学位論文

Human biomonitoring of phthalates and their effect on respiratory and allergic symptoms in Japanese children

（フタル酸エステル類のヒトバイオモニタリングおよび呼吸器・アレルギー症状との関連）

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I. Abstract

Phthalates are ubiquitous environmental contaminants used in building materials as polyvinyl chloride materials, in food packing, toys, personal care products, etc. Previous studies have reported phthalates association with adverse health effects including respiratory and allergic symptoms. Moreover, children are more vulnerable than adults to phthalates exposure and health effect mainly due to children's still developing organs and age-related behaviors. Previous epidemiological studies focused on examining individual phthalates exposure effect on respiratory and allergic symptoms. However, this approach has limitations as humans are exposed to several phthalates in their daily lives.

Thus, in this thesis, study 1 investigated the secular trend of five parent phthalates is using their metabolites in children aged 7 years old. In the study 2, associations of individual and mixture of phthalate metabolites with wheeze, rhino-conjunctivitis, and eczema symptoms in children and the hypothesis of mediating effect of oxidative stress in the association between phthalates and symptoms are examined.

From the ongoing Hokkaido birth cohort study, 400 first-morning spot urine samples collected from 2012 to 2017 of 7 years old children were included in this study. The ISAAC questionnaire completed by parents or guardians of the children was used to investigate demographic and allergic symptoms (wheeze, rhino-conjunctivitis, and eczema). (i) Ten urinary phthalate metabolites: MiBP, MnBP, MBzP, MEHP, MEOHP, MEHHP, MECPP, MINP, OH-MINP, and cx-MINP concentrations were measured by UPLC-MS/MS, (ii) the levels of three oxidative stress biomarkers, 8-OHdG, HEL, and HNE were measured, (iii)

statistical analysis, study 1: multivariable regression model was performed to assess the secular trend of metabolites from 2012-2017 and the association between phthalate metabolites and building characteristics. Study 2: Weighted quantile sum (WQS) and Bayesian kernel machine regression (BKMR) models were used to investigate the association of individual and mixture effects of phthalate metabolites with health outcomes. Baron and Kenny's regression approach was used to investigate the possible mediating effect of oxidative stress biomarkers on the association between phthalate exposure and health outcomes.

Study 1 results and discussion

All phthalate metabolites were detected in more than 96% of the children's urine, indicating high detection frequency. High detection was observed in MECPP, MnBP and MEHHP with median concentrations of 37.4 ng/mL, 36.8 ng/mL and 25.8 ng/mL, respectively. Comparing phthalate metabolites levels in similar aged children with other countries, DEHP metabolites (MEHP, MEOHP, MEHHP, MECPP) were higher in Japanese children than children in the USA and Germany. On contrary, metabolites MiBP, MnBP, MBzP, MINP, OH-MINP and cx-MINP were comparable or lower. A stable trend of phthalate metabolites was observed in children between 2012 and 2017. This stable or no change in phthalates exposure is despite the Japan's revised 2010 phthalates regulation which is only enforced to children and food products. Thus, the stable trend could be an indication of phthalates exposure from non-regulated products such as PVC floor/wall materials, and personal care products such as lotions, shampoo. Considering personal and building

characteristics; elevated levels of ∑DINP in children from low-income households, MnBP from those living in old buildings, and MiBP, MnBP, and ∑DEHP from those with window opening habits of ≥1 h.

Study 2 results and discussion

The individual linear regression analysis showed MECPP (OR = 1.41, 95% CI: 1.02–1.97) and cx-MINP (OR = 1.40, 95% CI: 1.07–1.86) were associated with wheeze. In the mixture analysis the WQS index had a significant association (OR = 1.46, 95% CI: 1.09–1.96) with wheeze and (OR = 1.40, 95% CI: 1.07–1.82) with eczema for which MINP and MEOHP, respectively, were the most highly weighted metabolites. Considering BKMR, DINP metabolites showed the highest group posterior inclusion probability (PIP). Among DINP metabolites, MINP in wheeze, cx-MINP in rhino-conjunctivitis and OH-MINP in eczema showed the highest conditional PIPs. The overall effect of phthalate metabolites mixture showed increasing association with eczema. These findings showed the association of individual and phthalate metabolites mixture with wheeze and eczema. More importantly, metabolites of DINP followed by DEHP are the main contributors to the associations. No mediation of oxidative stress in the association between phthalates and symptoms was observed, this could be attributed to low oxidative stress. Yet, as this study's participants are only 7 years old children, the result may change when they get older.

In conclusion, this study revealed a stable trend of phthalate exposure from 2012 to 2017. The health assessment from different models emphasizes individual metabolites MECPP and cx-MINP and mixture effect of DEHP and DINP metabolites are identified as the primary contributors to wheeze and eczema. In the future, trend biomonitoring of phthalates is important to surveil exposure level in different general population. More importantly, phthalates exposure is associated with respiratory and allergic symptoms thus reduction of exposure level in children is warranted.

II. Author's publications and conference presentations

Lists of publication based on this dissertation

1. Rahel Mesfin Ketema, Yu Ait Bamai, Atsuko Ikeda Araki, Takeshi Saito, Reiko Kishi. Secular trends of urinary phthalate metabolites in 7-year-old children and association with building characteristics: Hokkaido study on environment and children's health. International Journal of Hygiene and Environmental Health 234:113724 (2021). doi: 10.1016/j.ijheh.2021.113724.

2. Rahel Mesfin Ketema, Yu Ait Bamai, Chihiro Miyashita, Takeshi Saito, Reiko Kishi, Atsuko Ikeda-Araki. Phthalates mixture on allergies and oxidative stress biomarkers among children: The Hokkaido study. Environment International. 2022 Jan 17; 160:107083. doi: 10.1016/j.envint.2022.107083. Epub ahead of print. PMID: 35051840.

Lists of other publications

1. Reiko Kishi, Atsuko Ikeda-Araki, Chihiro Miyashita, Sachiko Itoh, Sumitaka Kobayashi, Yu Ait Bamai, Keiko Yamazaki, Naomi Tamura, Machiko Minatoya, Rahel Mesfin Ketema, Kritika Poudel, Ryu Miura, Hideyuki Masuda, Mariko Itoh, Takeshi Yamaguchi, Hisanori Fukunaga, Kumiko Ito, Houman Goudarzi & the members of The Hokkaido Study on Environment and Children's Health. Hokkaido birth cohort study on environment and children's health: cohort profile 2021. Environmental Health and Preventive Medicine 26(1):59 (2021). doi: 10.1186/s12199-021-00980-y.

2. Rahel Mesfin Ketema, Atsuko Araki, Yu Ait Bamai, Takeshi Saito, Reiko Kishi. Lifestyle behaviors and home and school environment in association with sick building syndrome among elementary school children: a cross-sectional study. Environmental Health and Preventive Medicine 25, 28 (2020). doi.org/10.1186/s12199-020-00869-2.

3. Shojiro Yamasaki, Tomomi Tomihara, Goh Kimura, Yukako Ueno, Rahel Mesfin Ketema, Shin Sato, Yuuka Mukai, Sikder Tajuddin, Masaaki Kurasaki, Toshiyuki Hosokawa, Takeshi Saito. Long-term effects of maternal resveratrol intake during lactation on cholesterol metabolism in male rat offspring. International journal of food sciences and nutrition. 71(2):226-234 (2020). doi: 10.1080/09637486.2019.1639638.

4. Tomomi Kita-Tomihara, Shin Sato, Shojiro Yamasaki, Yukako Ueno, Goh Kimura, Rahel Mesfin Ketema, Tae Kawahara, Masaaki Kurasaki & Takeshi Saito. Polyphenol-enriched azuki bean (Vina angularis) extract reduces the oxidative stress and prevents DNA oxidation in the hearts of streptozotocin-induced early diabetic rats. International Journal of Food Sciences and Nutrition. 70(7):845-855 (2019). doi: 10.1080/09637486.2019.1576598.

5. Reiko Kishi, Rahel Mesfin Ketema, Yu Ait Bamai, Atsuko Araki, Toshio Kawai, Tazuru Tsuboi, Ikue Saito, Eiji Yoshioka, Takeshi Saito. Indoor Environmental Pollutants and Their Association with Sick House Syndrome among Adults and

Children in Elementary School. Building and Environment, Volume 136, 15 May 2018, Pages 293-301.

6. Atsuko Araki, Michiel Bastiaensen, Yu Ait Bamai, Nele Van den Eede, Toshio Kawai, Tazuru Tsuboi, Rahel Mesfin Ketema, Adrian Covaci, Reiko Kishi. Association between allergic symptoms and phosphate flame retardants in dust and their urinary metabolites among school children. Environment International. 119:438-446. doi: 10.1016/j.envint.2018.07.018.

7. 荒木敦子、アイツバマイゆふ、ラヘル　メスフィン　ケテマ、岸玲子「室内環境中のハウスダストによる健康影響」日本衛生学雑誌.73(2):130-137. doi: 10.1265/jjh.73.130. 2018.

Book Chapter

1. Atsuko Araki, Rahel Mesfin Ketema, Yu Ait Bamai, and Reiko Kishi. In book part III. Factors Determining Indoor Air Qualities and Their Health Impacts. Aldehydes, Volatile Organic Compounds (VOCs), and Health. Kishi, Reiko, Norbäck, Dan, Araki, Atsuko (Eds.) Indoor Environmental Quality and Health Risk toward Healthier Environment for All. Springer 2019.

Domestic and international academic presentations

1. Rahel Mesfin Ketema, Yu Ait Bamai, Atsuko Ikeda Araki, Takeshi Saito, Reiko Kishi. Association of phthalates exposure and oxidative stress biomarkers with wheeze, rhino-conjunctivitis and eczema in children. International Society of Exposure Science. (Virtual Poster). August 30 - September 2. 2021.

2. Rahel Mesfin Ketema1, Yu Ait Bamai, Takeshi Saito, Reiko Kishi, and Atsuko Ikeda-Araki. Mixture effect of phthalate metabolites in children's wheeze, rhino-conjunctivitis and eczema: The Hokkaido study. (Virtual poster). The Fifth FHS International Conference. September 17-18. 2021.

3. Rahel Mesfin Ketema, Yu Ait Bamai, Atsuko Araki, Takeshi Saito, Reiko Kishi. Biomonitoring of Phthalate Metabolites in Children: The Hokkaido Study. (Hybrid oral). The 8th SaSSOH. Hokkaido University,Japan. September 16,2020.

4. Rahel Mesfin Ketema, Yu Ait Bamai, Atsuko Araki, Takeshi Saito, Reiko Kishi. Changing trends in urinary phthalate metabolites in elementary school children; 2012-2017. Poster. 32nd Annual conference of the International Society for Environmental Epidemiology. USA. Virtual poster. August 24-27,2020.

5. Rahel Mesfin Ketema, Yu Ait Bamai, Atsuko Araki, Takeshi Saito, Reiko Kishi. Urinary phthalate metabolites and oxidative stress biomarkers in 7 years old children from Hokkaido. (Poster) The Fourth FHS International Conference. July 5,2019, Sapporo, Japan.

III. Lists of abbreviations

8-OHdG: 8-hydroxy-2-deoxyguanosine

BBzP: butyl benzyl phthalate

BKMR: Bayesian kernel machine regression

BMI: body mass index

CE: creatinine excretion

CSH: charged surface hybrid

CV: coefficient of variation

cx-MINP: mono-carboxy-isononyl phthalate

DBP: dibutyl phthalate

DEP: diethyl phthalate

DEHP: di(2-ethylhexyl) phthalate

DEHTP: di(2-ethylhexyl) terephthalate

DIDP: di-iso-decyl phthalate

DINCH: cyclohexane-1,2-dicarboxylic acid-diisononyl ester

DINP: di-isononyl-phthalate

DnOP: di-octyl phthalate

EDCs: endocrine disrupting chemicals

ETS: Environmental tobacco smoke

G-EQUAS: German external quality assessment scheme

HEL: hexanoyl lysine

HMW: high molecular weight

HNE: 4-hydroxynonenal

HBM: Human biomonitoring

ISAAC: International study on asthma and allergies in childhood

LMW: low molecular weight

LOD: limits of detection

LOQ: limits of quantification

MBzP: mono-benzyl phthalate

MDA: malonaldehyde

MECPP: mono (2-ethyl-5-carboxypentyl) phthalate

MEHHP: mono (2-ethyl-5-hydroxyhexyl) phthalate

MEHP: mono (2-ethylhexyl) phthalate

MEOHP: mono (2-ethyl-5-oxohexyl) phthalate

MHLW: Ministry of Health, Labor and Welfare

MiBP: mono-isobutyl phthalate

MINP: mono-isononyl phthalate

MnBP: mono-n-butyl phthalate

MRM: multiple-reaction monitoring

OH-MINP: mono-hydroxy-isononyl phthalate

PIP: Posterior inclusion probability

PVC: polyvinyl chloride

SD: standard deviation

SDGs: Sustainable Development Goals

UN: United Nations

UPLC-MS/MS: ultra-performance liquid chromatography tandem mass spectrometry

WHO: World Health Organization

WQS: Weighted quantile sum

IV. General Introduction

In our daily life we are surrounded by thousands of synthetic chemicals. This is owing to global industrial development and modern lifestyle of humans to increase the production and use of numerous chemicals. The initial objective of the production and invention of synthetic chemicals was to sustain and enhance properties of industrial production. These chemicals are used in numerous aspects of human life such as in our foods or agriculture, house/building materials, personal care products, etc. However, despite their intent to make our daily life better and convenient, certain chemicals may have an unforeseen negative impact on human health and the environment. Nearly 4.9 million deaths were attributed to environmental exposure to chemicals in 2004 (Pruss-Ustun et al., 2011). Global efforts to reduce death and illness due to environmental chemical exposure are included in the United Nations (UN) target of Sustainable Development Goals (SDGs): Target 3.9 "Reduce the number of deaths and illnesses from dangerous chemicals and air, water, and soil pollution and contamination" (un.org, 2021).

Among synthetic chemicals, phthalates are a group of industrial compounds that includes dialkyl-alkyl/aryl esters of 1,2-benzenedicarboxylic acid in their chemical structure. Phthalates are used in wide range of industrial and daily consumer products as plasticizer to produce polyvinyl chloride (PVC), and thus enhance flexibility, transparency, and durability of rigid polymers. They are widely used in children's toys, plastic packaging, medical tubing, pharmaceuticals, automotive parts, etc. Phthalates are also used as a solvent in products such as paints, cosmetics, perfumes, shampoos etc.

The specific characteristics and the decomposing pattern of the phthalates depend on the length of the dialkyl or alkyl/aryl side chain. The more branched the phthalates are, the more isomeric forms are available and the more hydrophobic the single compound is. Table 1 shows a list of diester phthalates and the most common metabolites, including their abbreviations and Figure. 1 shows the chemical structures of some of the most common phthalates.

Table 1. Phthalates and their metabolites

Phthalates		Metabolites	
Dimethyl phthalate	DMP	Monomethyl phthalate	MMP
Diethyl phthalate	DEP	Monoethyl phthalate	MEP
Di-n-butyl phthalate ★	DBP	Mono-n-butyl phthalate ★	MBP
Di-iso-butyl phthalate ★	DiBP	Mono-iso-butyl phthalate ★	MiBP
Butylbenzyl phthalate ★	BBzP	Monobenzyl phthalate ★	MBzP
Di(2-ethylhexyl) phthalate ★	DEHP	Mono(2-ethylhexyl) phthalate ★	MEHP
		Mono(2-ethyl-5-hydroxyhexyl) phthalate ★	MEHHP
		Mono(2-ethyl-5-oxohexyl) phthalate ★	MEOHP
		Mono(2-ethyl-5-carboxypentyl) phthalate ★	MECPP
		Mono(2-carboxy-hexyl) phthalate	MCMHP
Di-iso-nonyl phthalate ★	DINP	Mono-iso-nonyl phthalates ★	MiNP
		Mono(hydroxy-iso-nonyl) phthalate ★	OH-MiNP
		Mono(carboxy-iso-octyl) phthalate ★	cx-MiNP
		Mono(oxo-iso-nonyl) phthalate	oxo-MiNP
Di-n-octyl phthalate	DnOP	mono-n-octylphthalate	MnOP
Di-isodecyl phthalate	DIDP	monocarboxyisononyl phthalate	MCiNP
		monooxoisodecyl phthalate	MOiDP
		monohydroxyisodecyl phthalate	MHiDP

★ Targeted phthalates and their metabolites in this thesis

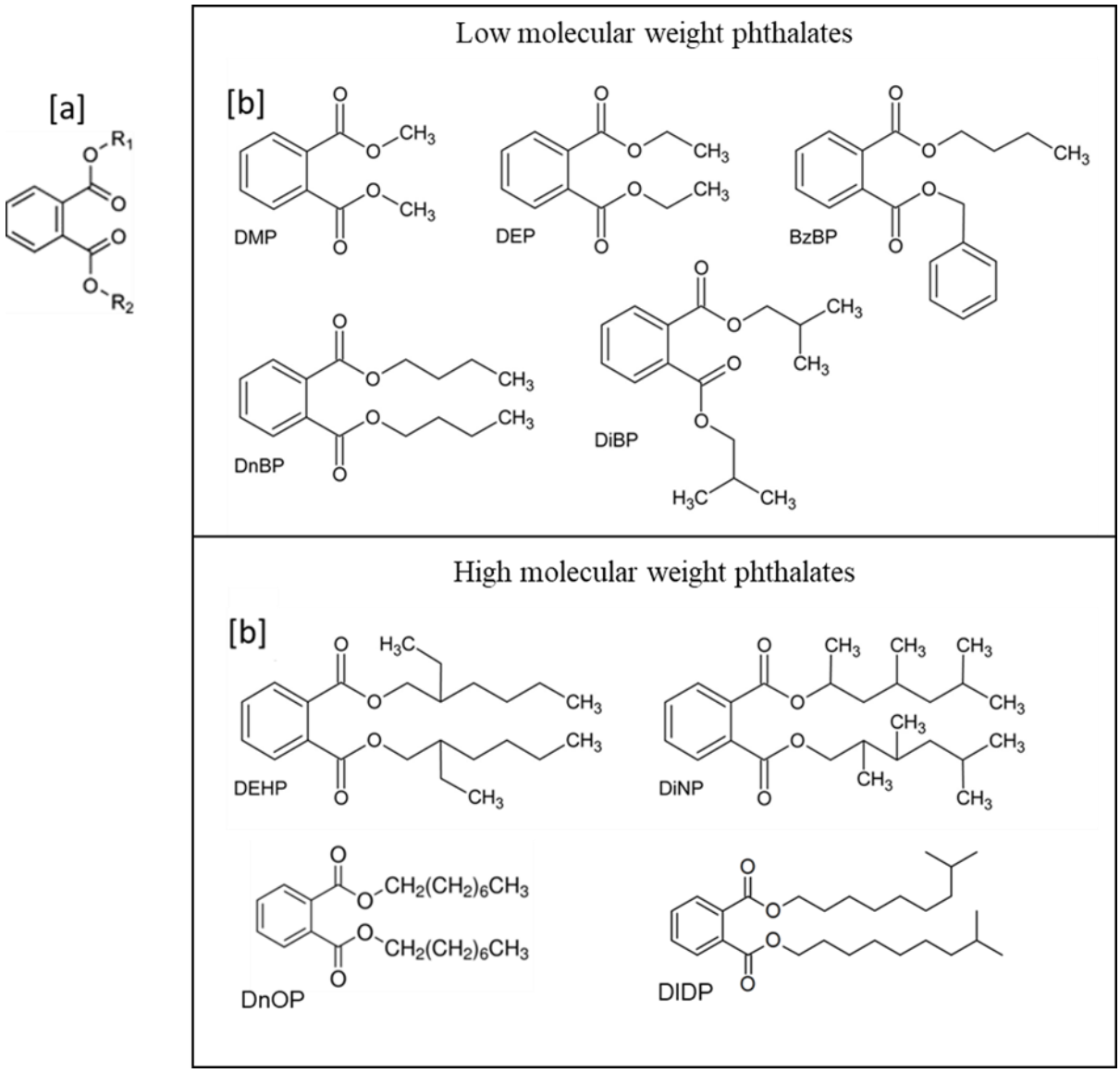

Figure 1. [a] General structure of phthalate (R_1 and R_2 = CnH2n+1; n = 4 – 15), [b] Chemical structures of phthalates

Due to modernization of human life as well as their cheap, versatile, and attractive manufacturing properties, phthalates dominate the plasticizer industry with 65% of global plasticizer consumption (CEH, 2018). Furthermore, phthalate production is forecasted to increase 1.3% annually from 4.9 million metric tons in 2017 to 5.2 million metric tons in 2022 (CEH, 2018). The application of phthalates in different products varies depending on the length of the carbon chain. Phthalates can be divided into low- and high- molecular weight phthalates based on their carbon chain. The high molecular weight (HMW) phthalates are commonly used as a plasticizer in building products such as PVC flooring or wall papers (Wormuth et al., 2006). The low molecular weight (LMW) phthalates are commonly used as a solvent in personal care products like shampoo, lotions, cosmetics, and perfumes (Wang et al., 2019; Wormuth et al., 2006). The LMW phthalates contain 3-6 carbon atom in their alkyl chain and includes diethyl phthalate (DEP), dibutyl phthalate (DBP), and butyl benzyl phthalate (BBzP). The HMW phthalates contain 7-13 carbon atoms in their alkyl chain and includes di(2-ethylhexyl) phthalate (DEHP), di-isononyl-phthalate (DINP), di-iso-decyl phthalate (DIDP), and di-octyl phthalate (DnOP).

