, the first BSID edition has been used in Japan for the developmental assessment of infants [33], and a high correlation was reported between BSID-II and the Kyoto Developmental Test, which is standardised in Japan. Furthermore, a study in Taiwan revealed high reproducibility using BSID-II despite cultural differences [34]. To improve the reliability of the BSID-II scores in our study, we limited our evaluation of development to 6-month-old infants, and every examiner scored each infant. Therefore, the BSID-II scores of the participants in this study are directly comparable with each other.

Antenatal depression, gestational age and infant mental development

In previous studies examining the association of antenatal depression with infant development, Deave et al. [6] used the EPDS and reported that antenatal depression has an adverse impact on infant development, whereas DiPietro et al. [11]. used the BSID-II and reported a positive impact. Surprisingly, our results are inconsistent with both of these studies and successfully added new findings to current knowledge. There are several possible reasons why our results differ from those reported previously, especially in terms of controlling for confounders. First, our study differed from these previous studies with respect to the confounding factors entered into the statistical analyses and the credibility of this information. Although many potential confounders were considered by Deave et al. [6], all but four (antenatal tobacco use, maternal age, postnatal life events, postnatal depression) were removed using a conceptual framework. DiPietro et al. [11] also removed the gestational age confounder in the final analyses. Moreover, DiPietro et al. do not describe how information on gestational age was obtained. In our study, all perinatal information was obtained from medical records, ensuring the reliability of the data. Second, there are differences in childcare factors and in infant age at assessment. In our study, the child care environment was taken into account as a considerable confounder. In addition, infant development was evaluated at 6 months to minimise the influence of other confounding factors after birth. In the Deave et al. [6] and DiPietro et al. [11] studies, child assessments were conducted much later (18 and 24 months, respectively), and childcare factors were not controlled for throughout all the steps of the analysis. DiPietro et al. [11] reported a high level of maternal education (median 17 years), but Deave et al. [6] provided no information on maternal education. Higher education levels can counteract various negative influences that occur during the perinatal period [35]. It is also likely that other aspects of the child care environment positively or negatively affect infant development [36]. In our study, the education levels of both parents were also analysed statistically as confounding variables. Third, there were differences in the measures used. Deave et al. [6] applied the DDST, which evaluates similar developmental abilities as the BSID-II, but depends on parental reporting. Depressed mothers may possibly perceive their children's abilities as being lower, and such a reporting bias would lead to the apparent statistical association between antenatal depression and child development in these author's study. In contrast, reporting bias in infant assessment was avoided in our study by using an objective and blinded assessment, which provided improved credibility.

We identified gestational age to be a considerable confounding variable; that is, infants of depressed mothers tended to be delivered earlier and to suffer cognitive developmental delays as a consequence. A higher EPDS score during pregnancy was related to shorter gestational age ($\beta = -0.25$, 95 % CI [−1.20, −0.17], $p = 0.010$), and shorter gestational age was related to lower scores of mental ($\beta = 0.23$, 95 % CI [0.00, 0.00], $p = 0.013$) as well as psychomotor ($\beta = 0.23$, 95 % CI [0.00, 0.00], $p = 0.012$) development in the adjusted linear regression analysis (Tables 5, 6). This finding supports the notion that the developmental delays were a consequence of early delivery brought on by maternal antenatal depression. Results from several studies substantiate the impact of maternal depression on the length of gestation by showing that antenatal depression is associated with either a reduced gestational age [37] or a greater incidence of preterm birth among severely depressed women compared to non-depressed women [38, 39]. The influence of antenatal depression on gestational age may be explained by a potential biological pathway. According to recent studies, cortisol increases the release of a placental corticotropin-releasing hormone (CRH) [39, 40], which plays a key role in triggering parturition [40–43]. Antenatal depression is related to a greater incidence of premature delivery and to elevated antenatal cortisol levels compared to non-depressed women [38]. Higher levels of cortisol and CRH have also been detected in women who delivered preterm infants compared with those who delivered at term [40]. In our study, it was not possible to analyse the relation between preterm birth and maternal depression during pregnancy due to only five women (3.2 %) in our cohort giving birth to preterm infants. However, it is important to explore the association between gestational age, preterm birth and maternal antenatal depression in greater detail in further studies.

Child care factors and infant psychomotor development

Infant PDI was related not only to gestational age but also to "avoidance of restriction and punishment" in this study. The positive association of "avoidance of restriction and

punishment" with PDI in our study agrees with the results of earlier studies. Some studies have reported that infants exposed to maltreatment have lower PDI scores than control groups [44, 45], confirming our findings. However, the authors of these studies also noted that maltreatment had an impact on the MDI scores [44, 45], which we did not see. The reason for this difference may be the difference in infant age at the time of assessment. Previous studies examined infants aged between 2 and 30 months, whereas we enrolled infants aged 6 months in our study. In one earlier study, the reported impact of maltreatment on MDI appeared only after 14 months of age [46]; thus, the lack of association at an earlier age is consistent with our results.

Study strengths and limitations

This study constitutes a prospective cohort study, which minimises recall bias. We collected infant development scores through constructed assessment by examiners blinded to other data, enabling us to control for reporting bias and observer bias. We also collected perinatal information on mothers and infants (such as disease history, pregnancy conditions, and birth weight and size) from medical records written by obstetricians, not from maternal reports, which further increased data reliability. Moreover, diverse confounding variables were controlled for during a series of statistical analyses.

Nonetheless, this study has the following limitations. First, our sample size was relatively small to represent the general population, even though it was larger than those in several previous studies [10–12]. However, despite the small sample size, the goodness of fit in all of the adjusted models of linear regression analyses indicated statistical significance ($p < 0.05$), thereby endorsing the validity of the study results. Second, our study may have suffered from selection bias as it was based on a cohort from one regional hospital treating pregnant women in Sapporo and the surrounding areas, and the participant rate was low (30 %). Since women who were not interested in or were unwilling to participate in our study were excluded from the study, there is a possibility that depressed women may have been less likely to be involved in this study. The follow-up rate was also slightly low in our cohort study (50 %). Of 298 women with a single birth (96 % of all study participants), those who did not complete or return the mailed EPDS questionnaire between 1 and 4 months after delivery (10 % of those of single birth) and those who did not attend the infant assessment during the period from 5 months and 16 days up to 6 months and 15 days after birth (42 % of those who were assessed the postnatal EPDS) were excluded from the study. Because the BSID-II has not been standardised for use in Japan, we strictly limited the period of assessment, which may have resulted in the low follow-up rate. In our study, the prevalence of SGA was also very small (1.9 %). Pregnant women may possibly have avoided participating in our cohort study or dropped out during the follow-up period because of depression itself, causing a selection bias that may slightly lower the prevalence of depressive symptoms, as well as that of SGA. These factors may limit the extrapolation of our results to the general population. However, there was no remarkable difference between the prevalence of antenatal depression in all participants at the beginning (309 women, 5.2 %) and that in the analysed women (154 women, 5.8 %); therefore, we conclude that the low follow-up rate was unlikely to have had a significant influence on the study results. Finally, information on antenatal psychological distress may have been insufficient; maternal depression was not based on clinical diagnosis, and the experience of stressful events and the other maternal psychological factors were collected using dichotomous questions based on unstandardised questionnaires. However, the EPDS is thought to be a well-validated scale and was used in the previous study by Deave et al. [6] in the absence of clinical diagnosis.

In conclusion, the results of our study suggest that delay in infant mental development may be related to a shorter gestational period resulting from maternal depression during pregnancy. Because impaired cognitive and motor functions present at 6 months can be reversed by school age, further follow-up monitoring should continue at least until school age, and additional studies are required to clarify this issue.

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MATERNAL-FETAL MEDICINE

First-trimester serum folate levels and subsequent risk of abortion and preterm birth among Japanese women with singleton pregnancies

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Abstract
Objectives To determine whether a low serum folate level during the first trimester predicts subsequent late abortion, preterm birth, or fetal growth restriction (FGR).
Study design A prospective cohort study involving 5,075 women whose serum folate levels were measured during the first trimester. The participants were informed of their serum folate levels.
Results The pregnancy duration, birthweight, rate of late abortion/preterm birth, and the rate of FGR did not differ significantly among the four groups classified according to folate status. The mean serum folate levels did not differ among quartiles classified according to the gestational week at the time of delivery. Nineteen of the 20 women with folate deficiency gave birth at term to infants with a birthweight of 3.132 ± 321 g; only one infant had FGR.
Conclusion Low serum folate levels during the first trimester were not associated with the risk of late abortion, preterm birth, or FGR.

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Keywords Abortion · Nutrition · Preterm birth · Serum folate

Introduction

Adequate folate status is important not only during the periconceptional period, but also throughout pregnancy to achieve a satisfactory pregnancy outcome [1]. Since unambiguous evidence has shown that folic acid protects against both the first occurrence and the recurrence of neural tube defects [2, 3], expert committees worldwide have issued folic acid recommendations. In 2000, the Japanese government also issued a recommendation that all women planning to conceive consume a daily supplement containing 400 μg of folic acid to reduce the risk of neural tube defects during pregnancy.

