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*Published in:*  
Environment International

*DOI:*  
[10.1016/j.envint.2018.09.037](https://doi.org/10.1016/j.envint.2018.09.037)

Published: 01/12/2018

*Document Version:*  
Publisher's PDF, also known as Version of record

[Link to publication](#)

*Citation for published version (APA):*  
Sun, X., Li, J., Jin, S., Li, Y., Liu, W., Zhao, H., Zhou, Y., Jiang, Y., Liu, H., Xia, W., Cai, Z., Xu, S., & Shen, X. (2018). Associations between repeated measures of maternal urinary phthalate metabolites during pregnancy and cord blood glucocorticoids. *Environment International*, 121, 471-479.  
<https://doi.org/10.1016/j.envint.2018.09.037>

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## Associations between repeated measures of maternal urinary phthalate metabolites during pregnancy and cord blood glucocorticoids

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### ARTICLE INFO

Handling Editor: Yong Guan Zhu

#### Keywords:

Phthalate  
Glucocorticoid  
Prenatal exposure  
Trimester-specific

### ABSTRACT

**Background:** Previous studies have suggested that phthalates might disrupt fetal steroidogenesis. However, the evidence of the effects of prenatal phthalate exposure across pregnancy on fetal glucocorticoids was insufficient. **Objective:** We investigated the associations between urinary phthalate metabolites across pregnancy and cord blood glucocorticoids in a prospective birth cohort.

**Methods:** Our study included 553 mother-infant pairs from a prospective birth cohort conducted in Wuhan, China. Maternal urine samples were collected at 14, 24 and 36 weeks of gestation (mean). Urinary phthalate metabolites and cord blood glucocorticoids (cortisol and cortisone) were measured. Generalized estimating equation models were conducted to explore the relationships of phthalate metabolite concentrations at each trimester and glucocorticoid levels.

**Results:** Among the participants, mono-benzyl phthalate (MBzP) in the first trimester was associated with higher cortisol/cortisone ratio concentrations, and mono-(2-ethyl-5-carboxypentyl) phthalate (MECPP) and mono-(2-ethyl-5-oxohexyl) phthalate (MEOHP) measured in the third trimester were associated with decreased cortisone. Moreover, the associations between phthalates and glucocorticoids varied by sex. Among the female infants, each 10-fold increase in several maternal urinary phthalate metabolite concentrations in 1st and 3rd trimester was associated with the increased glucocorticoid levels with percent changes ranged from 16.2%–55.9%. However, among male infants, each 10-fold increase in maternal urinary MECPP, mono-(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP) and MEOHP in 3rd trimester was associated with 20.8%–36.3% decreased cortisol and cortisone levels, respectively.

**Conclusion:** We have shown that prenatal phthalate exposure during early and late trimester disrupted the infant steroidogenesis and these associations might be modified by infant sex. To the best of our knowledge, this is the first study to evaluate phthalate exposure at three trimesters during pregnancy in relation to infant glucocorticoids.

### 1. Introduction

Glucocorticoids, including cortisol and its metabolite cortisone, play an integral role in regulating the homeostasis in metabolism and growth, especially for the maturation of the fetal brain and lungs (Moisiadis and Matthews, 2014). Cortisol in fetal circulation may be originated from the maternal adrenal gland during whole pregnancy, as well as from the fetal adrenal gland as early as week 8 of gestation

(Murphy et al., 2006; Goto et al., 2006). The respective contribution of the mother or the fetus to the production of glucocorticoid hormones remains unclear. However, it is well known that cortisol in cord blood is much lower than that in maternal blood, owing to the barrier enzyme 11 $\beta$ -hydroxysteroid dehydrogenase 2 (11 $\beta$ -HSD2) in the placenta which can metabolize cortisol to its inactive metabolite cortisone (Fowden et al., 2016). Glucocorticoids levels in umbilical cord blood, an indicator of the fetal stress response (Gitau et al., 2001), may be affected

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by maternal or fetal characteristics including gestational age and infant sex (Giesbrecht et al., 2016; Rog-Zielinska et al., 2014). Human health data have shown that insufficient or excess glucocorticoid in fetal development may have an impact on gene methylation and histone modification, which can exert long-lasting adverse effects on the cardiovascular system in later life (Wood, 2013). Previous studies have also reported that an elevated ratio of cortisol to cortisone, reflected the decreased activity of 11 $\beta$ -HSD2, might allow an increased access of maternal glucocorticoids to the fetus, further retard growth and lead to higher offspring systolic blood pressure (Huh et al., 2008). Therefore, maintaining fetal intra-uterine glucocorticoid homeostasis is critical for fetal development in later life.

Fetal hormone system homeostasis might be susceptible to early life endocrine disrupting chemicals exposure, such as phthalates (Scott et al., 2009). Phthalates, a kind of synthesized plasticizer, have been widely used in industry and consumer products, such as plasticizer, adhesives, food packaging plastics and personal care products (ATSDR, 2002). Because of the weak affinity towards other mixed substances, phthalates can easily leach from products into the environmental media. Phthalates have been widely measured in pregnant women all over the world (Cantonwine et al., 2014; Myridakis et al., 2015; Watkins et al., 2017a, 2017b; Zhu et al., 2016). Recently, prenatal phthalate exposure has been associated with some adverse effects, such as decreased (Zhang et al., 2018) birth weight, altered anogenital distance (Wenzel et al., 2018) and impaired children neurodevelopment (Kim et al., 2011; Whyatt et al., 2012) in the previous studies. The potential mechanisms of these toxicological effects on fetal growth might be due to the fact that phthalates might alter hormone system homeostasis through binding with some receptors which were related to fetal development, such as glucocorticoid and peroxisome proliferator-activated receptors (Sathyanarayana et al., 2017; Adibi et al., 2017; Hong et al., 2009).