Since phthalates do not have a covalent bond with the products, they can easily leach out of the products and diffuse into the air, dust, water, food, and soil etc (Wormuth et al., 2006; Heudorf et al., 2007). Thus, humans are exposed to them through inhalation, dermal contact, or ingestion. Although phthalates are useful industrial chemicals, used in several products, they are classified as endocrine disrupting chemicals (EDCs) (WHO, 2012). EDCs were defined by WHO in 2002: "An endocrine disruptor is an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub)populations". This is due to the fact that exposure to phthalates has been

reported in association with adverse health effects such as reproductive, neurodevelopment and respiratory diseases. Phthalates can interfere with hormonal balance by mimicking stimulation or inhibition of hormones (Diamanti-Kandarakis et al., 2009). After entering the human body, phthalates are transformed to their metabolites in at least two steps; a phase I hydrolysis followed by phase II conjugation (Fig. 2). In the first step, the diester phthalate is hydrolysed into the primary metabolite monoester phthalate in a step called detoxification and in the second step, conjugation takes place with phase II enzymes. However, studies have shown that diester phthalates become more bioactive when they are hydrolysed to monoester phthalates (Heindel et al., 1992).

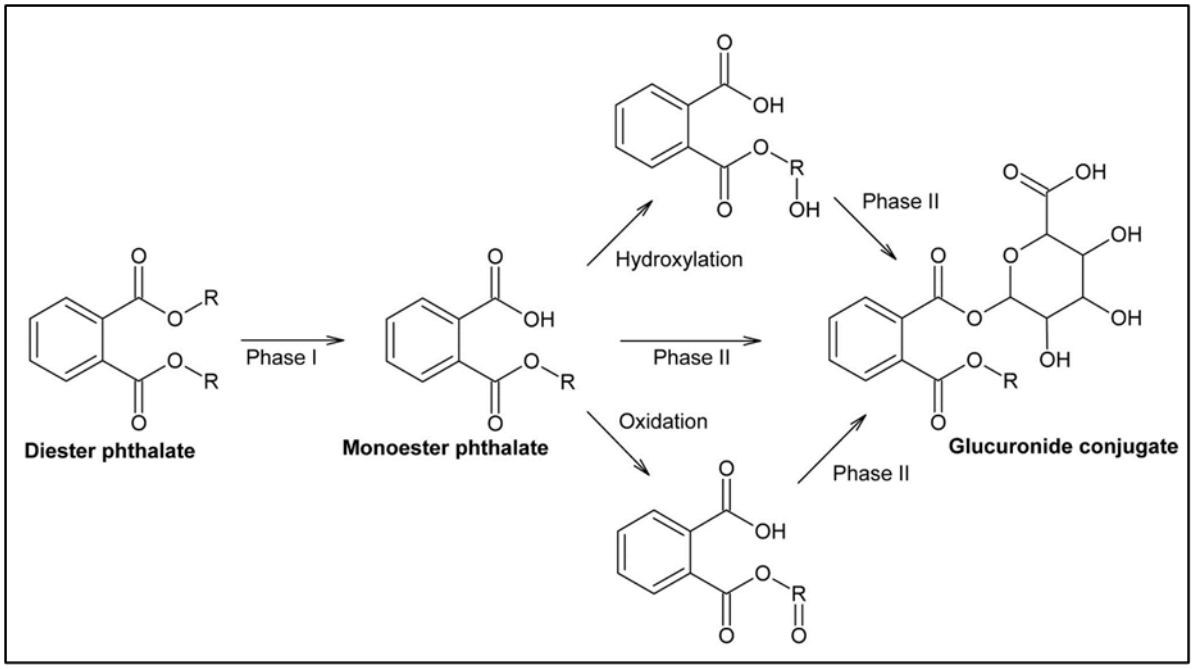

Figure 2. Metabolic pathways of phthalates

Phthalate metabolites can interact with the endocrine signaling system through mimicking, blocking, or interfering with hormones and resulting in adverse health effects. For instance, animal studies have reported exposure to phthalates can enhance the sensitization of respiratory and allergic responses (Guo, 2012). Although a clear mechanism on the association between phthalates exposure and allergic responses has not been established some *in vivo* and *in vitro* studies indicate there could be a mediation of oxidative stress (Dearman, 2008; Zhou, 2020). However, this hypothesis of mediating effect of oxidative stress in association between phthalates and respiratory and allergic response in human has not been explored well.

Health risks associated with exposure to phthalates, especially in susceptible population such as pregnant women and children, has been a global concern. Children are more vulnerable to exposure to environmental chemicals than adults. This is for a variety of reasons such as different body size, hand to mouth behaviors or daily activities (Hauptman M, Woolf AD et al., 2017). One previous study supported this type of vulnerability in children with a report on higher phthalates metabolites level in children than adults (Ait Bamai et al., 2015). Furthermore, children were more at risk to phthalate exposure-related asthma and allergies than adults (Ait Bamai et al., 2014).

Human biomonitoring (HBM) is essential for a comprehensive assessment of environmental chemicals such as phthalates. Accurate HBM is a useful tool to avoid bias when conducting epidemiological health-related risk assessments. Thus, it is important to use reliable analytical methods and biomarkers to determine the precise exposure level of chemicals such as phthalates in humans. After phthalates enter the human body, the body breaks them down into monoesters

called metabolites and excretes them in urine, and feces (Wang et al., 2015). Urine has been a commonly used matrix in biomonitoring phthalates exposure with their metabolites. This is due to phthalate's short half-life of 2-72 hours in humans and quick excretion in urine as monoester metabolites (Koch et al., 2012, 2003). Therefore, although the metabolites of phthalates can be detected in different bodily fluids such as plasma, saliva, amniotic fluid, breast milk, most epidemiological studies use urine as the preferable biomarker to determine the exposure of parent phthalates in human (Johns et al., 2015). This is partly due to the non-persistent characteristics of phthalates.

Given that phthalate regulation in certain products has been revised in Japan since 2010 (MHLW Notice No.370, 2010), changes in production and consumption may have an impact on exposure levels. Thus, subsequent phthalate exposure assessment is important. However, until now such trend analyses were not available in a Japanese population. Furthermore, previous epidemiological studies focused on the association between individual phthalate exposure and health outcomes such as respiratory and allergic symptoms. On contrary, humans are exposed to numerous chemicals simultaneously in their daily life indicating mixture exposure.

Therefore, this dissertation intends to address the following research questions/ hypothesis,

1. What is the status of consecutive years phthalates exposure level in Japanese children and explore whether personal and building factors could be related to the phthalate's exposure? Subsequent to the revised phthalates regulation, I hypothesis there could be phthalate exposure level change to the general population.
2. The key point of future epidemiological studies will be to look beyond the traditional individual chemical exposure to health outcome. Thus, I explore the individual and joint effect of phthalate mixture to respiratory and allergic symptoms?
3. *In vivo* and *in vitro* studies reported that phthalates can affect respiratory system via oxidative stress. However, only one human study investigated mediating effect of only 8-OHdG in association between phthalates and asthma, indicating lack of human epidemiological studies. Hence, in this research, I investigated mediating effect of oxidative stress using three markers in the association between phthalates and respiratory and allergic symptoms.

V. Objectives of this study

This study aims

In chapter one

a) To bio-monitor phthalates in Japanese children using consecutive urine samples collected between 2012 to 2017.
b) Assess phthalates exposure level in association with personal and building characteristics.

In chapter two

c) Investigate both individual and mixture effect of phthalate metabolites with respiratory and allergic symptoms in children.
d) Investigate weather oxidative stress mediate association between phthalates and health outcomes.

Chapter 1

Human biomonitoring of phthalates trend between 2012 and 2017 using 7-years old children urine

Abstract

The widespread commercial production and use of phthalates as plasticizers in consumer products have led to significant human exposure. Some phthalates are known to disrupt the endocrine system and result in adverse health outcomes. As such, they have been regulated in materials used for children's items and food packages. In this study, the secular trend of urinary phthalate metabolites in children and the association between metabolites and building characteristics were examined. In total, 400 first-morning spot urine samples of 7 years old children collected from 2012 to 2017 from an ongoing birth cohort study were examined. Parents provided information on demographics and building questionnaires. using ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/ MS) ten urinary phthalate metabolites from five phthalate diesters: MiBP, MnBP, MBzP, MEHP, MEOHP, MEHHP, MECPP, MINP, OH-MINP, and cx-MINP were analyzed. A multivariable regression model with creatinine-corrected metabolite levels was applied to assess secular trends during 2012–2017. The association between metabolite levels and building characteristics was investigated using a mutual-adjusted linear regression. The metabolites MnBP, MEHP, MEOHP, MEHHP, MECPP, and OH-MINP were detected in all samples. The highest median concentration was for MECPP 37.4 ng/mL, followed by MnBP and MEHHP at concentrations of 36.8 and 25.8 ng/mL, respectively. Overall, DBP, BBzP, and DINP metabolite concentrations in this study were

comparable to or lower than those in previous studies from Japan and other countries in a similar study period. Higher concentrations of DEHP metabolites were observed in this study than in children from the USA and Germany, as per previous reports. Despite updated phthalate regulations and reports of production volume change in Japan, all the measured metabolites showed a stable trend between 2012 and 2017. Higher phthalate metabolite levels were observed among children from households with low annual income, those who lived in old buildings, and those with window opening habits of ≥1 h than <1 h. In contrast, children in houses that vacuumed 4 or more days/week showed a lower level of MnBP than those in houses that vacuumed ≤3 days/week. This study demonstrates that the internal exposure level of phthalates in Japanese children was stable from 2012 to 2017. This study findings suggest that phthalate exposure in children is consistent. Thus, improvements in the indoor environment, such as frequent vacuuming, may reduce exposure. Follow-up biomonitoring of phthalates is also critical for elucidating their possible health effects and developing mitigation strategies to decrease exposure level.

Keywords

Urinary phthalate metabolites, Secular trend, Children, Human biomonitoring, Building characteristics

1.1. Introduction

Phthalates or phthalic acid esters are a group of synthetic chemicals with the chemical structure of dialkyl or alkyl aryl esters of 1,2-benzene dicarboxylic acid (Cao et al., 2010). Phthalates are widely used as plasticizers, solvents, and additives in products such as polyvinyl chloride (PVC) materials, children's toys, food packaging, pharmaceuticals, and personal care products (Shinohara et al., 2020; Wang et al., 2019; Duty et al., 2005; Hauser and Calafat, 2005). The increased use of phthalates in several products results in its ubiquitous presence in the environment and exposure to the general population through inhalation, ingestion, or dermal contact (Anderson et al., 2018; Hauser and Calafat, 2005; Latini, 2005). Phthalate exposure has been reported to have endocrine-disrupting effects in humans (Hauser and Calafat, 2005; WHO, 2012) and experimental studies (Lyche et al., 2009).

Owing to the reproductive toxicity of phthalates, the United States and European government regulations were enacted to ban or restrict the use of phthalates, such as di (2-ethylhexyl) phthalate (DEHP), dibutyl phthalate (DBP), and butyl benzyl phthalate (BBzP), in the production of items associated with children and cosmetics (DIRECTIVE 2005/84/EC, 2005; Public Law 110–314 H.R. 4040, 2008). Such government regulations aim to change the production and use patterns of phthalates. Trend analysis studies in the general population have been used to monitor changes in exposure, such as in Denmark, Germany, Italy, and the US (Frederiksen et al., 2020; Koch et al., 2017; Tranfo et al., 2018; Zota et al., 2016). For instance, a study from the US using the National Health Nutrition and Examination Survey (NHANES) from 2001 to 2010 reported a decrease in DnBP, BBzP, and DEHP metabolite concentrations and increased levels of DINP

in the general population of children and adults (CDC, 2019; Zota et al., 2014). Similar studies in Europe have also reported a decline in urinary metabolites of DBP, BBzP, and DEHP in adults (Frederiksen et al., 2020; Tranfo et al., 2018). Additionally, a biomonitoring study conducted in Germany reported a decline in DBP, DEHP, and DINP metabolites in samples collected between 1988 and 2005, mainly from students aged 20–29 years (Koch et al., 2017). Government regulations in the US and European countries have been considered effective, as decreased phthalate exposure has been attributed to them (Tranfo et al., 2018; Zota et al., 2014). In 2010, the Ministry of Health, Labor and Welfare (MHLW) in Japan updated restrictions on the use of DBP, BBzP, DEHP, di-iso-decyl phthalate (DIDP), di-isononyl-phthalate (DINP), and di-octyl phthalate (DnOP) in children's toys and food packaging materials (MHLW Notice No.370, 2010). A previous study in Japan have shown higher urinary phthalate metabolite concentrations in children than in adolescents or adults (Ait Bamai et al., 2015). Moreover, urinary phthalate metabolite levels were positively correlated with phthalate concentrations in house dust (AitBamai et al., 2016). Children are more vulnerable to the adverse effects of phthalate exposure on asthma and allergies than adults (Ait Bamai et al., 2014). Previous studies have indicated the importance of building characteristics and indoor environments on phthalate exposure (Ait Bamai et al., 2014; Hsu et al., 2017). Moreover, until now no prior study has been conducted on the biomonitoring of trends in consecutive years of phthalate exposure in the Japanese population. Therefore, I aimed to investigate the secular trend of phthalate exposure in Japanese children between 2012 and 2017 and examined the association between internal phthalate exposure levels and the building characteristics of their homes. Additionally, the daily intake of phthalates based on metabolite levels in the urine were estimated.

1.2. Materials and methods

1.2.1. Study population and data collection

This study was based on data from the Hokkaido Study on Environment and Children's Health, an ongoing prospective birth cohort study that recruited 20,926 women between 2003–2012 in their first trimester of pregnancy between 2003 and 2012 (Kishi et al., 2021, 2017, 2013, 2011). Of the 20,926 pregnant women initially recruited a total of 15,757 were eligible for follow-up after excluding participants who voluntarily withdrew from the study, were lost to follow-up, or had a stillbirth or twins. Then between 2012 and 2017, a follow-up questionnaire and request for urine samples from the child when they reached 7 years of age was sent to 10,655 participants. Of these, 6218 responded, and 2,541 provided a complete questionnaire and urine sample. Based on power estimations, from these 2,541 samples, three subsets of 100 children each displaying symptoms of either wheeze, rhino-conjunctivitis, or eczema were formed. However, some children had comorbidities, resulting in 250 symptomatic children in total. A further subset of 150 asymptomatic children was also randomly selected, giving 400 children in total. After excluding 14 children with insufficient urine samples or abnormal chromatography, 386 children were used in the final analysis. Of these, 142 children were in the asymptomatic group and 244 in the symptomatic group. In the latter, 109 children had wheeze, 101 children had rhino-conjunctivitis, and 116 children had eczema. Of the symptomatic children, 72 children had comorbidities. (A detailed breakdown of the final symptomatic and asymptomatic children in this study can be found in Figure 1.1).

The building questionnaire was completed by parents or guardians of the children. The questionnaire determined building age, annual household income, number of residents, housing type (single-family house/ multi-family house), structure (wood/concrete), newly built or renovated within 1 year (yes/no), ventilation in living and/or child room(s) (yes/no), condensation (yes/no), mold odor (yes/no), visible mold (yes/ no), water leakage (yes/no), humidity (yes/no), insecticide (yes/no), flooring (PVC/non-PVC), wall material (PVC/non-PVC), vacuum cleaning/week, duration of the window opening, and whether the house was on the main road (yes/no).

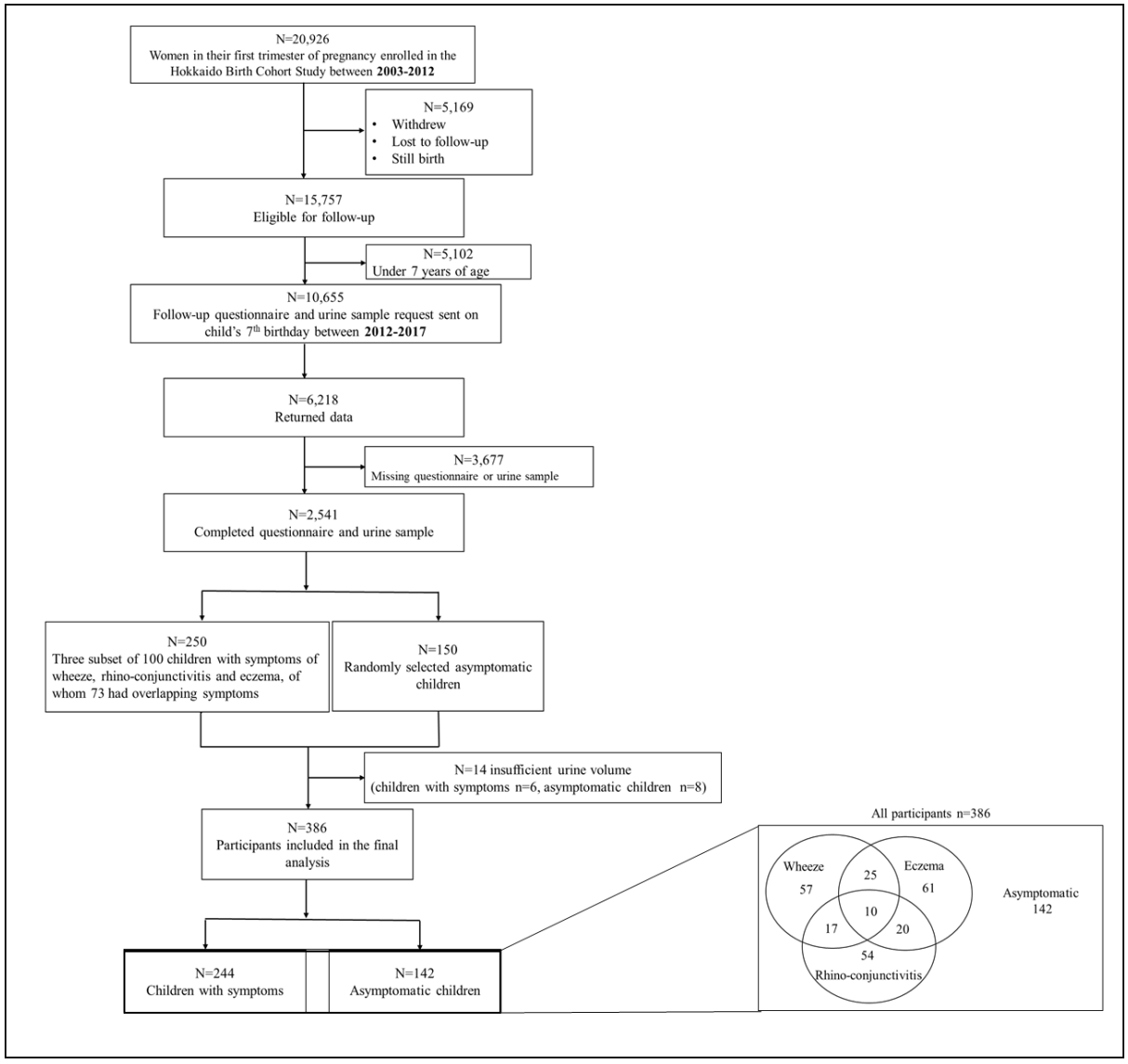

Figure 1.1. This study participants selection flow chart.

