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A Section 508–conformant HTML version of this article is available at http://dx.doi.org/10.1289/ehp.1408834.

The Association of Prenatal Exposure to Perfluorinated Chemicals with Maternal Essential and Long-Chain Polyunsaturated Fatty Acids during Pregnancy and the Birth Weight of Their Offspring: The Hokkaido Study

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Background: Fatty acids (FAs) are essential for fetal growth. Exposure to perfluorinated chemicals (PFCs) may disrupt FA homeostasis, but there are no epidemiological data regarding associations of PFCs and FA concentrations.

Objectives: We estimated associations between perfluorooctane sulfonate (PFOS)/perfluorooctanoate (PFOA) concentrations and maternal levels of FAs and triglyceride (TG) and birth size of the offspring.

Methods: We analyzed 306 mother–child pairs in this birth cohort between 2002 and 2005 in Japan. The prenatal PFOS and PFOA levels were measured in maternal serum samples by liquid chromatography–tandem mass spectrometry. Maternal blood levels of nine FAs and TG were measured by gas chromatography–mass spectrometry and TG E-Test Wako kits, respectively. Information on infants' birth size was obtained from participant medical records.

Results: The median PFOS and PFOA levels were 5.6 and 1.4 ng/mL, respectively. In the fully adjusted model, including maternal age, parity, annual household income, blood sampling period, alcohol consumption, and smoking during pregnancy, PFOS but not PFOA had a negative association with the levels of palmitic, palmitoleic, oleic, linoleic, α-linolenic, and arachidonic acids ($p < 0.005$) and TG (p-value = 0.016). Female infants weighed 186.6 g less with mothers whose PFOS levels were in the fourth quartile compared with the first quartile (95% CI: –363.4, –9.8). We observed no significant association between maternal levels of PFOS and birth weight of male infants.

Conclusions: Our data suggest an inverse association between PFOS exposure and polyunsaturated FA levels in pregnant women. We also found a negative association between maternal PFOS levels and female birth weight.

Citation: Kishi R, Nakajima T, Goudarzi H, Kobayashi S, Sasaki S, Okada E, Miyashita C, Itoh S, Araki A, Ikeno T, Iwasaki Y, Nakazawa H. 2015. The association of prenatal exposure to perfluorinated chemicals with maternal essential and long-chain polyunsaturated fatty acids during pregnancy and the birth weight of their offspring: the Hokkaido Study. Environ Health Perspect 123:1038–1045; http://dx.doi.org/10.1289/ehp.1408834

Introduction

Perfluorinated chemicals (PFCs) are ubiquitous and stable chemicals widely detected in humans and environment. Contamination of drinking water, house dust, foods, and fish products are the possible major exposure pathways of humans to PFCs (Lau et al. 2007). The most widely studied and detected PFCs are perfluorooctane sulfonate (PFOS) and perfluorooctanoate (PFOA). In 2009, PFOS was added to Annex B of the Stockholm Convention on Persistent Organic Pollutants (POPs) (United Nations Environment Programme 2007). PFOS and PFOA are being voluntarily phased out by several industries, and are being substituted by longer carbon-chain PFCs. Recently, we reported that plasma levels of PFOS and PFOA were generally decreasing in plasma of pregnant Japanese women; however, we observed an increased trend for PFCs with longer carbon chains (Okada et al. 2013). Additionally, PFOS and PFOA are still present in older products, and they are slowly eliminated from the human body, with mean half-lives of 5.4 and 3.8 years for PFOS and PFOA, respectively (Olsen et al. 2007).

Both PFOS and PFOA have been shown to have developmental and reproductive toxicity in animal studies, including early pregnancy loss, reduced fetal weight, and postnatal mortality (Abbott et al. 2007; Luebker et al. 2005). A strong correlation of these compounds has been demonstrated between maternal and cord blood samples in humans, indicating that neonates are exposed to PFCs via the placental passage (Inoue et al. 2004; Monroy et al. 2008). Some epidemiological studies have also reported an association between PFC exposure and poor birth outcomes including decreased birth size (Apelberg et al. 2007; Fei et al. 2007; Washino et al. 2009). In a prospective study, prenatal PFOA exposure was positively associated with the prevalence of overweight female offspring at 20 years of age (Halldorsson et al. 2012). However, Barry et al. (2014) reported no association between early-life PFOA exposure and overweight and obesity risk in adults 20–40 years of age.

Recent research has shown that PFCs perturb metabolic end points, including lipid metabolism, glucose homeostasis, and thyroid hormone balance, in animals (Seacat et al. 2003; Thibodeaux et al. 2003). Such effects might explain associations between PFCs and birth outcomes. Most epidemiological studies regarding the association between PFCs and lipids [triglyceride (TG) and cholesterol] have been conducted in nonpregnant participants. Although previous reports suggest a positive association between PFCs and cholesterol levels (Frisbee et al. 2010; Winquist and Steenland 2014), the reports regarding the association of PFCs and TG levels are inconsistent. In a targeted group of Inuit adults 18–74 years of age, Château-Degat et al. (2010) reported a significant negative association between high PFOS exposure levels and TG only in women. However, some groups reported no association between exposure to PFCs and TG levels (Fisher et al. 2013; Sakr et al. 2007) or even a positive association between exposure to PFCs and TG levels in nonpregnant women (Steenland et al. 2009). Therefore, the relevance of these findings is uncertain.

Fetal growth is dependent on maternal metabolic resources, and this is exemplified by the correlation between maternal and fetal

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Supplemental Material is available online (http://dx.doi.org/10.1289/ehp.1408834).

We are grateful to all of the participants for taking part in this study and to the staff at Sapporo Toho Hospital. Additionally, we thank Y.M. Ito for his kind support in data analysis.

This study was supported in part by a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science and the Japan Ministry of Health, Labour, and Welfare.

The authors declare they have no actual or potential competing financial interests.

Received: 16 June 2014; Accepted: 1 April 2015; Advance Publication: 3 April 2015; Final Publication: 1 October 2015.

TG and fatty acids (FAs) levels (Kitajima et al. 2001; Schaefer-Graf et al. 2011). Major physiologic changes in lipid metabolism take place during normal pregnancies. There is an increase in body fat depots during early pregnancy, whereas lipolysis of fat depots occurs in late pregnancy, resulting in hyperlipidemia. Maternal hyperlipidemia during late pregnancy facilitates the availability of lipid substrates to the fetus. Fetuses need essential fatty acids (EFAs) and long-chain polyunsaturated fatty acids (LCPUFAs) for growth and development, especially for nervous system development (Alvarez et al. 1996). EFAs include linoleic acid and α-linolenic acid, which are precursors of omega 6 and omega 3 LCPUFAs, respectively. These substances must be obtained from the maternal diet, and the maternal blood concentrations of EFAs determine the corresponding concentrations in the cord blood (Herrera and Ortega-Senovilla 2010).

PFCs resemble FAs structurally and they may disrupt the homeostasis of FAs (Hu et al. 2005). Physiologic hyperlipidemia in late pregnancy is essential for fetal growth. However, to our knowledge, the influence of PFCs on FA homeostasis in pregnant women has not been investigated. In the present analyses, we explored the relationship between PFOS/PFOA exposure and the levels of four families of FAs (saturated, monounsaturated, omega 3, and omega 6 polyunsaturated FAs) including nine FAs and TGs in the maternal blood samples and birth size of their offspring from a birth cohort study.

Methods

Study population. This study was a part of the Hokkaido Study on Environment and Children's Health conducted between July 2002 and October 2005, and the details have been previously described (Kishi et al. 2011, 2013). Of 1,796 potentially eligible women, the following subjects were excluded: women who decided to participate in the Japanese cord blood bank (22% of those approached), and women who decided to deliver at another hospital (3% of those approached). Of the remaining eligible subjects, 514 women (28.6% of those approached) agreed to participate in this study (Konishi et al. 2009). These pregnant women at 23–35 weeks of gestation registered during a routine gynecologic checkup and delivered at the Toho Hospital in Sapporo, Hokkaido, Japan. Ten registered women were excluded due to miscarriage and stillbirth (n = 2), relocation (n = 1), or voluntary withdrawal (n = 7) from the study before follow-up.

Questionnaires and medical records. A self-administered questionnaire survey was completed after the second trimester (Washino et al. 2009) containing information related to smoking, household income and educational levels, and alcohol and caffeine intake during pregnancy. Medical information including maternal age, maternal body mass index (BMI) before pregnancy, parity, gestational age, pregnancy complications, type of delivery, infant sex, and birth size (weight, length, chest, and head circumferences) were obtained from participant medical records. This study was conducted with the written informed consent of all participants, and the study protocol was approved by the institutional ethical board for epidemiological studies at the Graduate School of Medicine and Center for Environmental and Health Sciences, Hokkaido University.

Blood sampling and exposure assessments. A 40-mL blood sample was taken from a peripheral vein after the second trimester of pregnancy and was used to measure maternal serum levels of PFOS, PFOA, TG, and FAs. All samples were stored at –80°C until analysis. Detailed methods for the measurement of PFOS and PFOA have been described in our previous report (Nakata et al. 2009). In brief, serum samples (0.1 mL) were mixed with 0.2 mL internal standard ($^{13}C_4$-PFOS-Na^+ and $^{13}C_2$-PFOA) solution containing acetonitrile, centrifuged at 1,450 × g for 10 min, and the supernatant was transferred to a polypropylene tube. An aliquot of the filtered sample solution was subjected to column-switching liquid chromatography–tandem mass spectrometry. The PFOS values of all samples were detected, and for samples with PFOA levels below the detection limit (0.50 ng/mL) (n = 17, 5.5% of participants), we used a value of half the detection limit (0.25 ng/mL).

The TG and FA concentrations in maternal blood. The FA levels in nonfasting maternal blood specimens were determined by gas chromatography–mass spectrometry (GC-MS) as described previously in detail (Nakashima et al. 2013). Briefly, the FA levels in maternal blood were measured as follows: Lipid extracted from 25 μL of blood according to the method of Folch et al. (1957) was mixed with 1.2 mL methanol, 75 μL acetyl chloride, and 75 μL 10 μg/100 μL tricosanoic acid ethyl ester/methanol (internal standard). After adding n-hexane (500 μL) and centrifugation, the upper organic layer was collected and moved into another vial. The n-hexane extraction was repeated once more, and then the concentration of FA methyl ester contained in the n-hexane layer was measured by GC-MS. Finally, the nine FA species targeted for measurement, including the palmitic and stearic acids of saturated FAs, the palmitoleic and oleic acids of monounsaturated FAs, linoleic acid (LA) and arachidonic acid (AA) of the omega 6 FAs, and the α-linolenic acid (ALA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) of omega 3 FAs. The detection limits were 2.4 μL/mL for palmitic acid, 1.3 μg/mL for stearic acid, 0.069 μg/mL for palmitoleic acid, 3.6 μg/mL for oleic acid, and 2.0 μg/mL for the others. The detection rates for all FAs were > 99.0% (except for EPA, with a detection rate of 97.8%). Nonfasting blood TG levels were measured using TG E-Test Wako kits (Wako, Osaka, Japan) after lipid extraction according to the methods described by Folch et al. (1957).

Data analysis. For the analysis of associations between maternal PFOS and PFOA levels and birth size, the following subjects were excluded: women with pregnancy-induced hypertension (n = 11), women with diabetes mellitus (n = 1), mother–infant pairs with fetal heart failure (n = 1), and twins (n = 7). After the exclusion of these subjects, 428 mother–infant pairs had available PFOS and PFOA concentrations. We excluded subjects whose blood samples were obtained after delivery (n = 105). Additionally, TG and/or FA levels were not available for 17 subjects. The available sample size for statistical analysis after considering the exclusion criteria was 306 subjects. Because of the skewed distributions, we treated the levels of PFOS, PFOA, and lipids as a continuous variable on a $\log_{10}$ scale.

