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Multiple exposures to organophosphate flame retardants alter urinary oxidative stress biomarkers among children: The Hokkaido Study

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ABSTRACT

Organophosphate flame retardants (PFRs) are used as additives in plastics and other applications such as curtains and carpets as a replacement for brominated flame retardants. As such, exposure to PFR mixtures is widespread, with children being more vulnerable than adults to associated health risks such as allergies and inflammation. Oxidative stress is thought to be able to modulate the development of childhood airway inflammation and atopic dermatitis. To evaluate these associations, the present study investigated the relationship between urinary PFR metabolites, their mixtures and urinary oxidative stress biomarkers in children as part of the Hokkaido Study on Environment and Children's Health. The levels of the oxidative stress biomarkers, such as 8-hydroxy-2′-deoxyguanosine (8-OHdG), hexanoyl-lysine (HEL), and 4-hydroxynonenal (HNE), and of 14 PFR metabolites were measured in morning spot urine samples of 7-year-old children (n = 400). Associations between PFR metabolites or PFR metabolite mixtures and oxidative stress biomarkers were examined by multiple regression analysis and weighted quantile sum regression analysis, respectively. We found that the non-chlorinated PFR metabolites, 2-ethylhexyl phenyl phosphate (EHPHP), bis(2-butoxyethyl) phosphate (BBOEP), and diphenyl phosphate (DPHP) were associated with increased levels of oxidative stress biomarkers. Furthermore, the PFR metabolite mixture was associated with increased levels of HEL and HNE, but not 8-OHdG. The combination of elevated top 2 PFR metabolites was not associated with higher urinary oxidative stress marker levels. This is the first study to report associations between urinary PFR metabolites and oxidative stress biomarkers among children.

1. Introduction

Organophosphate flame retardants (PFRs) are semi-volatile organic compounds (SVOCs) that are mainly used as flame retardants and additives in plastics and polyurethane foams (PUFs), textiles, thermoset coatings, and polymer mixtures (van der Veen and de Boer, 2012). PFRs have replaced brominated flame retardants, following the latter's listing as persistent organic pollutants at the Stockholm Convention (UNEP, 2009). PFR production volumes are expected to increase continuously over the next years (Grand View Research, 2017). Chlorinated PFRs

Abbreviations: 3-HO-TPHP, 3-hydroxyphenyl diphenyl phosphate; 4-HO-DPHP, 4-hydroxyphenyl phenyl phosphate; 4-HO-TPHP, 4-hydroxyphenyl diphenyl phosphate; 5-HO-EHDPHP, 2-ethyl-5-hydroxyhexyl diphenyl phosphate; 8-OHdG, 8-hydroxy-2′-deoxyguanosine; BBOEHEP, 2-hydroxyethyl bis(2-butoxyethyl) phosphate; BBOEP, bis(2-butoxyethyl) phosphate; BCIPHIPP, 1-hydroxy-2-propyl bis(1-chloro-2-propyl) phosphate; BCIPP, bis(1-chloro-2-propyl) phosphate; BDCIPP, bis(1,3-dichloro-2-propyl) phosphate; BMI, body mass index; CI, confidence interval; DPHP, diphenyl phosphate; EHDPHP, 2-ethylhexyl phenyl phosphate; EHPHP, 2-ethylhexyl phenyl phosphate; ELISA, enzyme-linked immunosorbent assay; ETS, environmental tobacco smoke; HEL, hexanoyl-lysine; HNE, 4-hydroxynonenal; LOQ, limit of quantification; 3-HO-TBOEP, (3-hydroxy)-2-butoxyethyl bis(2-butoxyethyl) phosphate; PFR, organophosphate flame retardant; PUF, polyurethane foam; SVOC, semi-volatile organic compound; TBOEP, Tris (2-butoxyethyl) phosphate; TCEP, tris(chloroethyl) phosphate; TCIPP, tris(1-chloro-2-isopropyl) phosphate; TDCIPP, tris(2,3-dichloro-2-propyl) phosphate; TNBP, tri-n-butyl phosphate; TPHP, triphenyl phosphate; WQS, weighted quantile sum

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such as tris(2-chloroisopropyl) phosphate (TCIPP), tris(chloroethyl) phosphate (TCEP), and tris(2,3-dichloropropyl) phosphate (TDCIPP) are the main flame retardants in polyurethane foam (PUF), while non-chlorinated PFRs such as tri-n-butyl phosphate (TNBP), triphenyl phosphate (TPHP), and tris (2-butoxyethyl) phosphate (TBOEP) are mainly used as plasticisers and lubricants. TBOEP is present in floor polish and covering; TNBP is found in lubricants; and TPHP is included in resins and polyvinylchloride plastics (Stapleton et al., 2009; Van den Eede et al., 2011). SVOCs, such as PFRs, are not chemically bound and can be dispersed from products into the air and dust of the indoor environment, subsequently leading to human exposure.