1.2.2. Ethics

The research protocol regarding human sampling was reviewed and approved by the Institutional Review Board of the Hokkaido University Center for Environmental and Health Sciences with an institutional review board number (Kanken 20-122) before the study was conducted. The parents of all participants provided written informed consent to confirm their participation in this study.

1.2.3. Chemical analysis

Ten phthalate metabolites were targeted based on literature survey of major presence in the environment and health risks of their parent phthalates. The phthalate metabolites assessed included the DBP metabolites [mono-n-butyl phthalate (MnBP), mono-isobutyl phthalate (MiBP)], BBzP metabolite [mono-benzyl phthalate (MBzP)], DEHP metabolites [mono (2-ethylhexyl) phthalate (MEHP), mono (2-ethyl-5-hydroxyhexyl) phthalate (MEHHP), mono (2-ethyl-5-oxohexyl) phthalate (MEOHP), and mono (2-ethyl-5-carboxypentyl) phthalate (MECPP)], and the DINP metabolites [mono-isononyl phthalate (MINP), mono-hydroxy-isononyl phthalate (OH-MINP), and mono-carboxyisononyl phthalate (cx-MINP)]. Individual native phthalate metabolites MiBP, MnBP, MBzP, MEHP, MEHHP, MEOHP, MECPP, and MINP and their isotopically labeled standards D_4-MiBP, $^{13}C_4$-MnBP, $^{13}C_4$-MBzP, $^{13}C_4$-MEHP, $^{13}C_4$-MEHHP, $^{13}C_4$-MEOHP, $^{13}C_4$-MECPP, and $^{13}C_4$-MINP with purity > 99.9% were purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA). Native standard mono-(4-methyl-7-hydroxyoctyl) phthalate (7OH-MMeOP) and mono-(4-methyl-7-carboxyheptyl) phthalate (7cx-MMeHP) and their isotope-labeled internal standards D_4-7OH-MMeOP and D_4-7cx-MMeHP, respectively, were purchased from Institut

für Dünnschichttechnologie und Mikrosensorik e.V. (Teltow, Germany) (Table 1.2). LCMS-grade ultra-pure water, methanol, ammonium bicarbonate, acetic acid, and formic acid were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Nitric acid was obtained from Kanto Chemicals, Co., Inc. (Tokyo, Japan). Ammonium acetate was purchased from Sigma-Aldrich Co., Ltd. (St. Louis, MO, USA). β-Glucuronidase (Escherichia coli-K12) was purchased from Roche Diagnostics GmbH (Mannheim, Germany). Solid phase extraction (SPE) Oasis Max 96-well plate (30 mg of polymer, 30 µm particles) was purchased from Waters Corporation (Milford, MA, USA).

1.2.4. Instrumental analysis

The phthalate metabolites were quantified using the ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) Waters ACQUITY UPLC H-class equipped with Xevo TQ-S micro mass spectrometer (Waters Corporation, Milford, MA, USA). Chromatographic separation was achieved using an Acquity UPLC charged surface hybrid (CSH) Phenyl-Hexyl column (Waters; 1.7 µm, 2.1 mm × 100 mm). The mobile phase was constituted of 5 mM ammonium bicarbonate in Milli-Q water (A) and 5 mM ammonium bicarbonate in 95% methanol (B). The chemical analysis was performed in a negative ion electrospray ionization mode, and target compounds were determined by multiple-reaction monitoring (MRM). The UPLC-MS/MS condition and gradient ratio of mobile phase are summarized in Table 1.1. Detailed information including instruments, chromatographic conditions, and mass spectrometric conditions can be found below in the Table 1.1 and Table 1.2.

Table 1.1. Parameters of analysis condition of LC-MS/MS for determination of phthalate metabolites

Parameters		Conditions									
LC		ACQUITY UPLC H-class									
Analytical column		CSH Phenyl Hexyl column (Waters, 2.1 x 100mm, 1.7 µm)									
Guard column		CSH Phenyl Hexyl (Waters, 2.1 x 5 mm,1.7µm)									
Retention gap column		Atlantis T3 (Waters, 2.1 x 50 mm, 3µm)									
Column temperature		40 ˚C									
MS/MS		Xevo TQ-S micro tandem quadrupole:									
MS mode		Multiple Reactions Monitoring (MRM)									
Ionization mode		Electrospray in negative mode (ESI - Negative)									
Mobile phase A		5 mM Ammonium bicarbonate in water									
Mobile phase B		5 mM Ammonium bicarbonate in 95 % Methanol									
Mobile phase C		Methanol									
Flow rate		0.25 mL/min									
Injection volume		40 µL									
Total run time		30 minutes									
Gradient	Time (min)	0	0.5	1	10	11	15	15.1	23	23.1	30
	A (%)	90	90	70	65	55	52.5	35	25	90	90
	B (%)	10	10	30	35	45	47.5	65	72.5	10	10
	C (%)	0	0	0	0	0	0	0	2.5	0	0

Table 1.2. MRM parameters of ten phthalate metabolites and their isotopically labeled internal standards with instrument conditions.

Native compounds	Quantification Ion	Confirmation Ion 1	Confirmation Ion 2	Quantification Ion		Confirmation Ion	
	Precursor/Product (m/z)	Precursor/Product (m/z)	Precursor/Product (m/z)	Cone (V)	Collision (eV)	Cone (V)	Collision (eV)
MiBP	220.82>76.93	220.82>133.98		15	19	15	12
MnBP	220.82>76.93	220.82>70.93		10	17	10	14
MBzP	254.79>76.86	254.79>104.42		10	21	10	15
MEHP	277.05>133.91	277.05>126.95		9	14	9	18
MEOHP	290.98>143.03	290.98>120.89		18	12	18	16
MEHHP	292.93>145.03	292.93>120.88		10	13	10	18
MECPP	306.98>158.98	306.98>112.87		9	11	9	29
MINP	291.15>141.07	291.15>76.99		18	17	18	25
OH-MINP	307.27>120.95	307.27>159.1	307.27>76.99	18	18	18	16
cx-MINP	321.0>173.04	321.0>120.95	321.0>76.93	15	16	15	25
Labeled internal standards							
MiBP-d_4	224.82>80.96	224.82>138.00		15	19	15	12
MnBP-$^{13}C_4$	224.76>71.00	224.76>78.95		10	17	10	14
MBzP-$^{13}C_4$	258.84>106.95	258.41>76.41		10	21	10	15
MEHP-$^{13}C_4$	281.09>136.91	281.09>127.2		9	14	9	15
MEOHP-$^{13}C_4$	294.84>143.02	294.84>123.88		18	12	18	16
MEHHP-$^{13}C_4$	296.73>123.88	296.73>145.04		10	13	10	18
MECPP-$^{13}C_4$	310.97>159.04	310.97>113.01		9	11	9	29
MINP-$^{13}C_4$	294.7>141.13	294.7>78.95		18	17	18	25
OH-MINP-d_4	311.21>124.98	311.21>159.09		18	18	18	16
cx-MINP-d_4	325.06>173.09	325.06>124.98		15	16	15	25

1.2.5. Quality assurance

For each batch, two procedural blanks were analyzed to control for background contamination. For all target analytes, 12 calibration points ranging from 0 to 20 ng/mL were used to construct the calibration curves. A satisfactory correlation coefficient of calibration curves ≥0.998 was obtained for all measured metabolites. In each batch of 20 samples, replicated analysis of the calibration standard at a concentration of 5 ng/mL and known concentration sample of the German external quality assessment scheme (G-EQUAS) samples with reference value of 63 were conducted to determine both inter-and intra-day precision and were within acceptable limits with a coefficient of variation <10%. The limits of detection (LOD) and limits of quantification (LOQ) of individual phthalate metabolites were determined based on 7 repeated analyses of spiked ultra-pure water with 0.16 ng/mL for MEOHP, OH-MINP, and cx-MiNP; 0.32 ng/mL for MEHP, MINP, MEHHP, and MECPP; 0.8 ng/mL for MBzP; and 1.6 ng/mL for MiBP and MnBP. The standard deviation (SD) of these repeated analyses was calculated using the following formula: LOD = 2 × t (n-1, 0.05) × SD and LOQ = 10 × SD. Here, t is the student's *t*-value for the 95th percentile of n-1 degree of freedom, where n is the number of repeated samples. The metabolites LOD and LOQ ranged 0.05–0.95 ng/mL and 0.13–2.5 ng/mL, respectively. The recovery percentages of native and labeled internal standards spiked in pooled urine samples ranged from 81% to 120%. The detailed quality assurance (QA)/quality control (QC) results are shown in Table 1.3. In addition to internal quality control, the precision of the method was externally validated by G-EQUAS, and results are summarized in Table 1.4.

Table 1.3. Summary of internal method validation and quality control results

Metabolites	Retention window (min)	R^2	IDL (ng/mL)	IS recovery %	CV %	Native recovery %	CV%	LOD (ng/mL)	LOQ (ng/mL)	Inter-day mean (ng/mL)	CV %	Intra-day mean (ng/mL)	CV %
MiBP	7:0 - 8:2	0.998	0.03	99	4.5	92	8.0	0.95	2.5	5.2	5.7	5.1	2.7
MnBP	7:5 - 8:5	0.999	0.04	100	5.5	91	8.6	0.78	2.02	5.0	5.1	5.0	1.7
MBzP	10:0 - 10:4	0.999	0.02	89	2.7	100	6.1	0.1	0.26	4.9	5.8	5.0	3.1
MEHP	21:0 - 21:3	0.999	0.01	86	3.6	96	4.8	0.15	0.39	4.9	5.3	4.9	0.9
MEOHP	10:3 - 11:0	0.999	0.01	93	3.8	97	5.9	0.05	0.15	5.0	6.7	5.1	2.2
MEHHP	11:3 - 12:1	0.999	0.02	86	5.7	96	7.5	0.15	0.25	5.0	5.6	5.0	1.1
MECPP	4:0 - 5:0	0.999	0.01	113	2.4	97	8.2	0.12	0.38	4.9	5.4	5.0	1.7
MINP	21:1 - 22:0	0.999	0.01	83	3.9	95	4.9	0.09	0.31	5.0	5.6	5.0	1.7
OH-MINP	15:1 - 16:3	0.999	0.01	86	6.6	108	6.1	0.05	0.13	5.3	4.9	5.3	1.3
cx-MINP	7:3 - 10:0	0.999	0.01	94	6.0	91	5.5	0.11	0.33	4.8	7.4	4.8	1.6

R^2: Correlation Coefficient; IDL: Instrument detection limit; ng/mL: nanogram per milliliter; IS (Internal standard) mean recovery % based on 10 repeats of standard spiked in pooled children urine; native mean recovery % based on 10 repeats of individual native metabolites spiked in pooled children urine; CV: coefficient of variation; LOD: Limit of detection; LOQ: Limit of quantification; Inter-/ Intra-day precision were based on 4 replicates of the standard at a concentration of 5 ng/mL.

Table 1.4. Mean concentration (ng/mL) of phthalate metabolites in urine samples analytical method validation result from reference values G-EQUAS 63

	Control material 63-9A		Control material 63-9B	
Metabolites	This study measured value	Reference value (tolerance range)	This study measured value	Reference value (tolerance range)
MiBP	8.6	8.6 (6.2 - 11.0)	81.8	75.9 (60.3 - 91.5)
MnBP	9.4	14.1 (9.3 - 18.9)	28.3	42.2 (29.3 - 55.1)
MBzP	1.0	1.3 (0.7 - 1.9)	3.4	3.6 (2.7 - 4.5)
MEHP	2.8	3.4 (2.3 - 4.5)	9.2	11.8 (8.7 - 17.9)
MEOHP	13.0	11.8 (9.3 - 14.5)	32.3	29.4 (23.4 - 35.3)
MEHHP	18.9	17.5 (12.5 - 22.6)	43.4	39.8 (30.0 - 49.6)
MECPP	20.8	16.0 (10.7 - 21.3)	48.7	37.5 (26.5 - 48.6)

G-EQUAS: German external quality assessment scheme (http://www.g-equas.de/)

1.2.6. Urine sample collection and preparation

Parents of children were asked to collect the first morning void urine samples of their children in a polypropylene cup, and these were sent to Hokkaido University, Center for Environmental and Health Sciences, using a cool delivery service. When the shipped urine samples arrived at our center, the creatinine content was measured using an enzyme-linked immunosorbent assay at SRL, Inc. (Tokyo, Japan). On the same day, samples were transferred to glass test tubes with ground glass stoppers cleaned with acetone, sealed with fluoric tape, wrapped with aluminum foil, and kept at - 20 ◦C until the day of analysis.

The detailed urine preparation for phthalate metabolites measurement is summarized in figure 1.2. For sample preparation, 500 µL urine sample was spiked with 20 µL of a mixture of labeled internal standards and then buffered with 500 µL 100 mM ammonium acetate (pH 6.5), and deconjugated by 50 µL β-glucuronidase with incubation at 37 ^{0}C for 90 min. After incubation, 1 mL 0.5% ammonia water was added to each sample. After this, sample extraction was performed using solid-phase extraction (SPE) that was conditioned with 1 mL 0.05% nitric acid in 90% methanol, 1 mL methanol, and then with 1 mL 0.5% ammonia water to activate the cartridge. Samples were loaded onto the conditioned SPE cartridge and sequentially washed with 0.5 mL ultra-pure water, 0.5 mL methanol, 0.5 mL ultra-pure water, and 0.5 mL 40% methanol containing 0.2% formic acid. The samples were eluted using a mixture of 90% methanol containing 0.2% formic acid. The eluted mixture (250 µL) was transferred to a vial and diluted with 750 µL ultrapure water. To quantify phthalate metabolites, 40 µL of the sample from the vial was injected into a UPLC-MS/MS (ACQUITY

UPLC H-class) equipped with a Xevo TQ-S micro mass spectrometer (Waters Corporation, Milford, MA, USA).

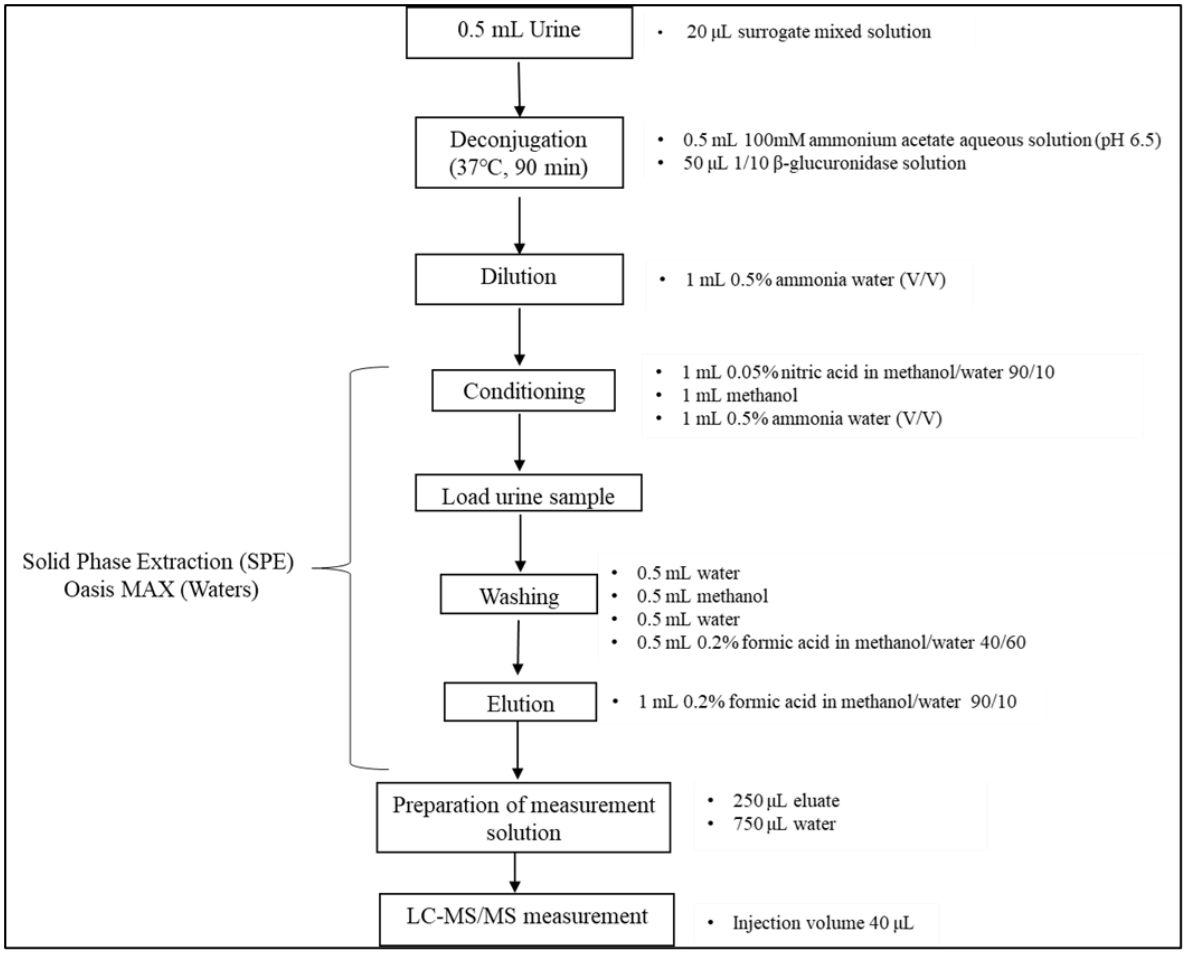

Figure 1.2. Sample preparation procedure for urinary phthalate metabolite

1.2.7. Daily intake estimation

Based on the measured concentrations of urinary phthalate metabolites, the daily phthalate intake was estimated for each subject using the following equation (Wittassek et al., 2007).

$$EDI = \frac{UE_{sum} \times CE_{smoothed}}{F_{UE} \times BW} \times MW_p$$

where EDI (μg/kg bw/day) is the estimated daily intake of phthalate; UEsum is the sum of the creatine-adjusted molar concentration of phthalate metabolites (μmol/g Cr); CEsmoothed (g/day) is the smoothed creatinine excretion (CE) rate; BW (kg) is the bodyweight; MWp (g/mol) is the molecular weight of the respective parent phthalate; and Fue is the urinary excretion factor of the parent phthalate, DnBP, DiBP, BBzP, DEHP, and DINP, which were set to 0.69, 0.69, 0.73, 0.62, and 0.39, respectively (Anderson et al., 2001; Koch et al., 2012; Wittassek et al., 2011). Gender-based values for urinary CE were determined using the following equations, where ht (cm) is the participant's height (Mage et al., 2008).

CE = ht × {6.265 + 0.0564 × (ht - 168)} × 103 (male)

CE = 2.045 × ht × exp {0.01552 × (ht - 90)} × 103(female)

1.2.8. Statistical analysis

For concentrations below the LOD, the LOD × detection frequency was assigned for statistical analysis (James et al., 2002). Additionally, the molar concentrations of DBP metabolites (MiBP and MnBP), DEHP metabolites (MEHP, MECPP, MEOHP, and MEHHP), and DINP metabolites (MINP, OH-MINP, and cx-MINP) were combined to estimate the parent compound exposure. The distribution of phthalate metabolites is presented as minimum, percentiles (25, 50, 75, and 95), and maximum values. A regression model with creatinine-corrected metabolite concentrations was applied to assess the secular trend from 2012 to 2017. Additionally, Dunnett's test, considering 2012 as a reference, was conducted to compare pairwise metabolite level mean differences by year. The *p*-values were adjusted using the Bonferroni correction. First a univariate analysis was conducted to analyze the distribution of urinary phthalate metabolite levels according to different building characteristics. Then associations between phthalate metabolite concentrations and the building characteristics that showed a significant difference in univariate analysis were investigated using mutual-adjusted linear regression. The percent difference calculated from the regression coefficient as $(e(\beta) - 1) \times 100\%$ with 95% CIs estimated as $(e(\beta \pm \text{critical value} \times SE) - 1)$, where β and SE are the estimated regression coefficient and standard error, respectively. Dampness indicators: condensation, mold odor, visible mold, water leakage, and humidity with response yes were assigned a value of 1 to compute the dampness index (1–5). Statistical analysis was performed using JMP Clinical 6.0, SAS.