Experiments *in vitro* have shown that phthalate inhibited rat and mouse 11 $\beta$ -HSD2 activities, indicating the possible disruption effects of phthalate exposure on glucocorticoids (Zhao et al., 2010; Hong et al., 2009). To date, only two human studies reported the association between prenatal phthalate exposure and glucocorticoid levels in the literature. Recently, Araki et al. reported an inverse association between maternal blood mono-2-ethylhexyl phthalate (MEHP) levels at 23–35 weeks of gestation, with cord blood cortisol and cortisone in a prospective birth cohort (Araki et al., 2017). However, Jensen et al. reported a positive association between amniotic fluid mono-(2-ethyl-5-carboxypentyl) phthalate (MECPP) and cortisol levels at the 2nd trimester in a population including cryptorchidism and hypospadias cases and controls (Jensen et al., 2015). Both of these findings have suggested that prenatal phthalate exposure might disrupt the regulation glucocorticoid metabolism in the fetus. However, these studies have focused on only a specific group of phthalate metabolites and used one-spot samples during pregnancy. The variations of phthalate during pregnancy raised a concern that a single spot sample might not be adequate to assess prenatal phthalate exposure (Braun et al., 2012).

Thus, in the present work, we analyzed maternal urinary nine phthalate metabolites during 1st, 2nd and 3rd trimesters and cord blood glucocorticoids, and applied generalized estimating equation model to explore the relationships between prenatal phthalate exposure and infant glucocorticoids in a prospective birth cohort. To the best of our knowledge, this is the first study to investigate phthalate exposure at three trimester during pregnancy in relation to infant glucocorticoids.

## 2. Methods

### 2.1. Study population and data collection

The participants in this study were selected between 2014 and 2015 at Wuhan Women and Children Medical Care Center, a major maternity hospital in Wuhan, China. Study inclusion criteria were shown as

**Table 1**  
Characteristics of the included (n = 553) and total (n = 856) mother-infant pairs [n (%)].

Characteristics	Included	Total
Age (years)		
18–24	97 (17.5)	124 (14.5)
25–29	290 (52.5)	451 (52.7)
30–34	140 (25.3)	227 (26.5)
$\geq 35$	26 (4.7)	54 (6.3)
Mean ( $\pm$ SD)	28.3 $\pm$ 3.2	28.6 $\pm$ 3.3
Pre-pregnancy BMI (kg/m <sup>2</sup> )		
Normal (18.5–23.9)	383 (69.3)	584 (68.2)
Underweight (< 18.5)	111 (20.1)	157 (18.3)
Overweight ( $\geq 24$ )	59 (10.6)	115 (13.4)
Mean ( $\pm$ SD)	20.7 $\pm$ 2.7	20.9 $\pm$ 2.8
Parity		
1	472 (85.4)	736 (86.0)
$\geq 2$	81 (14.6)	120 (14.0)
Educational level		
More than high school	456 (82.5)	671 (78.4)
High school and below	97 (17.5)	185 (21.6)
Alcohol consumption during pregnancy		
No	553 (100.0)	856 (100.0)
Yes	0 (0.0)	0 (0.0)
Smoking during pregnancy		
No	552 (99.8)	855 (99.9)
Yes	1 (0.2)	1 (0.1)
Passive smoking during pregnancy		
No	371 (67.1)	570 (66.6)
Yes	182 (32.9)	286 (33.4)
Mode of delivery		
Vaginal delivery	236 (42.7)	331 (38.7)
Cesarean delivery	317 (57.3)	525 (61.3)
Fetal sex		
Male	280 (50.7)	447 (52.2)
Female	272 (49.3)	409 (47.8)
Gestational age (week)		
Mean ( $\pm$ SD)	39.4 $\pm$ 1.1	39.4 $\pm$ 1.2
Birth weight (g)		
Mean ( $\pm$ SD)	3330.4 $\pm$ 399.3	3316.8 $\pm$ 409.2

Abbreviations: BMI, body mass index; SD, standard deviation.

follows: (1) residents of the Wuhan; (2) a singleton gestation with < 16 weeks pregnant at enrollment; (3) willing to have prenatal care and give birth in the study hospital. There were 856 participants who have donated urine samples at 1st, 2nd and 3rd trimester. A total of 610 cord blood samples were available for glucocorticoid measurements. Mothers (n = 57) who had pregnancy-induced hypertension or gestational diabetes mellitus were excluded from the analysis. Finally, 553 participants were included in the final analysis. All participants signed informed consents and registered in this cohort for enrollment. Ethical permission was taken from the ethics committees of the Women and Children Medical and Healthcare Center of Wuhan and Tongji Medical College, Huazhong University of Science and Technology.

We conducted face-to-face interviews with the participants to gather maternal demographic and socioeconomic characteristics (such as maternal age, ethnicity, and education) and life style in the pregnancy (alcohol consumption and smoking). The gestational age (calculated based on the last menstrual period) at birth, mode of delivery, as well as the infants' birth date, gender, and birth weight were obtained from medical records. The maternal pre-pregnancy body mass index (BMI) was computed by pre-pregnancy weight (extracted from the first prenatal visit records in the hospital) and height (measured with a stadiometer).

### 2.2. Phthalate metabolites measurement

Maternal urine samples were collected in polypropylene tubes at first trimester (13.1  $\pm$  1.2 weeks), second trimester (23.6  $\pm$  3.3 weeks) and third trimester (36.0  $\pm$  3.3 weeks) and were