We analyzed correlations between PFOS and PFOA concentrations and the characteristics of the mothers and infants using the Spearman correlation test, the Mann–Whitney U-test, and the Kruskal–Wallis test. The same statistical analyses were performed to find correlations between the maternal blood TG and FA levels and the characteristics of the mothers and infants. Additionally, we performed multiple regression analyses to determine the relationship between the maternal PFOS and PFOA levels and the lipid levels, and potential confounders selected according to the current results in this paper influencing exposure levels (maternal age, parity, smoking during pregnancy), lipid levels (alcohol intake during pregnancy), or both (blood sampling period). In addition, we included annual household income as an indicator of socioeconomic status. Therefore, the fully adjusted models included maternal age (years), smoking status and alcohol intake during pregnancy (yes/no), annual household income (categorical), parity (0/≥ 1), and the blood sampling period during pregnancy (categorical or continuous). The blood sampling period during pregnancy was categorized in model 1 as follows: 23–31 weeks of gestation (n = 137), 32–34 weeks of gestation (n = 82), and 35–41 weeks of gestation (n = 87) (Konishi et al. 2009). Due to the importance of the blood sampling time with respect to the PFC and TG/FA levels,

we included the blood sampling period as a continuous variable (by week of pregnancy) for tighter adjustment of model 2. Additionally, the daily inshore and deep-sea fish intake were included as potential covariates for the levels of EPA, DHA, and omega 3 FA levels (Grandjean et al. 2001). After log_{10}-transformation of PFOS and lipid levels (TG and fatty acids), we divided PFOS levels into four quartiles. Then, least square means (LSMs) and 95% confidence intervals (CIs) were calculated, and the LSMs and CIs were back transformed from log_{10} to normal values. For calculation of p for trend, we used linear contrast coefficients –3, –1, +1, +3 assigned to quartiles 1, 2, 3, and 4, respectively. The same approach was applied to find the association between the PFC levels and infant birth size, and confounders as reported by our group previously (Washino et al. 2009) were maternal age, annual household income, alcohol intake and smoking status during pregnancy, maternal BMI, parity, the blood sampling period (categorical), and gestational age (weeks). In addition, the least square means of birth weight for each quartile were compared using the Hsu–Dunnett method to accommodate for multiple comparisons. To examine the association with birth size, neonates were stratified by sex. We performed all of the statistical analyses using JMP pro 10 (SAS Institute Inc., Cary, NC, USA). The results were considered statistically significant at $p < 0.05$.

Results

Table 1 shows the maternal serum PFOS and PFOA concentrations in relation to the characteristics of the mothers and infants. The median (25–75 percentile) values of PFOS and PFOA were 5.6 ng/mL (4.0–7.5 ng/mL) and 1.4 ng/mL (0.9–2.0 ng/mL), respectively. The Spearman correlation coefficients detected a modest level of correlation between the PFOS and PFOA concentrations ($\rho = 0.223$, p-value < 0.001). We found statistically significant differences in the mean PFOS concentrations by parity, smoking during pregnancy, and the blood sampling period ($p \leq 0.001$). Additionally, we observed significant differences in the mean PFOA concentrations by parity, caffeine intake during pregnancy, and infant sex.

The maternal blood levels of TG and nine FA and their relation to the maternal and infant characteristics are shown in Table 2. The EPA levels increased with the higher frequency of inshore fish intake ($p < 0.001$). Maternal TG, palmitic acid, palmitoleic acid, and oleic acid increased significantly with increased gestation, but, inversely, DHA decreased with increased gestation. Maternal age, caffeine intake during pregnancy, and maternal education levels did not show significant associations with maternal lipids (data not shown). Table 3 presents the results of the univariate and multivariate regression analyses for the maternal blood TG and FAs on the log_{10}-transformed PFOS and PFOA concentrations. In the crude model, we found significant negative associations of PFOS exposure with TG, palmitic acid, and the palmitoleic and the oleic acids of monounsaturated FAs, LA and AA of the omega 6 family, and ALA of the omega 3 family in maternal blood samples ($p < 0.01$). We considered two models for adjustment; in both models, maternal age, smoking and alcohol intake during pregnancy, annual household income, parity, and the blood sampling period (categorical in model 1; continuous in model 2) were used as confounding factors. In model 2,

Table 1. Maternal blood PFOS and PFOA levels (ng/mL) in relation to the characteristics of the subjects participating in the Hokkaido Study on Environment and Children's Health, Sapporo, Japan, 2002–2005 (n = 306).

Characteristic	n (%)	PFOS [mean ± SD, median (25th–75th percentile), or correlation[a] (p-value)]	p-Value[b]	PFOA [mean ± SD, median (25th–75th percentile), or correlation[a] (p-value)]	p-Value[b]
Mean (± SD)	306 (100.0)	6.02 ± 2.67		1.52 ± 0.89	
Median (25th–75th percentile)	306 (100.0)	5.60 (4.0–7.5)		1.40 (0.9–2.0)	
Maternal characteristics					
Age (years)					
< 28	87 (28.4)	6.72 ± 0.28	0.020	1.68 ± 0.09	0.140
28–33	151 (49.4)	5.77 ± 0.21		1.43 ± 0.07	
> 34	68 (22.2)	5.69 ± 0.32		1.49 ± 0.10	
Prepregnancy BMI (kg/m^2)					
< 18.5	44 (14.4)	6.18 ± 0.40	0.519	1.55 ± 0.13	0.955
18.5–25	236 (77.1)	6.05 ± 0.17		1.51 ± 0.05	
> 25	26 (8.5)	5.56 ± 0.52		1.52 ± 0.17	
Parity[c]					
0	161 (52.7)	6.54 ± 0.20	0.001	1.80 ± 0.06	< 0.001
≥ 1	144 (47.2)	5.46 ± 0.21		1.20 ± 0.07	
Educational level (years)					
≤ 12	138 (45.0)	5.78 ± 0.22	0.195	1.50 ± 0.07	0.330
≥ 13	168 (54.9)	6.22 ± 0.20		1.53 ± 0.06	
Annual household income (million yen)[c]					
< 5	210 (69.0)	5.96 ± 0.18	0.567	1.54 ± 0.06	0.934
> 5	94 (30.9)	6.13 ± 0.27		1.47 ± 0.09	
Smoking during pregnancy					
Yes	130 (42.4)	5.41 ± 0.23	< 0.001	1.45 ± 0.07	0.353
No	176 (57.5)	6.48 ± 0.19		1.56 ± 0.06	
Alcohol consumed during pregnancy					
Yes	97 (31.6)	5.95 ± 0.27	0.868	1.45 ± 0.09	0.560
No	209 (68.3)	6.06 ± 0.18		1.55 ± 0.06	
Caffeine intake during pregnancy (mg/day)	144.3 ± 123	ρ = –0.058	0.310	ρ = –0.125	0.028
Fish intake during pregnancy					
Inshore fish					
≤ 1–2 times/month	166 (54.2)	6.00 ± 0.20	0.750	1.55 ± 0.06	0.996
≥ 1–2 times/week	140 (45.7)	6.05 ± 0.22		1.48 ± 0.07	
Deep-sea fish					
≤ 1–2 times/month	148 (48.3)	5.91 ± 0.22	0.223	1.52 ± 0.07	0.958
≥ 1–2 times/week	158 (51.6)	6.14 ± 0.21		1.51 ± 0.07	
Blood sampling period					
23–31 weeks during pregnancy	137 (44.7)	6.35 ± 0.22	< 0.001	1.59 ± 0.07	0.079
32–34 weeks during pregnancy	82 (26.7)	6.51 ± 0.28		1.49 ± 0.09	
35–41 weeks during pregnancy	87 (28.4)	5.05 ± 0.27		1.43 ± 0.09	
Type of delivery					
Vaginal	255 (83.3)	6.14 ± 0.16	0.109	1.52 ± 0.05	0.915
Cesarean section	51 (16.6)	5.45 ± 0.37		1.47 ± 0.12	
Gestational age (days)	276.7 ± 9.4	ρ = 0.057	0.314	ρ = 0.051	0.371
Infant characteristics					
Sex					
Male	141 (46.0)	6.18 ± 0.22	0.409	1.59 ± 0.07	0.040
Female	165 (53.9)	5.89 ± 0.20		1.45 ± 0.06	
Birth weight (g)					
Total	3076.9 ± 377.4	ρ = –0.067	0.238	ρ = –0.117	0.039
Male	3093.6 ± 374.2	ρ = 0.075	0.372	ρ = –0.076	0.367
Female	3062.5 ± 380.6	ρ = –0.185	0.017	ρ = –0.148	0.056

[a]Spearman's correlation (ρ) and p-value for continuous variables. [b]p-Values for Mann–Whitney U-test or Kruskal–Wallis test. [c]Missing data: parity (n = 1), annual household income (n = 2).

due to the importance of the blood sampling period for the PFC and lipid levels, blood sampling was included as a continuous variable (by week of pregnancy) for tighter adjustment. After adjusting models 1 and 2, for which the outcome and exposure levels were both $\log_{10}$ transformed, significant associations remained for TG and all six mentioned FAs. In model 2, PFOS was negatively associated with the TG/FAs levels as follows: TG (β = –0.130; 95% CI: –0.253, –0.011), palmitic acid (β = –0.175; 95% CI: –0.240, –0.044), palmitoleic acid (β = –0.168; 95% CI: –0.338, –0.058), oleic acid (β = –0.149; 95% CI: –0.242, –0.026), linoleic acid (β = –0.278; 95% CI: –0.745, –0.294), α-linolenic acid (β = –0.227; 95% CI: –0.739, –0.220), and arachidonic acid (β = –0.184; 95% CI: –0.555, –0.111). Additionally, the prenatal PFOS levels were significantly negatively associated with EFAs (β = –0.278; 95% CI: –0.745, –0.294) and omega 6 FAs (β = –0.272; 95% CI: –0.722, –0.277). No significant associations between PFOS and stearic acid and EPA were detected in any model. We did not observe any significant association between PFOA and lipid levels, except a positive association of prenatal PFOA levels with palmitic acid after full adjustment (β = 0.136; 95% CI: 0.009, 0.152).

We also examined the association between the plasma PFOS quartiles, TG and FAs. The PFOS concentrations were divided into quartiles: 1.5–4.0, 4.0–5.6, 5.6–7.5, and 7.5–16.2 ng/mL. In Figure 1 (see also Supplemental Material, Table S1), the quartile analysis after full adjustment showed decreasing trends for lipids in the fourth quartile of PFOS compared with the first quartile, with significant linear trend: TG (–16.1 mg/dL, p for trend < 0.003), palmitic acid (–422.1 μg/mL, p for trend < 0.001), palmitoleic acid (–32.4 μg/mL, p for trend < 0.001), oleic acid (–217.5 μg/mL, p for trend = 0.002), LA (–373.6 μg/mL, p for trend < 0.001), ALA (–5.2 μg/mL, p for trend < 0.001), AA (–24.9 μg/mL, p for trend < 0.001) and DHA (–6.4 μg/mL, p for trend < 0.03). In addition, increasing PFOS quartiles were negatively associated with EFA (–379.5 μg/mL, p for trend < 0.001) and omega 6 FAs (–399.7 μg/mL, p for trend < 0.001). However, a nonsignificant negative association was observed between the PFOS levels and omega 3 FAs (–7.3 μg/mL, p for trend = 0.068).

Maternal PFOS concentrations were negatively associated with birth weight among female infants but not male infants (Table 4). In the adjusted model, female infants whose mothers were in the highest quartile of PFOS concentration weighed –186.6 g less (95% CI: –363.4, –9.8) than female infants born to mothers with concentrations in the lower quartile. PFOS was not significantly associated with birth length, or with chest or head circumference in either sex (data not shown). Additionally, we did not observe a significant association between prenatal PFOA exposure and any of the birth size outcomes (data not shown).

Discussion

To our knowledge, this study is the first to address the association between PFCs and FAs in pregnant women. In our analysis, we found a significant negative association of relatively low PFOS levels with maternal TG and several FA levels including saturated (palmitic acid), monounsaturated (palmitoleic and oleic acids), omega 6 (LA and AA), and omega 3 (ALA) fatty acids during pregnancy in a Japanese birth cohort. There was a significant decreasing trend for TG, palmitic acid, palmitoleic acid, oleic acid, LA, ALA, AA, and DHA with increasing PFOS levels. Javins et al. (2013) reported a consistent decreasing trend of PFCs throughout all trimesters for pregnancy. Additionally, the levels of maternal TG and FAs increased in late pregnancy, consistent with the supply of the fetal levels (Herrera and Ortega-Senovilla 2010). To reduce the potential for bias due to confounding by sampling time, we included the blood sampling period as a continuous variable (by week of pregnancy) in the fully adjusted model (model 2), which had little influence on estimated associations. Our results provide important evidence regarding the association of low background levels of PFOS with the concentration of essential and long-chain polyunsaturated fatty acids. Furthermore, prenatal PFOS exposure was negatively associated with birth weight in female newborns. Unlike PFOS, the levels of PFOA did not have strong associations with the lipid levels or birth size.

Median concentrations for maternal PFOS and PFOA in the current study were

Table 2. Maternal blood TG and FA levels in relation to the characteristics of the mother–infant pairs (n = 306).

