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Levels and health risks of urinary phthalate metabolites and the association between phthalate exposure and unexplained recurrent spontaneous abortion: a large case-control study from China

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ABSTRACT

Phthalate acid esters (PAEs) are environmental endocrine disruptors that can interfere with endocrine processes and cause adverse reproductive outcomes. The link between PAE exposure and unexplained recurrent spontaneous abortion (URSA) remains unknown. In this study, nine urinary metabolites of PAEs (mPAEs) were measured in 594 URSA cases and 569 healthy controls. The measured mPAEs were ubiquitously detected and present at higher levels (median: 203 ng/mL) in the URSA cases than in the controls (median: 161 ng/mL). Multiple logistic regression analysis showed that URSA was associated with higher concentrations of mono (2-ethyl-5-hydroxyhexyl) phthalate (mEHHP), mono (2-ethylhexyl) phthalate (mEHP), and mono-ethyl phthalate (mEP) and lower concentrations of mono-isobutyl phthalate (miBP). Moreover, a quantile-based g-computation (QGC) model revealed a positive association between mPAEs mixture and URSA. The URSA cases showed significantly higher concentrations of di-(2-ethylhexyl) phthalate (DEHP) than the controls. This was consistent with the health risk assessment, which suggested that DEHP is the main contributors to potential non-carcinogenic risk. DEHP accounted for over 80% of total risk. The large case-control study results suggest that PAE exposure may increase the risk of URSA, and that policy-makers and public health experts should pay more attention to DEHP exposure.

1. Introduction

Phthalate acid esters (PAEs) are non-persistent synthetic chemicals that have been extensively used as plasticizers or solvents in polyvinyl chloride, plastic products (e.g., food packaging materials and toys), adhesives, paints, personal care products, and cosmetics (Gao and Wen, 2016; Huang et al., 2021). PAEs can be divided into high-molecular-weight phthalates, such as di-iso-nonyl phthalate (DiNP), butylbenzyl phthalate (BBzP), di-n-octyl phthalate (DnOP), and di-(2-ethylhexyl) phthalate (DEHP), along with low-molecular-weight phthalates, including di-iso-butyl phthalate (DiBP), di-n-butyl phthalate (DBP), di-ethyl phthalate (DEP), and di-methyl phthalate (DMP) (Wang et al., 2015a). When added as an additive, PAEs non-covalently bond to the original product. This characteristic, along with extensive

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use across multiple industries, mean that PAEs widely occur in various environmental media, including food (2.23 mg/kg in vegetables), surface water (91.22 μg/L in Yangtze River), soil (559 ng/g in Yangtze River Delta), and the air (34.8 ng/m^3 in $PM_{2.5}$ in Shenzhen) (Gao et al., 2018; Lu et al., 2018; Net et al., 2015; Wang et al., 2015b). This has resulted in ubiquitous human exposure to PAEs through inhalation, ingestion, and dermal contact (Huang et al., 2021; Wang et al., 2019).

Recurrent spontaneous abortion (RSA), which is defined as miscarriage prior to the 20th week of gestation for two or more consecutive pregnancies, affects approximately 2%–5% of women in reproductive age (El Hachem et al., 2017). Currently, endocrine and immunologic disturbances, parental genetic abnormalities, and uterine anomalies are listed as etiologies of RSA (El Hachem et al., 2017; Rai and Regan, 2006). However, only half of total RSA cases can be explained. This means that the other half of RSA cases, termed unexplained RSA (URSA), cannot be attributed to a precise cause (Garrido-Gimenez and Alijotas-Reig, 2015). There is mounting evidence that environmental chemicals, especially endocrine disruptors, may play an important role in URSA (Krieg et al., 2016).

The pathways by which PAEs may adversely affect URSA are not fully understood. Experimental and epidemiological studies have shown significant associations between increased exposure to PAEs and inhibited meiotic maturation (Absalan et al., 2017; Kalo et al., 2019), placental malfunction (Zong et al., 2015), disrupted reproductive hormones and the biomarkers of oxidative stress and inflammation (Hirosawa et al., 2006; Sathyanarayana et al., 2017; Ferguson et al., 2014; van 't Erve et al., 2019), which could lead to pregnancy loss.

Toxicologic studies have demonstrated that high doses of DEHP and DiBP can cause significant embryonic lethality and teratogenicity, reduce the number of litters, and increase the incidence of abortion (Ema et al., 1995; Saillenfait et al., 2006; Schmidt et al., 2012). In humans, numerous studies had linked the urinary mPAEs to multiple adverse reproductive outcomes, including endometriosis, polycystic ovary syndrome, and pregnancy loss (Kay et al., 2013; Zhang et al., 2020). To the best of our knowledge, only three reports have investigated the association between PAE exposure and URSA (Chang et al., 2021; Liao et al., 2018; Peng et al., 2016). A case-control study by Peng et al. (2016) revealed that URSA cases showed higher concentrations of mono (2-ethylhexyl) phthalate (mEHP) than control samples. Another two studies, conducted among reproductive-aged women from Taiwan, China, reported that ΣDBP, BBzP, and DEHP levels increased the risk of URSA (Chang et al., 2021; Liao et al., 2018). However, these studies had relatively small sample size of cases, ranging from 30 to 260. Moreover, previous studies on URSA examined the effects of mPAEs in isolation, i.e., the research did not consider the potential associations of multiple metabolites, and nonlinear or non-additive interactive effects of the individual chemicals.

Therefore, the present study conducted a large case-control study to demonstrate that human exposure to PAEs is associated with URSA. In the present case, over one thousand urine samples were collected from women of childbearing age; the urinary levels of mPAEs, as well as the health risks of PAE exposure, were analyzed. A set of well-defined single pollutant and multipollutant models were used to address knowledge gaps and explore whether urinary levels of mPAEs were associated with the occurrence of URSA. The results provide insight into PAE exposure as an etiology of URSA and, as such, the present study is a valuable basis for future risk assessment of PAEs.