Several studies have reported experimental and epidemiological evidence on the health effects following PFR exposure. TBOEP, TCIPP, and TDCIPP are classified as mild-to-moderate irritants of rabbit skin (WHO, 1998, 2000), butylated TPHP and tricresyl phosphate have shown reproductive toxicity in rats (Latendresse et al., 1994), and TPHP is toxic to murine dendritic cells (Canbaz et al., 2017). However, only a few studies have reported an association between PFR exposure and adverse health effects in humans. Higher maternal and paternal preconception urinary PFR metabolite levels were associated with reduced fertilisation (Carignan et al., 2017; Carignan et al., 2018), and higher urinary PFR metabolite levels have been linked to reduced sperm quality (Meeker et al., 2013). However, it has been reported that inconsistent observations of the associations between PFR metabolites and semen parameters were reported from the same group (Ingle et al., 2018). In a matched case–control study, higher levels of TCEP in dust were correlated with increased risk of papillary thyroid cancer (Hoffman et al., 2017), and higher maternal urinary TPHP metabolite concentrations were associated with decreased intelligence quotient scores at 7 years of age (Castorina et al., 2017). We previously demonstrated that elevated TCIPP and TDCIPP concentrations in dust were linked to atopic dermatitis and that TNBP was associated with asthma in the Japanese population (Araki et al., 2014). Furthermore, higher levels of TDCIPP in dust and of TDCIPP, TBOEP, and TCIPP metabolites in urine were associated with children's allergy symptoms, such as eczema and rhino-conjunctivitis (Araki et al., 2018). On the other hand, there was no relationship between PFRs in mattress dust and the development of childhood asthma (Canbaz et al., 2016).

Exposure to TCEP, TNBP, and TPHP was shown to increase the levels of 8-hydroxy-2′-deoxyguanosine (8-OHdG), a marker of 2,4-dinitrophenol-induced oxidative stress, in a population living close to an electronic (e-)waste site (Lu et al., 2017). Oxidative stress contributes to asthma and inflammation (Finkel and Holbrook, 2000; Sugiura and Ichinose, 2008) by modulating the response of Toll-like receptor to allergens, leading to the production of inflammatory factors and lung damage (Mishra et al., 2018). Moreover, oxidative stress biomarkers are involved in the development and progression of childhood atopic dermatitis (Tsukahara et al., 2003). However, there is no information on the relationship between exposure to PFRs and oxidative stress biomarkers in children, and the impact of PFR mixtures on oxidative stress biomarkers is not known. Significantly higher levels of urinary PFR metabolites were found in children compared with adults (Van den Eede et al., 2015), suggesting that children are more vulnerable than adults to the effects of PFRs. Therefore, we investigated the association between urinary PFR metabolites, PFR mixtures and urinary oxidative stress biomarkers in children.

2. Materials and methods

2.1. Study population

This study was part of the Hokkaido Study on Environment and Children's Health, Hokkaido cohort. Subject recruitment has been described elsewhere (Kishi et al., 2017). Among 20,926 children who were enrolled from February 2003 to March 2012, 5102 children dropped out (including spontaneous abortion, stillbirth, loss to follow-up and voluntary withdrawn) before August 2017. After excluding twins (n = 172), 10,655 singleton children (67.6%) who had reached the age of 7 by August 2017 were included in the study. A follow-up questionnaire was sent to 10,655 children and 6218 responses (58.4%) were received. There were 2451 children for whom both the completed questionnaire and urinary sample were available. The study population was selected based on an ongoing nested case–cohort study design to investigate the association between urinary PFR metabolites and allergies in 7 years old children. Allergies were defined based on the criteria of the International Study on Asthma and Allergies in Childhood: wheezing, allergic rhino-conjunctivitis, and eczema. According to the power of sample size estimation, an *n* of 100 was required for case and cohort groups (sub-cohort); we therefore randomly selected 100 children for each allergic symptom (wheeze, allergic rhino-conjunctivitis, and eczema) from 2451 potential subjects; of these, 60 children had at least two symptoms of allergy. Therefore, 160 children were randomly selected as a sub-cohort group (without any of symptoms). In total, 400 children (240 cases and 160 sub-cohort) were included in the study (Fig. 1). In the present study, we aimed to investigate for the first time whether there are any associations between urinary PFR metabolites levels and urinary oxidative stress biomarkers among children who are included in an ongoing nested case–cohort study.

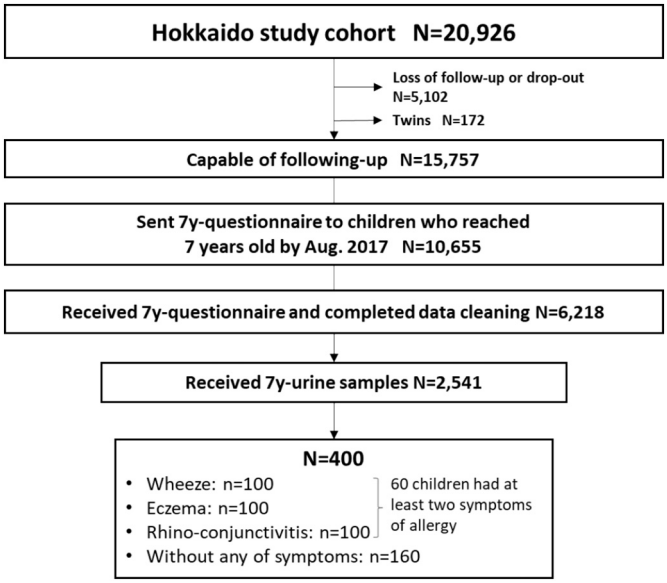

Fig. 1. Study population. The participants in this study were selected based on an ongoing nested case–cohort study to investigate the association between urinary PFR metabolites and allergies in 7 years old children. Among 20,926 children who were enrolled from February 2003 to March 2012, excluding dropped out children (n = 5102) and twins (n = 172), 10,655 singleton children who had reached the age of 7 by August 2017 were included. Among them, 2451 children for whom both the completed follow-up questionnaire (n = 6218) and urinary sample were available. According to the power of sample size estimation, an n of 100 was required for each case (wheeze, rhino-conjunctivitis, and eczema) and without any of symptoms groups; In total, 400 children were included in the study.

