Association between prenatal bisphenol A and phthalate exposures and fetal metabolic related biomarkers: The Hokkaido Study on Environment and Children's Health

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Abstract

Bisphenol A and phthalates are widely detected in human urine, blood, breast milk, and amniotic fluid. Both bisphenol A and phthalates have been suggested as playing a role in obesity epidemics. Exposure to these chemicals during fetal development, and its consequences should be concerning because they can cross the placenta. Thus, this study aimed to assess the association between prenatal exposure to bisphenol A and phthalates, and cord blood metabolic-related biomarkers. Maternal serum was used during the first trimester, to determine prenatal exposure to bisphenol A and phthalates. Levels of metabolic-related biomarkers in the cord blood were also determined. Linear regression models were applied to the 365 participants with both, exposure and biomarker assessments, adjusted for maternal age, pre-pregnancy body mass index, parity, education, and sex of the child. The level of bisphenol A was negatively associated with the leptin level (β=-0.06, 95% confidence interval [CI]: -0.11, -0.01), but was positively associated with the high-molecular-weight adiponectin level, with marginal significance (β=0.03, 95%CI: 0.00, 0.06). The mono-isobutyl phthalate (MiBP), mono-n-butyl phthalate (MnBP), mono-(2-ethylhexyl) phthalate (MEHP), and summation of MEHP and MECPP to represent DEHP exposure (∑DEHPm) levels were inversely associated with the leptin levels (β=-0.14, 95%CI: -0.27, -0.01; β=-0.12, 95%CI: -0.24, 0.00 with marginal significance; β=0.08, 95%CI: -0.14, -0.03; and β=-0.09, 95%CI: -0.16, -0.03, respectively). The present study provided some evidence that prenatal exposure to bisphenol A and

certain phthalates may modify fetal adiponectin and leptin levels.

Keywords

Bisphenol A, phthalates, metabolic related biomarkers, birth cohort, prenatal exposure

Funding information

This work was supported by Grant-in-Aid for Scientific Research from the Japanese Ministry of Health, Labor, and Welfare (JSPS KAKENHI Grant Numbers JP25253050 and 16K16619) and the Environment Research and Technology Development Fund (5-1454) from the Ministry of the Environment, Japan.

Introduction

Bisphenol A and phthalates are both known as endocrine disruptors, and there is a growing concern about exposure to these chemicals and their adverse health outcomes on humans. Bisphenol A and phthalates have been detected in the urine, blood, breast milk, and amniotic fluid (Vandenberg et al. 2009, Dobrzynska 2016). Bisphenol A is widely used in polycarbonate products, such as epoxy resins used as coatings on the inside of many food and beverage cans (Vandenberg et al. 2007). Various phthalates are used in the manufacture of consumer products, such as food packages, polyvinyl chloride floor materials, shampoo, lotion, and fragrances. The consequences of exposure to these chemicals during fetal development should be considered because they can cross the placenta (Mose et al. 2007, Balakrishnan et al. 2010).

There is growing evidence that the *in-utero* environment programs fetal obesity risk. As molecular mechanism and epigenetic programing during fetal development may permanently affect adipogenesis and metabolism throughout the life, gestational period is highly susceptible to these environmental chemicals (Newbold et al. 2009). Bisphenol A and phthalates along with other environmental obesogenic chemicals have particularly played a role in obesity epidemic recently. Experimental studies have demonstrated that exposure to bisphenol A and phthalates modified the regulation of metabolism (Grun and Blumberg 2009) via peroxisome proliferator (PPAR)-modulated

pathways (Desvergne et al. 2009), adipogenesis (Chamorro-Garcia et al. 2012), and alternation of pancreatic β-cell function (Ropero et al. 2008, Lin et al. 2011, Soriano et al. 2012). However, whether levels of human exposure sufficiently induce such effects is unknown. Examining the disruption of metabolic regulations in newborns possibly induced by exposure to environmental chemicals during fetal development is difficult because only limited outcomes (i.e., birth size) are available and symptoms of metabolic dysfunction cannot be observed in newborns. Thus, conducting epidemiological investigations on prenatal exposures to these chemicals and interpreting the findings were challenging. Despite the importance of understanding in utero exposures and their consequences on the regulation of fetal metabolism, the evidence is insufficient.

Metabolic-related biomarkers such as adiponectin, leptin, tumor necrosis factor alpha (TNF-α), and interleukin 6 (IL-6) can be used to detect and monitor metabolic dysfunctions (Srikanthan et al. 2016). Adiponectin and leptin are adipokines produced by adipocytes. Leptin is also produced by the placenta in pregnant women (Srikanthan et al. 2016). TNF-α and IL-6 are pro-inflammatory cytokines secreted by the adipose tissues. In epidemiological studies, these biomarkers have been measured in cord blood samples (Chou et al. 2011, Ashley-Martin et al. 2014, Hui et al. 2016, Huang et al. 2017). Several studies presented adverse health effects in neonates associated with increased levels of IL-6 and TNF-α (Amarilyo et al. 2011, Catarino et al. 2012,

Lausten-Thomsen et al. 2014, Sorokin et al. 2014).