2. Materials and methods

2.1. Reagents and materials

The nine standards of mPAEs, including mono (2-ethyl-5-hydroxyhexyl) phthalate (mEHHP), mono (2-ethylhexyl) phthalate (mEHP), mono-ethyl phthalate (mEP), mono (2-ethyl-5-oxohexyl) phthalate (mEOHP), mono-n-butyl phthalate (mBP), mono-isobutyl phthalate (miBP), mono-methyl phthalate (mMP), mono-n-octyl phthalate (mOP), and mono-benzyl phthalate (mBzP), as well as their isotope-labeled internal standards, $^{13}C_4$-mEHP, $^{13}C_4$-mEOHP, $^{13}C_4$-mEHHP, $^{13}C_4$-mEP, $^{13}C_4$-mBP, d_4-miBP, $^{13}C_4$-mMP, $^{13}C_4$-mOP, and $^{13}C_4$-mBzP, were purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA). Acetonitrile was purchased from Merck (Darmstadt, Germany), while β-glucuronidase/sulfatase was purchased from Sigma Aldrich (St. Louis, MO, USA).

2.2. Study design and sample collection

A multicenter, hospital-based case-control study was conducted to evaluate how environment pollutants may affect URSA. Cases and controls were recruited from Shanghai and Zhejiang, China, from May 2014 to December 2016. Information regarding the criteria for URSA cases and controls were presented elsewhere (Ao et al., 2021). Briefly, the criteria for URSA cases was set as follows: a) two or more consecutive RSA before 20 weeks of gestation; b) normal chromosome of both partners; c) normal anatomic reproductive tract; d) negative autoantibodies (i.e. anticardiolipin, antiphospholipid or antinuclear) and TORCH infection (i.e. toxoplasmosis, rubella virus, cytomegalovirus, herpes simplex virus, hepatitis viruses, and human immunodeficiency virus) in female; e) no endocrine disorders, including thyroid disease, polycystic ovary syndrome, endometriosis and hyperprolactinemia; f) a normal male partner without known reproductive problems. Controls were non-pregnant women who had at least one normal birth and came for preconception care.

Initially, a total of 1331 cases and 454 controls were recruited in Shanghai. Considering the costs and time of mPAE measurements, 454 cases without previous live birth were randomly used in this study. Among them, 40 cases and 13 controls were excluded for lack of urine sample, leaving us with 414 cases and 441 controls for Shanghai site. For Zhejiang site, 263 cases and 252 controls were initially recruited, and 34 cases and 124 controls were excluded for lack of urine samples. We further excluded the cases with previous live birth which left us with 180 cases and 128 controls for Zhejiang site. Therefore, a total of 594 cases and 569 controls were used and analyzed in the present study.

Information on demographic and lifestyle characteristics, along with reproductive and medical history, was collected using a standardized questionnaire. Clinical information was abstracted from medical records. Urine samples were collected at least one month after the miscarriage event and stored at −20 °C before use. This study was approved by the Ethics Committees of all involved research institutions and hospitals (the Xinhua Hospital Ethics Committee Affiliated to Shanghai Jiao Tong University School of Medicine (Approval Number: XHEC-C-2015-046). Written informed consent was obtained from each subject.

2.3. Sample analysis

The pretreatment of urine samples and instrument analysis was performed as outlined in a previous report (Li et al., 2021). In brief, following enzymatic hydrolysis, urinary protein was removed by centrifugation and the supernatant was analyzed using on-line solid phase extraction-liquid chromatography tandem mass spectrometry (online SPE-HPLC-MS/MS). Detailed information about this procedure can be found in the Supplementary material.

The sample treatment and instrumental analysis included strict quality assurance and quality control measures. The intra- and inter-assay coefficients of variation (CV) for mPAEs were all below 10%. The limits of detection (LOD) for mEHP, mEHHP, mEOHP, mEP, mBP, miBP, mOP, mBzP, and mMP were 0.008, 0.150, 0.023, 0.246, 0.016, 0.051, 0.004, 0.102, and 4.28 ng/mL, respectively. Any concentrations that fell below the LOD were replaced by the LOD divided by the square root of 2. Urinary creatinine concentrations were measured using an automatic biochemical analyzer (7100, Hitachi Inc., Tokyo, Japan) and

used to adjust for urine dilutions of the analytes.

2.4. Health risk assessments

To assess the non-carcinogenic risks of PAE exposure, the total estimated daily intake (TEDI) and hazard quotient (HQ) for each PAE were calculated; the hazard index (HI) was calculated as the sum of the HQ values. The formulae were as follows:

$$TEDI = \frac{C_u \times V_u \times MW_p}{f \times BW \times MW_m} \quad (1)$$

$$HQ = \frac{TEDI}{RfD} \quad (2)$$

$$HI = \Sigma\, HQ \quad (3)$$

where TEDI is the estimated daily intake of a PAE compound (μg/kg-bw/day); C_u is the concentration of an individual urinary mPAE (μg/L); V_u is the total volume of urine excreted per day, with 2.0 L/day used in the present study (Guo et al., 2011b); BW is body weight (kg); MW_p and MW_m are the molecular weights of the PAE and its corresponding metabolite (g/mol); f (dimensionless) represents the urine excretion ratio of PAE to the corresponding mPAE; RfD is the reference dose (μg/kg-bw/day). In this study, the f values for mEP, mEHP, mEHHP, mEOHP, mBP, miBP, mBzP, mMP and mOP were 69.0%, 6.2%, 14.9%, 10.9%, 70.0%, 70.3%, 73.0%, 69.0% and 13.0%, respectively (Yu et al., 2021), while the RfDs of DEP, DEHP, DBP, DiBP, BBzP, and DnOP were 800, 20, 100, 100, 200, and 800 μg/kg-bw/day, respectively (USEPA – U.S. Environmental Protection Agency, 1993a; 1993b; 1993c, 1990).

