学 位 論 文

Exposure to Organophosphate Flame Retardants and its Associations with Allergies among School-aged Children

（学童期におけるリン系難燃剤曝露とアレルギーとの関連）

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I. Abstract

Exposure to organophosphate flame retardants and plasticizers (PFRs) has been reported to increase the risk of asthma and allergies. However, little is known about its association with type 2 inflammation (T2) biomarkers and oxidative stress biomarkers, which were associated with the development of allergies. The objective of this thesis was to identify the association between PFR exposure and allergic symptoms, T2 biomarkers, and oxidative stress biomarkers. The data and samples were collected between 2017 and 2020, including school children (n=427) aged 9–12 years living in Sapporo City, Japan, among the participants of "The Hokkaido Study on Environment and Children's Health." Thirteen urinary metabolites of five PFR were measured by LC-MS/MS. Allergic symptoms were assessed using the International Study of Asthma and Allergies in Childhood questionnaire. For T2 biomarkers, the peripheral blood eosinophil counts, fraction of exhaled nitric oxide level (FeNO), and serum total immunoglobulin E (IgE) level were measured. For oxidative stress biomarkers, 4-hydroxynonenal (HNE), hexanoyl-lysine (HEL), and 8-hydroxy-2'-deoxyguanosine (8-OHdG), were measured in spot urine samples.

Chapter 1 investigated the biomonitoring of PFRs metabolites and the determinants of PFRs exposure in children aged 9-12 years old. The metabolites of bis(1,3-dichloro-2-

propyl) phosphate (BDCIPP), 1-Hydroxy-2-propyl bis(1-chloro-2-propyl) phosphate (BCIPHIPP), and diphenyl phosphate (DPHP) were detectable for more than 60% of participants. The highest concentration of PFR was Σtris(1-chloro-isopropyl) phosphates (ΣTCIPP) (Median:1.20 nmol/L). Comparisons with children and adolescents from other countries showed that urinary concentrations of PFRs were relatively lower among Japanese children. Determinants analysis found boys, higher BMI, higher annual household income, and experiencing environment tobacco smoking were associated with significantly higher levels of PFR metabolites. Higher age was associated with lower ΣTPHP and higher ΣEHDPHP.

Chapter 2 investigated associations among urinary PFR metabolite concentrations, allergic symptoms, and T2 biomarkers using both individual and mixture analyses. Multiple logistic regression analysis, quantile-based g-computation (qg-computation), and Bayesian kernel machine regression (BKMR) were used to examine the associations between the health outcomes of the individual PFRs and the PFR mixtures. Tris(1,3-dichloro-2-propyl) phosphate (TDCIPP) was significantly associated with a high odds ratio (OR, 95% CI:1.36, 1.07 - 1.72) for wheeze. TDCIPP (1.19, 1.02 - 1.38), Σtriphenyl phosphate (ΣTPHP) (1.81, 1.40 - 2.37), and Σtris(2-butoxyethyl) phosphate (ΣTBOEP) (1.40, 1.13 - 1.74) were significantly associated with increased odds of FeNO (≥35 ppb).

ΣTPHP (1.44, 1.15 - 1.83) was significantly associated with high eosinophil counts (≥300/μL). For the PFR mixtures, a one-quartile increase in all PFRs (1.48, 1.18 - 1.86) was significantly associated with high FeNO (≥35 ppb) in the qg-computation model. The PFR mixture was positively associated with high FeNO (≥35 ppb) and eosinophil counts (≥300/μL) in the BKMR models. These results may suggest that exposure to PFRs increases the probability of asthma, allergies, and T2 inflammation.

In Chapter 3, associations of individual PFRs and the PFR mixtures with oxidative stress biomarkers in children were investigated, and the hypothesis of the mediation effect of oxidative stress on the associations between PFRs and T2 biomarkers are examined. The median for HNE, HEL, and 8-OHdG were 23.5 (μg/mL), 99.3 (nmol/L), and 9.2 (ng/mL), respectively. For the individual PFRs, a natural-log unit increase in TDCIPP (β, 95% CI: 0.12, 0.03 - 0.22), ΣTPHP (0.52, 0.40 - 0.64), ΣTBOEP (0.29, 0.16 - 0.41), and ΣEHDPHP (0.16, 0.003 - 0.32) were significantly associated with higher HNE. Higher TDCIPP (0.04, 0.003 - 0.08), ΣTPHP (0.10, 0.05 - 0.15), and ΣTBOEP (0.07, 0.02 - 0.12) were associated with higher HEL. Higher ΣTPHP (0.05, 0.03 - 0.08), and ΣTBOEP (0.05, 0.03 - 0.08) were significantly associated with higher 8-OHdG. For the PFR mixtures, a one-quartile increase in all PFRs was associated with a significant increase in HNE (0.55, 0.36 - 0.73), HEL (0.08, 0.01 - 0.15), and 8-OHdG (0.07, 0.04 - 0.12) in the qg-

computation model. The PFR mixture was positively associated with all three oxidative stress biomarkers in the BKMR models. No mediation effect of oxidative stress biomarkers among PFR exposure and levels of eosinophil and FeNO were found. PFR exposures were associated with oxidative stresses of DNA damage and lipid peroxidation among the general population of 9–12-year-old children. Future study including other environmental chemicals such as phthalates, bisphenols, and nonylphenol is needed to evaluate the mediation effect of oxidative stress for environmental chemicals exposure and asthma.

This thesis found that over half of school children are exposed to PFRs. The exposure concentrations were relatively low in Japanese school aged children compared with children from other countries, and the estimated daily intake levels were well below the oral reference doses. The study results suggest that exposure to PFRs may increase the probability of asthma, allergies, T2 inflammation, and oxidative stress. Future studies focused on lifestyle and usage of daily necessities, as well as the source of PFRs, will be helpful in finding effective ways to utilize the chemicals in daily life and finally reduce PFR exposure effectively.

II. Lists of abbreviations

adjusted odds ratio (AOR)

bayesian kernel machine regression (BKMR)

bis(1,3-dichloro-2-propyl) phosphate (BDCIPP)

bis(1-chloro-2-propyl) phosphate (BCIPP)

bis(2-butoxyethyl) 3'-hydroxy-2-butoxyethyl phosphate (3-HO-TBOEP)

bis(2-butoxyethyl) phosphate (BBOEP)

body mass index (BMI)

bronchoalveolar lavage fluid (BAL)

confidence interval (CI)

di-n-butyl phosphate (DNBP)

diphenyl phosphate (DPHP)

enzyme-linked immunosorbent assay (ELISA)

estimated daily intakes (EDI)

environmental tobacco smoke (ETS)

fractional of exhaled nitric oxide (FeNO)

hexanoyl-lysine (HEL)

4-hydroxynonenal (HNE)

hydroxyphenyl diphenyl phosphate (HO-TPHP)

immunoglobulin E (IgE)

International Study of Asthma and Allergies in Childhood (ISAAC)

international unit (IU)

limits of quantification (LOQ)

organophosphate flame retardants and plasticizers (PFRs)

oral reference doses (RfD)

parts per billion (ppb)

quality control (QC)

quantile-based g-computation (qg-computation)

specific gravity (SG)

tri-n-butyl phosphate (TNBP)

tris(1,3-dichloro-2-propyl) phosphate (TDCIPP)

tris(chloroethyl) phosphate (TCEP)

type 2 helper T (Th2) cells

type 2 inflammation (T2) biomarkers

type 2 innate lymphoid (ILC2) cells

8-hydroxy-2'-deoxyguanosine (8-OHdG)

Σ 2-Ethylhexyldiphenyl phosphate (ΣEHDPHP) metabolites (EHPHP and 5-HO-EHDPHP)

Σ tris(1-chloro-isopropyl) phosphate (ΣTCIPP) metabolites (BCIPP and BCIPHIPP)

Σ triphenyl phosphate (ΣTPHP) metabolites (DPHP, HO-TPHP, and 4HO-DPHP)

Σ tris(2-butoxyethyl) phosphate (ΣTBOEP) metabolites (BBOEP, BBOEHEP, and 3-HO-TBOEP)

1-hydroxy-2-propyl bis(1chloro-2-propyl) phosphate (BCIPHIPP)

2-ethyl-5-hydroxyhexyl diphenyl phosphate (5-HO-EHDPHP)

2-ethylhexyl phenyl phosphate (EHPHP)

2-hydroxyethyl bis (2-butoxyethyl) phosphate (BBOEHEP)

4-hydroxyphenyl phenyl phosphate (4-HO-DPHP)

III. General Introduction

In our contemporary daily life, a myriad of synthetic chemicals envelops us. The convergence of global industrial development and the evolving lifestyle preferences of humanity, leading to an exponential surge in the production and utilization of diverse chemicals. They have an indispensable role in daily life and are used as industrial chemicals, agricultural practices, building materials pharmaceuticals, and personal care products. However, despite the ostensibly beneficial intentions behind their creation, aiming to render our lives more convenient and enhance our well-being, certain chemicals have been found to harbor unforeseen negative consequences, posing potential threats to both human health and the environment. These chemicals can enter the environment during production, use, and waste stages and finally lead to human exposure. Approximately 220 million tons of synthetic compounds used in industrial and consumer products are released into the environment each year (Nagesh et al., 2022). It was estimated that over one-third (35%) of ischemic heart disease (the leading cause of deaths worldwide), and about 42% of stroke (the second largest contributor to global mortality), could be prevented by reducing or removing exposure to hazardous chemicals from ambient environment (OECD 2012). Some of these chemicals including organofluoride compounds, phthalates, and bisphenols, interfere with the development of the endocrine

system and organs that respond to endocrine signals in organisms (Colborn et al., 1993). International conventions have been released to regulate those environmental hazards including air pollution, pesticides, heavy metals, persistent organic pollutants. However, new chemicals are emerging as alternatives, yet the health risks of these chemicals are not well known.

Flame retardants are commonly added to building materials and consumer products to meet fire safety standards and regulations (Van der Veen and De Boer 2012, Hoffman et al., 2015). Polybrominated diphenyl ethers (PBDEs) were well-known and widely used flame retardants. Due to their persistency and toxicity, PBDEs have been listed in the Stockholm Convention and have largely been phased out globally (UNEP 2009). The organophosphate flame retardants and plasticizers (PFRs), which are less persistent and bio-accumulative, have been proposed as alternatives of PBDEs (Van der Veen and De Boer 2012). PFRs are widely used as flame retardants and plasticizers in consumer products such as textile, furniture, electronics, polyvinyl chloride plastic, and polyurethane foam (Wei et al., 2015a). In Western Europe, there was a significant rise in the utilization of PFRs, with quantities increasing from 58,000 tons in 1998 to 83,000 tons in 2001, further climbing to 85,000 tons in 2005, and reaching 91,000 tons in 2006 (Reemtsma et al., 2008). The consumption of PFRs anticipates further growth in response

to the limitations imposed on brominated flame retardants.

There are around 20 commonly used PFRs in the market (Greaves and Letcher 2017, Zhang et al., 2022). Table 1 summarizes the names and abbreviations of the most common PFRs. Phosphate triester is the base structure of PFR compounds (Greaves and Letcher 2017). PFRs are classified into alkyl-, chlorinated alkyl-, aryl- and oligomeric ones according to the structure of branched-chain. PFRs are classified as semi-volatile organic compounds, as the vapor pressure at 25 ℃ of these PFRs ranged from 0.85 to 2.06×10^{-8} mmHg. They are not chemically bound to materials used in consumer products, resulting in a slow release into the ambient environment via abrasion, leaching, and volatilization (Wei et al., 2015b), which finally leads to human exposure. PFRs have a short biological half-life ranging from several hours to days and are rapidly metabolized and eliminated from the body (Greaves et al., 2016, Völkel et al., 2018). In order to measure internal exposure levels, a biomonitoring method for urinary metabolites of PFRs has recently been established (Van Den Eede et al., 2013, Van Den Eede et al., 2015, Bastiaensen et al., 2018). Seven PFRs reported in this thesis were highlighted with grey color in Table 1.

Previous epidemiological studies have shown the health concerns about exposure to PFRs. Exposure to PFRs potentially impacts child neurodevelopment through decreased intelligence quotient and working memory scores (Castorina et al., 2017), associated with

inflammatory responses through high levels of oxidative stress among children (Ait Bamai et al., 2019). Previous studies measured external PFRs exposure, found that PFRs in house dust significantly increased the odds ratio of wheeze, eczema, and allergic rhinoconjunctivitis (Araki et al., 2014b, Ait Bamai et al., 2018, Araki et al., 2018). Moreover, a study that measured the PFR metabolites in urine reported a significant increase in children's allergic symptoms with higher concentrations of PFR metabolites (Araki et al., 2018). However, there is still a lack of clarity about the relationship between PFR exposure and allergic diseases. To our knowledge, there is no epidemiological evidence of an association between exposure to PFRs and T2 biomarkers, oxidative stress biomarkers, which could better clarify the relationships among PFRs, asthma, and allergies. Furthermore, previous epidemiological studies focused on the associations between individual PFR exposure and health outcomes. Considering the overall health effects of multiple PFRs exposure is important, as humans are simultaneously exposed to different chemicals (Meek et al., 2011).

Therefore, this thesis intends to address the following research questions:

1. What is the internal exposure level (distribution of urinary PFR metabolites) of PFRs among Japanese children? What is the estimated daily intake of PFR in the studied population, and how does it compare with data from other relevant studies? What are the

determinants contributing to elevated concentrations of PFR exposure?

2. Does higher levels of PFR exposure positively associated with a higher risk of allergic symptoms and higher levels of T2 biomarkers?

3. Does higher levels of PFR exposure positively associated with higher levels of oxidative stress biomarkers? Does the oxidative stress mediate the relationship between PFR exposure and the presence of allergic symptoms and T2 biomarkers?

Name	Abbreviation	MW
Trimethyl phosphate	TMP	140.80
Triethyl phosphate	TEP	182.15
Tripropyl phosphate	TPRP	224.23
Tri-n-butyl phosphate	**TNBP**	**266.31**
Tri-iso-butyl phosphate	TIBP	266.32
Tris(2-butoxyethyl) phosphate	**TBOEP**	**398.47**
Tris(2-ehylhexyl) phosphate	TEHP	434.63
Tris(chloroethyl) phosphate	**TCEP**	**285.49**
Tris(1-chloro-isopropyl) phosphate	**TCIPP**	**327.57**
Tris(1,3-dichloro-2-propyl) phosphate	**TDCPP**	**430.89**
Triphenyl phosphate	**TPHP**	**326.29**
Trimethylphenyl phosphate	TMPP	368.36
2-Ethylhexyl diphenyl phosphate	**EHDPHP**	**362.40**
Cresyl diphenyl phosphate	CDPP	340.30
Isodecyl diphenyl phosphate	IDDP	390.40
t-Butylphenyl diphenyl phosphate	BPDP	382.30
Tris(p-tert-butylphenyl) phosphate	TBPHP	494.00
Resorcinol bis(diphenyl phosphate)	RDP	574.50
Tetrakis(2-chloroethyl) dichloroisopentyl diphosphate	V6	582.90
Bisphenol A bis (diphenyl phosphate)	BDP	692.60
Tris(2,4-di-tert-butylphenyl) phosphate	AO168=O	662.9
Bis(2,4-di-tert-butylphenyl) pentaerythritol diphosphate	A0626=O_2	636.7
Triisodecyl phosphate	TiDeP	518.8
Tris(2-nonylphenyl) phosphate	TNPP	705.0

IV. Objectives of this study

In chapter 1

a) Define levels of PFR metabolites among children aged 9-12 years old.

b) Assess the estimated daily intake of PFR. Compared PFR exposure level with other studies.

c) Identify the determinants of elevated PFR metabolite concentrations.

In chapter 2

d) Investigate the associations among urinary PFR metabolite concentrations and allergic symptoms.

e) Investigate the associations among urinary PFR metabolite concentrations and T2 biomarkers.

f) Examine the abovementioned associations using both individual analyses (multiple logistic regression analyses) and mixture analyses models (quantile-g computation and bayesian kernel machine regression analysis).

In chapter 3

g) Investigate the associations among urinary PFR metabolite concentrations and

oxidative stress biomarkers.

h) Examine the mediation effect of oxidative stress on the association between PFRs and allergic symptoms as well as T2 biomarkers.

Chapter 1: Human Biomonitoring of Exposure to Organophosphate Flame Retardants and Plasticizers among School-aged Children

ABSTRACT

Organophosphorus flame retardants and plasticizers (PFRs) have been widely used in a variety of consumer products, and anticipate further growth in the future. A previous study found median values for bis(1,3-dichloro-2-propyl) phosphate (BDCIPP), 1-Hydroxy-2-propyl bis(1-chloro-2-propyl) phosphate (BCIPHIPP), diphenyl phosphate (DPHP) were higher in 2012-2017 compared with 2009-2010 among Japanese children. Keeping human biomonitoring of exposure to PFRs is essential to track changes in exposure levels and evaluate the potential health risks. In this study, concentrations of thirteen PFR metabolites were measured in urine samples from 427 Japanese school-aged children. The Samples were collected between 2017 and 2020, including children aged 9–12 years living in Sapporo City, Japan, among the participants of "The Hokkaido Study on Environment and Children's Health." Urinary PFR metabolites were measured by LC-MS/MS. The metabolites of BDCIPP, BCIPHIPP, and DPHP were detectable for more than 60% of participants. The highest concentration of PFR was Σtris(1-chloro-isopropyl) phosphates (ΣTCIPP) (Median:1.20 nmol/L). Comparisons with study populations from

other countries showed that urinary concentrations of PFRs were relatively lower in this study. Determinants analysis found boys, higher BMI, higher annual household income, and experiencing environment tobacco smoking were associated with significantly higher levels of PFR metabolites. Higher age was associated with lower ΣTPHP and higher ΣEHDPHP. Although the estimated daily intakes (EDI) of school-aged children in Japan are all well below the current oral reference doses (RfD), future studies are needed to verify the potential health risks of low-level PFRs exposure.

Keywords: Urinary metabolites, PFR, Children, Human biomonitoring, Estimated daily intake

1.1. Introduction

Organophosphate flame retardants and plasticizers (PFRs) are commonly employed in numerous industries, such as plastics, textile, furniture, electronics, construction, vehicle, and petroleum industries (Marklund et al., 2003). PFRs are frequently presented in materials including polyurethane foam, paints, lubricants, floor waxes, electronic and electrical products, textiles, plastics, and food packaging (Stapleton et al., 2009, Kajiwara et al., 2011, Van der Veen and De Boer 2012, Xu et al., 2016). Tris(chloroethyl) phosphate (TCEP), tris(2-chloroisopropyl) phosphate (TCIPP), tris(2,3-dichloropropyl) phosphate (TDCPP), Triphenyl phosphate (TPHP) are commonly employed in polyurethane foam applications. Meanwhile, PFRs are also utilized as plasticizers and lubricants, such as 2-ethylhexyldiphenyl phosphate (EHDPHP) in food packaging, tris(2-butoxyethyl) phosphate (TBOEP) in floor wax, and tri-n-butyl phosphate (TNBP) in hydraulic fluids (Bastiaensen et al., 2018).

