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

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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.

Keywords:

- Children
- Phthalate metabolites
- Mother-child pairs
- Father-child pairs
- Phthalate exposure

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

1 MTBSTFA, N-methyl-N-(tert-butyldimethylsilyl)-trifluoroacetamide

2 MW_p, molecular weights of parent phthalate

3 NHANES, National Health and Nutrition Examination Survey

4 PCP, personal care product

5 PVC, polyvinyl chloride

6 RfD, Reference Dose

7 SIM, selective ion mode

8 TDI, Tolerable Daily Intake

1. 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 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.

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; Commission 2008; Japan Ministry of Health 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. (2008) 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.

2. Materials and Methods

2.1. 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., 2012; 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.

2.2. Phthalate metabolites in urine

2.2.1. 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).

2.2.2. 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. ß-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.

2.2.3. 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□ 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□ for l hour. The adherence of

sample to the tube was resolved with 30 μL of ethyl acetate. The derivatisation processes 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□ 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.

2.2.4. 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 □ 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.

2.2.5. 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 (Figure 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 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.

2.3. 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(Koch et al. 2007) and Wittassek et al. (2007):

$DI = (C \times CE) / (F_{ue} \times BW \times MW_p)$

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; Koch et al. 2005; Kohn et al. 2000), respectively. BW (kg) is body weight. MW_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 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 2008(Mage et al. 2004; Mage et al. 2008).

more than 18 years old

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

3–18 years old

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

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

2.4. 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).

3. 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 2. 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 Figure 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. 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 Figure 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 3. 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.

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 (Commission 2004; Koch et al. 2012). Although DnBP and DiBP have been banned in cosmetics because of reproductive toxicity in Europe (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 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). 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. 2013; Song et al. 2013), the levels of the metabolites of DEHP, MEHP, MEOHP, and MECPP were similar or higher than in other studies (Figure 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; Koch et al. 2011; Lin et al. 2011; Wittassek et al. 2007), as shown in Figure 5. On the other hand, despite our 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 U.S. 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 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.

5. 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.

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Figure 1

Chromatograms of each phthalate metabolite.