A fairly large number of epidemiological studies have shown a significant association between urinary levels of bisphenol A and various phthalates, and obesity and obesity-related disorders (Lind et al. 2012, Wang et al. 2012, James-Todd et al. 2016, Vafeiadi et al. 2016); however, the etiology is still largely unknown. Only limited number of studies have investigated the association between bisphenol A and phthalate exposures, and metabolic-related biomarkers. Several cross-sectional studies investigated the association between maternal bisphenol A levels, and TNF-α and IL-6; however, their findings were inconsistent (Watkins et al. 2015, Ferguson et al. 2016). Cross-sectional studies on adults and children demonstrated relationships between bisphenol A and various phthalate exposures and adipokine levels (Menale et al. 2016, Choi et al. 2017), yet studies that elicit a conclusion were insufficient. Thus, this prospective cohort study aimed to investigate the association between prenatal exposure to bisphenol A and phthalates and cord blood metabolic-related biomarkers.

Methods

Study design

This was one of the follow-up studies on the Hokkaido Study on Environment and Children's Health, a prospective birth cohort study. Details of the cohort profile can be found

elsewhere (Kishi et al. 2011, Kishi et al. 2013, Kishi et al. 2017). Briefly, the whole cohort consisted of 20,926 participants enrolled from 2003 to 2012, and sub-cohort population (23.3% of the whole cohort population) designated for exposure and/or biomarker assessments was randomly selected from the whole cohort population. Defining sub-cohort population strategy was effective to avoid additional costs and time of processing the exposure assessment of all participants. Details of the sub-cohort population can be found elsewhere (Kishi et al. 2017). Participants were recruited during early pregnancy (<13 weeks of gestational age). The baseline questionnaire including information on demographic characteristics, smoking history, alcohol consumption, and medical history was filled by pregnant women during the recruitment. Perinatal information including birth weight, infant sex, mode of delivery, and diagnosis of congenital anomalies were obtained from medical records completed by obstetricians. This follow-up study targeted cohort study participants who were born between April 2008 and May 2011 (n=5,695) and those who have reached 5 years old. Questionnaires to assess child neurobehavioral development such as strength and difficulties questionnaire and attention deficit and hyperactivity rating scale were distributed via mail to the subpopulation with child aging 5 and 6 years. A total of 3,223 valid responses were received at the end of May 2016. The response rate was 56.6%. Among the 3,223 sub-cohort population, 820 were included. Among the 820 sub-cohort population, those who did not have maternal serum or cord blood samples and no available medical records during delivery were excluded. A total of 419

participants presented with bisphenol A and phthalate metabolite levels in the first-trimester serum, and 375 presented with metabolic-related biomarkers in the cord blood. For the statistical analysis, those who had both exposure assessment and metabolic-related biomarker measurement were included (n=365, Figure 1).

This study was conducted after obtaining written informed consents from all participants. The protocol used in this study was approved by the Institutional Ethical Board for epidemiological studies at the Hokkaido University Graduate School of Medicine and Hokkaido University Center for Environmental and Health Sciences.

Measurements of bisphenol A and phthalates

Maternal serum in the first trimester was collected and stored at –80 °C until the analyses. Serum samples were analyzed for bisphenol A and seven variants of phthalate metabolites: mono-n-butyl phthalate (MnBP), mono-isobutyl phthalate (MiBP), mono-(2-ethylhexyl) phthalate (MEHP), mono-benzyl phthalate (MBzP), mono-(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP), mono-(2-ethyl-5-carboxypentyl) phthalate (MECPP), and mono-(4-methyl-7-carboxyheptyl) phthalate (cx-MiNP) using isotope-diluted liquid chromatography-tandem mass spectrometry (LC-MS/MS) for bisphenol A analysis and ultra-performance LC-MS/MS for phthalate metabolite analyses. The method detection limits (MDLs) of bisphenol A, MnBP, MiBP, MBzP, MEHP, MEHHP, MECPP, and cx-MiNP were 0.011 ng/ml, 0.57 ng/ml, 0.44 ng/ml, 0.19 ng/ml, 0.31 ng/ml, 0.23 ng/ml,

0.11 ng/ml, and 0.12 ng/ml, respectively. All analyses were conducted at Idea Consultants, Inc. (Shizuoka, Japan).