The presence of PFRs has been widely demonstrated in environmental matrices, including air, water, soil, and indoor dust (Araki et al., 2014b, Tajima et al., 2014, He et al., 2019, Zhang et al., 2022). Using the products containing PFRs, inhaling or ingesting PFR-contaminated environmental matrices could ultimately result in human exposure. The “pseudo-persistent” pollutants refer to compounds that are continuously introduced

into the environment, with new molecules constantly replacing those being eliminated (Daughton 2003). Owing to their widespread distribution and continuous exposure, PFRs can be considered pseudo-persistent, which raises concerns regarding potential risks to human health.

To measure internal exposure, biomonitoring of metabolites of PFRs in urine, has been recently established (Van Den Eede et al., 2013, Van Den Eede et al., 2015, Bastiaensen et al., 2018). The levels of urinary metabolites can reflect internal exposure not only from dust, but also from other exposure sources, including diet (Araki et al., 2018, Doherty et al., 2019). A previous study found median values for bis(1,3-dichloro-2-propyl) phosphate (BDCIPP), 1-Hydroxy-2-propyl bis(1-chloro-2-propyl) phosphate (BCIPHIPP), diphenyl phosphate (DPHP) were higher in 2012-2017 compared with 2009-2010 among Japanese seven years of age children (Bastiaensen et al., 2019a, Bastiaensen et al., 2020). Therefore, it is important to keep biomonitoring the PFR exposure and assess its health effects. In this study, we aimed to biomonitoring PFR metabolites in children aged 9-12 years old. Additionally, we explored the determinants that might have a significant influence on PFR exposure level and assessed whether current exposure levels raise any concerns by calculating estimated daily intakes (EDI) and comparing them to the available reference doses (RfD).

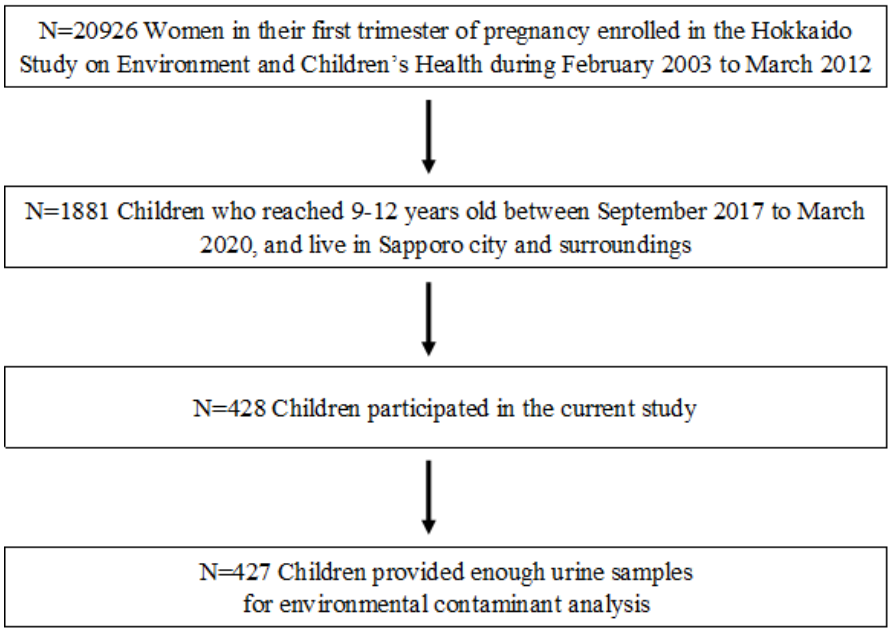
1.2. Materials and methods

1.2.1. Study population

This cross-sectional study was part of the “Hokkaido Study on Environment and Children’s Health”, Hokkaido cohort (Kishi et al., 2011, Kishi et al., 2013, Kishi et al., 2017, Kishi et al., 2021). A flowchart of the participant selection process is presented in Figure 1.1. This ongoing birth cohort study included 20,926 pregnant women between 2003 and 2012 in Hokkaido prefecture, Japan. The face-to-face survey was conducted from September 2017 to March 2020 to collect data and samples. We contacted a total number of 1881 children born between April 2006 to January 2010, who were 9–12 years old and residing in Sapporo City and its surrounding areas at the time points of the survey. 428 of them agreed to participate in a face-to-face survey and visited selected pediatric clinics with their parents (Goudarzi et al., 2023). A questionnaire survey, urine sampling, blood sampling, FeNO measurement, and anthropometric measurements were done on the same day. In total, 427 children, available with information from the questionnaire and spot urine samples, were included in the final analysis. Parents answered a questionnaire to collect information on their children's demographics. Demographic characteristics included sex, age, physical exercise frequency, environmental tobacco smoke (ETS) status, annual household income, and maternal educational level. ETS

(yes/no) is defined as a child's current exposure to passive smoke from a mother, father, or other household members who smoke tobacco in places where the child lives.

Figure 1.1. Flow chart of participants selection.



1.2.2. Measurement of PFR metabolites in urine

Spot urine samples of children were collected using a polypropylene cup by the research staff at pediatric clinics on the same day as the survey, and samples were transported to Hokkaido University Center for Environmental and Health Sciences in a cool box. Upon arrival, the samples were immediately dispensed into a glass tube that was cleaned with acetone, sealed with fluoric tape, wrapped in aluminum foil, and stored at −30 °C until the day of analysis. The specific gravity (SG) of urine samples was

determined at room temperature using a handheld refractometer (ATAGO T3-SE, ATAGO Co., Ltd. Japan).

Urinary metabolites of PFRs were measured at the Toxicological Center of the University of Antwerp, Belgium. The details of the PFR analysis are explained in the previous report (Bastiaensen et al., 2020). Thirteen PFR metabolites (Table 1.1), di-n-butyl phosphate [DNBP], bis(1,3-dichloro-2-propyl) phosphate [BDCIPP], tris(chloroethyl) phosphate [TCEP], bis(1-chloro-2-propyl) phosphate [BCIPP], 1-hydroxy-2-propyl bis(1chloro-2-propyl) phosphate [BCIPHIPP], diphenyl phosphate [DPHP], hydroxyphenyl diphenyl phosphate [HO-TPHP], 4-hydroxyphenyl phenyl phosphate [4-HO-DPHP], bis(2-butoxyethyl) phosphate [BBOEP], 2-hydroxyethyl bis (2-butoxyethyl) phosphate [BBOEHEP], bis(2-butoxyethyl) 3'-hydroxy-2-butoxyethyl phosphate [3-HO-TBOEP], 2-ethylhexyl phenyl phosphate [EHPHP], and 2-ethyl-5-hydroxyhexyl diphenyl phosphate [5-HO-EHDPHP]), were measured in urine. Urine was analyzed using an Agilent 1290 Infinity II UPLC system coupled to a 6495C triple quadrupole mass spectrometer (Agilent Technologies, Santa Clara, CA, USA).

Table 1.1. List of organophosphate flame retardants and plasticizers (PFRs) metabolites considered in this study, with their parent compound.

Parent compound	MW (g/mol)	PFR metabolite	Abbreviation	MW (g/mol)
Tri-n-butyl phosphate (TNBP)	266.31	Di-n-butyl phosphate	DNBP	210.21
Tris(1,3-dichloro-2-propyl) phosphate (TDCIPP)	430.89	Bis(1,3-dichloro-2-propyl) phosphate	BDCIPP	319.96
Tris(chloroethyl) phosphate (TCEP)	285.5	Tris(chloroethyl) phosphate	TCEP	285.5
Tris(1-chloro-isopropyl) phosphate (TCIPP)	327.60	Bis(1-chloro-2-propyl) phosphate	BCIPP	251.05
		1-Hydroxy-2-propyl bis(1-chloro-2-propyl) phosphate	BCIPHIPP	309.12
Triphenyl phosphate (TPHP)	326.29	Diphenyl phosphate	DPHP	250.19
		Hydroxyphenyl diphenyl phosphate	HO-TPHP	342.28
		4-Hydroxyphenyl phenyl phosphate	4-HO-DPHP	265.18
Tris(2-butoxyethyl) phosphate (TBOEP)	398.5	Bis(2-butoxyethyl) phosphate	BBOEP	298.31
		2-Hydroxyethyl bis(2-butoxyethyl) phosphate	BBOEHEP	342.37
		Bis(2-butoxyethyl) 3'-hydroxy-2-butoxyethyl phosphate	3-HO-TBOEP	414.50

2-Ethylhexyldiphenyl phosphate (EHDPHP)	362.4	2-Ethylhexyl phenyl phosphate	EHPHP	286.30
		2-Ethyl-5-hydroxyhexyl diphenyl phosphate	5-HO-EHDPHP	378.40

Abbreviations: molecular weight (MW).

The following quality control (QC) measures were used during the sample preparation and analytical analysis to ensure the accuracy of the results. Procedural blanks consisting of 1 mL Milli-Q water were made before sample preparation and analyzed in parallel with urine and QC samples. For every 20 urine samples, two procedural blanks, one QC-low, and one QC-medium were added. The values of the analytes measured in the procedural blanks were subtracted from those measured in the samples. Field blanks were not used, as it was not feasible to create representable field blanks during the face-to-face survey, and contamination with urinary metabolites from the environment was unlikely. External QC was ensured by successful participation in inter-laboratory comparison exercises, such as the Human Biomonitoring for EurPFR External Quality Assurance Scheme (HBM4EU ICI/EQUAS, 2018–2021) and the External Quality Assessment Scheme for Organic Substances in Urine (OSEQAS, 2018–2023). The performance of QC measures for the PFR metabolites during the analysis of the sample baches is summarized in Table 1.2.

Table 1.2. Quality control (QC) measures for the PFR metabolites during the analysis of the sample batches.

	Spiking level 0.8 ng/mL (n=6)		Spiking level 4.0 ng/mL (n=6)		HBM4EU QC (n=13)		OSEQAS 2018-2019 (n=8)		OSEQAS 2020 (n=6)	
	Accuracy (%)	Precision (%)	Accuracy (%)	Precision (%)	Accuracy (%)	Precision (%)	Accuracy (%)	Precision (%)	Accuracy (%)	Precision (%)
BCIPP	100	16	92	5	102	9				
DPHP	102	14	87	2	86	12	106	7	98	7
BDCIPP	99	8	91	2	93	10	88	9	106	12
4-HO-DPHP	97	9	106	10						
DNBP	118	11	102	5						
BBOEP	76	13	79	13						
BCIPHIPP	89	10	104	7						
TCEP	103	6	99	1						
EHPHP	110	10	116	10						
BBOEHEP	94	9	99	7						
3-HO-TBOEP	83	10	72	10						
3/4-HO-TPHP	95	8	104	10						
5-HO-EHDPHP	114	19	104	17						

Spiking MilliQ water at level of 0.8 and 4.0 ng/mL.

HBM4EU QC samples were used during various rounds of Intercomparison exercises organized in the frame of the EU H2020 project HBM4EU (n=13).

OSEQAS samples were used in the various rounds of the Interlaboratory tests organized in 2018 and 2019 by the Institute of Toxicology (Quebec) (n=8).

Results from the Interlaboratory tests organized in 2020 by the Institute of Toxicology (Quebec) (n=6).

1.2.3. Statistical analysis

For the concentrations below the limits of quantification (LOQ), the value was imputed by the LOQ × detection frequency (James et al., 2002). DNBP and TCEP were excluded from further analysis because of the low detection frequencies (<10%). The Σ metabolite concentrations were estimated using the molar concentrations of ΣTCIPP metabolites (BCIPP and BCIPHIPP), Σ triphenyl phosphate (ΣTPHP) metabolites (DPHP, HO-TPHP, and 4HO-DPHP), ΣTBOEP metabolites (BBOEP, BBOEHEP, and 3-HO-TBOEP), Σ 2-Ethylhexyldiphenyl phosphate (ΣEHDPHP) metabolites (EHPHP and 5-HO-EHDPHP). The concentrations of Σ metabolites were used as exposure variables for assessing exposure to parent PFRs. The concentrations were corrected for individual SG values using the following formula:

$$\text{SG corrected concentration} = \text{uncorrected concentration} \times [(SG_m - 1) \div (SG_i - 1)]$$

where the SG_m is the median SG of this study (1.025), and SG_i is the individual SG (Pearson et al., 2009, Meeker et al., 2012). Levels of PFR > LOQ were corrected using the individual SG concentration. Levels < LOQ were corrected using the median SG concentration of all children. SG reflects the ratio between the density of the urine sample and pure water. SG correction positively reduces the variability of urinary concentrations

of non-persistent organic chemicals (Roggeman et al., 2022). Urinary creatinine is a chemical by-product generated from muscle metabolism, which may introduce bias to the data analysis among children owing to the developmental growth of muscle mass (Pearson et al., 2009). Conversely, SG is less likely to be influenced by individual factors compared to creatinine (Bastiaensen et al., 2021b).

The estimated daily intakes (EDI in ng/kg bw/day) of five PFR parent components were determined by computing the urinary concentrations of metabolites using the equation below (Fromme et al., 2014, Chen et al., 2018):

$$\text{EDI} = \left(\frac{c_{meta} \times V_{urine}}{F_{UE} \times bw}\right) \times \frac{MW_p}{MW_m}$$

where c_{meta} is the SG corrected metabolite concentration (in ng/mL SG); V_{urine} is the daily excreted volume of urine (estimated at 700 mL/day for 10 years old children) (Valentin 2002); F_{UE} is the urinary excretion factor, estimated based on results from in vitro human liver microsomes (HLM) and S9 fractions experiments for TDCIPP, TCIPP, TPHP, and TBOEP (Van Den Eede et al., 2013). No Fue is available in literature, therefore low and high Fue scenario were used for EHDPHP; bw is the body weight of the participant (in kg); and MWp and MWm are the molecular weight of the parent compound and its metabolite respectively (in g/mol, Table 1.1). The available oral reference doses (RfD) values were provided to compare the EDI of PFRs (Poma et al., 2018).

The Mann-Whitney U test was used to assess the statistical significance of differences in the levels of PFRs and basic characteristics including sex, annual household income, physical exercise frequency, and ETS. Spearman's rank correlation coefficient was calculated to evaluate the association between each PFR and child age as well as child BMI. All statistical analyses were performed using R (Version 4.2.3).

1.2.4. Ethics

After explaining the research objectives and methods to the participants, written consent was obtained from all parents, and permission was obtained from all children. The research review boards of Hokkaido University Graduate School of Medicine and Hokkaido University Center for Environmental and Health Sciences approved this study (21–136).

1.3. Results

1.3.1. Characteristics of study participants

The demographic characteristics and health outcomes of the study participants are shown in Table 1.3. A total of 427 children aged 9–12 years were included in the study. The children in this study were 9 to 12 years old. The gender participation was nearly balanced with boys (53.9%) and girls (46.1%). 39.1% of them were currently experiencing environmental tobacco smoke.

Table 1.3. Characteristics of study participants (n=427).

Variable		n	%
Sex	Boys	230	53.9
	Girls	197	46.1
Age	9	140	32.8
	10	171	40.0
	11	45	10.5
	12	71	16.6
BMI (kg/m^2) (mean ± SD)			17.8 ± 2.97
Physical exercise	yes	314	73.5
Environmental tobacco smoke	yes	167	39.1
Annual household income	< 5 million	126	29.5
(Japanese Yen/year)	5 - 8 million	180	42.2
	> 8 million	97	22.7
	missing	24	5.6

1.3.2. Organophosphate flame retardants and plasticizers (PFRs) metabolites

The distributions of the urinary concentrations of the PFR metabolites by raw weight (before SG correction, ng/mL) and Σ weight (nmol/L) are shown in Table 1.4. The detection frequency for BDCIPP, BCIPHIPP, and DPHP were 63.9%, 78%, and 84.5%. The level of ΣTCIPP was the highest (median value of 1.20 nmol/L), followed by ΣTPHP (median value of 1.10 nmol/L). Significant correlations ($p < 0.05$) were found among all PFR metabolite groups, with Spearman's ρ ranging from 0.10−0.35, as shown in Figure. 1.2.

Table 1.4. Distribution of organophosphate flame retardants and plasticizers (PFRs) metabolites in urine (n=427).

Metabolites	LOQ	> LOQ (%)	min	25th	50th	75th	95th	max
Raw concentration (Before specific gravity correction, ng/mL)								
DNBP	0.20	6.8				<LOQ	0.25	1.01
BDCIPP	0.05	63.9		<LOQ	0.11	0.29	2.61	42.9
TCEP	0.10	0.5					<LOQ	0.31
BCIPP	0.40	22.5				<LOQ	1.96	39.9
BCIPHIPP	0.05	78.0	<LOQ	0.06	0.20	0.64	2.87	83.5
DPHP	0.10	84.5	<LOQ	0.14	0.27	0.54	1.73	40.7
HO-TPHP	0.05	0.9					<LOQ	0.20
4HO-DPHP	0.20	0.0						<LOQ
BBOEP	0.05	58.1		<LOQ	0.07	0.19	0.70	4.19
BBOEHEP	0.05	53.6		<LOQ	0.06	0.23	1.31	8.32
3-HO-TBOEP	0.10	20.6				<LOQ	0.19	1.75
EHPHP	0.20	59.7		<LOQ	0.24	0.48	1.12	6.01
5-HO-EHDPHP	0.05	17.8				<LOQ	0.13	0.45
ΣMetabolites concentration (nmol/L)								
TDCIPP (based on BDCIPP)			0.10	0.10	0.34	0.91	8.17	134.0
ΣTCIPP (Σ of BCIPP, BCIPHIPP)			0.48	0.57	1.20	3.49	15.47	429.2
ΣTPHP (Σ of DPHP, HO-TPHP, 4HO-DPHP)			0.34	0.58	1.10	2.17	6.92	162.8
ΣTBOEP (Σ of BBOEP, BBOEHEP, 3-HO-TBOEP)			0.23	0.23	0.63	1.61	5.85	30.3
ΣEHDPHP (Σ of EHPHP, 5-HO-EHDPHP)			0.34	0.44	0.89	1.78	4.33	22.05
Specific gravity		100	1.003	1.018	1.025	1.029	1.034	1.044

Abbreviations: limits of quantification (LOQ).