2.5. Statistical analysis

Differences in baseline characteristics and distributions of mPAEs between the URSA and control groups were assessed using a Wilcoxon rank sum test or Chi-squared test. Jonckheere-Terpstra tests were applied when exploring the associations between levels of urinary mPAEs and the studied variables. Three chemicals (i.e., mOP, mMP, and mBzP) were not included in association analyses due to low detection frequencies (54.5%, 32.8%, and 32.9% for them, respectively). All statistical analyses were conducted in SAS 9.4 software (SAS Institute Inc., Cary, NC, USA) and R 3.6 (The R Foundation, Vienna, Austria). R packages "qgcomp" and "splines" were used to conduct quantile-based g-computation (QGC) analyses and restricted cubic spline (RCS) analyses. Statistical significance was set at 0.05 (two-tails).

In the association analyses, multiple logistic regression models were applied to estimate the odds ratios (OR) and 95% confidence intervals (CI) for PAE exposure and URSA. The mPAE levels were first analyzed as continuous values, after which the values were natural log-transformed to improve normality. Next, the mPAE values were categorized into quartiles according to distributions among the control group, and the lowest quartile was assigned as the reference group. Moreover, RCS models with knots at the 10th, 50th, and 90th percentiles of each mPAE were fitted to identify potential non-linear associations between mPAEs and URSA. Spearman correlation analysis was used to determine the correlations among individual mPAEs. Considering the potential co-exposure of mPAEs, QGC model was applied to explore the joint effects of mPAEs mixture (Keil et al., 2020). To allow for the potential nonlinear effects of a specific component in the joint effects, a natural cubic spline term (degrees of freedom (df) = 3) was incorporated for mPAEs that presented significant nonlinear relationships with URSA in the RCS analyses. Each component was categorized into quartiles, estimates and the corresponding 95% CIs were generated after 200 bootstrapping iterations.

To replace the missing values of covariates before modeling, a multiple imputation procedure was conducted. Less than 9.1% participants had one or more missing values. A total of five imputed datasets were generated, and the final estimates were pooled across the five imputed datasets (Yuan, 2001). The necessary set of potential confounders was inferred based on previous studies and a directed acyclic graph (DAG) (Fig. S1), including educational level (less than a Bachelor's degree, Bachelor's degree, above a Bachelor's degree), body mass index (BMI, continuous), age (continuous), age at menarche (continuous), alcohol consumption (Yes/No), and creatinine (ln-transformed).

Several sensitivity analyses were performed to test the robustness of the results. First, stratified analyses by BMI and age were conducted because both of these factors were reported to be associated with miscarriage and, as such, could potentially modify the associations between PAE exposure and URSA (El Hachem et al., 2017; Cavalcante et al., 2019). Second, site-specific association analyses were conducted to evaluate whether samples from different study sites could be pooled. Third, the regression models were rerun after excluding control samples with a history of one sporadic miscarriage (n = 59). Fourth, the regression analyses were rerun using two common methods for urine dilution adjustment recommended by a previous study (O'Brien et al., 2016): one was dividing the mPAEs concentration by the urinary creatinine concentration (Barr et al., 2005), another was covariate-adjusted standardization approach where creatinine (ln-transformed) was first regressed on covariates that might affect creatinine concentrations directly and chronically (e.g., age, race/-ethnicity, BMI), then mPAEs concentration was divided by creatinine ratio, which was calculated via dividing observed creatinine concentration by fitted creatinine values in the previous model (O'Brien et al., 2016).

3. Results and discussion

3.1. Characteristics of the subjects and occurrence of mPAEs in urine

The demographic and socioeconomic characteristics of the study population are shown in Table 1. The average age of women representing URSA cases was 29 years, which was significantly lower than the average age of the control group (30 years). Most women did not have educational qualification higher than a Bachelor's degree (87.1%), but the control group had a generally higher educational level than the women who had experienced URSA ($p < 0.01$). Moreover, women in the control group had significantly higher income levels than women who had experienced URSA ($p < 0.01$). There were no significant between-

Table 1
Characteristics of women representing URSA cases and the control group.

Demographic characteristics	URSA case (n = 594)	Control (n = 569)	*P*-value [a]
	n (%) or mean (SD)	n (%) or mean (SD)	
Age	29 ± 3.4	30 ± 3.7	<0.01
Body mass index (BMI, kg/m^2)	21.6 ± 2.9	21.3 ± 2.9	0.08
Age at menarche (years)	14 ± 1.3	14 ± 1.3	0.25
Educational level			<0.01
Less than Bachelor's degree	297 (50.4)	149 (26.4)	
Bachelor's degree	246 (41.8)	312 (55.3)	
Above Bachelor's degree	46 (7.8)	103 (18.3)	
Income (10^3 Chinese Yuan, CNY)			<0.01
<15	325 (54.7)	116 (20.4)	
15–30	114 (19.2)	291 (51.2)	
>30	40 (6.7)	105 (18.4)	
Missing	115 (19.4)	57 (10.0)	
Active smoking (Yes)	3 (0.5)	12 (2.1)	<0.01
Alcohol consumption (Yes)	86 (14.8)	206 (36.4)	<0.01

Note: SD, standard deviation. Missing data, age (n = 16), age at menarche (n = 69), BMI (n = 8), educational level (n = 10), income (n = 173), alcohol consumption (n = 14). [a] Wilcoxon test, Chi-squared or Fisher's exact test were used to calculate the statistical significance of between-group differences.

group differences in the BMI or age at menarche. The women representing URSA cases were less likely to smoke and consumer alcohol than women in the control group.