Characteristic	n	TG (mg/dL)	Palmitic acid (μg/mL)	Palmitoleic acid (μg/mL)	Stearic acid (μg/mL)	Oleic acid (μg/mL)	Linoleic acid (μg/mL)	α-Linolenic acid (μg/mL)	Arachidonic acid (μg/mL)	EPA (μg/mL)	DHA (μg/mL)
All samples	306	90.8 ± 46.1	2064.8 ± 858.8	126 ± 78.5	573.1 ± 206.3	1214.5 ± 564.5	721.2 ± 419.7	10.7 ± 8.3	71.2 ± 42.4	10.2 ± 8.4	30.2 ± 21.6
Maternal characteristics											
Parity[a,b]											
0	161	88.5 ± 3.6	2063.0 ± 67.8	123.4 ± 6.2	582.0 ± 16.2	1207.4 ± 44.6	711.9 ± 33.1	10.5 ± 0.6	71.0 ± 3.3	10.1 ± 0.6	29.0 ± 1.7
≥ 1	144	92.7 ± 3.8	2070.1 ± 71.7	129.2 ± 6.5	564.5 ± 17.2	1223.5 ± 47.1	729.7 ± 35.0	10.9 ± 0.6	71.4 ± 3.5	10.2 ± 0.7	31.4 ± 1.8
Smoking during pregnancy[a]											
Yes	130	95.7 ± 4.0	2121.9 ± 75.3	133.3 ± 6.8	563.5 ± 18.1	1268.9 ± 49.4	740.5 ± 36.8	10.9 ± 0.7	69.0 ± 3.7	9.1 ± 0.7	27.4 ± 1.8
No	176	87.1 ± 3.4	2022.6 ± 64.7	120.6 ± 5.9	580.2 ± 15.5	1174.4 ± 42.4	707.0 ± 31.6	10.6 ± 0.6	72.8 ± 3.1	11.0 ± 0.6	32.2 ± 1.6
Alcohol intake during pregnancy[a]											
Yes	97	83.8 ± 4.6	2020.1 ± 87.2	118.4 ± 7.9	573.7 ± 20.9	1200.5 ± 57.4	745.0 ± 42.6	11.4 ± 0.8	75.2 ± 4.3	10.2 ± 0.8	32.2 ± 2.2
No	209	94.0 ± 3.1*	2085.6 ± 59.4	129.5 ± 5.4	572.8 ± 14.2	1221.0 ± 39.1	710.2 ± 29.0	10.4 ± 0.5	69.4 ± 2.9	10.2 ± 0.5	29.3 ± 1.5
Fish intake during pregnancy[a]											
Inshore fish											
≤ 1–2 times/month	166	91.0 ± 3.5	2054.1 ± 66.7	127.6 ± 6.1	566.3 ± 16.0	1217.7 ± 43.8	708.5 ± 32.6	10.2 ± 0.64	71.8 ± 3.2	8.8 ± 0.64	28.6 ± 1.6
≥ 1–2 times/week	140	90.5 ± 3.9	2077.5 ± 72.6	124.0 ± 6.6	581.1 ± 17.4	1210.8 ± 47.7	736.4 ± 35.5	11.3 ± 0.70	70.5 ± 3.5	11.8 ± 0.7**	32.1 ± 1.8
Deep-sea fish											
≤ 1–2 times/month	148	91.6 ± 3.7	2114.6 ± 70.5	132.9 ± 6.4	586.9 ± 16.9	1248.7 ± 46.4	723.7 ± 34.5	10.7 ± 0.6	72.0 ± 3.4	9.8 ± 0.6	28.8 ± 1.7
≥ 1–2 times/week	158	90.0 ± 3.6	2018.1 ± 68.3	119.5 ± 6.2	560.2 ± 16.4	1182.5 ± 44.9	718.9 ± 33.4	10.7 ± 0.6	70.5 ± 3.3	10.5 ± 0.6	31.5 ± 1.7
Blood sampling period (categorical)[c]											
23–31 weeks during pregnancy	137	81.2 ± 3.8	1928.7 ± 72.7	110.7 ± 6.6	562.7 ± 17.4	1095.8 ± 47.3	686.3 ± 35.8	10.3 ± 0.7	70.9 ± 3.5	10.5 ± 0.7	32.3 ± 1.8
32–34 weeks during pregnancy	82	93.5 ± 5.0	2107.2 ± 94.0	129.5 ± 8.5	624.2 ± 22.5	1252.1 ± 61.2	726.6 ± 46.3	10.9 ± 0.9	80.1 ± 4.6	10.1 ± 0.9	32.0 ± 2.3
35–41 weeks during pregnancy	87	103.2 ± 4.8**	2239.2 ± 91.2*	146.7 ± 8.2*	541.2 ± 21.9**	1366.2 ± 59.4**	771.3 ± 44.9	11.1 ± 0.8	63.4 ± 4.5	9.7 ± 0.9	25.2 ± 2.3*
Blood sampling period (week of pregnancy)[d]	306	ρ = 0.198**	ρ = 0.176**	ρ = 0.208**	ρ = 0.055	ρ = 0.223**	ρ = 0.086	ρ = 0.035	ρ = –0.042	ρ = –0.009	ρ = –0.122*
Infant characteristics											
Birth weight[d]	306	ρ = –0.007	ρ = –0.055	ρ = –0.076	ρ = –0.074	ρ = –0.011	ρ = 0.021	ρ = –0.022	ρ = 0.010	ρ = 0.052	ρ = 0.031

Abbreviations: DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; TG, triglyceride. Data are means ± SD or correlations unless otherwise indicated.
*p < 0.05. **p < 0.01. p-Values were calculated by [a]Mann–Whitney U-test, [c]Kruskal–Wallis test, or [d]Spearman's correlation (ρ); [b]missing data: parity (n = 1);

5.60 and 1.40 ng/mL, respectively, which are lower than the median values of cohorts conducted in the United States (PFOS: 8.2 ng/mL, PFOA: 2.9 ng/mL) (Stein et al. 2012), Canada (PFOS: 16.6 ng/mL, PFOA: 2.1 ng/mL) (Monroy et al. 2008), Denmark (PFOS: 21.5 ng/mL, PFOA: 3.7 ng/mL) (Halldorsson et al. 2012), Norway (PFOS: 13 ng/mL, PFOA: 2.2 ng/mL) (Starling et al. 2014), Korea (PFOS: 9.3 ng/mL, PFOA: 2.6 ng/mL) (Lee et al. 2013), and China (PFOS: 6.7 ng/mL, PFOA: 4 ng/mL) (Jiang et al. 2014) among pregnant women.

To our knowledge, the association between PFCs and human FA levels has not been described prior to this study. Two studies described the association between PFCs and TG during pregnancy. In the Baltimore THREE Study, Apelberg et al. (2007) reported a negative association between cord blood PFOS and PFOA levels (median, 5 ng/mL and 1.6 ng/mL, respectively) and birth weight, the ponderal index, and head circumference in a cross-sectional study. However, they did not find an association between PFCs and cord blood TG and total cholesterol levels. Additionally, the associations between PFOS/PFOA and birth size persisted after adjustment for cord serum TG and cholesterol concentrations. In another cross-sectional study in Norway, no associations between maternal PFOS (median, 13 ng/mL) and PFOA (median, 2.2 ng/mL) and TG levels during pregnancy were found (Starling et al. 2014).

In contrast with our findings, Steenland et al. (2009) reported that serum TGs were positively associated with higher deciles of PFOS and PFOA in a cross-sectional study of adults living in close proximity to a chemical plant, with no statistical evidence of effect modification by sex. However, exposure levels in the previous study were substantially higher (median values of 19.6 and 26.6 ng/mL for PFOS and PFOA, respectively) than the low background levels in our study population (median values of 5.6 ng/mL and 1.4 ng/mL, respectively). In contrast, Château-Degat et al. (2010) reported a significant negative association between PFOS exposure levels (arithmetic mean, 23.1 ng/mL) and TG levels among nonpregnant women in an Inuit population of Nunavik (n = 723). Similarly, we observed a negative association between low PFOS exposure and maternal TG levels during pregnancy. In rodent studies, PFOS exposures were reported to reduce TG serum concentrations (Seacat et al. 2002, 2003; Thibodeaux et al. 2003), consistent with our findings.

A mode of action(s) that might explain the correlation between the PFC exposure and lipids concentration is not fully understood. Although there is weak activation of PPAR (peroxisome proliferator–activated receptor) γ, the most likely target of PFCs has been shown to be PPARα (Vanden Heuvel et al. 2006; Takacs and Abbott 2007). Curran et al. 2008 showed in rats that PFOS activated PPARα and its downstream genes involved in lipid metabolism, thus resulting in decreased serum triglyceride. Therefore, further studies are needed to clarify the effects of PPAR family polymorphisms on human lipid profile. Unlike PFOS, our estimates did not support an association between *in utero* exposure to PFOA and the levels of FAs (except a positive association with the levels of palmitic acid). Experimental studies suggested that PFOA is a stronger agonist than PFOS for the transactivation of PPARα and PPARγ in mouse and human

Table 3. The regression coefficients (95% CIs) between PFOS/PFOA concentrations (ng/mL) and the levels of TG and FAs in the maternal blood (n = 306).

Dependent variable	PFOS [β (95% CI)]	p-Value	PFOA [β (95% CI)]	p-Value
TG				
Crude	−0.197 (−0.313, −0.087)	< 0.001	0.008 (−0.078, 0.090)	0.888
Model 1	−0.147 (−0.272, −0.027)	0.016	0.059 (−0.045, 0.134)	0.333
Model 2	−0.130 (−0.253, −0.011)	0.032	0.066 (−0.039, 0.138)	0.273
Palmitic acid				
Crude	−0.215 (−0.264, 0.085)	< 0.001	0.084 (−0.016, 0.117)	0.138
Model 1	−0.204 (−0.264, −0.067)	0.001	0.126 (0.002, 0.147)	0.043
Model 2	−0.175 (−0.240, −0.044)	0.004	0.136 (0.009, 0.152)	0.027
Palmitoleic acid				
Crude	−0.227 (−0.397, −0.138)	< 0.001	−0.008 (−0.104, 0.090)	0.886
Model 1	−0.195 (−0.371, −0.088)	0.001	0.048 (−0.063, 0.146)	0.436
Model 2	−0.168 (−0.339, −0.058)	0.005	0.059 (−0.052, 0.155)	0.333
Stearic acid				
Crude	0.047 (−0.046, 0.112)	0.410	0.068 (−0.022, 0.093)	0.229
Model 1	0.017 (−0.074, −0.098)	0.779	0.055 (−0.034, 0.091)	0.374
Model 2	0.056 (−0.047, 0.126)	0.372	0.058 (−0.033, 0.094)	0.352
Oleic acid				
Crude	−0.214 (−0.291, −0.093)	< 0.001	0.051 (−0.040, 0.107)	0.369
Model 1	−0.179 (−0.270, −0.052)	0.003	0.101 (−0.013, 0.147)	0.102
Model 2	−0.149 (−0.242, −0.026)	0.014	0.112 (−0.055, 0.153	0.067
Linoleic acid				
Crude	−0.264 (−0.701, −0.292)	< 0.001	0.036 (−0.104, 0.205)	0.524
Model 1	−0.295 (−0.777, −0.326)	< 0.001	0.051 (−0.100, 0.240)	0.420
Model 2	−0.278 (−0.745, −0.294)	< 0.001	0.055 (−0.095, 0.245)	0.385
α-Linolenic acid				
Crude	−0.222 (−0.705, −0.237)	< 0.001	0.010 (−0.158, 0.192)	0.849
Model 1	−0.248 (−0.782, −0.263)	< 0.001	0.021 (−0.160, 0.226)	0.735
Model 2	−0.227 (−0.739, −0.220)	< 0.001	0.026 (−0.152, 0.234)	0.675
Arachidonic acid				
Crude	−0.146 (−0.467, −0.062)	0.010	−0.017 (−0.173, 0.126)	0.756
Model 1	−0.201 (−0.584, −0.142)	0.001	−0.030 (−0.204, 0.123)	0.628
Model 2	−0.184 (−0.555, −0.111)	0.003	−0.031 (−0.206, 0.123)	0.619
EPA				
Crude	0.111 (−0.0004, 0.426)	0.054	0.017 (−0.133, 0.180)	0.764
Model 1[a]	0.085 (−0.072, 0.398)	0.175	0.007 (−0.160, 0.179)	0.908
Model 2[a]	0.101 (−0.041, 0.426)	0.107	0.011 (−0.154, 0.186)	0.854
DHA				
Crude	−0.062 (−0.339, 0.098)	0.278	−0.040 (−0.218, 0.102)	0.477
Model 1[a]	−0.120 (−0.472, 0.005)	0.052	−0.055 (−0.253, 0.096)	0.377
Model 2[a]	−0.103 (−0.440, 0.040)	0.102	−0.055 (−0.255, 0.097)	0.377
Essential fatty acids				
Crude	−0.264 (−0.700, −0.291)	< 0.001	0.036 (−0.104, 0.205)	0.521
Model 1	−0.295 (−0.776, −0.326)	< 0.001	0.051 (−0.100, 0.240)	0.419
Model 2	−0.278 (−0.745, −0.294)	< 0.001	0.055 (−0.095, 0.246)	0.384
Omega 6				
Crude	−0.256 (−0.676, −0.272)	< 0.001	0.032 (−0.108, 0.196)	0.571
Model 1	−0.289 (−0.753, −0.309)	< 0.001	0.045 (−0.106, 0.228)	0.474
Model 2	−0.272 (−0.722, −0.277)	< 0.001	0.049 (−0.102, 0.234)	0.440
Omega 3				
Crude	−0.041 (−0.225, 0.104)	0.473	−0.034 (−0.158, 0.083)	0.543
Model 1[a]	−0.090 (−0.314, 0.047)	0.149	−0.047 (−0.182, 0.081)	0.452
Model 2[a]	−0.069 (−0.283, 0.079)	0.268	−0.044 (−0.180, 0.085)	0.479

Abbreviations: DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; TG, triglyceride. Data are $\log_{10}$-transformed exposure and outcomes. Units: TG (md/dL); FAs (μg/mL). Model 1 was adjusted for maternal age, smoking and alcohol intake during pregnancy, annual household income, parity, and blood sampling period (categorical). Model 2 was adjusted for maternal age, smoking and alcohol intake during pregnancy, annual household income, parity, and blood sampling period (by week of pregnancy).
[a]For EPA, DHA, and omega 3 FAs, adjusted model 1 and 2 also included fish intake.

cells (Vanden Heuvel et al. 2006). PFOS has the highest inhibitory effect on the binding affinity of the liver fatty acid–binding protein for liver FAs whereas PFOA had the lowest potency among the examined PFCs (Luebker et al. 2002). Therefore, PFOS and PFOA may regulate lipid homeostasis with different potencies and modes of action. The maternal serum concentration of PFOA in this study was low, and the differences in the PFOS and PFOA concentrations could also be an alternative explanation.