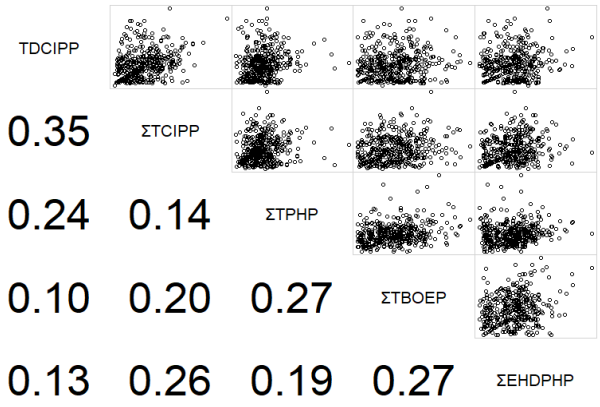


Figure 1.2. The correlations between concentrations of organophosphate flame retardants and plasticizers (PFRs). The upper panel shows the scatter diagrams and the lower panel shows the correlation coefficient (ρ values) calculated by Spearman's rank correlation test. Significant correlations ($p < 0.05$) were found among all ($n = 427$).

1.3.3. Estimated Daily Intake (EDI) of PFRs

Among these five PFRs, TPHP had the highest average daily intake dose with a median value of 45.7 ng/kg bw/day when using Fue 9 in the formula (Table 1.5). That was followed by TBOEP (42.0 ng/kg bw/day) and EHDPHP (41.3 ng/kg bw/day). The median EDIs of TDCIPP were observed to be tenfold lower than those of the other PFRs (TCIPP, TPHP, TBOEP, EHDPHP). Notably, the calculated maximum EDI values for TCIPP, TPHP, and EHDPHP were found to be between 8 and 36 times higher than the 95th percentile value, suggesting the presence of exceptionally elevated levels of these

metabolites in certain participants' urine. In evaluating the potential health risks associated with these estimated intakes, oral reference doses (RfD) for human exposure were used (Poma et al., 2018), the EDIs of all participants were determined to be well below the RfDs established for the investigated PFRs, with the 95th percentile EDI values ranging from 28 times lower (TCIPP) to 220 times lower (TDCIPP) than the respective

Table 1.5. Estimated daily intakes (EDI in ng/kg bw/day) to organophosphate flame retardants and plasticizers calculated based on urinary metabolite concentrations.

EDIs (ng/kg bw/day)	F_{UE}		Min	25th	50th	75th	95th	Max	RfD
TDCIPP (Based on BDCIPP)	S9	0.48	0.88	2.10	5.05	12.5	90.5	1501	2.0×10^{4}
	HLM	0.71	1.30	3.11	7.47	18.5	134	2219	
TCIPP (Based on BCIPHIPP + BCIPP)	S9	5.95	10.0	17.8	36.1	98.4	354	4809	1.0×10^{4}
	HLM	5.05	8.50	15.1	30.6	83.5	300	4081	
TPHP (Based on DPHP + HO-TPHP)	S9	6.54	13.5	26.4	45.7	83.5	247	8936	2.0×10^{4}
	HLM	3.03	6.24	12.2	21.2	38.7	115	4141	
TBOEP (Based on BBOEP + BBOEHEP + TBOEP-HO)	S9	5.82	9.91	21.1	42.0	87.6	307	1466	1.5×10^{4}
	HLM	1.15	1.96	4.17	8.30	17.3	60.6	290	
EHDPHP (Based on EHPHP + 5-HO-EHDPHP)	High F_{UE}	8.59	12.5	24.0	41.3	69.5	162	1366	1.5×10^{4}
	Low F_{UE}	2.15	3.12	6.00	10.3	17.4	40.5	341	

HLM: human liver microsome fraction; S9: S9 fraction; bw: bodyweight; per: percentile.

F_{UE} estimations taken from (Van Den Eede et al., 2013).

No Fue of EHDPHP is available in the literature, therefore low and high Fue scenario were used.

Oral reference doses (RfDs) taken from (Poma et al., 2018).

RfDs, and the max EDI values ranging from 2 times lower (TCIPP) to 13 times lower (TDCIPP) than the respective RfDs.

1.3.4. Determinants of exposure

The basic characteristics of participants could be related to the measured internal exposure. Associations between participants' characteristics and urinary concentration of PFRs are summarized in Table 1.6. Boys have significantly higher levels of TCIPP compared with girls. Although not statistically significant, urinary concentrations of other PFRs were relatively higher in boys than girls. Higher level of annual household income was significantly associated with higher level of TBOEP exposure. Although not statistically significant, lower level of annual household income was associated with higher level of TCIPP exposure. Significantly higher TDCIPP, TCIPP, TBOEP, and EHDPHP were found among children who are currently experiencing environment tobacco smoking compared with those who are not. Higher age is associated with lower TPHP but higher EHDPHP. Higher BMI is significantly associated with higher levels of TDCIPP, TCIPP, TPHP, TBOEP, and EHDPHP.

Table 1.6. Associations between participants' characteristics and urinary concentration of PFRs.

	TDCIPP		∑TCIPP		∑TPHP		∑TBOEP		∑EHDPHP	
	mean	***p***	**mean**	***p***	**mean**	***p***	**mean**	***p***	**mean**	***p***
Sex										
Boys	0.364	0.393	1.636	0.007	1.124	0.376	0.770	0.060	0.893	0.390
Girls	0.319		1.119		0.970		0.570		0.797	
Annual household income (Japanese yen/year)										
< 5 million	0.378	0.459	1.695	0.062	1.024	0.301	0.587	0.048	0.856	0.410
5 - 8 million	0.312		1.277		0.950		0.605		0.776	
> 8 million	0.362		1.202		1.224		0.845		0.938	
Physical exercise										
yes	0.381	0.877	1.416	0.925	0.935	0.346	0.770	0.307	0.843	0.960
no	0.331		1.362		1.097		0.638		0.850	
Environment tobacco smoking										
yes	0.431	0.018	1.675	0.008	1.188	0.223	0.828	0.012	1.006	0.019
no	0.297		1.213		0.973		0.587		0.761	
	ρ	***p***	**ρ**	***p***	**ρ**	***p***	**ρ**	***p***	**ρ**	***p***
Age	0.002	0.970	0.048	0.320	-0.095	0.050	-0.004	0.938	0.103	0.034
BMI (kg/m^2)	0.152	0.002	0.176	0.000	0.097	0.046	0.201	<.0001	0.141	0.004

The p values were calculated by Mann–Whitney U test and Spearman's rank correlation test for the categorical and continuous variables respectively. The ρ values were the correlation coefficient calculated by Spearman's rank correlation test.

1.4. Discussion

1.4.1. Comparison of urinary metabolite levels with other countries

We conduct a comparison of the median concentrations of PFR metabolites reported

in this study and other countries in Table 1.7. The exposure levels of PFRs in the current data are found to be comparable to those reported in previous studies among 7-year-old and school-aged Japanese children (Bastiaensen et al., 2019a, Bastiaensen et al., 2020). These samples were taken from 2009 to 2020, which may prove that the exposure levels of PFRs in Japanese children remained stable during these years. TDCIPP, TCIPP, and TPHP are commonly employed in polyurethane foam applications, which have been frequently detected in house dust at relatively high levels (Kanazawa et al., 2010, Meeker et al., 2013, Hoffman et al., 2017). BDCIPP, BCIPHIPP, and DPHP are the three main metabolites from these parent components, which have been most studied in human urine. In comparison, the urine concentrations of BDCIPP, BCIPHIPP, and DPHP detected in this study were lower than those reported in the urine of children or adolescents in China, USA, Australia, Germany, Norway and Belgium (Fromme et al., 2014, Cequier et al., 2015, He et al., 2018b, Ospina et al., 2018, Ding et al., 2019, Bastiaensen et al., 2021a, Li et al., 2022, Yu et al., 2022a, Louis et al., 2023). TBOEP was utilized as plasticizers and lubricants in floor wax, and comparable level of BBOEHEP and BBOEP was found in this study and other developed countries including Australia and Germany (Fromme et al., 2014, He et al., 2018a, He et al., 2018b). BBOEP level was relatively low in the 2015

Table 1.7. Comparison of median concentrations of PFR metabolites (ng/mL) reported in various studies.

				TNBP	EHDPHP		TBOEP			TPHP		TCIPP		TDCIPP	
Country	n	Sampling	Population	DNBP	5-HO-EHDPHP	EHDPHP	3-HOTBEP	BBOEP	BBOEHEP	4-HODPHP	DPHP	BCIPP	BCIPHIPP	BDCIPP	Reference
Japan	427	2017-2020	Children (9–12 y)	<LOQ	<LOQ	0.24	<LOQ	0.07	0.06	<LOQ	0.27	<LOQ	0.2	0.11	This study
Japan	400	2012-2017	Children (7 y)	<LOQ	0.01	0.31	<LOQ	0.11	0.11	<LOQ	0.46	<LOQ	0.4	0.13	(Bastiaensen et al., 2020)
Japan	128	2009-2010	Children (7–12 y)	<LOQ	0.04	<LOQ	<LOQ	0.2	0.36	0.22	0.32	<LOQ	0.2	0.07	(Bastiaensen et al., 2019a)
China	411	2015	Children (8–12 y)	0.12				0.05			0.28	0.2		0.05	(Chen et al., 2018)
China	306	2015	Adolescents (12–15 y)	2.54				0.11	<LOQ	<LOQ	0.42	0.2	1.5	6.17	(Ding et al., 2019)
China	227	2016	Children (0–5 y)	0.06				0.04			0.27	0.7		0.08	(Zhang et al., 2018)
China	180	2016-2017	Adults and children	0.003				0.1			0.07	<LOQ		<LOQ	(Sun et al., 2018)
China	929	2018	Children and adolescents (6–18 y)	<LOQ				0.16		<LOQ	0.22		0.4	0.19	(Yu et al., 2022a)
USA	147	2009-2013	Children (5–12 y)								1.62			1.38	(Louis et al., 2023)
USA	592	2011-2014	Adolescents (12–19 y)								1.25	0.2		1.14	(Li et al., 2022)
USA	427	2013-2014	Adolescents (12–19 y)	0.21							1.44	0.2		1.43	(Ospina et al., 2018)
USA	203	2014-2016	Children (3–6 y)								2.3	0.5	1.3	6.0	(Hoffman et al., 2018)
USA	90	2015	Children (3–5 y)								3.2	0.9	0.6	2.6	(Gibson et al., 2019)
USA	40	2015	Children (0–3 y)								2.5	<LOQ	2	7.4	(Butt et al., 2014)
Australia	400	2014-2015	Children (0–5 y)	0.18			0.03	0.32	0.08		25	0.9	0.4	2.6	(He et al., 2018b)
Australia	51	2015-2016	Children (0–2 y)	0.15			0.13	0.1	0.08		1	0.7	0.9	3.3	(He et al., 2018a)
Germany	312	2011-2012	Children (2–6 y)	0.2				2			0.8				(Fromme et al., 2014)
Norway	54	2012	Children (6–12 y)	<LOQ				<LOQ			1			0.23	(Cequier et al., 2015)
Belgium	582	2017-2018	Adolescents (14–15 y)	<LOQ	0.01	4.07	<LOQ	<LOQ	0.04	<LOQ	1.37	<LOQ	0.7	0.34	(Bastiaensen et al., 2021a)

Blank represented the median concentration of the metabolite that was not measured in that study.

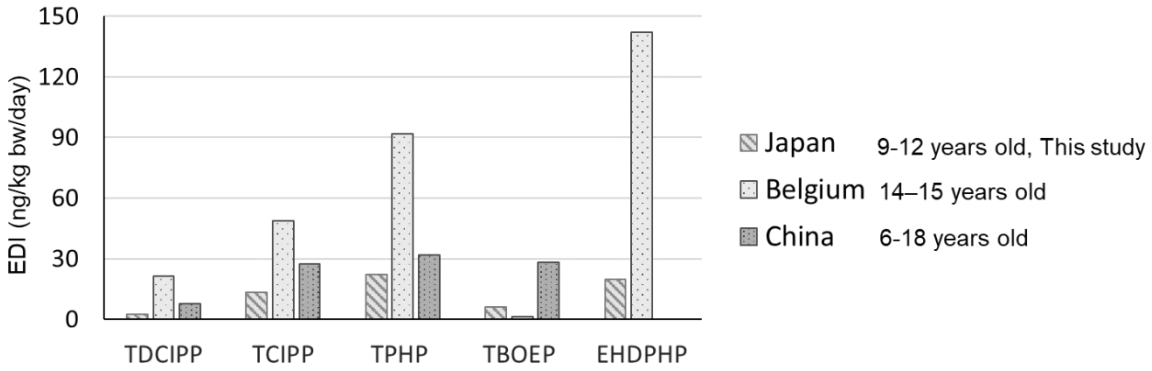
sampling results of China, but became comparable to Japan from the 2018 results (Chen et al., 2018, Yu et al., 2022b). EHDPHP is used as a flame retardant and plasticizer for polyvinyl chloride (PVC), as well as in coatings, rubbers, adhesives, and food packaging materials (Ballesteros-Gómez et al., 2015). However, limited studies have evaluated the internal exposure level of EHDPHP. Unlike Belgian adolescents who were mainly exposed to EHDPHP, Japanese children were exposed at a level 17 times lower than Belgian adolescents (Bastiaensen et al., 2021a). TNBP is used in hydraulic fluids. The concentration for Japanese children is below the detectable level (LOQ = 0.2 ng/mL), which is relatively lower compared with China and the USA (Ospina et al., 2018, Ding et al., 2019). Combined with those findings, the concentrations of urinary PFR metabolites in Japanese children were relatively lower than those in other countries.

1.4.2. Estimated daily exposure

The EDI could represent the cumulative PFRs exposure over all known or unknown exposure routes and sources. Our study results suggest that Japanese school-aged children are well below the risk threshold. Then, the bar graph below shows the comparison of EDI with other studies (Figure 1.3). The EDI level of PFRs is relatively lower in Japanese children compared to those from Belgium and China (Bastiaensen et al., 2021a, Yu et al.,

2022a). In this study, we exclusively discussed EDI results from the FUE-S9 fraction, as it includes both phase I and phase II metabolites. PFR metabolites are excreted as glucuronide conjugates in both phase I and phase II, while HLM only contains phase I enzymes (Van Den Eede et al., 2013, Richardson et al., 2016). However, it is important to note that daily intakes calculated here and these RfD values were determined based on limited toxicological data and should be interpreted within the context of their limitations. RfD values do not take into account reference dose for children who are more vulnerable to chemical exposure, or the potential overlapping health effects of combined exposures to multiple PFRs. Therefore, the daily intake calculated here should be considered as an estimate and should be interpreted with caution. Although RfD values were higher than the EDI levels of children, it is still very important to verify the potential health risks of low-level PFR exposure.

Figure 1.3. Comparison of EDIs with other studies.



1.4.3. Determinants of exposure

In this study, we investigated the association between basic characteristics and urinary concentration of PFRs. Results of univariate testing are shown in Table 1.5. The urinary TCIPP was significantly higher in school-aged boys compared with girls. Although the results were not significant, the frequently detected PFRs including TPHP, TBOEP, TDCIPP, and EHDPHP were measured at higher concentrations for boys in this study. Consist sex differences have been observed in previous research that boys have higher 3-HO-TBOEP and BBOEHEP than girls among Japanese school children (Bastiaensen et al., 2019a). In contrast, Chinese girls aged 12-15 years and American girls aged 5-12 years have higher levels of TPHP compared to boys (Ding et al., 2019, Louis et al., 2022). This association is likely due to behavioral differences between boys and girls, as well as cross-cultural differences. TPHP was present at the highest concentrations in laptop computers, PC boards, keyboard tops, LCD panels, and electrical outlets (Kajiwara et al., 2011). Moreover, nail polish has been identified as an exposure source of TPHP (Mendelsohn et al., 2016). Investigating the lifestyles of those who use these potential sources of exposure on a daily basis may help to further understand sex-related differences.

Age was significantly positively associated with EHDPHP, but significantly negatively

associated with the TPHP in this study. Thus, the older children tended to have higher concentrations of EHDPHP but lower concentrations of TPHP. A previous research focused on 7-12 years old Japanese children, reported a significant decreasing association of urinary TCEP with age (Bastiaensen et al., 2019a). Studies conducted in other countries also have reported a decrease in urinary concentrations of certain PFR metabolites with increasing age (Van Den Eede et al., 2015, Chen et al., 2018, Sun et al., 2018). With increasing age, metabolic and ventilation rates become lower and the body surface area smaller, which may lead to lower exposures (Van Den Eede et al., 2015). In contrast, increased use of electronic devices, personal care products, and cosmetics with age may also increase PFR exposure. As previously stated, future studies about lifestyle and usage of daily necessities as well as the exposure source will deepen our understanding of this topic.

Annual household income was an important determinant of TBOEP and TCIPP concentrations in urine. Higher annual household income was associated with higher TBOEP. It was reported that TBOEP was used as a component of floor polisher and wax as a leveling agent (WHO, 2000), and significantly higher levels of TBOEP were detected in the house dust from the wooden floors than for other types of flooring, such as carpeting, tile, stone, or tatami (Araki et al., 2014a). Wooden floors cost more compared to other

floors, it is more affordable for higher income families. Floor polisher and wax are often used for surface protection of wooden floors (Mizouchi et al., 2015), which may be the reason for children with higher annual household incomes are exposed to higher levels of TBOEP.

Significant positive correlations between TCIPP, TPHP, TBOEP, TDCIPP, EHDPHP, and BMI were observed in this study. However, inconsistent evidence has been reported that children with higher BMI had significantly lower levels of BBOEP, DPHP, and EHPHP (Bastiaensen et al., 2021a). Previous studies focused on 7-12 years old children did not find significant differences in BMI. The reason for the different findings may be that the inter-day variations in PFR metabolite concentrations are influenced by BMI. Individuals who are overweight or obese are more prone to experiencing significant fluctuations in urinary PFR metabolite levels, which might be attributed to differences in dietary consumption, physical activity, and metabolic kinetics (Wang et al., 2019, Wang et al., 2021, Roggeman et al., 2022).

Limited studies have reported the association between tobacco smoking and PFRs exposure. Children currently exposed to ETS have higher levels of PFRs concentration compared to those who are not in this research. Previous research focused on general participants reported that smoking behavior is significantly correlated with urinary DPHP

(Van Den Eede et al., 2013). In contrast, the presence of smoking in the household was significantly associated with a lower likelihood of di-p-cresylphosphate (DPCP) concentrations in American children (Louis et al., 2022). Lower levels of DPHP and DNBP was found among participants who were smoker and past-smoker compared with non-smoker in Chinese adults and children (Sun et al., 2018). A possible reason for the inconsistent results of existing studies is that they focus more on active smoking but do not consider exposure to tobacco smoke in the environment. Both active and passive smoking habits for the participants will help them understand the relationship between smoking and PFR exposure.