2.2. Measurement of PFR metabolites in urine

The mothers of the participating children were instructed to collect first morning urine samples from their children in a cup and send it to Hokkaido University, Center for Environmental and Health Sciences using a cool delivery service. The samples were immediately dispensed into a stoppered glass test tube that had been cleaned with acetone and sealed with fluoric tape, wrapped in aluminium foil, and stored at −20 °C until analysis at the Toxicological Center of the University of

Antwerp, Belgium.

Fourteen PFR metabolites (di-n-butyl phosphate [DNBP], diphenyl phosphate [DPHP], 4-hydroxyphenyl phenyl phosphate [4-HO-DPHP], 3-hydroxyphenyl diphenyl phosphate [3-HO-TPHP], 4-hydroxyphenyl phenyl phosphate [4-HO-TPHP], 2-ethylhexyl phenyl phosphate [EHPHP], 2-ethyl-5-hydroxyhexyl diphenyl phosphate [5-HO-EHDPHP], bis(2-butoxyethyl) phosphate [BBOEP], 2-hydroxyethyl bis (2-butoxyethyl) phosphate [BBOEHEP], 2-(3-hydroxy)butoxyethyl bis (2-butoxyethyl) phosphate [3-HO-TBOEP], 1-hydroxy-2-propyl bis(1-chloro-2-propyl) phosphate [BCIPHIPP], bis(1-chloro-2-propyl) phosphate [BCIPP], tris(chloroethyl) phosphate [TCEP], and bis(1,3-dichloro-2-propyl) phosphate [BDCIPP]) were measured in urine as previously described (Bastiaensen et al., 2019). Briefly, 2 mL of urine were spiked with mass-labelled reference standards (IS, 5 ng), followed by addition of 1.5 mL phosphate buffer (1 M, pH 6) and 100 μL β-glucuronidase enzyme solution (*E. coli*, 2 mg/mL in phosphate buffer, pH 6) and incubation for 2 h at 37 °C. Target analytes were acidified with 100 μL formic acid and extracted by solid phase extraction, and the samples were loaded onto Bond-Elut C18 cartridges (3 mL, 200 mg, Agilent Technologies, Santa Clara, CA, USA), conditioned with 3 mL methanol followed by 2 mL ultrapure water. Samples were washed with 1 mL of ultrapure water and the washing solvent was transferred to the cartridge, which was washed with 1.5 mL ultrapure water and eluted with 3 mL methanol. Before evaporating the eluates to near-dryness under a gentle stream of nitrogen, 50 μL ultrapure water was added as the keeper solvent. Final extracts were then reconstituted in 100 μL of ultrapure water:methanol (50/50, v/v), passed through 0.2-μm nylon centrifugal filters (VWR International, Leuven, Belgium), and transferred to glass vials for liquid chromatography–tandem mass spectrometry analysis. Instrumental analysis was carried out on a 1290 Infinity liquid chromatography system coupled to a 6460 triple quadrupole mass spectrometer (both from Agilent Technologies, Santa Clara, CA, USA). Metabolites were separated on a Kinetex biphenyl reversed-phase column (2.1 × 100 mm, 2.6 μm; Phenomenex, Torrance, CA, USA) at a column temperature of 40 °C. Mobile phases consisted of ultrapure water with 2% methanol (A) and methanol with 2% ultrapure water (B), both with 5 mM ammonium acetate. The mobile phase gradient started at 5% (B), which increased to 50% at 3.5 min, to 65% at 7.5 min, reached 97% at 9.5 min, was held for 4 min, and finally equilibrated for 3.5 min at 5%. The injection volume was set at 5 μL and the flow rate at 0.35 mL/min. Samples were quantified using an 8-point calibration curve ranging from 0.04 to 10 ng/mL for all compounds, except for BCIPP and 4-HO-DPHP, for which the calibration ranged from 0.2 to 50 ng/mL in neat solvents. Urinary creatinine levels were determined by enzyme-linked immunosorbent assay (ELISA) by SRL (Tokyo, Japan).

2.3. Measurement of oxidative stress biomarkers in urine

The levels of the oxidative stress biomarkers, 8-OHdG, hexanoyllysine (HEL), and 4-hydroxynonenal (HNE) were determined in each urine sample. 8-OHdG is a product of oxidative DNA damage formed by hydroxyl radicals; its concentration was measured using the 8-OHdG Check ELISA kit (Japan Institute for the Control of Aging, Nikken SEIL Co., Shizuoka, Japan). HEL, a lipid hydroperoxide-modified lysine residue, is a marker of the initial stage of lipid peroxidation. HNE is a major fatty acid oxidation product. Measurement of HEL and HNE was performed by MACROPHI (Kagawa, Japan) using a HEL ELISA kit (Japan Institute for the Control of Aging, Nikken SEIL Co.) (Sakai et al., 2014) and the OxiSelect HNE Adduct Competitive ELISA kit (Cell Biolabs, San Diego, CA, USA), respectively. The coefficient of variation (CV) and the recovery rates of 8-OHdG was ranged from 7.0%–8.4% and 95%–114%, respectively (Saito et al., 2000). The calibration curves of HEL and HNE showed good linearity ($R^2 > 0.997$). CVs of calibration curves of HEL and HNE were ranged from 3.4%–10.4% and 1.0%–5.4%, respectively.