1.3. Results and discussion

1.3.1. Study population

Due to insufficient sample volume or sample preparation error, 14 samples were excluded, and 386 samples were included in this study. All children in this study were seven years old; the gender participation was nearly balanced with males (52.6%) and girls (47.4%). The data represent the phthalate exposure trend of six consecutive years as children's urine samples were collected each year from 2012 to 2017. Nearly 60% of the participants lived in a mechanically ventilated house. Compared to a previous study in Sapporo children (Kishi et al., 2018), more participants in this study lived in houses with PVC flooring (16.9% vs. 7%) and slightly older buildings (median: 13 years vs. 10.5 years). The participants' demographic and building characteristics with urine collection years are presented in Table 1.5.

To ensure that the study population (n = 386) was representative of the original cohort, a comparison with a sub-cohort that included 243 participants was conducted. The results showed similarity in the distribution of demographic characteristics, building characteristics (Tables 1.5). Thus, minimal probable bias for this study population (n = 386) can be anticipated.

Table 1.5. Demographic and building characteristics of this study participants and this study sub-cohort participants

		This study (N= 386)	This study sub-cohort (N=243)
Gender	Boys	203 (52.6)	115 (47.3)
	Girls	183 (47.4)	128 (52.7)
Height	cm	119.8 (102.0-150.0)	119.2 (115.8 - 121.7)
Weight	Kg	22.0 (14.8-42.3)	21.7 (20.0 - 24.0)
Urine sample collection year	2012	62 (16.1)	39 (16.0)
	2013	65 (16.8)	43 (17.7)
	2014	54 (14.0)	34 (13.9)
	2015	74 (19.1)	48 (19.7)
	2016	86 (22.2)	47 (19.3)
	2017	45 (11.6)	32 (13.1)
Annual household income (JPY)	< 3 Million	48 (12.4)	28 (12.2)
	≥ 3 Million	321 (83.2)	201 (87.8)
Number of residents	≤4	251 (65.0)	153 (63.0)
	≥5	135 (35.0)	90 (37.0)
Home type	Detached	269 (70.0)	170 (69.9)
	Apartment	116 (30.0)	73 (30.1)
	Wooden	269 (69.5)	169 (70.0)
House structure	Concrete	114 (29.5)	72 (30.0)
Renovation within the past 1 year	Yes	22 (5.7)	13 (5.3)
	No	364 (94.3)	230 (94.7)
Mechanical ventilation system in living and/or child room	Yes	229 (59.3)	149 (61.3)
	No	157 (40.7)	94 (38.7)
Use of insecticide	Yes	125 (32.4)	80 (32.9)
	No	261 (67.6)	163 (67.1)
PVC flooring	Yes	65 (16.9)	45 (18.5)
	No	321 (831)	198 (81.5)
PVC wall material	Yes	310 (80.3)	198 81.4)
	No	76 (19.7)	45 (18.6)
Vacuum cleaning/week	≤3 times	199 (54.8)	114 (50.8)
	4-7 times	164 (45.2)	10 (49.2)
Duration of window opening/day	<1 hour	247 (64.0)	158 (65.0)
	≥1 hour	139 (36.0)	85 (35.0)
Main road	< 50 meters	75 (19.5)	40 (16.5)
	No or ≥ 50 meters	310 (80.5)	202 (83.5)
Building age (years)	Continuous	13 (<1- 50)	12 (7 - 22)
Dampness index (0-5)	Continuous	2 (1 - 5)	2 (1 - 2)

1.3.2. Concentration and secular trend of urinary phthalate metabolites

The distribution of urinary phthalate metabolite concentrations along with creatinine (Cr)-corrected levels in children is summarized in Table 1.6. Phthalate metabolites MnBP, MEHP, MEOHP, MEHHP, MECPP, and OH-MINP were detected in all samples. The highest concentration was found among the DEHP metabolites MECPP and MEHHP, followed by MnBP. The creatinine-corrected concentrations showed a similar trend. Furthermore, the distribution of phthalate metabolites comparison between this study and sub-cohort showed no difference (Table 1.6). All creatinine-corrected urinary phthalate metabolites in this study showed a significant positive Spearman's correlation (Table 1.7). The highest correlation was found between DEHP and DINP metabolites. Although many studies have reported seasonal variations in phthalate exposure (Bi et al., 2018; Li et al., 2019), in this study, no association was observed between the sample collection seasons and phthalate metabolite levels (Table 1.8).

Table 1.6. Distribution of urinary phthalate metabolite concentrations in this study and comparison with sub-cohort in 7 years old children.

Metabolites (ng/mL)	LOD	%>LOD	This study (N= 386)	This study sub-cohort (N=243)
MiBP	0.95	99.7	12.1 (7.1- 27.4)	11.8 (7.2 - 25.2)
MnBP	0.78	100	35.1 (20.7 - 58.8)	33.6 (20.5 - 56.0)
MBzP	0.10	98.9	1.5 (0.7 - 3.5)	1.4 (0.7 - 3.2)
MEHP	0.15	100	4.1 (2.7 - 7.0)	3.9 (2.3 - 7.2)
MEOHP	0.05	100	20.5 (12.3 -33.2)	20.4 (11.4 - 32.8)
MEHHP	0.15	100	26.7 (16.4 - 43.8)	26.1 (15.6 - 43.5)
MECPP	0.12	100	38.4 (23.3 - 67.1)	37.2 (20.2 - 64.7)
MINP	0.09	96.9	0.6 (0.4 -1.2)	0.6 (0.3 - 1.1)
OH-MINP	0.05	100	4.1 (2.2 - 7.5)	3.8 (2.1 - 7.2)
cx-MINP	0.11	99.7	2.4 (1.3 - 4.6)	2.3 (1.3 - 4.6)
∑DEHP [a] (µmol/L)	n.a	n.a	0.29 (0.18 - 0.49)	0.29 (0.17 - 0.49)
∑DINP [b] (µmol/L)	n.a	n.a	0.02 (0.01 – 0.05)	0.02 (0.01 - 0.04)
Creatinine corrected (µg/g Cr)				
MiBP			13.3 (8.4 - 25.0)	13.0 (8.3 - 23.9)
MnBP			39.1 (26.3 - 59.2)	36.1 (26.3 - 58.2)
MBzP			1.7 (0.7 - 3.9)	1.4 (0.7 - 3.4)
MEHP			4.5 (2.9 - 7.4)	4.4 (2.9 - 7.6)
MEOHP			22.4 (14.9 - 32.5)	21.3 (13.9 - 31.2)
MEHHP			28.7 (19.3 - 43.7)	27.8 (19.2 - 42.2)
MECPP			42.8 (27.0 - 68.5)	42.0 (26.8 - 62.7)
MINP			0.7 (0.4 - 1.3)	0.7 (0.4 - 1.2)
OH-MINP			4.5 (2.8 - 7.4)	4.3 (2.7 - 7.2)
cx-MINP			2.7 (1.6 - 4.9)	2.7 (1.5 - 4.8)
∑DEHP [a] (µmol/L Cr)			0.34 (0.22 - 0.51)	0.32 (0.21 – 0.48)
∑DINP [b] (µmol/L Cr)			0.02 (0.01 - 0.03)	0.02 (0.01 – 0.04)

Data are expressed as median and interquartile range (IQR).

[a] ΣDEHP: sum of molar concentrations metabolites [MEHP + MEOHP + MEHHP + MECPP]

[b] ΣDINP: sum of molar concentrations metabolites [MINP + OH-MINP + cx-MINP]

Abbreviations; LOD: Limit of detection; Max: maximum; Min: minimum; P: percentiles; n.a: not applicable; MiBP: mono-isobutyl phthalate, MnBP: mono-n-buty phthalate, MBzP: mono-benzyl phthalate, MEHP: mono (2-ethylhexyl) phthalate, MEOHP: mono (2-ethyl-5-oxohexyl) phthalate, phthalate, MEHP: mono (2-ethylhexyl) phthalate, MEOHP: mono (2-ethyl-5-oxohexyl) phthalate, MEHHP: mono (2-ethyl-5-hydroxyhexyl) phthalate, MECPP: mono (2-ethyl-5-carboxypentyl) phthalate, MINP: mono-isononyl phthalate, OH-MINP: mono-hydroxy-isononyl phthalate, cx-MINP: mono(carboxy-isononyl phthalate)

Table 1.7. Spearman's correlations of ten phthalate metabolites analyses (μg/g creatinine)

	MiBP	MnBP	MBzP	MEHP	MEOHP	MEHHP	MECPP	MINP	OH-MINP	cx-MINP
MiBP	1	0.079***	0.015***	0.140***	0.250***	0.233***	0.235***	0.077***	0.133***	0.057***
MnBP		1.000	0.061***	0.122***	0.155***	0.147***	0.135***	0.035***	0.039***	0.044***
MBzP			1.000	0.091***	0.135***	0.112***	0.093***	0.319***	0.276***	0.220***
MEHP				1.000	0.703***	0.710***	0.686***	0.540***	0.369***	0.306***
MEOHP					1.000	0.966***	0.936***	0.464***	0.534***	0.477***
MEHHP						1.000	0.949***	0.502***	0.582***	0.509***
MECPP							1.000	0.454***	0.557***	0.548***
MINP								1.000	0.827***	0.693***
OH-MINP									1.000	0.863***

Table 1.8. Seasonal variation of phthalate metabolites level in children

Metabolites	Season	25%	Median	75%	P value
MiBP	Winter	7.55	13.07	21.97	0.115
	Spring	9.43	15.86	38.90	
	Summer	9.61	15.86	26.38	
	Autumn	6.64	11.59	23.56	
MnBP	Winter	24.58	40.17	59.14	0.121
	Spring	30.09	45.67	68.10	
	Summer	27.99	38.81	60.28	
	Autumn	22.54	34.21	56.76	
MBzP	Winter	0.72	1.71	4.02	0.555
	Spring	0.87	1.68	5.74	
	Summer	0.73	1.73	3.38	
	Autumn	0.71	1.39	3.72	
MEHP	Winter	2.91	4.39	7.37	0.598
	Spring	3.33	5.19	7.55	
	Summer	3.07	4.38	6.87	
	Autumn	2.85	3.93	8.31	
MEOHP	Winter	14.63	22.38	31.06	0.613
	Spring	17.19	24.05	34.98	
	Summer	14.98	21.92	33.69	
	Autumn	14.71	21.09	32.18	
MEHHP	Winter	19.03	29.32	42.73	0.690
	Spring	23.09	31.76	45.89	
	Summer	19.90	28.05	42.93	
	Autumn	18.72	26.38	45.32	
MECPP	Winter	26.57	42.26	64.73	0.955
	Spring	30.40	45.00	71.72	
	Summer	27.92	39.79	73.39	
	Autumn	26.98	44.62	67.92	
∑DEHP	Winter	0.21	0.34	0.51	0.822
	Spring	0.25	0.36	0.56	
	Summer	0.22	0.32	0.52	
	Autumn	0.21	0.34	0.50	
MINP	Winter	0.50	0.76	1.40	0.880
	Spring	0.45	0.69	1.39	
	Summer	0.41	0.66	1.34	
	Autumn	0.47	0.81	1.24	
OH-MINP	Winter	2.96	4.50	7.29	0.931
	Spring	2.58	3.67	7.26	
	Summer	2.78	4.49	8.21	
	Autumn	2.72	4.58	6.70	
cx-MINP	Winter	1.68	2.83	4.81	0.200
	Spring	1.42	2.19	3.82	
	Summer	1.71	3.03	5.63	
	Autumn	1.50	2.63	4.32	
∑DINP	Winter	0.02	0.03	0.04	0.809
	Spring	0.01	0.02	0.04	
	Summer	0.02	0.03	0.05	
	Autumn	0.02	0.02	0.03	

The secular trend of the evaluated urinary phthalate metabolite levels is shown in Figure. 1.3 A. Regression analysis showed that all measured metabolites were stable throughout the study period. Additionally, the trend of this study sub cohort data (n=243) also showed similar stable trend and ensures this study data (n=386) can be a representative of the cohort (Figure1.3 B). Thus far, this is the first human biomonitoring study to investigate internal phthalate exposure trends in the Japanese population. In 2010, the regulation of phthalates in children's toys and food packaging materials was revised in Japan. Consequently, changes in the production and exposure to phthalates have been reported in Japan, for example, from 2012 to 2017, the production of DEHP decreased by 13.3% (135,000 to 117,000 tons). In contrast, the production of DINP increased by 43.2% (67,000 to 96, 000 tons) (IHS Markit, 2018; VEC, 2018). However, urinary phthalate metabolites showed a stable trend. This suggests that the reported changes in chemical production do not reflect children's exposure. A plausible explanation for the lack of a trend in the current study could be the limited scope of phthalate regulation. The regulation only concerns toys meant for children under 6 years of age and food containers containing fats and oils but excludes materials such as PVC flooring, wall and ceiling coverings (common in modern Japanese houses and apartments), and personal care products, which have been reported as potential phthalate exposure sources (Bornehag et al., 2005; Carlstedt et al., 2013; Husoy et al., 2020). Higher levels of DEHP in indoor dust in Japanese households than in other EU countries and the USA have been reported (Ait Bamai et al., 2014). Thus, the stable trend in exposure levels observed in this study can be attributed to phthalates emitted from non-regulated materials (Ait Bamai et al., 2014; Carlstedt et al., 2013; Husoy et al., 2020).

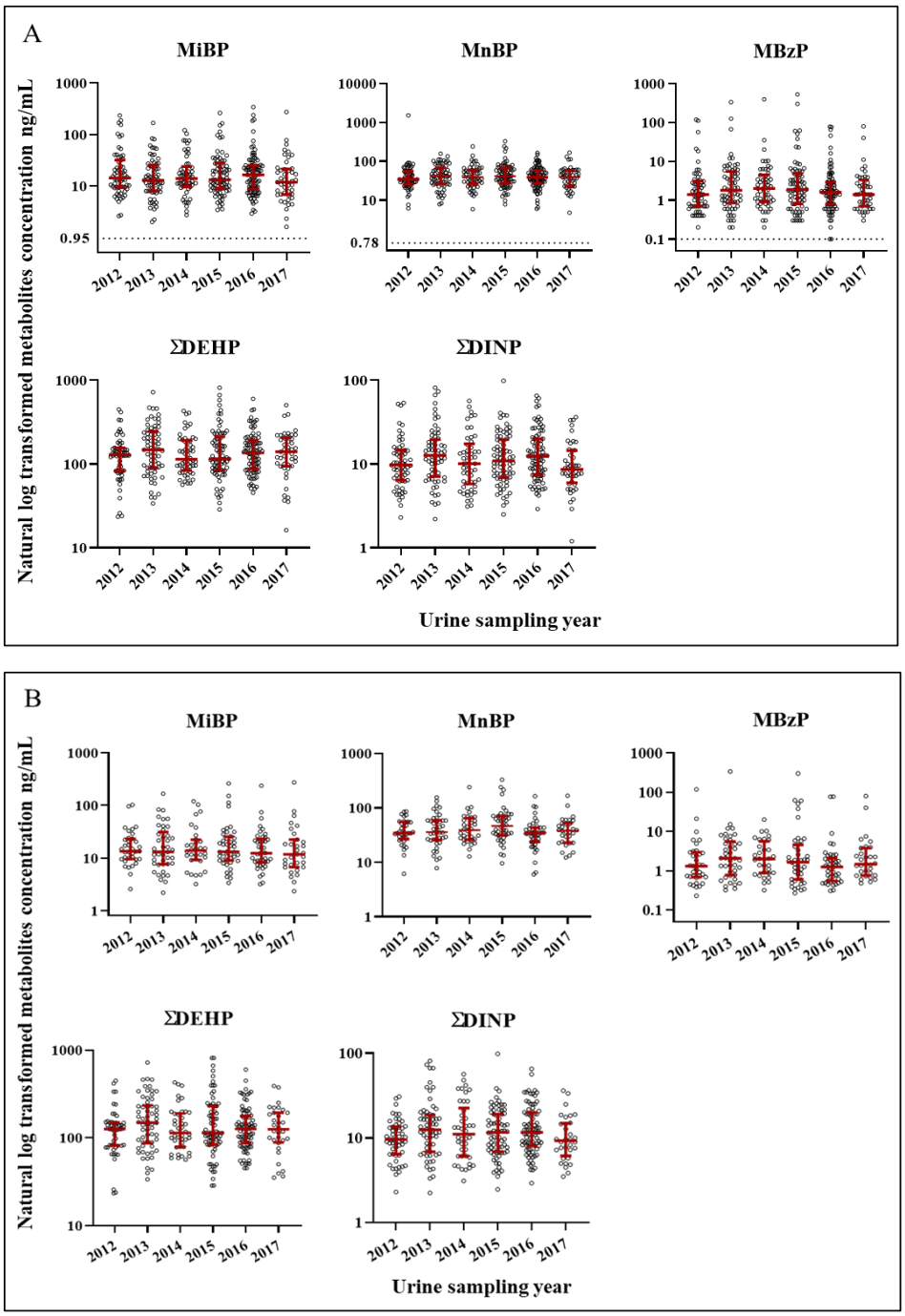

Figure 1.3. Natural log transformed creatinine corrected concentration trend of urinary phthalate metabolites for (A) this study participants n=386; (B) this study sub-cohort participants n=243 for comparison. Bars represent interquartile ranges and median. Bars represent interquartile ranges and median. Points on dotted line indicates samples with concentration limit of detection (<LOD)

Comparing the results of this study and previous study in Japan (Ait Bamai et al., 2015) children showed a relatively higher median concentration for MiBP, MBzP, MEHP, MEOHP, and lower MnBP in the previous study than in the current study (Figure. 1.4). However, this comparison should be interpreted with caution because the method of analysis was different for the two studies. The previous study used derivatization and was measured by GC-MS, while the current study used LC-MS/MS. The concentrations of urinary phthalate metabolites in children among comparable age groups show variations simil ar to those in the findings from other countries over a similar period (Ait Bamai et al., 2015; Becker et al., 2009; CDC, 2019; Hartmann et al., 2015; Liao et al., 2018; Schwedler et al., 2020; Song et al., 2013; Wang et al., 2015; Weng et al., 2017). For instance, a similar level of MiBP was observed in children from the US (CDC, 2019), while a 2- to 3-fold higher median concentration was observed in Germany (Schwedler et al., 2020) and China (Liao et al., 2018; Wang et al., 2015). A noticeably higher level of DEHP metabolites was observed in this study participants than in Germany (Schwedler et al., 2020) and the USA (CDC, 2019) (Fig. 1.4). This indicates that Japanese children still have high exposure to DEHP despite production regulations and efforts to replace DEHP (Rowdhwal and Chen, 2018; VEC, 2018). Ait Bamai, 2016 form Japan previously reported that DEHP and BBzP concentrations in house dust are positively correlated with urinary metabolite concentrations (Ait Bamai et al., 2016). Additionally, a review revealed that house dust ingestion is a significant exposure pathway for phthalates such as DEHP in Japan (Takagi and Yoshinaga, 2009). Thus, the high levels of urinary DEHP metabolites in this study might be due to non-regulated consumer products, such as PVC building materials, which can release phthalates.

Considering the DINP in this study, consistent trends in its metabolites were observed. This might be due to the wide use of DINP in PVC wallpapers, wire, and cable insulation jacketing in Japan, which are less likely to be changed/installed frequently (IHS Markit, 2018). Moreover, most of the children in this study lived in houses with a mean age of 13 years (Table 1.7), which was built before the regulation and increased production of DINP, could explain the stable trend observed in this study. Biomonitoring studies conducted in the early 2010s reported increased levels of DINP in Germany, Italy, and the USA (Tranfo et al., 2018; Wittassek et al., 2007; Zota et al., 2016). DINP has been subjected to regulation due to its various health risks and is substituted with alternatives such as di(2-ethylhexyl) terephthalate (DEHTP) and cyclohexane-1,2-dicarboxylic acid-diisononyl ester (DINCH), resulting in a decline in DINP exposure in recent years (CDC 2019; Frederiksen et al., 2020; Schwedler et al., 2020). Since the current study did not measure urinary DINP metabolites before the 2010 revised phthalate regulations, exposure levels of DINP in Japanese children before the regulation are uncertain. This warrants follow-up studies with a large population size to elucidate exposure changes over time.