1.4.4. Strengths and limitations

The strength is that the exposure assessment included 13 PFR metabolites using a validated biomonitoring method. This research is part of the Hokkaido Study, a well-established birth cohort. The cohort study collected detailed information on mother-child pairs, enabled the long-term follow-up of the participants, continued exposure assessment of changes in exposure level, and evaluation of the short- and long-term health effects.

This study had some limitations. Firstly, PFR metabolites were measured from a single spot urine sample. Due to the short biological half-life and frequent exposure, levels of

PFR metabolites tend to vary, and urine concentrations measured from a single urine sample may not accurately reflect the exposure variance of PFRs. Nonetheless, the short-term reproducibility of PFR metabolite concentrations improved significantly when the values were corrected for SG (Bastiaensen et al., 2021b, Roggeman et al., 2022). Secondly, DPHP can be a metabolite of both TPHP and EHDPHP (Van Den Eede et al., 2016), which raises the possibility of overestimating TPHP and underestimating EHDPHP. Thirdly, due to the lack of in toxicokinetic data of PFRs in humans, and the F_{UE} was estimated using only one vitro HLM and S9 experiment, the calculated EDI may be over- or underestimated. Finally, the health risk assessment using RfD value did not account for the potential additive effect on the health of combined exposure to multiple PFRs and/or other toxic chemicals.

1.5. Conclusion

This study reported the concentration of 13 PFR urinary metabolites originating from 7 parent compounds among Japanese school-aged children. The metabolites of BDCIPP, BCIPHIPP, and DPHP were detectable for more than 60% of participants. Sex, age, BMI, annual household income, and environment tobacco smoking were the determinants significant for several PFR metabolites. Although the EDI level of PFRs for Japanese school-aged children were all well below the oral reference doses, future study is needed

to verify the potential health risks of low level PFR exposure.

Chapter 2: Exposure to Organophosphate Flame Retardants and Plasticizers and its Associations with Allergic Symptoms and Type 2 Inflammation (T2) Biomarker Levels

ABSTRACT

Exposure to organophosphate flame retardants and plasticizers (PFRs) has been reported to increase the risk of asthma and allergies. The type 2 inflammation biomarkers, such as fractional exhaled nitric oxide (FeNO), blood eosinophils, and total immunoglobulin E (IgE), are recommended for the diagnosis and management of allergies. However, little is known about its association with levels of T2 biomarkers. This chapter investigated associations among urinary PFR metabolite concentrations, allergic symptoms, and T2 biomarkers. The data and samples were collected between 2017 and 2020, including school children (n=427) aged 9–12 years living in Sapporo City, Japan, among the participants of "The Hokkaido Study on Environment and Children's Health." Thirteen urinary PFR metabolites were measured by LC-MS/MS. Allergic symptoms were assessed using the International Study of Asthma and Allergies in Childhood questionnaire. For T2 biomarkers, the peripheral blood eosinophil counts, FeNO, and serum total IgE level were measured. Multiple logistic regression analysis, quantile-based

g-computation (qg-computation), and Bayesian kernel machine regression (BKMR) were used to examine the associations between the health outcomes of the individual PFRs and the PFR mixtures. The highest concentration of PFR was Σtris(1-chloro-isopropyl) phosphates (ΣTCIPP) (Median:1.20 nmol/L). Tris(1,3-dichloro-2-propyl) phosphate (TDCIPP) was significantly associated with a high odds ratio (OR, 95%CI:1.36, 1.07-1.72) for wheeze. TDCIPP (OR, 95%CI:1.19, 1.02-1.38), Σtriphenyl phosphate (ΣTPHP) (OR, 95%CI:1.81, 1.40-2.37), and Σtris(2-butoxyethyl) phosphate (ΣTBOEP) (OR, 95%:1.40, 1.13-1.74) were significantly associated with increased odds of FeNO (≥35 ppb). ΣTPHP (OR, 95%CI:1.44, 1.15-1.83) was significantly associated with high eosinophil counts (≥300/μL). For the PFR mixtures, a one-quartile increase in all PFRs (OR, 95%CI:1.48, 1.18-1.86) was significantly associated with high FeNO (≥35 ppb) in the qg-computation model. The PFR mixture was positively associated with high FeNO (≥35 ppb) and eosinophil counts (≥300/μL) in the BKMR models. These results may suggest that exposure to PFRs increases the probability of asthma, allergies, and T2 inflammation.

Keywords: PFRs, FeNO, Serum total IgE, Peripheral blood eosinophils, BKMR, Qg-computation

2.1. Introduction

Health concerns about exposure to PFRs are increasing. Exposure to PFR potentially impacts child neurodevelopment through decreased intelligence quotient and working memory scores (Castorina et al., 2017). It is also associated with inflammatory responses through high levels of oxidative stress among children (Ait Bamai et al., 2019). Previous studies measured the PFRs in house dust from floor or multi-surfaces. We found that tri-n-butyl phosphate (TNBP), tris(1-chloro-isopropyl) phosphate (TCIPP), and tris(1,3-dichloro-2-propyl) phosphate (TDCIPP) significantly increased the odds of wheeze, eczema, and allergic rhinoconjunctivitis (Araki et al., 2014b, Ait Bamai et al., 2018, Araki et al., 2018). However, different results have been reported in studies conducted in the United States and Sweden. No positive associations were observed between childhood asthma and the PFRs in dust from household filter systems (Bi et al., 2018) and dust from mothers' mattresses (Canbaz et al., 2016). This inconsistency may be due to the different sources of the dust samples, and the concentration of PFRs in house dust represents external exposure but not internal exposure.

To measure internal exposure, biomonitoring of metabolites of PFRs in urine, has been recently established (Van Den Eede et al., 2013, Van Den Eede et al., 2015, Bastiaensen et al., 2018). The levels of urinary metabolites can reflect internal exposure not only from

house dust, but also from other exposure sources, including diet (Araki et al., 2018, Doherty et al., 2019). A previous study on the measurement of PFR metabolites in urine reported a significant increase in children's allergic symptoms with higher concentrations of metabolites from TDCIPP, TCIPP, and tris(2-butoxyethyl) phosphate (TBOEP) (Araki et al., 2018). However, the association between urinary PFR metabolite concentration and allergic disease remains unclear.

The pathophysiology of allergic diseases includes an overreaction of type 2 immune responses driven by type 2 helper T (Th2) cells and type 2 innate lymphoid (ILC2) cells (Lloyd and Snelgrove 2018). The measurement of T2 biomarkers, such as fractional exhaled nitric oxide (FeNO) levels, blood eosinophils, and total immunoglobulin E (IgE), is recommended for the diagnosis and management of allergies (Pavord et al., 2018, Reddel et al., 2022, Goudarzi et al., 2023). An experimental study reported that eosinophil counts in mouse bronchoalveolar lavage fluid increased dramatically after inhaling TNBP (Meng et al., 2022). However, this experimental evidence was based on high doses of a single PFR exposure, whereas human exposure involves multiple PFRs at much lower levels. Whether these experimental findings can be validated in humans remains to be studied. To our knowledge, there is no epidemiological evidence of an association between exposure to PFRs and T2 biomarkers, which could better clarify the relationships

among PFRs, asthma, and allergies.

The evaluation of the health effects of combined exposure to multiple chemicals has been highlighted, as humans are simultaneously exposed to different chemicals (Meek et al., 2011). Certain challenges must be addressed when considering the overall health effects of multiple chemicals. Traditional regression analysis models may be biased due to collinearity and interactions among different chemicals. Quantile-based g-computation (qg-computation) and Bayesian kernel machine regression (BKMR) analyses can identify linear and nonlinear effects, respectively, and assess the health effects of multiple exposures that were multicollinear or highly correlated (Bobb et al., 2015, Bobb et al., 2018, Keil et al., 2020). Using the abovementioned analyses, this study investigated the association among urinary individual PFR metabolites, PFR mixtures, allergic symptoms, and T2 biomarkers in 9–12 years old children. We hypothesized that increased internal exposure to PFRs would be associated with a high probability of allergic symptoms and high levels of T2 biomarkers.

2.2. Materials and methods

2.2.1. Study population

The study participants for this study are the same children described in Chapter 1

shown in flow chart figure 1.1.

2.2.2. Questionnaire

Parents answered a questionnaire to collect information on their children's demographics and allergic symptoms. Demographic characteristics included sex, age, physical exercise frequency, environmental tobacco smoke (ETS) status, annual household income, and maternal educational level. ETS (yes/no) is defined as a child's current exposure to passive smoke from a mother, father, or other household members who smoke tobacco in places where the child lives. Allergic symptoms of the children, including wheeze, eczema, and allergic rhinoconjunctivitis, were defined using the International Study of Asthma and Allergies in Childhood (ISAAC) questionnaire (Asher et al., 1995, Asher et al., 2006). Wheeze was a positive “yes” answer to the question “Has your child wheezed or whistled in the chest in the last 12 months?,” and eczema symptoms were defined based on a “yes” answer to all the following three assertions: (a) “Presence of an itchy rash that comes and goes for at least 6 months” and (b) “Presence of an itchy rash on below-mentioned areas in the last 12 months” and (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.” Allergic rhinoconjunctivitis symptoms were defined by positive responses 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) “Was this problem accompanied by itchy or watery eyes?”.

2.2.3. Measurement of T2 markers and PFR metabolites

The analytical methods for T2 biomarkers have been described in a previous study (Goudarzi et al., 2023). Peripheral blood samples were collected from 417 children, and FeNO levels were measured from 422 children in pediatric clinics. Peripheral blood was then sent for analysis by a cool delivery service. Peripheral blood eosinophil counts (/μL) was measured at Daiichi Kishimoto Clinical Laboratories, Inc. (Sapporo, Japan). Serum total IgE levels (international unit, IU/mL) were measured using enzyme-linked immunosorbent assay (ELISA) at SRL, Inc. (Tokyo, Japan). FeNO levels were measured with an electrochemical sensor NIOX VERO® (Aerocrine, Stockholm, Sweden). A single-breath online method measured gas-phase FeNO (parts per billion, ppb) (American Thoracic Society and European Respiratory Society 2005, Dweik et al., 2011). In this study, we used cut-off values to define high levels of T2 biomarkers. FeNO ≥35 ppb in children younger than 12 years of age was described to indicate eosinophilic inflammation and to support measuring airway inflammations, including asthma (Dweik et al., 2011). There is no gold standard for children’s cut-off values of total IgE level and

peripheral blood eosinophil counts yet. Shimazu (1994) reported that the total IgE level for healthy children (7 years and older) is less than 170 IU/ml, which has been used as a cut-off value in a national cohort study in Japan recently (Shimazu 1994, Saito-Abe et al., 2021). Nakamura (2020) reported that the eosinophil counts higher than 300/μL is related to an increased risk of asthma (Nakamura et al., 2020). The concentration of PFR metabolites was measured as described in Chapter 1.

2.2.4. Statistical analysis

The SG corrected PFR concentrations were converted to natural log scales for the downstream analyses to obtain better normality. The random forest method was used to fill in the missing values of the annual household income (missing rate: 5.6%) (Breiman 2001). Differences and correlations between demographic characteristics, PFR concentrations, and health outcomes were analyzed using the Mann–Whitney *U*, chi-square, and Spearman's rank correlation tests. Covariates retained in the adjusted models included sex, age, annual household income, body mass index (BMI), and ETS, which were selected based on previous study (Goudarzi et al., 2023). These covariates were associated with PFR exposure, allergic symptoms, and T2 biomarkers in the bivariate tests ($p < 0.1$).

Multiple logistic regression models were used to assess the relationships between

individual PFRs, wheeze, eczema, allergic rhinoconjunctivitis, and T2 biomarkers. Qg-computation and BKMR examine the partial and joint effects of the PFR mixture. Qg-computation adapts the weighted quantile regression approach and enhances the causal inferential aspects using g-computation (Keil et al., 2020). Qg-computation utilizes the following equation (Keil et al., 2020):

$$Y_i = \beta_0 + \sum_{j=1}^{d} \beta_j X_{ji}^{q} + \epsilon_i$$

where Y_i is the health outcome for individual i (i = 1, 2, 3…n), β_0 denotes the model intercept, X_{ji}^{q} is a quartile version of j^{th} chemical exposure, $\sum_{j=1}^{d} \beta_j$ is the weighted quantile sum, which estimates the combined effect of increasing every exposure in the PFR mixture by one quantile simultaneously, and ϵ_i is the error term. The qg-computation also enables the evaluation of the individual contributions of the mixture and simultaneously estimates the positive or negative weight. The sum of positive and negative weights is defined to be 1.0. Each model was run for 500 iterations using bootstrapping.

Compared with qg-computation, BKMR is a non-parametric method for estimating the overall mixture effect and individual chemical impact on health outcomes. The BKMR model was executed using a kernel machine regression equation (Bobb et al., 2015, Bobb et al., 2018):

$$Y_i = h(z_{il}, \ldots, z_{im}) + x_i\beta + \epsilon_i$$

where Y_i is the health outcome for individual i (i = 1, 2, 3…n), z_{im} denotes the m^{th} chemical exposure, h is the function that fits the exposure and the outcome considering nonlinear interactions between the exposures, x_i represents the potential confounder, β is the effect of the covariates, and ϵ_i is the residual. The cumulative effect of the PFR mixture on allergic symptoms and T2 biomarkers was assessed by comparing the values when all chemical components were at their 50th percentile with values when all chemical components were at a particular percentile between the 25th and 75th percentile. The individual chemical impacts were evaluated while keeping all the remaining exposures in the mixture fixed at their median concentration. The interaction effects among PFRs on health outcomes were quantified by comparing a single PFR health risk when all other exposures were fixed at their 25th percentile to when all PFRs were fixed at their 75th percentile (Bobb et al., 2015, Bobb et al., 2018). Models were run for 10,000 iterations using a Markov-chain Monte Carlo sampler. Two-tailed tests were used for all statistical analyses, and a p-value of less than 0.05 was considered statistically significant. We consider a BKMR analysis statistically significant when its 95% credible interval does not overlap with zero. All statistical analyses were performed using R (Version 4.2.3) with the packages "randomForest" (version 4.7-1.1), "glm2" (version 1.2.1), "qgcomp"

(Version 2.10.1), “bkmr” (Version 0.2.2) for missing value imputations, logistic regression analysis, qg-computation, and BKMR, respectively.

2.2.5. Ethics

This study ethical approval and parents’ consent was obtained as described in Chapter 1.

2.3. Results

2.3.1. Allergic symptoms and level of T2 biomarkers

The prevalence of allergic symptoms and level of T2 biomarkers of study participants were shown in Table 2.1. The number of children with allergic symptoms of wheeze, eczema, and allergic rhinoconjunctivitis was 32 (7.5%), 96 (23%), and 92 (22%), respectively. For the T2 biomarker, the median (interquartile range) for FeNO, total IgE, and eosinophil counts were 17.5 (9–37) ppb, 129 (37–394) IU/mL, and 234 (130–422) counts/μL, respectively. The number of children showing FeNO ≥35 ppb, total IgE ≥170 IU/mL, and eosinophil counts ≥ 300/μL was 116 (27%), 183 (44%), and 162 (39%), respectively.

Table 2.1. Allergic symptoms and level of T2 biomarkers of study participants (n=427)

Variable		n	%
Wheeze	yes	32	7.5
Eczema	yes	96	22.5
Allergic rhinoconjunctivitis	yes	92	21.6
FeNO (high)(n=422)	≥35 ppb	116	27.5
Serum total IgE (high) (n=417)	≥170 IU/mL	183	43.9
Peripheral blood eosinophils (high) (n=417)	≥300 counts/μL	162	38.8

2.3.2. Allergic symptoms, T2 biomarkers, and individual PFR

In multiple logistic regression models (Figure. 2.1A, Table 2.2, and Table 2.3), for wheeze, a natural log unit increase in TDCIPP was significantly associated with a high adjusted odds ratio (AOR) (95% CI) of 1.36 (1.07–1.72). For FeNO ≥35 ppb, TDCIPP, ΣTPHP, and ΣTBOEP were significantly associated with high AOR (95% CI) of 1.19 (1.02–1.38), 1.81 (1.40–2.37) and 1.40 associated with a high AOR (95% CI) of 1.44 (1.15–1.83). No significant associations were observed between the concentrations of PFR metabolites and eczema, allergic rhino-conjunctivitis, or total IgE ≥170 IU/mL. The results of the categorical models represent the dose response relationships (Figure 2.2). A positive significant trend was observed among TDCIPP and wheeze; TDCIPP, TPHP, and FeNO; TPHP and eosinophil.

Table 2.2. Associations between allergic symptoms and urinary organophosphate flame retardants (PFRs) in multiple logistic regression models.

	Wheeze		Eczema		Allergic rhinoconjunctivitis	
	AOR (95% CI)	p	AOR (95% CI)	p	AOR (95% CI)	p
BDCIPP	1.36 (1.07, 1.72)	0.011	1.00 (0.84, 1.19)	0.960	1.04 (0.88, 1.23)	0.640
BCIPP	1.27 (0.95, 1.67)	0.097	1.00 (0.82, 1.22)	0.970	0.92 (0.74, 1.13)	0.439
BCIPHIPP	1.13 (0.87, 1.46)	0.336	0.94 (0.79, 1.11)	0.472	1.02 (0.85, 1.21)	0.847
DPHP	1.24 (0.85, 1.74)	0.224	1.08 (0.83, 1.40)	0.541	1.25 (0.97, 1.60)	0.082
HO-TPHP	1.38 (0.90, 2.00)	0.095	0.77 (0.49, 1.12)	0.216	1.17 (0.84, 1.59)	0.316
BBOEP	1.30 (0.93, 1.80)	0.118	1.14 (0.91, 1.43)	0.251	1.19 (0.94, 1.49)	0.139
BBOEHEP	0.92 (0.68, 1.20)	0.536	0.95 (0.79, 1.14)	0.591	1.12 (0.94, 1.33)	0.215
3-HO-TBOEP	1.00 (0.65, 1.47)	0.992	1.04 (0.80, 1.35)	0.757	1.02 (0.77, 1.32)	0.913
EHPHP	0.81 (0.50, 1.28)	0.385	0.73 (0.54, 0.99)	0.047	0.97 (0.71, 1.29)	0.814
5-HO-EHDPHP	0.73 (0.45, 1.09)	0.159	0.98 (0.76, 1.25)	0.886	0.99 (0.77, 1.26)	0.950
TDCIPP	1.36 (1.07, 1.72)	0.011	1.00 (0.84, 1.19)	0.960	1.04 (0.88, 1.23)	0.640
ΣTCIPP	1.22 (0.90, 1.64)	0.189	0.96 (0.78, 1.18)	0.690	1.01 (0.81, 1.23)	0.956
ΣTPHP	1.25 (0.86, 1.75)	0.217	1.08 (0.83, 1.39)	0.548	1.25 (0.97, 1.60)	0.082
ΣTBOEP	1.19 (0.84, 1.67)	0.309	1.08 (0.85, 1.36)	0.520	1.22 (0.97, 1.54)	0.088
ΣEHDPHP	0.80 (0.49, 1.27)	0.360	0.75 (0.55, 1.02)	0.071	0.97 (0.72, 1.31)	0.866

Table 2.3. Associations between T2 biomarkers and urinary organophosphate flame retardants (PFRs) in multiple logistic regression models.

