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Interaction of melamine and di-(2-ethylhexyl) phthalate exposure on markers of early renal damage in children: The 2011 Taiwan food scandal

Chia-Fang Wu [a, b, 1], Chao A. Hsiung [c, 1], Hui-Ju Tsai [d, e], Yi-Chun Tsai [e, f], Hui-Min Hsieh [a], Bai-Hsiun Chen [e, g], Ming-Tsang Wu [a, b, e, h, *]

[a] Department of Public Health, College of Health Sciences, Kaohsiung Medical University, Kaohsiung, Taiwan
[b] Research Center for Environmental Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan
[c] Institute of Population Health Sciences, National Health Research Institutes, Zhunan, Taiwan
[d] Department of Family Medicine, Kaohsiung Municipal Ta-Tung Hospital, Kaohsiung Medical University Hospital, Kaohsiung Medical University, Kaohsiung, Taiwan
[e] Graduate Institute of Clinical Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan
[f] Division of Nephrology, Department of Internal Medicine, Kaohsiung Medical University Hospital, Kaohsiung Medical University, Kaohsiung, Taiwan
[g] Department of Laboratory Medicine and Pediatrics, Kaohsiung Medical University Hospital, Kaohsiung, Taiwan
[h] Department of Community Medicine, Kaohsiung Medical University Hospital, Kaohsiung Medical University, Kaohsiung, Taiwan

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ABSTRACT

Melamine and phthalate, mainly di-(2-ethylhexyl) phthalate (DEHP), are ubiquitously present in the general environment. We investigated whether urine melamine levels can modify the relationship between DEHP exposure and markers of early renal damage in children. A nationwide health survey for Children aged ≤12 years possibly exposed to phthalates were enrolled between August 2012 and January 2013. They were administered questionnaires to collect details regarding past DEHP exposure to phthalate-tainted foodstuffs. Urine samples were measured melamine levels, phthalate metabolites and biomarkers of renal damage, including urine microalbumin/creatinine ratio (ACR), N-acetyl-beta-D-glucosaminidase (NAG), and β2-microglobulin. The study included 224 children who had a median urine melamine level (μg/mmol creatinine) of 1.61 ranging 0.18–47.42. Positive correlations were found between urine melamine levels and urine ACR as well as urine NAG levels (both Spearman correlation coefficients $r = 0.24$, $n = 224$, $p < .001$). The higher the past DEHP exposure or urine melamine levels, the higher the prevalence of microalbuminuria. An interaction effect was also found between urine melamine levels and past DEHP exposure on urine ACR. Melamine levels may further modify the effect of past DEHP exposure on urine ACR in children.

1. Introduction

The 2008 melamine baby formula scandal in China caused kidney-related diseases and kidney failure in children (Ingelfinger, 2008). Although the threat of added melamine to baby formula subsided, we should be reminded that the chemical melamine remains ubiquitously present in our environment (Ingelfinger, 2008; Panuwet et al., 2012; Lin et al., 2013). It has been detected in most urine samples obtained from the general populations of the USA and Taiwan, where chronic low-dose exposure to environmental melamine has been associated with risk of renal stone formation and early renal damage (Wu et al., 2010, 2015; Liu et al., 2011).

News of another food scandal, one involving phthalate-tainted food, broke out in Taiwan in 2011 (Wu et al., 2012). Phthalates, mainly di-(2-ethylhexyl) phthalate (DEHP), were intentionally added to a variety of foodstuffs, including multiple vitamins and probiotics, regularly taken by infants and children as nutrient supplements (Wu et al., 2012, 2013a,b, 2014; Tsai et al., 2016a,b). One of our recent studies found that DEHP may be associated with a higher prevelance of microalbuminuria in children exposed daily to

* Corresponding author. Department of Public Health, College of Health Sciences, Kaohsiung Medical University, Room 721, CS Building, No.100, Shih-Chuan 1st Road, Kaohsiung 807, Taiwan.
E-mail address: 960021@ms.kmuh.org.tw (M.-T. Wu).
[1] These authors contributed equally to this work.

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higher amounts of phthalate-tainted foods (Tsai et al., 2016a).

Although the USA and European Union established recommended tolerable daily intakes (TDIs) of common environmental chemicals such as melamine and DEHP in humans, their estimates were based largely on the results of animal experiments which they extrapolated to the humans, including sensitive subpopulations such as children (EPA 1987; EFSA 2005; WHO, 2009). Another problem with recommended TDIs is that they are usually established for one chemical alone, which is hardly the case in the real world, where people are potentially exposed to a mixture of chemical toxins. In this study, we investigated the interactive effect of two common environmental chemicals, melamine and DEHP, on biomarkers of renal injury in a unique cohort of children.

2. Materials and methods

2.1. Study participants

The slightly modified design used for this study has been described in detail in an earlier study (Tsai et al., 2016a). Briefly, we recruited children whose families had complained to the Taiwan Consumers' Foundation about their being possible victims of the phthalate-tainted foods and had filed a lawsuit for compensation between August 2012 and January 2013 (Wu et al., 2012; Tsai et al., 2016a). Those who lodged complaints and who were willing to participate in this study were referred to one of three special phthalate clinics, located in the northern, central, and southern Taiwan. The current study revisits this cohort focusing on children aged $\leq$12 yrs. After agreeing to participate in this study, the main caregiver (mostly the mother) of each child was administered a questionnaire collecting detailed information from which we could estimate each child's exposure to phthalate-tainted foods. All participants were well-being without diabetes mellitus, urinary tract obstruction and other systemic diseases. The children also received physical examinations and blood and urine workups. The measurements of all clinical and laboratory parameters have been described previously (Tsai et al., 2016a). The protocol for this study was approved by the institutional review boards (IRBs) of National Health Research Institutes (NHRI), the Ministry of Health and Welfare Hospitals (MHWHs), and Kaohsiung Medical University Hospital (KMUH). The reference number of the approvals was NHRI-EC100090 and KMUH-IRB-2012-11-01. The methods were carried out in accordance with the approved guidelines. Written informed consent was obtained from the main caregiver of each child studied.