The detailed sample preparation for bisphenol A analysis can be found in our previous report (Yamamoto et al. 2016). In each serum sample, bisphenol A-d_{16} spiking solution was added and mixed by shaking, and then β-glucuronidase and 0.2 M acetate buffer solution (pH 5.0) were also added. Samples were stored in an incubator at 37 °C for 1.5 h, followed by solid-phase extraction. The detailed phthalate metabolite analyses and their serum samples were prepared as follows: MnBP-d_4, MiBP-d_4, MBzP-d_4, MEHP-d_4, MEHHP-$^{13}C_4$, MECPP-$^{13}C_4$, and cx-MiNP-d_4 were added as internal standard, and then 90 µl of 1M phosphoric acid was added to the serum sample (0.5 ml). After mixing using the vortex and ultrasonic irradiated for 10 min, 940 µl of acetonitrile was consequently added and centrifuged with 3,500 rpm for 5 min. Supernatants were transferred into the new tubes, and 1000 µl of ammonium acetate buffer solution (100 mM, pH 9.1), 3,000 µl of ammonium acetate buffer solution (100 mM, pH 6.5), and 10 µl of β-glucuronidase were added to each sample for the enzymatic hydrolysis of phthalate metabolite conjugates. Samples were stored in an incubator at 37 °C for 1.5 h, followed by solid phase-extraction using Oasis MAX 96-well plate (30 mg, 30 µm, Waters, Milford, MA, USA). After the solid-phase extraction, a 500 µl of elution was transferred into sample vials, and 500 µl of ultra-pure water was added and analyzed using UPLC (ACQUITY UPLC H-Class, Milford, MA, USA), along with triple quadrupole tandem MS (QTRAP 6500,

AB SCIEX, Framingham, MA). The insoluble particulates were filtered using in-line filters (2.1×5 mm, 1.7 µm, Vanguard Phenyl column, Waters, Tokyo, Japan) preceding the BEH Phenyl column (2.1×50 mm, 1.7 µm, Waters, Tokyo, Japan). The retention gap technique was used by installing retention gap columns Atlantis T3 (2.1×50 mm, 3 µm, Waters, Tokyo, Japan), which improved phthalate metabolite sensitivity by trapping mobile-phase phthalate metabolites in the retention gap column. The temperature column was 40 °C. The analytes were quantified using ESI-negative SRM mode. The total UPLC cycle time was 20 min including column re-equilibration. The calibration curve was linear over a concentration ranging from 0.02 ng/ml to 20 ng/ml with a coefficient correlation (r^2) of >0.999. Detailed information can be found in the Appendix. The MDLs of bisphenol A and phthalate metabolites were calculated based on the procedure manual on the Chemical Analyses by the Ministry of Environment of Japan (Ministry of the Environment 2009).

All necessary precautions were taken to avoid contamination with BPA and phthalate during sample preparation. All possible external sources of contamination were eliminated using the baked glassware for sample storage. Additionally, background levels were measured and confirmed that the influences of external contamination were null.

MEHP and MECPP were combined and expressed as the summation of DEHP metabolites (ΣDEHPm). MEHHP was also a DEHP metabolite; however, in this study population, the detection rate was low, and thus, it was not included in the summation of DEHP metabolites. To combine the

metabolites, the summation of each metabolite expressed in molar concentration was multiplied with their respective parent molecular weight (MW) as follows: Σ DEHPm = $((C_{MEHP}/MW_{MEHP})+(C_{MECPP}/MW_{MECPP})) \times MW_{DEHP}$

where C is the measured concentration (ng/ml) and MW is the molecular weight (ng/nmol) (Hart et al. 2014).

Measurements of metabolic-related biomarkers

Cord blood samples were obtained during delivery and stored at −80 °C until the analysis. The total and high-molecular-weight (HMW) adiponectin and leptin, TNF-α, and IL-6 levels in the cord blood were measured. HMW adiponectin has large multimers of 12–18 subunits of oligomeric complexes of adiponectin. Both total and HMW adiponectin levels were determined by enzyme-linked immunosorbent assay using the Human Adiponectin Assay kit (Sekisui Medical Co. Ltd., Tokyo, Japan). Leptin levels were determined by radioimmunoassay (RIA) using Human Leptin RIA kit (Linco Research Inc., St. Charles, MO, USA). TNF-α and IL-6 levels were determined by chemiluminescent enzyme immunoassay (CLEIA) using Quanti Glo Human TNF-α Chemiluminescent Immunoassay 2nd generation kit (R&D systems, Minneapolis, MN, USA). All analyses were conducted based on the operation manual in LSI Medience (Tokyo, Japan). The limit of detections (LODs) were 0.39 μg/ml for adiponectin, 0.5 ng/ml for leptin, 0.55 ng/ml for TNF-α, and 0.3 ng/ml for IL-6. Intra- and inter-assay CVs were 7.6–9.1% and 7.8–10.1% for total

adiponectin, 6.0–9.2% and 6.8–11.6% for HMW adiponectin, 2.8–5.3% and 6.3–8.1% for leptin, 3.2–3.6% and 5.6–6.2% for TNF-α, and 1.5–7.4% and 3.5–10.5% for IL-6, respectively.