Because folate is essential for DNA and RNA biosynthesis, an increased demand for folate exists during pregnancy because of the cellular proliferation occurring in the placenta and fetus. Indeed, the amount of folate breakdown products in the urine doubles during the second trimester and returns to the baseline level postpartum, suggesting an extra demand for dietary folate during pregnancy [4]. Since the calculated total fetal and placental folate content is 800 μg at term [5], the increased demand for folate may be due not only to fetal transfer, but also to the accelerated breakdown of the vitamin through folate's participation in DNA synthesis [6]. This hypothesis suggests a possible relation between the folate status in the mother and fetal growth. Indeed, the effects of folate on increases in birthweight have been observed with some consistency among poorly nourished women [7, 8].

Folate is also required for homocysteine metabolism. Folate is the major determinant of homocysteine

concentrations, with well-established evidence that supplementation with folic acid has a marked homocysteine-lowering effect [9]. Folic acid supplementation during pregnancy decreases the plasma total homocysteine level [10]. Thus, the serum homocysteine level can be used as a marker of folate status. Hyperhomocysteinemia is associated with pregnancy complications and an adverse pregnancy outcome, including miscarriage [11], placental abruption [12], and severe preeclampsia [13]. Since all these complications may shorten the length of gestation, the preterm birth rate may be higher among women with folate deficiency than among those with a normal folate status.

Indeed, according to the results of a previous study [14], women with serum folate levels <16.3 ng/mL (<37.3 nmol/L) during the second trimester have a higher risk of preterm birth than those with folate levels ≥16.3 ng/mL; however, no information regarding frequency of preeclampsia or placental abruption has been provided.

Whether the folate status during the first trimester is associated with the risk of preterm birth or inadequate fetal growth remains unknown. Accordingly, we conducted this prospective cohort study to examine whether the first-trimester serum folate level is associated with preterm birth or fetal growth.

Methods

This study was performed after receiving the approval of our institutional review board and obtaining informed consent from all the participants. The study participants were native Japanese pregnant women recruited from an ongoing birth cohort: the Hokkaido study on environment and children's health. The cohort was started in February 2003 and recruitment is ongoing; the details of the study have been described previously elsewhere [15]. All pregnant indigenous Japanese women who were booked to receive antenatal care at any of 37 participating health facilities in Hokkaido were considered eligible for the cohort study. This report analyzed data from 5,075 participants who were recruited during their first trimester between February 2003 and March 2006. Data on the demographic characteristics were obtained from baseline self-administered questionnaires at the time of recruitment and the infant's hospital birth records. We were able to collect information including maternal age, number of prior births, body height, prepregnancy body weight, folate supplementation, smoking, alcohol ingestion, annual income, gestational week at delivery and infant birthweight. Small for gestational age (SGA) was defined as a birthweight below the tenth percentile for gestational age for Japanese infants [16].

Serum folate concentrations were assayed in the blood specimens obtained during the first trimester [gestational week (GW) of $5^{-0/7}$ to $13^{-6/7}$] using an automated competitive protein-binding chemiluminescent enzyme immunoassay (CLEIA) technique according to the assay manufacturer's instructions; the measurements were performed by a commercial laboratory (SRL Inc., Tokyo, Japan). Analyses were conducted in batches because of the continuous recruitment of the study participants. The laboratory personnel were blinded to the folate supplement status of the participants whose biological specimens were assayed. Each participant's physician informed the participant of her serum folate level, approximately 3 weeks after the blood sample collection.

Statistical analyses were performed using the JMP8 statistical software package (SAS, Cary, NC). Data are reported as mean ± SD, unless otherwise stated. The Mann–Whitney U and the Kruskal–Wallis tests were used when comparing two or three groups, respectively. Differences in the means were tested using the Tukey–Kramer HSD (honestly significant difference) test between each group, and categorical variables were compared using Pearson's Chi-squared test. A p value of less than 0.05 was considered to indicate statistical significance.

Results

Among the 5,075 women analyzed in the present study, the mean serum folate level determined at GW 10.7 ± 1.5 was 18.4 ± 21.6 nmol/L (Table 1). Forty-eight (1.0 %) and 220 (4.3 %) women experienced a late abortion between GW $14^{-0/7}$ and $21^{-6/7}$ and preterm birth between GW $22^{-0/7}$ and $36^{-6/7}$, respectively. The intake of folate supplements significantly increased the serum folate levels (Table 1) and the number of women with higher serum folate levels (Fig. 1). Serum folate levels (nmol/L) were 16.9 ± 13.8 in 4,499 women with no folate supplementation, 29.2 ± 59.2 in 389 women who began to take folate supplementation after conception, and 36.0 ± 19.0 in 129 women who initiated folate supplementation before conception (Table 1). Serum folate levels increased with advancing maternal age (Table 1; Fig. 2). In addition, the prepregnancy maternal body mass index, use of infertility treatment, alcohol ingestion, and annual income were associated with serum folate levels. Women with smoking habit had a significantly lower serum folate level than nonsmokers. Multiple linear regression analysis was performed with serum folate concentration as the dependent variable and maternal age, prepregnancy BMI, infertility treatment, folate supplementation, smoking, alcohol intake, and annual income as independent variables, with the result that only folate supplementation was a statistically significant variable ($p = 0.0004$). None of the maternal

characteristics were significantly associated with the rate of late abortion or preterm birth (Table 1).

The effect of folate status during the first trimester on the subsequent preterm birth and fetal development was examined. The rate of late abortion/preterm birth, the mean gestational week at delivery, the mean birthweight, the SGA rate, or the female-to-male ratio did not differ significantly among quartiles divided according to folate status (Table 2). Unexpectedly, the mean serum folate level was significantly higher among women who gave birth at a GW of 28–36 than among women who gave birth at term (Table 3). The outcomes of the 20 women with folate deficiency (<6.8 nmol/L) were investigated individually. One woman miscarried at GW 20, but the remaining 19 women gave birth to infants with a birthweight of 3,132 ± 321 g (mean ± SD) at term (GW 39.5 ± 1.1). Only one of them gave birth to an SGA infant weighing 2,550 g at GW 38.

Table 1 Relationships between demographic characteristics, serum folate levels, late abortion, and preterm birth

	Serum folate level (*n*) (nmol/L)	*p* value[a]	Late abortion (%)	*p* value (χ^2)	Preterm delivery (%)	*p* value (χ^2)
Maternal age (years)						
≤19	46 (13.2 ± 4.0)	<0.0001	0 (0.0)	0.0825	2 (4.4)	0.3626
20–29	2,370 (17.2 ± 8.1)[b]		18 (0.8)		90 (3.8)	
30–39	2,565 (19.6 ± 29.2)		27 (1.1)		124 (4.8)	
≥40	94 (19.5 ± 10.7)		3 (3.2)		4 (4.3)	
Prepregnancy BMI (kg/m2)						
<18.5	899 (17.8 ± 11.0)	0.0019	9 (1.0)	0.6611	42 (4.7)	0.1617
18.5–24.9	3,528 (18.8 ± 25.0)		31 (0.9)		140 (4.0)	
≥25.0	550 (17.3 ± 8.2)		7 (1.3)		37 (5.6)	
Parity						
0	1,075 (18.2 ± 9.4)	0.4237	8 (0.7)	0.1587	46 (4.3)	0.8739
≥1	2,570 (18.3 ± 24.2)		33 (1.3)		107 (4.2)	
Infertility treatment						
No	4,871 (18.3 ± 21.9)	0.0006	45 (0.9)	0.3367	208 (4.3)	0.4556
Yes	185 (20.8 ± 11.1)		3 (1.6)		10 (5.4)	
Folate supplementation						
No	4,499 (16.9 ± 13.8)	<0.0001	43 (1.0)	0.9888	194 (4.3)	0.4694
After conception	389 (29.2 ± 59.2)[c]		4 (1.0)		19 (4.9)	
Before conception	129 (36.0 ± 19.0)[d]		1 (0.8)		5 (3.9)	
Smoking						
No	2,207 (19.5 ± 26.3)	<0.0001	18 (0.8)	0.3691	99 (4.5)	0.6460
Yes	2,820 (17.7 ± 17.2)		30 (1.1)		119 (4.2)	
Alcohol ingestion						
No	1,866 (17.8 ± 16.9)	0.0060	18 (1.0)	0.9512	92 (5.0)	0.0971
Yes	3,167 (18.8 ± 18.0)		30 (1.0)		125 (4.0)	
Annual income (USD)						
<36,585	889 (17.2 ± 9.7)	<0.0001	11 (1.2)	0.0817	33 (3.7)	0.7985
36, 586–60, 974	1,927 (18.4 ± 20.1)		11 (0.6)		87 (4.5)	
60, 975–97, 560	1,092 (19.7 ± 35.5)		16 (1.5)		47 (4.3)	
>97,561	323 (20.2 ± 9.7)		4 (1.2)		13 (4.0)	
Overall	5,075 (18.4 ± 21.6)		48 (1.0)		220 (4.3)	