In this study, we found a significant negative association between the levels of PFOS and female infant birth weight, but not among male infants. However, prenatal PFOA levels did not show significant association with birth weight. Apelberg et al. (2007) found a negative association between the cord blood levels of PFOS and PFOA and the birth weight of infants. Additionally, Maisonet et al. (2012) reported a negative association between the prenatal PFOS and PFOA concentrations and the birth weight of British girls. It is difficult to compare our results with those of other studies because of the differences in the biological samples used to measurement exposure, and the genetic background of our population; however, we found a negative association between prenatal PFOS levels and birth weight, especially in females, consistent with some previous reports (Apelberg et al. 2007; Maisonet et al. 2012). TG and FAs are sources of energy for fetuses, and FA deficiency during fetal development results in metabolic and energy programming

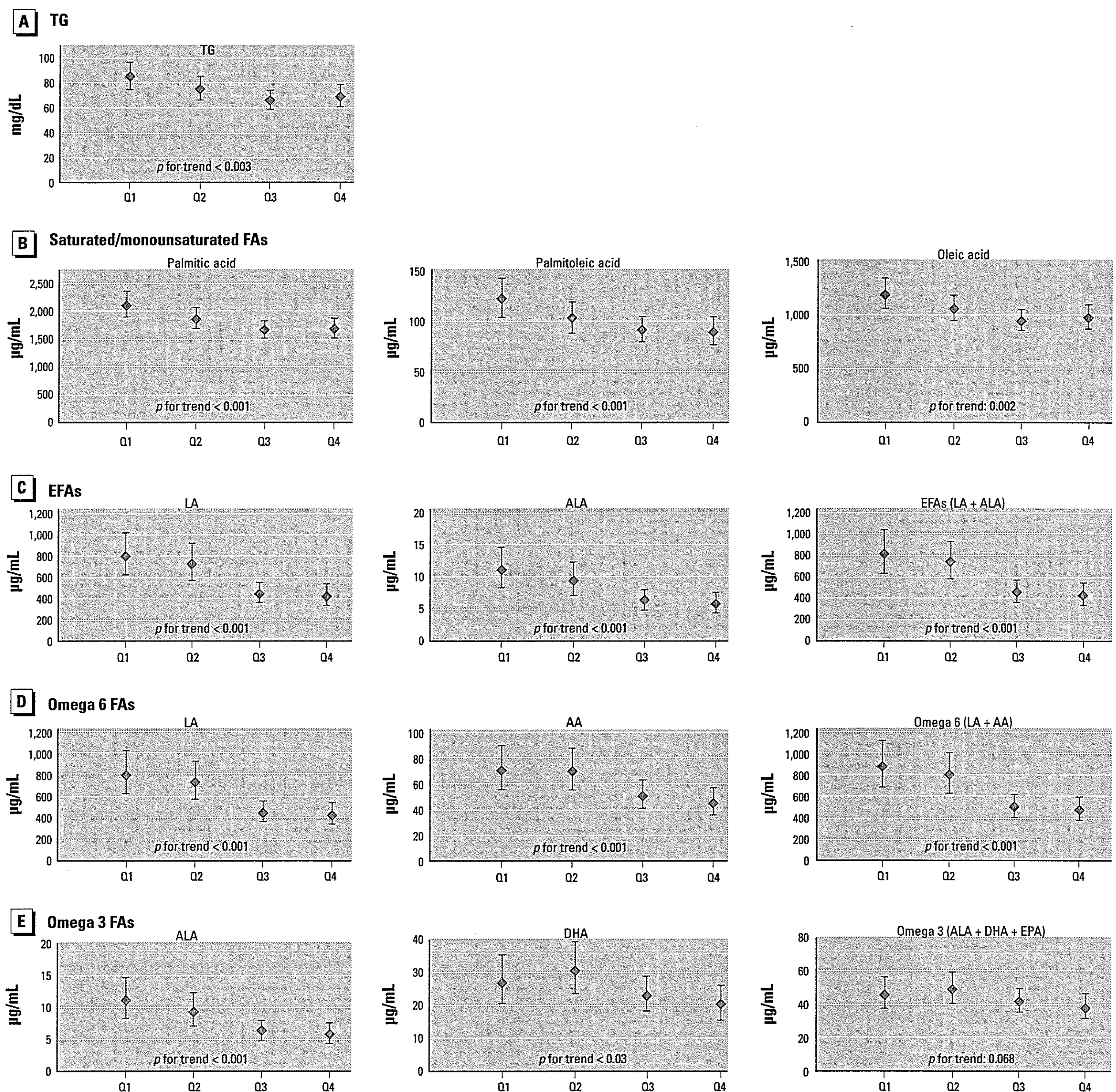

Figure 1. The dose–response relationship between the quartiles (Q) of PFOS and reduced levels of TG (*A*) and FAs (*B–E*) in maternal serum samples, Sapporo, Japan, 2002–2005 ($n = 306$). The LSMs were adjusted for maternal age, smoking and alcohol intake during pregnancy, annual household income, parity, and the blood sampling period (categorical). For EPA, DHA, and omega 3 FAs, the adjusted model also included fish intake. The LSMs were back transformed from $\log_{10}$ to normal values, and the error bars depict 95% CIs.

Table 4. The crude and adjusted least square means (LSMs) and regression coefficients (β) for the birth weight (g) of male and female infants by quartiles of PFOS.

	Crude		Adjusted[a]	
PFOS in quartiles (ng/mL)	LSM	β (95% CI)	LSM	β (95% CI)
Male infants (n = 141)				
Quartile 1 (1.60–4.35)	3059.5	Reference	3069.2	Reference
Quartile 2 (4.35–5.80)	3010.6	–48.9 (–267.6, 169.8)	3012.4	–56.7 (–255.9, 142.4)
Quartile 3 (5.80–7.45)	3151.3	91.7 (–123.3, 306.8)	3099.7	30.5 (–169.7, 230.8)
Quartile 4 (7.45–16.20)	3144.1	84.5 (–136.9, 306.0)	3165.1	95.9 (–116.5, 308.4)
p for trend	0.182		0.187	
Female infants (n = 165)				
Quartile 1 (1.50–3.80)	3147.0	Reference	3125.4	Reference
Quartile 2 (3.80–5.50)	3034.6	–112.4 (–310.5, 85.6)	3055.2	–70.1 (–242.5, 102.2)
Quartile 3 (5.50–7.65)	3120.1	–26.9 (–217.7, 163.9)	3086.3	–39.1 (–216.1, 137.8)
Quartile 4 (7.65–13.30)	2934.6	–212.3 (–400.7, –24.0)*		–186.6 (–363.4, –9.8)*
p for trend	0.030		0.031	

[a]Adjusted for gestational age, maternal age, prepregnancy BMI, smoking and drinking during pregnancy, parity, annual household income, and blood sampling period (categorical). *$p < 0.05$ compared with quartile 1 calculated by Hsu–Dunnett method.

(Innis 2011). Although the extent to which these decreases in maternal blood FAs during the gestational period are clinically important to mothers is not apparent, the consequences of hypolipidemia during pregnancy on the growth, neurodevelopment, and metabolic end points of infants should be examined in the future.

This study has some potential limitations. Our study employed only a single measurement of TG and FA concentrations for each participant. Another potential limitation of this study is that we measured the lipids in nonfasting blood samples, and fasting is a routine process before the measurement of lipid profiles. However, recent data show that lipoproteins and lipids, including TG, change minimally following normal food intake (Langsted et al. 2008). Although this study is part of a birth cohort study, the analysis of maternal FAs and TG during pregnancy is cross-sectional in nature, thereby limiting causal inference. Potential selection bias may have occurred because this cohort was based in one hospital that cared for pregnant women in Sapporo and the surrounding areas. Additionally, the participation rate was low due to the exclusion of eligible women who decided to participate in the Japanese cord blood bank. Additionally, before data analysis, we excluded subjects whose blood samples were obtained after delivery (n = 105). The excluded subjects had annual household income levels, maternal education levels, and alcohol consumption similar to those of participants in this study, but the incidence of smoking and multiparity was higher, which may suggest the possibility of selection bias.

Previously, our group reported time trends of 11 types of PFCs between 2003 and 2011 in plasma samples of pregnant women in Hokkaido (Okada et al. 2013). The results indicated that PFOS and PFOA concentrations declined, whereas long-chain PFNA and PFDA levels increased. Thus, focusing on the effects of PFCs with longer carbon chain—which may be more potent than ones with shorter chains on TGs, FAs, and other metabolic end points in pregnant women and their offspring—should be included in future studies.

Conclusions

This study supports the association between PFOS and lipid levels during pregnancy. We found that relatively low PFOS levels had a significant negative association with TG and saturated (palmitic acid), monounsaturated (palmitoleic and oleic acids), omega 6 (linoleic and arachidonic acids), and omega 3 (α-linolenic acid and DHA) FAs.

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HPV vaccination crisis in Japan

Free vaccination against the human papillomavirus (HPV) began in December, 2010, for Japanese girls aged 12–16 years and since April, 2013, the vaccine was included in the national immunisation programme. However, in June, 2013, the Japanese Ministry of Health, Labour, and Welfare suspended proactive recommendations for the HPV vaccine after unconfirmed reports of adverse events following vaccination appeared in the media.[1] In January, 2014, the Vaccine Adverse Reactions Review Committee investigating these adverse events concluded that there was no evidence to suggest a causal association between the HPV vaccine and the reported adverse events after vaccination, but they still did not reinstate proactive recommendations for its use.[2] We report the resulting effects of such a decision by presenting data from Sapporo, a city of 2 million people in northern Japan.

Before public funding was introduced, we investigated correlates of HPV vaccine acceptance in mothers with adolescent daughters living in Sapporo. Although cost was a large barrier, with only 1·5% of parents willing to pay the full vaccine price, recommendation from a physician was a strong motivator (odds ratio 12·2, 95% CI 7·1–21·1).[3] In the years between becoming free (2011 in Sapporo) and before the suspension of recommendations (2013), rates of HPV vaccination in Sapparo ranged from 73·6–77·2% at initiation and 68·4–74·0% for three dose completion in girls in the 1994–98 birth cohorts (figure). However, in the first birth cohort of 7705 girls eligible for vaccination after suspension, completion rates plummeted to just 0·6%, with only 49 girls finishing the dosing course despite the vaccine still being part of the national immunisation programme and free.

We believe that Japan, whose uptake rates for cervical cancer screening have stagnated at about 30%, might have lost a real opportunity to decrease morbidity and mortality associated with cervical cancer. Other countries with successful HPV vaccination programmes (such as Australia and the UK), who have also dealt with similar adverse event crises, are already documenting substantial reductions in precancerous cervical lesions in those vaccinated.[4,5]

No vaccine safety signal has been recorded in Japan. Instead, individuals who have the misfortune to be unwell with rare or difficult to treat disorders have been encouraged by antivaccination advocates to blame the HPV vaccine, especially in an unrestrained media environment and with little reassurance and systematic addressing of these events by the government.

According to the Global Advisory Committee on Vaccine Safety, "Allegations of harm from vaccination based on weak evidence can lead to real harm when, as a result, safe and effective vaccines cease to be used." Sadly, this is what has transpired in Japan.

We thank Keisuke Tamori and Julia Brotherton for their advice.

SJBH reports lecture fees from Japan Vaccine and the Japanese Expert Board for the Eradication of Cervical Cancer. EY, YI, and RK declare no competing interests.