2.4. Statistical analysis

PFR metabolites detected in < 60% of samples were excluded from statistical analyses. Concentrations of oxidative stress biomarkers higher than the upper calibration point were also excluded. The correlation between urinary PFR metabolites and biomarkers was calculated with Spearman's correlation test and each correlation coefficient is presented with a ρ value. To determine the association between urinary PFR metabolites and oxidative stress biomarkers, multiple regression analysis was performed to obtain an effect estimate (β and 95% confidential interval [CI]) of each oxidative stress biomarker for an increase in PFR metabolite concentration. Both oxidative stress biomarkers and PFR metabolites were corrected for individual creatinine levels and converted to natural log scales. Based on significant associations in multiple regression analysis, we carried out categorical models to estimate the least-square mean (LSM) and 95% CI. Exposure levels were categorised into quartiles. P-values for trends were calculated from the linearities from 1st quartile to 4th quartile. Metabolites detected in < 75% of samples were divided into dichotomized categories (< LOQ, ≥LOQ). P-values were calculated for the (< LOQ) as reference.

To assess the association between the mixture of PFR metabolites and oxidative stress biomarkers, we used weighted quantile sum (WQS) regression, which can be used to combine highly correlated exposure into an index that is used to estimate the association between co-exposure and outcome (Carrico et al., 2015). In this study, the index was determined from quartiles of individual urinary PFR metabolites and is shown with the effect estimate (β and 95% CI), which can be interpreted as an estimate of the effect of the mixture of urinary PFR metabolites on oxidative stress biomarkers. Furthermore, WQS weights (%) were estimated to determine the relative contributions of mixture components to the overall association (Carrico et al., 2015). Urinary PFR metabolites with higher WQS weights represent a greater effect on the outcome of interest than those with lower WQS weights. Based on significant associations in WQS analyses, we carried out mixture analyses using the top two PFR metabolites with the strongest weights (%). Each PFR metabolite was categorised as “Low” or “High” based on tertile concentrations of PFR metabolites. The first and second tertiles were categorised as “Low” and the third tertile as “High”. The metabolites with the top two strongest weights were combined as Low × Low, Low × High, High × Low, and High × High before calculating the least square means of each combination. The significance of P-values was evaluated based on differences between Low × Low and High × High categories.

Sex, body mass index (BMI), and exposure to environmental tobacco smoke (ETS) were selected as confounders based on a > 10% change in the estimate in the model. Both the multiple regression and multiple WQS regression models were adjusted for these parameters. A two-tailed test and 5% level of significance were used for all analyses, which were performed using JMP Pro 10 for Macintosh (SAS Institute, Cary, NC, USA) and R package WQS (0.0.1) to fit WQS models using quartiles of urinary PFR metabolite levels with 100 bootstrap runs.

2.5. Ethics

This study was approved by the Institutional Ethical Review Board for Epidemiological Studies at Hokkaido University Graduate School of Medicine, Hokkaido University Center for Environmental and Health Sciences, and was carried out in accordance with the principles outlined in the Declaration of Helsinki. The cohort study was conducted after obtaining written informed consent from all the participants during the time of pregnancy. Additional written informed consent was provided for the collection of urine from all parents of 7 years old children.

Table 1
Characteristicis of participants N = 400.

		7 years old children in this study (n = 400)		7 years old children in the original cohort (n = 2541)	
		N	%	N	%
Gender	Boy	214	53.5	1330	52.3
	Girl	186	46.5	1211	47.7
Birth year	2003	4	1.0	26	1.0
	2004	60	15.0	422	16.6
	2005	60	15.0	399	15.7
	2006	51	12.8	402	15.8
	2007	61	15.3	352	13.9
	2008	74	18.5	379	14.9
	2009	66	16.5	389	15.3
	2010	24	6.0	172	6.8
BMI	Mean, SD	15.9	2.1	15.7	4.0
ETS	Yes	121	30.4	876	34.5
	No	277	69.6	1665	65.5
Maternal age at delivery	Mean, SD	31.4	4.2	31.6	4.4
Maternal history of allergies	Yes	248	62.0	1448	57.5
	No	152	38.0	1069	42.5
Household income	< 300	51	13.3	271	11.2
	≥300	331	86.4	2069	88.3

BMI: body mass index, ETS: environmental tobacco smoke.

3. Results

3.1. Characteristics of the study population

The two sexes were almost equally represented in the study population (53.5% boys; Table 1). The distribution of birth year, BMI, ETS, and maternal age at delivery was the same as in our original cohort, while the maternal history of allergies and household annual income were slightly higher in the study population.

3.2. Urinary PFR metabolites and oxidative stress biomarkers

Table 2 shows the distribution of PFR metabolites and oxidative stress biomarkers in children's urine. Oxidative stress biomarkers 8-OHdG, HEL, and HNE were detected in all samples. The levels of 8-OHdG in this study were similar or slightly lower compared to other studies with children, while HEL levels were higher (Omata et al., 2001; Tanuma et al., 2008). Urinary PFR metabolites DPHP, EHPHP, BBOEHEP, and BCIPHIPP were detected in > 90% of children. The most abundant metabolite was DPHP, followed by BCIPHIPP and EHPHP. On the other hand, 4-HO-DPHP, 3-HO-TPHP, 4-HO-TPHP, 3-HO-TBOEP, and BCIPP had a detection frequency below 10% and DNBP was detected in < 15% of children.