Covariates

Baseline information such a medical history, lifestyle, and socioeconomic status were obtained from questionnaires, which were filled out by pregnant women during the first trimester. Potential confounders considered based on the previous literature were maternal age (continuous variable), maternal pre-pregnancy body mass index (BMI) (continuous variable), parity (nulliparous, parous), maternal education (≦12 years, ≧13 years), and annual family income (≧5 M, <5 M) (Ashley-Martin et al. 2014). Alcohol drinking and smoking during pregnancy were also considered as possible confounders; however, these variables were not correlated with fetal metabolic-related biomarker levels or did not change the beta values in the statistical models and thus were not included as covariates (Table S1).

Statistical analysis

Maternal serum bisphenol A and phthalate metabolite levels and cord blood metabolic-related biomarker levels were used as continuous variables for statistical analyses. As bisphenol A and phthalate metabolite levels were not normally distributed, these levels were $\log_{10}$ transformed for the statistical analyses. Similarly, cord blood metabolic-related biomarker levels were not normally distributed; these values were $\log_{10}$ transformed. Values below the LODs or

MDLs were replaced with half of the values of LODs or MDLs. Linear regression analyses adjusted for maternal age, maternal pre-pregnancy BMI, parity, maternal education, and sex of the child were conducted to investigate the association between maternal bisphenol A and phthalate metabolite levels (dependent variables), and cord blood metabolic-related biomarker levels (independent variables). Three phthalates metabolites (MBzP, MEHHP, and cx-MiNP) were not examined in the linear regression analyses due to the low detection rate (MBzP = 7.1%, MEHHP = 1.7%, and cx-MiNP = 0.0%). Statistical analyses were performed using the SPSS 22.0J (IBM Japan, Tokyo, Japan). P-value of <0.05 was considered statistically significant.

Results

Table 1 presents the characteristics of the participants and fetal metabolic-related biomarker levels. The mean (±SD) maternal age and pre-pregnancy BMI were 31.9 ± 4.4 years, and 20.8 ± 2.7 kg/m^2. Approximately 12.1% and 5.2% of pregnant women reported to have drank alcohol and smoked during pregnancy, respectively. The mean (±SD) of gestational age, birth weight, and length were 275 ± 8 days, 3040 ± 352 g, and 48.9 ± 1.9 cm, respectively. Girls showed significantly higher levels of total (p = 0.002) and HMW adiponectin (p = 0.001) and leptin (p = 0.002) levels in the cord blood compared to boys (data not shown).

Table 2 shows the distributions of maternal bisphenol A and phthalate metabolite levels.

Sample size varied from 346 to 356 due to available blood sample volume. Bisphenol A was detected from nearly all the participants (99.1%). MnBP and MiBP were detected from all the participants. MEHP and MEHHP, the primary and secondary metabolite of di-(2-ethylhexyl) phthalate (DEHP), were detected from 97.2% and 87.1% of the participants.

Table 3 shows the cord blood metabolic-related biomarker levels associated with maternal bisphenol A and phthalate metabolite levels, stratified by sex of the child. Overall, bisphenol A level was negatively associated with leptin level (β =-0.06, 95% confidence interval (CI): -0.11, -0.01). Bisphenol A level was positively associated with HMW adiponectin level with marginal significance (β =0.03, 95%CI: 0.00, 0.06).

MiBP and MnBP levels were inversely associated with leptin level (β =-0.14, 95%CI: -0.27, -0.01 and β =-0.12, 95%CI: -0.24, 0.00 with marginal significance, respectively). MEHP level was also inversely associated with leptin level (β =-0.08, 95%CI: -0.14, -0.03). The association between ΣDEHPm (summation of MEHP and MECPP to represent DEHP exposure) and metabolic-related biomarkers was also investigated. ΣDEHPm level was inversely associated with leptin levels (β =-0.09, 95%CI: -0.16, -0.03). Any prenatal exposure and IL-6 level were insignificantly associated.

Even though some of the statistical significance were only found in either boys or girls, prenatal exposures to bisphenol A and phthalates on metabolic-related biomarkers of cord blood levels were essentially insignificant. In addition, the interaction effects of bisphenol A and phthalate

metabolites were tested and showed no such effects.

Discussion

In this study, cord blood metabolic-related biomarker levels were examined in association with bisphenol A and phthalate levels of first trimester maternal blood. We observed that bisphenol A, MiBP, and MEHP levels were inversely associated with leptin level.

The cord blood biomarker levels in this study were comparable to the previous studies among Asian population (Chou et al. 2011, Hui et al. 2016, Huang et al. 2017). However, the leptin levels were lower compared to the values reported by Ashley-Martin et al. (Ashley-Martin et al. 2014).