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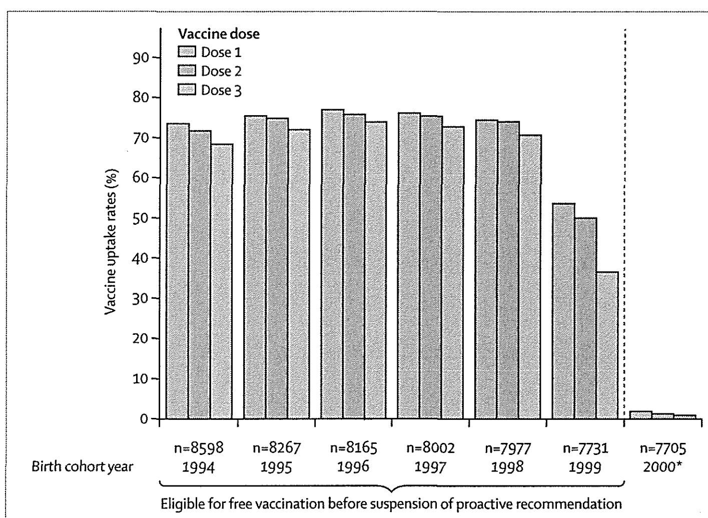

***Figure:* Uptake rates for the human papillomavirus vaccine in Sapporo, Japan, as of March, 2014**
Data are from the Department of Infection Control, Sapporo Health Board (Sapporo, Japan). n=number of girls in cohort. *The first birth cohort who were eligible for free vaccination after suspension of proactive recommendation.

For the **Global Advisory Committee on Vaccine Safety's statement on HPV vaccination** see http://www.who.int/vaccine_safety/committee/topics/hpv/GACVS_Statement_HPV_12_Mar_2014.pdf

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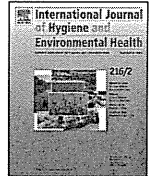
International Journal of Hygiene and Environmental Health 218 (2015) 461–470

Contents lists available at ScienceDirect

International Journal of Hygiene and Environmental Health

journal homepage: www.elsevier.com/locate/ijheh



Comparisons of urinary phthalate metabolites and daily phthalate intakes among Japanese families

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ARTICLE INFO

Article history:
Received 1 December 2014
Received in revised form 25 March 2015
Accepted 26 March 2015

Keywords:
Children
Phthalate metabolites
Mother–child pairs
Father–child pairs
Phthalate exposure

ABSTRACT

We measured urinary phthalate metabolites, including di-*n*-butyl phthalate (DnBP), di-isobutyl phthalate, benzyl butyl phthalate (BBzP), and di(2-ethylhexyl) phthalate (DEHP), from 178 school-aged children and their 284 family members using gas chromatography-mass spectrometry, and we calculated daily phthalate intakes. The highest median levels of phthalate metabolites were for mono-isobutyl phthalate in all participants except schoolchildren, where the highest levels were for mono-(2-ethyl-5-oxohexyl) phthalate (MEOHP). Comparing the schoolchildren with their parents, the schoolchildren had significantly higher urinary metabolites for MEOHP, mono-(2-ethyl-5-carboxypentyl) phthalate, and ΣDEHP. Regarding daily intakes, the schoolchildren had significantly higher daily intakes of DnBP, BBzP, and ΣDEHP. All phthalate metabolite and sums of metabolite levels in the schoolchildren were positively correlated with their mothers' levels, except for MEHP, whereas fathers were less correlated with their children. The DEHP intake in this study was higher than that of most other studies. Moreover, 10% of the children and 3% of the adults exceeded the Reference Dose (RfD) value (20 μg/kg/day) of the U.S. Environmental Protection Agency, which indicates that it is important to focus on children's DEHP exposure because the children exceeded the RfD more than adults among the same families who shared similar exposure sources. Our results will contribute to considerations of the regulations for some phthalates and the actual phthalate exposure levels in the Japanese population.

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Introduction

Phthalates are widely used as plasticisers for consumer products, such as toys, food containers, furniture, personal care products (PCPs), coatings of medications, and electric cables. The most commonly used phthalate is di(2-ethylhexyl) phthalate (DEHP) in Japan (Japan Plasticizer Industry Association and Ministry of Economy TAI, 2014), and its house dust level is higher in Japan than other countries (Ait Bamai et al., 2014). According to the Chemical Economics Handbook (Bizzari, 2013), DEHP is still the dominant plasticizer/phthalate in Japan, while dibutyl phthalate (DBP) is consumed less in Japan than in Europe; DEHP consumption in Japan decreased slightly from 224 kilotons (kt) in 2000 to 161 kt in 2012. At the same time, consumption of DEHP decreased considerably in Europe and also in the US, from 395 kt in 2000 to 80 kt in 2012 and from 129 kt in 2000 to 70 kt in 2012, respectively. Therefore, higher DEHP dust levels in Japan reflect the characteristics of the Japanese market.

Abbreviations: BBzP, benzyl butyl phthalate; BSTFA, *N,O*-bis(trimethylsilyl)-trifluoro acetamide; BW, body weight; CE, creatinine clearance rate; DBP, dibutyl phthalate; DEHP, di(2-ethylhexyl) phthalate; DiBP, di-iso-butyl phthalate; DiNP, di-iso-nonyl phthalate; DMP, dimethyl phthalate; DnBP, di-*n*-butyl phthalate; DI, daily intake; EPA, Environmental Protection Agency; GC/MS, gas chromatography/mass spectrometry; ISAAC, International Studies of Asthma and Allergies on Childhood; LOD, limit of detection; LOQ, limit of quantification; MBzP, mono-benzyl phthalate; MCNP, mono(carboxynonyl) phthalate; MCPP, mono(3-carboxypropyl) phthalate; MECPP, mono(2-ethyl-5-carboxypentyl) phthalate; MEHP, mono(2-ethylhexyl) phthalate; MEHHP, mono(2-ethyl-5-hydroxyl-hexyl) phthalate; MEOHP, mono(2-ethyl-5-oxohexyl) phthalate; MiBP, mono-isobutyl phthalate; MnBP, mono-*n*-butyl phthalate; MTBSTFA, *N*-methyl-*N*-(*tert*-butyldimethylsilyl)-trifluoroacetamide; M_{W_p}, molecular weights of parent phthalate; NHANES, National Health and Nutrition Examination Survey; PCP, personal care product; PVC, polyvinyl chloride; RfD, reference dose; SIM, selective ion mode; TDI, tolerable daily intake.

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http://dx.doi.org/10.1016/j.ijheh.2015.03.013
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Humans are exposed to phthalates through food ingestion, inhalation, and dermal absorption throughout their lifetime beginning in foetal stages. In particular, the main source of exposure for high molecular weight phthalates is foodstuffs, while the source of exposure for low molecular weight phthalates seems to be very diffuse (Koch et al., 2013). Based on the endocrine-disrupting effects of phthalates in animal studies (Gray et al., 1982; Oishi, 1993), the adverse effects of phthalates became a matter of international concern. In recent decades, DEHP and di-iso-nonyl phthalate (DiNP) were banned for use in toys and child-care products in the EU, USA, and Japan because of their reproductive toxicity (2005/84/EC 2005; USCPS Commission, 2008; Japan Ministry of Health Law, 2002). DBP and benzyl butyl phthalate (BBzP) are also banned for use in cosmetics in the EU (2004/93/EC 2004). However, phthalates, including DEHP, DBP, and BBzP, are still detected from human specimens, such as urine, serum, saliva, and breast milk (Hines et al., 2009; Koch et al., 2011; Silva et al., 2004).

Urinary phthalate metabolites are used as biomarkers of human exposure to phthalates as non-persistent chemicals with short half-lives (Calafat and McKee, 2006). Currently, although several epidemiological studies have reported urinary phthalate metabolite levels among mother–child pairs, only three studies have reported the associations of children's urinary phthalate metabolites and their mothers for the same urine sampling period (Kasper-Sonnenberg et al., 2012; Sathyanarayana et al., 2008a; Song et al., 2013). Kasper-Sonnenberg et al. (2012) measured urinary phthalate metabolite levels in mother/school-aged child pairs in Germany and found that metabolites of dimethyl phthalate (DMP), di-iso-butyl phthalate (DiBP), di-*n*-butyl phthalate (DnBP), DEHP, and DiNP were correlated between the mothers and children (Kasper-Sonnenberg et al., 2012). Conversely, Song et al. (2013) measured the urinary metabolite levels from mother/0–6-year-old child pairs in Korea and found that only mono-(2-ethylhexyl) phthalate (MEHP) was correlated between the mothers and children. Moreover, children had a faster relative metabolic rate of DEHP metabolism from MEHP to mono-(2-ethyl-5-hydroxyl-hexyl) phthalate (MEHHP) than mothers, especially younger children, who were the fastest (Song et al., 2013). Sathyanarayana et al. (2008a,b) measured urinary metabolite levels from mother/1–37-month-old child pairs in the USA and found that correlation coefficients between mothers and their children increased with decreasing age of the children (Sathyanarayana et al., 2008a). However, to the best of our knowledge, there are no studies that measure urinary phthalate metabolite levels both in children and all of their family members, including their mother, father, and siblings, and that assess the differences of phthalate exposure levels among family members. Therefore, this study aimed to present the differences in phthalate exposure between children and adults among families that are thought to share lifestyle and home environments. We measured seven phthalate metabolite levels in urine samples from Japanese elementary schoolchildren and their family members. Next, we estimated daily phthalate intakes from urinary phthalate metabolite levels. In addition, we considered whether the phthalate exposure contributions among children were more correlated with their mothers or fathers.

Materials and methods

Study population

This study was based on the second phase of a home environment and allergies study: a baseline questionnaire survey in 2008 and questionnaire and environmental measurements survey conducted between 2009 and 2010. The details of the baseline questionnaire survey in 2008 have been reported previously (Ukawa et al., 2013; Ait Bamai et al., 2014). Briefly, all 6393 schoolchildren from 12 public elementary schools in Sapporo were asked to participate in the study, of which 4408 children responded to the questionnaire (response rate 69.0%). A total of 951 children (832 households) agreed to allow a home visit to conduct environmental measurements. In 2009 and 2010, we contacted children who were still attending the same elementary school as in 2008, excluding those who left blanks on the baseline questionnaires regarding their gender, grade, and allergies for ISAAC (International Studies of Asthma and Allergies on Childhood). This selection procedure identified a total of 128 households who allowed home visits for environmental measurements, dust collection, spot urine collection, and questionnaire in October and November of 2009 and 2010. During the home visit survey, we collected 479 urine samples and questionnaires from the family members of the 128 households. The questionnaire included questions on gender, age, body height, weight, and time spent at home. We selected 471 participants who had data for the urine sample and data for their gender, age, body height, and weight. From these, we excluded grandparents ($n = 9$). Finally, a total of 462 study participants from the 128 households were included in this study.

All participants provided their written informed consent. The parents provided informed consent for participation in this study if their children were under 12 years old. The study protocol was approved by the ethics board for epidemiological studies at Hokkaido University Graduate School of Medicine.

Phthalate metabolites in urine

Collection of urine samples

Parents were asked to collect the morning spot urine for the home visit and refrigerate the sample until our visit. Each urinary sample was dispensed into a stoppered glass test tube, which had been cleaned by acetone in our laboratory and sealed with fluoric-tape, wrapped with aluminium foil, and kept at −20 °C until the day of analysis. All 462 urinary samples were assayed for creatinine using an enzyme-linked immunosorbent assay at SRL, Inc. (Tokyo, Japan).

Standards and reagents

Mono-*n*-butyl phthalate (MnBP), MiBP, mono(3-carboxypropyl) phthalate (MCPP), mono-benzyl phthalate (MBzP), MEHP, mono(2-ethyl-5-oxohexyl) phthalate (MEOHP), and mono(2-ethyl-5-carboxypentyl) phthalate (MECPP) standards and MEHP-d4 were purchased from Cambridge Isotope Laboratories, Inc., Massachusetts, USA. Acetonitrile and hydrochloric acid were purchased from Kanto Chemical Co., Inc., Tokyo, Japan. Acetic acid, ethyl acetate, and sodium sulphate were purchased from Wako Pure Chemical Industries, Ltd., Osaka, Japan. β-Glucuronidase/Arylsulfatase from Helix pomatia (30/60 unit) was purchased from Merck & Co., Inc., Darmstadt, Germany. *N*-methyl-*N*-(*tert*-butyldimethylsilyl)-trifluoroacetamide (MTBSTFA) was purchased from GL Sciences Inc., Tokyo, Japan.

Sample preparation

For the sample preparation, 50 μL of acetic acid (1 M; pH 4.8), buffer, and 50 μL of MEHP-d_4 (2 μg/mL) were added to 0.5 mL of sample urine. Solutions were incubated with 10 μL of β-glucuronidase/arylsulphatase (30/60 unit) at 36 °C for 24 h. The mixture was extracted with 100 μL of hydrochloric acid (2 M). Ethyl acetate (2 mL) was added with vortexing for 30 s and then centrifuged (3000 rpm for 10 min). This extraction procedure was repeated twice. Supernatants were transferred into new tubes and dried at 36 °C for 1 h. The adherence of sample to the tube was resolved with 30 μL of ethyl acetate. The derivatisation processes

Table 1
Mass transitions and recovery for each phthalate metabolite and internal standard ($n = 5$).