2.2. Past DEHP exposure estimated based on intake of phthalate-tainted food items by questionnaire

Before each child received a phlebotomy, his or her main caregiver was interviewed using a standardized questionnaire to collect information on the child's consumption of any government-identified phthalates-tainted food items. The phthalate concentrations in these phthalate-tainted food items were extracted from publications produced by Taiwan's Food and Drug Administration (TFDA) and the Bureau of Health of Kaohsiung City (KBOH) (Wu et al., 2012). With this information, we were able to construct daily DEHP intake (DDI, mg/kg body weight (bw)/day) based on amount of DEHP they were exposed to (mg per time) and frequency (times per day) divided by body weight of each child (Wu et al., 2013a,b; Tsai et al., 2016a). Our previous study has demonstrated a high correlation between estimated DDI by questionnaire and oxidative DEHP metabolites in urine collected during that incident (Wu et al., 2013b). Among the 23 affected children with exposure information about that incident and the baseline of urine specimens collected during that incident, we found that the Spearmen correlation coefficient between estimated daily DEHP intake by questionnaire and by urinary oxidative DEHP metabolites using creatinine-based models was 0.466, which was significantly correlated ($p = .025$).

2.3. Urine collection

One-spot morning urine sample in each child was collected after the interview of questionnaire. Part of each sample was used for routine urinary analysis and the rest of urine sample was aliquoted and stored at −20 °C for the subsequent detection and measurement of melamine, phthalate metabolites, and biomarkers of renal injury.

2.4. Measurement phthalate metabolites in urine

We measured nine phthalate metabolites in the urine samples. The metabolites were mono-methyl phthalate (MMP), mono-ethyl phthalate (MEP), mono-n-butyl phthalate (MnBP), mono-benzyl phthalate (MBzP), mono-(2-ethylhexyl) phthalate (MEHP), mono-(2-ethyl-5-oxohexyl) phthalate (MEOHP), mono (2-ethyl-5-hydroxyhexyl) phthalate (MEHHP), mono-isobutyl phthalate (MiBP) and monoisononyl phthalate (MiNP), seven of which are the most common phthalates found in the environment (DEHP, DnBP, DiBP, BBzP, DMP, DEP, and DINP). Liquid chromatography/electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) was used to measure the nine phthalate metabolites in urine, following methods described in detail previously (Kuo et al., 2015; Tsai et al., 2016a). In brief, to prepare urine samples for analysis, 1 mL of each sample was thawed, transferred to a glass tube, and spiked with a mixture of isotopic (^{13}C4) phthalate monoester standards (10 μL). Then, they were buffered with ammonium acetate (250 μL, 1 M, pH = 6.5) and β-glucuronidase enzyme (3 μL, 200 U/mL), and were incubated in a 37 °C water bath for 90 min. After hydrolysis, each sample was acidified by adding 2 mL phosphate buffer (0.14 M NaH2PO4 in 0.85% H3PO4), vortex-mixed, and centrifuged at 3500 rpm for 10 min. The supernatant was loaded into a solid-phase extraction cartridge (NEXUS, Varian, Inc., Palo Alto, CA, U.S.A.). Formic acid (2 mL) and water (2 mL) were added to remove hydrophilic compounds, and then acetonitrile (1 mL) and ethyl acetate (1 mL) were added to elute metabolites. The combined elutes were concentrated under a stream of dry nitrogen at 55 °C. Finally, the residues were reconstituted with water and subjected to LC-MS/MS for analysis.

For method validation, the calibration was performed by using standard solutions of phthalate metabolites in pooled urine samples described in the previous studies (Kuo et al., 2015; Tsai et al., 2016a). The corresponding rings labeled analogs were used as internal standard (IS). The calibration range of each metabolite was divided into two: 1–50 ng/mL for the low one and 50–1000 ng/mL for the high one. The correlation coefficients (R^2) of these calibrations were 0.998–0.999 for MEHP, 0.996–0.998 for MEHHP, 0.998–1 for MEOHP, 0.994–0.998 for MnBP, 0.996–0.999 for MiBP, 0.991–0.996 for MEP, 0.993–0.999 for MBzP, 0.993–0.997 for MMP, and 0.998–1 for MiNP, which were all higher than 0.9950. Internal quality control was performed by analyzing both low (10 ng/mL) and high (100 ng/mL) levels spiked in urine in each batch. The accuracy for all calibration concentration curves and internal quality controls was within the range from 95.2 to 104.6% and with the precision expressed as a coefficient of variance (CV) ranging from 1.2 to 7.4% ($n = 5$). The intra- and inter-day relative standard deviation (RSD) ranged from 0.50 to 9.10% and 2.50–11.30% respectively. The averaged IS recovery in urine mixture was 80–115.0%, except for MEHP, MMP, and MiNP that showed IS recovery about 50%.

During the analyses of urine samples, each analytical run had 1-2 reagent blanks added, a low and a high concentration quality control sample, and 1–2 duplicated known samples. Calibration checks were also run every 20 samples to ensure instrumental stability throughout the entire analyses. Quantification of the calibration concentrations was within 15% of the theoretical value with a CV less than 15%, and therefore met performance criteria. The method of detection limit (MDL), determined by using a urine sample spiked with standard was 0.2 ng/mL for 5 phthalate metabolites (MEOHP, MEHHP, MBzP, MMP, and MiNP) and 1.0 ng/mL for others (MEHP, MnBP, MiBP, and MEP). The measurement below MDL was treated as half of MDL. Because MiNP was not detectable in all urine samples of our study subjects, we only presented the findings of the rest of the eight urinary phthalate metabolites.

2.5. Current DEHP exposure estimated based on DEHP metabolites in urine

Each child's current daily DEHP intake was also estimated based on a creatinine excretion-based model from the urinary DEHP metabolites including MEHP, MEHHP and MEOHP. The calculation of this value has been previously described (Wu et al., 2013b; Tsai et al., 2016b). In brief, the creatinine excretion-based model followed the equation: DEHP (μg/kg/day) = [UE_{sum}(μmol/g creatinine) × $CE_{smoothed}$ (g creatinine/day)/F_{UE} × bw (kg)] × MW_{DEHP}, where UE_{sum} (μmol/g creatinine) was the molar urinary excretion sum of MEHP, MEOHP, and MEHHP adjusted for creatinine. $CE_{smoothed}$ (g creatinine/day) was the daily creatinine excretion rate according to the study of Remer et al. (2002). MW represented the molecular weight of a given DEHP metabolite. F_{UE} (44.2%) was the sum of the proportions of MEHP, MEHHP, and MEOHP excreted in urine following the ingested DEHP.