**Table 2**  
SG-corrected urinary phthalate metabolite concentrations during each trimester.

Phthalate metabolites (ng/mL)	DR	GM	Percentiles				
			5th	25th	50th	75th	95th
<b>MEHP</b>							
1st trimester	91.9	2.2	< LOD	1.2	3.1	6.2	27.8
2nd trimester	89.2	1.4	< LOD	0.8	2.1	4.6	14.3
3rd trimester	85.4	1.2	< LOD	0.6	2.0	4.3	17.6
<b>MECPP</b>							
1st trimester	99.3	10	2.7	6.2	10.0	17.3	45.5
2nd trimester	99.6	7.9	2.3	4.6	7.7	12.5	40.2
3rd trimester	98.9	7.6	2.1	5.2	8.0	13.0	28.0
<b>MEHHP</b>							
1st trimester	99.5	6.8	1.5	3.9	6.7	12.0	37.8
2nd trimester	99.6	4.7	1.1	2.5	4.6	7.7	24.1
3rd trimester	99.1	4.8	1.3	2.9	5.1	8.4	17.2
<b>MEOHP</b>							
1st trimester	99.3	4.9	1.1	2.9	5.0	8.8	27.4
2nd trimester	99.5	3.7	0.8	2.0	3.9	6.2	17.7
3rd trimester	99.1	4.0	1.0	2.6	4.1	6.9	14.4
<b>ΣDEHP (nmol/L)</b>							
1st trimester	–	92.0	22.1	53.9	86.9	154.4	471.1
2nd trimester	–	66.2	18.1	36.4	63.6	104.3	341.4
3rd trimester	–	68.1	19.4	41.8	68.2	110.9	256.4
<b>MBzP</b>							
1st trimester	79.0	0.1	< LOD	0.0	0.1	0.2	0.9
2nd trimester	73.2	0.1	< LOD	< LOD	0.1	0.1	0.6
3rd trimester	75.4	0.1	< LOD	0.0	0.1	0.1	0.6
<b>MEP</b>							
1st trimester	98.0	10.4	2.3	5.5	10.5	21.3	93.5
2nd trimester	98.7	8.1	1.4	3.8	7.4	18.4	76.9
3rd trimester	98.0	8.0	1.2	4.0	7.9	18.8	81.0
<b>MiBP</b>							
1st trimester	93.1	11.0	< LOD	8.9	16.7	33.0	90.9
2nd trimester	90.2	6.7	< LOD	4.9	11.8	23.7	80.0
3rd trimester	90.2	8.1	< LOD	6.2	14.2	28.1	91.5
<b>MnBP</b>							
1st trimester	97.1	39.5	5.3	20.6	45.4	102.2	383.9
2nd trimester	96.4	27.9	1.2	13.3	35.2	83.7	371.4
3rd trimester	97.1	45.2	3.4	22.1	56.7	141.9	440.9
<b>MMP</b>							
1st trimester	36.0	0.3	< LOD	< LOD	< LOD	10.8	49.1
2nd trimester	30.0	0.2	< LOD	< LOD	< LOD	5.9	31.8
3rd trimester	35.3	0.2	< LOD	< LOD	< LOD	7.4	30.1
<b>ΣLMW (nmol/L)</b>							
1st trimester	–	446.5	76.9	238.8	435.6	892.5	2709.6
2nd trimester	–	332.7	55.8	179.5	341.5	681.7	2272.5
3rd trimester	–	445.5	75.6	249.5	484.5	962.6	2620.9
<b>ΣPAEs (nmol/L)</b>							
1st trimester	–	609.9	136.7	330.7	570.1	1101.6	3032.5
2nd trimester	–	455.3	93.2	251.1	447.5	809.6	2785.4
3rd trimester	–	567.4	111.6	322.9	585.7	1102.3	2824.7

Abbreviations: SG, specific gravity; DR, detection rate; GM, geometric mean; LOD: limit of detection.

**Table 3**  
Intraclass correlation coefficients (ICC) of SG-corrected phthalate metabolite concentrations (ng/mL) in different trimester.

Phthalate metabolites	ICC	95% CI
MEHP	0.17	0.12, 0.23
MECPP	0.33	0.28, 0.39
MEHHP	0.08	0.04, 0.15
MEOHP	0.49	0.44, 0.54
ΣDEHP (nmol/L)	0.31	0.25, 0.36
MBzP	0.10	0.06, 0.17
MEP	0.25	0.20, 0.31
MiBP	0.18	0.13, 0.24
MnBP	0.08	0.04, 0.15
MMP	0.06	0.02, 0.14
ΣLMW (nmol/L)	0.08	0.04, 0.15
ΣPAEs (nmol/L)	0.25	0.20, 0.31

frozen at  $-20\text{ }^{\circ}\text{C}$  before analysis. The sample preparation procedure was referred to our previous study (Zhao et al., 2017). The phthalates metabolites were detected by negative-ion electrospray ionization mass spectrometry and multiple reaction monitoring (MRM) mode using a Thermo Scientific™ TSQ Quantiva™ Triple Quadrupole mass spectrometer coupled to an Ultimate 3000 Ultra-high performance liquid chromatography system (Dionex, Sunnyvale, CA, USA) (Supplementary Tables S1 and S2). We measured nine phthalate metabolites in this study, including MEHP, MECPP, mono-(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP) and mono-(2-ethyl-5-oxohexyl) phthalate (MEOHP), mono-benzyl phthalate (MBzP), mono-ethyl phthalate (MEP), mono-methyl phthalate (MMP), mono-isobutyl phthalate (MiBP), and mono-*n*-butyl phthalate (MnBP), which accounted for the main phthalate exposure in Chinese population (Gao et al., 2016). MEHP, MECPP, MEHHP and MEOHP were primary metabolites of DEHP, thus the total concentration of DEHP (ΣDEHP) was obtained by summing the molar concentrations of the four metabolites. We also calculated low molecular weight phthalates (ΣLMW) by summing of the molar concentrations of the four metabolites (MEP, MnBP, MiBP and MMP) which relative molecular weight < 250. Sum of the molar concentrations of measured metabolites was computed as the total phthalate metabolite concentrations. When the phthalate metabolite concentrations were lower than the limits of detection (LOD), the phthalate metabolite concentrations were replaced by values of 1/2 LOD.

We detected the maternal urinary specific gravity (SG) using a handheld digital refractometer (Atago, Tokyo, Japan). The variation of urine dilutions was controlled according to the following formula:  $P_s = P \times [(SG_m - 1) / (SG_i - 1)]$ , where  $P_s$  is the SG-corrected metabolite concentration (ng/mL),  $P$  is the observed metabolite concentration (ng/mL),  $SG_i$  is the individual urinary SG, and  $SG_m$  is the median SG for urine samples of each trimester.