All urinary PFR metabolites included in statistical analyses were significantly correlated among themselves (Spearman ρ 0.18–0.75; $P < 0.01$) and with three oxidative stress biomarkers in the range of 0.20–0.54 ($P < 0.01$). DPHP and EHPHP showed strong correlations ($\rho > 0.40$) with 8-OHdG, HEL, and HNE (Supplemental Table 1).

3.3. Associations between urinary PFR metabolites and oxidative stress biomarkers

Table 3 shows the effect estimated with oxidative stress biomarker concentrations for an increase in PFR metabolite concentration. EHPHP and BBOEP were significantly associated with higher 8-OHdG [β (95% CI): EHPHP: 0.100 (0.042–0.158), $P = 0.001$; BBOEP: 0.040 (0.001–0.080), $P = 0.044$]; DPHP was significantly associated with higher HEL [0.137 (0.063–0.211), $P < 0.001$]; and EHPHP was significantly associated with higher HNE [0.206 (0.113–0.300), $P < 0.001$].

In categorical models (Fig. 2), significant and positive dose-response relationships were found between EHPHP and 8-OHdG (P for trend = 0.002) and HNE (P for trend < 0.001) and between DPHP and HEL (P for trend < 0.001). As BBOEP was detected in about half of the samples, the data for BBOEP were categorised as not detected (≤ limit of detection [LOD]) or detected (> LOD). 8-OHdG levels were higher in children with detected BBOEP in their urine than in those without ($P < 0.001$).

3.4. Associations between urinary PFR metabolite mixtures and oxidative stress biomarkers

WQS regression analysis was used to assess the effect of multiple exposures to PFR metabolites [BBOEP, BBOEHEP, EHPHP, BCIPHIPP, bis(1,3-dichloro-2-propyl) phosphate (BDCIPP), and DPHP] on urinary oxidative stress biomarkers (Fig. 3). The WQS index of PFR metabolites

Table 2
Distributions of urinary PFR metabolites and oxidative stress biomarkers.

		N	LOQ (ng/mL)	Detection frequency (%)	Mean	SD	Min	25	50	75	Max
PFR metabolites (ng/mL)											
TNBP	DNBP	400	0.15	12.0	0.071	0.27	< LOQ	< LOQ	< LOQ	0.018	4.3
TPHP	DPHP	400	0.1	96.8	0.67	0.89	< LOQ	0.28	0.46	0.73	13
	4-HO-DPHP	400	0.5	1.0	0.02	0.1	< LOQ	< LOQ	< LOQ	< LOQ	1
	3-HO-TPHP	400	0.01	2.0	0.002	0.02	< LOQ	< LOQ	< LOQ	< LOQ	0.2
	4-HO-TPHP	400	0.01	0.8	0.0005	0.005	< LOQ	< LOQ	< LOQ	< LOQ	0.07
EHDPHP	EHPHP	400	0.05	93.3	0.49	0.58	< LOQ	0.18	0.31	0.58	4.8
	5-HO-EHDPHP	400	0.01	52.5	0.02	0.08	< LOQ	< LOQ	0.01	0.02	2.0
TBOEP	BBOEP	400	0.05	62.0	0.25	0.4	< LOQ	< LOQ	0.1	0.3	5
	BBOEHEP	400	0.01	99.3	0.5	0.8	< LOQ	0.09	0.2	0.5	8
	3-HO-TBOEP	400	0.01	7.5	0.003	0.009	< LOQ	< LOQ	< LOQ	< LOQ	0.1
TCIPP	BCIPHIPP	400	0.04	98.0	0.9	2	< LOQ	0.2	0.4	0.9	15
	BCIPP	400	1.00	0.3	0.007	0.09	< LOQ	< LOQ	< LOQ	< LOQ	1.8
TCEP	TCEP	400	0.04	57.8	0.05	0.02	< LOQ	< LOQ	< LOQ	0.06	0.1
TDCIPP	BDCIPP	400	0.05	74.0	0.3	1	< LOQ	< LOQ	0.1	0.3	23
Urinary biomarkers											
	8-OHdG (ng/mL)	398		100	10.6	4.9	2.5	7.3	10.1	13.1	40.4
	HEL (nmol/L)	381		100	150	140	21.9	74.7	113	167	1230
	HNE (ug/mL)	351		100	59.5	47.1	1.3	22.6	45.7	85.5	198
	Creatinine (ug/mL)	400		100	95.9	38.6	10.2	67.7	91.8	121	219

LOQ: limit of quantification; SD: standard deviation.
Concentrations of oxidative stress biomarkers higher than the upper calibration point were excluded.

Table 3
Association between urinary PFR metabolites and oxidative stress biomarkers.