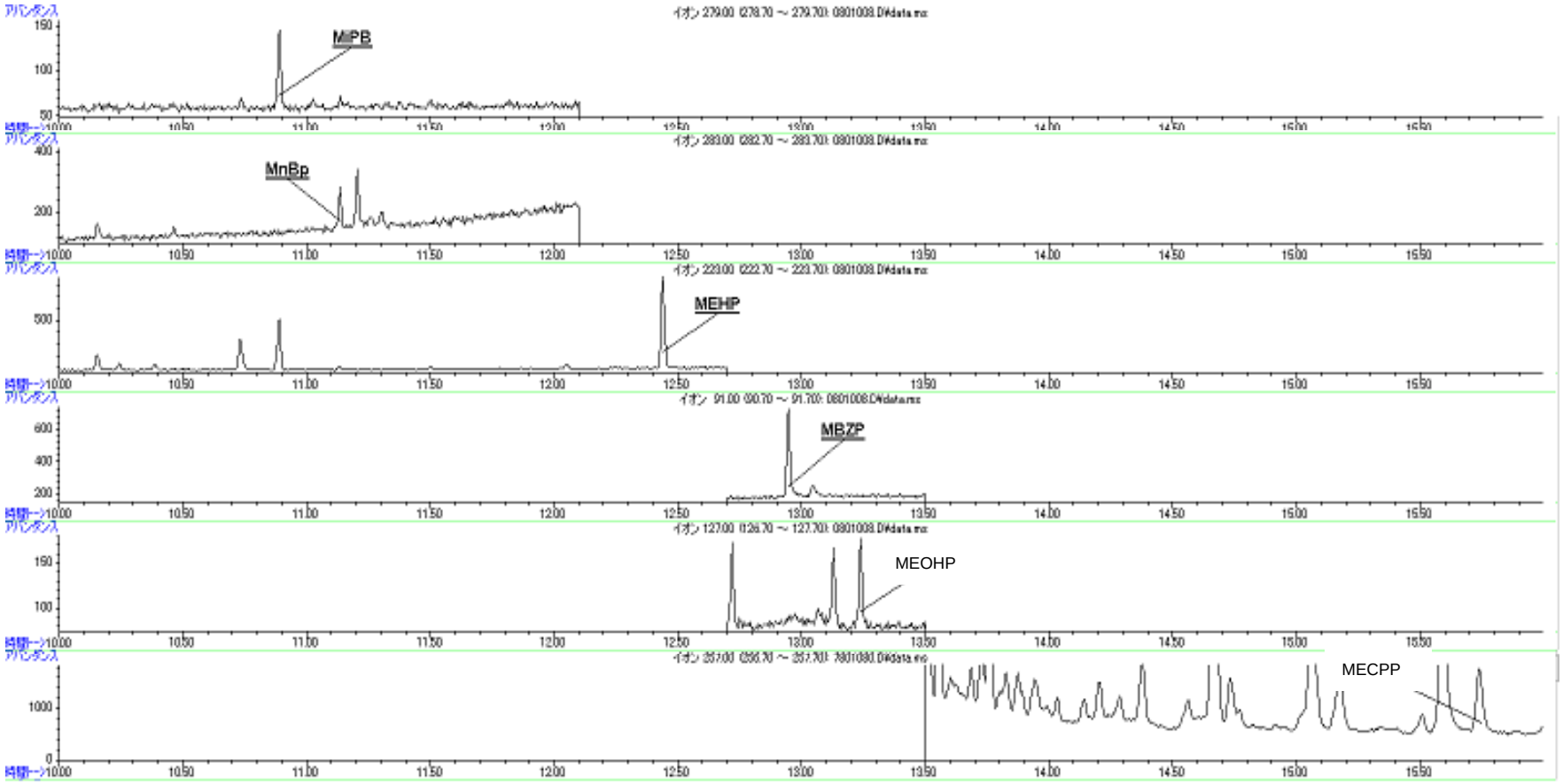
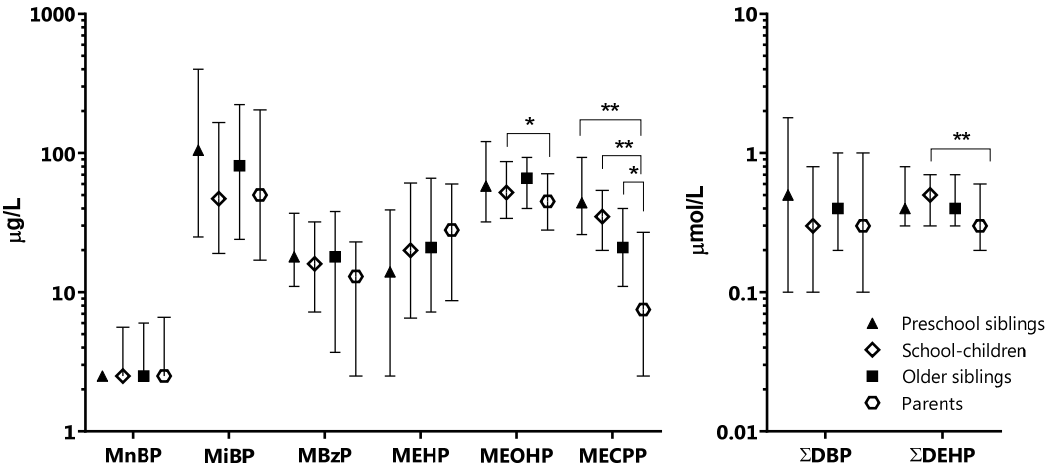


Figure 2

Phthalate metabolite levels in different age groups.