**2.3. Measurement of glucocorticoids**

Cord blood was collected from the umbilical vein at delivery. The samples were centrifuged at 4000 rpm for 15 min to retrieve serum and frozen at  $-80\text{ }^{\circ}\text{C}$  for further analysis. Thawed cord serum (150 μL) spiked with cortisol-d4 (45 μL, 100 ng/mL) (Sigma-Aldrich, St. Louis, MO) was vortex-mixed for 0.5 min, and then extracted with acetonitrile (750 μL) by vortexing for 5 min. After a centrifugation at 12,000 rpm for 10 min, the supernatant was transferred into a new tube and evaporated to dryness under nitrogen and reconstituted with 150 μL methanol/water (1/1, v/v).

Cord serum cortisol and cortisone were measured using a Waters 2695 high-performance liquid chromatography system coupled to a Waters XEVO® triple quadrupole mass spectrometer (HPLC-MS/MS) (Waters, Manchester, UK). Waters SymmetryShield RP18 column (3.5 μm, 150 × 4.6 mm) was used to achieve chromatographic separation with a mobile phase gradient with formic acid/water (1:1000, v/v) (mobile A) and formic acid/methanol (1:1000, v/v) (mobile B) (Supplementary Table S3). Positive-ion electrospray ionization mass spectrometry and multiple reaction monitoring mode were used for quantitative analysis. Along with 12 cord blood samples, blank and quality control sample were processed in each batch of samples to monitor the instrument performance. The collision voltage (V) and cone voltage (V) of the monitored MRM transitions for each analyte optimized for maximum signal intensity are shown in Table S4. The accuracy of each analyte in mid-point calibration standard is within 15%.

**2.4. Statistical analyses**

All analyses were conducted using SAS (version 9.4; SAS Institute Inc., Cary, NC, USA). We calculated the distributions of SG-adjusted urinary phthalate metabolites. Phthalate metabolites, which were detected in > 70% urine samples, were studied. To examine reproducibility in urinary phthalate levels in subject, intra-class correlation

**Table 4**

Adjusted percent changes [%Δ (95% CI)] in cord blood glucocorticoid concentrations in relation to SG-corrected urinary phthalate metabolite concentrations (n = 553).

Phthalate metabolites	Cortisol	Cortisone	Cortisol/cortisone
<b>MEHP</b>			
1st trimester	8.4 (−6.3, 25.4)	9.4 (0.0, 19.8)	−1.0 (−12.9, 12.6)
2nd trimester	−0.3 (−13.3, 14.5)	1.7 (−6.7, 10.9)	−2.0 (−13.3, 10.7)
3rd trimester	14.2 (0.6, 29.5)*	4.2 (−3.6, 12.7)	9.5 (−2.0, 22.4)
<b>MECPP</b>			
1st trimester	6.2 (−15.8, 34.0)	3.2 (−10.6, 19.2)	2.9 (−16.1, 26.2)
2nd trimester	1.4 (−21.2, 30.4)	−2.9 (−16.9, 13.5)	4.4 (−16.3, 30.2)
3rd trimester	−8.2 (−26.1, 14.0)	−14.4 (−25.2, −2.1)*	7.3 (−11.3, 29.8)
<b>MEHHP</b>			
1st trimester	10.1 (−14.2, 41.3)	3.9 (−11.0, 21.3)	6.0 (−14.9, 31.9)
2nd trimester	−3.4 (−25.3, 24.8)	−4.1 (−18.2, 12.4)	0.7 (−19.6, 26.1)
3rd trimester	−8.4 (−28.8, 17.9)	−14.4 (−26.7, 0.1)	7.0 (−14.3, 33.5)
<b>MEOHP</b>			
1st trimester	13.8 (−10.7, 45.0)	4.4 (−10.1, 21.4)	9.0 (−12.0, 34.8)
2nd trimester	0.8 (−21.6, 29.5)	−2.3 (−16.4, 14.0)	3.2 (−17.2, 28.6)
3rd trimester	−8.6 (−28.7, 17.2)	−15.5 (−27.6, −1.4)*	8.2 (−13.0, 34.6)
<b>ΣDEHP (nmol/L)</b>			
1st trimester	12.1 (−15.0, 47.8)	8.7 (−8.4, 29.1)	3.1 (−19.1, 31.4)
2nd trimester	−1.5 (−24.4, 28.4)	−2.4 (−17.2, 15.0)	0.9 (−20.0, 27.3)
3rd trimester	10.5 (−15.1, 43.8)	−10.4 (−23.9, 5.5)	23.3 (−2.1, 55.4)
<b>MBzP</b>			
1st trimester	17.5 (−4.4, 44.5)	−5.8 (−17.1, 7.1)	24.7 (4.1, 49.5)*
2nd trimester	4.9 (−15.7, 30.7)	5.0 (−8.4, 20.3)	0.0 (−17.5, 21.2)
3rd trimester	16.8 (−5.3, 44.0)	2.7 (−9.9, 16.9)	13.8 (−5.3, 36.7)
<b>MEP</b>			
1st trimester	3.2 (−13.8, 23.5)	−4.3 (−14.4, 6.9)	7.9 (−7.9, 26.2)
2nd trimester	11.4 (−8.8, 36.0)	−1.2 (−12.7, 11.9)	12.7 (−5.4, 34.3)
3rd trimester	2.2 (−14.5, 22.2)	−3.2 (−13.3, 8.2)	5.6 (−9.7, 23.4)
<b>MiBP</b>			
1st trimester	4.3 (−8.6, 19.0)	−1.4 (−9.2, 7.0)	5.8 (−5.8, 18.8)
2nd trimester	2.7 (−8.9, 15.8)	0.7 (−6.5, 8.4)	2.0 (−8.1, 13.3)
3rd trimester	0.7 (−10.6, 13.5)	−4.2 (−11.0, 3.2)	5.1 (−5.3, 16.8)
<b>MnBP</b>			
1st trimester	10.2 (−5.1, 27.9)	−1.3 (−10.0, 8.3)	11.6 (−2.1, 27.2)
2nd trimester	6.9 (−7.0, 22.9)	2.9 (−5.6, 12.2)	3.9 (−8.1, 17.4)
3rd trimester	1.8 (−11.8, 17.6)	−0.6 (−9.1, 8.6)	2.5 (−9.6, 16.3)
<b>ΣLMW (nmol/L)</b>			
1st trimester	−0.8 (−20.4, 23.7)	−1.7 (−14.2, 12.7)	0.9 (−16.9, 22.4)
2nd trimester	17.4 (−5.8, 46.3)	−0.6 (−13.3, 14.0)	18.0 (−2.7, 43.2)
3rd trimester	11.6 (−9.3, 37.4)	−1.3 (−13.2, 12.3)	13.1 (−5.7, 35.7)
<b>ΣPAEs (nmol/L)</b>			
1st trimester	−1.8 (−25.0, 28.5)	1.3 (−14.3, 19.7)	−3.1 (−23.4, 22.7)
2nd trimester	5.9 (−18.4, 37.5)	−2.8 (−17.3, 14.2)	9.0 (−13.3, 37.0)
3rd trimester	10.5 (−13.0, 40.3)	−4.5 (−17.7, 10.7)	15.7 (−6.2, 42.8)