	8-OHdG				HEL				HNE			
	β	(95% CI)		P value	β	(95% CI)		P value	β	95% CI		P value
DPHP	−0.017	(−0.085,	0.050)	0.613	**0.136**	**(0.062,**	**0.210)**	**< 0.001**	0.030	(−0.070,	0.131)	0.551
EHPHP	**0.100**	**(0.042,**	**0.158)**	**0.001**	0.035	(−0.030,	0.101)	0.292	**0.161**	**(0.074,**	**0.248)**	**< 0.001**
BBOEP	**0.041**	**(0.001,**	**0.081)**	**0.043**	0.010	(−0.034,	0.055)	0.652	0.044	(−0.013,	0.102)	0.128
BCIPHIPP	−0.018	(−0.060,	0.023)	0.389	0.024	(−0.022,	0.071)	0.303	−0.002	(−0.062,	0.059)	0.958
BBOEHEP	0.028	(−0.010,	0.067)	0.152	0.018	(−0.025,	0.061)	0.416	0.053	(−0.004,	0.109)	0.069
BDCIPP	0.004	(−0.040,	0.048)	0.865	0.043	(−0.006,	0.092)	0.084	0.023	(−0.040,	0.086)	0.473

Adjusted for sex, BMI, ETS, and presence of allergies.
All PFR and inflammation biomarker levels were natural log-transformed.
PFR metabolite levels below LOQ were corrected for median creatinine levels. PFR metabolite levels above LOQ were corrected for individual creatinine levels.

was significantly associated with increased levels of HEL [WQS β (95% CI) 0.106 (0.049–0.163), P < 0.001], with DPHP having the strongest weight (62.3%), followed by BDCIPP (12.2%). The index was also significantly associated with increased level of HNE [WQS β (95% CI), P = 0.140 (0.062–0.217), P < 0.001], with EHPHP having the strongest weight (49.7%), followed by BDCIPP (17.7%). However, the WQS index of PFR metabolites was not significantly associated with 8-OHdG (P = 0.183).

Given the significant associations in WQS analyses, we carried out mixture analyses using the two PFR metabolites with the strongest weights (Fig. 4). Children with higher levels of both EHPHP and BDCIPP (High × High) had significantly higher levels of HNE than those with low levels (Low × Low) (P = 0.020).

4. Discussion

In this study, we investigated the association between urinary PFR metabolites, their mixtures and urinary oxidative stress biomarkers in 7-year-old children. Significant positive associations were found between urinary EHPHP and BBOEP and 8-OHdG concentrations; DPHP and HEL concentrations; and EHPHP and HNE concentrations. Furthermore, the WQS index was significantly and positively associated with urinary HEL and HNE, but not 8-OHdG. DPHP and BDCIPP were the top two urinary PFR metabolites contributing to HEL levels, and EHPHP and BDCIPP to HNE levels. In the combination analysis of the top two PFR metabolites, children in the "High × High" category did not show increased levels of either urinary HEL or HNE. To our knowledge, this is the first study investigating the associations between PFR metabolite mixtures and oxidative stress biomarkers in children's urine.

To investigate these associations, we examined one marker of oxidative DNA damage (8-OHdG) and two markers of lipid peroxide-modified proteins (HEL and HNE). 8-OHdG is excreted in urine as a result of oxidative damage to DNA. HEL and HNE are oxidised products of fatty acids that serve as lipid peroxidation markers. HEL was identified as a lipid hydroperoxide-modified lysine residue (Minato et al., 2005), while HNE can modify and/or cross-link proteins and is a biomarker for various diseases including early-stage cancer, atherosclerosis, and inflammation (Fritz et al., 2012; Khan et al., 2016). These oxidative stress biomarkers can modulate airway inflammation (Mishra et al., 2018) and the development of inflammatory processes in childhood atopic dermatitis (Tsukahara et al., 2003).

In single-exposure models, higher urinary levels of EHDPHP and TBOEP metabolites (EHPHP and BBOEP, respectively) were associated with higher urinary 8-OHdG. Higher urinary levels of TPHP and EHDPHP metabolites (DPHP and EHPHP, respectively) were associated with higher urinary HEL and HNE, respectively. In previous experimental studies, TPHP and TCEP induced oxidative stress in Leydig cells (Chen et al., 2015b) and mouse liver (Chen et al., 2015a), while TDCIPP induced lipid peroxidation in cultured PC12 cells (Dishaw et al., 2011). TDCIPP and TPHP have an immunocytotoxic effect on the expression of major histocompatibility complex class II (Canbaz et al., 2017). Only one epidemiological study has reported that urinary metabolites of TCIPP, TCEP, TPHP, and TNBP were positively correlated with urinary 8-OHdG levels in a population living in an e-waste area (Lu et al., 2017). However, in our study, the TDCIPP metabolite BDCIPP and TCIPP metabolite BCIPHIPP did not show any significant associations with DNA oxidation or lipid peroxidation, whereas our results of TPHP are in partial agreement with those of experimental and epidemiological studies. These findings may suggest that TPHP induce or increase oxidative stress.

In the WQS regression model, urinary DPHP and BDCIPP were the top two urinary PFR metabolites contributing to HEL levels. According to this model, contributions to urinary HEL level were 62.3% for DPHP