Exchange rate: 1 USD/82.00 Japanese Yen

USD united states dollar, *BMI* body mass index

[a] The Mann–Whitney *U* and the Kruskal–Wallis tests were used when comparing the means in two or three groups, respectively

[b] $p = 0.0005$ versus age 30–39 years

[c] $p = 0.0089$ versus no folate supplementation

[d] $p < 0.0001$ versus no folate supplementation and folate supplementation after conception

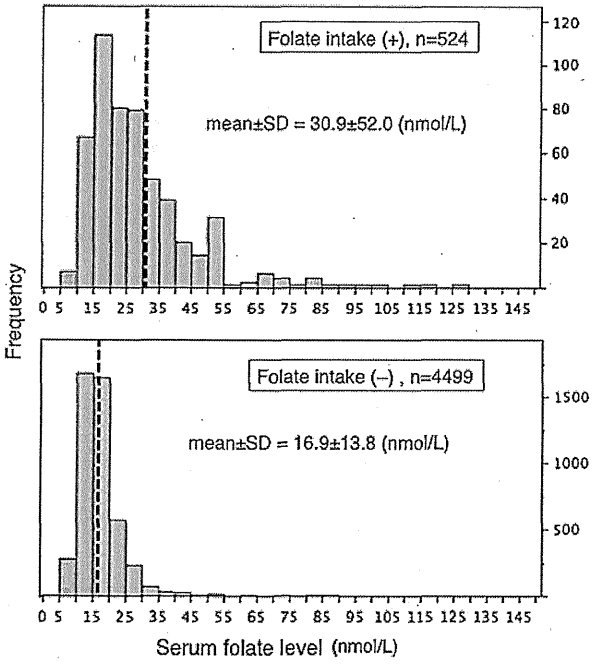

Fig. 1 Frequency of serum folate levels among women with or without folate supplementation

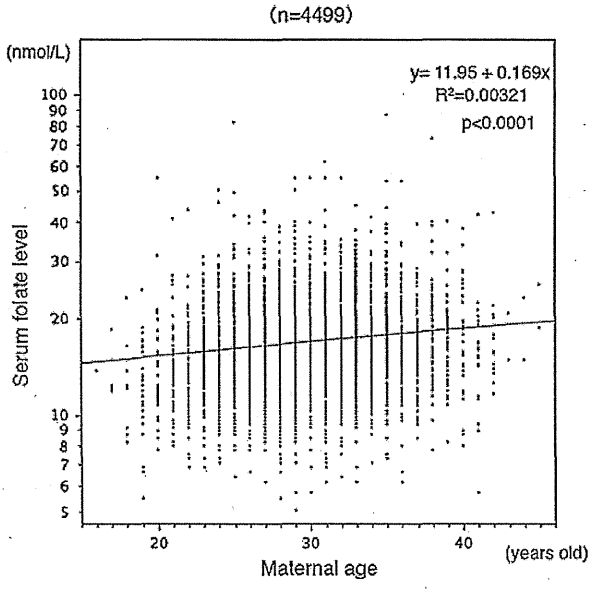

Fig. 2 Relationship between maternal age and serum folate levels

Comment

Unexpectedly, the present study indicated that the serum folate level during the first trimester was not associated with either preterm birth, including late abortion, or fetal growth.

With regard to the possible relationship between serum folate levels and preterm delivery, a series of previous reports have suggested that a low serum folate level during pregnancy increases the risk of premature delivery [14, 17, 18]. The total homocysteine (tHcy) level measured in serum can be a marker of folate status, suggesting an inverse relationship between serum folate and total homocysteine levels [19]. Methylenetetrahydrofolate reductase (*MTHFR*) is involved in the metabolism of folate and homocysteine: a polymorphism (677C → T) in the gene of *MTHFR* is associated with lower serum folate levels and higher tHcy levels [20, 21]. In the Hordaland homocysteine study using a cohort of 5,883 women, a higher plasma tHcy concentration, measured among women aged 40–42 years, was associated with significantly higher incidences of previous preeclampsia and previous premature delivery [17, 18]. In addition, in a prospective study of 2,026 women with a mean (SD) serum folate level of 21.1 ± 11.0 ng/mL (median 20.1 ng/mL; 5th–95th percentile, 6.4–41.1 ng/mL) during the second trimester, a significantly larger number of women with serum folate levels <16.3 ng/mL subsequently gave birth at preterm, compared with women whose serum folate levels were ≥16.3 ng/mL [14]. Although the Hordaland homocysteine study [17, 18] did not measure the plasma folate levels, all these reports have suggested the possibility that low plasma folate levels during the first trimester predict the risk of preterm birth.

The preterm premature rupture of membranes (PPROM) explains up to one-third of all preterm births [22]. However, the serum level of homocysteine or folic acid measured in patients with PPROM does not differ from that in matched control women [23, 24], raising questions regarding the association between the folate status and preterm birth. In the present study, the participants were not blinded to their folate levels determined during the first trimester: the folate levels were sent to the participants' physicians approximately 3 weeks after blood collection and no rules were defined as to whether the physicians should inform the participants of their folate levels or how to explain the folate level results. Some participants may have begun to take folate supplements after being informed about their serum folate level or after the blood collection independently of their folate status, although information regarding this issue was not available in the present study. If we assume that the women who had a low serum folate status during certain periods of their pregnancies would have a higher risk of preterm birth, such behavioral changes among the participants may have modified the present results, although the effect of folate fortification on the risk of preterm birth has not been demonstrated to date.

As to the possible relationship between the serum folate levels and fetal growth, many studies have suggested an association between these factors [18, 25–28]. The folate levels in cord blood were significantly lower in infants with

Table 2 Preterm birth, gestational week at delivery and fetal growth according to serum folate levels

Serum folate level (nmol/L) (*n*)	Late abortion/ preterm delivery (%)	*p* value[a]	Gestational age at delivery (week)	*p* value[b]	Birthweight (g)	*p* value[b]	SGA (<10 percentile)	*p* value[a]	Girl/ boy	*p* value[a]
≤13.1 (1,244)	71 (5.7)	0.6928	38.5 ± 3.0	0.2531	3,007 ± 474	0.2372	5.5	0.1135	1.052	0.3968
13.2–16.1 (1,303)	72 (5.5)		38.5 ± 3.1		3,008 ± 528		7.6		0.940	
16.2–20.0 (1,273)	60 (4.7)		38.7 ± 2.4		3,042 ± 447		6.8		1.003	
≥20.1 (1,255)	65 (5.2)		38.5 ± 2.8		3,021 ± 487		8.4		1.065	
Overall (5,075)	268 (5.3)		38.5 ± 2.9		3,020 ± 485		7.1		1.012	

SGA small for gestational age

[a] χ^2 test

[b] ANOVA

Table 3 Serum folate levels according to gestational week at delivery

Gestational week at delivery (week) (*n*)	Serum folate level (nmol/L)
14–21 (48)	17.3 ± 12.0
22–27 (12)	20.4 ± 10.9
28–36 (208)	22.3 ± 55.8
≥37 (4,807)	18.2 ± 18.8[a]
Overall (5,075)	18.4 ± 21.6

[a] $p < 0.05$ vs. 28–36 weeks

FGR than in normal birthweight infants [25]. The percentage of FGR decreases as the serum folate concentration at 30 weeks of gestation increases, in addition to significantly larger birthweight infants born to mothers with a serum folate level above the median value, compared with their counterparts [26]. The risk of FGR increases with the number of T alleles in the (677C → T) polymorphism of the *MTHFR* gene [27, 28] in addition to an increased risk of FGR among women with higher serum tHcy levels in the Hordaland homocysteine study [29]. Conflicting results have also been obtained in other studies [29–32]. An elevated tHcy level did not increase the risk of FGR [29, 30]. A maternal 677TT genotype for *MTHFR* was not associated with the risk of FGR [31, 32], but a maternal 1298CC genotype for *MTHFR* reduced the risk of FGR (odds ratio 0.49; 95 % CI 0.25–0.93) [32]. A recent study suggested that a maternal *MTHFR* 1298AA genotype was associated with a low folate status, and only smokers with this genotype had a significantly reduced infant birthweight; meanwhile, carrying the T allele of the *MTHFR* (677C → T) genotype was significantly associated with a low serum folate level, but not with the offspring's birthweight [33], suggesting that smoking and the *MTHFR* (1298A → C) polymorphism have a greater impact on fetal growth than the *MTHFR* (677C → T) polymorphism. These findings may explain why conflicting results have been obtained regarding the relation between the maternal serum folate/tHcy status and fetal growth.