(a) Non-creatinine-adjusted



(b) Creatinine-adjusted

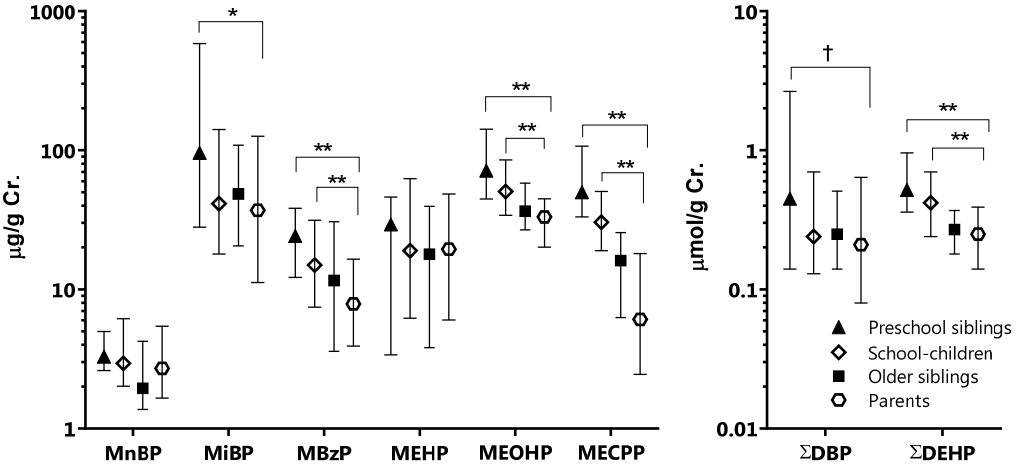


Figure 3.

Median daily intake of phthalates in different age groups.

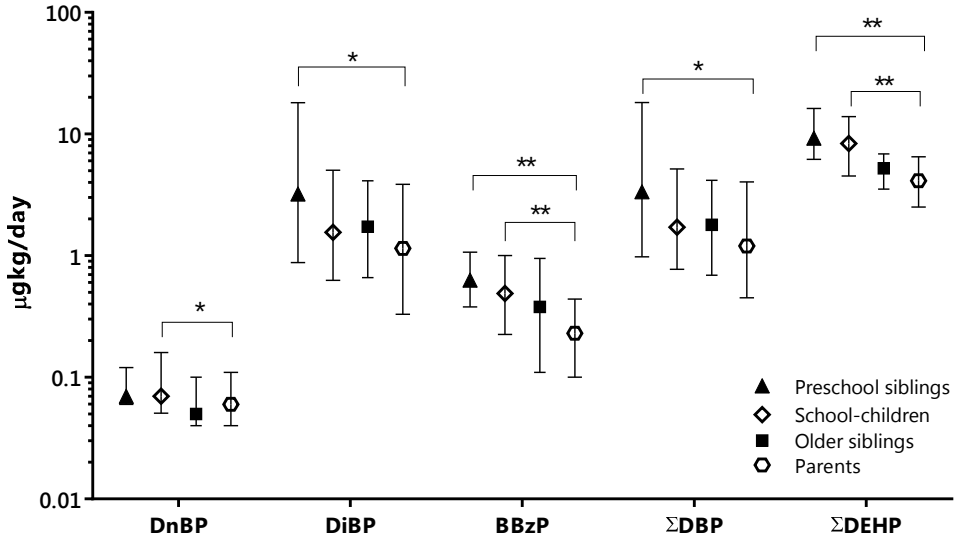
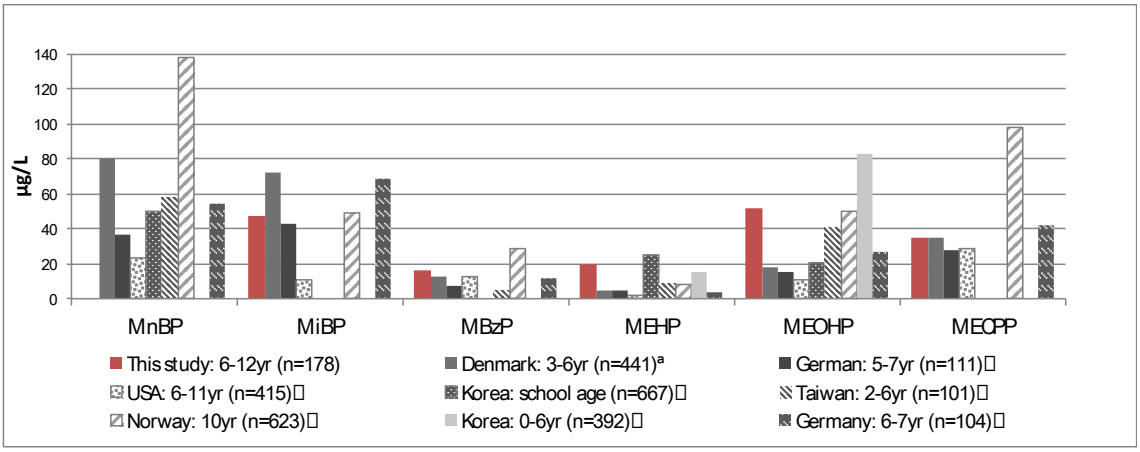