2.6. Measurement of melamine in urine

One separately stored urine sample was used to measure melamine levels, as described previously (Wu et al., 2010; Liu et al., 2011). Briefly, the elute of 1 mL urine sample collected using an Oasis® MCX SPE cartridge (Waters Corp., Malford, MA, USA) was dried under nitrogen gas. The residues were then reconstituted in 200 μL mobile phase and subjected into LC-MS/MS for analysis. The method of detection limit (MDL) in urine was 0.8 ng/mL (ppb) (Hornung and Reed, 1990; Wu et al., 2010). The measured values of all urine samples were all above MDL.

Urinary creatinine was quantified using spectrophotometry (U-2000; Hitachi, Tokyo, Japan) at a wavelength of 520 nm to measure the creatinine–picrate reaction. Urinary melamine concentrations were expressed as μg/mmol creatinine. The technicians who measured urinary melamine and phthalate metabolite levels were blind to the concentrations of phthalate-tainted foodstuffs consumed by the children.

2.7. Measurement of biomarkers of renal injury in urine

Urine microalbumin, N-acetyl-beta-D-glucosaminidase (NAG), and β2-microglobulin, all clinical biomarkers of renal injury, were measured according to previously described methods (Tsai et al., 2016a). Briefly, urinary microalbumin was measured by radioimmunoassay (RIA) using an albumin RIA kit (Beckman Coulter, Immunotech, Prague, Czech Republic) (Comper and Osicka, 2005). NAG was measured using the NAG assay kit from Diazyme (Diazyme Laboratories, Poway, CA) and β2-microglobulin by N Latex β2-microglobulin assay from Siemens (Siemens Healthcare Diagnostics, Marburg, Germany). Because most of the urine samples were found to have β2-microglobulin below its method of detection limit (MDL, 0.206 mg/L), we omitted this marker from subsequent analysis (Tsai et al., 2016a). The concentrations of microalbumin and NAG in each urine sample were corrected by creatinine and expressed as mg/mmol creatinine for microalbumin and as U/mmol creatinine for NAG. Microalbuminuria was defined as having a urine microalbumin/creatinine ratio (ACR) higher than 3.5 mg/mmol.

2.8. Statistical analyses

The baseline characteristics, clinical parameters, past DEHP exposure (estimated daily DEHP intake from DEHP-tainted foods by questionnaire), and current DEHP exposure (estimated from urine DEHP metabolites) of all study children were categorized and analyzed by urine melamine level quartiles (1st quartile: ≤ 1.05, 2nd quartile: > 1.05, ≤1.61, 3rd quartile: > 1.61, ≤2.82, 4th quartile: > 2.82 μg/mmol creatinine). Continuous variables were expressed as mean ± SD and median (25th, 75th percentile), whereas categorical variables were expressed as percentages. Spearman correlation was performed to analyze the association between urine melamine levels and urinary biomarkers of early renal injury (microalbumin and NAG).

Multiple linear regression and logistic regression with modified stepwise procedures in two modeling steps were used to examine the association between each renal injury marker (corrected by urinary creatinine) with urine melamine levels (quartile or continuous) before and after adjusting for other covariates. Because urine ACR and NAG were not normally distributed, log10-transformation was performed. We categorized the study children into normal and microalbuminuria groups using the clinical cut-point of microalbuminuria, ACR >3.5 mg/mmol creatinine (>30 μg/mg creatinine). Urine NAG levels were dichotomized based on median (1.61 μg/mmol creatinine) for logistic regression analyses. Because the results based on urine melamine levels analyzed by continuous or quartile were similar, we only showed results based on quartiles.

In our multivariable models, we first adjusted for age, sex, body mass index, MAP, insulin resistance, serum cholesterol and uric acid levels, as suggested by our previous study (Tsai et al., 2016a). We forced two variables (past DEHP exposure from intake of phthalate-tainted food items and current DEHP exposure estimated from urine samples) into the final multivariate models. Past DEHP exposure from intake of phthalate-tainted food items (mg/kg/day) were categorized into high exposure (>0.05), moderate exposure (≤0.05 and > 0.02) and mild exposure (≤0.02) based on the recommended tolerable daily intake (TDI) of DEHP from the USEPA (TDI <0.02 mg/kg/day) and EFSA (TDI <0.05 mg/kg/day) (Tsai et al., 2016a), whereas current DEHP exposure was treated as a continuous variable. The interaction between urine melamine levels and past DEHP exposure from intake of phthalate-tainted food items was also investigated. All statistical operations were performed using SPSS version 18. All p-values were two-sided and considered significant if < 0.05.

2.9. Sensitivity analysis

Approximately 15.6% (35 out of 224) study children were known to be exposed to phthalate-tainted foodstuffs but we did not have access to exact DEHP concentration levels. We used the multiple imputation method to check the robustness of our original analyses with missing data (Rubin, 1987; Li et al., 2015). Multiple imputation with four imputations for those samples were performed following standard rules described in a study by Rubin to achieve 96%–97% relative efficiency to ensure in-range values (Rubin, 1987). A similar imputation approach was also applied to other covariates with missing data.

3. Results

3.1. Study children

Between August 2012 and January 2013, we enrolled 224 children who were ≤12 yrs, who had complete questionnaire data, and who had provided urine samples for the measurement of melamine (Fig. 1). An additional six children were added to the previous cohort to create the current cohort because we extended the age range to ≤12 yrs from ≤10 yrs (Tsai et al., 2016a). We categorized 35 children who had been exposed to phthalate-tainted foods with unknown DEHP concentrations into a mild DEHP exposure group (DDI ≤ 0.02 mg/kg/day) (n = 3), a moderate DEHP exposure group (DDI ≤ 0.05 and > 0.02 mg/kg/day) (n = 12), and a high DEHP exposure group (DDI > 0.05 mg/kg/day) (n = 20), respectively, using a multiple imputation method.