The prevalence of preterm births at <37 weeks of gestation was relatively low (4.3 %) in the present study, with a prevalence of 7.3 % reported in the Hordaland homocysteine study [18] and 14.2 % reported in a study performed in the USA [14]. During the study period, the number of preterm singleton infants born at or after 22 weeks of gestation was approximately 55,000–57,000 annually in Japan, accounting for approximately 5.0 % of all newborn infants (tabulated information released annually by the Japanese Ministry of Health, Labour and Welfare). Thus, although Japan has a relatively low incidence rate of preterm births compared with other countries, the reason why the preterm birthrate is low in Japan remains unknown. Approximately 0.3 % of pregnant women do not receive adequate antenatal care in Hokkaido, and such women with no adequate antenatal care have an increased risk of preterm birth [34]. In the present study, the study subjects were pregnant women who received regular antenatal care from an early stage of pregnancy. This situation may have provided a favorable bias, resulting in a smaller number of women with preterm births in the present study compared with the general statistics for Japan (4.3 % vs. approximately 5.0 %).

In conclusion, the prospective cohort study involving 5,075 pregnant women with known first-trimester folate levels demonstrated that the pregnancy duration, birthweight, rate of late abortion/preterm birth and the rate of FGR did not differ significantly among four groups classified according to the first-trimester folate status. In addition, the mean serum folate levels did not differ among quartiles classified according to the gestational week at the time of delivery. These results suggested that low serum folate levels during the first trimester were not associated with the risk of late abortion, preterm birth or FGR. However, the present study did not preclude a possibility that active folate intake at and after the second trimester in women with lower folate levels modified the pregnancy duration and or fetal growth.

Conflict of interest We declare that we have no conflict of interest.

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Temporal trends of perfluoroalkyl acids in plasma samples of pregnant women in Hokkaido, Japan, 2003–2011 ☆,☆☆

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ABSTRACT

Perfluoroalkyl acids (PFAAs) are persistent organic pollutants that are used in a wide range of consumer products. Recent epidemiological studies have shown that prenatal exposure to toxic levels of PFAAs in the environment may adversely affect fetal growth and humoral immune response in infants and children. Here we have characterized levels of prenatal exposure to PFAA between 2003 and 2011 in Hokkaido, Japan, by measuring PFAA concentrations in plasma samples from pregnant women. The study population comprised 150 women who enrolled in a prospective birth cohort study conducted in Hokkaido. Eleven PFAAs were measured in maternal plasma samples using simultaneous analysis by ultra-performance liquid chromatography coupled to triple quadrupole tandem mass spectrometry. At the end of the study, in 2011, age- and parity-adjusted mean concentrations of perfluorooctanoic acid (PFOA), perfluorononanoic acid (PFNA), perfluorodecanoic acid (PFDA), perfluoroundecanoic acid (PFUnDA), perfluorododecanoic acid (PFDoDA), perfluorotridecanoic acid (PFTrDA), perfluorohexane sulfonate (PFHxS), and perfluorooctane sulfonate (PFOS) were 1.35 ng/mL, 1.26 ng/mL, 0.66 ng/mL, 1.29 ng/mL, 0.25 ng/mL, 0.33 ng/mL, 0.28 ng/mL, and 3.86 ng/mL, respectively. Whereas PFOS and PFOA concentrations declined 8.4%/y and 3.1%/y, respectively, PFNA and PFDA levels increased 4.7%/y and 2.4%/y, respectively, between 2003 and 2011. PFUnDA, PFDoDA, and PFTrDA were detected in the vast majority of maternal samples, but no significant temporal trend was apparent. Future studies must involve a larger population of pregnant women and their children to determine the effects of prenatal exposure to PFAA on health outcomes in infants and children.

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1. Introduction

Perfluoroalkyl acids (PFAAs) are used in a broad range of consumer products because of their surface properties, which include insulation and water resistance. These compounds are persistent and widespread organic pollutants within the environment, wildlife, and humans (Lau et al., 2007). Contamination of drinking water, foodstuffs such as seafood, leaching from food packaging and non-stick cookware, and household dust are known major routes of human exposure (Fromme et al., 2009). Potential health effects associated with PFAA exposure in humans are made worse by both bioaccumulation and persistence. In 2002, after 50 years of production, the 3M Company phased out the production and distribution of perfluorooctane sulfonate (PFOS) (Renner, 2001). PFOS has subsequently been regulated by the governments of the United States (Significant New Use Rules, United States Environmental Protection Agency, 2000), Canada (Schedule 1 of CEPA 1999 in Environment Canada, 2006), and the European Union (Directive 76/769/EEC, European Commission, 2006). PFOS was also included in

Abbreviations: PFAAs, perfluoroalkyl acids; PFCAs, perfluorinated carboxylic acids; PFSAs, perfluoroalkane sulfonates; PFHxA, perfluorohexanoic acid; PFHpA, perfluoroheptanoic acid; PFOA, perfluorooctanoic acid; PFNA, perfluorononanoic acid; PFDA, perfluorodecanoic acid; PFUnDA, perfluoroundecanoic acid; PFDoDA, perfluorododecanoic acid; PFTrDA, perfluorotridecanoic acid; PFTeDA, perfluorotetradecanoic acid; PFHxS, perfluorohexane sulfonate; PFOS, perfluorooctane sulfonate; BEH, ethylene-bridged hybrid; UPLC-MS/MS, ultra-performance liquid chromatography coupled to triple quadrupole tandem mass spectrometry; MDL, method detection limit; CI, confidence interval; GM, geometric mean.

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☆☆ Ethics approval: This study was conducted with written informed consent from all patients and was approved by the institutional ethics board for epidemiological studies at the Hokkaido University Graduate School of Medicine.

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Annex B of the 2009 Stockholm Convention on Persistent Organic Pollutants (UNEP, 2007; Wang et al., 2009). United States Environmental Protection Agency (2006) launched a 2010/2015 PFOA Stewardship Program to voluntarily reduce perfluorooctanoic acid (PFOA) emissions. Recent studies have indicated that concentrations of PFOS and PFOA are declining in the general human population (Kato et al., 2011; Olsen et al., 2012; Sundström et al., 2011; Wang et al., 2011). In contrast, concentrations of long-chain perfluorinated carboxylic acids (PFCAs) in the general human population are increasing (Wang et al., 2011).

PFOS and PFOA pass the placental barrier and are transferred to the fetus in humans (Midasch et al., 2007; Monroy et al., 2008). Previous epidemiological studies have reported a negative association between prenatal PFOS or PFOA exposure and birth weight (Andersen et al., 2010; Chen et al., 2012; Fei et al., 2007; Washino et al., 2009). Moreover, maternal PFOS levels correlate negatively with antibody concentrations in children aged 5 years (Grandjean et al., 2012). However, the effects of prenatal exposure to other PFAAs [e.g., long-chain perfluorinated carboxylic acids (PFCAs), such as perfluorononanoic acid (PFNA), perfluorodecanoic acid (PFDA), perfluoroundecanoic acid (PFUnDA), and perfluorododecanoic acid (PFDoDA)] remain unclear. PFCAs with chains longer than those in PFOA have high bioconcentration factors, suggesting their environmental persistence (Martin et al., 2003). It is necessary to measure, therefore, levels of exposure of pregnant women to PFOS, PFOA, and other PFAAs. It is also critical to determine whether environmental levels of these compounds are changing over time.

Here we have measured the concentration of 11 PFAAs in blood samples taken from pregnant women in Hokkaido, Japan. Analysis of samples from 2003 to 2011 allowed us to assess temporal trends associated with changes in the levels of these compounds.

2. Materials and methods

2.1. Study population

Study participants included 150 pregnant women, between 28 and 32 weeks of gestation, who were enrolled in a prospective birth cohort study (the Hokkaido Study on Environment and Children's Health). This ongoing cohort study was initiated in February 2003, and details have been described (Kishi et al., 2011). Briefly, subjects were considered eligible if they were indigenous Japanese women who had antenatal care at one of 37 participating hospitals within Hokkaido during the first trimester of pregnancy. Healthcare personnel introduced the study and provided each potential participant with an invitation, which included a consent form and a baseline questionnaire. All participants provided written informed consent. Among the 20,737 women that were registered between February 2003 and December 2011, only patients associated with a consent form, a baseline questionnaire, medical records at birth, and a maternal blood sample were included in this study. This represented 1944 women selected during 2003, 2459 women selected during 2005, 1820 women selected during 2007, 1274 women selected during 2009, and 1103 women selected during 2011. From these populations, 30 women from each year were randomly selected for analysis. The protocol used in this study was approved by the institutional ethics board for epidemiological studies at the Hokkaido University Graduate School of Medicine.

2.2. Standards and reagents

Acetonitrile, methanol, ultrapure water, and an HPLC-grade ammonium acetate solution (1 mol/L) were purchased from Wako Pure Chemical Inc., Osaka, Japan. Bulk ENVI-Carb sorbent was purchased from Supelco, Bellefonte, PA, USA. Acetic acid (purity: 99.7%) was purchased from Kanto Chemicals, Tokyo, Japan. Perfluorohexane sulfonate (PFHxS; >98%), PFOS (>98%), and a mixture of native PFCAs [perfluorohexanoic acid (PFHxA; >98%), perfluoroheptanoic acid (PFHpA; >98%), PFOA (>98%), PFNA (>98%), PFDA (>98%), PFUnDA (>98%), PFDoDA (>98%), perfluorotridecanoic acid (PFTrDA; >98%), and perfluorotetradecanoic acid (PFTeDA; >98%)] were obtained from Wellington Laboratories, Inc., Guelph, Ontario, Canada. Wellington Laboratories also supplied $^{13}C_3$-labeled PFHxS (≥99%), $^{13}C_4$-labeled PFOS (≥99%), and a mixture of ^{13}C-labeled PFCAs [$^{13}C_2$-PFHxA (≥99%), $^{13}C_4$-PFOA (≥99%), $^{13}C_5$-PFNA (≥99%), $^{13}C_2$-PFDA (≥99%) and $^{13}C_2$-PFUnDA (≥99%)].