Figure 4.

Levels of urinary phthalate metabolites (μg/L) in this study, compared with several previous studies.



a: Langer et al., 2013

b: Koch et al., 2011

c: CDC, 2013

d: Cho et al., 2010

e: Hsu et al., 2010

f: Bertelsen et al., 2013

g: Song et al., 2013

h: Kasper-Sonnenberg et al., 2012

Figure 5.

Levels of daily phthalate intake (μg/kg/day) in this study, compared with several previous studies.

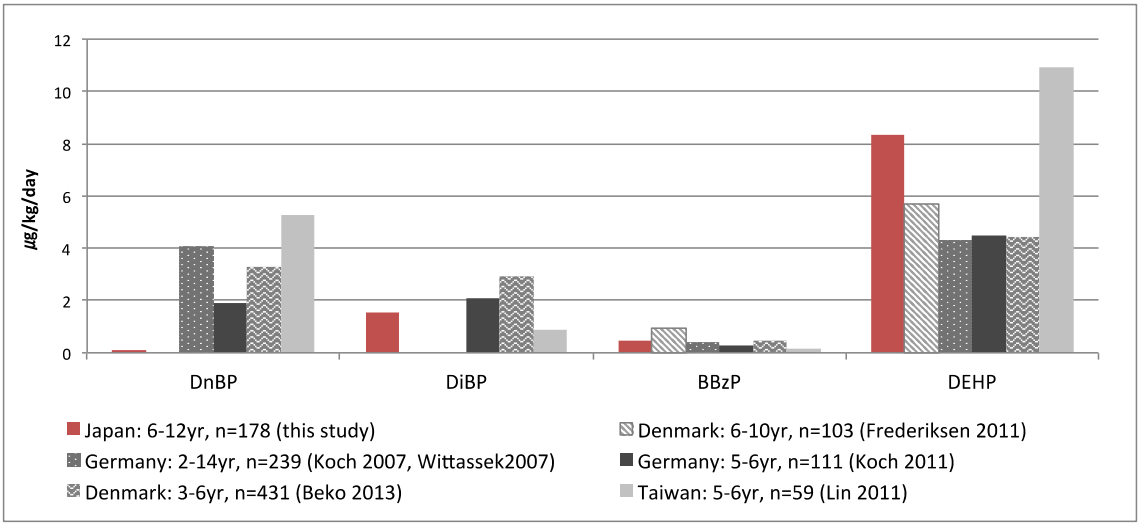


Figure legends

Fig. 1. Chromatograms of each phthalate metabolite.

X-axis shows the retention time. Y-axis shows the peak level.

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.

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.

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).

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).

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.

Table 2.
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-749.90	<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	- 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		0.04-2	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

Table 3
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). Table 2-6. Average body weight and height, <http://www.mhlw.go.jp/toukei/youran/indexyk_2_1.html>