Each phthalate metabolite was introduced into the model separately and adjusted for maternal age, parity, pre-pregnancy BMI, gestational age, mode of delivery and infant sex. Phthalate metabolites (ng/mL) were log<sub>10</sub>-transformed and glucocorticoids were natural log-transformed.

\* P < 0.05.

coefficients (ICC) and 95% confidence intervals (CI) were calculated using linear mixed model. The distributions of nine phthalate metabolites, as well as glucocorticoid levels were skewed and the data were log<sub>10</sub>-transformed for phthalates and natural log-transformed for glucocorticoid. We explored the associations between repeated measures of urinary phthalate metabolites and cord blood glucocorticoids using generalized estimating equation models (GEE). In GEE models, the trimester was treated as a dummy variable and artificial multivariate outcomes were created for each individual by repeating the outcome, thus the trimester-specific beta were estimated based on the beta of the 1st trimester (Sánchez et al., 2011; Horton et al., 1999). Covariates were added based on biological considerations or they could result in main effect estimates changes > 10%. Full models were adjusted for maternal age, pre-pregnancy BMI, parity, gestational age, mode of delivery and fetal sex. Only one mother in our study has reported smoking during pregnancy so we did not adjust for maternal smoking. We calculated the percent changes of glucocorticoids for each 10-fold increase in maternal urinary phthalate metabolite concentrations using the formula: percent change (%Δ) = (10<sup>β</sup> − 1) × 100, where β was coefficient from the GEE models (Barrera-Gomez and Basagana, 2015).

Considering sex differences in fetal-placental response to glucocorticoid (Scott et al., 2011) and hormone activity of phthalate (Wenzel et al., 2018; Zhang et al., 2018; Zhu et al., 2018), two approaches were used to explore the potential effect modification by fetal sex: 1) we stratified the models by fetal sex; and 2) we included an interaction term between each phthalate metabolite and trimester and fetal sex in the models. For those who had significant association, we further evaluated the dose-response relation with quartiles of SG-corrected phthalates and restricted cubic spline regressions. Two-sided P-values < 0.05 was considered statistically significant.

### 3. Results

Main characteristics of participants are summarized in Table 1. The average age of the mothers at birth was 28.3 years (standard deviation: 3.2). Among the pregnant women, 69.3% women had a normal pre-pregnancy BMI, 85.4% were nulliparous, and 82.5% had high education levels (more than high school). The average birth weight and gestational age at delivery were 3330 g and 39.4 weeks. Mother–infants pairs included in the present analysis had similar basic characteristics