2.3. Sample preparation

A 10-mL blood sample was taken from the maternal peripheral vein between 28 and 32 weeks of pregnancy. All samples were stored at −80 °C before analysis. An internal standard, which consisted of $^{13}C_3$-labeled PFHxS, $^{13}C_4$-labeled PFOS, and $^{13}C_4$-labeled PFCAs (2.5 ng of each), was added to each human plasma sample (0.5 mL). Samples were extracted with 2 mL acetonitrile by vortexing for 30 s. After centrifugation (3000 ×*g* for 15 min), supernatants were transferred into new tubes containing 25 mg ENVI-Carb and 50 μL acetic acid. Solutions were mixed by vortexing for 30 s. After centrifugation (3000 ×*g* for 15 min), each supernatant taken from above the ENVI-Carb was concentrated to 0.25 mL under nitrogen, and 0.25 mL methanol was added with subsequent mixing.

2.4. Ultra-performance liquid chromatography coupled to triple quadrupole tandem mass spectrometry (UPLC-MS/MS)

Extracted solutions were analyzed using UPLC-MS/MS instrumentation. The ACQUITY UPLC system (Waters, Tokyo, Japan) was used with ethylene-bridged (BEH) C18 columns (1.7 μm, 2.1 × 50 mm). The retention gap technique was used by installing retention gap columns [BEH C18 columns (1.7 μm, 2.1 × 100 mm)], which improved PFAA sensitivity by trapping mobile-phase PFAAs (contaminants) in the retention gap column. The column temperature was 55 °C, and the column oven was maintained at 57 °C. A Micromass Quattro Premier tandem quadruple mass spectrometer (Waters) was used for MS/MS. Conditions for MS/MS were as follows: desolvation and source temperatures were set at 350 °C and 120 °C, respectively. The capillary was held at a potential of 3.5 kV relative to the counterelectrode in the negative-ion mode for all compounds. Cone and desolvation gas flow rates were 50 and 800 L/h, respectively. Cone and collision voltages, and monitored transition ions are listed in Table 1. Analytes were eluted from the column with a linear gradient involving solvent A (2 mM ammonium acetate in water) and solvent B (2 mM ammonium acetate in acetonitrile) as follows: 10% B for the initial 0.2 min, then a gradient of 10–100% B from 0.2 min to 9 min. The effluent was maintained at 100% B from 9 min to 12 min. The total UPLC cycle time was 15 min including column re-equilibration. An eluent flow rate of 0.3 mL/min was employed for all analyses. The injection volume was 5 μL.

2.5. Quality control

Levels of PFHxA, PFHpA, PFOA, PFNA, PFDA, PFUnDA, PFDoDA, PFTrDA, PFTeDA, PFHxS, and PFOS were measured. Calibration curves were prepared using calibration standards that consisted of seven concentrations (between 0.1 and 10 ng/mL) prepared in 1:1 acetonitrile/methanol. Each calibration standard also contained the internal standard (5 ng/mL). Calibration curves were constructed to perform linear regressions (1/× weighting) that compared plots of peak area/internal standard area versus standard concentration/internal standard concentration. Plasma samples were quantified using calibration curves that showed good linearity and correlation coefficients (R^2) > 0.995 for all compounds. Quantification was performed using a relative-response ratio to an internal standard that most structurally matched the target analyte (Table 1).

Recoveries and relative standard deviations were evaluated using five replicate fortifications (fortified to 10 times the original

Table 1
Mass transitions, MS/MS conditions, recovery, and detection limits for each PFAA and internal standard.

Compound	Precursor ion (m/z)	Product ion (m/z)	Cone (V)	Collision (eV)	Recovery (%)	RSD[a] (%)	IDL[b,c] (ng/mL)	MDL[d,e] (ng/mL)	SRM 1957	
									This study[f] (ng/mL) (SE)	Reference values[g] (μg/kg)
PFCAs										
PFHxA	313	269	10	9	94.9	(9.29)	0.1	0.1	<0.1	–
$^{13}C_2$-PFHxA	315	270	10	9	–	–	–	–	–	–
PFHpA	363	319	16	10	93.1	(9.09)	0.1	0.1	0.338 (0.022)	0.305
PFOA	413	368	17	11	94.9	(6.41)	0.1	0.2	4.76 (0.23)	5.00
$^{13}C_4$-PFOA	417	372	17	11	–	–	–	–	–	–
PFNA	463	419	15	11	92.9	(5.74)	0.1	0.3	0.924 (0.049)	0.88
$^{13}C_5$-PFNA	468	423	15	11	–	–	–	–	–	–
PFDA	513	469	15	13	94.5	(4.90)	0.1	0.1	0.267 (0.053)	0.39
$^{13}C_2$-PFDA	515	470	15	13	–	–	–	–	–	–
PFUnDA	563	519	15	13	85.8	(4.60)	0.1	0.1	0.165 (0.046)	0.174
$^{13}C_2$-PFUnDA	565	520	15	13	–	–	–	–	–	–
PFDoDA	613	569	20	13	90.1	(5.55)	0.1	0.1	0.141 (0.007)	–
PFTrDA	713	669	22	15	85.7	(5.59)	0.1	0.1	0.110 (0.009)	–
PFTeDA	663	619	15	14	100.0	(4.23)	0.1	0.1	<0.1	–
PFSAs										
PFHxS	399	80	50	30	91.5	(6.13)	0.2	0.2	4.01 (0.27)	4.00
$^{13}C_3$-PFHxS	402	80	50	30	–	–	–	–	–	–
PFOS	499	80	45	40	75.2	(4.76)	0.2	0.3	22.3[h] (1.09)	21.1
$^{13}C_4$-PFOS	503	80	45	40	–	–	–	–	–	–

[a] RSD: relative standard deviation.
[b] IDL: instrument detection limit.
[c] 5-μL injection.
[d] MDL: method detection limit.
[e] 0.5-mL plasma sample.
[f] 0.5-mL serum sample of NIST SRM 1957.
[g] Reference values in NIST, Certificate of Analysis, SRM® 1957.
[h] PFOS concentration reflects integration of peak areas for both the branched and linear isomers.

concentration) of a human plasma sample with low levels of contamination (Table 1). The procedural blank levels were determined using 0.5 mL of ultrapure water. Instrumental detection limits were defined as the mass of analyte that produced a peak with a signal-to-noise ratio of 3. These values ranged from 0.1 ng/mL (PFCAs) to 0.2 ng/mL (PFSAs). The method detection limit (MDL) was defined as the mass of analyte that produced a peak with a signal-to-noise ratio of 10. These values ranged from 0.1 ng/mL (PFHxA, PFHpA, PFDA, PFUnDA, PFDoDA, PFTrDA and PFTeDA) to 0.3 ng/mL (PFNA and PFOS) (Table 1). Chromatographic resolution of branched and linear isomers in plasma samples was achieved using UPLC-MS/MS. For quantification of PFOS, the total area of the branched and linear isomer peaks was integrated.

To assess potential inter-laboratory differences, we analyzed NIST standard reference material (SRM) 1957 (Table 1). Our methods yielded reliable data given that values for PFHpA to PFUnDA, PFHxS, and PFOS were comparable to those measured during inter-laboratory comparisons (Harada et al., 2011; Keller et al., 2010; National Institute of Standards & Technology, Certificate of Analysis, SRM 1957).

2.6. Statistical analysis

Because our data did not fall into a normal distribution, PFAA concentrations were converted to a natural log scale. For participants with PFAA concentrations below the MDL, a value equal to half of the MDL was assigned for statistical analyses, except for PFHxA, PFHpA, and PFTeDA. We did not include PFHxA, PFHpA, and PFTeDA in the statistical analysis because these compounds were detected very infrequently. Given that the age of pregnant women correlates negatively with PFAA concentrations and that the concentrations measured for multiparous women are lower than for primiparous women (Fei et al., 2007; Okada et al., 2012; Washino et al., 2009), age- and parity-adjusted means were calculated using a least squares mean obtained from analysis of covariance. We used linear regression to analyze temporal trends between 2003 and 2011 and calculated change per year through the period assessed from the discrete rate of change (termed lambda). To assess the temporal trends of proportion for PFAAs, we performed the Cochran–Armitage trend test. Correlations between different PFAAs were assessed using the Spearman's rank correlation coefficient (ρ). All statistical analyses were performed using the Statistical Package for Social Science (SPSS) for Windows, version 16.0J (Japanese version; SPSS, Inc., Chicago, IL, USA) and JMP 10 Statistical Discovery Software for Windows (S.A.S. Institute Inc., Cary, North Carolina). Differences were considered statistically significant at $p < 0.05$.