Compounds	Quantitation (m/z)	Confirmation (m/z)	Retention times (min)	Recovery (%)	RSD (%)
MnBP	283	227	11.13	88.6	26.3
MiBP	279	223	10.89	86.2	8.6
MBzP	91	166	12.95	116.3	26.8
MEHP	223	335	12.44	99.2	22.1
MEOHP	127	201	13.24	86.8	16.7
MECPP	337	147	15.75	136.2	9.3
MCPP	201	267	14.04	115.1	31.7
MEHP-d4	227	167	12.43	–	–

RSD: relative standard deviation.

for each metabolite were conducted using 30 μL of MTBSTFA (Ito et al., 2005). After addition of MTBSTFA, the solutions were mixed by vortexing at 70 °C for 30 min. All solutions were transferred into inserted vials, and 1 μL was injected into GC/MS. MEHHP was not measurable because the derivatisation of MEHHP did not work well.

Gas chromatography mass spectrometry (GC/MS)

Extracted solutions were analysed using GC/MS instrumentation. An HP GC6890 (Agilent Technologies Inc., Palo Alto, CA, USA) and Agilent 5973N MSD (Agilent Technologies Inc., Palo Alto, CA, USA) were used for analysis of 8 phthalate metabolites and MEHP-d_4. The column was a DB-5MS (30 m × 0.25 mm i.d. × 0.25 μm; Agilent Technologies, Inc., Santa Clarita, California, USA). Helium was used as the carrier gas (70 kPa, constant flow mode). The oven temperature was programmed as follows: 80 °C for 2 min, followed by 20 °C/min up to 300 °C for 20 min. The injector was operated in the split mode at 280 °C (1 μL injection volume). The detector was operated in the selective ion mode (SIM) at a temperature of 230 °C.

Quality control

The levels of MiBP, MnBP, MCPP, MBzP, MEHP, MEOHP, and MECPP were measured. Each calibration curve was prepared using 0.5 mL of standard pooled urine samples from healthy volunteers with added calibration standards that consisted of six concentrations (0, 0.01, 0.05, 0.1, 0.5, and 1.0 μg/mL) prepared in 20% acetonitrile water. Each calibration standard also contained the internal standard (IS) (0.2 μg/mL). Calibration curves were constructed to perform linear regressions of the peak area ratio between the standard and MEHP-d4 versus six concentrations. Urine samples were quantified using calibration curves that presented 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 of a human urine sample with 0.01 μg/mL of standard solution (Table 1). The procedural blank levels were determined using 1 mL of ultrapure water. In this study, the LOD values were determined as 0.005 μg/mL (5 μg/L) for all metabolites. Because it was suspected that <0.01 μg/mL of quantitative assessment was acceptable, according to the chromatogram of the peaks and signal-to-noise ratios of each metabolite. In addition to this assessment, >0.005 μg/mL of quantitative assessment was acceptable in accordance with these ratios (Fig. 1). However, the RSD value for MCPP was too high (31.7%); therefore, we excluded MCPP from further analysis. Positive correlations between primary DEHP metabolites of MEHP and secondary DEHP metabolites of MEOHP and MECPP were obtained ($p < 0.01$). For quantification of phthalate metabolites, the total area of the branched and linear isomer peaks was integrated. Calibration curves were constructed before and after each batch (approximately 50 samples) to maintain the quality of the analysis. All instruments for sample collection, preparation, and GC/MS analysis were washed with acetone and covered with aluminium foil

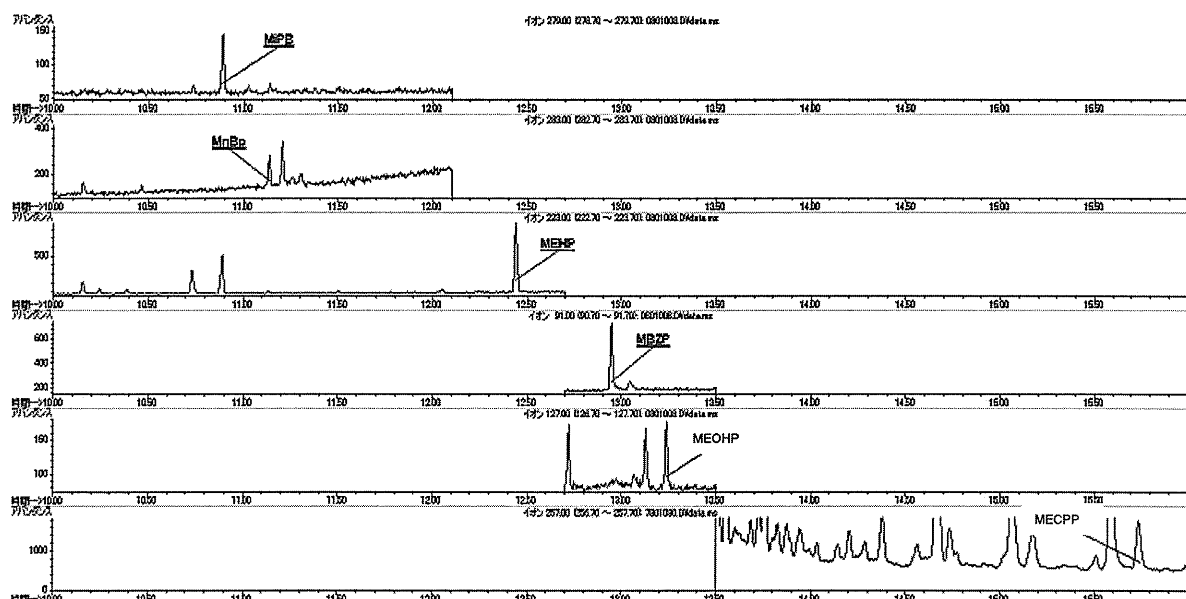


Fig. 1. Chromatograms of each phthalate metabolite. *X*-axis shows the retention time. *Y*-axis shows the peak level.

Table 2
Basic characteristics of schoolchildren and their family members.

	Preschool siblings $n=29$		School-children $n=178$		Older siblings $n=40$		Mothers $n=125$		Fathers $n=90$	
	Median	Range	Median	Range	Median	Range	Median	Range	Median	Range
Gender (male/female)	15/14		100/78		19/21		0/126		90/0	
Age	5	3–6	9	7–12	15	13–24	41	27–51	42	31–56
Height (cm)	108.6*	94.8–121	135	119–167	158	147–170	158	147–170	170	155–187
Weight (kg)	18*	14–24	30	19–56	47.5	34–87	52	41–82	68	52–90
Creatinine in urine (μg/mL)	794	283–1765	1031	307–2754	1518	274–4392	1300	344–3434	1662	630–3349

* Values were obtained from data of the Ministry of Health, Labour and Welfare (2013). Tables 2–6. Average body weight and height, (http://www.mhlw.go.jp/toukei/youran/indexyk_2_1.html).

until use to prevent phthalate contamination. To confirm that there was no phthalate contamination from the materials used for sampling, the sampling receptacles were extracted with acetone, and the blank values were examined. The blank value for MEHP was 0.0017 μg/mL (range, 0.0016–0.0019 μg/mL). The blank values of other metabolites were not detected.

Estimated daily intake calculations

The daily intake of the target phthalates was calculated for each participant from the phthalate metabolite concentration in the urine. In the present paper, the calculations of the total daily intake of phthalates were estimated using the following model based on Koch et al. (2007) and Wittassek et al. (2007):

$$\mathrm{DI} = \frac{(C \times \mathrm{CE})}{\left(F_{\mathrm{ue}} \times \mathrm{BW} \times M_{W_p}\right)}$$

where DI (μg/kg/day) is the total daily intake of phthalate normalised for body weight; C (μmol/gCr) is the urinary phthalate metabolite concentration; CE (g/day) is the creatinine clearance rate; and F_{ue} is the urinary excretion factor, which describes the molar ratio between the excreted amount of a metabolite in relation to the intake of the parent phthalate: MiBP, MnBP, MBzP, MEHP, MEOHP, and MECPP, which were reported to be 0.69, 0.69, 0.73, 0.059, 0.15, and 0.185 (Koch et al., 2004a, 2005; Kohn et al., 2000), respectively. BW (kg) is body weight. M_{W_p} (g/mol) is the molecular weights of the parent phthalate. The information on gender, age, body height, and weight for each participant was obtained from the self-reported questionnaire, except for preschoolers aged less than six years. We used the values of Japanese gender-age-specific body height and weight (Japan Ministry of Health Law, 2013) for the preschoolers because the preschoolers were not asked to complete the questionnaire, and the calculations for the daily intake of phthalates required information regarding gender, age, body height, and weight. CE was estimated following the model based on Mage et al. (2004) and Mage et al. (2008).

more than 18 years old.

- $\mathrm{CE} = 1.93 \times (140 - \mathrm{Age}) \times \mathrm{BW}^{1.5} \times \mathrm{ht}^{0.5} \times 10^6$ ... male
- $\mathrm{CE} = 1.64 \times (140 - \mathrm{Age}) \times \mathrm{BW}^{1.5} \times \mathrm{ht}^{0.5} \times 10^6$... female

3–18 years old

- $\mathrm{CE} = \mathrm{ht} \times \{6.265 + 0.0564 \times (\mathrm{ht} - 168)\} \times 10^3$ ...ht < 168 cm male
- $\mathrm{CE} = \mathrm{ht} \times \{6.265 + 0.2550 \times (\mathrm{ht} - 168)\} \times 10^3$ ...ht > 168 cm male
- $\mathrm{CE} = 2.045 \times \mathrm{ht} \times \exp\{0.01552 \times (\mathrm{ht} - 90)\} \times 10^3$ ... female

where Age (years old) is the participant's age. ht (cm) is height.

Data analysis and statistics

For values below the LOD, we assigned a value of the LOD divided by 2 (LOD/2). The metabolites of DBP and DEHP were combined into their sum of the individual metabolite concentrations as ΣDBP (MnBP and MiBP) and ΣDEHP (MEHP, MEOHP, and MECPP). To simplify the interpretations of the sums of the phthalate metabolite levels, urinary phthalate metabolite levels were converted to molecular concentration (μmol/L).

We classified participants into five groups: "preschool siblings (3–6 years old)", "schoolchildren (7–12 years old)", "older siblings (13–24 years old)", "mothers", and "fathers". Then, when comparing the differences in the phthalate metabolite and daily intake levels between family members, mothers and fathers were categorised using one variable, "parents". The data for phthalate metabolite and daily intake levels were not normally distributed according to the Shapiro–Wilk W-test ($p > 0.05$). The differences in the phthalate metabolite and daily intake levels with parents as a referent were analysed using the Steel test. For the multiple comparisons, the statistical significance of the p-value was $p < 0.017$ based on Bonferroni's correction. The other statistical significance of the p-value was $p < 0.05$. For statistical analyses, a two-tailed test and a 5% level of significance were used. All analyses were performed using JMP Pro 10 for Macintosh (SAS Institute Inc., Cary, NC).

Results

The present study is based on data from 462 participants (224 males and 238 females) aged from 3 to 56 years. The distribution of each age group was as follows: 29 preschool siblings, 178 schoolchildren, 40 older siblings, 125 mothers, and 90 fathers (total 215 parents) (Table 2).

The distributions of urinary phthalate metabolite levels (with non-creatinine-adjusted and creatinine-adjusted values) in the schoolchildren and their family members are shown in Table 3. All phthalate metabolites, except MnBP, were detected in more than 50% of the urine samples. The most frequently detected metabolite was MEOHP (98.9%), followed by MiBP, MEHP, MBzP, MECPP, and MnBP. MECPP was detected in 93.1%, 92.7%, and 80.6% of urine samples among preschool siblings, schoolchildren, and older siblings, whereas they were detected in 56.7% and 57.6% of mothers and fathers, respectively. The highest median levels of phthalate metabolites were found for MiBP in all participants except for schoolchildren. Instead, MEOHP had the highest median level in schoolchildren. For most of the metabolites, the range between the minimum and maximum levels was 2–3 orders of magnitude.

The phthalate metabolite levels of schoolchildren and their family members are shown in Fig. 2 for (a) non-creatinine-adjusted data and (b) creatinine-adjusted data. When comparing different groups with parents (mothers and fathers) as a control (a), preschool siblings had a significantly higher urinary metabolite level for MECPP. Schoolchildren had significantly higher urinary metabolite levels for MEOHP, MECPP, and ΣDEHP. When adjusting for creatinine values (b), the results showed stronger and clearer associations; preschool siblings had significantly higher urinary metabolite levels for MiBP, MBzP, MEOHP, MECPP, and ΣDEHP.

Table 3
Distribution of urinary phthalate metabolite levels (with non-creatinine-adjusted values) in schoolchildren and their family members.