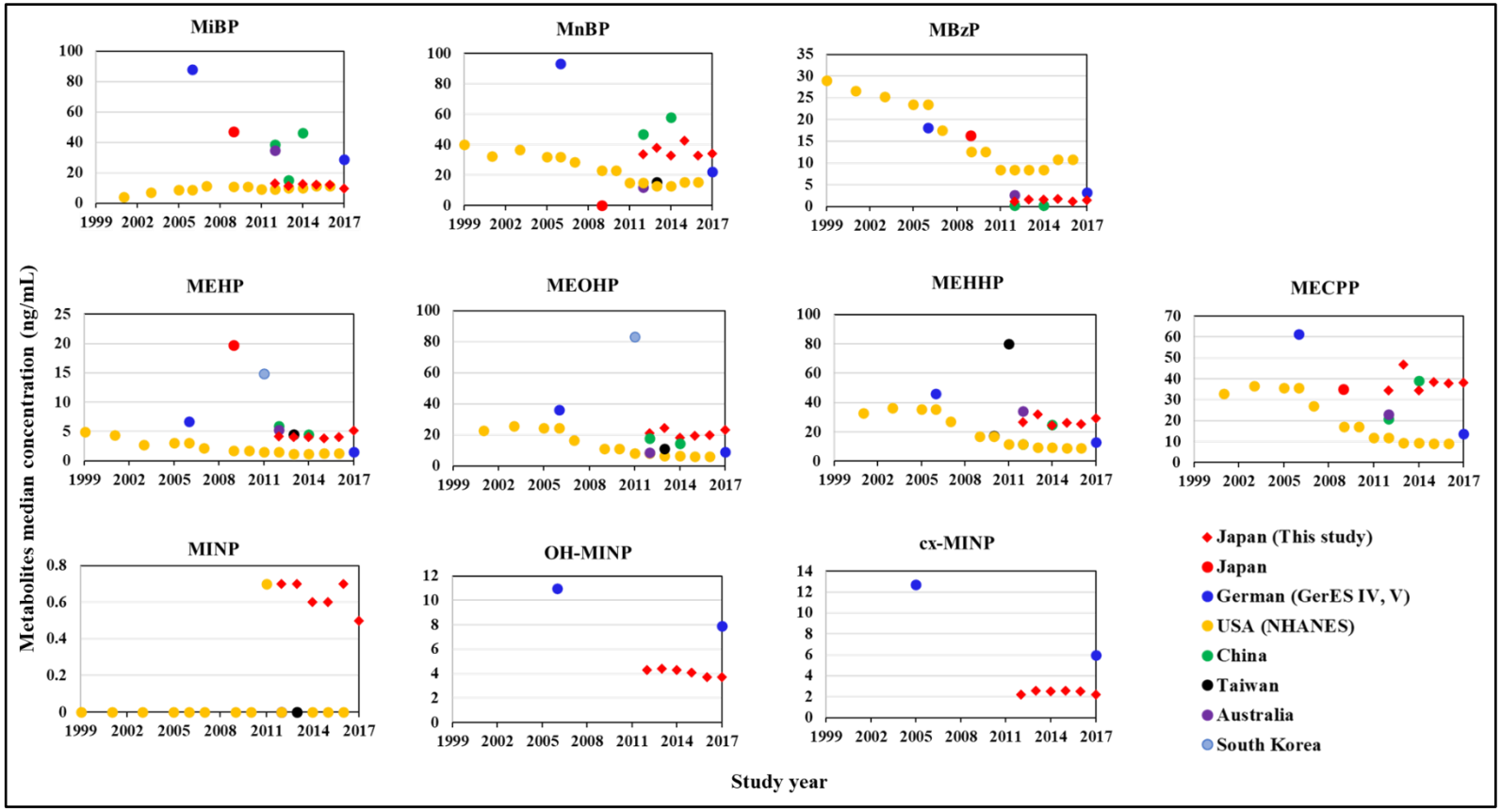

Figure 1.4. Secular trends and comparison of phthalate metabolites median concentration in ng/mL from children in different countries.

1.3.3. Urinary phthalate metabolite levels and building characteristics

Despite the government regulations regarding phthalates in Japan, this study trend analysis showed a stable level of phthalate metabolites. Additionally, the high detection frequency of urinary phthalate metabolites indicates that children are still widely exposed, probably from non-regulated products such as building materials. Thus, potential phthalate exposure based on household characteristics and daily habits were evaluated (Table 1.9). The mutually adjusted regression model revealed a significant positive association between lower household income and OH-MiNP ($\beta = 0.138$) and $\sum$DINP ($\beta = 0.127$). This result is in agreement with those of previous studies that reported that lower socioeconomic status (SES) families tend to show a higher level of urinary phthalate metabolites, such as MBzP and DEHP metabolites (Koo et al., 2002; Navaranjan et al., 2020). This may have several precipitating factors, such as the influence of SES on dwelling characteristics or fast-food consumption habits resulting from parental education levels, which have previously been reported as variables causing increased phthalate exposure (Ait Bamai et al., 2014; Zota et al., 2016).

In the present study, MnBP levels showed a significant positive association with building age. Furthermore, the stratified analysis revealed an increased beta value of MnBP ($\beta > 0$) for older buildings, indicating an increased level of MnBP as the building age increased. Building age has been reported as a common predictor of indoor phthalate levels in the dust (Bornehag et al., 2005). There is evidence that DnBP parent compound of MnBP was used as a plasticizer in PVC materials in the 1980s (Kavlock et al., 2002). Thus, the use

of DnBP in interior materials for older buildings could explain this study finding of a significant relationship between MnBP and building age.

Metabolite MnBP was lower in children who lived in houses that were vacuumed 4–7 times/week than in those that were vacuumed ≤3 days/week. This result can be explained by the frequent vacuuming association with decreasing dust accumulation, resulting in lower phthalate levels (Wilson and VanSnick, 2017), as phthalates emitted from vinyl building materials can be absorbed into house dust (Liang and Xu, 2014).

Children in houses with ≥1 h/day window opening habits showed significantly elevated levels of MiBP, MnBP, and DEHP metabolites compared to children in houses with <1 h/day window opening habits. This result is unpredicted, as opening windows are expected to facilitate air exchange and decrease phthalate levels in the indoor environment (Smie´łowska et al., 2017). A possible reason for this contradictory result could be the confounding effect of building age on the association between window opening and metabolite levels. Higher metabolite levels in children living in older buildings was observed (Table 1.9). Hence, this finding raises the question: "Did the families with fewer open windows live in newer houses with air-conditioning installed?". Unfortunately, this study did not collect data on the use/installation of air-conditioning, but in this study area Hokkaido, it was uncommon for households to have air-conditioning due to the climate being cool. Moreover, no significant difference ($p > 0.786$) was observed in building age between less window opening houses (median 15.9 year) and more window opening houses (median 16.8 year). Therefore, building age was not found to be a confounding factor in the relationship between window

opening and metabolites. Another study that examined the impact of open and closed windows on indoor air composition reported that emission of semi-volatile compounds such as phthalates was enhanced when windows were open rather than closed (Fortenberry et al., 2019). Additionally, Xu et al. (2010) revealed that the emission rate of DEHP from vinyl flooring increased at a high ventilation rate because of the higher air velocity near the surfaces and consequently results in an increase in the mass-transfer coefficients that promote the emission of DEHP. This suggests that window opening enhances ventilation and increases emissions to the indoor environment. Subsequently, the internal exposure level of the DEHP increased.

Table 1.9. Percent difference (95% CI) in phthalate metabolites concentrations with demographic and building characteristics of children house (N=386)

Variables	Categories	MiBP	MnBP	MBzP	∑DEHP	∑DINP
Annual household income (in JPY)	≥ 3 Million	Ref	Ref	Ref	Ref	Ref
	<3 Million	8.2 (-7.0,25.9)	3.6 (-7.0,15.3)	-3.6 (-22.0,20.0)	10.3 (-0.6,22.4)	13.9 (1.1,28.3) *
Building age (years)	Continuous	-0.6 (-1.6,0.4)	1.0 (0.3,1.7) **	0.8 (-0.7,2.3)	0.1 (-0.6,0.8)	-0.5 (-1.3,0.2)
Vacuum cleaning/week	≤3 times	Ref	Ref	Ref	Ref	Ref
	4-7 times	-8.5 (-17.2,1.0)	-7.2 (-13.5, -0.4) *	-7.3 (-19.7,7.1)	-5.0 (-11.3,1.7)	4.7 (-3.2,13.3)
Duration of window opening	<1 hour	Ref	Ref	Ref	Ref	Ref
	≥1 hour	11.6 (0.6,23.8) *	9.7 (1.9,18.1) *	-1.4 (-15.2,14.6)	12.3 (4.6,20.6) **	-1.0 (-8.8,7.4)
Ventilation in living or child room	Yes	Ref	Ref	Ref	Ref	Ref
	No	3.0 (-7.6, 14.7)	-1.0 (-8.3,6.9)	-6.5 (-22.7,13.2)	0.4 (-6.7,8.0)	-4.0 (-11.8,4.5)
Dampness index (0-5)	Continuous	1.6 (-6.8,10.9)	5.3 (-1.0,12.0)	-4.7 (-16.0,8.1)	0.1 (-5.7,6.3)	0.8 (-5.9,8.0)

Ref: reference, *P<0.05, ** P<0.01, Phthalate metabolites in urine were natural log transformed and corrected for creatinine level before analysis. General regression analysis conducted with phthalate metabolites concentration as dependent variable and the all building characteristics were mutually adjusted. DEHP metabolites MEHP, MEOHP, MEHHP and MECPP showed similar estimates thus in this table ∑ DEHP is presented. DINP metabolites MINP, OH-MINP, and cx-MINP showed similar direction estimate thus in this table ∑ DINP is presented.

1.3.4. Estimated daily intake (EDI) of phthalates

Hereafter, the daily intake of phthalates in children were estimated, as shown in Figure 1.5. Daily intake of DEHP had the highest median EDI value of 3.7 μg/kg/day. The EDIs of DnBP were slightly higher in boys than girls, with a mean value of 1.8 and 1.4 μg/kg/day, respectively. Based on the European Food Safety Authority (EFSA), tolerable daily intake (TDI) reference values for individual phthalates DnBP and DEHP, one child in each phthalate exceeded the reference values of 10 and 50 μg/kg/day, respectively (EFSA, 2005a; 2005c). However, none of the children in this study exceed the TDI of DiBP, BBzP and DINP reference values 10, 500, and 150 μg/kg/day, respectively (EFSA, 2005a; 2005b; 2005d). Considering the updated EFSA risk assessment of combined exposure to DBP, BBzP, DEHP, and DINP at a group-TDI level of 50 μg/kg/day, two children with one child at a marginal level were observed (EFSA 2019). Considering the US reference dose (RfD) of 20 μg/kg/day for DEHP (US. EPA, 1991), two children exceeded the RfD value and another 2 were on the reference borderline, representing 1.03% of the participants. Comparing this study's median EDI values with those of other studies in children, DiBP, DnBP, BBzP, DEHP, and DINP were lower or comparable (Ait Bamai et al., 2015; Kasper-Sonnenberg et al., 2014; Yoshida et al., 2020). In contrast, the EDI value of DINP in this study was higher than that of Taiwanese children with a median of 0.5 and 0.2 μg/kg/day, respectively (Chang et al., 2017). However, caution should be taken when interpreting phthalate EDI comparisons, since variations in participant characteristics or study methods may alter EDI among different studies and countries.

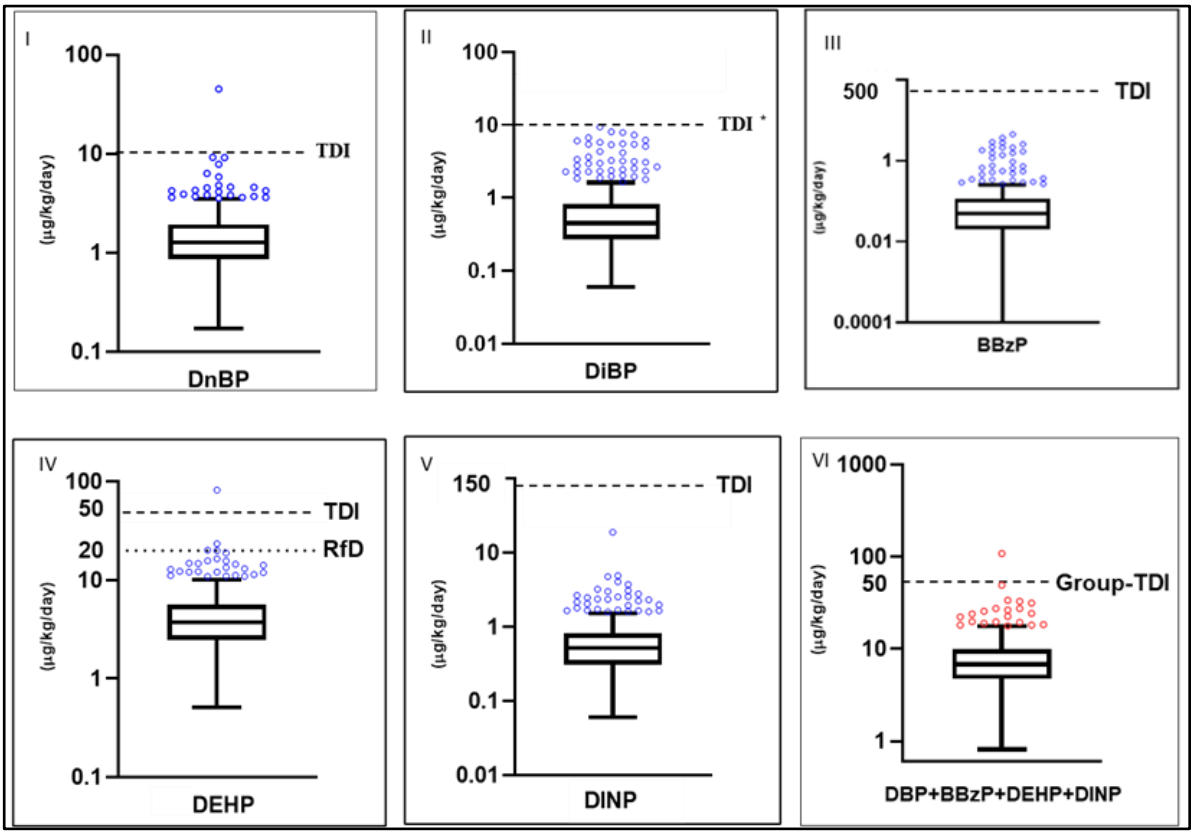

Figure 1.5. Estimated daily intake in µg/kg/day

In the future, the use of PVC gloves and rubbing alcohol during the COVID-19 pandemic is likely to increase exposure risk among the general population, which further highlights the need for phthalate biomonitoring.

1.4. Strengths and limitations

Selecting participants of the same age (7 years) in this study allowed for a better comparison by eliminating age as a confounding factor. Additionally, the accuracy of phthalate metabolite measurement method was validated by the external quality assessment scheme GEQUAS, which strengthens the reliability of this study results (Table 1.4). The building characteristics data in this study also strengthened the investigation by facilitating the identification of possible phthalate exposure sources. The primary limitation of this study is the small sample size. However, this data still provides valuable evidence on changes in phthalate exposure during 2012–2017 in Japanese school aged children. A secondary limitation is the participants' selection bias of including children with allergies, which could limit the generalizability of this study. However, since the distribution of children with allergies in each year was similar, approximately 16%. Thus, the inclusion of children with allergies would have a minimal effect on any probable bias in the trend analysis. Another limitation is that the urine samples were collected only once, which may not represent variance in urinary phthalate metabolite excretion based on individual activity, diet, personal care product use, cleanliness, and seasonal dust accumulation with an open window. Thus, to reduce the variability of metabolite levels during the day, the first morning void urine samples were used.

1.5. Conclusions

This study is the first to document the consecutive stable trend of the internal exposure level of phthalates in Japanese children between 2012 and 2017, indicating that consistent phthalate exposure exists even after the regulation update of phthalates. Furthermore, correlations between a high exposure level of MnBP among children in old buildings, DINP metabolites among those with lower household income, and MiBP, MnBP, and DEHP metabolites among those with long window opening habits were identified. Frequent vacuum cleaning was associated with lower MnBP levels in children. Finally, other personal care and protective equipment that are known to increase phthalate exposure risk should be evaluated in future studies.

Chapter 2

Phthalates mixture on allergies and oxidative stress biomarkers among children

Abstract

Background: Exposure to individual phthalates and the mediation effect of oxidative stress in association with asthma and allergic symptoms have been studied previously. Little is known about the mixture effect of phthalates on health outcomes. Thus, we investigated the effect of a mixture of ten phthalate metabolites in association with wheeze, rhino-conjunctivitis, and eczema. The mediating effect of three oxidative stress biomarkers was also assessed.

Methods: Levels of 10 phthalate metabolites and 3 oxidative stress biomarkers were measured in 386 urine samples from 7-year-old children. Parents reported demographic and allergic symptoms using ISAAC questionnaires. Logistic regression for individual metabolites and mixture analysis weighted quantile sum (WQS) and Bayesian kernel machine regression (BKMR) were fitted to examine the association between phthalate metabolite exposure and health outcomes. Baron and Kenny's regression approach was used for mediation analysis.

Results: In logistic regression model showed mono (2-ethyl-5-carboxypentyl) phthalate (MECPP) (OR = 1.41, 95% CI 1.02–1.97) and mono carboxy-isononyl phthalate (cx-MINP) (OR = 1.40, 95% CI 1.07–1.86) were associated with wheeze. The WQS index had a significant association (OR = 1.46, 95% CI 1.09–1.96) with wheeze and (OR = 1.40, 95% CI 1.07–1.82) with eczema. Mono-isononyl phthalate (MINP) and mono-(2-ethyl-5-oxohexyl) phthalate (MEOHP) were the most highly weighted metabolites. In the BKMR model, diisononyl phthalate (DINP) metabolites showed the highest group posterior inclusion probability (PIP). Among DINP metabolites, MINP in wheeze, cx-MINP in rhino-conjunctivitis and OH-MINP in eczema showed the highest conditional PIPs. The overall metabolites mixture effect was associated with eczema. We did not find any mediation of oxidative stress in the association between phthalates and symptoms. No significant association between phthalate metabolites and oxidative stress was observed in this study.

Conclusion: Mixture of phthalate metabolites were associated with wheeze and eczema. The main contributors to the association were DEHP and DINP metabolites. No mediation of oxidative stress was observed.

Keywords

Phthalates; chemical mixtures effect; children; weighted quantile sum; Bayesian kernel machine regression; oxidative stress

2.1. Introduction

Wheeze, rhino-conjunctivitis, and eczema are common global chronic respiratory and allergic symptoms in children (Langan et al., 2020; WHO, 2007). A nationwide survey conducted between 2005 and 2015 in Japanese children suggested a stabilizing trend of wheeze (10.2%) and eczema (14.6%) prevalence and increasing rhino-conjunctivitis (18.7%) (Sasaki et al., 2019). Phthalates are used as plasticizers and additives in a variety of industrial and commercial products. Given that phthalates are omnipresent and migrate from products, people can be exposed via inhalation, diet, or dermal absorption, depending on the industrial application (Wittassek et al., 2011; Wormuth et al., 2006).

Previous studies have reported that phthalate exposure is associated with different health outcomes in children (Ait Bamai et al., 2018, 2016, 2014; Choi et al., 2014; Hsu et al., 2017). Previously, Ait Bamai, 2014 reported association between increased levels of DMP, DINP, DEHP, DIBP, and BBzP are associated with elevated allergic rhinitis, conjunctivitis, and atopic dermatitis prevalence (Ait Bamai et al., 2014).

Most previous studies have investigated the association between single phthalate exposure and health outcomes. However, in real life, humans are simultaneously exposed to several chemical mixtures. Thus, statistical approaches such as weighted quantile sum (WQS) regression and Bayesian kernel machine regression (BKMR) have been used to comprehensively assess the combined effect of mixed chemical exposure on health outcomes (Bobb et al., 2018; Carrico et al., 2015; Zhang et al., 2019), and provide further insight for identifying the important components of the mixture. Recent research on the association

between prenatal phthalate exposure and wheezing and asthma in children reported that MEP and MBzP were highly weighted metabolites that increased symptoms, whereas an inverse association was observed between the mixture of DEHP metabolites and asthma (Adgent et al., 2020).