The detection frequencies and urinary concentrations of mPAEs are shown in Table 2. The target mPAEs were detected from all of the subjects, which clearly demonstrates widespread exposure to PAEs among women. The median Σ_9mPAEs (sum of nine mPAE compounds) for all subjects was 184 ng/mL, with miBP and mBP showing the highest concentrations (44.9 and 39.9 ng/mL, respectively). The median concentrations of DEHP metabolites (mEHP, mEHHP, and mEOHP), mBP, and miBP were higher than what has been reported for women from Korea, Thailand, Germany, UK, Denmark, Canada, and the United States, with up to 10-fold differences noted in the maximum concentrations (Table S2) (CDC, 2019; Exley et al., 2015; Frederiksen et al., 2013; Kasper-Sonnenberg et al., 2012; Lee et al., 2020; Mok et al., 2021). On the other hand, the mEP and mBzP concentrations presented in this study were lower than what has been reported for women from European and American countries (Arbuckle et al., 2014; CDC, 2019; Frederiksen et al., 2013; Kasper-Sonnenberg et al., 2012). The differences may be caused by different lifestyles, the contents of PAEs in daily consumer products, and discrepancies in how these chemicals are in regulated in various countries (Colacino et al., 2010; Guo et al., 2011a; Shin et al., 2020).

3.2. Composition profiles and potential sources

The composition profiles of urinary mPAEs are shown in Fig. S2. The predominant compound was miBP, which accounted for 35.7% of mPAEs, followed by mBP, which accounted for 31.8% of mPAEs. The metabolites of DEHP, i.e., mEHP, mEHHP, and mEOHP, accounted for 22.7% of the total compounds, which indicates that the parent compounds DBP, DiBP, and DEHP are the three main PAEs that Chinese women are exposed to. Similar results have been found in other studies from China (Chen et al., 2019; Yu et al., 2021).

To understand the possible sources of both the parent PAEs and mPAEs, Spearman correlation coefficients were calculated for the six main mPAEs detected from urine samples. The mPAEs were moderately correlated, demonstrating correlation coefficients of 0.13–0.70 (Table S3). The significant correlations observed for mEHP, mEHHP, and mEHOP were expected, as these compounds represent the primary metabolite and two oxidative metabolites of DEHP. Moreover, significant correlations were found between mEOHP and both mBP and miBP, and between mBP and miBP ($p < 0.01$), indicating similar sources for DEHP, DBP, and DiBP.

To further understand the possible sources of the detected metabolites, a principal component analysis (PCA) was conducted. The results, which are shown in Fig. S3, demonstrate that two factors (PC 1 and PC 2) explain 61.6% of the total variance. PC1 included mEHP, mEHHP, and mEOHP, the metabolites of DEHP, which are mainly used in residential building materials, polyvinyl chloride products, and food packaging as a plasticizer. As reported in the literature, diet is usually the main source for human exposure to DEHP, as DEHP may migrate from packaging materials to food (Dong et al., 2017; Guo et al., 2014; He et al., 2019). PC2 included mBP, miBP, and mEP, with the parent compounds (DEP, DBP, and DiBP) mainly occurring in personal care products, cosmetics, paints, and adhesives (Abdel daiem et al., 2012; Guo et al., 2014; Guo and Kannan, 2013). Berman et al. (2009) reported that urinary concentrations of mEP increase among women who have used personal care products in the past 48 h, and that the concentrations are associated with the number of personal care products used (Berman et al., 2009). Therefore, PC1 and PC2 could be interpreted as the dietary and dermal intake of PAEs. It should be noted that a previous study found the air levels of DEP and DBP to be correlated with urinary mEP and mBP levels (Adibi et al., 2003); hence, PC2 may also partly reflect PAE exposure via inhalation.

3.3. Factors influencing PAE exposure

Understanding which factors influence PAE exposure is important for predicting human exposure to these chemicals. Therefore, the present study analyzed the relationships between demographic characteristics (age, income, educational level, BMI, alcohol consumption, etc.) and the urinary concentrations of mPAEs (Fig. 1A and Table S4) among all subjects (case and control groups). Among these factors, age, income, and alcohol consumption were significantly correlated with the urinary concentrations of mPAEs.

Women under the age of 30 years showed significantly higher median Σ_9mPAEs values than women over the age of 30 years (204 vs. 169 ng/mL, respectively). Further analysis found that this difference was mainly explained by discrepancies in mEP and mBP levels between the age groups (Table S4). As discussed above, these two chemicals fall under PC2, which indicates dermal intake following the use of cosmetics and personal care products by younger women. A previous study reported that a woman's age is negatively associated with the consumption of personal care products (such as perfume and nail polish) (Mok et al., 2021).

A subject's income was also significantly associated with PAE exposure ($p < 0.01$). For example, women with an income of less than 15,000 Chinese Yuan (CNY) showed the highest Σ_9mPAEs values (218 ng/mL), while women with an income of more than 30,000 CNY showed the lowest Σ_9mPAEs values (161 ng/mL). Similar relationships were also reported in some (Arbuckle et al., 2014; Wenzel et al., 2018), but not all (Wang et al., 2014; Yao et al., 2020), previous studies. As Table S4 shows, the differences between income groups could be explained by

Table 2
Concentrations of urinary mPAEs (ng/mL) in the URSA (n = 594) and control (n = 569) groups.

mPAE	LOD	Total		URSA		Control		*p*-value [a]
		DF (%)	median (P25, P75)	DF (%)	median (P25, P75)	DF (%)	median (P25, P75)	
mEHP	0.008	76.2	6.69 (0.30, 16.6)	83.3	8.42 (1.89, 18.4)	68.7	4.24 (0.01, 14.7)	<0.01
mEHHP	0.150	97.9	14.7 (7.36, 22.0)	97.8	18.6 (14.2, 25.7)	98.1	8.45 (4.27, 15.7)	<0.01
mEOHP	0.023	98.6	7.09 (3.50, 12.8)	98.8	7.21 (3.34, 14.0)	98.4	7.07 (3.66, 11.7)	0.44
mEP	0.246	94.2	11.9 (4.97, 29.9)	96.8	12.3 (5.67, 31.3)	91.4	11.2 (4.03, 29.1)	0.02
mBP	0.016	88.0	39.9 (12.8, 100)	87.4	50.2 (13.9, 115)	88.6	33.4 (12.2, 83.9)	<0.01
miBP	0.051	96.0	44.9 (20.2, 90.5)	94.6	44.2 (19.8, 81.3)	97.4	46.4 (21.0, 99.3)	0.06
mOP	0.004	54.5	0.43 (<LOD, 1.66)	60.9	1.12 (<LOD, 2.58)	47.8	<LOD (<LOD, 0.98)	<0.01
mMP	4.280	32.8	<LOD (<LOD, 16.3)	22.7	<LOD (<LOD, <LOD)	43.4	<LOD (<LOD, 20.0)	<0.01
mBzP	0.102	32.9	<LOD (<LOD, 3.08)	27.1	<LOD (<LOD, 2.22)	39.0	<LOD (<LOD, 5.49)	0.09
∑mPAEs			184 (97.6, 315)		203 (110, 328)		161 (82.7, 294)	<0.01