The 224 study children had a mean age of 5.5 yrs, were 58.9% boys, and had urine melamine levels ranging from 0.18 to 47.42 μg/mmol creatinine (Table 1, Table 2). Categorizing urine melamine levels into four groups by quartile ranges, we found significant differences in urine ACR and NAG, but not in past and current DEHP exposure, across the four groups (Table 1, Table S2). The Spearman correlation between urinary phthalate metabolites, anthropometric measurements, and urinary markers of kidney injury was shown in Table S3.

3.2. Relationship between melamine, DEHP, and biomarkers of early renal injury

Significant positive correlations were found between urine melamine levels and urine ACR and urine NAG levels (both Spearman correlation coefficient $r = 0.24$, $n = 224$, $p < .001$) (Fig. 2A, Fig. 2B). However, past DEHP exposure was significantly associated with urine ACR only, but not urine NAG (Fig. S1).

After adjustment, urine melamine levels were significantly and positively associated with urine ACR and NAG levels (Table S4, Table S5). However, current DEHP exposure was not found to be significantly associated with either urine ACR or NAG (Table S4, Table S5).

Seventeen children had microalbuminuria (urine ACR > 3.5 mg/mmol creatinine). Similar to the positive and significant association found between past DEHP exposure and microalbuminuria in our previous study (Tsai et al., 2016a), our current study also found that the higher the melamine level quartile, the higher the prevalence of microalbuminuria (Table 3, Table S6), after adjusting for other covariates, although the significance is borderline (urinary melamine level, the highest quartile *vs.* the lowest quartile, $p = .07$). Dichotomizing urine NAG by median (1.61 μg/mmol creatinine), we found a significant and positive association between urine NAG binary and urine melamine level quartile, but not past DEHP exposure.

3.3. The interactive effect of urine melamine levels and past DEHP exposure on biomarkers of renal injury

The higher the past DEHP exposure, the higher the prevalence of microalbuminuria, the trend becoming more pronounced in the groups with high urine melamine levels (Fig. 3, Fig. S2). Among those with past DEHP exposures >0.05 mg/kg bw/day and urine melamine levels >1.61 μg/mmol creatinine, ~19% children (7/37)

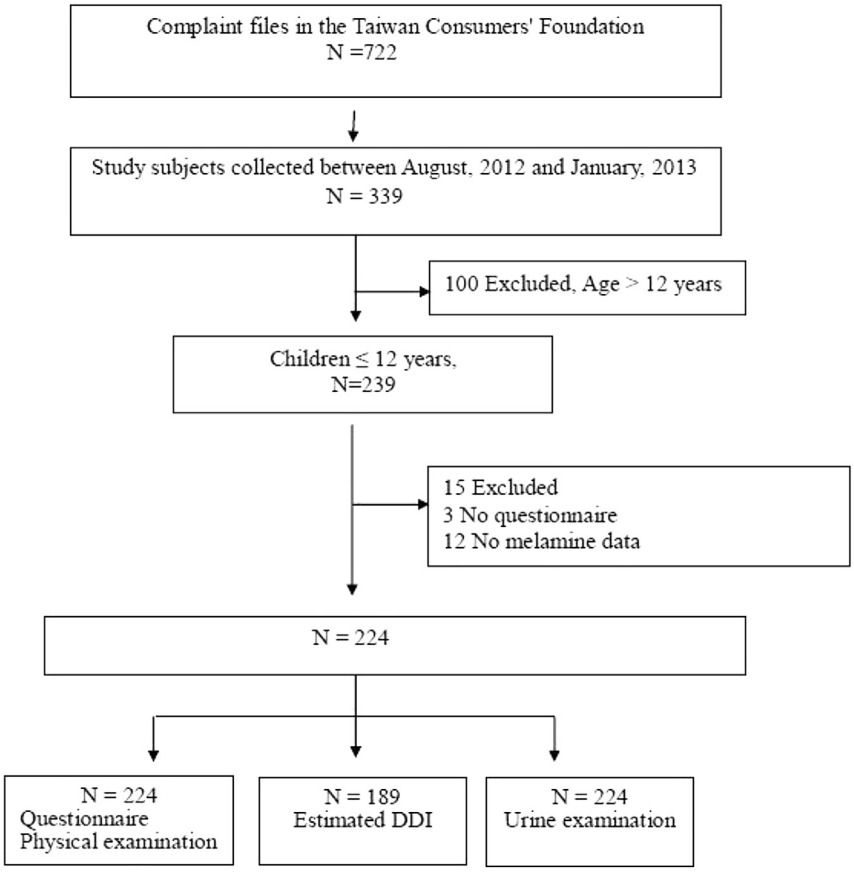

Fig. 1. Study flowchart.

Table 1
Demographic characteristics and clinical parameters categorized by the quartile of urine melamine levels.[a]