Compared to the previous studies that measured maternal blood levels of bisphenol A (Lee et al. 2008, Kosarac et al. 2012, Aris 2014), maternal bisphenol A levels in this study were relatively lower. Despite the higher detection rate, maternal exposure to bisphenol A was estimated to be not as high as previously reported population. The MEHP levels were comparable that of the previous reports on the median serum MEHP levels of US adults (Silva et al. 2004), Swedish elderly population (Olsen et al. 2012), young Danish (Frederiksen et al. 2011), and Australian pregnant women (Hart et al. 2014),. Thus, exposure to DEHP in this study population was similar to the levels in the previous studies.

Two of the previous studies reported that increased maternal bisphenol A levels were associated with lower adiponectin, particularly among boys (Chou et al. 2011, Ashley-Martin et al. 2014). One study found a positive association between maternal bisphenol A levels and cord leptin (Chou et al. 2011). Our findings were inconsistent with these previous studies. Chou et al. measured bisphenol A levels in maternal blood during delivery, whereas the first-trimester maternal blood was used in this study. Their geometric mean was 2.5 ng/ml, which was considerably higher than this study population. Ashley-Martin et al. used the first-trimester urine samples, while we used the maternal blood. This inconsistency can be due to the differences in bisphenol A levels, bio-samples used for assessment, and timing of exposure. Recently, the cord blood TNF-α and IL-6 levels and third-trimester maternal urine bisphenol A were investigated and insignificantly associated (Huang et al. 2017). Our finding was consistent with the finding from this study. However, epidemiological studies investigating this association were insufficient; thus, further studies are required to elucidate the relationship between bisphenol A and these biomarkers.

Two prospective studies investigated the prenatal phthalate exposures and cord biomarkers. Ashley-Martin et al. showed that increased odds of high leptin were associated with mono-(3-carboxypropyl) phthalate among males (Ashley-Martin et al. 2014). Our study did not measure the MCPP levels, and thus findings from their study were incomparable. We found that DBP and DEHP metabolite levels were negatively associated with cord leptin levels, whereas they

found that DOP metabolite and leptin levels were positively associated. This may be interpreted that the association with leptin level was metabolite specific. Another study reported that maternal third-trimester blood MEHP level was positively associated with cord blood adiponectin levels in boys and was negatively associated with leptin levels in girls (Minatoya et al. 2017). The present study confirmed the findings of MEHP levels and decreased leptin levels found in the previous study.

Adverse birth outcomes such as small for gestational age (SGA) and preterm birth associated with lower cord blood leptin and adiponectin levels (Romano et al. 2014, Yeung et al. 2015) have been reported. Cord adipokines are not only related to birth outcomes but also can predict early childhood body size and adiposity according to the previous prospective studies (Mantzoros et al. 2009, Karakosta et al. 2016).

The mechanisms in which prenatal bisphenol A and phthalate exposures may disrupt metabolic-related biomarkers of neonates are still uncertain. One possible mechanism is via peroxisome proliferator-activated receptors (PPARs). Both animal and in vitro studies have demonstrated that bisphenol A and phthalates can alter PPAR-α and PPAR-γ (Hurst and Waxman 2003). Additionally, PPAR activity was reported to be different by sex (Jalouli et al. 2003), which may potentially explain some of the significant association found only in certain sex in this study.

Increased HMW adiponectin levels in the cord blood should be cautiously interpreted.

Increased adiponectin levels in adult studies normally indicate better health outcomes; however, in the cord blood, increased adiponectin may have different indications. For example, the recent study found that the cord blood adiponectin concentration was considerably higher in the gestational diabetes mellitus (GDM) group compared to non GDM group (Aramesh et al. 2017). This suggested that increased levels of cord adiponectin may possibly be an indicator of adverse health outcome. Additionally, one previous literature investigated the trajectory of adipokine levels from birth to school age and reported that some evidence of certain adiponectin trajectories may exist (Volberg et al. 2013).

Leptin can also affect the central nervous system with subsequent alternations in psychological functions (Stieg et al. 2015), and low leptin levels have been associated with increased risk of developing dementia (Stieg et al. 2015). Decreased leptin at birth may remain low and may potentially influence the adverse outcomes on neuro- and psycho-development. Longitudinal studies should be conducted to help understand the connection between changes in the cord blood adiponectin and leptin levels and childhood anthropometric and neurobehavioral development.

The limitations of this study should be discussed. First, our investigation relied on the one-time measurement of exposures at the first trimester of pregnancy. This may not represent the critical window of fetal adipocyte development. We assumed that prenatal exposure levels were

based on this one-time measurement, and thus bisphenol A and phthalates can be misclassified due to their short half-lives. Variability of urinary phthalate metabolite and bisphenol A concentrations before and during pregnancy was examined by Braun et al. (Braun et al. 2012). The magnitude of variability was biomarker specific, and a single spot urine sample may reasonably classify MEP and MBP concentrations during pregnancy; however, more than one sample may be necessary for MBzP, DEHP, and bisphenol A (Braun et al. 2012). This variability may have caused misclassification.