Note: LOD, limit of detection; DF: detection frequency; P25–P75, 25th–75th percentiles; mEHP, mono(2-ethylhexyl) phthalate; mEHHP, mono(2-ethyl-5-hydroxyhexyl) phthalate; mEOHP, mono(2-ethyl-5-oxohexyl) phthalate; mEP, mono-ethyl phthalate; mBP, mono-n-butyl phthalate; miBP, mono-isobutyl phthalate; mOP, mono-n-octyl phthalate; mBzP, monobenzyl phthalate; mMP, monomethyl phthalate; ∑mPAEs, summation of the urinary concentrations of all nine PAE metabolites. [a] Wilcoxon test was used to calculate the statistical significance of between-group differences.

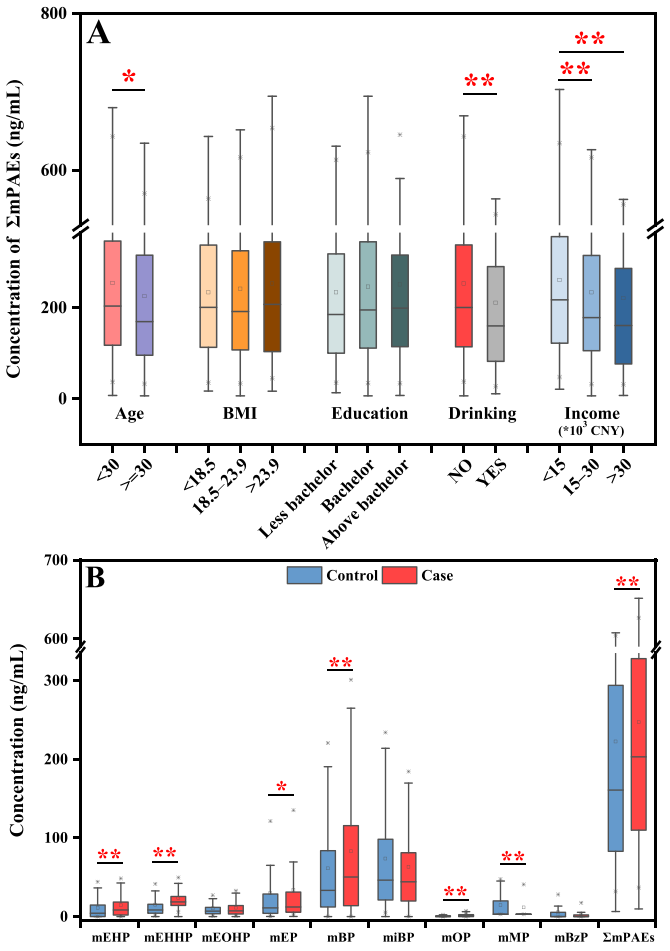

Fig. 1. Urinary concentrations of mPAEs in different groups of subjects (categorized based on age, BMI, education, drinking, and income) (A), and across the URSA and control groups (cases and controls) (B). $^{**}p < 0.01$ level (2-tailed); $^{*}p < 0.05$ level (2-tailed).

discrepancies in mEHHP, mEOHP, mBP, and mEP levels; these compounds belong to both PC1 and PC2. This leads us to postulate that women from the high-income group might use cosmetics and personal care products that contain less PAEs, while women from the low-income group might consume more foods contaminated by PAEs during processing and packaging (Cao et al., 2020; He et al., 2019; Kobrosly et al., 2012).

In contrast to what has been reported in previous studies, we found that the concentrations of mPAEs in women who drank alcohol were significantly lower than those who did not drink (Table S4). Earlier research has found alcohol consumption to be positively correlated with the urinary levels of low-molecular-weight PAE metabolites (Dong et al., 2017; Tranfo et al., 2013). Moreover, a survey of the PAEs in Chinese spirits showed high frequencies and concentrations of both DBP and DEHP (Wang et al., 2015c). To understand the possible explanation for our finding, we examined the differences in drinking habits between different income and age groups. Women aged <30 years were less likely to consume alcohol than those aged >30 years (22.4% vs. 29.8%), and women with an income of less than 15,000 CNY per month were less likely to consume alcohol in comparison to those with monthly salaries over 15,000 CNY (17.6% vs. 30.9%). As mentioned above, age and income were influencing factors for PAE exposure. Based on the multivariable regression models, income, age, and consumption of alcohol were all significantly negatively correlated with urinary mPAEs levels. As such, younger women with low incomes and who did not consume alcohol had a higher risk of PAE exposure than other women (Table S5). Therefore, non-drinking women might have other potential exposure sources of PAEs.

Regarding the other two factors (educational level and BMI), no significant differences between urinary mPAE levels and educational level or BMI were observed, although there were obvious differences in two mPAE compounds. Furthermore, previous literature has provided inconsistent results. For example, Zhu et al. (2016) found that educational level was positively correlated with mEP and mEOHP concentrations. However, other studies have found inverse correlations between PAE exposure and educational level among women (He et al., 2019; Ye et al., 2008). In the present case, women with lower educational levels showed significantly higher median mEOHP concentrations, yet significantly lower miBP levels, than women with higher educational levels. This might be a result of educational level influencing a women's lifestyle, which is linked with PAE exposure. BMI was not found to significantly affect the Σ_9mPAEs value. However, women with a BMI higher than 23.9 showed the highest median mEHHP concentrations, while mEHHP concentrations decreased at lower BMI values ($p < 0.05$). Dong et al. (2017) showed that dietary intake accounted for 50% of DEHP (parent of mEHHP) exposure. Hence, overeating could partly explain the higher mEHHP levels observed in higher-BMI subjects.