3. Results

The mean age of all pregnant women included in the study was 30.3 ± 4.8 years (Table 2). Concentrations of maternal plasma PFAAs for each year are provided in Table 3. Sum-total PFAA concentrations (ΣPFAAs) are also provided. PFOA, PFNA, PFDA, PFUnDA, and PFOS were detected in all samples. Detection rates for the other compounds were: PFHxA (13.3%), PFHpA (23.3%), PFDoDA (73.3%), PFTrDA (97.3%), PFTeDA (28.0%), and PFHxS (80.7%). Age- and parity-adjusted mean concentrations at the end of the study (in 2011) were: PFOA

Table 2
The study population.

Year	n	Age (years)			Parity	
		Mean	(SD[a])	Range	Primiparous	Multiparous
2003	30	29.7	(4.8)	23–39	15	15
2005	30	29.2	(4.8)	19–37	17	13
2007	30	30.6	(3.8)	24–37	18	12
2009	30	29.6	(5.8)	19–38	16	14
2011	30	32.5	(3.9)	25–40	9	21

[a] SD: standard deviation.

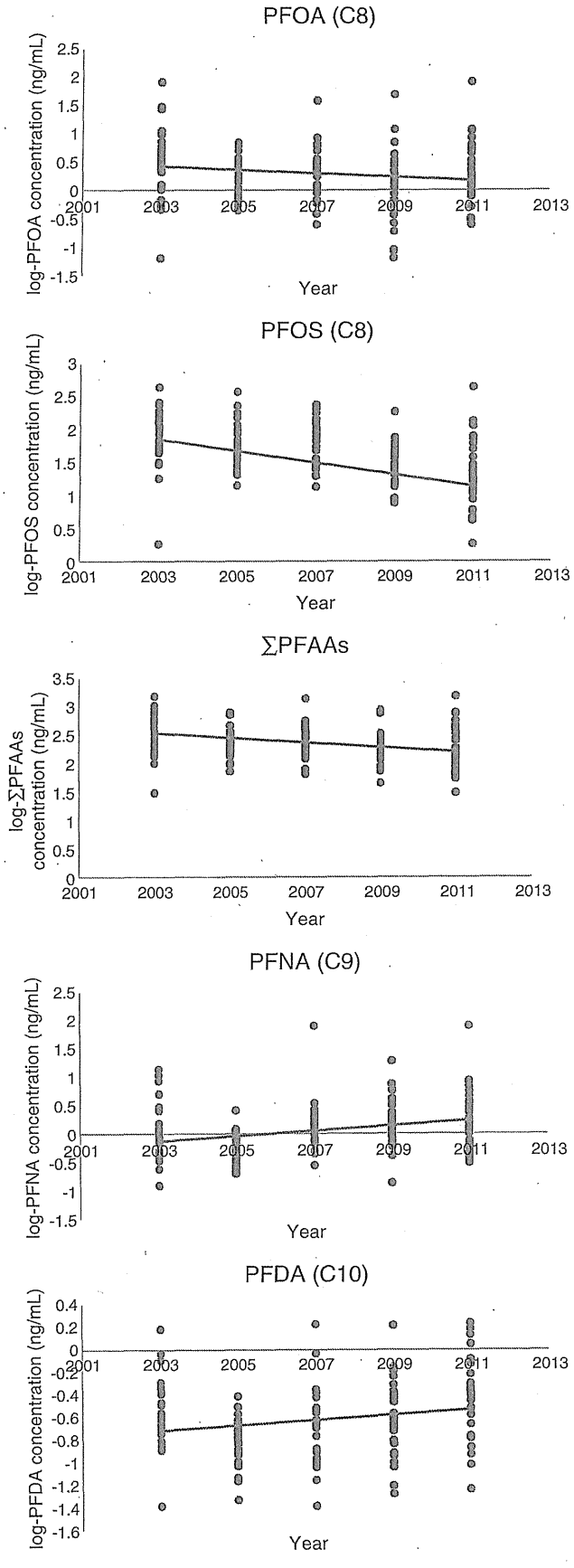
(1.35 ng/mL), PFNA (1.26 ng/mL), PFDA (0.66 ng/mL), PFUnDA (1.29 ng/mL), PFDoDA (0.25 ng/mL), PFTrDA (0.33 ng/mL), PFHxS (0.28 ng/mL), PFOS (3.86 ng/mL), and ΣPFAAs (10.13 ng/mL).

Scatter-plots and linear regressions of age- and parity-adjusted concentrations of PFOA, PFOS, ΣPFAAs, PFNA, and PFDA from 2003 to 2011 are shown in Fig. 1. Statistical analyses of these trends are

Table 3
Concentration of each PFAA compound in plasma collected from pregnant women from 2003 to 2011.

Compound	Year	Detection		Concentration (ng/mL)						Adjusted concentration (ng/mL)[a]		p for trend[c]
		No.	(%)	Range	Mean	(SD[b])	25th	50th	75th	Mean	(95%CI)	
PFCAs												
PFHxA (C6[e])	2003	8	(26.7)	<0.1–0.16	<0.1	–	<0.1	<0.1	0.11	<0.1	–	–
	2005	5	(16.7)	<0.1–0.14	<0.1	–	<0.1	<0.1	<0.1	<0.1	–	
	2007	1	(3.3)	<0.1–0.12	<0.1	–	<0.1	<0.1	<0.1	<0.1	–	
	2009	0	(0)	<0.1–<0.1	<0.1	–	<0.1	<0.1	<0.1	<0.1	–	
	2011	6	(20.0)	<0.1–0.13	<0.1	–	<0.1	<0.1	<0.1	<0.1	–	
PFHpA (C7[e])	2003	17	(56.7)	<0.1–0.26	<0.1	–	<0.1	0.11	0.16	<0.1	–	–
	2005	1	(3.3)	<0.1–0.11	<0.1	–	<0.1	<0.1	<0.1	<0.1	–	
	2007	0	(0)	<0.1–<0.1		–		<0.1	<0.1	<0.1	–	
	2009	2	(6.7)	<0.1–0.14	<0.1	–		<0.1	<0.1	<0.1	–	
	2011	15	(50.0)	<0.1–0.20	<0.1	–		<0.1	<0.1	<0.12	–	
PFOA (C8[e])	2003	30	(100)	0.71–6.88	2.05	(1.26)	1.33	1.93	2.18	1.77	1.51–2.08	0.023
	2005	30	(100)	0.70–2.35	1.25	(0.44)	0.88	1.16	1.56	1.15	0.98–1.35	
	2007	30	(100)	0.55–4.89	1.56	(0.78)	1.09	1.44	1.71	1.36	1.16–1.60	
	2009	30	(100)	0.30–5.45	1.36	(0.99)	0.72	1.19	1.68	1.09	0.93–1.27	
	2011	30	(100)	0.54–2.93	1.42	(0.63)	0.98	1.27	1.77	1.35	1.13–1.61	
PFNA (C9[e])	2003	30	(100)	0.41–3.14	1.13	(0.66)	0.72	0.92	1.14	0.98	0.84–1.13	<0.001
	2005	30	(100)	0.49–1.52	0.81	(0.25)	0.65	0.74	0.91	0.75	0.64–0.86	
	2007	30	(100)	0.57–6.74	1.31	(1.06)	0.90	1.19	1.33	1.11	0.96–1.28	
	2009	30	(100)	0.42–3.57	1.32	(0.59)	0.98	1.24	1.45	1.20	1.04–1.39	
	2011	30	(100)	0.60–2.54	1.34	(0.57)	0.82	1.26	1.73	1.26	1.07–1.48	
PFDA (C10[e])	2003	30	(100)	0.41–1.20	0.60	(0.18)	0.49	0.56	0.68	0.58	0.52–0.65	0.016
	2005	30	(100)	0.26–0.66	0.42	(0.09)	0.36	0.40	0.48	0.41	0.36–0.46	
	2007	30	(100)	0.25–1.25	0.54	(0.20)	0.40	0.52	0.61	0.50	0.45–0.56	
	2009	30	(100)	0.28–1.24	0.57	(0.20)	0.42	0.51	0.67	0.53	0.48–0.60	
	2011	30	(100)	0.29–1.27	0.71	(0.26)	0.50	0.69	0.89	0.66	0.59–0.75	
PFUnDA (C11[e])	2003	30	(100)	0.71–2.22	1.34	(0.41)	1.09	1.26	1.64	1.27	1.10–1.47	0.876
	2005	30	(100)	0.55–1.64	1.08	(0.31)	0.79	1.13	1.30	1.03	0.89–1.19	
	2007	30	(100)	0.47–2.28	1.37	(0.52)	0.84	1.44	1.87	1.24	1.08–1.44	
	2009	30	(100)	0.42–2.90	1.19	(0.53)	0.77	1.21	1.46	1.09	0.95–1.26	
	2011	30	(100)	0.43–3.40	1.45	(0.70)	0.90	1.30	1.80	1.29	1.10–1.52	
PFDoDA (C12[e])	2003	30	(100)	0.17–0.35	0.24	(0.05)	0.21	0.23	0.27	0.24	0.21–0.29	0.913
	2005	14	(46.7)	<0.1–0.15	0.08	(0.04)	<0.1	<0.1	0.13	<0.1	–	
	2007	19	(63.3)	<0.1–0.30	0.12	(0.06)	<0.1	0.11	0.16	<0.1	<0.1–0.12	
	2009	18	(60.0)	<0.1–0.21	0.11	(0.06)	<0.1	0.12	0.15	<0.1	<0.1–0.11	
	2011	29	(96.7)	<0.1–0.51	0.25	(0.08)	0.22	0.24	0.30	0.25	0.21–0.30	
PFTrDA (C13[e])	2003	30	(100)	0.25–0.81	0.41	(0.12)	0.32	0.40	0.47	0.40	0.35–0.47	0.055
	2005	29	(96.7)	<0.1–0.40	0.24	(0.08)	0.18	0.24	0.29	0.23	0.20–0.27	
	2007	29	(96.7)	<0.1–0.80	0.27	(0.14)	0.18	0.25	0.34	0.25	0.21–0.29	
	2009	28	(93.3)	<0.1–0.48	0.25	(0.11)	0.17	0.25	0.34	0.23	0.20–0.27	
	2011	29	(96.7)	<0.1–0.78	0.36	(0.14)	0.28	0.33	0.44	0.33	0.28–0.39	
PFTeDA (C14[e])	2003	16	(53.3)	<0.1–0.16	<0.1	–	<0.1	0.11	0.12	<0.1	–	–
	2005	0	(0)	<0.1–<0.1	<0.1	–	<0.1	<0.1	<0.1	<0.1	–	
	2007	0	(0)	<0.1–<0.1	<0.1	–	<0.1	<0.1	<0.1	<0.1	–	
	2009	0	(0)	<0.1–<0.1	<0.1	–	<0.1	<0.1	<0.1	<0.1	–	
	2011	4	(13.3)	<0.1–0.15	0.11	(0.03)	0.11	0.12	0.13	<0.1	–	
PFSAs												
PFHxS (C6[e])	2003	29	(96.7)	<0.2–0.60	0.40	(0.11)	0.34	0.40	0.49	0.37	0.30–0.45	0.106
	2005	22	(73.3)	<0.2–0.53	0.27	(0.13)	<0.2	0.26	0.35	0.22	0.18–0.27	
	2007	24	(80)	<0.2–0.61	0.28	(0.13)	0.22	0.27	0.35	0.24	0.20–0.29	
	2009	23	(76.7)	<0.2–0.77	0.30	(0.16)	<0.2	0.29	0.39	0.25	0.21–0.30	
	2011	23	(76.7)	<0.2–0.78	0.33	(0.18)	<0.2	0.33	0.46	0.28	0.22–0.35	
PFOS (C8[e])[d]	2003	30	(100)	3.53–14.1	7.76	(2.46)	5.72	7.66	9.50	7.66	6.92–8.39	<0.001
	2005	30	(100)	3.17–13.1	6.20	(2.24)	4.84	5.37	7.37	5.99	5.25–6.74	
	2007	30	(100)	3.11–10.8	6.23	(2.05)	4.37	6.30	7.59	6.08	5.34–6.81	
	2009	30	(100)	2.43–9.69	4.54	(1.48)	3.31	4.48	5.30	4.43	3.70–5.16	
	2011	30	(100)	1.31–8.46	3.90	(1.87)	2.49	3.52	5.05	3.86	3.04–4.68	
ΣPFAAs	2003	–	–	7.51–24.33	14.19	(3.90)	11.37	13.97	16.19	14.05	12.90–15.20	<0.001
	2005	–	–	6.40–18.01	10.51	(2.73)	8.78	9.65	12.24	10.18	9.01–11.35	
	2007	–	–	6.15–23.30	11.83	(3.29)	9.60	11.76	13.41	11.52	10.36–12.67	
	2009	–	–	5.23–18.83	9.80	(2.92)	8.03	9.21	11.11	9.64	8.50–10.78	
	2011	–	–	4.42–18.22	10.02	(3.57)	7.37	9.07	12.70	10.13	8.85–11.42	