Previous *in vivo* and *in vitro* studies have reported that phthalate exposure enhances the sensitization of respiratory and allergic responses to oxidative stress as a mediator (Deaman et al., 2008, 2008; Guo et al., 2012; Kang et al., 2018; Nishioka et al., 2012; Zhou et al., 2020). For instance, after exposure of mice to DBP, a significant increase in oxidative stress biomarkers (8-hydroxy-2-deoxyguanosine (8-OHdG) and malonaldehyde (MDA) levels were observed (Zhou et al., 2020); the aforementioned conditions were reported as indicators in asthmatic patients (Sandeep et al., 2010). However, in humans, the mechanism underlying the mediation of oxidative stress to respiratory and allergic symptoms due to phthalate exposure has not yet been elucidated. Thus far, only one study has investigated the association between phthalate exposure and doctor-diagnosed asthma in adolescents by considering the oxidative stress biomarker 8-OHdG as a mediator (Franken et al., 2017). However, the study did not find a mediation effect of 8-OHdG. Franken et al. hypothesized that the lack of mediation could be attributed to 8-OHdG not being an optimal oxidative stress biomarker. This could be due to oxidative stress changes that can be displayed not only with DNA-damaged cell structures but also with altered nucleic acids, carbohydrates, lipids, or proteins (Birben et al., 2012). For instance, increased lipid peroxidation, expressed with hexanoyl lysine (HEL) and 4-hydroxynonenal (HNE) has been reported in patients with atopic keratoconjunctivitis patients (Wakamatsu et al., 2010). Additionally, *in vitro* studies

on lipid peroxidation in association with immune and airway inflammatory responses by inducing dysfunction in small airway epithelial cells (Galam et al., 2015; Liu et al., 2020) or by the regulation of NF-κB (Yadav and Ramana, 2013) have been carried out. Due to the heterogeneity of respiratory and allergic diseases, it is important to explore various oxidative stress biomarkers such as oxidative stress biomarkers 8-OHdG for DNA damage, as well as HEL and HNE since lipid peroxidation is associated with allergic responses.

Thus, in this study investigated (i) the possible individual and mixed effects of phthalate metabolites using logistic regression, WQS regression, and BKMR models on children's wheeze, rhino-conjunctivitis, and eczema, and (ii) the mediating effect of oxidative stress biomarkers, 8-OHdG, HEL, and HNE, on the association between phthalate exposure and allergic diseases.

2.2. Materials and methods

2.2.1. Study population

The study participants for this study are the same children described in chapter one shown in flow chart figure 1.1 A.

2.2.2. Ethics

This study ethical approval and parents' consent was obtained as described in chapter 1.

2.2.3. Questionnaire

Wheeze, rhino-conjunctivitis, and eczema were defined by the International Study on Asthma and Allergies in Childhood (ISAAC) questionnaire (Asher et al., 1995) completed by parents of the children. The children were classified as having wheezing based on the yes answer to the question: 'Has your child had wheezing or whistling in the chest in the last 12 months?'. Rhino-conjunctivitis was defined by the 'yes' response to both of the following questions: (a) 'Has your child experienced sneezing or a runny/stuffy nose in the absence of a cold or flu in the last 12 months?' and (b) 'This child's nose problem was accompanied by itchy or watery eyes?'. Eczema was defined based on 'yes' answer to all the following three assertions (a) "Presence of an itchy rash that come and go for at least 6 months," or (b) "In the last 12 months presence of an itchy rash on below-mentioned areas" or (c) "Presence of an itchy rash on one or several of the following areas: around the neck, ears, and eyes, the folds of inside the elbows, on the back of knees, in front of the ankles, or under the buttocks." Personal and building characteristics were also collected, including sex, parental history of allergy, body mass index (BMI), environmental tobacco smoke (ETS), building age, and

dampness index (0–5) in each house was determined by adding the "yes" responses given a value of 1 for each dampness indicators, namely condensation, water leakage, mold odor, visible mold, and humidity.

2.2.4. Analytical methods of phthalate metabolites and oxidative stress biomarkers

Measured phthalate metabolites data described in chapter 1 was used in this chapter 2. Determination of the three oxidative stress biomarkers, 8-OHdG, HEL, and HNE, in urine samples has also been reported in (Ait Bamai et al., 2019). Briefly, 8-OHdG levels within the range of 0.5–200 ng/mL were measured using an 8-OHdG Check ELISA kit (Japan Institute for the Control of Aging, Nikken SEIL Co., Shizuoka, Japan). HEL in a range of 2–700 nmol/L was analyzed using the HEL ELISA kit (Japan Institute for the Control of Aging, Nikken SEIL Co.) and the OxiSelect HNE Adduct Competitive ELISA kit (Cell Biolabs, San Diego, CA, USA) was used to measure HNE in a range of 1.56–200 μg/mL. HEL and HNE analyses were performed by MACROPHI Inc. Kagawa, Japan. The precision of the quantitative assay for oxidative stress biomarkers was determined. The CVs ranged from 7.0% to 8.4% for 8-OHdG, from 3.4% to 10.4% for HEL, and from 1.0% to 5.4% for HNE. The recovery rate of 8-OHdG in urine samples was between 95% and 114%. The calibration curves for HEL and HNE showed good linearity, with $R^2 > 0.997$ (Ait Bamai et al., 2019).

2.2.5. Statistical analysis

For descriptive analyses, continuous variables were described as mean ± standard deviation, and categorical variables as numbers and percentages. The chi-square test and t-test were used to analyze categorical and continuous variables, respectively. The Mann-Whitney *U-test* was used to compare phthalate metabolites and oxidative stress biomarker levels between children with and without symptoms. Statistical analyses were conducted with their molar sum as ∑DBP for (MiBP and MnBP), ∑DEHP for (MEHP, MEOHP, MEHHP, and MECPP), and ∑DINP for (MINP, OH-MINP, and cx-MINP).

Logistic regression analysis was conducted to analyze the association of individual phthalates and oxidative stress biomarkers with health outcomes. The odds ratios (ORs) and 95% confidence intervals (95% CIs) of the outcomes were computed using the JMP clinical software (version 6.0; SAS Institute, Inc., Cary, NC, USA).

To comprehensively analyze the joint effect of 10 phthalate metabolites on health outcomes (wheeze, rhino-conjunctivitis, and eczema), WQS regression and BKMR models were used. The WQS regression model calculated both positive and negative associations weighted linear indices and identified chemicals of concern in the mixture. The corresponding weight index based on quartiles of all the metabolites in the mixture showed the contribution of a particular chemical to the WQS index (Carrico et al., 2015). WQS regression utilizes the following equation:

$$g(\mu) = \beta_0 + \beta_1 \left(\sum_{i=1}^{c} \omega_i q_i \right) + z' \varphi$$

where $g()$ is the link function, μ is the mean of the outcome, ω_i is the weight associated with the ith component, q_i refers to different quantiles, $(\sum_{i=1}^{c} \omega_i q_i)$ represents the weight quantile sum of the c components in the mixture, z' is the vector of covariates, and φ is the coefficient for the covariates vector of parameters associated with the covariates. The weights associated with each component in the mixture were estimated as the average of 386 bootstrap samples. A higher contribution to the weighted index was illustrated by metabolites with higher weights. Because of the small sample size, the data was not split into test and validation sets to allow for stable weight estimates.

BKMR is a non-parametric approach that allowed us to explore the joint effects of chemical exposure on health outcomes (Bobb et al., 2018). Thus, to examine phthalate metabolite exposure with wheeze, rhino-conjunctivitis, and eczema outcomes, the BKMR model was executed using a kernel machine regression equation.

$$Y_i = h(z_i) + x_i\beta + \epsilon_i$$

where Y_i is the health outcome for individual i (i = 1, 2, 3…n), z_i is the chemical exposure, x_i is the potential confounder, β represents the effect of the covariates, ϵ_i is the residual that obeys the normal distribution, and h() is the function that fits the exposure and the outcome considering nonlinear interactions between the exposures. Phthalate metabolites with the same parent phthalate were grouped into four groups (group 1: MiBP and MnBP; group 2: MBzP; group 3: MEHP, MEOHP, MEHHP, and MECPP; and group 4, MINP, OH-MINP, and cx-MINP). A hierarchical variable selection method with 30,000 iterations was employed to estimate the posterior inclusion probability of highly correlated variables. The

interaction of each phthalate metabolite was evaluated by comparing a single metabolite health risk when all other exposures were fixed to their 25th percentile to when all metabolites were fixed to their 75th percentile. The overall metabolite mixture effect on wheeze, rhino-conjunctivitis, and eczema was evaluated by comparing all metabolites at a percentile when all of them were at their 50th percentile as the reference. The probability of the group and the chemical in each group were indicated by the group probabilities of inclusion (groupPIP) and conditional PIP (condPIP). Both WQS (version 3.0.4) and BKMR (version 0.2.0) were implemented using R statistical software (version 3.5.1).

Baron and Kenny's (1986) regression was used for mediation analysis to investigate the possible mediating effect of oxidative stress biomarkers in the association of phthalates with wheeze, rhino-conjunctivitis, and eczema using SPSS (Version 27.0, IBM SPSS Inc., USA). Bootstrap approach with 5,000 replications was used for indirect mediation of oxidative stress biomarkers in the association between phthalates and health outcomes in the PROCESS macro for SPSS.

All analytic models used ln-transformed phthalate metabolites and oxidative stress levels adjusted for confounders: sex, parental history of allergy, and creatinine. To select confounders for adjustment, first all significant variables listed in Table 1 and literature-based covariates were included with each outcome independently. Then covariates that did not make an estimated change by more than 10% were eliminated (VanderWeele, 2019). Statistical significance was set at $p \leq 0.05$, and a *p*-value between 0.051 and 0.100 was considered marginally significant.

2.3. Results

The demographic and housing characteristics of the study participants are presented in Table 2.1. A total of 386 children aged 7 years were included in this study. Children with wheezing had a higher BMI and were more likely to live in old and damp buildings compared to children with no wheezing. Children with rhino-conjunctivitis had a higher prevalence of parental allergies. No significant gender differences were observed in the BMI (boy's median: 15.5 kg/m^2; girl's median: 15.4 kg/m^2) of the study participants.

Table 2.1. The Prevalence of wheeze, rhino-conjunctivitis and eczema symptoms in different personal and building characteristics (n=386)

		Total n (%)	Wheeze		Rhino-conjunctivitis		Eczema	
			Yes (N=109)	No (N=277)	Yes (N=101)	No (N=285)	Yes (N=116)	No (N=270)
Sex	Boys	203 (52.6)	62 (56.8)	141 (50.9)	60 (59.4)	143 (50.2)	63 (54.3)	140 (51.8)
	Girls	183 (47.4)	47 (43.2)	136 (49.1)	41 (40.6)	142 (49.8)	53 (45.7)	130 (48.2)
Parental history of allergy	Yes	118 (30.6)	40 (36.7)	78 (28.2)	41 (40.6)	77 (27.0) *	43 (37.1)	75 (27.8)
	No	268 (69.4)	69 (63.3)	199 (71.8)	60 (59.4)	208 (73.0)	73 (62.9)	195 (72.2)
ETS	Yes	122 (31.6)	38 (34.9)	84 (30.3)	26 (25.7)	96 (33.7)	36 (31.0)	86 (31.8)
	No	264 (68.3)	71 (65.1)	193 (69.1)	75 (74.3)	189 (66.3)	80 (68.9)	211 (68.2)
Annual household income (Japanese Yen)	< 3 Million	48 (12.4)	16 (15.1)	32 (12.2)	14 (14.3)	34 (12.5)	15 (13.4)	33 (12.8)
	≥ 3 Million	321 (83.2)	90 (84.9)	231 (87.8)	84 (85.7)	237 (87.5)	97 (86.6)	224 (87.2)
	Missing	17 (4.4)	-	-	-	-	-	-
Building age (Years)	Median (IQR)	13 (7 – 24)	19 (9 – 25)	12 (7 – 22) *	15 (7 – 23)	12 (7 – 24)	12 (7 – 25)	13 (7 – 25)
BMI (kg/m^2)	Mean ± SD	15.9 ± 2.1	16.5 ± 2.4	15.7 ± 1.8 *	15.8 ± 2.0	16.0 ± 2.1	16.1 ± 2.1	15.8 ± 2.0
Urinary creatinine (g/l)	Mean ± SD	0.9 ± 0.4	0.9 ± 0.3	0.9 ± 0.4	0.9 ± 0.3	0.9 ± 0.4	0.9 ± 0.3	0.9 ± 0.4
Dampness index (0-5)	Mean ± SD	1.6 ± 1.2	1.9 ± 1.3	1.5 ± 1.2*	1.7 ± 1.3	1.6 ± 1.2	1.7 ± 1.3	1.7 ± 1.2

ETS: Environmental tobacco smoke

The distributions of phthalate metabolites and oxidative stress biomarkers among children stratified by symptoms are presented in Table 2.2. The results showed significantly ($p < 0.05$) higher MINP and cx-MINP concentrations in children with wheeze and MEOHP and MECPP in children with eczema. Additionally, marginally ($p < 0.1$) high concentrations of MEOHP, MEHHP, MECPP, ∑DEHP, and OH-MINP in children with wheeze and MEHHP, ∑DEHP, OH-MINP and ∑DINP in children with eczema were observed.

Table 2.2. Urinary concentration of phthalate metabolites and oxidative stress biomarkers in Median (IQR) of children with symptoms compared with controls (N=386)

Parent phthalates	Phthalate metabolites (ng/mL)	Wheeze		Rhino-conjunctivitis		Eczema	
		With (N=109)	Without (N=277)	With (N=101)	Without (N=285)	With (N=116)	Without (N=270)
DBP	MiBP	12.2 (6.9 – 28.1)	12.1 (7.0 – 27.2)	12.4 (6.7 – 26.4)	12.1 (7.1 – 12.0)	11.8 (6.8 – 32.5)	12.4 (7.1 – 28.8)
	MnBP	36.1 (22.3 – 59.1)	34.5 (20.3 – 58.8)	33.6 (21.8 – 57.7)	35.3 (20.5 – 59.8)	43.4 (24.1 – 61.1)	33.7 (20.4 – 58.4)
	∑DBP (µmol/L)	0.24 (0.14 – 0.43)	0.22 (0.13 – 0.41)	0.23 (0.14 – 0.45)	0.23 (0.13 – 0.42)	0.27 (0.15 – 0.48)	0.22 (0.13 – 0.40)
BBzP	MBzP	1.4 (0.7 – 3.4)	1.4 (0.6 – 3.7)	1.5 (0.7 – 3.6)	1.4 (0.7 – 3.4)	1.6 (0.6 – 3.7)	1.4 (0.7 – 3.4)
DEHP	MEHP	4.1 (2.3 – 6.8)	4.0 (2.4 – 7.2)	4.4 (2.3 – 7.2)	3.9 (2.4 – 6.9)	4.3 (2.7 – 7.7)	3.9 (2.2 – 6.8)
	MEOHP	20.6 (12.1 – 34.8)	20.4 (12.3 – 32.7) +	20.6 (13.6 – 32.1)	20.4 (11.6 – 34.6)	25.2 (14.4 – 35.9)	19.6 (11.0 – 31.3) *
	MEHHP	26.4 (15.8 – 46.2)	26.7 (16.5 – 43.5) +	28.5 (17.1 – 41.9)	26.0 (15.8 – 44.9)	30.5 (17.5 – 47.3)	24.9 (15.7 – 43.5) +
	MECPP	39.8 (23.3 – 63.6)	37.8 (23.3 – 63.6) +	38.1 (25.8 – 68.6)	38.5 (21.3 – 65.9)	45.9 (28.8 – 71.2)	36.1 (20.2 – 63.4) *
	∑DEHP (µmol/L)	0.31 – 1.17 – 0.51)	0.29 (0.18 – 0.49) +	0.30 (0.20 – 0.48)	0.29 (0.17 – 0.51)	0.37 (0.21 – 0.54)	0.28 (0.16 – 0.47) +
DINP	MINP	0.9 (0.5 – 1.4)	0.6 (0.4 – 1.2) *	0.6 (0.4 – 1.4)	0.6 (0.3 – 1.1)	0.7 (0.4 – 1.3)	0.6 (0.4 – 1.2)
	OH-MINP	4.7 (2.2 – 8.4)	3.9 (2.3 – 7.1) +	3.8 (2.0 – 7.5)	4.3 (2.2 – 7.6)	4.3 (2.7 – 8.3)	4.0 (2.0 – 7.1) +
	cx-MINP	2.6 (1.5 – 4.9)	2.3 (1.3 – 4.5) *	2.5 (1.2 – 4.2)	2.4 (1.4 – 4.7)	2.5 (1.5 – 5.5)	2.4 (1.3 – 4.2)
	∑DINP (µmol/L)	0.03 (0.01 – 0.05)	0.02 (0.01 – 0.04)	0.02 (0.01 – 0.05)	0.02 (0.01 – 0.04)	0.02 (0.01 – 0.04)	0.02 (0.01 – 0.04) +
Oxidative stress biomarkers							
	8-OHdG (ng/mL)	9.4 (6.8 – 12.3)	10.4 (7.5 – 13.5)	9.7 (7.1 – 12.2)	10.5 (7.4 – 13.5)	10.1 (7.9 – 12.6)	10.2 (7.1 – 13.5)
	HEL (nmol/L)	121.0 (81.1 – 177.3)	115.1 (75.9 – 178.3) +	110.0 (77.9 – 164.5)	116.6 (74.9 – 181.6)	120.9 (81.4 – 184.9)	115.8 (74.2 – 167.8)
	HNE (µg/mL)	49.4 (24.4 – 91.3)	51.2 (24.0 – 111.3)	46.3 (22.6 – 102.9)	52.8 (25.1 – 105.5)	55.2 (26.3 – 100.2)	48.0 (23.8 – 107.3)

Abbreviations; MiBP: mono-isobutyl phthalate, MnBP: mono-n-buty phthalate, MBzP: mono-benzyl phthalate, MEHP: mono (2-ethylhexyl) phthalate, MEOHP: mono (2-ethyl-5-oxohexyl) phthalate, MEHHP: mono (2-ethyl-5-hydroxyhexyl) phthalate, MECPP: mono (2-ethyl-5-carboxypentyl) phthalate, MINP: mono-isononyl phthalate, OH-MINP: mono-hydroxy-isononyl phthalate, cx-MINP: mono(carboxy-isononyl phthalate), ∑DBP: dibutyl phthalate: sum of metabolites MiBP and MnBP, ∑DEHP: di (2-ethylhexyl) phthalate sum of metabolites MEHP, MEOHP, MEHHP and MECPP, ∑DINP: diisononyl phthalate sum of metabolites MINP, OH-MINP, and cx-MINP, 8-OHdG: 8-hydroxy-2′-deoxyguanosine, HEL: hexanoyllysine, and HNE: 4-hydroxynonenal. P values were calculated based on cr corrected metabolites with non-parametric Mann-Whitney U test. * $p<0.05$, + $p<0.1$

The detection frequencies of the measured oxidative stress biomarkers were 100% for 8-OHdG, 98.1% for HEL, and 95.8% for HNE (data not shown). Among them, HEL showed a marginally increased level in children with wheeze. The oxidative stress biomarker HNE was higher in boys and children with a parental history of allergy and a marginal increase in children with < 3 million yen annual household incomes (Table 2.3).