Second, the disadvantages of using blood samples for exposure assessment were observed. We should note that measurable levels are much higher in the urine compared to blood samples for bisphenol A and phthalate metabolites. Besides, urinary measurements of bisphenol A are considered to be more robust to contamination than plasma or serum (Koch et al. 2012). However, according to the NIH Round Robin study, bisphenol A concentration in blood samples can be accurately assayed without contamination (Vandenberg et al. 2014). The Round Robin study concluded that bisphenol A contamination can be controlled during sample collection and inadvertent hydrolysis of bisphenol A conjugates can be avoided during sample handling. In the present study, baked glassware was used for sample storage and glass cartridge instead of polypropylene was used to avoid external contamination; thus, we consider that external contamination was well controlled. For phthalate metabolites, many researchers advocate the use

of urine samples for phthalate exposure assessment. However, in this study, urine samples were not available. Hydrolytic enzymes are present in blood samples and may be responsible for diester to monoester conversion after drawing the blood sample (Hines et al. 2009). The speed depending on the length of alkyl chain and longer alkyl chains such as DEHP takes significantly longer to convert (Kato et al. 2003). The best way to inhibit enzyme activity is to add acid immediately after the sample collection. However, in this study, the blood samples were stored without adding acid. To avoid the influence of enzyme activity, using secondary metabolites of phthalates was recommended. In the present study, secondary metabolites of DEHP were measured. Additionally, contamination during the process of blood collection and storage was likely minor in this study based on relatively low levels of observed primary metabolites, which were consistent with the range in the previous report (Hart et al. 2014).

Third, the population included in the present study was limited to those with preschool-age children, who responded to the follow-up questionnaire, and had first-trimester maternal blood and cord blood samples available. This may potentially result in a selection bias. The basic characteristics such as maternal age, pre-pregnancy BMI, and parity were comparable between the population in the follow-up study (n=5,600) and the present study population (n=365). The percentage of smokers during pregnancy was low, and the annual family income and maternal education level were slightly higher in the present study population, as compared to the general

population; this indicates that the present study population might be wealthier than the general population (Table S3). However, these factors were not correlated with cord blood biomarkers (Table S1); therefore, the slight difference unlikely affected our results. Smoking during pregnancy and annual family income were positively correlated with MnBP and MiBP, respectively (Table S2). Lower percentage of smokers in the present study may possibly contribute to lower MnBP levels and may underestimate the effects of MnBP exposure.

Lastly, exposure to other environmental chemicals, such as POPs and heavy metals and air pollution during pregnancy, was not considered in the present study. However, some previous literatures indicated associations with metabolic-related biomarkers (Ashley-Martin et al. 2015, Lavigne et al. 2016, Ashley-Martin et al. 2017). Significant associations may be possibly identified due to the number of chemicals and biomarkers tested in this study. However, three out of four significant findings in this study would pass the Bonferroni correction for multiple testing. DEP, one of the most widely used phthalates, was not assessed in this study. However, MEP, a metabolite of DEP, is a frequently detected phthalate metabolite; therefore, we should explore the association between DEP exposure and metabolic-related biomarkers in the future work.

Conclusion

This study provided some evidence that prenatal exposure to bisphenol A and certain

variants of phthalates may modify fetal adiponectin and leptin levels. Further investigation to elucidate the association between prenatal exposure to environmental chemicals and long-term metabolic-related outcomes is warranted.

Acknowledgments

We thank all the study participants and staff at the Hospitals.