N	Total	1st quartile	2nd quartile	3rd quartile	4th quartile	P value[b]
	224	56	56	56	56	
Mean ± SD (Median, IQR) or N (%)						
Age (yrs)	5.5 ± 2.3 (5.0, 4.0–7.0)	6.5 ± 2.6 (6.8,4.4–8.0)	5.7 ± 2.3 (5.0, 4.0–7.0)	5.3 ± 2.2 (5.0, 3.1–6.4)	4.6 ± 1.7 (4.0, 3.0–6.0)	<.001
Gender, male	132 (58.9)	30 (53.6)	35 (62.5)	36 (64.3)	31 (55.4)	.60
Height (cm)	114.6±15.0[c] (113.0, 103.0–123.6)	119.6 ± 15.9 (118.4, 106.0–131.8)	115.4±15.1[c] (113.0, 103.6–123.8)	113.5 ± 16.0 (112.2, 101.9–121.6)	109.8 ± 11.3 (110.3, 102.1–116.8)	.01
Weight (kg)	21.9±7.9[c] (19.6, 16.5–25.0)	24.6 ± 7.8 (24.1, 18.2–29.5)	21.5±7.5[c] (19.6, 16.4–23.4)	21.9 ± 9.6 (18.7, 16.9–23.9)	19.5 ± 5.3 (18.5, 16.1–22.3)	.003
BMI (kg/m^2)	16.2±2.2[c] (15.8, 14.9–17.2)	16.8 ± 2.4 (16.3, 15.4–17.7)	15.7±1.7[c] (15.3, 14.7–16.2)	16.4 ± 2.6 (16.0, 14.9–17.3)	16.0 ± 1.9 (15.6, 14.9–17.1)	.03
SBP (mmHg)	101.8±12.8[d] (100.5, 93.0–108.0)	102.0±14.5[f] (101.5, 92.5–108.3)	104.0±11.5[c] (103.0, 96.0–112.0)	98.9±10.9[c] (97.5, 91.0–105.0)	102.2 ± 13.7 (100.5, 93.3–107.8)	.14
DBP (mmHg)	65.1±10.1[d] (64.8, 59.0–70.0)	64.7±11.2[f] (64.0, 59.0–68.0)	66.5±9.0[c] (67.0, 60.0–71.0)	63.8±8.6[c] (64.0, 57.0–71.0)	65.6 ± 11.2 (64.0, 60.0–69.8)	.34
MAP (mmHg)	77.3±10.3[d] (76.3, 70.9–81.9)	77.0±11.7[f] (76.2, 70.7–79.9)	78.9±9.2[c] (78.5, 72.7–85.7)	75.5±8.6[c] (76.0, 68.1–82.0)	77.6 ± 11.4 (75.9, 71.4–82.0)	.26
Past DEHP exposure (μg/kg/day)	48.4±67.2[e] (35.1, 5.1–62.8)	52.5±107.4[g] (28.9, 3.0–55.6)	48.6±46.9[h] (43.1, 4.7–68.9)	39.2±42.7[g] (30.2, 6.1–53.3)	53.2±53.8[i] (49.7, 5.1–74.1)	.42
Current DEHP exposure (μg/kg/day)	5.9±5.0[d] (4.7, 3.2–6.9)	5.0±3.6[f] (3.7, 2.9–6.1)	6.7±6.3[c] (5.2, 3.3–7.4)	6.0 ± 4.3 (4.8, 3.2–7.0)	6.0±5.4[c] (4.5, 3.5–6.9)	.21
Urine ACR (mg/mmol)	1.1 ± 1.8 (0.6, 0.3–1.2)	1.0 ± 2.7 (0.4, 0.2–0.8)	1.0 ± 1.0 (0.6, 0.2–1.5)	0.8 ± 0.9 (0.6, 0.3–1.2)	1.6 ± 2.0 (0.8, 0.4–1.7)	.002
Urine NAG (U/mmol)	0.8 ± 1.0 (0.5, 0.3–0.8)	0.5 ± 0.4 (0.4, 0.2–0.5)	0.9 ± 1.0 (0.5, 0.3–0.7)	0.9 ± 1.0 (0.5, 0.4–0.9)	1.1 ± 1.3 (0.6, 0.3–1.4)	.003

Abbreviation: ACR: albumin-to-creatinine ratio; BMI: body mass index; Cr: creatinine; DBP: diastolic blood pressure; DEHP: di-(2-ethylhexyl) phthalate; IQR: Interquartile range; MAP: mean arterial pressure; NAG: N-acetyl-beta-D-glucosaminidase; SBP: Systolic blood pressure.
[a] Urine melamine levels categorized by quartile: 1st quartile: ≤ 1.05, 2nd quartile: > 1.05, ≤1.61, 3rd quartile: > 1.61, ≤2.82, 4th quartile: > 2.82 μg/mmol Cr.
[b] Kruskal-Wallis H or Fisher's exact test among the four groups.
[c] One missing data.
[d] Four missing data.
[e] Thirty-five missing data.
[f] Two missing data.
[g] Ten missing data.
[h] Seven missing data.
[i] Eight missing data.

Table 2
Distribution of urine melamine and phthalate metabolites, estimated DEHP intake, and renal injury markers in study children.

Variables	Percentiles							
	N	Min	5th	25th	50th	75th	95th	Max
Melamine (μg/mmol Cr.)								
All study children	224	0.18	0.46	1.05	1.61	2.82	7.74	47.42
Children with intake of DEHP-tainted foods	170	0.18	0.45	1.06	1.57	2.80	7.62	19.86
Past DEHP exposure (μg/kg/day)								
All study children	189	0	0	5.12	35.13	62.81	156.06	705.34
Children with intake of DEHP-tainted foods	170	0.23	1.22	15.23	41.48	67.34	158.21	705.34
Current DEHP exposure (μg/kg/day)								
All study children	220	0.20	1.76	3.18	4.66	6.90	16.55	40.46
Children with intake of DEHP-tainted foods	168	0.20	1.70	3.21	4.55	6.67	15.69	40.46
Urine (μg/g Cr.)								
MMP	224	0.08	2.63	8.46	14.54	27.15	68.30	578.29
MEP	224	0.12	0.37	6.81	13.98	28.53	105.56	573.64
MnBP	224	6.01	16.69	30.35	46.38	76.15	164.51	344.68
MBzP	224	0.06	0.12	1.48	2.84	5.38	15.80	231.58
MEHP	224	0.19	0.52	4.90	10.06	18.10	37.06	99.93
MEHHP	224	1.83	16.56	30.74	45.77	65.16	151.59	404.89
MEOHP	224	0.11	10.82	21.67	32.20	46.78	107.72	271.35
MiBP	224	2.22	7.00	15.55	24.32	40.20	79.52	641.20
Urine ACR (mg/mmol Cr.)	224	0.03	0.07	0.29	0.59	1.19	4.24	19.53
Urine NAG (U/mmol Cr.)	224	0.05	0.16	0.33	0.49	0.77	3.25	5.20

Abbreviations: ACR = albumin-creatinine ratio; Cr. = creatinine; DEHP = di-(2-ethylhexyl) phthalate; Max = maximum; MBzP = mono-benzyl phthalate; MEHHP = mono-(2-ethyl-5-oxohexyl)phthalate; MEHP = mono-(2-ethylhexyl)phthalate; MEOHP = mono-(2-ethyl-5-hydroxylhexyl)phthalate; MEP = mono-ethyl phthalate; MiBP = mono-iso-butyl phthalate; Min = minimum; MiNP = mono-isononyl phthalate; MMP = Mono-methyl phthalate; MnBP = mono-n-butyl phthalate.