	FeNO ≥35 ppb		Total IgE ≥170 IU/mL		Eosinophil ≥300 /μL	
	AOR (95% CI)	p	AOR (95% CI)	p	AOR (95% CI)	p
BDCIPP	1.19 (1.02, 1.38)	0.028	0.99 (0.86, 1.13)	0.833	1.02 (0.88, 1.18)	0.773
BCIPP	1.12 (0.92, 1.35)	0.242	0.99 (0.83, 1.17)	0.875	1.07 (0.90, 0.25)	0.431
BCIPHIPP	1.15 (0.98, 1.36)	0.080	0.96 (0.83, 1.11)	0.583	1.11 (0.96, 0.25)	0.174
DPHP	1.80 (1.40, 2.35)	0.000	1.21 (0.97, 1.51)	0.095	1.44 (1.15, 0.60)	0.002
HO-TPHP	1.41 (1.05, 1.93)	0.025	1.47 (1.09, 2.07)	0.017	1.26 (0.94, 0.53)	0.121
BBOEP	1.42 (1.14, 1.76)	0.002	1.16 (0.96, 1.42)	0.124	1.14 (0.93, 0.33)	0.197
BBOEHEP	1.20 (1.02, 1.41)	0.031	1.02 (0.88, 1.18)	0.814	1.00 (0.86, 0.15)	0.971
3-HO-TBOEP	0.93 (0.72, 1.20)	0.590	0.89 (0.71, 1.11)	0.292	0.84 (0.66, 0.05)	0.135
EHPHP	1.06 (0.80, 1.39)	0.691	0.83 (0.64, 1.06)	0.133	0.96 (0.74, 0.21)	0.738
5-HO-EHDPHP	1.12 (0.89, 1.40)	0.321	0.95 (0.77, 1.16)	0.595	1.05 (0.85, 0.26)	0.641
TDCIPP	1.19 (1.02, 1.38)	0.028	0.99 (0.86, 1.13)	0.833	1.02 (0.88, 1.18)	0.773
ΣTCIPP	1.19 (0.98, 1.44)	0.082	0.97 (0.81, 1.16)	0.742	1.17 (0.98, 1.39)	0.091
ΣTPHP	1.81 (1.40, 2.37)	0.000	1.21 (0.97, 1.52)	0.091	1.44 (1.15, 1.83)	0.002
ΣTBOEP	1.40 (1.13, 1.74)	0.002	1.08 (0.89, 1.32)	0.423	1.05 (0.86, 1.28)	0.639
ΣEHDPHP	1.06 (0.80, 1.40)	0.662	0.83 (0.64, 1.06)	0.137	0.96 (0.74, 1.23)	0.739

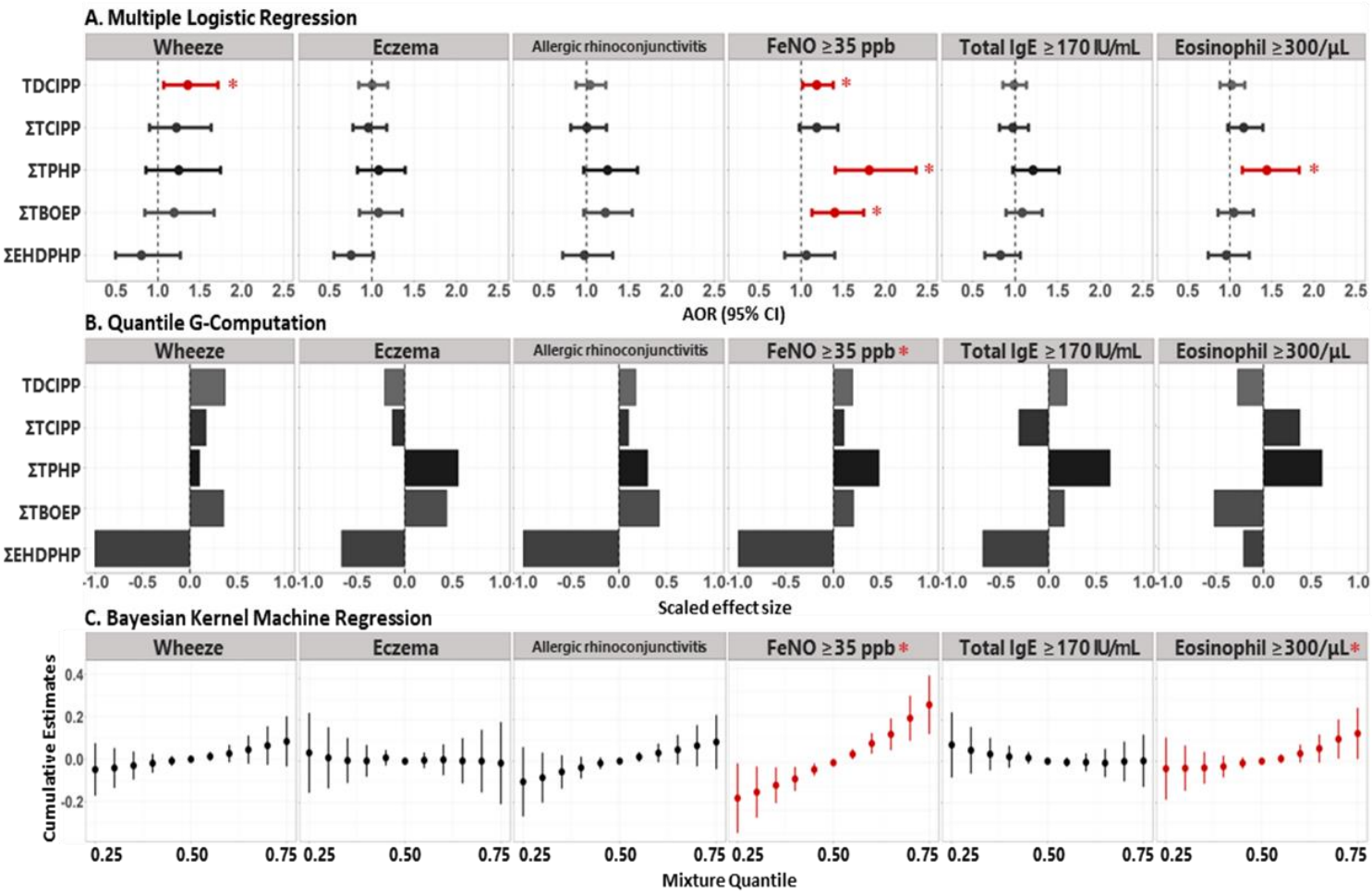


Figure 2.1. Associations between allergic symptoms and type 2 inflammation (T2) biomarkers and urinary organophosphate flame retardants and plasticizers (PFRs) among school children in Hokkaido study, 2017-2020.

(A)shows the results of multiple logistic regression models. Adjusted odds ratio and 95% confidence interval (CI) indicates changes in allergic symptoms and T2 biomarkers in association with a natural-log unit increase in individual PFRs; (B)shows the results of quantile g-computation. Positive and negative weights represent the partial contribution of PFRs in the mixture on health outcomes; (C)shows the results of Bayesian kernel machine regression (BKMR). The cumulative effect of PFR mixture on health outcomes was estimated when all chemical components were at their 50th percentile.

All models were adjusted for sex, age, annual household income, BMI, and environmental tobacco smoke. The concentrations of urinary PFR metabolites were specific gravity adjusted and natural log transformed. An asterisk is marked next to the significant result.

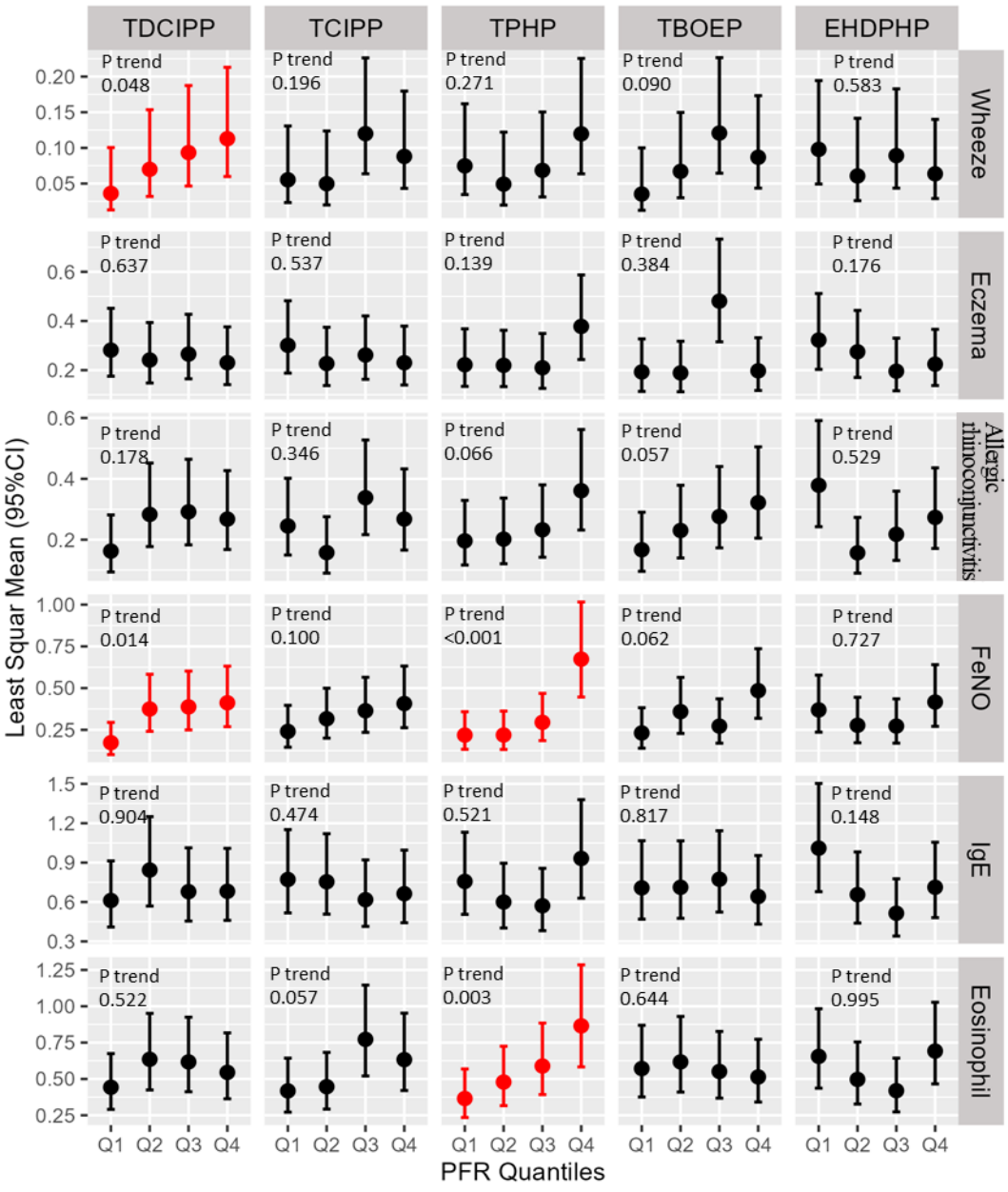


Figure 2.2. Associations between urinary PFRs and allergic symptoms and T2 biomarkers in categorical model. Y-axis represents least square mean (LSM) and 95% confidential interval (95% CI) of allergic symptoms and T2 biomarkers, shown as black squares and whiskers, respectively. X-axis represents the levels of urinary PFR metabolites in quartiles. LSMs were adjusted for sex, age, household income, and environmental tobacco smoke. P for trends show the linearities from 1st quartile to 4th quartile. Significant positive P trends were highlighted with red color.

2.3.3. Allergic symptoms, T2 biomarkers, and PFRs mixture

QG-computation was used to analyze the mixture effect of the PFRs. One-quartile increase in the PFRs index was associated with significantly high odds of exceeding the cut-off level for FeNO, with values ≥35 ppb (AOR=1.48, 95% CI=1.18–1.86). No significant association was observed between the PFR mixture, wheeze, eczema, allergic rhinoconjunctivitis, total IgE, or eosinophil counts. The individual contributions of the mixtures to each chemical are shown in Figure 2.1B and Table 2.4. For FeNO, the chemicals with the highest weight (%) in the positive direction were ΣTPHP (47.9%),

Table 2.4. Associations between allergic symptoms, T2 biomarkers, and organophosphate flame retardant and plasticizer (PFRs) mixture in quantile g-computation.

Quantile g-computation	Adjusted OR (95% CI)	p-value	The sum of positive coefficient	The sum of negative coefficient
Wheeze	1.59 (0.91 , 2.80)	0.105	0.77	-0.26
Eczema	1.02 (0.77 , 1.34)	0.901	0.34	-0.31
Allergic rhinoconjunctivitis	1.27 (0.94 , 1.71)	0.121	0.50	-0.18
FeNO	**1.48 (1.18 , 1.86)**	**0.001**	**0.69**	**-0.09**
Total IgE	0.96 (0.81 , 1.14)	0.664	0.13	-0.19
Eosinophil	1.17 (0.96 , 1.43)	0.124	0.52	-0.25

The adjusted odds ratio is interpreted as the effect on allergic symptoms and type 2 inflammation biomarkers (T2) of increasing every one quantile unit of exposure to the PFR mixture. The models were adjusted for sex, age, annual household income, BMI, and environmental tobacco smoke.

Abbreviations: odds ratio (OR), confidence interval (CI). Bold letters indicate statistical significance ($p<0.05$).

ΣTBOEP (21.0%), and TDCIPP (20.1%), while ΣEHDPHP was identified as the only one with negative weight.

The results of another mixed chemical model were analyzed using BKMR. The PFR mixture was positively associated with FeNO levels and eosinophil counts (Figur 2.1C). The univariate estimation of the exposure-response functions on the relationship between PFRs and health outcomes is shown in Figure 2.2. When all other metabolites were at their median levels, ΣTPHP was positively associated with the possibilities of FeNO ≥35 ppb and eosinophil counts ≥ 300/μL. Figure 2.3 shows the interaction effects among urinary PFRs exposure to health outcomes. The interactive effects were the change in the single-exposure health effects when all of the remaining exposures are fixed at their 25th percentile as compared to when they are fixed at their 75th percentiles. After subtracting the red points value from the blue points in Figure 2.3, no interaction was found between the PFRs, allergic symptoms, and T2 biomarkers. A sensitivity analysis was conducted to test the robustness of the main findings. The random forest method was used to fill in all missing annual household income values. We assessed the extent to which filled data could affect the findings by removing all data containing missing values and found that the results remained consistent with the original findings (data not shown).

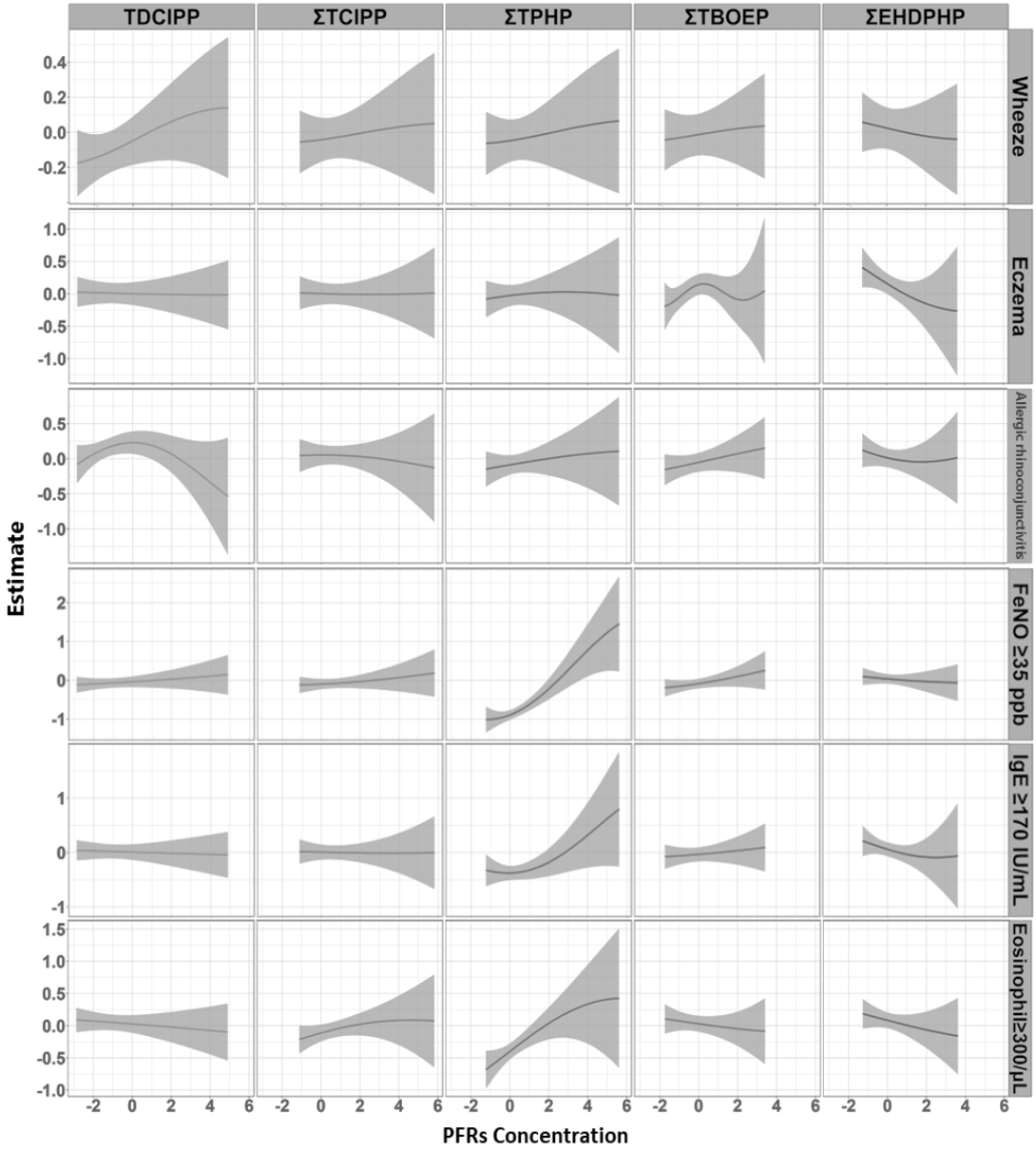


Figure 2.3. Univariate exposure-response functions for the change in allergic symptoms, type 2 Inflammation (T2) biomarkers and urinary organophosphate flame retardants and plasticizers (PFRs). Univariate exposure-response functions and 95 % credible intervals for the change in allergic symptoms, T2 biomarkers and urinary PFRs while holding all the remaining exposures in the mixture fixed at their median concentration, estimated using Bayesian kernel machine regression (BKMR). The models were adjusted for sex, age, annual household income, BMI, and environmental tobacco smoke.