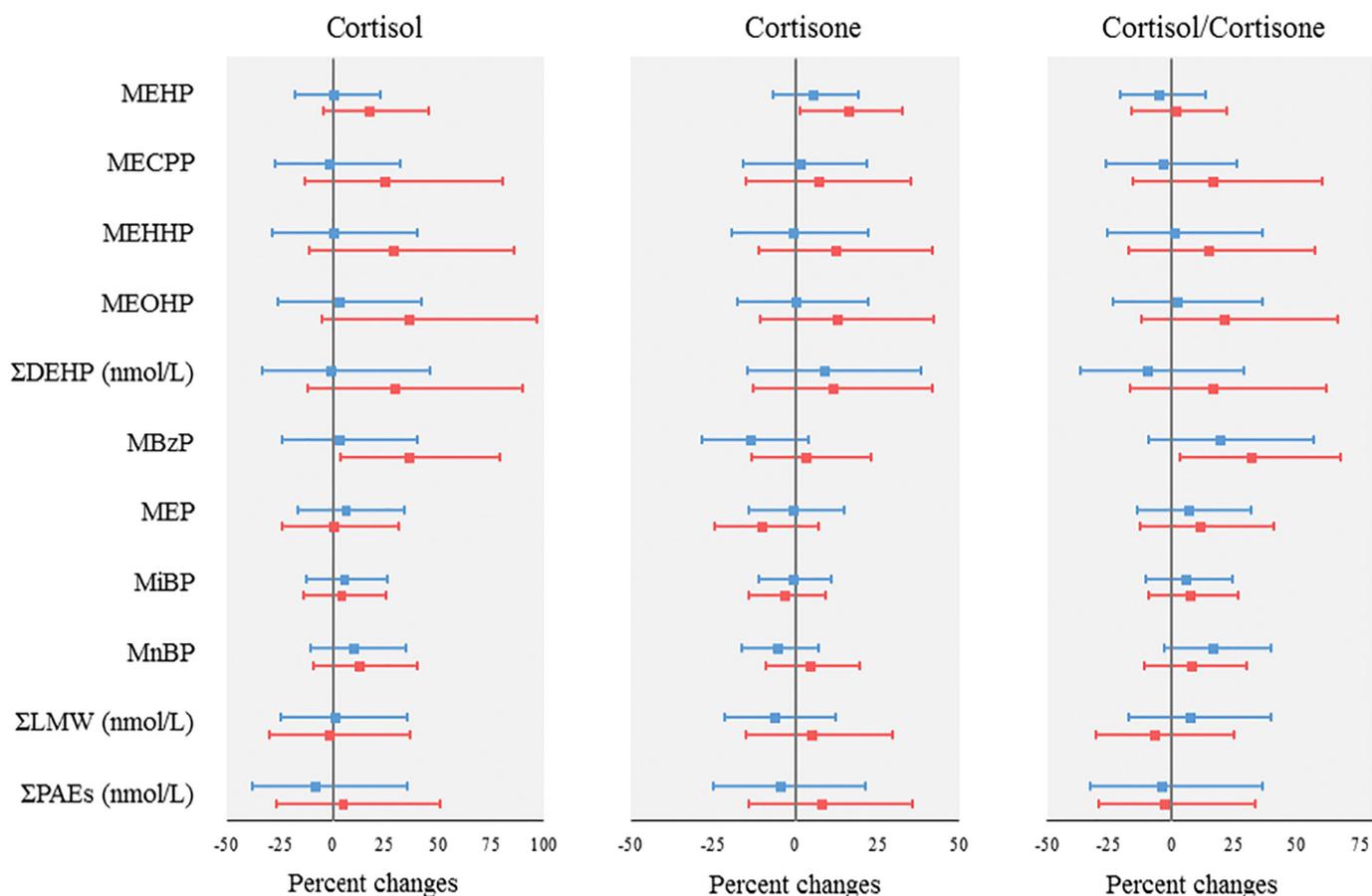


Fig. 1. Percent changes for cord blood glucocorticoid concentrations in relation to 1st trimester SG-corrected urinary phthalate metabolite concentrations (ng/mL). Adjusted for maternal age, parity, pre-pregnancy BMI, gestational age and mode of delivery. Phthalate metabolites were log<sub>10</sub>-transformed and glucocorticoids were natural log-transformed. Male infants in blue (n = 280) and female infants in red (n = 273). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

with the total population. Glucocorticoids were detected in all of the cord blood samples. Median (25th, 75th percentile) values of cortisol, cortisone and cortisol/cortisone ratio were 38.4 (21.6, 87.8) ng/mL, 79.2 (53.5, 121.3) ng/mL, and 0.5 (0.3, 0.8), respectively.

Total population phthalate metabolite geometric means and percentiles (SG-corrected) in three gestation periods are presented in Table 2. Eight phthalate metabolites were above 70% of their LOD, and those metabolites were subjected to further analysis in our study. Among the metabolites, DEHP metabolite concentrations were lower than LMW phthalate metabolite concentrations. The ICC for SG-corrected phthalate metabolites were ranged from 0.06 (MMP) to 0.49 (MEOHP), indicating phthalate metabolites varied across trimesters (Table 3). Generally, the ICC for DEHP phthalate metabolites were slightly higher than those for LMW phthalate metabolites.

Table 4 shows the associations between the repeated measures of phthalate metabolites (SG-corrected, ng/mL) and glucocorticoids. After adjusting other covariates, each 10-fold increment of MBzP in 1st trimester was associated with 24.7% (95% CI: 4.1%, 49.5%) increase of cortisol/cortisone ratio level. Maternal urinary phthalate metabolites in the 2nd trimester had no relationship with glucocorticoid levels. In the 3rd trimester, each 10-fold increment of MEHP was associated with 14.2% (95% CI: 0.6%, 29.5%) increased cortisol level. However, there were inverse associations between phthalate metabolites and cortisone, with significant relationships observed for MECPP [percent changes (%Δ) = -14.4% (95% CI: -25.2%, -2.1%)] and MEOHP [%Δ = -15.5% (95% CI: -27.6%, -1.4%)].

Sex-stratified analyses were conducted to explore the potential effect modification by fetal sex. The correlations between phthalates and

glucocorticoids were sex-specific (Figs. 1, 2 and 3). For female infants, maternal urinary phthalate metabolites exhibited a significant positive relationship with glucocorticoid levels. Each 10-fold increase in MEHP and MBzP concentrations in 1st trimester was associated with 16.2%–36.1% increase in glucocorticoid levels. In the 3rd trimester, each 10-fold increase in DEHP metabolites and total phthalates metabolite concentrations was also in relation to increased glucocorticoid levels with percent change ranged from 24.7% to 55.9%. However, for male infants, we observed a significant inverse association between maternal urinary phthalate metabolite concentrations in the 3rd trimester with glucocorticoid levels, where each 10-fold increase in MECPP, MEHHP and MEOHP were associated with 20.8%–36.3% reduction of cortisol. The above associations between phthalate exposure and glucocorticoid levels and sex interaction were examined. No significant interactions between phthalate exposure and fetal sex were identified ( $P > 0.05$ ) (Supplementary Table S5).

The results of the dose-response relation with quartile concentrations of urinary phthalate metabolites confirmed similar associations with those from model treated the exposure as continuous variables (Table S6). In addition, there were no significant non-linear relationships ( $P > 0.10$ ) when tested by restricted cubic spline regressions (Table S6).