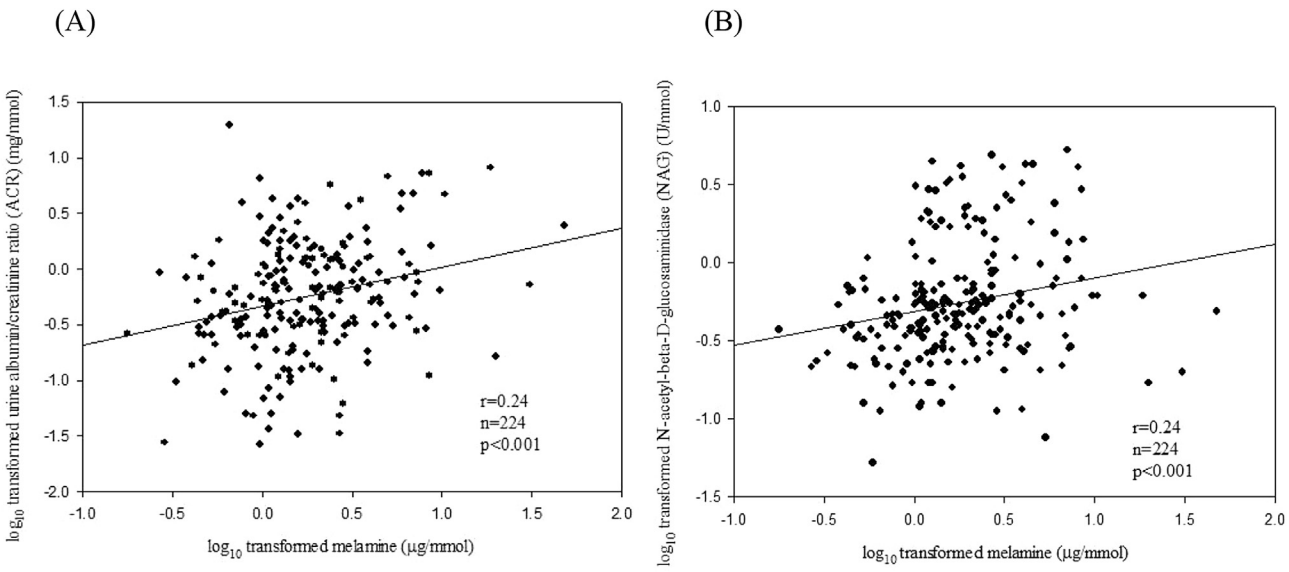

Fig. 2. The Spearman correlation between urine log-transformed melamine and log-transformed biomarkers of renal injury. (A) Urine melamine and ACR; (B) Urine melamine and NAG.

Table 3
Relationship of melamine and DEHP exposures with microalbuminuria and urine dichotomized NAG in logistic regression models.[a]

	Microalbuminuria		Crude			Model[b]			Model[c]		
	Yes	No	OR	95% CI	*P*	OR	95% CI	*P*	OR	95% CI	*P*
	N (%)										
Urine melamine											
1st quartile	3 (5.4)	53 (94.6)	1	–	–	1	–	–	1	–	–
2nd quartile	3 (5.4)	53 (94.6)	1.00	0.19–5.18	1.00	1.06	0.19–6.03	0.95	1.94	0.15–24.99	0.61
3rd quartile	2 (3.6)	54 (96.4)	0.85	0.11–4.07	0.65	0.78	0.11–5.38	0.80	2.25	0.17–29.13	0.54
4th quartile	9(16.1)	47 (83.9)	3.38	0.85–13.24	0.08	3.97	0.87–18.04	0.08	8.12	0.85–77.75	0.07
Past DEHP exposure (μg/kg/day)											
≤ 0.02,	1 (1.4)	68 (98.6)	1	–	–				1	–	–
≤ 0.05, >0.02	4 (8.0)	46 (92.0)	5.91	0.64–54.61	0.12				6.52	0.62–68.43	0.12
> 0.05	9 (12.9)	61 (87.1)	10.03	1.24–81.50	0.03				8.74	0.93–81.95	0.06
Current DEHP exposure (μg/kg/day)[d]	16 (7.3)	204 (92.3)	0.99	0.89–1.10	0.85				0.97	0.83–1.13	0.72
	NAG		Crude			Model[b]			Model[c]		
	≥median	< median	OR	95% CI	*P*	OR	95% CI	*P*	OR	95% CI	*P*
	N (%)										
Urine melamine											
1st quartile	16 (28.6)	40 (71.4)	1	–	–	1	–	–	1	–	–
2nd quartile	31 (55.4)	25 (44,6)	3.10	1.42–6.79	.005	3.66	1.52–8.80	.004	5.34	1.93–14.73	.001
3rd quartile	33 (58.9)	23 (41.1)	3.59	1.63–7.88	.001	4.37	1.81–10.55	.001	5.53	2.00–15.28	.001
4th quartile	32 (57.1)	24 (42.9)	3.33	1.52–7.31	.003	3.44	1.43–8.30	.006	4.70	1.67–13.24	.003
Past DEHP exposure											
≤ 0.02,	28 (40.6)	41(59.4)	1	–	–				1	–	–
≤ 0.05, >0.02	27 (54.0)	23 (46.0)	1.72	0.82–3.59	.15				1.40	0.59–3.30	.45
> 0.05	39 (55.7)	31 (44.3)	1.84	0.94–3.61	.08				1.38	0.62–3.08	.44
Current DEHP exposure (μg/kg/day)[d]	111 (50.5)	109 (49.5)	0.97	0.92–1.02	.26				0.93	0.86–1.00	.05

Abbreviations: ACR: albumin-to-creatinine ratio; DEHP: di-(2-ethylhexyl) phthalate; NAG: N-acetyl-beta-D-glucosaminidase.
[a] Microalbuminuria was defined as urine ACR > 3.5 mg/mmol creatinine, whereas the median of urine NAG was 0.49 U/mmol creatinine.
[b] Model 1 included age, gender, body mass index, blood pressure, insulin resistance, cholesterol, uric acid, exposure to second-hand smoke, activity level, and urine melamine by quartile.
[c] Model 2 included age, gender, body mass index, blood pressure, Insulin resistance, cholesterol, uric acid, exposure to second-hand smoke, activity level, urine melamine by quartile, past DEHP exposure estimated from questionnaire, and current DEHP exposure estimated from urine DEHP metabolites.
[d] Four missing data.

had microalbuminuria (Fig. 3B). We investigated the interactive effect of urine melamine and past DEHP exposure on biomarkers of renal injury by stratifying the study children into four groups based on median urine melamine levels (1.61 μg/mmol creatinine) and USEPA TDI (0.02 mg/kg/day) for past DEHP exposure. We found a significant dose-response relationship between both urine melamine levels and past DEHP exposure and urine ACR (Table 4, Table S7), after adjusting for other covariates. In contrast, high urine melamine levels, but not past DEHP exposure, had a significant effect on urine NAG (Table 4, Table S7).