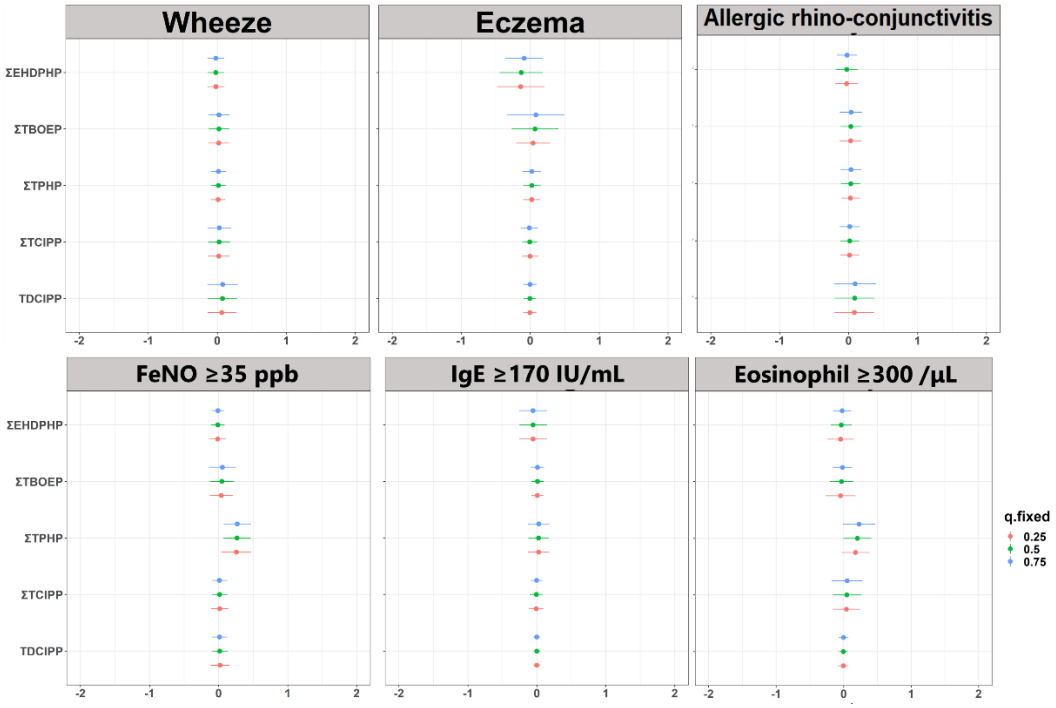


Figure 2.4. Interaction effects among urinary organophosphate flame retardants and plasticizers (PFRs) to health outcomes. Interaction effects were quantified by comparing a single PFR health risk when all other exposures are fixed to their 25th percentile to when all PFRs are fixed to their 75th percentile.

2.4. Discussion

This study explored the associations of five individual PFRs and their mixture with allergic symptoms and T2 biomarkers using three statistical analytical methods. For individual PFRs, positive associations were observed between TDCIPP and wheeze; TDCIPP, ΣTPHP, ΣTBOEP, and FeNO ≥35 ppb; ΣTPHP and peripheral blood eosinophil counts ≥ 300 /μL. The exposure to a higher concentration of PFR mixture was positively associated with higher odds of having FeNO and the eosinophil counts exceeding the cut-off level, and ΣTPHP exhibited the highest contribution to the increase in FeNO and the

eosinophil counts. No association was observed with the prevalence of eczema, allergic rhinoconjunctivitis symptoms, or a serum total IgE ≥170 IU/mL in either individual or mixture analyses. Using mixture analyses, which consider multiple exposures that exhibit multicollinearity or high correlation, can potentially reduce bias compared to single exposure analyses in determining health effects. Despite the different statistical approaches to mixtures, our findings from the qg-computation and BKMR models were generally in line with the multiple regression analysis results, verifying the robustness of our findings.

2.4.1. PFR exposure and allergic symptoms

Positive associations were observed between TDCIPP and wheeze in school children aged 9–12. This result aligns with that of previous studies examining the association between PFRs and allergic diseases. Previous research within the Hokkaido Study reported that TDCIPP in house dust is associated with increased odds of wheeze among 7-year-old children (Ait Bamai et al., 2018). Another study focused on a different population of Sapporo City focused on house dust and urinary metabolites of elementary school children. Here, we found that higher levels of TDCIPP in house dust increased the odds of eczema. For urinary results, TCIPP was positively associated with allergic rhinoconjunctivitis and at least one symptom of allergy; TBOEP increased the odds of

eczema; and BDCIPP increased the odds of at least one symptom of allergy (Araki et al., 2018). A recent study that examined 9 PFR metabolites of school children with asthma in the United States, found that higher levels of urinary DPHP were positively associated with at least one of the daytime symptoms of asthma (Louis et al., 2023). These results indicate that PFR exposure is positively associated with allergic symptoms among children in both the general and asthmatic populations.

2.4.2. PFR exposure and T2 biomarkers

This study observed a significant positive association between the mixture of urinary PFRs and FeNO ≥35 ppb, while the partial effects were the highest for ΣTPHP. Many studies have found that exposure to phthalates, bisphenols, hazardous metals, and parabens can increase FeNO levels in the exhaled breath (Kim et al., 2018, Mamuya et al., 2018, Junge et al., 2022, Sung et al., 2022, Wu et al., 2022). Besides the abovementioned environmental contaminants, our results provide epidemiological evidence that PFRs are associated with higher FeNO levels.

Our study found a significant positive association between the ΣTPHP and high eosinophil counts, which aligns with experimental evidence. An *in vivo* study reported that the number of eosinophils, macrophages, and neutrophils in the bronchoalveolar lavage fluid (BAL) increased drastically in asthmatic mice after TNBP exposure (Meng

et al., 2022). Another experimental study also reported increased eosinophils and lymphocytes in BAL of asthmatic mice after TBOEP exposure (Win-Shwe et al., 2022). These findings indicate that PFRs may cause airway inflammation and hyperresponsiveness by increasing the eosinophil counts. They may also have a potential exacerbating effect on existing airway inflammatory diseases, particularly in individuals with asthma. Future studies in asthmatic patients are needed to validate this hypothesis.

2.4.3. Combined effect of PFRs mixture

Humans are simultaneously exposed to multiple chemicals. Both phthalates and PFRs are used as additives in consumer products and are found in house dust (Kanazawa et al., 2010, Ait Bamai et al., 2014, Araki et al., 2014b, Ballesteros-Gómez et al., 2015). An experimental study reported that mice that received ovalbumin with mono-n-butyl phthalate had higher eosinophil cell counts than those that received only ovalbumin (Quoc et al., 2022). We recently examined the combined exposure to PFRs and phthalates and their associations with allergic symptoms, in which PFRs contributed more to increasing wheeze and allergic symptoms than phthalates, even though the concentration of PFRs is much lower than that of phthalates (Araki et al., 2020). This evidence indicates the need to measure the combined exposure including other environmental chemicals, and its association with eosinophils, as well as other T2 biomarkers in future research.

2.4.4. Strengths and limitations

There are several strengths in our research. This is the first study investigating the association between urinary PFR metabolite mixtures and T2 biomarker levels in children. We conducted qg-computation and BKMR analyses to explore the associations between the mixture of PFR exposure levels, three allergic symptoms, and three T2 biomarkers. The ISAAC questionnaire defines allergy by asking about symptoms in the past year, answer of parents may have recall bias due to the large timeframe. In addition to the questionnaire results, we obtained the FeNO levels, peripheral blood eosinophil counts, and serum total immunoglobulin E level for T2 biomarkers, which are objective values used in managing

This study had some limitations. Firstly, it is important to note that, due to the cross-sectional design of this study, causal relationships between exposure and outcomes could not be demonstrated. Secondly, PFR metabolites were measured from a single spot urine sample, which may not reflect the variance of exposure because of the short biological half-lives and frequent exposure. Thirdly, mixture models alone do not provide information on toxicological measures such as toxicity thresholds or dose-response relationships of individual PFR. Fourthly, DPHP can be a metabolite of both TPHP and EHDPHP (Van Den Eede et al., 2016), which raises the possibility of overestimating

TPHP and underestimating EHDPHP. Fifthly, anti-inflammatory asthma medication histories were not included because very few children took Inhaled Corticosteroid (n=2) and other drugs (n=2), possibly affecting Th2 biomarkers. Sixthly, we could not incorporate potential confounders, including household dampness and mold in this study. Lastly, the questionnaire was answered by the child's parents. Of those we approached, the participation percentage was 22.8%. The selection bias may exist as the included participants were the individuals who responded to the tracking of the Hokkaido Study until the age of 9–12 and who were more convenient to visit the selected clinics. To mitigate the potential for selection bias, we approached all children aged 9–12 years, and we deliberately avoided specifying allergic diseases as study outcomes in the invitation letters. Moreover, previous research has demonstrated that the participants in this study exhibit similar characteristics (child sex, birth weight) and allergic disease prevalence as those in the original cohort (Goudarzi et al., 2023).

2.5. Conclusion

Our study revealed positive associations between TDCIPP and wheeze, TDCIPP, ΣTPHP, ΣTBOEP and FeNO, ΣTPHP, and peripheral eosinophil counts from individual analyses. The PFR mixture was positively associated with FeNO and eosinophil counts

above the cut-off levels. These findings suggest that exposure to PFRs increases the possibility of asthma and allergies by enhancing the expression of T2 biomarkers. Regulating PFR-containing products, particularly in populations with higher exposure levels, may reduce the prevalence of asthma and allergies. Further investigation is needed to assess the combined exposure effects, including other environmental chemicals that have similar effects on allergic diseases, such as phthalates and bisphenols.

Chapter 3: Organophosphate Flame Retardants Mixture Associated with Increased Urinary Oxidative Stress Biomarkers

ABSTRACT

Exposure to organophosphate flame retardants (PFRs) has been reported to be associated with increased risks of asthma and allergies. Oxidative stress contributes to the development of asthma, allergic diseases, and inflammation. This study examined the association of individual and mixture exposure to PFRs with urinary oxidative stress biomarkers among school children. Moreover, the hypothesis of the mediation effect of oxidative stress on the association between PFRs and T2 biomarkers are examined. The study is a part of the Hokkaido Study on Environment and Children's Health. School children aged 9-12 years living in Sapporo and its suburbs were included. The concentration of 13 PFRs metabolites and 3 oxidative stress biomarkers including 4-hydroxynonenal (HNE), hexanoyl-lysine (HEL), and 8-hydroxy-2'-deoxyguanosine (8-OHdG), were measured in spot urine samples (n=412). Multiple regression analysis, Quantile-based g-computation (qg-computation), Bayesian kernel machine regression (BKMR), and Bayesian Kernel Machine Regression-Causal Mediation Analysis (BKMR-CMA) were used to analyze the associations. The median level for HNE, HEL, and 8-

OHdG were 23.5 (μg/mL), 99.3 (nmol/L), and 9.2 (ng/mL), respectively. For the individual PFRs, a natural-log unit increase in TDCIPP [β (95% CI): 0.124 (0.034, 0.215)], TPHP [0.520 (0.395, 0.644)], TBOEP [0.288 (0.164, 0.412)], and EHDPHP [0.162 (0.003, 0.322)] were significantly associated with higher HNE. Higher TDCIPP [0.039 (0.003, 0.076)], TPHP [0.099 (0.046, 0.152)], and TBOEP [0.071 (0.021, 0.122)] were associated with HEL. Higher TPHP [0.054 (0.027, 0.081)], and TBOEP [0.053 (0.028, 0.079)] were significantly associated with 8-OHdG. For the PFR mixtures, one-quartile increase in all PFRs was associated with a significant increase in HNE [0.545 (0.362, 0.729)], HEL [0.081 (0.012, 0.150)], and 8-OHdG [0.077 (0.037, 0.117)] in the qg-computation model. The PFR mixture was positively associated with all three oxidative stress biomarkers in the BKMR models. No mediation effect of oxidative stress biomarkers among PFR exposure and levels of eosinophil and FeNO. PFR exposures were associated with oxidative stresses of DNA damage and lipid peroxidation among the general population of 9–12-year-old children. Future study including other environmental chemicals such as phthalates and bisphenols is needed to evaluate the mediation effect of oxidative stress for environmental chemicals exposure and asthma.

Keywords: Organophosphate Flame Retardants, Mixtures analysis, Oxidative stress, Children's environmental health, Environmental epidemiology

3.1. Introduction

Organophosphate flame retardants and plasticizers (PFRs) are types of semi-volatile chemicals, which have been widely used in consumer products. PFRs were added to materials to prevent combustion and meet fire safety standards, and used as a plasticizer and lubricant (Van der Veen and De Boer 2012, Wei et al., 2015b). They are not chemically bound to those products, can be slowly released out and finally lead to human exposure. The occurrence of PFRs, such as metabolites of triphenyl phosphate (TPHP), tris(1-chloro-isopropyl) phosphate (TCIPP), and tris(1,3-dichloro-2-propyl) phosphate (TDCIPP) has been frequently detected in humans (Bastiaensen et al., 2021a, Li et al., 2022, Yu et al., 2022a). The human health burden of PFRs exposure has been reported, such as the adverse associations between reproductive function, positive associations with papillary thyroid cancer, allergic disease, and oxidative stress (Carignan et al., 2017, Hoffman et al., 2017, Araki et al., 2018, Carignan et al., 2018a, Ait Bamai et al., 2019).

Oxidative stress occurs from the imbalance between reactive oxygen and nitrogen species production and antioxidant defenses, which damages cells including proteins, nucleic acid, and lipids (Sies 2015, Liguori et al., 2018). Biomarkers are used as proxies, such as protein carbonylation represents the protein oxidation, 8-hydroxy-2'-deoxyguanosine (8-OHdG) represents DNA damage, and malondialdehyde (MDA), 4-

hydroxynonenal (HNE), hexanoyl-lysine (HEL) represent the lipid peroxidation (Cooke et al., 2003, Michaeloudes et al., 2022). Among the experimental studies, higher levels of triphenyl phosphate (TPP) exposure induced the MDA levels in mouse liver, tri-n-butyl phosphate (TNBP) and tris(2-butoxyethyl) phosphate (TBOEP) exposure induced the MDA levels in mouse lung tissue (Chen et al., 2015, Meng et al., 2022). Exposure to long-chain PFRs including triisobutyl phosphate (TIBP), tris (2-ethylhexyl) phosphate (TEHP), and TNBP increased the DNA damage in A549 cells (Yuan et al., 2020). TDCIPP exposure increased 8-OHdG levels in Chinese rare minnows (Chen et al., 2019). Suggesting the positive associations between PFRs exposure and the occurrence of lipid peroxidation and DNA damage.

In previous epidemiology studies, higher PFRs metabolites were positively associated with 8-OHdG levels in e-waste recyclers (Lu et al., 2017) and pregnant women (Ingle et al., 2020, Yao et al., 2021). Only one case-cohort study focused on children with allergy and without allergy, reported significant positive association between PFRs exposure and urinary 8-OHdG, HNE, and HEL levels (Ait Bamai et al., 2019). However, participants in these studies had relatively higher levels of oxidative stress compared with the general population. Increased oxidative stress level was associated with pregnancy due to enhanced metabolism, high consumption of oxygen, and utilization of fatty acids

(Hussain et al., 2021); as well as associated with allergic disease due to over-production of reactive oxygen species oxygen by infiltrating immune cells, and deregulated nicotinamide adenine phosphate oxidases activity by irritants exposure and inflammatory mediators (Michaeloudes et al., 2022). A research gap exists for the association between PFR exposure and oxidative stress levels among the general population.

Oxidative stress causes tissue damage and deregulation of cell signaling, which contributes to the pathogenesis of numerous diseases, including asthma (Michaeloudes et al., 2022). Accompany the inflammatory response in asthma, oxidative damage promotes airway remodeling and hyperresponsiveness (Belanger et al., 2016, Sies and Jones 2020). Previous research found urinary PFRs concentration positively associated with higher peripheral blood eosinophil counts and fraction of exhaled nitric oxide level (FeNO) (Zeng et al., 2023). Eosinophil counts and FeNO level are biomarkers that are recommended for the diagnosis and management of asthma (Pavord et al., 2018, Reddel et al., 2022). PFRs may be associated with asthma through increased oxidative stress. This indicates a need to understand the potential mediation effect for oxidative stress among the association between PFR exposure and eosinophil counts and FeNO levels.

Considering the overall health effects of multiple PFRs exposure is important, as humans are simultaneously exposed to different chemicals (Meek et al., 2011). Quantile-

based g-computation (qg-computation) and Bayesian kernel machine regression (BKMR) analyses can assess the overall health effects of the highly correlated chemicals, using the linear and nonlinear methods respectively (Bobb et al., 2015, Bobb et al., 2018, Keil et al., 2020). Moreover, Bayesian kernel machine regression-causal mediation analysis (BKMR-CMA) is a framework that implement the BKMR model, allowing to simultaneously estimate the total effect and mediation effects of PFR chemicals (Devick et al., 2022). Using these analyses, this study examined the association of individual and mixture exposure to PFRs with urinary oxidative stress biomarkers among school children. Another purpose of this study was to assess the mediation effect of oxidative stress on the association between PFR exposure and eosinophil counts and FeNO level.

3.2. Materials and methods

3.2.1. Study population

The study participants for this study are the same children described in Chapter 1. From the 427 participants, a total of 423 participants who provided sufficient urine samples for oxidative stress biomarkers analyses were included in the final analyses of this study.

3.2.2. Questionnaire

The information of the questionnaire for this study is the same as described in Chapter 2.