Table 2.3. Relation of oxidative stress biomarkers with personal and building characteristics in median (IQR)

		N (%) or mean ± SD	8-OHdG	HEL	HNE
Gender	Boys	203 (52.6)	10.4 (9.0 - 12.5)	130.8 (100.5 - 165.2)	62.9 (39.2 - 102.7) **
	Girls	183 (47.4)	10.7 (8.9 - 13.2)	123.3 (96.1 - 164.2)	48.8 (31.3 - 78.0)
Parental history of allergy	Yes	118 (30.6)	10.5 (8.8 - 12.7)	122.4 (96.1 - 169.7)	49.5 (34.3 - 69.0) *
	No	268 (69.4)	10.7 (9.0 - 12.9)	126.7 (101.8 - 162.3)	62.2 (35.7 - 95.1)
ETS	Yes	122 (31.6)	10.6 (9.1 - 12.5)	124.1 (98.7 - 166.1)	63.1 (39.8 - 92.6)
	No	264 (68.3)	10.6 (8.6 - 13.1)	129.9 (98.3 - 164.0)	52.4 (34.4 - 88.0)
Annual household income (Japanese Yen)	< 3 Million	48 (12.4)	10.0 (8.9 - 12.4)	135.6 (100.1 - 180.1)	69.2 (38.1 - 109.2) +
	≥ 3 Million	321 (83.2)	10.7 (9.0 - 12.8)	127.2 (98.6 - 166.4)	54.5 (34.9 - 86.3)
BMI	kg/m2	15.9 ± 2.1	-0.07 [a]	0.02 [a]	0.05 [a]
Building age	Years	16.2 ±11.2	0.05	0.00	-0.01
Dampness index	0-5	1.6 ± 1.2	0.12	0.01	-0.02

IQR: inter quartile range, ETS: environmental tobacco smoke, BMI: body mass index, 8-OHdG: 8-hydroxy-2-deoxyguanosine, HEL; hexanoyl lysine, and HNE: 4-hydroxynonenal. Oxidative stress biomarkers were creatinine corrected, [a]: Spearman's correlation **p <0.001, * p<0.05, +p<0.1

The results of multivariable logistic regression analysis to assess the individual effects of phthalate metabolites and oxidative stress biomarkers on wheeze, rhino-conjunctivitis, and eczema are presented in Table 2.4. The results showed a positive significant ($p < 0.05$) association between MECPP, cx-MINP, and ∑DINP with wheeze. Additionally, marginal positive associations ($p < 0.1$) were observed between MEHHP, ∑DEHP, MINP, and OH-MINP with wheeze and MEOHP, OHMINP, cx-MINP and ∑DINP with eczema. Among the oxidative stress biomarkers, HEL showed marginally positive association with wheeze and eczema. No association was observed between phthalate metabolites and oxidative stress biomarkers (Table 2.5).

Table 2.4. Association between allergic symptoms with individual phthalates metabolites and oxidative stress biomarkers

Parent Phthalates	Phthalate metabolites	Wheeze AOR (95% CI)	Rhino-conjunctivitis AOR (95% CI)	Eczema AOR (95% CI)
DBP	MiBP	1.06 (0.84 - 1.33)	1.13 (0.89 - 1.44)	1.02 (0.61 - 2.04)
	MnBP	1.22 (0.88 - 1.69)	1.07 (0.77 - 1.50)	1.09 (0.79 - 1.49)
	∑DBP	1.19 (0.87 - 1.63)	1.19 (0.86 - 1.64)	1.02 (0.75 - 1.39)
BBzP	MBzP	1.07 (0.90 - 1.25)	1.09 (0.92 - 1.28)	1.10 (0.81 - 1.49)
DEHP	MEHP	1.15 (0.84 -1.59)	1.25 (0.90 - 1.73)	1.21 (0.89 - 1.65)
	MEOHP	1.32 (0.93 - 1.86)	1.18 (0.83 - 1.69)	1.33 (0.95 - 1.88) +
	MEHHP	1.33 (0.95 - 1.89) +	1.21 (0.85 - 1.72)	1.26 (0.90 - 1.77)
	MECPP	1.41 (1.02 - 1.97) *	1.21 (0.87 - 1.70)	1.29 (0.94 - 1.77)
	∑DEHP	1.36 (0.96 - 1.94) +	1.26 (0.88 - 1.82)	1.31 (0.93 - 1.84)
DINP	MINP	1.28 (0.97 - 1.68) +	1.21 (0.91 - 1.60)	1.08 (0.83 - 1.41)
	OH-MINP	1.30 (0.98 - 1.74) +	1.10 (0.82 - 1.47)	1.31 (0.99 - 1.73) +
	cx-MINP	1.40 (1.07 - 1.86) *	1.03 (0.77 - 1.36)	1.25 (0.96 - 1.64) +
	∑DINP	1.35 (1.01 - 1.83) *	1.13 (0.83 - 1.53)	1.29 (0.96 - 1.72) +
Oxidative stress biomarkers				
	8-OHdG (ng/mL)	1.05 (0.48 - 2.30)	0.68 (0.30 - 1.52)	0.85 (0.39 - 1.84)
	HEL (nmol/L)	1.52 (0.98 - 2.35) +	1.06 (0.67 - 1.67)	1.42 (0.94 - 2.15) +
	HNE (µg/mL)	1.25 (0.84 - 1.86)	1.00 (0.66 - 1.48)	0.99 (0.69 - 1.43)

All phthalate metabolites, sum of phthalates and oxidative stress biomarkers were ln transformed concentrations Adjusted with sex, parental history of allergy, creatinine. * $p<0.05$, + $p<0.1$.
Abbreviations used: AOR: adjusted odds ratio, CI: confidence interval, MiBP: mono-iso-butyl phthalate, MnBP: mono-n-butyl phthalate, ∑DBP: dibutyl phthalate sum of metabolites MiBP and MnBP, MBzP: monobenzyl phthalate, MEHP: mono(2-ethylhexyl) phthalate, MEHHP: mono(2-ethyl-5-hydroxyhexyl) phthalate, MEOHP: mono(2-ethyl-5-oxohexyl), MECPP: mono(2-ethyl-5-carboxypentyl) phthalate, MINP: mono-isononyl phthalate, OH-MINP: mono-hydroxy-isononyl phthalate, cx-MINP: mono-carboxy-isononyl phthalate (carboxy-isononyl phthalate), ∑DEHP: di (2-ethylhexyl) phthalate sum of metabolites MEHP, MEOHP, MEHHP and MECPP, ∑DINP: diisononyl phthalate sum of metabolites MINP, OH- MINP, and cx-MINP, 8-OHdG: 8-hydroxy-2′-deoxyguanosine, HEL: hexanoyllysine, and HNE: 4-hydroxynonenal.

Table 2.5. Association between phthalate metabolites and oxidative stress biomarkers

Parent phthalates	Phthalate Metabolites (ng/mL)	8-OHdG (ng/mL) AOR (95% CI)	HEL (nmol/L) AOR (95% CI)	HNE (µg/mL) AOR (95% CI)
DBP	MiBP	0.97 (0.92 - 1.02)	1.01 (0.90 - 1.12)	0.94 (0.82 - 1.06)
	MnBP	1.01 (0.94 - 1.08)	1.02 (0.97 - 1.08)	0.98 (0.82 - 1.14)
	∑DBP (µmol/L)	1.02 (0.98 – 1.06)	1.04 (0.97 – 1.11)	0.96 (0.88 – 1.04)
BBzP	MBzP	1.00 (0.97 - 1.04)	0.93 (0.82 - 1.03)	1.02 (0.93 - 1.10)
DEHP	MEHP	0.95 (0.88 - 1.01)	1.00 (0.88 - 1.11)	0.88 (0.72 - 1.04)
	MEOHP	0.96 (0.88 - 1.03)	0.99 (0.88 - 1.11)	0.94 (0.76 - 1.11)
	MEHHP	0.95 (0.88 - 1.03)	1.02 (0.91 - 1.12)	0.95 (0.76 - 1.13)
	MECPP	0.97 (0.90 - 1.04)	1.02 (0.91 - 1.12)	0.96 (0.80 - 1.13)
	∑DEHP (µmol/L)	0.96 (0.88 – 1.04)	1.00 (0.88 – 1.12)	0.94 (0.76 – 1.11)
DINP	MINP	1.00 (0.93 - 1.06)	0.95 (0.86 - 1.05)	0.94 (0.79 - 1.08)
	OH-MINP	1.01 (0.95 - 1.07)	1.00 (0.90 - 1.10)	1.00 (0.85 - 1.15)
	cx-MINP	1.01 (0.95 - 1.07)	0.99 (0.90 - 1.09)	0.97 (0.83 - 1.11)
	∑DINP (µmol/L)	1.01 (0.95 – 1.08)	0.99 (0.89 – 1.09)	0.98 (0.83 – 1.13)

Phthalate metabolites and oxidative stress biomarkers were ln transformed concentration. Adjusted with sex, parental history of allergy, creatinine. Abbreviations used: AOR: adjusted odds ratio, CI: confidence interval, MiBP: mono-iso-butyl phthalate, MnBP: mono-n-butyl phthalate, MBzP: monobenzyl phthalate, MEHP: mono(2- ethylhexyl) phthalate, MEHHP: mono(2-ethyl-5-hydroxyhexyl) phthalate, MEOHP: mono(2-ethyl-5 -oxohexyl), MECPP: mono(2-ethyl-5-carboxypentyl) phthalate, MiNP: mono-isononyl phthalate, OH-MiNP: mono-hydroxy-isononyl phthalate, cx-MiNP: mono-carboxy-isononyl phthalate (carboxy-isononyl phthalate), ∑DBP: dibutyl phthalate: sum of metabolites MiBP and MnBP, ∑DEHP: di (2-ethylhexyl) phthalate sum of metabolites MEHP, MEOHP, MEHHP and MECPP, ∑DINP: diisononyl phthalate sum of metabolites MINP, OH-MINP, and cx-MINP, 8-OHdG: 8-hydroxy-2′-deoxyguanosine, HEL: hexanoyllysine, and HNE: 4-hydroxynonenal.

Furthermore, WQS and BKMR analyses were performed to examine the effects of chemical mixtures on health outcomes. Figure 2.1 shows the WQS regression analysis of the association between the combined ten phthalate metabolites and allergic symptoms. A significantly positive association was observed in the WQS index of wheeze and eczema. For wheeze (OR = 1.46; 95% CI: 1.09–1.96, p = 0.011), MINP (28%) was predominant in the WQS index, followed by MECPP (18%) and cx-MINP (14%) (Figure 2.1 A). As shown in Figure 2C, the WQS index was positively associated with eczema (1.40; 1.07–1.82, p = 0.011) and MEOHP contributed one-fourth of the association (25%), followed by OH-MINP (21.0%) and MECPP (19.8%). The WQS index was not significant for rhino-conjunctivitis (1.23; 0.93–1.63, p = 0.131) (Figure 2.1 B). The negative WQS models for all symptoms were not statistically significant (data not shown).

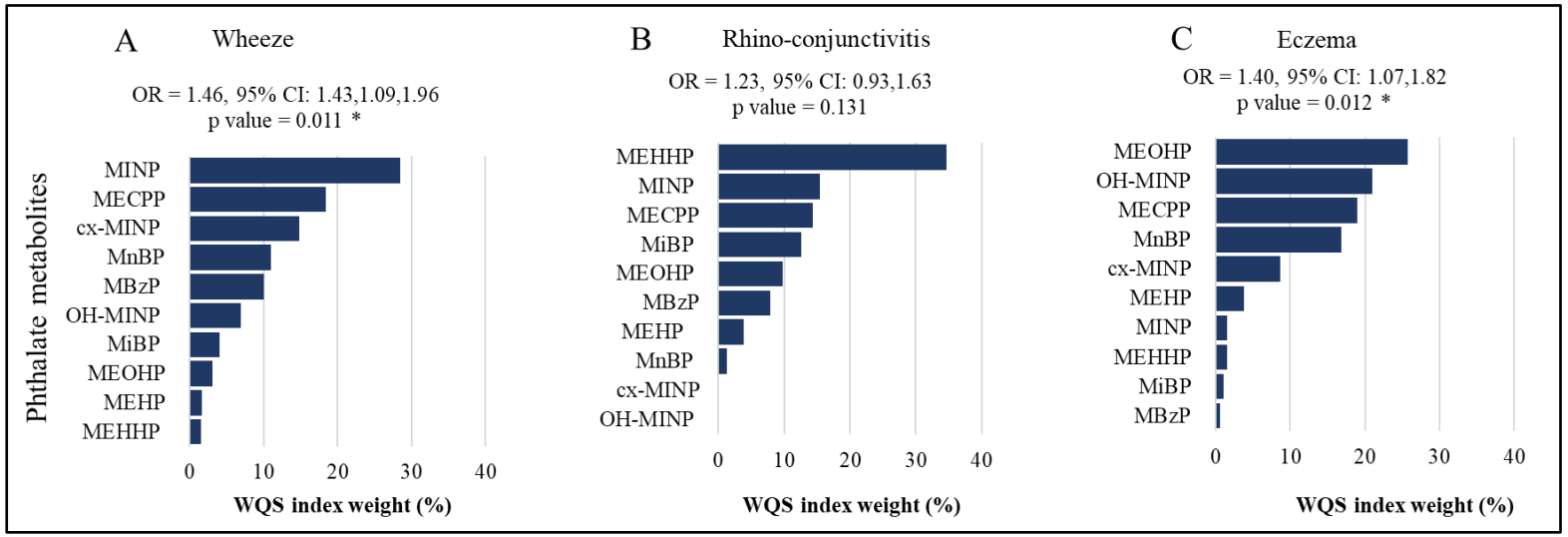

Figure 2.1. WQS regression analysis on association of ln transformed urinary phthalate metabolites with A: wheeze, B: rhino-conjunctivitis, and C: eczema. Bar graphs show the magnitude of WQS weight for each metabolite. Models are adjusted with sex, parental history of allergy, and creatinine for the odds ratios (95% confidence interval) for association between phthalate metabolites with allergies. *$p < 0.05$.

Table 2.6 shows the posterior inclusion probabilities (PIPs) for the four groups (group PIPs) and (condPIPs) obtained from the BKMR model for the three outcomes. In all allergies, the DINP metabolites group showed the highest group PIPs, followed by DEHP metabolites. Among the group of DINP metabolites, high condPIPs were observed in MINP for wheeze and cx-MINP for rhino-conjunctivitis and OH-MINP for eczema. As for the DEHP metabolites, MECPP for wheeze and MEHP for rhino-conjunctivitis and MEOHP for eczema showed high condPIPs.

Table 2.6. Bayesian kernel machine regression model of PIPs for group PIPs and condPIPs

Parent phthalates	Phthalate metabolites	Group	Wheeze		Rhino-conjunctivitis		Eczema	
			group PIPs	condPIPs	group PIPs	condPIPs	group PIPs	condPIPs
DBP	MiBP	1	0.61	0.47	0.31	0.51	0.39	0.32
	MnBP	1	0.61	0.53	0.31	0.49	0.39	0.68
BBzP	MBzP	2	0.58	1.0	0.36	1.0	0.42	1.00
DEHP	MEHP	3	0.67	0.21	0.35	0.27	0.51	0.13
	MEOHP	3	0.67	0.24	0.35	0.22	0.51	0.41
	MEHHP	3	0.67	0.23	0.35	0.24	0.51	0.16
	MECPP	3	0.67	0.31	0.35	0.26	0.51	0.29
DINP	MINP	4	0.72	0.35	0.48	0.26	0.56	0.27
	OH-MINP	4	0.72	0.29	0.48	0.34	0.56	0.46
	cx-MINP	4	0.72	0.34	0.48	0.40	0.56	0.27

Adjusted with sex, parental allergy, and creatinine. PIPs: posterior inclusion probabilities, condPIPs: conditional PIPs. MBzP in grouped PIPs was converted to molar concentration to make uniform with molar sum concentrations of DBP, DEHP, DINP metabolites

The univariate estimation of exposure-response functions on the relationship between phthalate metabolites and health outcomes is shown in Figure 2.2. When all other metabolites were at their median levels, MECPP, and OH-MINP were positively associated with wheeze (Figure 2.2 A), while no noticeable trend was observed in rhino-conjunctivitis (Figure 2.2 B). Regarding eczema, OH-MINP was positively associated (Figure 2.2 C). Figure 2.3 shows no interactions between phthalate metabolites and health outcomes. The effect of the overall metabolite mixture on health outcomes was assessed by comparing metabolites at a particular percentile with all metabolites at the 50^{th} percentile. In this study, although not statistically significant due to the wide tail above the 50^{th} percentile, the overall metabolite mixture showed a positive association with eczema (Figure 2.4 C).

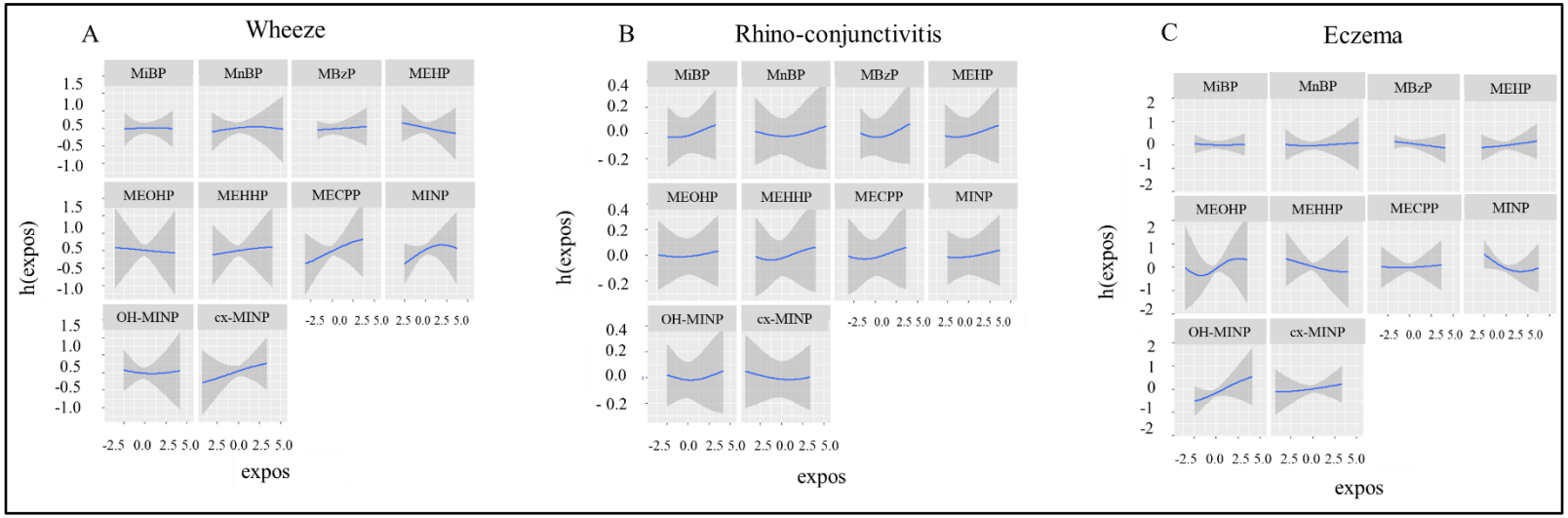

Figure 2.2. BKMR univariate estimation of exposure-response relationships for the associations between phthalate metabolite levels with wheeze, rhino-conjunctivitis and eczema by holding other phthalate metabolite levels at their median. The models were adjusted for sex, parental history of allergy, and creatinine.