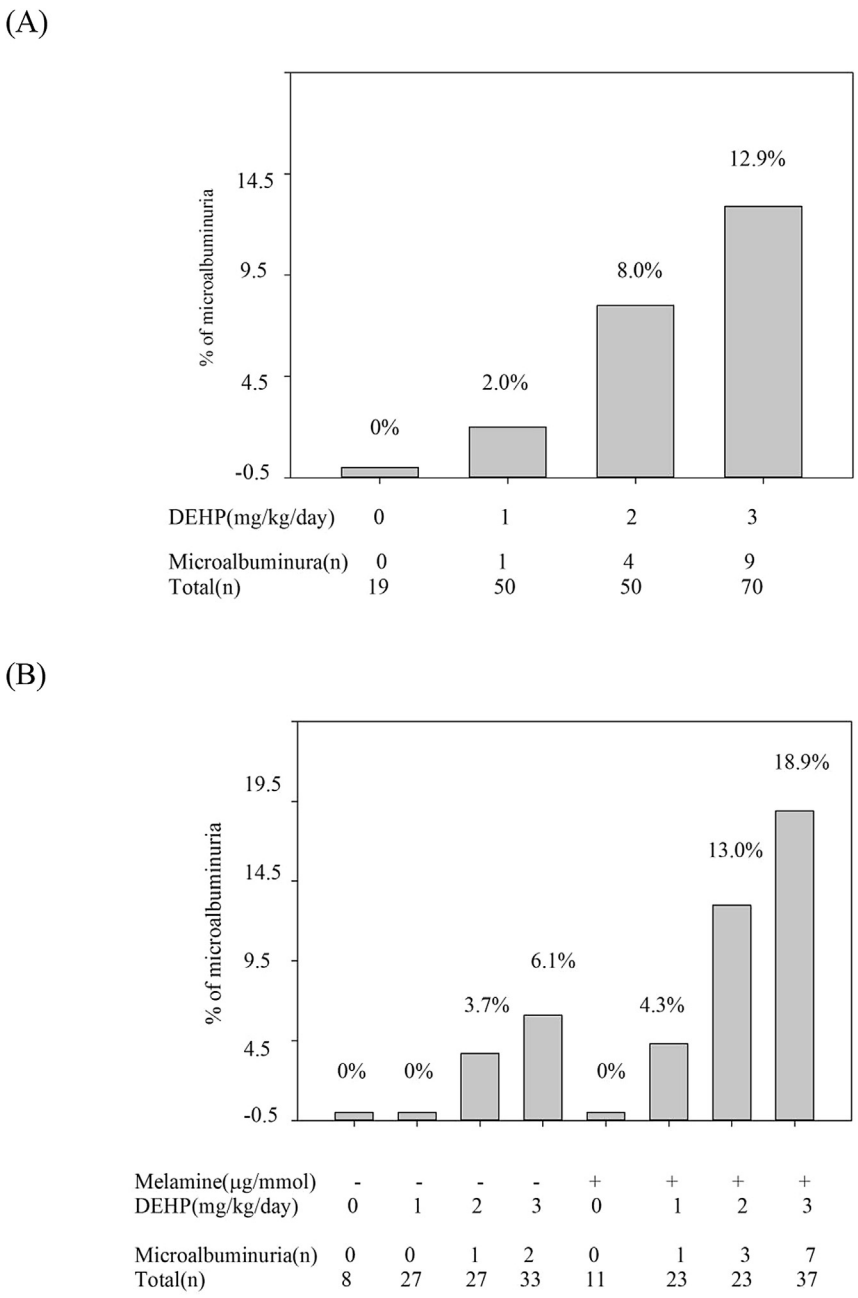

Fig. 3. The prevalence of microalbuminuria categorized by past DEHP exposure based on the recommended TDI of DEHP from the U.S. Environmental Protection Agency and European Food Safety Authority and median of urine melamine. (A) Categorized by DEHP; (B) categorized by DEHP and melamine. DEHP (mg/kg/day):0 indicates no known exposure to phthalate-tainted foods; 1 indicates ≤ 0.02 mg/kg/day, > 0 mg/kg/day; 2 indicates ≤ 0.05 mg/kg/day, > 0.02 mg/kg/day; and 3 indicates > 0.05 mg/kg/day; urine melamine levels (μg/mmol creatinine): indicates ≤ 1.61 and + indicates > 1.61.

4. Discussion

This study found that melamine levels can further influence the effect of past DEHP exposure on urine ACR in children. However, only urine melamine level, but not past DEHP exposure, was associated with urine NAG. Current DEHP exposure, estimated by DEHP metabolites in urine, was not associated with either urine ACR or urine NAG.

After the 2008 melamine-tainted toxic-milk scandal in China, some epidemiological studies surveyed urine melamine levels in various communities in an effort to understand environmental exposure to this chemical (Kong et al., 2011; Panuwet et al., 2012; Lin et al., 2013). The present study found that the median urine melamine level in this cohort of children was 1.5–1.6 μg/mmol creatinine (Table 2). These levels were similar to those reported by community children studies from Taiwan and Hong Kong (~0.78–1.7 μg/mmol creatinine) (Kong et al., 2011; Lin et al., 2013). One study from USA also detected melamine (MDL = 0.66 ng/mL) in 76% of 492 urine samples from the general population there (Panuwet et al., 2012), although the authors of that study did not provide the ages of their study subjects. These findings suggest that melamine is ubiquitously present in the environment and that most of the general population, including children, are likely to be exposed to it.