[a] Adjusted concentrations were evaluated with respect to the average maternal age of 30.32 years and parity by analysis of covariance.
[b] SD: standard deviation.
[c] Linear regressions to detect temporal trends from 2003 to 2011.
[d] PFOS concentration reflects integration of peak areas for both the branched and linear isomers
[e] C: carbon chain length.



provided in Table 3. Through the period 2003 to 2011, the rate of change per year for the concentrations of PFOA, PFOS, and ΣPFAAs exhibited a statistically significant decrease: PFOA = −3.1%, PFOS = −8.4%, and ΣPFAAs = −4.0%. In contrast, the rate of change per year for PFNA and PFDA levels increased significantly: PFNA = +4.7%; and PFDA = +2.4%.

The proportion of ΣPFAA that was accounted for by each PFAA is shown in Fig. 2. Each year of collected samples is represented separately. A comparison of the 2003 and 2011 data showed that relative levels of PFOS (as a percentage of ΣPFAA) dropped from 57.1% to 40.7%, but this apparent temporal trend of the proportion was not statistically significant. Although the proportion of other PFAAs also did not exhibit a significant trend over time, the relative levels of PFNA (7.3% to 13.3%), PFDA (4.3% to 7.0%), and PFUnDA (9.5% to 13.6%) increased comparing 2003 with 2011.

Correlation coefficients among PFOA, PFNA, PFDA, PFUnDA, PFDoDA, PFTrDA, PFHxS, and PFOS for all samples are listed in Table 4. The level of each PFAA correlated significantly with that of the corresponding PFAA having a different chain length. Significant correlation coefficients (ρ) were found for the following pairs: PFNA and PFDA (0.702), PFDA and PFUnDA (0.698), PFDA and PFDoDA (0.616), PFUnDA and PFTrDA (0.675), and PFDoDA and PFTrDA (0.707). In general, strong correlations were measured between PFAAs with similar chain length.

4. Discussion

Here we determined the concentration of 11 PFAAs in plasma samples of pregnant women in Hokkaido, Japan. Detectable levels of PFNA, PFDA, PFUnDA, and PFOS were found in all samples. Between 2003 and 2011, plasma concentrations of PFOS and PFOA decreased, whereas PFNA and PFDA concentrations increased. To our knowledge, this is the first report that has investigated maternal levels of 11 PFAA in Japan.

The declining levels of PFOS and PFOA in humans are consistent with previous studies (Calafat et al., 2007; Harada et al., 2011; Olsen et al., 2008, 2012). In our study, the proportion of PFOS in ΣPFAA also decreased as compared with 2003 and 2011. This is because the 3M Company phased out their manufacture and distribution of PFOS in 2002, following 50 years of production (Renner, 2001). Given that PFOS subsequently ceased to accumulate in the environment and indeed human exposure to this compound decreased, PFOS concentrations in human plasma decreased dramatically between 2003 and 2005. Further reductions in PFOS levels by 2011 may be explained by two developments. First, PFOS was listed on Annex B of the Stockholm Convention on Persistent Organic Pollutants in 2009 (UNEP, 2007; Wang et al., 2009). Second, Japan designated PFOS a Type I Specified Chemical Substance (characterized by persistence, bioaccumulation, and long-term human toxicity) in the Act on the Evaluation of Chemical Substances and Regulation of Their Manufacture, etc., in 2010 (Ministry of Health, Labour and Welfare, Ministry of Economy, Trade and Industry and Ministry of the Environment, Japan, 2010). A similar decline in PFOA concentrations may have resulted from phasing out of the manufacture and use of PFOA in 2006 as stipulated by the U.S. Environmental Protection Agency via the 2010/2015 PFOA Stewardship program.

Between 2003 and 2011, concentrations of PFNA and PFDA increased by 4.7% and 2.4% per year, respectively. These results are consistent with recent reports. For example, a study of Swedish primiparous women between 1996 and 2010 revealed that PFNA and PFDA concentrations increased by 4.3% and 3.8% per year, respectively (Glynn et al.,

Fig. 1. Temporal trends associated with changes in PFOA, PFOS, ΣPFAA, PFNA, and PFDA, levels in maternal plasma adjusted for age and parity between 2003 and 2011. Solid lines denote the predicted fit from linear regression adjusted for age and parity (log-PFOA = −0.031 year + 0.447; log-PFOS = −0.088 year + 1.943; log-ΣPFAAs = −0.040 year + 2.574; log-PFNA = 0.046 year − 0.171; and log-PFDA = 0.024 year − 0.747). The letter 'C' indicates the carbon chain length of each compound.