	Preschool siblings (n = 29)					Schoolchildren (n = 178)					Older siblings (n = 40)					Mothers (n = 125)					Fathers (n = 90)				
	>LOD%	Range	25th	Median	75th	>LOD%	Range	25th	Median	75th	>LOD%	Range	25th	Median	75th	>LOD%	Range	25th	Median	75th	>LOD%	Range	25th	Median	75th
Phthalate metabolites (μg/L)																									
MnBP	10.3	<LOD-74	<LOD	<LOD	<LOD	29.2	<LOD -36	<LOD	<LOD	5.62021	30.6	<LOD-13	<LOD	<LOD	6.0	33.9	<LOD-37	<LOD	<LOD	6.0	39.1	<LOD-57	<LOD	<LOD	8.3
MiBP	100	9.90-8302	24.6	105.0	401.3	96.6	<LOD-6995	18.9	47.0	166.5	97.2	<LOD-4008	24.1	81.3	222.7	95.3	<LOD-6946	17.3	47.3	169.7	92.4	<LOD-2084	14.6	52.6	263.4
MBzP	86.2	<LOD-327	11.0	17.8	36.9	80.3	<LOD-1616	7.2	16.3	32.5	75	<LOD-981	3.7	18.3	38.0	74.0	<LOD-445	<LOD	11.6	23.1	73.9	<LOD-269	<LOD	13.4	22.7
MEHP	65.5	<LOD-144	<LOD	14.1	39.4	81.5	<LOD-522	6.5	19.7	60.9	83.3	<LOD-450	7.2	20.5	65.9	85.8	<LOD-416	10.3	28.6	59.9	73.9	<LOD-597	<LOD	24.6	62.0
MEOHP	96.6	<LOD-316	32.0	58.4	121.5	99.4	<LOD-3000	33.9	51.5	87.4	100	5.83913-193	39.8	66.4	93.1	98.4	<LOD-199	27.4	47.3	71.9	98.9	<LOD-205	28.4	44.6	66.0
MECPP	93.1	<LOD-245	25.5	44.1	93.2	92.7	<LOD-5357	19.6	34.9	54.2	80.6	<LOD-127	10.7	20.9	39.8	56.7	<LOD-182	<LOD	7.5	26.7	57.6	<LOD-419	<LOD	7.5	27.5
Phthalate sums (μmol/L)																									
ΣDBP		0.02-37	0.1	0.5	1.4		0.02-32	0.1	0.2	0.8		0.02-3	0.1	0.4	1.1		0.02-31	0.1	0.2	0.8		0.02-9	0.1	0.3	1.0
ΣDEHP		0.1-2	0.3	0.5	0.7		0.08-29	0.3	0.4	0.7		0.09-3	0.3	0.4	0.7		0.04-s2	0.2	0.3	0.6		0.06-2	0.2	0.3	0.6

LOD: limit of detection.
ΣDBP: sum of MnBP and MiBP; ΣDEHP: sum of MEHP, MEOHP, and MECPP.

Schoolchildren had significantly higher urinary metabolite levels for MBzP, MEOHP, MECPP, and ΣDEHP.

The median daily intakes of phthalate of schoolchildren and their family members are shown in Fig. 3. Comparing different groups with parents as a control, preschoolers had significantly higher daily intakes of DiBP, BBzP, ΣDBP, and ΣDEHP. Schoolchildren had significantly higher daily intakes of DnBP, BBzP, and ΣDEHP. Taking into account the family relatedness, associations between phthalate metabolite and daily intake levels and family members were evaluated using multi-level analysis, and some of the significant values decreased; however, the tendencies did not change.

The correlations between schoolchildren and their mothers or fathers in terms of urinary phthalate metabolites and daily intake of phthalates are shown in Table 4. All phthalate metabolite and sums of metabolite levels in schoolchildren were positively correlated with their mothers' levels, except for MEHP. For fathers, only MiBP, MBzP, MECPP, and ΣDBP were correlated with their schoolchildren. In addition, all daily intakes of phthalates were positively correlated with mothers, whereas for fathers, only DiBP, BBzP, and ΣDBP were correlated. After adjustment for creatinine, these results did not change (Table 4).

Discussion

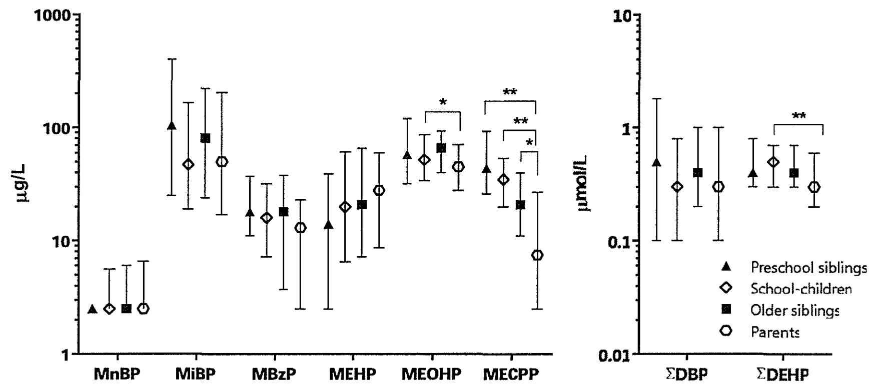
We simultaneously measured seven urinary phthalate metabolite levels in Japanese elementary schoolchildren and their family members (siblings, mother, and father) using the GC/MS method. The present study revealed that preschool siblings and schoolchildren had higher levels of phthalate metabolites in their urine compared with their parents. Moreover, preschool siblings and schoolchildren also had higher levels of daily intake of most phthalates than their parents. All phthalate metabolite levels in schoolchildren were positively correlated with their mothers, except for MEHP, whereas the levels in fathers were less correlated with their children.

Distributions of urinary phthalate metabolites

We measured the urinary levels of six phthalate monoesters using the GC/MS method in a population of elementary school children and their family members in Sapporo. However, we could not detect MEHHP using our method because derivatisation of MEHHP did not work well. The detection of MiBP, MEHP, and MEOHP was achieved for >80% of samples measured in this population, and the detection of MBzP and MECPP was achieved for >75%. MnBP was detected in 31.4% of the samples. The maximum and 75th percentile urinary ΣDBP levels were higher than ΣDEHP levels. The parent phthalate of MiBP, DiBP, is used for coatings of medications and cosmetics as a substitute for DnBP (E. Commission, 2004; Koch et al., 2012). Although DnBP and DiBP have been banned in cosmetics because of reproductive toxicity in Europe (E. Communities, 1993), there are no regulations for cosmetics and medications for both DnBP and DiBP in Japan. It is possible that the high individual levels of ΣDBP may be associated with medications or high frequencies of PCP use; the population of this study was based on a study of children's allergies, which had a high prevalence of asthma and allergies. However, this association cannot be ascertained in this study because the use of PCPs and medications was not assessed.

Exposure to phthalates occurs not just from diet, but also from other routes, such as medications, PCPs, cosmetics, and indoor air and dust. DEHP is the most widely used plasticiser in Japan, and we previously reported that DEHP had the highest phthalate level in dust among the same study population (Ait Bamai et al., 2014). The sum of the urinary DEHP metabolites (ΣDEHP: MEHP, MEOHP, and

(a) Non-creatinine-adjusted



(b) Creatinine-adjusted

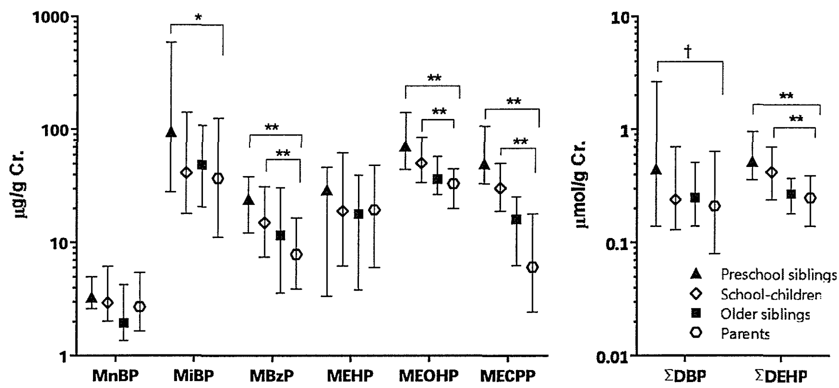


Fig. 2. Creatinine-adjusted phthalate metabolite levels (a) and sums of metabolites (b) in different age groups. Lower and upper error bars indicate the 25% and 75% percentiles, and the median is indicated by the symbol. The comparisons between the parents and different age groups were analysed using the Kruskal–Wallis test, and p values were adjusted using Bonferroni's correction. †: $p<0.017$; *: $p<0.01$; **: $p<0.001$. ΣDBP: sum of MnBP and MiBP; ΣDEHP: sum of MEHP, MEOHP, and MECPP.

MECPP) in school children was positively correlated with DEHP in house dust (Supplementary Table 4). MiBP and/or MnBP were not correlated with their parent compounds in house dust. Both dust concentrations and detection frequencies of phthalates in house dust in this study are in line with our previous findings (Ait Bamai et al., 2014). The contribution of indoor exposure to DEHP is higher in dust than in the gas phase, whereas the contributions of DnBP and DiBP are higher in the gas phase than in dust (Beko et al., 2013).

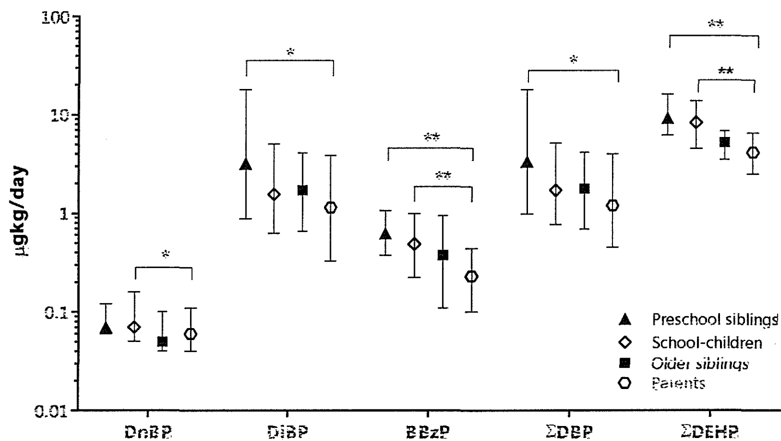


Fig. 3. Median daily intake of phthalates in different age groups. Lower and upper error bars indicate the 25% and 75% percentiles, and the median is indicated by the symbol. The comparisons between the parents and different age groups were analysed using the Kruskal-Wallis test, and p values were adjusted using Bonferroni's correction. *: $p<0.01$; **: $p<0.001$. ΣDBP: sum of MnBP and MiBP; ΣDEHP: sum of MEHP, MEOHP, and MECPP.

Table 4
Spearman coefficients of the correlations of urinary phthalate metabolites and daily intake of phthalates between schoolchildren and their mothers or fathers.

	Phthalate metabolite (μg/L)		Daily intake of phthalate (μg/kg/day)		
	Mother (n = 125)	Father (n = 90)		Mother (n = 125)	Father (n = 90)
MnBP	0.23**	0.08	DnBP	0.21*	0.11
MiBP	0.25**	0.29**	DiBP	0.27**	0.22*
MBzP	0.17*	0.40**	BBzP	0.29**	0.41**
MEHP	0.60	−0.04			
MEOHP	0.25**	0.02			
MECPP	0.38**	0.28**			
ΣDBP	0.25**	0.29**	ΣDBP	0.29**	0.22*
ΣDEHP	0.32**	0.05	ΣDEHP	0.30**	0.02

Spearman's ρ. ΣDBP: sum of MnBP and MiBP; ΣDEHP: sum of MEHP, MEOHP, and MECPP.
* $p<0.05$.
** $p<0.01$.

Therefore, DEHP in dust might also contribute to the sum of the DEHP urinary levels even though foods and other staff are the main sources of exposure to DEHP.

Urinary phthalate metabolites and daily phthalate intakes among family members

We observed that levels of urinary MEOHP, MECPP, and ΣDEHP, and daily intakes of DnBP, DiBP, BBzP, ΣDBP, and ΣDEHP were higher in preschool siblings and/or schoolchildren than in parents, suggesting that younger children have higher levels of exposure to these phthalates. When phthalate metabolite levels were adjusted for individual creatinine values, the results showed stronger and clearer associations because creatinine is strongly dependent on age and gender (Barr et al., 2005).

As for gender differences, there were no differences between mother and father for both phthalate metabolite levels and daily intake levels (Supplementary Table 2). Guo et al. (2011) reported that no gender-specific differences were found in DEHP metabolites (Guo et al., 2011), which is consistent with our results. In contrast, studies from the German Environmental Survey IV and U.S. population of the National Health and Nutrition Examination Survey (NHANES) reported that levels of urinary MEP, MnBP, and MBzP among adults were higher in females than in males because of the use of PCPs such as cosmetic products and fragrances (Koch et al., 2003; Silva et al., 2004). As for children (preschoolers and schoolchildren), MBzP levels and daily BBzP intake in boys were higher than in girls (p = 0.007 and p = 0.006, respectively) (Supplementary Table 3). Several research groups have reported consistent results indicating that levels of DEHP metabolites, MBP, and MBzP were higher in boys than in girls (Becker et al., 2009; Boas et al., 2010; Frederiksen et al., 2011), and daily BBzP intake tended to be higher in boys than in girls (Frederiksen et al., 2011). In contrast, the NHANES reported that the median values tend to be slightly higher in girls than in boys for MEP, MBP, MBzP, and DEHP metabolites (MEHP, MEOHP, and MEHHP) (Hatch et al., 2008).