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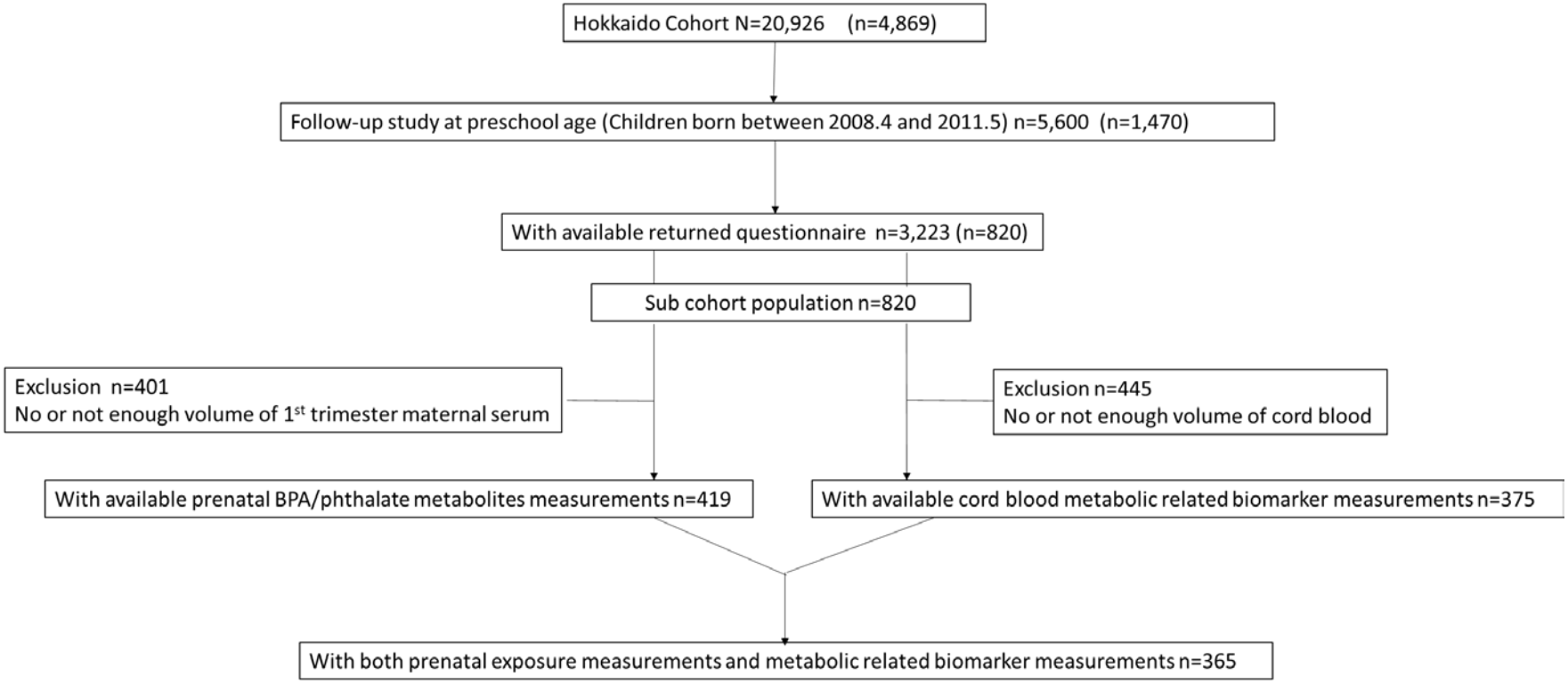

Figure 1. Flowchart of selecting study population.

Numbers in parentheses are the number of sub-cohort population.

Sub-cohort population; The sub-cohort population was randomly selected from whole cohort population to represent the whole cohort population and was designated for exposure and/or biomarker assessments.

Table 1 Characteristics of participants.

		Mean ± SD, n (%), median (IQR)
Maternal		
Age (years)		31.9 ± 4.4
Pre-pregnancy BMI (kg/m^2)		20.8 ± 2.7
Parity	Nulliparous	148 (40.5)
	Parous	201 (55.1)
	Missing	16 (4.4)
Drinking alcohol during pregnancy	Yes	44 (12.1)
	No	313 (85.8)
	Missing	8 (2.2)
Smoking during pregnancy	Yes	19 (5.2)
	No	276 (75.6)
	Missing	70 (19.2)
Annual family income (JPY)	< 5M	187 (51.2)
	≧ 5M	132 (36.2)
	Missing	46 (12.6)
Education (years)	≦ 12	140 (38.4)
	≧ 13	221 (60.5)
	Missing	4 (1.1)
Child		
Sex	Girl	176 (48.2)
	Boy	189 (51.8)
Gestational age (days)		275 ± 8
Birth weight (g)		3040 ± 352
Birth length (cm)		48.9 ± 1.9
Total adiponectin (μ g/ml)		17.14 (12.75, 21.17)
HMW adiponectin (μ g/ml)		11.28 (8.07, 15.10)
Leptin (ng/ml))		4.8 (3.1, 8.1)
TNF-α (pg/ml)		2.46 (1.93, 3.28)
IL-6 (pg/ml)		1.04 (0.61, 2.46)

SD: standard deviation, IQR: inter quartile range, BMI: body mass index, JPY: Japanese yen, HMW: high-molecular-weight, TNF-a: tumor necrosis factor alpha, IL-6: interleukin 6

Table 2 Bisphenol A and phthalate metabolite levels in maternal blood.

Chemical	n	MDL (ng/ml)	Detection rate (%)	Median (IQR) (ng/ml)
Bisphenol A	346	0.011	99.1	0.060 (0.023, 0.250)
MnBP	336	0.57	100	26.0 (17.0, 37.0)
MiBP	336	0.44	100	6.95 (4.60, 9.58)
MBzP	336	0.19	7.1	< MDL (<MDL, <MDL)
MEHP	356	0.31	97.2	1.50 (0.82, 9.35)
MEHHP	356	0.23	1.7	< MDL (< MDL, < MDL)
MECPP	356	0.11	87.1	0.22 (0.13, 0.32)
cx-MiNP	356	0.12	0.0	< MDL (< MDL, < MDL)

MDL: method detection limit, IQR: inter quartile range

Table 3 Cord blood metabolic related biomarker levels in association with maternal Bisphenol A and phthalate metabolites levels.