3.2.3. Analytical methods of oxidative stress biomarkers, PFR metabolites and T2 biomarkers

Three oxidative stress biomarkers including 4-hydroxynonenal (HNE), hexanoyl-lysine (HEL), and 8-hydroxy-2'-deoxyguanosine (8-OHdG) were measured in the urine samples. The concentration of 8-OHdG was measured at Japan Institute for the Control of Aging, Nikken SEIL Co. using the 8-OHdG Check ELISA kit (Japan Institute for the Control of Aging, Nikken SEIL Co., Shizuoka, Japan). Measurement of HEL and HNE was conducted at MACROPHI Inc. (Kagawa, Japan). The concentration of HEL was measured using an ELISA kit from Nikken SEIL Co. (Sakai et al., 2014). The concentration of HNE was measured using the OxiSelect HNE Adduct Competitive ELISA kit (Cell Biolabs, Inc.). Regarding the performance of the assays, the coefficient of variation (CV) rates for 8-OHdG, HEL, and HNE is 5.6%, 11.6%, and 7.2%, respectively. The concentration of PFR metabolites was measured as described in Chapter 1. The analytical method of FeNO and peripheral blood eosinophil count was described

in Chapter 2.

3.2.4. Statistical analysis

The concentrations of Σ metabolites were used as exposure variables for assessing exposure to parent PFRs. The concentrations of both PFR metabolites and oxidative stress biomarkers were corrected for individual SG values. The Σ metabolites of PFR, oxidative stress biomarkers, and T2 biomarkers were transformed into natural log scales for downstream analyses to improve normality. Multiple regression models were used to assess the relationships between the level of PFR metabolites and oxidative stress biomarkers. To explain the dose response relationships, we conducted categorical models to estimate the least-square mean (LSM) and 95% CI. The concentration of PFR metabolites were categorized into quartiles. P-values for trends calculated the linearities from the 1st quartile to the 4th quartile. To estimate the partial and joint effect of the PFRs mixture, we applied the quantile-based g-computation (qg-computation) and Bayesian kernel machine regression (BKMR). The detailed explanation and formula of qg-computation and BKMR are the same as described in Chapter 2.

Bayesian Kernel Machine Regression-Causal Mediation Analysis (BKMR-CMA) was used to investigate the mediation effect of oxidative stress biomarkers for PFR exposure

and airway inflammation. The BKMR–CMA tool enables to testing the mixture effect of chemical exposure using a causal mediation analysis based on the counterfactual approach (Devick et al., 2022). Health outcomes were biomarkers for airway inflammation including FeNO level and peripheral blood eosinophil counts. Mediators were the oxidative stress biomarkers. BKMR-CMA is a framework implements BKMR model, allows to simultaneously estimate the total effect and mediation effects of PFR mixtures (Devick et al., 2022). The BKMR model was executed using a kernel machine regression equation (1) (Bobb et al., 2015, Bobb et al., 2018):

$Y_i = h(Z_i) + C_i^T\beta + \epsilon_i$ (1)

where Y_i is the health outcome for individual i (i = 1, 2, 3…n), Z_i denotes the chemical exposure, h is the function that fits the exposure and the outcome considering nonlinear interactions between the exposures, $Ci = (C_{1i}, \dots, C_{pi})^T$ is a vector of additional covariates,$\cdot\beta$ is the effect of the covariates, and ϵ_i is the residual.

We estimate the total effect, natural direct effects, and natural indirect effects using BKMR-CMA based on these formulas:

$M_i = h_M(Z_i) + C_i^T\beta + \epsilon_{Mi}$ (2)

$Y_i = h_Y(Z_i, M_i) + C_i^T\theta + \epsilon_{Yi}$ (3)

$Y_i = h_{TE}(Z_i) + C_i^T\gamma + \epsilon_{TEi}$ (4)

Where Mi is the mediator. We modeled the association between the exposure mixture and mediator using the BKMR model (2). We accounted for exposure-mediator interactions when modeling the health outcome in (3). To model the total effect of the exposure mixture on the health outcome, we consider the BKMR model (4). For each dataset, we conducted 10,000 iterated BKMR runs and produced posterior mean estimates and 95% credible intervals.

All statistical analyses were performed using R (Version 4.2.3) with the packages "randomForest" (version 4.7-1.1), "glm2" (version 1.2.1), "multcomp" (version 1.4-23), "emmeans" (version 1.8.6), "qgcomp" (Version 2.10.1), "bkmr" (Version 0.2.2) for missing value imputations, regression analysis, p-values for trends calculation, LSM estimation, qg-computation, and BKMR, respectively. The R code that implements BKMR-CMA is assessed on GitHub (https://github.com/kdevick/BKMR-CMA).

3.2.5. Ethics

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

3.3. Results

3.3.1. Distribution of oxidative stress biomarkers and PFR metabolites

The basic characteristics and health outcomes of the study participants are shown in Table 3.1. The median and interquartile range (IQR) of FeNO and peripheral blood eosinophil count were 17.5 (9-37) parts per billion (ppb), and 232 (126-429) /μL respectively.

The distributions of the urinary oxidative stress biomarker, the PFR metabolite concentrations (ng/mL), and the EDI level of PFRs (nmol/L) are shown in Table 3.2. The median and IQR of HNE, HEL, and 8-OHdG were 23.5 (11.7-39.7) μg/mL, 99.3 (60.1-151) nmol/L, 9.2 (5.80-11.9) ng/mL, respectively. We measured the concentration for 13 PFR urinary metabolites originating from 7 parent compounds. The detection frequency for BDCIPP, BCIPHIPP, and DPHP were 64.5%, 77.8%, and 84.4%. DNBP and TCEP were excluded from further analysis because of the low detection frequencies (<10%). Among the Σ metabolites concentration, ΣTCIPP was the highest (median value of 1.20 nmol/L), followed by ΣTPHP (median value of 1.10 nmol/L).

Table 3.1. Characteristics of study participants (N = 423).

Variable		n	%
Gender	boys	228	53.9
	girls	195	46.1
Age	9	140	33.1
	10	167	39.5
	11	45	10.6
	12	71	16.8
Environmental tobacco smoke	yes	164	38.8
Annual household income	< 5 million	125	29.6
(Japanese yen/year)	5 - 8 million	180	42.6
	> 8 million	95	22.5
	missing	23	5.4
		Mean ± SD	
BMI (kg/m^2)			17.78 ± 2.97
		IQR	
FeNO (parts per billion, ppb) (n=418)			17.5 (9-37)
Peripheral blood eosinophil count (/μL) (n=413)		232 (126-429)	

Abbreviation: SD, standard deviation. IQR, Interquartile range.

Table 3.2. Distribution of oxidative stress biomarkers and PFR metabolites (n=423).

Oxidative stress biomarkers	**LOD**	**> LOD (%)**	**Min**	**25th**	**50th**	**75th**	**95th**	**Max**
4-hydroxynonenal (HNE) (µg/mL)	**1.56**	**99.8**	<LOD	11.7	23.5	39.7	91.1	195
hexanoyl-lysine (HEL) (nmol/L)	**2**	**100**	3.66	60.1	99.3	151	302	2449
8-hydroxy-2′-deoxyguanosine (8-OHdG) (ng/mL)	**0.5**	**100**	0.70	5.80	9.20	11.9	17.1	30.2
Specific gravity			1.007	1.007	1.018	1.029	1.034	1.044
PFR metabolites (ng/mL)								
BDCIPP	0.05	64.5		<LOQ	0.11	0.29	2.65	42.9
BCIPP	0.40	22.7				<LOQ	2.02	39.9
BCIPHIPP	0.05	77.8	<LOQ	0.06	0.20	0.64	2.89	83.5
DPHP	0.10	84.4	<LOQ	0.14	0.27	0.54	1.74	40.7
HO-TPHP	0.05	0.95					<LOQ	0.20
4HO-DPHP	0.20	0						<LOQ
BBOEP	0.05	57.7		<LOQ	0.07	0.19	0.70	4.19
BBOEHEP	0.05	53.2		<LOQ	0.06	0.23	1.32	8.32
3-HO-TBOEP	0.10	20.3				<LOQ	0.19	1.75
EHPHP	0.20	59.8		<LOQ	0.25	0.49	1.12	6.01
5-HO-EHDPHP	0.05	17.7				<LOQ	0.12	0.45
DNBP	0.20	6.86				<LOQ	0.25	1.01
TCEP	0.10	0.47					<LOQ	0.31
ΣMetabolites concentration (nmol/L)								
TDCIPP (based on BDCIPP)			0.10	0.10	0.34	0.91	8.17	134.0
ΣTCIPP (Σ of BCIPP, BCIPHIPP)			0.48	0.57	1.20	3.49	15.47	429.2
ΣTPHP (Σ of DPHP, HO-TPHP, 4HO-DPHP)			0.34	0.58	1.10	2.17	6.92	162.8
ΣTBOEP (Σ of BBOEP, BBOEHEP, 3-HO-TBOEP)			0.23	0.23	0.63	1.61	5.85	30.3
ΣEHDPHP (Σ of EHPHP, 5-HO-EHDPHP)			0.34	0.44	0.89	1.78	4.33	22.05

LOQ: limit of quantification; LOD: limit of detection

3.3.2. Individual PFR and oxidative stress biomarkers

The results of multiple regression analyses using the continuous models were shown in table 3.3 A natural-log unit increase in TDCIPP [β (95% CI): 0.124 (0.034, 0.215)], TPHP [0.520 (0.395, 0.644)], TBOEP [0.288 (0.164, 0.412)], and EHDPHP [0.162 (0.003, 0.322)] were significantly associated with higher HNE. Higher TDCIPP [0.039 (0.003, 0.076)], TPHP [0.099 (0.046, 0.152)], and TBOEP [0.071 (0.021, 0.122)] were significantly associated with higher HEL. A natural-log unit increase in TPHP [0.054 (0.027, 0.081)], and TBOEP [0.053 (0.028, 0.079)] were significantly associated with higher 8-OHdG. The results of the categorical models represent the dose response relationships (Figure 3.1). A positive significant trend was observed among TDCIPP, TCIPP, TPHP, TBOEP, and HNE; TDCIPP, TPHP, TBOEP, and HEL; TPHP, TBOEP, and 8-OHdG.

Table 3.3. Association between concentrations of PFR metabolites and oxidative stress biomarkers.

	HNE				HEL				8-OHdG			
	β	95% CI		*P* value	β	95% CI		*P* value	β	95% CI		*P* value
TDCIPP	**0.124**	**0.034**	**0.215**	**0.007**	**0.039**	**0.003**	**0.076**	**0.035**	0.001	-0.018	0.019	0.923
TCIPP	0.098	-0.016	0.213	0.094	-0.011	-0.057	0.035	0.642	0.021	-0.003	0.044	0.081
TPHP	**0.520**	**0.395**	**0.644**	**< 0.001**	**0.099**	**0.046**	**0.152**	**< 0.001**	**0.054**	**0.027**	**0.081**	**< 0.001**
TBOEP	**0.288**	**0.164**	**0.412**	**< 0.001**	**0.071**	**0.021**	**0.122**	**0.006**	**0.053**	**0.028**	**0.079**	**< 0.001**
EHDPHP	**0.162**	**0.003**	**0.322**	**0.047**	0.046	-0.018	0.110	0.156	0.026	-0.007	0.058	0.124

All models were adjusted for sex, age, annual household income, and environmental tobacco smoke.

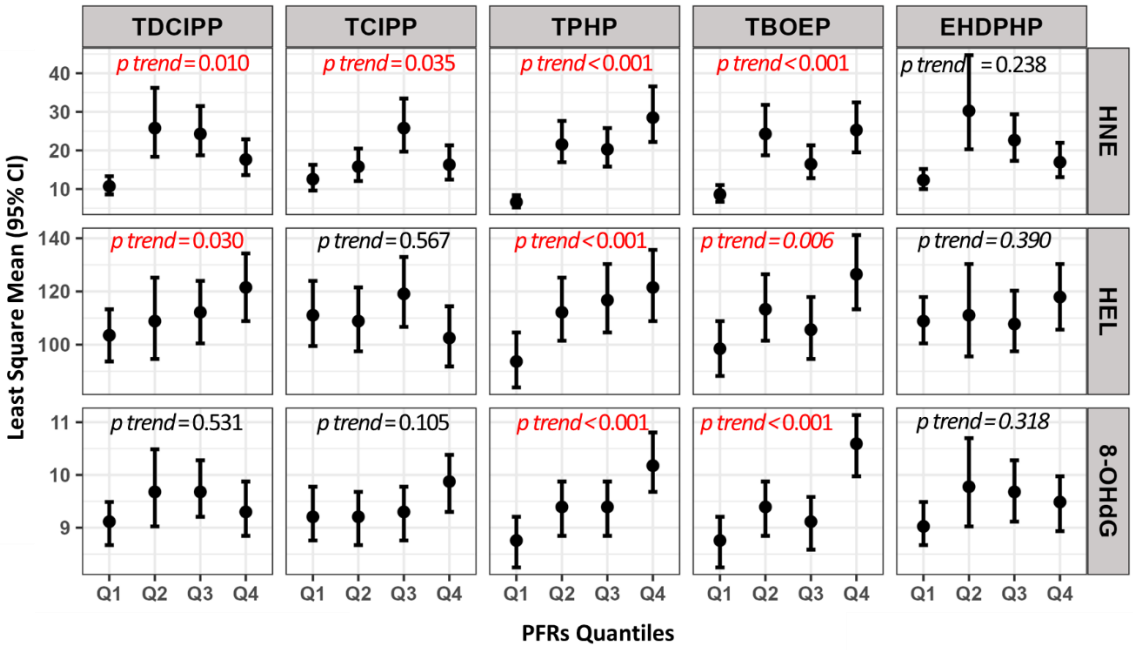


Figure 3.1. Associations between urinary PFRs and oxidative stress biomarkers in categorical model. Y-axis represents least square mean (LSM) and 95% confidential interval (95% CI) of urinary oxidative stress biomarkers are shown as black squares and whiskers, respectively. X-axis represents the levels of urinary PFR metabolites in quartiles. LSMs were adjusted for sex, age, household income, and environmental tobacco smoke. P for trends show the linearities from 1st quartile to 4th quartile.

3.3.3. PFRs mixture and oxidative stress biomarkers

The mixture effect of PFRs on oxidative stress and the individual contributions of the mixtures to each chemical were analyzed using Qg-computation (Figure 3.2). One-quartile increase in all PFRs was associated with a significant increase in HNE [β (95% CI): 0.545 (0.362, 0.729)], HEL [0.081 (0.012, 0.150)], and 8-OHdG [0.077 (0.037, 0.117)]. For HNE, the individual contributions of the mixtures with the highest weight (%) in the positive direction were ΣTPHP (58.0%). For HEL, chemicals with the highest weight in the positive direction were TDCIPP (32.6%). For 8-OHdG, ΣTBOEP has the highest weight (47.6%) in the positive direction. The association between PFRs mixture and oxidative stress were also analyzed with a non-parametric method using BKMR

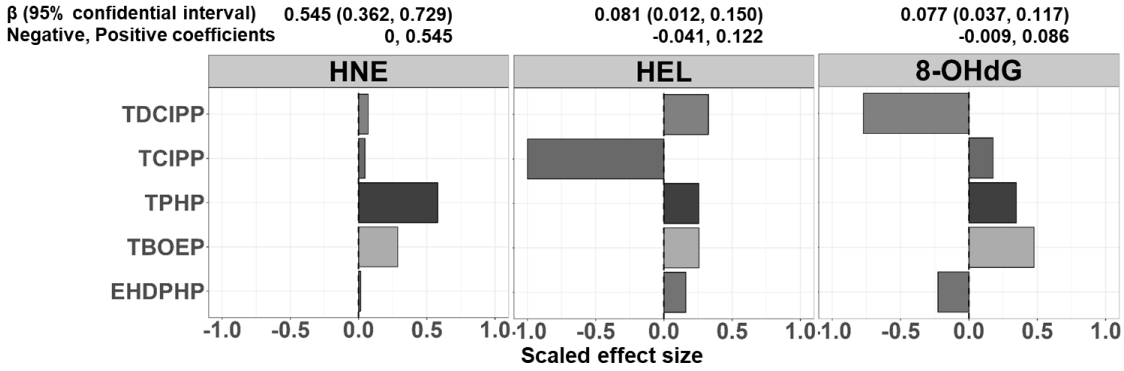


Figure 3.2. Associations between PFRs mixture and oxidative stress in quantile g-computation. The β are interpreted as the effect on oxidative stress biomarkers of increasing every one quantile unit of exposure of PFR mixture. Negative and positive coefficients reprenst the sum coefficients of the PFR mixture. The bar graph represent the negative and positive contribution weight (%) of individual PFR on health outcomes. The models were adjusted for sex, age, annual household income, and environmental tobacco smoke.

(Figure 3.3). The cumulative effects of PFR mixtures are shown in Figure 3A and the univariate exposure–response functions of individual PFR are shown in Figure 3B. PFRs mixture was positively associated with HNE, HEL, and 8-OHdG levels.