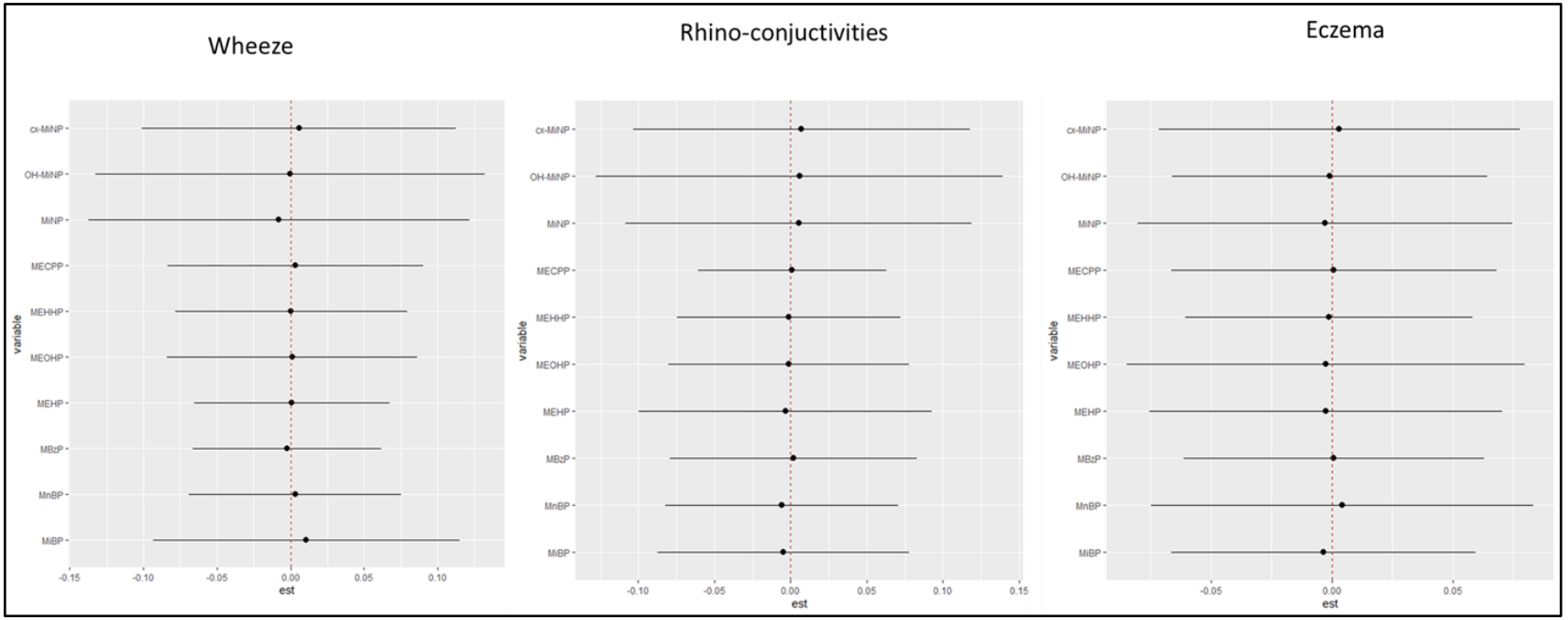

Figure 2.3. Interaction among phthalate metabolites. Interaction was employed by comparing a single metabolite health risk when all other exposures are fixed to their 25th percentile to when all metabolites are fixed to their 75th percentile. Abbreviations: MiBP: mono-iso-butyl phthalate, MnBP: mono-n-butyl phthalate, MBzP: monobenzyl phthalate, MEHP: mono (2- ethylhexyl) phthalate, MEHHP: mono(2-ethyl-5-hydroxyhexyl) phthalate, MEOHP: mono(2-ethyl-5-oxohexyl), MECPP: mono(2-ethyl-5-carboxypentyl) phthalate, MINP: mono-isononyl phthalate, OH-MINP: mono-hydroxy-isononyl phthalate, cx-MINP: mono-carboxy-isononyl phthalate (carboxy-isononyl phthalate),

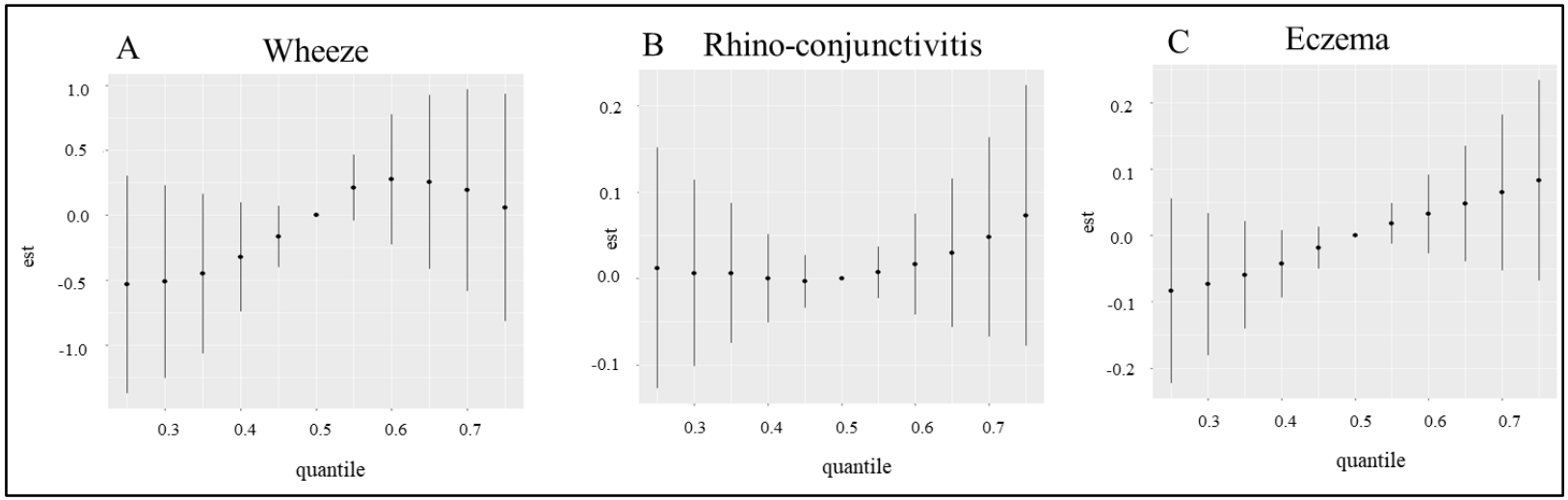

Figure 2.4. Overall effect of mixture on A: wheeze, B: rhino-conjunctivitis, and C: eczema when all metabolites at their 50th percentile by BKMR. The models were adjusted for sex, parental history of allergy, and creatinine.

In this study, considering the results from logistic regression, WQS, and BKMR, the metabolites of DEHP and DINP were associated with wheeze and eczema. Thus, the mediation effect of oxidative stress on the association of ∑DEHP and ∑DINP with wheeze and eczema are investigated. However, the result showed no mediation effect of oxidative stress in the association of ∑DEHP and ∑DINP with wheeze and eczema (Figures 2.5 A, B).

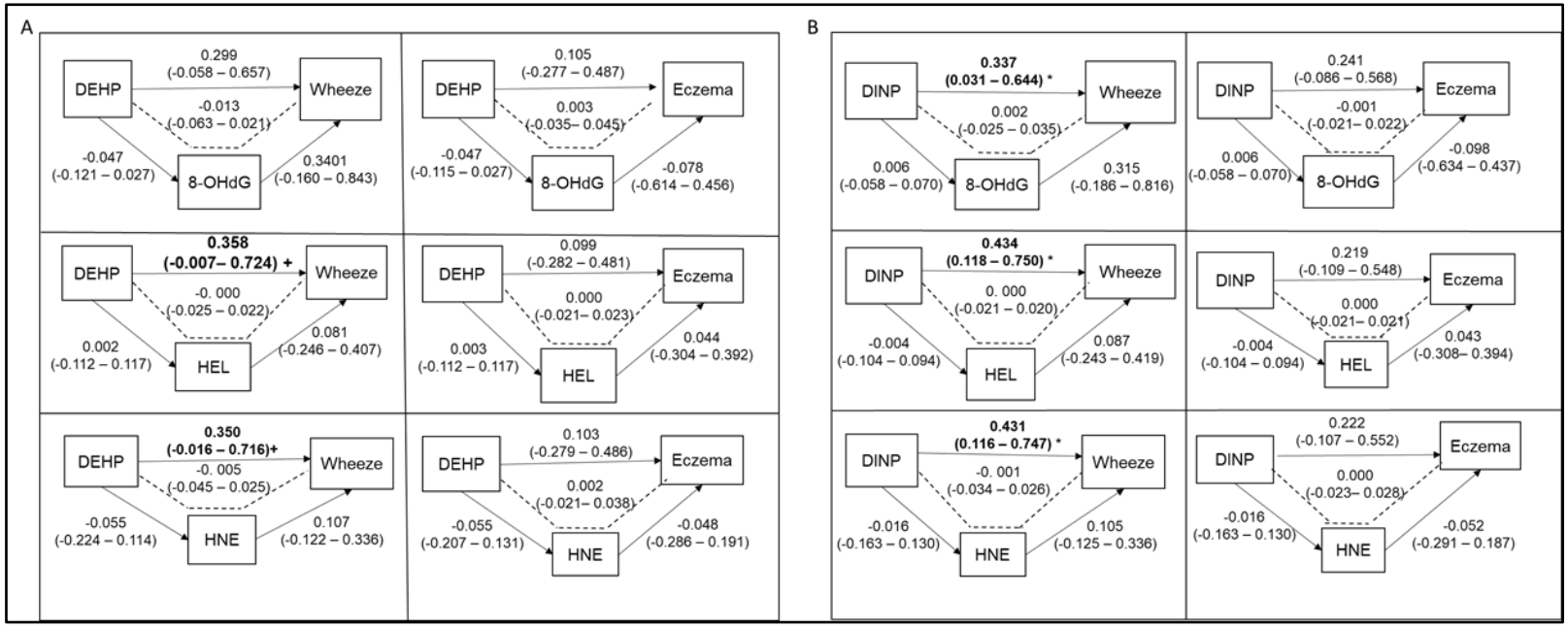

Figure 2.5. Mediation analysis to evaluate association between phthalates (A: DEHP, B: DINP) with wheeze and eczema. Models were adjusted with sex, BMI and creatinine. Molar concentration of phthalates was ln transformed. Abbreviations: 8-hydroxy-2′-deoxyguanosine (8-OHdG), hexanoyl lysine (HEL), and 4-hydroxynonenal (HNE), di(2-ethylhexyl) phthalate (DEHP), diisononyl phthalate (DINP). Brocken lines indicate the mediation or indirect effect of oxidative stress biomarkers in association between phthalate and health outcome. * Indicates $p<0.05$, + indicates $p<0.1$.

2.4. Discussion

This study explored individual metabolites and a mixture of phthalate metabolites associated with wheeze, rhino-conjunctivitis, and eczema in children using different statistical methods. The main findings are as follows: 1) individual metabolites of MECPP and cx-MINP and ΣDINP showed a significant positive association with wheeze, and 2) the WQS index demonstrated a significant positive association between wheeze and eczema with MINP and MEOHP as the highest weighted metabolites. 3) In the BKMR analysis, the highest group PIPs were observed in DINP followed by DEHP metabolites with all allergies, 4) the overall effect of metabolite mixtures showed a positive trend with eczema, and 5) the oxidative stress biomarker HEL showed a marginal positive trend with wheeze. However, oxidative stress did not have any mediating effect on the association between phthalates and allergies (wheeze, rhino-conjunctivitis, and eczema).

Logistic regression analysis was performed using individual or similar groups of chemicals to investigate adverse health effects. In this study, MECPP, cx-MINP, and ∑DINP were positively associated with wheeze. Moreover, humans are exposed to a mixture of chemicals at a time, and it is essential to further explore the effects of chemical mixtures on health outcomes. However, traditional regression analysis models may exhibit estimation bias, mainly in the case of multicollinearity or highly correlated chemicals (Adeboye et al., 2014). Thus, the WQS and BKMR models to estimate a mixture of phthalate metabolites for health outcomes were used. The WQS regression provides a weighted index estimation effect of the chemical mixture on health outcomes. The indexes are simple to interpret and identify

chemicals of concern in association with health outcomes (Carrico et al., 2015). The BKMR approach estimates different mixtures of chemical effects associated with health outcomes including interactions among chemicals (Bobb et al., 2018). Using both WQS and BKMR allowed estimation of the environmental exposure effect of chemical mixtures on children's respiratory and allergic symptoms. Thus, using WQS and BKMR, individual metabolites in the mixture to examine each metabolite's role in driving the association with health outcomes were assessed.

In the WQS analysis, significant positive associations were observed between the mixture of metabolites with wheeze and eczema, at which the MINP and MEOHP metabolites were highly weighted, respectively.

Compared to WQS, BKMR analysis can identify the exposure–response relationship of an individual chemical in the mixture with the allergies when other chemicals are at fixed levels. In our BKMR analysis, although it does not tell if these findings are significant or not,

positive trend were observed in MECPP, MINP, and cx-MINP for wheeze, as well as in OH-MINP for eczema. These BKMR univariate estimation results are noticeably in line with WQS analysis, as the metabolites that showed positive association were among the highest weighted metabolites in WQS analysis. This consistency strengthens this study finding on evaluating which metabolites play important roles to drive the association with health outcomes. Additionally, BKMR analysis of the overall effect allowed us to examine the influence of the mixture chemicals on different effect directions when the chemicals were at a certain level. The results showed that the overall phthalate metabolite mixture showed an

increasing trend in eczema. Overall, the three models identified in this study highlight that DEHP and DINP metabolites are the primary drivers of the mixture effect in association with wheeze and eczema, which is in line with previous studies that used individual chemical regression and reported the association of wheeze and eczema with DEHP and DINP exposure in humans (Ait Bamai et al., 2018, 2014; Callesen et al., 2014).

Among the three measured oxidative stress biomarkers, only HEL showed a marginally positive association with wheeze. Paredi et al. (2000) also showed that lipid peroxidation in asthmatic patients is positively associated with asthma severity (Paredi et al., 2000). Although significant associations between phthalate metabolites and allergies were found, no significant association was observed between phthalate metabolites and oxidative stress biomarkers (Data not shown). This result is different from previous studies reporting the association between phthalates and oxidative stress, particularly 8-OHdG in children (Jacobson et al., 2020, Lee et al., 2019, Rocha et al., 2017). This could be due to differences in study design and study subjects, or it could be a chance null finding. For instance, phthalate metabolite levels in children aged 6-14 in Rocha et al. (2017) were more than double, while their 8-OHdG levels were nearly three times lower than those in the current study. To date, there is only one study in humans that investigated the mediating effect of the oxidative stress biomarker 8-OHdG on the association between phthalate metabolites and asthma (Franken et al., 2017). The result reported no mediation effect of oxidative stress due to the lack of a significant association between 8-OHdG and asthma. In line with this result, no mediating effect of the three measured oxidative stress biomarkers in the association between phthalate exposure and health outcomes was observed. Compared with adolescents' participants in

Frank et al. (2017), this study participants were younger and had lower 8-OHdG levels. Furthermore, studies have found increased oxidative stress with aging (Cui et al., 2012, Liguori et al., 2018). Hence, this participants' lower oxidative stress could be due to their young age and indicate that follow-up studies are needed to examine changes as they get older. Additionally, a previous experimental study demonstrated that low and moderate oxidative stress levels can enhance T cell receptors and inhibit p38 mitogen-activated protein kinases (Hehner et al., 2000); which has been reported to remodel and improve allergic airway responses in asthmatic patients (Bhavsar et al., 2010). Another possible reason for this study's finding of direct associations between metabolites and symptoms may be the adjuvant effect of phthalates in enhancing the immune system response (Larsen et al., 2002), but not through the mediation of oxidative stress. However, further investigation of the physicochemical characteristics of phthalates is needed to elucidate the causal relationship between oxidative stress and the adjuvant effect of phthalates. Moreover, as there is emerging evidence of the association of alternative plasticizers such as DINCH metabolite monoester mono-isononyl-cyclohexane-1,2-dicarboxylate (MINCH) with increased reactive oxygen species and oxidative stress (Schaffert et al., 2021), future studies are warranted to elucidate these associations.

2.5. Strengths and limitations

The strength of this study is that it provides data on the association of individual phthalate metabolites and their mixture with wheeze, rhino-conjunctivitis, and eczema in children, evaluated using different statistical methods such as the WQS and BKMR approaches; this was, the effect of phthalate mixture exposure was evaluated comprehensively the significance of individual and mixed phthalates on health outcomes. The current study investigated three different oxidative stress biomarkers (8-OHdG, HEL, and HNE), notably examining less studied HEL and HNE. Nevertheless, other oxidative stress markers such as MDA should be considered in future studies. As no difference was observed in comparison of personal and building characteristics between the current study participants and the 2541 eligible children (Ait Bamai et al., 2019), it strengthens the representativeness of the current study to the available cohort data. There are some potential limitations to this study. The generalizability of this study may be limited, especially as this study included only 7-year-old children; young children are still in the developing stage; therefore, oxidative stress effects and allergies may not be observed but could be found as the children become older (Ali et al., 2018). Additionally, given the cross-sectional nature of this study, could not establish a relationship between oxidative stress and symptoms. Urine samples were collected only once and in different years between 2012–2017; this could have caused variations in phthalate metabolite levels due to the children's daily activity or diet. Thus, first-morning void urine was used to minimize daily variability, additionally no seasonal variation in urinary phthalate metabolite levels was shown in chapter 1. Another limitation

to this study is allergic outcomes were determined based on questionnaire and were completed by children's parents may lead to misclassification. However, using Japanese translated widely accepted standardized ISAAC questionnaire can minimize the misclassification and be available to compare with other studies. Both WQS and BKMR models have limitations and can produce imprecise estimates and erroneous interpretations. The WQS limitation is that the regression analysis assumes no interaction between exposures, and the assumption of directional homogeneity may cause estimation bias in the outcome (Keil et al., 2020, Carrico et al., 2015). Additionally, the fixing of chemicals at certain levels in the BKMR model could underestimate the effects of co-exposure at high and low levels of chemicals (Lazarevic et al., 2019. Zhang et al.,2019).

2.6. Conclusions

As conclusion from individual regression, MECPP and cx-MINP were associated with wheeze in children. Mixture of phthalate metabolites was associated with wheeze and eczema. DEHP and DINP metabolites were the main contributors to the mixture effect in the association with wheeze and eczema. In this study, we did not find a mediating effect of oxidative stress on the association between phthalates and allergic symptoms. Young children are still in the developing stage; therefore, follow-up is needed to elucidate the possible effects of oxidative stress and allergies resulting from phthalate exposure.

VIII. Summaries and future perspectives

In this PhD thesis, the biomonitoring and health effects of phthalates exposure in Japanese children has been reported and discussed. Below are summaries of the studies

- Despite the 2010 revised Japan regulation of phthalates in children and food packaging materials, high detection frequency (>96%) of all phthalate metabolites and stable exposure level from 2012-2017 was observed. This finding indicates further biomonitoring to different general population is necessary to examine the phthalate exposure level and elucidate exposure sources probably from non-regulated products.
- Increased ∑DINP in low household income children, MINP in those living in old building, and MiBP, MnBP, ∑DEHP in those with a longer window opening habit. While children living in houses that frequently vacuum decreased level of MiBP, and MnBP. In which reveals phthalates exposure could be associated with personal and building characteristics. As certain confounding factors such as socio-economic status, building age, vacuuming, indoor air exchanging habits are associated with exposure of phthalate has also been reported in previous studies. Thus, relevant improvement in buildings such as renovation of older buildings, installation of ventilations, and constant cleaning habits may be needed to minimize exposure of phthalates to the general population.
- The individual analysis showed metabolites MECPP and cx-MINP are associated with wheeze. The joint or mixture effect WQS index revealed within the assessed metabolites MINP and MEOHP are the highly weighted in association with wheeze

and eczema, respectively. The BKMR mixture model found the DINP metabolites group showed the highest PIP and among them MECPP and cx-MINP for wheeze and eczema and OH-MINP for rhino-conjunctivitis were the most important metabolites to drive the association with health outcomes. In this study consistent results from individual and mixture models as DEHP and DINP being important factors to drive the association with wheeze and eczema was observed. This may suggest consideration of future phthalates regulations to emphasize/revision on DEHP and DINP.

- Although direct effect between phthalates and health outcomes were evident in this study, no mediation effect of oxidative stress (8-OHdG, HEL, and HNE) was observed. This adds to the scarce studies in human about mediation effect of oxidative stress in relation between phthalates and health outcomes. Yet, as this epidemiological study is limited to elucidate mechanism of actions on causal relation future studies are required to establish the associations.

Therefore, as future perspectives in biomonitoring, decreasing exposure of phthalate and their health effects to the general population the following actions are required. In the future consecutive biomonitoring of phthalates exposure in different population and upscale of study participants including assessment of exposure source is essential. This will provide scientific evidence future perspectives to assist in phthalates regulation decisions. As the global and national plasticizer market is changing new alternatives to phthalates such as DINCH and DEHTP are being introduced to the market. In which

these alternatives toxicity and possible health effects are not well explored, thus it is essential to evaluate such alternatives in the aspects of exposure level as well as toxicity. While experimental studies *in vivo* and *in vitro* provide insightful mechanisms of chemicals health effect, there is still a lack of human epidemiological studies. Thus, future subsequent human epidemiological studies are needed as chemicals exposure level in experimental and environment and health effect are different and needs to be addressed accordingly. Moreover, epidemiological studies in health assessments of phthalates in national and international collaborations will advantage the global effort to achieve SDGs. For instance, such epidemiological biomonitoring and health assessment studies generate reliable baseline scientific data to measure the progress of SDGs such as "Good health and Wellbeing" and "Sustainable cities and communities". It also assists national and international regulations to reduce environmental chemicals related health effects.

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