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

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

Supplementary Table 1.
Distribution of urinary phthalate metabolite levels (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)			
	Range	25th	Median	75th	Range	25th	Median	75th	Range	25th	Median	75th	Range	25th	Median	75th	Range	25th	Median	75th
Creatinine-adjusted phthalate metabolites (μg/gCr)																				
MnBP	<LOD-105	<LOD	<LOD	<LOD	<LOD -38	<LOD	<LOD	6.2	<LOD -14	<LOD	<LOD	4.3	<LOD-31	<LOD	<LOD	5.4	<LOD-37	<LOD	<LOD	6.3
MiBP	11.5-10636	28.1	95.8	586.3	<LOD - 131177	18.0	41.4	141.0	<LOD -5958	20.6	48.6	108.6	<LOD-8264	12.5	37.9	120.4	<LOD-1281	8.1	36.1	161.4
MBzP	<LOD - 881	12.2	24.3	38.3	<LOD - 860	7.5	15.0	31.5	<LOD -223	3.6	11.6	30.6	<LOD-309	<LOD	9.4	17.3	<LOD-103	<LOD	6.9	14.5
MEHP	<LOD - 181	<LOD	29.3	46.2	<LOD - 484	6.2	19.0	62.5	<LOD -313	3.8	17.9	39.6	<LOD-615	9.4	22.8	52.7	<LOD-306	<LOD	12.7	47.2
MEOHP	<LOD - 524	44.6	71.7	142.3	<LOD - 2147	34.1	50.5	85.4	10.3457-120	26.8	36.6	58.1	<LOD-466	21.1	35.9	48.5	<LOD-168	19.4	26.4	41.7
MECPP	<LOD - 251	33.2	50.0	107.3	<LOD-3833	19.0	30.4	50.5	<LOD -73	6.3	16.1	25.6	<LOD-151	<LOD	7.1	21.0	<LOD-207	<LOD	5.1	15.5
Creatinine-adjusted sum of phthalates (μmol/gCr)																				
ΣDBP	0.06-48	0.1	0.5	2.6	0.03-59	0.1	0.2	0.7	0.03-27	0.1	0.3	0.5	0.02-37	0.1	0.2	0.6	0.02-6	0.1	0.2	0.7
ΣDEHP	0.14-3	0.4	0.5	1.0	0.06-21	0.2	0.4	0.7	0.06-2	0.2	0.3	0.4	0.05-4	0.2	0.3	0.4	0.03-1	0.1	0.2	0.3

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

Supplementary Table 2.
Comparisons of urinary phthalate metabolites (μg/L or μmol/L) and daily phthalate intakes (μg/kg/day) between mothers and fathers.

	Mothers (n=125)			Fathers (n=90)			p value
	25th	Median	75th	25th	Median	75th	
Phthalate metabolites							
MnBP	<LOD	<LOD	6.20	<LOD	<LOD	8.13	n.s
MiBP	16.86	44.87	172.04	13.94	52.56	271.99	n.s
MBzP	<LOD	11.41	23.57	<LOD	13.38	22.70	n.s
MEHP	10.43	28.60	60.82	<LOD	24.80	63.28	n.s
MEOHP	27.79	47.26	71.57	27.79	44.19	65.07	n.s
MECPP	<LOD	7.48	26.34	<LOD	7.41	26.14	n.s
ΣDBP	0.09	0.21	0.79	0.09	0.25	1.24	n.s
ΣDEHP	0.20	0.34	0.59	0.22	0.32	0.55	n.s
Daily intakes							
DnBP	0.04	0.05	0.10	0.04	0.06	0.17	n.s
DiBP	0.34	1.06	3.19	0.31	1.52	6.21	n.s
BBzP	0.10	0.25	0.40	0.10	0.22	0.53	n.s
ΣDBP	0.43	1.10	3.29	0.48	1.67	6.42	n.s
ΣDEHP	2.41	4.10	5.99	2.60	4.16	7.10	n.s

Mann-Whitney U-test
LOD: limit of detection
ΣDBP: sum of MnBP and MiBP; ΣDEHP: sum of MEHP, MEOHP, and MECPP

Supplementary Table 3.

Comparisons of urinary phthalate metabolites (μg/L or μmol/L) and daily phthalate intakes (μg/kg/day) between boys and girls.