		β (95% CI)				
		Total adiponectin	HMW adiponectin	Leptin	TNF-α	IL-6
Bisphenol A	All[a)]	0.02 (0.00, 0.42)	0.03^{+} (0.00, 0.06)	-0.06* (-0.11, -0.01)	0.00 (-0.03, 0.05)	0.00 (-0.12, 0.12)
	Girls[b)]	0.01 (-0.02, 0.03)	0.00 (-0.04, 0.04)	-0.10* (-0.17, -0.03)	0.00 (-0.06, 0.06)	-0.12 (-0.28, 0.05)
	Boys[b)]	0.03^{+} (0.00, 0.07)	0.05* (0.01, 0.10)	-0.04 (-0.10, 0.03)	0.01 (-0.04, 0.06)	0.10 (-0.06, 0.27)
MnBP	All[a)]	-0.06^{+} (-0.12, 0.00)	-0.07 (-0.15, 0.01)	-0.12^{+} (-0.24, 0.00)	0.06 (-0.04, 0.1)	0.10 (-0.21, 0.42)
	Girls[b)]	-0.02 (-0.10, 0.06)	-0.06 (-0.17, 0.05)	-0.13 (-0.31, 0.06)	0.08 (-0.07, 0.24)	0.14 (-0.32, 0.60)
	Boys[b)]	-0.09^{+} (-0.18, 0.00)	-0.08 (-0.20, 0.04)	-0.12 (-0.28, 0.04)	0.04 (-0.11, 0.19)	0.02 (-0.43, 0.47)
MiBP	All[a)]	-0.05 (-0.11, 0.02)	-0.06 (-0.15, 0.03)	-0.14* (-0.27, -0.01)	0.03 (-0.09, 0.14)	0.02 (-0.33, 0.36)
	Girls[b)]	-0.01 (-0.11, 0.09)	-0.06 (-0.19, 0.07)	-0.15 (-0.37, 0.06)	0.06 (-0.12, 0.24)	0.23 (-0.31, 0.77)
	Boys[b)]	-0.06 (-0.16, 0.03)	-0.05 (-0.18, 0.07)	-0.13 (-0.30, 0.03)	0.00 (-0.15, 0.16)	-0.17 (-0.64, 0.29)
MEHP	All[a)]	-0.01 (-0.04, 0.02)	-0.01 (-0.05, 0.03)	-0.08*(-0.14, -0.03)	0.04 (-0.01, 0.09)	0.02 (-0.13, 0.17)
	Girls[b)]	-0.03 (-0.06, 0.01)	-0.04^{+} (-0.10, 0.01)	-0.11* (-0.19, -0.02)	0.01 (-0.06, 0.08)	0.04 (-0.18, 0.26)
	Boys[b)]	0.01(-0.04, 0.05)	0.02 (-0.04, 0.07)	-0.06 (-0.14, 0.02)	0.08* (0.01, 0.15)	0.00 (-0.21, 0.22)
MECPP	All[a)]	-0.04 (-0.09, 0.02)	-0.03 (-0.10, 0.05)	-0.03(-0.14, 0.08)	0.00 (-0.09, 0.10)	-0.05 (-0.33, 0.23)
	Girls[b)]	-0.05 (-0.11, 0.01)	-0.07 (-0.15, 0.02)	-0.05 (-0.19, 0.10)	-0.04 (-0.16, 0.08)	-0.06 (-0.42, 0.30)
	Boys[b)]	-0.01 (-0.10, 0.08)	0.04 (-0.08, 0.16)	0.02 (-0.14, 0.18)	0.05 (-0.09, 0.20)	-0.07 (-0.52, 0.38)
Σ DEHPm	All[a)]	-0.01 (-0.06, 0.03)	-0.01 (-0.04, 0.02)	-0.09* (0.15, -0.03)	0.04 (-0.01, 0.10)	0.00 (-0.16, 0.17)
	Girls[b)]	-0.03 (-0.07, 0.01)	-0.05^{+} (-0.10, 0.01)	-0.12* (-0.21, -0.03)	0.00 (-0.07, 0.08)	0.03 (-0.20, 0.26)
	Boys[b)]	0.01 (-0.04, 0.06)	0.02 (-0.05, 0.08)	-0.07 (-0.15, 0.02)	0.08* (0.01, 0.16)	-0.01 (-0.24, 0.23)

[a)] Adjusted for maternal age, maternal pre-pregnancy BMI, parity, maternal education and child sex.

[b] Adjusted for maternal age, maternal pre-pregnancy BMI, parity and maternal education.

[*] $p < 0.05$, [+] $p < 0.10$

Σ DEHPm: Summation of MEHP and MECPP corrected for molecular weight, CI: confidence interval, HMW: high-molecular-weight, TNF-a: tumor necrosis factor alpha, IL-6: interleukin 6