3.4. Association between PAE exposure and URSA

The concentrations of target mPAEs in the URSA case and control groups are shown in Table 2 and Fig. 1B. The median Σ_9mPAEs value for the URSA cases was 203 ng/mL, which was significantly higher than what was observed for the control group (161 ng/mL). Similar results were observed for mEHP, mEHHP, mEP, and mBP. After adjusting for covariates, the risk of URSA increased as the urinary concentrations of mEP (OR = 1.10, 95%CI: 1.01, 1.20), mEHHP (OR = 2.08, 95%CI: 1.78, 2.43), and mEHP (OR = 1.11, 95%CI: 1.07, 1.15) increased. However, according to logistic regression analysis, the risk of URSA decreased as the urinary concentration of miBP increased (OR = 0.84, 95%CI: 0.77, 0.91) (Table 3). When mPAEs were grouped by quartiles, a monotonic increase in OR was observed for mEHP and mEHHP (*P*-trend < 0.01), while an opposite result was observed for miBP (*P*-trend < 0.01). Furthermore, certain quartiles of mEOHP (ORs for 3rd and 2nd vs 1st quartile: OR = 0.49, 95%CI: 0.33, 0.73; and OR = 0.62, 95%CI: 0.43, 0.90, respectively) and mBP (OR for 2nd vs 1st quartile: OR = 0.59, 95% CI: 0.40, 0.87) were negatively associated with URSA. These trends were also confirmed by the restricted cubic splines model (Fig. S4). The joint effect of increasing all six metabolites by one quartile on risk of URSA was 1.43 (95%CI: 1.17, 1.75). In the QGC model, four out of six chemicals, namely mEHHP, mEHP, mBP, and mEP, showed positive weights, with mEHHP having the greatest proportional positive contribution (Fig. S5). In contrast, mEOHP and miBP showed negative weights in the model, with mEOHP assigned the largest negative weight.

These results suggest that exposure to mPAEs, especially mEHHP, mEHP, mBP and mEP, may be an important risk factor for URSA. These results were partly supported by a systematic review and meta-analysis of four case-control studies and four longitudinal cohorts with a total of 651 cases and 4062 controls, which reported that certain mPAEs, such as mBP, mEHP, and mEHHP, were associated with increased risks of spontaneous pregnancy loss (Zhang et al., 2020). In addition, these findings also agreed with prior researches by Peng et al. (2016), Liao et al. (2018), and Chang et al. (2021), where higher risks of URSA were observed for increased mEHP, mEP, and ΣDEHP.

In contrast, negative weights in the QGC model for mEOHP and miBP were observed, which indicated negative associations with URSA. Similarly, Liao et al. (2018) reported a negative, yet non-significant, association between mEOHP levels and the risk of URSA. Furthermore, Jukic et al. (2016) revealed a negative association between mEOHP levels and early pregnancy loss (<6 gestational weeks, n = 33), whereas a positive correlation between mEOHP levels and pregnancy

Table 3
Associations between mPAE levels and URSA.

mPAE	URSA case/control	Crude OR (95%CI)	Adjusted OR (95% CI)[a]
mPAEs mixture[a]	594/569	1.98 (1.60, 2.40)	1.43 (1.17, 1.75)
mEHP[b]			
Continuous		1.12 (1.08, 1.15)	1.11 (1.07, 1.15)
Q1	99/178	Ref	Ref
Q2	102/107	1.71 (1.35, 2.08)	1.63 (1.09, 2.44)
Q3	210/141	2.68 (2.35, 3.00)	2.35 (1.64, 3.37)
Q4	183/143	2.30 (1.97, 2.63)	2.17 (1.51, 3.12)
p-trend		<0.01	<0.01
mEHHP[b]			
Continuous		2.32 (2.17, 2.47)	2.08 (1.78, 2.43)
Q1	25/143	Ref	Ref
Q2	17/141	0.69 (0.03, 1.35)	0.69 (0.35, 1.37)
Q3	167/143	6.68 (6.20, 7.16)	6.37 (3.82, 10.62)
Q4	385/142	15.51 (15.04, 15.97)	15.42 (9.25, 25.73)
p-trend		<0.01	<0.01
mEOHP[b]			
Continuous		1.04 (0.95, 1.14)	0.97 (0.86, 1.08)
Q1	164/143	Ref	Ref
Q2	130/142	0.80 (0.47, 1.13)	0.62 (0.43, 0.90)
Q3	106/141	0.66 (0.32, 0.99)	0.49 (0.33, 0.73)
Q4	194/143	1.18 (0.87, 1.49)	0.85 (0.58, 1.24)
p-trend		0.41	0.46
mEP[b]			
Continuous		1.14 (1.07, 1.21)	1.10 (1.01, 1.20)
Q1	89/143	Ref	Ref
Q2	192/141	2.19 (1.85, 2.53)	1.83 (1.26, 2.68)
Q3	153/143	1.72 (1.37, 2.07)	1.45 (0.97, 2.17)
Q4	160/142	1.81 (1.46, 2.16)	1.48 (0.99, 2.19)
p-trend		0.02	0.26
mBP[b]			
Continuous		1.02 (0.98, 1.06)	0.99 (0.95, 1.04)
Q1	142/142	Ref	Ref
Q2	94/142	0.66 (0.31, 1.01)	0.59 (0.4, 0.87)
Q3	149/142	1.05 (0.72, 1.38)	0.94 (0.64, 1.38)
Q4	209/143	1.46 (1.15, 1.78)	1.14 (0.77, 1.68)
p-trend		<0.01	0.13
miBP[b]			
Continuous		0.91 (0.85, 0.98)	0.84 (0.77, 0.91)
Q1	162/143	Ref	Ref
Q2	142/142	0.88 (0.56, 1.21)	0.62 (0.43, 0.9)
Q3	181/141	1.13 (0.82, 1.45)	0.82 (0.57, 1.19)
Q4	109/143	0.67 (0.34, 1.01)	0.46 (0.3, 0.69)
p-trend		0.12	<0.01