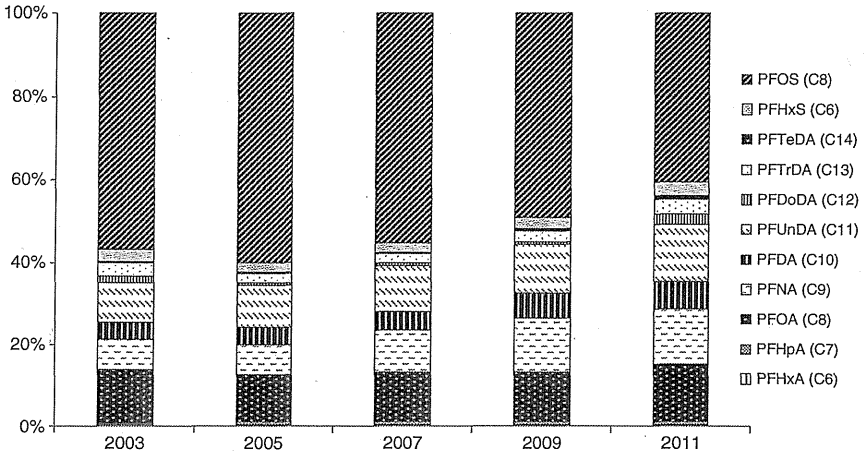


Fig. 2. The composition of total PFAA levels between 2003 and 2011. Relative levels of each PFAA (with ΣPFAA concentration set to 100%) were determined every other year between 2003 and 2011. The letter 'C' indicates the carbon chain length of each compound.

2012). In the National Health and Nutrition Examination Survey (NHANES), which evaluated the United States population, elevated levels of PFNA were measured when samples collected from females during 1999–2000 were compared with samples collected during 2003–2004 [geometric mean (GM) values of 0.5 ng/mL and 0.9 ng/mL, respectively] (Calafat et al., 2007). In Sendai, Japan, PFNA concentrations in samples from females increased from 1.01 ng/mL in 2003 to 1.80 ng/mL in 2008; moreover, an increase in PFDA concentration was also observed in these samples (0.52 ng/mL to 0.72 ng/mL) (Harada et al., 2011). Here we measured comparable PFNA and PFOA concentrations in samples collected during 2011. Moreover, the proportion of long-chain PFCAs generally increased over time. This result may be explained by higher environmental persistence (Martin et al., 2003) and longer half-lives of long-chain PFCAs (Ohmori et al., 2003), which generally have longer chains than PFOA. The toxicity of PFCAs has been correlated with the length of the carbon chain and the nature of the functional group (Liao et al., 2009; Wolf et al., 2008). Given the increased levels of PFNA and PFDA detected in human blood samples around the world, it is important to evaluate the potential health effects of PFCAs with chains longer than those in PFOAs.

PFUnDA, PFDoDA, and PFTrDA are frequently detected in maternal plasma. Olsen et al. (2012) found that the relative levels of long-chain PFCAs in humans could be ordered as follows: PFOA > PFNA > PFDA ≈ PFUnDA > PFHpA > PFDoDA. In our study, PFCA relative levels were similar to those reported by Olsen et al.: PFOA > PFNA > PFUnDA > PFDA > PFTrDA > PFHxA ≈ PFHpA ≈ PFDoDA. PFUnDA, PFDoDA, and PFTrDA levels were higher than seen in many countries but lower than reported for other areas of Japan (Harada et al., 2011). Total levels of long-chain PFCAs are equal to or greater than PFOA levels, and long-chain PFCA levels seem to be increasing in Japan and Korea (Harada et al., 2011). The composition of long-chain PFCAs within human blood samples can be used like a fingerprint to identify residents of East-Asian countries, including Japan (Harada et al., 2011). PFNA, PFUnDA, and PFTrDA are manufactured primarily in Japan via the oxidation of a mixture of linear fluorotelomer olefins (Prevedouros et al., 2006). Industrial application of these PFCAs may have contributed to our observed increase in PFNA concentrations over time and the accumulation of PFNA in East-Asian populations. In our study, although PFUnDA and PFOA concentrations were comparable in 2011 samples, no temporal trends were observed for PFUnDA, PFDoDA, or PFTrDA. Given that the ΣPFAA concentration was generally lower than for other regions in Japan, ΣPFAA may not be increasing over time. Strong correlations between PFAAs of similar chain length were detected, particularly between long-chain PFCAs. As such, sources of exposure for different long-chain PFCAs may be quite similar. In addition, it is likely that long- and short-chain PFCA sources of exposure are different.

Table 4
Correlations between levels of PFAA compounds with different chain lengths.

	PFOA (C8[a])		PFNA (C9[a])		PFDA (C10[a])		PFUnDA (C11[a])		PFDoDA (C12[a])		PFTrDA (C13[a])		PFHxS (C6[a])		PFOS (C8[a])
	ρ		ρ		ρ		ρ		ρ		ρ		ρ		ρ
PFCAs															
PFOA (C8[a])	1.00														
PFNA (C9[a])	0.492	***	1.00												
PFDA (C10[a])	0.480	***	0.702	***	1.00										
PFUnDA (C11[a])	0.201	*	0.482	***	0.698	***	1.00								
PFDoDA (C12[a])	0.288	***	0.271	***	0.616	***	0.459	***	1.00						
PFTrDA (C13[a])	0.128		0.227	**	0.548	***	0.675	***	0.707	***					
PFSAs															
PFHxS (C6[a])	0.268	***	0.202	*	0.239	**	0.291	***	0.264	**	0.329	***	1.00		
PFOS (C8[a])	0.404	***	0.128		0.166	*	0.273	***	0.043		0.202	*	0.408	***	1.00

ρ: Spearman's rank correlation coefficient.
[a] C: carbon chain length.
* $p < 0.05$
** $p < 0.01$
*** $p < 0.001$.

There is little data, however, concerning long-chain PFCA concentrations in different human populations around the world (particularly PFCAs that have a longer-chain than PFDA). It is important to evaluate long-term trends associated with long-chain PFCAs in human samples and to continue to monitor levels of these compounds. Further investigation of the source of human exposure to longer-chain PFCAs is needed to evaluate in detail the effects of longer-chain PFCA levels in East-Asian populations.

The maternal PFOS and PFOA concentrations we measured were generally lower than those measured in other parts of the world. These previous epidemiological studies include the NHANES study conducted in the United States (GM concentrations were 12.29 ng/mL PFOS and 2.6 ng/mL PFOA; Woodruff et al., 2011), the Danish National Birth Cohort study (mean concentrations were 35.3 ng/mL PFOS and 5.6 ng/mL PFOA; Fei et al., 2007), and the Family Study in Canada (mean concentrations were 18.31 ng/mL PFOS and 2.54 ng/mL PFOA; Monroy et al., 2008). Moreover, our previous study conducted in Sapporo City in Hokkaido between 2002 and 2005 also revealed low PFOS and PFOA concentrations (5.2 ng/mL and 1.3 ng/mL, respectively; Okada et al., 2012; Washino et al., 2009). All of Hokkaido, therefore, is an area where human exposure to PFAAs is relatively low. Note that the time of blood sampling during pregnancy could have affected concentrations owing to increased maternal blood volume during gestation (i.e., a dilution effect). The times of blood sampling during pregnancy in previous studies were as follows: the NHANES (first–third trimester), the Danish National Birth Cohort study (4–14 weeks of gestation), the Family Study in Canada (24–28 weeks of gestation), and the study in Sapporo City (23–35 weeks of gestation). As compared with those studies, the blood sampling time in our study was relatively late, i.e., between 28 and 32 weeks of gestation, although it was comparable to the study in Sapporo City. Also, levels of PFCAs in human breast milk in Kyoto in 2010 were reportedly higher than those in other East-Asian countries—GM concentrations were 0.083 ng/mL PFOA, 0.027 ng/mL PFNA, 0.017 ng/mL PFDA, and 0.030 ng/mL PFUnDA (Fujii et al., 2012). Levels of PFOA and PFNA in breast milk in the Sapporo City study were 0.089 ng/mL and 0.035 ng/mL, respectively, and these were similar to those of in Kyoto in 2010 (Nakata et al., 2009). However, because it was collected between 2002 and 2005, the breast milk concentrations of present Hokkaido may be lower than this. Although the levels of maternal PFOS and PFOA are low in Sapporo City, there is still a negative association between maternal PFOS/PFOA levels and birth weight or cord-blood IgE levels (Okada et al., 2012; Washino et al., 2009). As such, it remains important to assess risks associated with prenatal exposure to PFAAs even when environmental levels are low.

A limitation of our study is that we did not analyze samples taken from individuals over time (i.e., temporal trends within individuals were not assessed). Given that the participants were selected at random, however, our temporal-trend measurements reflect the general population of Hokkaido, Japan. A strength of our study is that it represents a large-scale cohort study of the general population of Hokkaido, Japan.

In conclusion, maternal plasma samples obtained from Hokkaido, Japan, contained lower levels of PFOS and PFOA than has been measured for similar samples from other regions of Japan and around the world. Whereas concentrations of PFOS and PFOA decreased over time, levels of PFNA and PFDA, which are longer-chain PFCAs, increased between 2003 and 2011. Future studies must continue to monitor long-term human-exposure trends associated with long-chain PFCAs and assess the effects of prenatal exposure to these compounds.

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