The percentage fraction of MEHP was significantly lower, and MECPP was higher in younger age groups (preschoolers and schoolchildren) than in parents. Our results are consistent with previous studies (Becker et al., 2004; Koch et al., 2004b; Song et al., 2013), which suggests the excretion of oxidative metabolites of DEHP is elevated in children compared with adults. However, as our analytic methods did not allow detection of the secondary DEHP metabolite of MEHHP, the fraction of MEHHP could not be assessed in this study.

Correlation of phthalate metabolites between schoolchildren and their mothers and fathers

Most of the phthalate metabolites and daily intakes among schoolchildren were more strongly correlated with that of their mothers compared with their fathers. MBzP and ΣDBP among schoolchildren were correlated with both those of mothers and fathers, whereas ΣDEHP was correlated with that of mothers only. The main exposure routes of DEHP and DBP are diet and PCPs, respectively (Colacino et al., 2010; Sathyanarayana et al., 2008b; Serrano et al., 2014). This may indicate that children share greater phthalate exposure with their mothers than with their fathers because of surrounding environment and lifestyle factors, such as the same diet and same PCP use. However, we did not assess diet and use of PCP. Therefore, further studies assessing daily diet and frequency of PCP use may contribute to knowledge of mother–child pair associations.

Several studies have reported urinary phthalate metabolite levels in mother/child pairs (Casas et al., 2011; Huang et al., 2009; Kasper-Sonnenberg et al., 2012; Lin et al., 2011; Sathyanarayana et al., 2008a; Song et al., 2013). However, there are only three studies that have sampled mother/child pair urine at the same time (Kasper-Sonnenberg et al., 2012; Song et al., 2013), and there are no previous studies that have included all family members, including mothers, fathers, and siblings.

The percentage fractions of the DEHP metabolites of MEHP and MECPP were significantly higher in younger age groups (preschool siblings and schoolchildren) than in parents ($p<0.001$), which is consistent with previous studies (Kasper-Sonnenberg et al., 2012; Koch et al., 2006; Song et al., 2013).

Comparisons of urinary phthalate metabolite and daily phthalate intake levels with different studies

When comparing children's phthalate metabolite levels with those in previous studies (Bertelsen et al., 2013; CDC, 2013; Cho et al., 2010; Hsu et al., 2012; Kasper-Sonnenberg et al., 2012; Koch et al., 2011; Langer et al., 2014; Song et al., 2013), the levels of the metabolites of DEHP, MEHP, MEOHP, and MECPP were similar or higher than in other studies (Fig. 4). The MBzP level was similar or higher than that reported in Germany, Denmark, USA, and Taiwan. We previously reported that the BBzP level in house dust among this study population was quite lower than that reported in other countries (Ait Bamai et al., 2014). However, the urinary MBzP level was positively correlated with BBzP in house dust (Supplementary Table 4), which suggests that BBzP in dust contributes to urinary MBzP levels despite low levels of BBzP in house dust. One previous study reported that indoor air BBzP levels were significantly correlated with urinary MBzP (Adibi et al., 2003), suggesting that inhalation may also be an important route of exposure to BBzP.

When comparing phthalate daily intakes among schoolchildren with other children's studies, the DiBP and BBzP intakes were similar, whereas the DnBP intake was quite lower than that reported in other children's studies (Beko et al., 2013; Frederiksen et al., 2011; Koch et al., 2007, 2011; Lin et al., 2011; Wittassek et al., 2007), as shown in Fig. 5. On the other hand, despite our

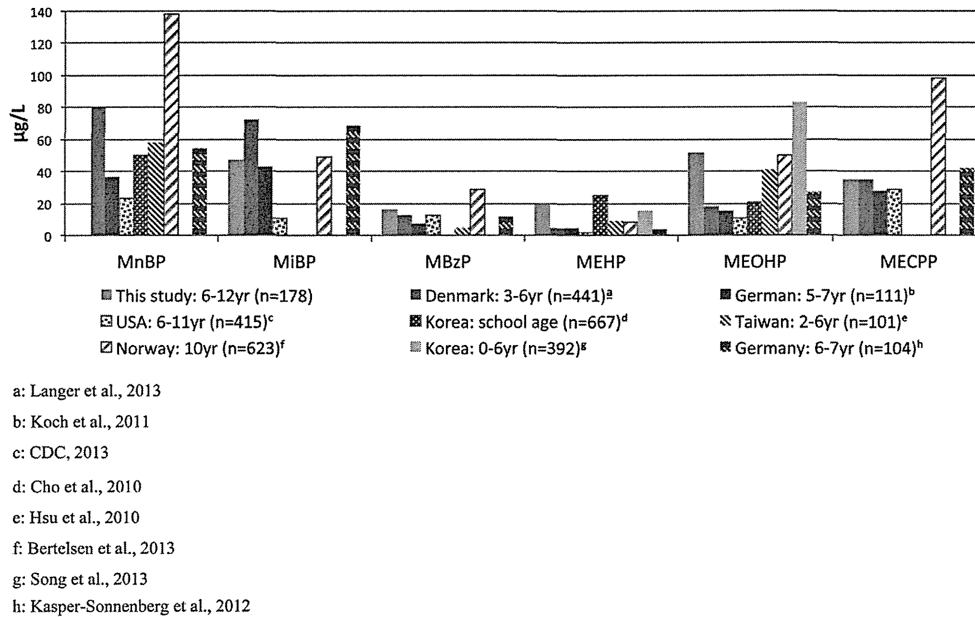


Fig. 4. Levels of urinary phthalate metabolites (μg/L) in this study, compared with several previous studies. *X*-axis shows the phthalate metabolites. *Y*-axis shows the urinary phthalate metabolite levels (μg/L).

underestimated daily DEHP intake because of a lack of the value of MEHHP, our DEHP intake was higher than in other studies except for a study in Taiwan (Lin et al., 2011). We previously reported that high levels of DEHP in house dust were detected from this study population with polyvinyl chloride (PVC) flooring, and the DEHP level in dust was remarkably higher than in other studies (Ait Bamai et al., 2014). In addition, our data showed positive correlations between DEHP in house dust and daily DEHP intake and ΣDEHP metabolites in schoolchildren's urine, but not correlated with mothers/fathers urine (Supplementary Table 4). It again may suggest that for the children in this study population, dust much contributes to total DEHP exposure than foods and other exposure sources. In contrast, it has been reported that DEHP levels in house dust (Becker et al., 2004) and indoor air (Adibi et al., 2008) are not correlated with DEHP metabolites in urine. Koch et al. (2013) also concluded that house dust/air does not seem to be a significant route of DEHP exposure. Therefore, these inconsistent results may be caused by high level of DEHP in house dust. When comparing the daily DEHP intake in this population to the Tolerable Daily Intake (TDI) value (50 μg/kg/day) of the EU and the Reference Dose (RfD) value (20 μg/kg/day) of the US Environmental Protection Agency (US EPA), 2 (0.4%) and 27 (5.8%) of the 462 participants exceed the TDI and the RfD, respectively. Of that number, 1 and 20 were children (preschoolers plus schoolchildren), which means approximately 0.5% and 10% of children and 0.4% and 3% of adults exceed the TDI and RfD values, respectively. Therefore, it is important to examine children's DEHP exposure because exceedance of the RfD is occurring more in children than in adults in the same families sharing similar exposure sources.

Interpretation of these comparisons should be cautious because the methods for collecting urine differ in each of the studies. Although production and use of DEHP, BBzP, and DnBP are

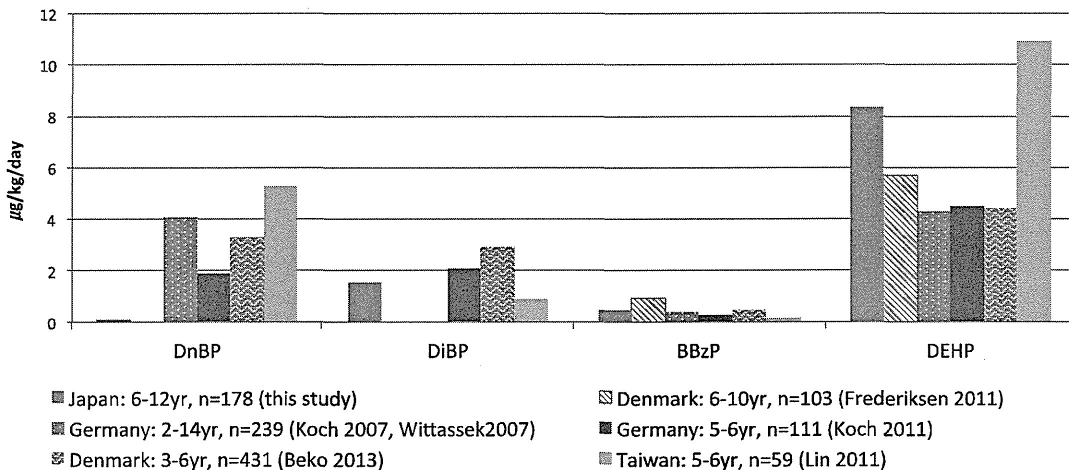


Fig. 5. Levels of daily phthalate intake (μg/kg/day) in this study, compared with several previous studies. *X*-axis shows the phthalate metabolites. *Y*-axis shows the daily phthalate intake levels (μg/kg/day).

temporally decreasing in recent years due to government regulations, leading to temporal decline of these urinary metabolite levels (CDC, 2013; Silva et al., 2004; Zota et al., 2014), the level in 10% of children in this study exceeded the RfD of the US EPA. Therefore, it is necessary to continue monitoring exposure to phthalates and to consider RfD with the health effects for children in mind.

Limitations

There are several limitations to this study. First, we collected urine samples only once from the first morning void. Moreover, we did not analyse samples taken from individuals over time. Therefore, it is possible that phthalate metabolite levels in this study were high or low by chance. Second, the distributions of age groups in this study were not even, with the number of schoolchildren and adults being 178 and 219, respectively, whereas the number of preschoolers and adolescents was 29 and 36, respectively. Therefore, statistical comparisons between age groups may be affected by this imbalance. Participation in this study was based on a study assessing the associations between elementary schoolchildren's allergies and their indoor environment, which means that children aged 6–12 years and their parents and siblings were the main participants. This study population has a propensity to have an interest in indoor air quality and their health (Ait Bamai et al., 2014), which may result in differences from the general Japanese population phthalate levels. Third, our GC/MS analysis procedures could not achieve detection of MEHHP, which accounts for a large portion of DEHP metabolites, from urine samples because the derivatisation of MEHHP did not work well. Thus, we may have underestimated our ΣDEHP and daily DEHP intake. Comparisons of our urinary phthalate metabolite levels and daily intakes to other studies should be made cautiously. We used MTBSTFA as a derivatisation reagent. Since Kim et al. (2014) recently reported that the GC/MS method using *N*,O-bis(trimethylsilyl)-trifluoro acetamide (BSTFA) as a derivatisation reagent allowed detection of MEHHP (Kim et al., 2014), although our study did not use BSTFA, further study needs to use BSTFA as a derivatisation reagent to detect MEHHP. However, using this method also allowed detection of seven phthalate monoesters in human urine samples of a large sample size with practical analysis cost. Fourth, the use of PCPs and medications, food consumption, and other behavioural patterns were not assessed in this study. Therefore, we could not ascertain the exposure source. Further studies are needed to advance our understanding of phthalate exposure.

Conclusions

We measured six urinary phthalate metabolite levels using a GC/MS method in Japanese (Sapporo) elementary schoolchildren and their family members. Most phthalate metabolite levels in children were similar or higher than in other studies. The daily intake of DEHP was higher, whereas the DnBP intake was quite lower than in other children's studies. DEHP metabolites and the daily intake of DEHP were especially high in preschool siblings compared with their parents. All phthalate metabolite levels in schoolchildren were positively correlated with the levels of their mothers except for MEHP, whereas the levels in fathers were less correlated with that of their children. Although there is decreasing production and use of phthalates, 10% of children and 3% of adults still exceeded the RfD value for DEHP, which indicates an important need to focus on children's DEHP exposure. Active endocrine phthalates act in a common fashion. However, the Japanese population is probably exposed to other phthalates that were not measured in the present study, e.g., diethyl phthalate, DMP, DiNP, and DiDP. Therefore, the cumulative exposure has to be taken into account in future studies. Our results will contribute to considerations of the regulations for some phthalates and the actual phthalate exposure levels in the Japanese population.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijheh.2015.03.013.

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