Note: Ref, Reference; mPAEs, phthalate metabolites. The effect estimate was expressed as an odds risk (OR) and the corresponding 95% confidence interval (95% CI). [a] QGC model was used to estimate the association between mPAEs mixture and URSA. [b] Logistic regression was used to estimate the associations between individual mPAEs and URSA; mPAEs were divided into quartiles according to the mPAE distributions in the control group. Both models were adjusted for maternal BMI (continuous), maternal age (continuous), age at menarche (continuous), educational level (categorical), study site (categorical), alcohol consumption (categorical) and urinary creatinine (continuous, ln-transformed).

loss has been reported in other studies (Gao et al., 2017; Messerlian et al., 2016). In addition, our finding for miBP is inconsistent with most of the previous studies, where negative or null associations of miBP with pregnancy loss or URSA were reported (Liao et al., 2018; Mu et al., 2015; Peng et al., 2016).

These inconsistencies in the results may be attributable to sample size, study design, composition profiles of PAEs, and type of pregnancy loss. The exposure profiles of PAEs varied across studies (Table S6). In the present study, the associations of mEOHP and miBP with URSA were different between two study sites (Table S9). The concentrations of mEOHP in Shanghai (case/control, 8.30/7.97 ng/mL) were by and large similar to those in the study by Liao et al. (2018) (case/control, 6.88/5.85 ng/mL), but higher than those in Zhejiang (case/control, 5.09/3.21 ng/mL) and in a study by Gao et al. (2017) (2.80 ng/mL). The concentration of miBP in Shanghai (case/control, 53.83/60.70 ng/mL) was several times higher than those in Zhejiang (case/control, 30.03/21.64 ng/mL) and the studies by Liao et al. (2018) (case/control, 9.81/5.29 ng/mL) and Peng et al. (2016) (case/control, 3.82/2.10 ng/mL), which might have contributed to the differences in the directionality of the associations. In addition, most of the previous studies focused on sporadic miscarriage rather than URSA, with these subtypes of miscarriage showing distinct pathophysiologies (Du et al., 2018).

In addition, the results of the BMI-stratified analyses suggested that positive effect estimates between continuous mPAEs (including mEHHP, mEOHP, and mEP) and URSA were more apparent among overweight and obese women (Table S7). A previous meta-analysis reported that women who had experienced RSA showed significantly higher BMI values than women in the control group (Cavalcante et al., 2019). However, the stratified analyses were imprecise for a small sample size of overweight and obese women, and the association need to be confirmed using a larger sample. Additionally, substantial differences in URSA incidence were not found in the age-stratified analyses or when the controls with a history of miscarriage were excluded (Table S7 and S8). Furthermore, the associations of mPAEs with URSA in different study sites did not change appreciably (Table S9). Three different approaches for creatinine adjustment did not materially change the present results (Table S10). This indicates that maternal age, previous history of miscarriage, creatinine-adjustment method and study site did not affect the present results.

URSA is a multifactorial disease and the exact causes are not well elucidated. Epidemiologic studies revealed that 50%–60% of URSA were due to embryonic chromosomal abnormalities (Du et al., 2018; Sugiura-Ogasawara et al., 2012), particularly numerical alterations, which mostly occur *de novo* (Carp et al., 2006). In vitro studies, they showed a dose-dependent relationship between mEHP and inhibited meiotic maturation in bovine (Anas et al., 2003) and mouse (Absalan et al., 2017). Kalo et al. (2019) further demonstrated that exposing bovine oocytes to mEHP was associated with decreased proportion of embryos that progressed up to the blastocyst stage and impaired transcriptomic expression in the embryo. The underlying mechanisms warrant further investigation.

Sugiura-Ogasawara et al. (2012) found that in RSA women with normal embryonic karyotype, 24.5% of cases were truly unexplained. Experiment study showed that exposing rats to DEHP, the parent compound of mEHP, mEHHP, and mEOHP, decreased levels of estradiol (E_2) and progesterone (Hirosawa et al., 2006). Rodent models also demonstrated that DEHP disrupted the growth and development of the placenta via inhibiting proliferation and inducing the apoptosis in placental cells (Zong et al., 2015). Additionally, studies reported positive associations of DEHP metabolite exposure with oxidative stress (i.e., 8-hydroxydeoxyguanosine and 8-isoprostane) and inflammation (i.e., IL-1β, IL-6, and IL-10), which might lead to pregnancy loss (Ferguson et al., 2014; van 't Erve et al., 2019). However, these mechanisms cannot explain the negative association of mEOHP with pregnancy loss. More research specifically on mEOHP is needed.

As for the negative association between miBP and URSA, *in vitro* studies showed that exposure to DiBP, the parent compound of miBP, increased estrogenic activity in ovarian granulosa cells, while DEHP has been related to reduce estrogenic activity in breast cancer cell lines (Gupta et al., 2010; Hannon et al., 2015; Harris et al., 1997). Further, Sathyanarayana et al. (2017) reported a positive association of miBP with estrone (E_1) and E_2 in 591 pregnant women with early pregnancy. DEHP and DiBP may have different toxicity on female reproduction. However, the present study cannot prove the causality. The results should be considered as preliminary.

3.5. Health risk assessment of PAE exposure

To understand the health risks of PAE exposure and screen which PAE was associated with the highest risk, the HI and HQ values were

calculated based on the total estimated daily intake of the chemicals and RfDs considering the non-carcinogenic endpoint (Table S11 and S12, and Fig. 2). The median HI value for all subjects was 0.36, which did not exceed the limit for health risk, i.e., one unit. However, 18.7% of the women exposed to PAEs had HI values higher than one unit. Regarding the URSA and control groups, the HI values ranged from 0 to 7.50 and 0 to 15.0, with median values of 0.43 and 0.29, respectively; this indicates that women in the URSA group had a higher health risk of PAE exposure than women in the control group (Fig. 2A). Although the median HI value for the URSA group was less than one unit, 21.8% of women in the case group had HI values exceeding one unit (Fig. S6); this suggests that exposure to PAEs could have potential health risks.