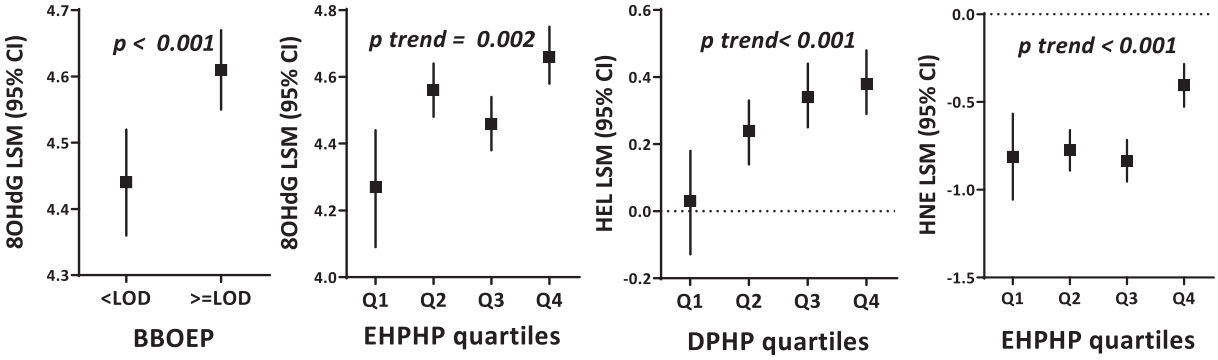

Fig. 2. Associations between urinary PFR metabolites and oxidative stress biomarkers in categorical model. Y-axis represents least square mean (LSM) and 95% confidential interval (95% CI) of urinary oxidative stress biomarkers are shown as black squares and whiskers, respectively. X-axis represents the levels of urinary PFR metabolites in quartiles or dichotomized (< LOD, ≥LOD). LSMs were adjusted for sex, body mass index, environmental tobacco smoke, and presence of allergies. P for trends show the linearities from 1st quartile to 4th quartile, and P value was calculated for the 1st dichotomize as reference.

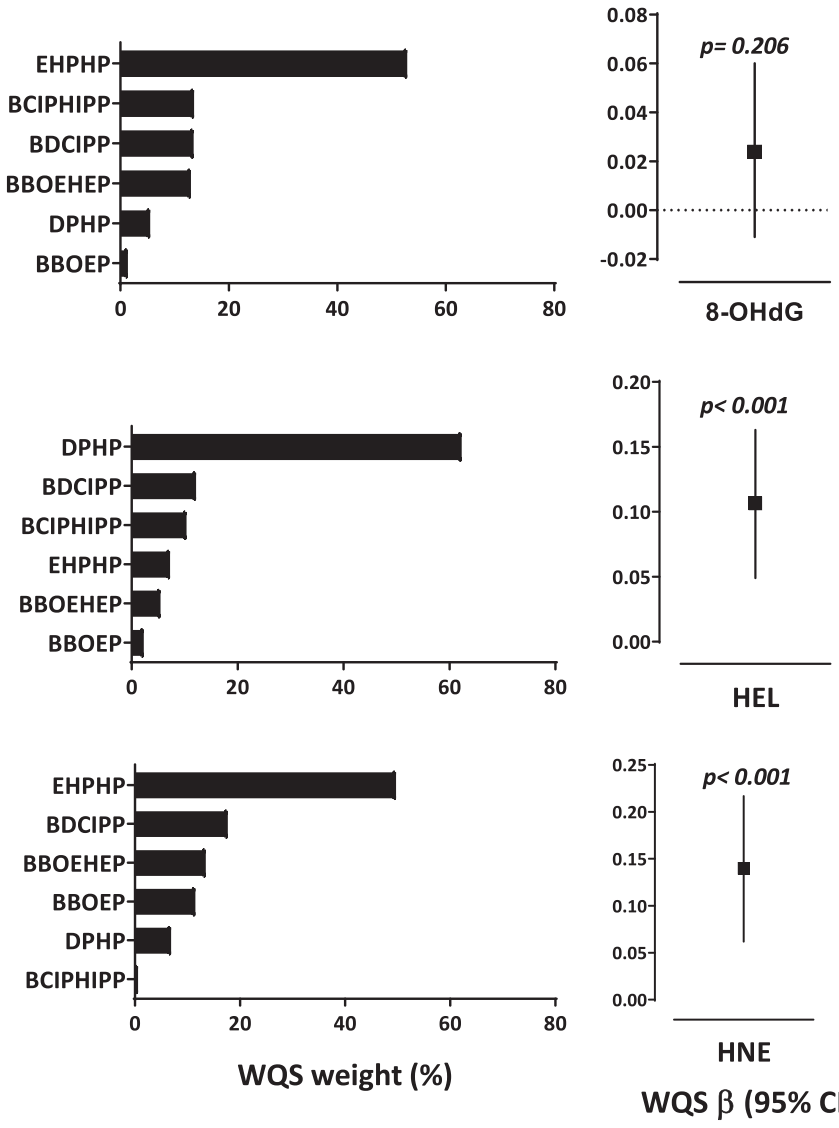

Fig. 3. WQS beta (95% CI) and WQS regression of urinary PFR metabolite index and urinary oxidative stress biomarkers among children. Weighted Quantile Sum (WQS) beta and 95% CI are calculated using WQS regression model and shown as black squares and whiskers, respectively. X-axis shows WQS weights (%) of each urinary PFR metabolites. Y-axis shows the estimated beta and 95% CI of urinary PFR metabolites of mixture. WQS weights (%) represent the relative contributions of mixture components to the overall association. WQS beta and WQS weight were adjusted for sex, body mass index, environmental tobacco smoke, and presence of allergies.