#### 4. Discussion

In this prospective cohort study, we examined the variability of maternal urinary phthalate metabolite concentrations during pregnancy and explored the possible window of phthalate exposure on

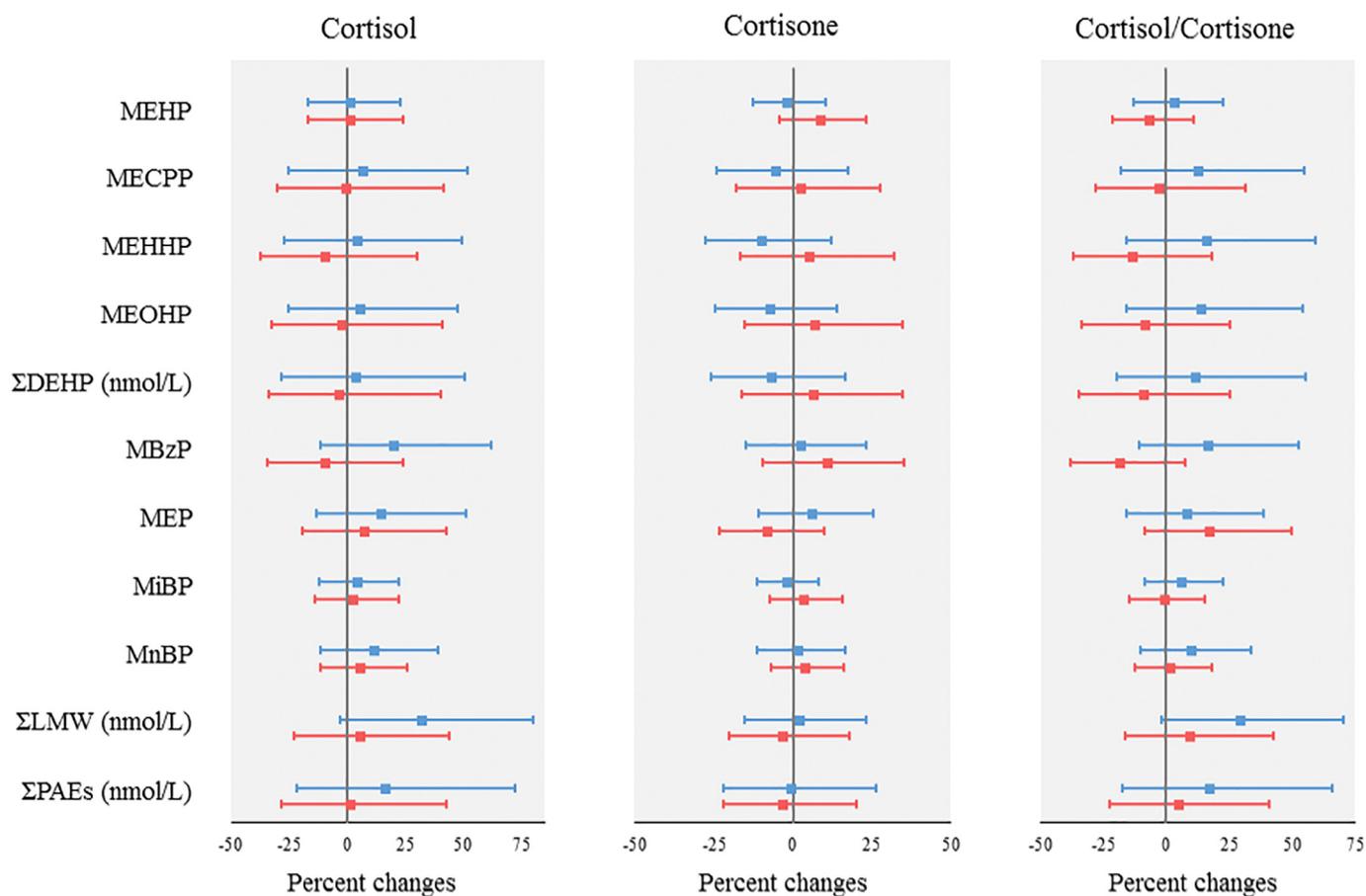


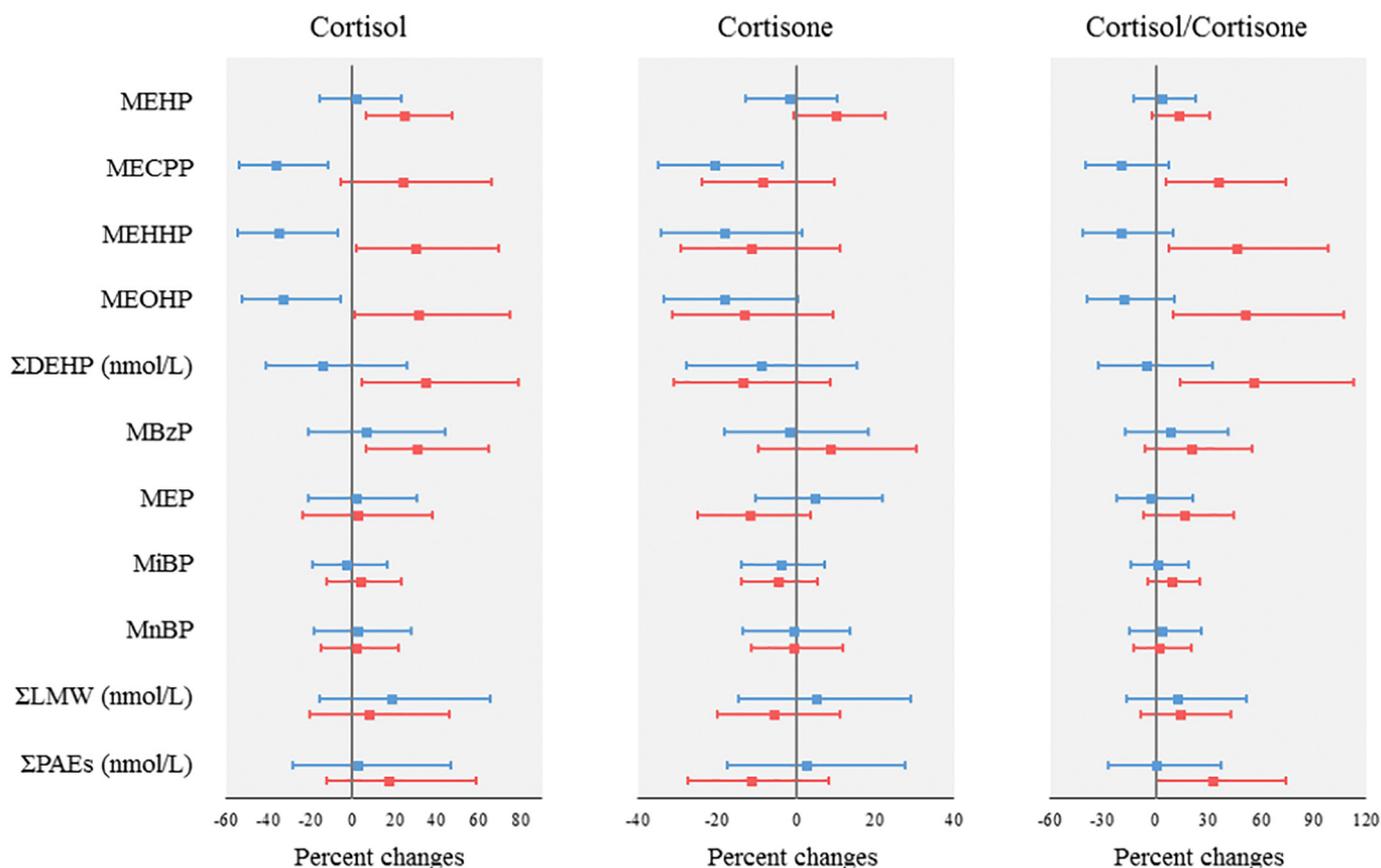
Fig. 2. Percent changes for cord blood glucocorticoid concentrations in relation to 2nd trimester SG-corrected urinary phthalate metabolite concentrations (ng/mL). Adjusted for maternal age, parity, pre-pregnancy BMI, gestational age and mode of delivery. Phthalate metabolites were log<sub>10</sub>-transformed and glucocorticoids were natural log-transformed. Male infants in blue (n = 280) and female infants in red (n = 273). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