To further reveal which one of the mPAEs was associated with the highest health risk, the contributions of individual mPAEs to overall risk were analyzed (Fig. 2B). Among the target mPAEs, mEHP, the metabolites of DEHP, accounted for 82.6% of the total risk (Fig. 2A). This is interesting, as the parent compounds of these metabolites – DEHP – has been linked to URSA in previous studies. The results indicate that policymakers should pay attention to the potential health risks of DEHP exposure, i.e., plausible non-carcinogenic risk and the association between PAE exposure and URSA incidence.

3.6. Strengths and limitations

The present study had several strengths. First, the research included a relatively large number of URSA cases. Second, all of the participants had undergone a complete check-up and laboratory tests before the diagnosis. Third, the QGC method was employed to investigate the overall associations between mPAEs mixture and URSA. When compared to weighted quantile sum regression, QGC has several advantages, i.e., it allows for directional heterogeneity, nonlinear or non-additive effects of various mPAEs, as well as the nonlinear effect of co-exposure to mPAEs, which improves computation efficiency through faster convergence.

This study also had certain limitations. First, mPAEs were quantified from single-spot urine samples, measurements in urine could vary greatly over time due to variable exposure, potentially leading to exposure misclassification. Second, it is difficult to draw a conclusion of a causal relationship between mPAE levels and URSA incidence given the case-control design. Therefore, our findings are purely suggestive, and the causality needs to be confirmed through prospective epidemiological studies and animal experiments. Third, information on embryonic karyotype in the URSA cases was not available. The inclusion of cases with abnormal embryonic karyotype may have resulted in biased estimates because embryonic chromosomal abnormalities are the most common cause for URSA (Du et al., 2018). Hence, the association between mPAEs and URSA could be underestimated if mPAE exposure does not cause chromosomal abnormalities. Fourth, the potential health risks can vary when different reference limit values (i.e., tolerable daily intake (TDI), RfD, and RfD-AA) are used (Huang et al., 2021; Kortenkamp and Faust, 2010; Lu et al., 2020); this could skew the results for DEHP, DBP, and DiBP, which were the predominant PAEs in the present case. Finally, the present results might be partly confounded by other unmeasured or unknown confounders, such as toxins, the frequency of using plastic products and personal care products.

4. Conclusions

This large case-control study investigated PAE exposure in reproductive age women in China, as well as how this exposure influenced the incidence of URSA. mPAEs in the urine samples indicated that humans are constantly exposed to various PAEs. The major mPAEs were miBP, mBP, mEP, mEHP, mEHHP, and mEOHP, with miBP present at the highest levels. Factors such as age, income, and alcohol consumption were found to influence the urinary concentrations of mPAEs. Certain mPAEs, especially the various metabolites of DEHP, were positively associated with an increased risk of URSA; this suggests that PAE exposure may increase the risk of URSA, though there were some inconsistencies in the associations between certain metabolites and URSA incidence. The health risk assessment demonstrated that DEHP posed a potential non-carcinogenic risk to approximately 20% of women in the case group, and contributed to over 80% of the total risk of the studied mPAEs. Nevertheless, further research, ideally including multiple, prospectively collected urine samples and information on embryonic karyotype, is needed to confirm the presented findings. Moreover, mechanistic studies are warranted to elucidate the possible biological mechanisms through which PAE exposure increases the risk of USRA.

5. Declaration of competing financial interests

There were no potential competing financial interests.

Ethics approval

This study was approved by the Ethics Committees of all involved research institutions and hospitals (the Xinhua Hospital Ethics Committee Affiliated to Shanghai Jiao Tong University School of Medicine (Approval Number: XHEC-C-2015-046). Written informed consent was obtained from each subject.

Consent to participate

All donors involved in this study signed an informed consent form before sample collection.

CRediT authorship contribution statement

Ruxianguli Aimuzi: Methodology, Formal analysis, Writing –

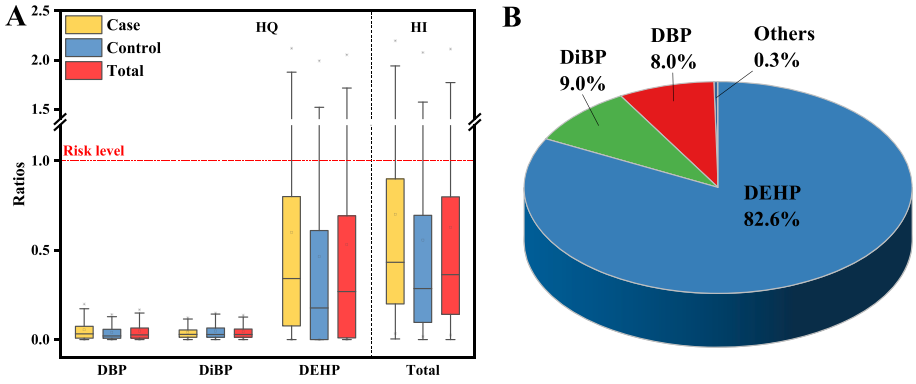

Fig. 2. The relative contributions of individual mPAEs to the HI value (A) and the non-carcinogenic risk quotients of PAE exposure (B).

original draft. **Senyuan Huang:** Methodology, Formal analysis, Writing – original draft. **Kai Luo:** Data curation, Formal analysis. **Shengtao Ma:** Methodology. **Xiaona Huo:** Resources, Investigation. **Guiying Li:** Methodology. **Ying Tian:** Resources, Investigation. **Jun Zhang:** Methodology, Supervision, Writing – review & editing. **Yingxin Yu:** Methodology, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envres.2022.113393.

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