and 12.2% for BDCIPP. The contribution to the urinary HNE level was 49.7% for EHPHP and 17.7% for BDCIPP. In the combination analyses, the associations "Low × Low" and "High × High" were not statistically significant. On the other hand, the associations "Low × Low" and "High × Low" were significant. This could be because DPHP for HEL and EHPHP for HNE make large (approximately 50% or over) contributions to HEL and HNE, respectively. Furthermore, both DPHP and EHPHP in single-exposure models, which significantly increased the urinary levels of HEL and HNE, respectively, implying that it acts alone to modulate urinary HEL and HNE levels rather than as part of a mixture and/or through combination effects. This suggests that DPHP and EHPHP modulate urinary HNE levels more in single exposure rather than as co-exposure to DPHP - BDCIPP and EHPHP – BDCIPP, respectively. However, owing to the lack of evidence on the effects of multiple

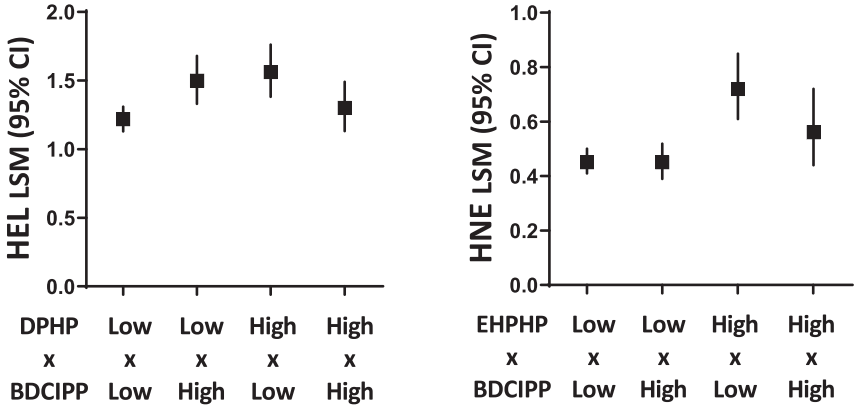

Fig. 4. Combination analysis of two urinary PFR metabolites and oxidative stress biomarkers. X-axis represents the combinations of two highest contributed PFR metabolites. High represents 3rd tertile and Low represents 1st and 2nd tertiles based on tertile concentrations of PFR metabolites. Y-axis represents LSM and 95% CI of urinary oxidative stress biomarkers are shown as black squares and whiskers, respectively. LSMs were adjusted for sex, body mass index, environmental tobacco smoke, and presence of allergies. Significant P values were calculated between "Low x Low" and "High x High".

exposures to PFR metabolites and how they might influence oxidative stress, we cannot speculate regarding the underlying mechanism.

The levels of urinary PFR metabolites in this study were similar to those measured in Japanese elementary school children in our previous report (Araki et al., 2018; Bastiaensen et al., 2019). TBOEP is present in floor polish, wax, and covering. We have also reported that TBOEP levels in house dust in Japan were higher than the other countries (Araki et al., 2014; Tajima et al., 2014). The levels of TBOEP metabolites (BBOEP and BBOEHEP) in this study were higher than those recorded in a study of Australian children (He et al., 2018) and Chinese 0–5-year-old children (Zhang et al., 2018). Thus, the Japanese population may be exposed to higher levels of TBOEP than people from other countries. On the other hand, BDCIPP and DPHP levels were lower than those measured in studies on toddlers (Thomas et al., 2017) and adults in the U.S (Carignan et al., 2018; Ingle et al., 2018), but higher than those measured in study on Chinese children (Zhang et al., 2018). However, given the differences in study population (children, adults, or pregnant women), type of biological sample (spot or pooled urine), and measurement protocols across studies, direct comparisons of the data are difficult.

This study had some limitations. Firstly, PFRs are chemicals with a short half-life; therefore, exposure data obtained from a single spot urine sample may not reflect long-term exposure and may have been misclassified. However, intraclass correlation coefficients (ICC) analysis of 11 PFR metabolites in creatinine-adjusted spot urine concentrations has shown that urinary PFR metabolite concentrations are relatively reproducible over time (range from 0.35 for BBOEP to 0.68 for BDCIPP) (Wang et al., 2019). Furthermore, as we measured both urinary PFR metabolites and oxidative stress biomarkers in the same urine sample, even for single measurements, the results suggest an association between urinary PFR metabolites and oxidative stress biomarkers. Secondly, participants in this study were limited to 7 year old children who could be followed-up and provided both questionnaire and urine sample which means that their parents, who are continuing the cohort study on their behalf, will generally have high health-awareness. Therefore, the study population in this study may lead to a healthier effect compared to other populations. As a result, the findings of our study can not be generalized for other age groups, such as toddlers, adolescents, or the elderly population. Thirdly, owing to the cross-sectional study design, a causal relationship could not be established from our data. Moreover, the individual metabolites that could be associated with oxidative stress biomarkers in opposite directions cannot be included in the same model through WQS regression.

In summary, to the best of our knowledge, this is the first study to examine the associations between urinary PFR metabolite mixtures and oxidative stress biomarkers in children. In a single-exposure model, non-chlorinated PFR metabolites such as EHPHP, BBOEP, and DPHP were associated with oxidative stress biomarkers. Exposure to a PFR metabolite mixture was associated with increased levels of HEL and HNE, but not of 8-OHdG. The combination of elevated top 2 PFR metabolites was not associated with higher urinary oxidative stress marker levels. It may imply that PFR metabolite mixtures act to modulate urinary oxidative stress markers, but not just highly contributed PFR metabolites. However, there is insufficient experimental and epidemiological evidence for the effects of PFR exposure on oxidative stress biomarkers; therefore, additional studies are needed to confirm these findings.

Declaration of Competing Interest

There are no conflicts of interest to declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2019.105003.

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