Table 4
The interactive effect of urine melamine levels and past DEHP exposure on biomarkers of renal injury in multivariate linear regression models.

	N	Log_{10}ACR				Log_{10}NAG			
		Crude		Adjusted[b]		Crude		Adjusted[b]	
		β (SE)	*P*	β (SE)	*P*	β (SE)	*P*	β (SE)	*P*
Low melamine & low DEHP[a]	35	–	–	–	–	–	–	–	–
High melamine & low DEHP	34	0.07 (0.12)	.55	0.08 (0.13)	.53	0.20 (0.09)	.04	0.26 (0.10)	.01
Low melamine & high DEHP	60	0.12 (0.10)	.26	0.11 (0.11)	.35	0.08 (0.08)	.31	0.10 (0.09)	.26
High melamine & high DEHP	60	0.39 (0.10)	<.001	0.34 (0.11)	.003	0.23 (0.08)	.005	0.24 (0.09)	.01

AbbreviationsACR: albumin-creatinine ratio; DEHP: di-(2-ethylhexyl) phthalate; NAG: N-acetyl-beta-D-glucosaminidase.
[a] The cut-off values were 1.61 μg/mmol creatinine for urine melamine levels and 0.02 mg/kg/day for past DEHP exposure.
[b] Adjusting for age, gender, body mass index, blood pressure, cholesterol, insulin resistance, uric acid (categorical), exposure to second-hand smoke exposure, activity level, and current DEHP exposure (continuous).

Our previous studies have found an association between environmental low-dose melamine exposure and the risk of renal stones as well as increases in renal tubular injury markers in urine, such as NAG and β2-microglobulin, in adults (Wu et al., 2010, 2015; Liu et al., 2011). The renal tubular injury caused by low and chronic melamine is probably due to increases in cellular reactive oxygen species, decreases in antiapoptotic/proapoptotic protein ratio, and cell apoptosis in renal tubular cells prior to renal morphological change, as was found in our previous *in-vitro* study (Hsieh et al., 2012). In the current study, we found that urine melamine levels resulting from exposure to the chemical in the general environment affected urine NAG levels in children affected by the 2011 Taiwan phthalate food scandal, a finding that was consistent with that of our prior studies of adults (Wu et al., 2015).

Previously, we found a positive and significant association between the intake of DEHP from phthalate-tainted foods and urine ACR (Tsai et al., 2016a). The current study found urine melamine level to be another independent risk factor for urine ACR. We also found an interactive effect between current melamine levels and past DEHP exposure, but not current DEHP exposure, on urine ACR. While it is known that the high melamine levels found in children exposed to toxic milk during the 2008 melamine incident caused kidney-related diseases and kidney failure, to the best of our knowledge, this study is the first to find an independent and interaction effect between environmental and low-dose melamine exposure on microalbuminuria, one clinical important biomarker of renal glomerular damage.

Based on our current findings combined with those of our previous study (Tsai et al., 2016a), the RfD (Reference dose) of 0.02 mg/kg bw/day DEHP recommended by U.S. Environmental Protection Agency (EPA) is probably more protective against renal damage than the TDI of 0.05 mg/kg bw/day recommended by European Food Safety Authority (EFSA). Agencies from the USA and European Union used DEHP exposure data from the different rodent experiments to estimate the risk to human health and establish their standards (EPA 1987; EFSA 2005). The ESFA's recommended TDI of 0.05 mg/kg bw/day DEHP for humans was based on the testicular toxicity and developmental toxicity from rodent experiments (rats) with the no-observable-adverse-effect level (NOAEL) of 5 mg/kg bw/day, considering an uncertainty factor of 100 (EFSA 2005). The USEPA's recommended RfD of 0.02 mg/kg bw/day DEHP for humans was based on the subchronic-to-chronic oral assay in guinea pigs, which found a significant increase of liver weights at 19 mg/kg bw/day at the lowest-observed-adverse-effect level (LOAEL), considering an uncertainty factor of 1000 (EPA 1987; Carpenter et al., 1953). After the 2011 food scandal, Taiwan's Department of Health, formerly the Ministry of Health and Welfare, adopted the limit recommended in European Union and officially announced the maximum TDI of DEHP to be < 0.05 mg/kg bw/day in July 2011, believing that it would be more favorable to economic growth (Wu et al., 2012). The findings of the current study in humans can be used as an additional reference for the governmental agencies considering future revisions to their DEHP regulations.

This study has several limitations. In addition to those of the previous studies on which part of this study is based, this study is limited by its cross-sectional design, which made it difficult to avoid the possibility of reverse causality between urine melamine levels and urine ACR and NAG levels, though the likelihood of reversed bias is reduced, because we did not find a positive correlation between urine phthalate metabolites and urine ACR or NAG levels. Another potential limitation is no external comparison group which was not exposed to any environmental chemicals related to renal injury in children in this study. Because this is an observational study, unmeasured confounding factors cannot be fully controlled in the regression models. Still another limitation is that only one-spot samples were used to measure markers of renal damage and to define microalbuminuria, which might be transient in nature.

5. Conclusions

In conclusion, urine melamine levels were significantly and independently associated with expression of markers of early renal damage, including urine ACR and NAG. Past DEHP exposure from phthalate-tainted foods modified the risk of urine melamine on urine ACR. Further studies are needed to evaluate whether the renal injury is transient or persistent after the discontinuation of exposure to phthalate-tainted foods and to follow the long-term health consequences in children.

Author contributions statement

Study concept and design: All authors.
Acquisition, analysis, or interpretation of data: All authors.
Experiment analysis: Dr. C.-F.W.
Drafting of the manuscript: Dr. C.-F.W., Professor C.-A.H.
Critical revision of the manuscript for important intellectual content: All authors.
Statistical analysis: Dr. C.-F.W., Dr. H.-J.T., Dr. H.-M.H.
Obtained funding: Professor M.-T.W.
Administrative, technical, or material support: Professor C.-A.H., Dr. H.-J.T., Dr. Y.-C.T., Professor B.-H.C., Professor M.-T.W.
Study supervision: Professor M.-T.W.

Conflicts of interest

None. The authors declare they have no competing financial interests.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.envpol.2017.12.107.

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