	Boys (n=115)			Girls (n=92)			p value
	25th	Median	75th	25th	Median	75th	
Phthalate metabolites							
MnBP	<LOD	<LOD	6.86	<LOD	<LOD	<LOD	n.s
MiBP	20.38	46.57	148.20	17.97	53.37	187.31	n.s
MBzP	10.23	19.14	39.54	<LOD	14.94	26.87	0.007
MEHP	5.40	18.49	58.98	7.90	21.53	66.10	n.s
MEOHP	38.13	63.57	90.33	33.12	48.13	81.83	n.s
MECPP	19.16	35.15	55.51	15.22	28.17	49.95	n.s
Σ.sP	0.11	0.23	0.69	0.09	0.25	0.85	n.s
Σ.sHP	0.28	0.46	0.67	0.25	0.39	0.70	n.s
Daily intakes							
DnBP	0.08	0.05	0.16	0.07	0.04	0.17	n.s
DiBP	1.53	0.70	4.96	1.62	0.56	5.15	n.s
BBzP	0.55	0.27	1.15	0.38	0.11	0.79	0.006
Σ.00	1.66	0.80	5.10	1.73	0.65	5.23	n.s
Σ.sHP	8.90	4.86	14.34	7.12	4.23	12.42	n.s

Mann-Whitney U-test
LOD: limit of detection

ΣDBP: sum of MnBP and MiBP; ΣDEHP: sum of MEHP, MEOHP, and MECPP

Supplementary Table 4.

Spearman's correlations between levels of phthalates in house dust and levels of phthalate metabolites in urine.

Dust	Metabolite/daily intake	School children	Mothers	Fathers
$DnBP_{floor}$	MnBP (µg/L)	-0.03	-0.12	-0.02
	ΣDBP (µmol/L)	0.04	0.00	-0.03
	DI MnBP (µg/kg/day)	0.03	-0.02	0.04
	DI ΣDBP (µg/kg/day)	0.05	0.05	0.00
$DnBP_{multi-surface}$	MnBP (µg/L)	0.03	-0.04	0.14
	ΣDBP (µmol/L)	-0.04	0.05	0.13
	DI MnBP (µg/kg/day)	0.03	0.06	0.09
	DI ΣDBP (µg/kg/day)	-0.03	0.10	0.12
$DiBP_{floor}$	MiBP (µg/L)	-0.08	0.00	-0.01
	ΣDBP (µmol/L)	-0.09	0.02	0.00
	DI MiBP (µg/kg/day)	-0.04	-0.03	0.06
	DI ΣDBP (µg/kg/day)	-0.04	-0.02	0.08
$DiBP_{multi-surface}$	MiBP (µg/L)	-0.10	0.01	-0.07
	ΣDBP (µmol/L)	-0.11	0.01	-0.08
	DI MiBP (µg/kg/day)	-0.09	-0.01	-0.04
	DI ΣDBP (µg/kg/day)	-0.10	-0.01	-0.04
$BBzP_{floor}$	MBzP (µg/L)	**0.30****	**0.22***	**0.22***
	DI MBzP (µg/kg/day)	**0.27****	**0.23****	0.17
$BBzP_{multi-surface}$	MBzP (µg/L)	**0.17***	0.11	0.07
	DI MBzP (µg/kg/day)	**0.19***	**0.18***	0.04
$DEHP_{floor}$	MEHP (µg/L)	0.14	0.13	0.14
	MEOHP (µg/L)	0.08	0.01	-0.04
	MECPP (µg/L)	**0.16***	0.14	0.12
	ΣDEHP (µmol/L)	**0.17***	0.10	0.05
	DI ΣDEHP (µg/kg/day)	**0.24****	0.11	0.16
$DEHP_{multi-surface}$	MEHP (µg/L)	0.07	-0.03	0.07
	MEOHP (µg/L)	0.13	0.06	0.01
	MECPP (µg/L)	**0.23****	**0.21***	0.01
	ΣDEHP (µmol/L)	**0.17***	0.07	-0.02
	DI ΣDEHP (µg/kg/day)	0.11	0.03	0.07

Spearman's correlation; *: $p<0.05$; **: $p<0.01$

Σ DBP: sum of MnBP and MiBP; ΣDEHP: sum of MEHP, MEOHP, and MECPP.

Dust data has already reported in Ait Bamai et al., 2014

Preschool and older siblings are not shown here because of number of participants are no enough to calculate correlations.