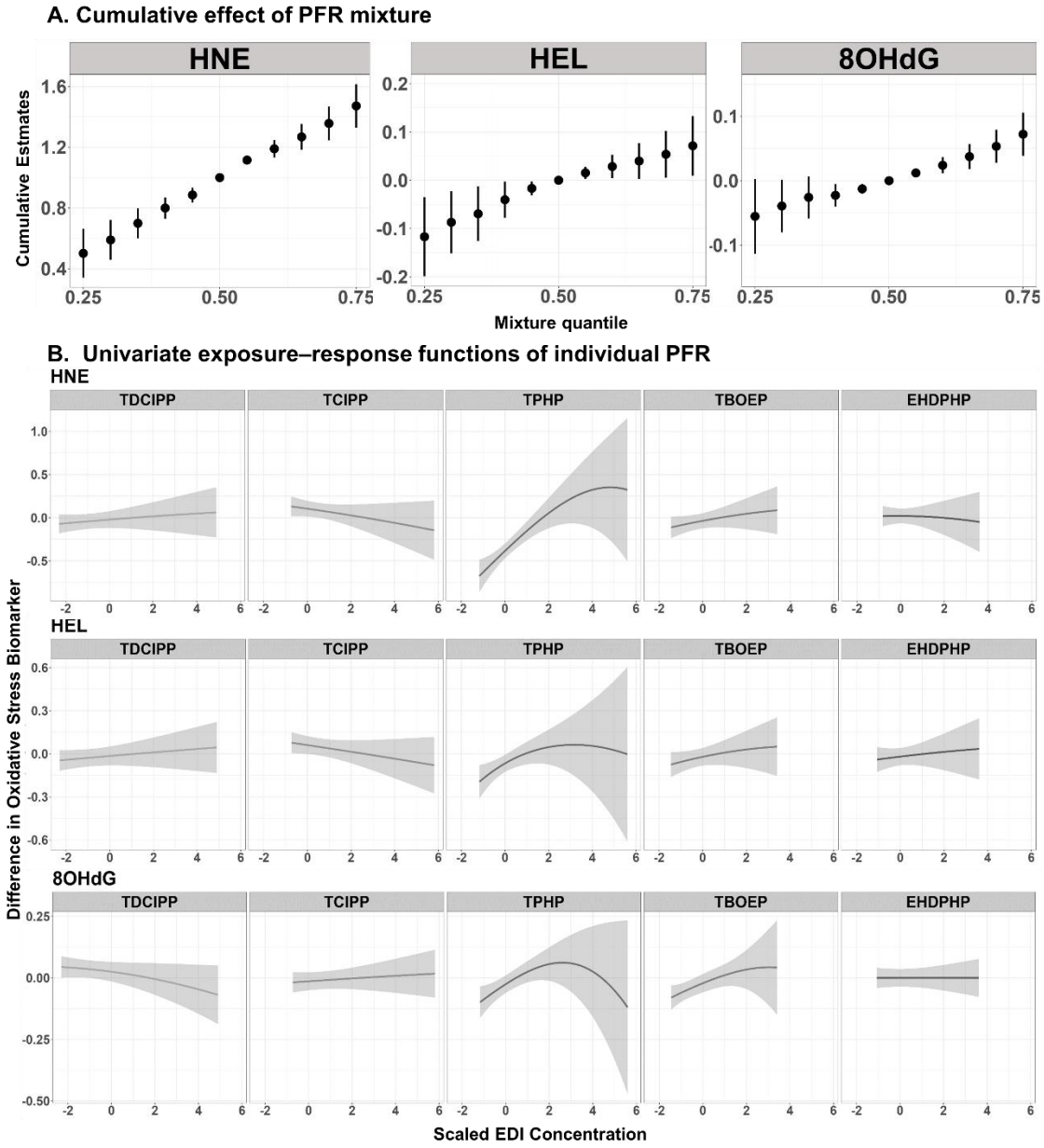


Figure 3.3. Associations between PFRs mixture and oxidative stress in BKMR. The cumulative effect of PFR mixture on oxidative stress biomarkers were estimated when all chemical components were at their 50th percentile. Univariate exposure–response functions and 95 % credible intervals for the change in oxidative stress biomarkers and individual PFR while holding all the remaining exposures in the mixture fixed at their median concentration. The models were adjusted for sex, age, annual household income, and environmental tobacco smoke.

3.3.4. Mediation effect of oxidative stress biomarkers

Table 3.4 shows the mediation analysis results using BKMR-CMA. For example, the total effect of the PFRs mixture on the FeNO level was [posterior mean estimates (95% credible intervals): 0.107 (-0.054, 0.248)]. The natural direct effect represents the effect of the PFRs mixture on FeNO level not through HNE, which was [0.105 (-0.063, 0.261)]. The natural indirect effect represents the effect that goes through the path of the HNE, of [0.002 (-0.152, 0.165)]. There was no evidence of mediation by oxidative stress biomarkers. The results of NIE were null across all three oxidative stress biomarkers, which reflects the indirect association of the PFRs mixture with FeNO and eosinophil mediated through HNE, HEL, and 8-OHdG were null.

Table 3.4. The natural direct effect of PFR mixture on FeNO and indirect effects of each oxidative stress biomarkers.

			Total Effect			Natural Direct Effect			Natural Indirect Effect		
			mean	lower	upper	mean	lower	upper	mean	lower	upper
Exposure	**Health outcome**	**Mediator**									
PFRs mixture	FeNO level	HNE	0.107	-0.054	0.248	0.105	-0.063	0.261	0.002	-0.152	0.165
		HEL	0.106	-0.062	0.253	0.106	-0.061	0.276	0.000	-0.176	0.164
		8OHdG	0.106	-0.062	0.253	0.102	-0.051	0.258	0.004	-0.168	0.163
Exposure	**Health outcome**	**Mediator**									
PFRs mixture	Eosinophil counts	HNE	0.010	0.000	0.146	0.010	-0.114	0.150	0.000	-0.131	0.125
		HEL	0.013	0.000	0.179	0.002	-0.120	0.147	0.012	-0.121	0.124
		8OHdG	0.013	0.000	0.179	0.004	-0.105	0.154	0.009	-0.128	0.115

Sensitivity analyses were conducted to test the robustness of the main findings. The random forest method was used to fill in all missing annual household income values. We assessed the extent to which filled data could affect our findings by removing all data containing missing values. We used the concentrations of PFR metabolites in the regression model and mixture analyses. We evaluated whether the findings will change by using the EDI of PFRs. Moreover, we stratified participants into two groups using wheeze symptoms to conduct the mediation analysis. We evaluated whether the mediation effect would be different among the children with wheeze symptom whose eosinophil counts and FeNO levels were higher than the children without wheeze. We found that the results remained consistent with our original findings.

3.4. Discussion

This cross-sectional study explored the health effects of low-dose PFR exposure in general populations of children. In single-exposure models, dose-response relationships were found among PFRs exposure and biomarkers of lipid peroxidation (HNE and HEL) and oxidative DNA damage (8-OHdG). For individual PFR, significant positive trends were observed between TDCIPP, TCIPP, TPHP, TBOEP, and HNE; TDCIPP, TPHP, TBOEP, and HEL; TPHP, TBOEP, and 8-OHdG in the categorical model using the

regression analyses. EHDPHP was positively associated with HNE in the continuous model (Table 3.3), but was no longer significant when considering the positive trend in the categorical model. Mixture analyses enable us to estimate the overall effect of the PFR mixture. Consistent with single-exposure models, positive associations were observed between the PFRs mixture and biomarkers of lipid peroxidation and oxidative DNA damage from both qg-computation and BKMR models.

3.4.1. PFRs exposure and lipid peroxidation

The epidemiological study on the association between PFRs and lipid peroxidation was very limited. It was first explored in a previous study, urinary EHPHP was positively associated with a higher level of HNE, and DPHP was positively associated with higher HEL among 7-year-old Japanese children (Ait Bamai et al., 2019). More recently, among Chinese pregnant women, an IQR increase in DPHP was associated with 0.129 log-unit increase in MDA, which is a biomarker for oxidative stress of lipid peroxidation (Yao et al., 2021). Our results of a positive association between TPHP and HEL were in line with previous studies. However, the result of EHDPHP and HNE was in partial disagreement with the previous finding. The reason for the inconsistent findings may be the difference in the participants, with the present study focusing on the general population, whereas the previous study used a case-cohort design in which half of the children had allergic

symptoms. The median level of HNE was 45.7 ug/mL in the case-cohort study (Ait Bamai et al., 2019), which was about 2 times higher than this study (23.5 ug/mL). The health effects on HNE level of PFR exposure may differ in children with or without allergies.

3.4.2. PFRs exposure and DNA damage

More findings have supported that exposure to PFRs are associated with higher 8-OHdG. It was initially reported among Chinese e-waste recyclers, that higher BCIPP, BCEP, DBP, and DPHP were significantly correlated with higher 8-OHdG levels (Lu et al., 2017). Then previous case-cohort study reported that urinary levels of TPHP, and TBOEP were positively associated with higher 8-OHdG in Japanese school-aged children (Ait Bamai et al., 2019). Moreover, in studies focused on pregnant women, positive associations were found between levels of 8-OHdG with urinary BCEtP, BCPP, and DPHP in Puerto Rico (Ingle et al., 2020), and with DBP, and DPHP in China (Yao et al., 2021). As mentioned in the introduction, these previous study participants inevitably had higher levels of oxidative stress due to pregnancy and allergies (Hussain et al., 2021, Michaeloudes et al., 2022). Our study provides further evidence that PFRs exposure is associated with oxidative DNA damage among the general population of Children. Evidence on the general population not only excludes the influences of confounders due to pregnancy and allergies, but also has a higher potential for generalization of findings.

3.4.3. Mediation effect of oxidative stress

The mediation effect of oxidative stress on the association between PFRs and levels of eosinophil and FeNO is null in this study. Similar null results were found in studies examining the association between phthalates exposure and asthma and allergies by considering the oxidative stress biomarker as a mediator (Franken et al., 2017, Ketema et al., 2022). The oxidative stress was identified as a redox imbalance of increased reactive oxygen species (ROS) production and attenuated antioxidant protection (Sies 2015, Liguori et al., 2018). The ROS takes part in the development of asthma including allergen sensing, immune responses, and airway remodeling, and the redox signaling is complex due to inter-organelle cross talk and feedback regulation mechanisms (Comhair and Erzurum 2010, Michaeloudes et al., 2022). It may be clearer to look at this association not only at the cellular level but also the pathogenesis of redox signaling in asthma. Another possible reason may be because these studies included only one class of chemicals. Humans are exposed to diverse chemicals at the same time, significant positive associations between oxidative stress levels and other environmental chemicals were reported. Positive associations were reported between BPF and 8-OHdG (Gys et al., 2020); dibutyl phthalate (DBP) and di(2-ethylhexyl) phthalate (DEHP) and benzyl butyl phthalate (BBzP) and 8-OHdG (Hsia et al., 2022); nonylphenol exposure, HEL and HNE

(Ringbeck et al., 2022). Therefore, overall mediation effect on the oxidative stress need evaluated including other environmental chemicals.

3.4.4. Strengths and limitations

The strength of this study was that we investigate the association between urinary PFRs metabolite and oxidative stress levels in general population of children. The exposure assessment included 13 PFRs metabolites using a well-validated biomonitoring method. Qg-computation and BKMR analyses were used to explore the overall effect of the PFR mixture on oxidative stress level. To the best of our knowledge, this is the first study that investigated the association between PFRs exposure and levels of eosinophil and FeNO by considering the oxidative stress biomarkers as a mediator.

This study has some limitations. First, causal relationships between exposure and outcomes could not be demonstrated due to the cross-sectional design. However, dose response relationships were observed in some of the categorical models in this study. The use of the prospective cohort study enables follow-up research and may provide longitudinal results and causal relationship evidence in the future. Second, a single spot urine sample was used for PFR metabolites measurement, which may not reflect the exposure variance of PFRs due to the short biological half-lives and frequent exposure. However, specific gravity correction significantly improved the short-term

reproducibility of PFR metabolite level (Bastiaensen et al., 2021b). Thirdly, there is a possibility of TPHP overestimation and EHDPHP underestimation, because DPHP was a metabolite of both TPHP and EHDPHP (Van Den Eede et al., 2016). Fourth, mixture analyses do not provide information on toxicological measures of PFRs.

3.5. Conclusion

In this study, PFRs were positively associated with HEL, HNE, and 8-OHdG after examining both individual and mixture exposure models, but no mediation effect of oxidative stress biomarkers among PFR exposure and levels of eosinophil and FeNO. Suggested that PFR exposures were associated with oxidative stresses of DNA damage and lipid peroxidation among the general population of 9–12-year-old children. Notion needs to be paid that the PFRs exposure level was relatively lower compared with other countries in the world. Continued biomonitoring the PFRs exposure among children and more broad research in other countries is needed. Although the mediation effect of oxidative stress for PFRs and asthma is null in this study, a future study including other environmental chemicals such as phthalates and bisphenols is needed to evaluate this topic.

V. Summaries and future perspectives

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

In chapter 1

a) This study reported the concentration of 13 PFR urinary metabolites originating from 7 parent compounds among Japanese school-aged children. The metabolites of BDCIPP, BCIPHIPP, and DPHP were detectable for more than 60% of participants.

b) The estimated daily intakes of PFRs for Japanese school-aged children were all well below the oral reference doses. The concentrations of urinary PFRs metabolites in Japanese children were relatively lower than those in other countries.

c) Boys, higher BMI, higher annual household income, and experiencing environment tobacco smoking were associated with significantly higher levels of PFR metabolites. Higher age was associated with lower TPHP and higher EHDPHP.

In chapter 2

d) Positive associations were observed between TDCIPP and wheeze in school children aged 9–12.

e) Positive associations were found between TDCIPP, ΣTPHP, ΣTBOEP, and FeNO ≥35

ppb; ΣTPHP and peripheral blood eosinophil counts ≥ 300 /μL. These findings suggest that exposure to PFRs increases the possibility of asthma and allergies by enhancing the expression of T2 biomarkers.

f) The PFR mixture was positively associated with FeNO and eosinophil counts above the cut-off levels. Consist results were found from individual analyses (multiple logistic regression analyses) and mixture analyses models (quantile-g computation and bayesian kernel machine regression analysis).

In chapter 3

g) PFRs were positively associated with HEL, HNE, and 8-OHdG after examining both individual and mixture exposure models. PFR exposures were associated with oxidative stresses of DNA damage and lipid peroxidation.

h) No mediation effect of oxidative stress was observed on the association between PFRs and levels of eosinophil and FeNO.

Although the EDI levels of PFR exposure were all well below the oral reference doses and the concentrations of urinary PFR metabolites in Japanese children were relatively lower than that in other countries around the world, health effects were observed in this

study. These finding indicates that continuing the biomonitoring with more broad research in other country is necessary to examine the PFR exposure level and its health effect. The association between PFR and total IgE, the mediation effect of oxidative stress remains unknown, study focused on asthmatic patients as well as the cytokine test may help understand this issue. As humans are exposed to many chemicals at one time, it is needed to evaluate the combined health effects of environmental chemicals including not only PFR, but also other environmental chemicals that have similar effects on allergic diseases, such as phthalates and bisphenols.

Several actions could be taken to control PFR exposure and to lessen the health burden. For example, products such as carpets and curtains that are labeled with fire-resistant are made to have high flame retardant properties which may contain more flame retardants compared with ordinary curtains. Reduce the use of such products according to actual needs is recommended. Previous research reported that rooms that are more air-tight and have thicker insulation tend to have higher PFR concentrations (Kanazawa et al., 2010, Araki et al., 2014a). Keeping the room well-ventilated will be helpful in reducing the level of PFR concentration. House dust can be considered a primary exposure source of environmental chemicals, because a significant association has been found between house dust levels and urinary metabolite levels (Araki et al., 2014b, Bastiaensen et al., 2019b).

Frequent cleaning to reduce dust in the room can also reduce exposure to PFR exposure. Moreover, future studies focused on lifestyle and usage of daily necessities will be helpful to reduce the PFR exposure effectively.

The issue of PFR exposure and environmental chemicals is an important part of achieving Sustainable Development Goals (SDGs) such as “Good health and wellbeing”, “Clean water”, and “Sustainable cities and communities”. As we strive to meet the demands of a growing market, finding an equilibrium between economic interests and safeguarding human health necessitates collaborative efforts across industries, disciplines, and borders. Moreover, issues related to environmental chemicals were not only associated with human health but also the “One Health” concept. Chemical exposure can affect animals and place an additional burden on the environment, demanding a thorough assessment of these far-reaching consequences. Developing effective countermeasures requires a synthesis of scientific, regulatory, and societal efforts to achieve the broader goal of promoting harmonious coexistence between humans and the environment.

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VII. Author's publications and conference presentations

I. Publications

<Peer-reviewed original articles>

1. Zeng, Y., Goudarzi, H., Ait Bamai, Y., Ketema, R. M., Roggeman, M., den Ouden, F., Gys, C., Miyashita, C., Ito, S., Konno, S., Covaci, A., Kishi, R., & Ikeda-Araki, A. (2023). Exposure to organophosphate flame retardants and plasticizers is positively associated with wheeze and FeNO and eosinophil levels among school-aged children: The Hokkaido study. *Environment International*, 181, 108278.
2. Zeng, Y., He, J., Hao, M., Han, W., & Yamauchi, T. (2023). Correlating Caretakers' Knowledge, Attitudes and Practices of Hygiene and Continued Breastfeeding with Infants' Gross Motor Development Delay. *Hong Kong Journal of Paediatrics* (New Series), 28(2), 99-108.
3. Hao, M., He, J., Zeng, Y., Han, W., Sai, A., & Yamauchi, T. (2022). A comprehensive assessment of hand washing: knowledge, attitudes and practices (KAP) and hand-washing behaviors among primary school students in northeast China. *Sanitation Value Chain*, 6(1), 13-22.
4. He, J., Zeng, Y., Hao, M., & Yamauchi T. (2020). Knowledge, Attitudes and

Practices of Sanitation and Hygiene among Primary School Students in Rural Area of Northeast China. *Sanitation Value Chain*, 4(1), 39-50.

<Thesis>

5. Water, Sanitation, and Hygiene Related Risk Factors for Infant Health and Motor Development in a Suburban Area of China(中国郊外に暮らす乳幼児の健康と運動発達に影響を与える飲用水、サニテーション、衛生に関わる要因; 2020 年度修士論文)
6. Exposure to Organophosphate Flame Retardants and its Associations with Allergis among School-aged Children (学童期におけるリン系難燃剤曝露とアレルギーとの関連; 2023 年度博士論文)

II. Conference presentations

＜International conferences＞

1. Zeng, Y., Goudarzi, H., Ait Bamai, Y., Ketema, R. M., Roggeman, M., den Ouden, F., Gys, C., Miyashita, C., Ito, S., Konno, S., Covaci, A., Kishi, R., & Ikeda A. The Association between Urinary Phthalates and Use of Plastic Utensils in

School Children: Hokkaido Study on Environment and Children's Health. *The 6th FHS International Conference,* Sapporo, October 20, 2023.

2. Zeng, Y., Ait Bamai, Y., Goudarzi, H., Ketema, R. M., Roggeman, M., den Ouden, F., Gys, C., Miyashita, C., Ito, S., Konno, S., Covaci, A., Kishi, R., & Ikeda A. Organophosphate Flame Retardants Mixture Associated with Increased Urinary Oxidative Stress Biomarkers among School Children: The Hokkaido Study, *The 35th Annual Conference of International Society for Environmental Epidemiology (ISEE),* Taiwan, September 17-21, 2023.
3. Zeng, Y., He, J., Hao, M., Han, W., & Yamauchi, T. The Household Environment and Caretaker's Hygiene Practices for Infants in a Suburban Area of China: Focus on Water, Sanitation and Hygiene, *The 36th Congress of Japan Association for International Health* Online, November 27-28, 2021.
4. Zeng, Y., He, J., Hao, M., Han, W., & Yamauchi, T. Association Between Hygiene Practices and Fecal Contamination in the Household Environment of Children in a Suburban Area of China. *The 5th FHS International Conference*. Sapporo, Hokkaido, September 17-18, 2021.
5. Zeng, Y., He, J., Hao, M., Han, W., & Yamauchi, T. Risk factors affecting gross motor development delay among children in a suburban area of China: Focus on

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