infant glucocorticoid levels. Our results demonstrated that phthalate exposure at 1st and 3rd trimester might disrupt the infant glucocorticoid levels. Moreover, maternal urinary phthalate metabolite levels have a positive relationship with glucocorticoids in female infants but have an inverse relationship with glucocorticoid levels in male infants, indicating that phthalates might have a sex-specific effect on these associations.

We have measured the urinary phthalate at multiple time points in pregnancy, which allowed for the evaluation of variability in urinary phthalate metabolites across the course of pregnancy. ICC for phthalate metabolite concentrations among all mothers ranged from 0.08 to 0.49, indicating phthalates within-individual variability. ICC for SG-adjusted DEHP metabolites were higher and the other metabolites were lower in our population compared with other studies (Braun et al., 2012; Ferguson et al., 2014; Fisher et al., 2015; Watkins et al., 2017a, 2017b). These differences may be owing to the variations of personal care products usage, eating habits and the living environments of pregnant women in different regions (Guo and Kannan, 2011). In addition, maternal urinary phthalate metabolite levels in our study were lower than those reported in the US (Ferguson et al., 2014; Fisher et al., 2015), Mexico (Watkins et al., 2017a, 2017b) and Puerto Rico (Cantonwine et al., 2014), which might also result in the difference of ICC.

Previous studies have suggested that glucocorticoids played a crucial role in maintaining the stability of the intrauterine environment, inadequacy or oversupply of glucocorticoids might have adverse impacts on postnatal development (Kapoor et al., 2008; Rog-Zielinska et al., 2014). Fetal glucocorticoids may be originated from mothers and fetal adrenal gland. Glucocorticoid levels were much lower in cord

blood than in maternal blood. However, the correlation between glucocorticoids in maternal and cord blood samples was still inconsistent (Stirrat et al., 2017; Travers et al., 2018). Therefore, cord blood glucocorticoids might be a suitable predictor for the fetal endocrine milieu. The levels of cord blood glucocorticoid in our population were comparable with those reported in Japan [Median (ng/mL): cortisol, 40.5; cortisone: 96.9] (Araki et al., 2017; Goudarzi et al., 2017), but relatively lower than those reported in France [Median (ng/mL): cortisol, 52.0; cortisone: 148.1] (Travers et al., 2018). A plausible explanation would be that few mothers in our study have reported smoking or alcohol assumption during pregnancy, and there is a significant association between smoking and cord blood cortisol (Varvarigou et al., 2009). Previous studies have shown that fetal glucocorticoid concentrations were also correlated with gestational age (Ishimoto and Jaffe, 2011; Rog-Zielinska et al., 2014) and delivery mode (Vogl et al., 2006). Both of them were adjusted as predictors of the outcome in models during the data analysis. To assess whether preterm infants may be contributing to our findings, we excluded preterm infants (gestational age < 37 weeks, n = 13) from the analysis. The results did not change, suggesting that these findings might not be biased by gestational age as a contributing covariate of infant glucocorticoids. Same analyses stratified by mode of delivery were also performed to assess the effect of delivery mode on the associations between phthalates and glucocorticoids. We observed almost same direction of associations in two subgroups which were consistent with those in total population. (Table S7). We are aware that only two studies have previously investigated the potential phthalate-associated alterations in infant glucocorticoid levels. A Danish case-control study included cases of cryptorchidism and



**Fig. 3.** Percent changes for cord blood glucocorticoid concentrations in relation to 3rd trimester SG-corrected urinary phthalate metabolite concentrations (ng/mL). Adjusted for maternal age, parity, pre-pregnancy BMI, gestational age and mode of delivery. Phthalate metabolites were log<sub>10</sub>-transformed and glucocorticoids were natural log-transformed. Male infants in blue (n = 280) and female infants in red (n = 273). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

hypospadias and control boys, measured phthalate metabolites and steroid hormones in amnion fluid in the second trimester. This study observed a 13% higher cortisol in the highest MECPP tertile compared to the lowest tertile among the combined population of cases and controls (Jensen et al., 2015). Our study has also found that most of the urinary phthalate metabolites in late pregnancy were related to the increase of cortisol, but the association did not reach statistical significance. However, a prospective cohort study conducted in 202 mother-infant pairs in Japan has reported that the increased maternal blood MEHP level at 23–35 weeks of gestation was associated with decreased cord blood cortisol and cortisone concentrations, whereas cortisol/cortisone ratios increased (Araki et al., 2017). In addition, the authors assessed effect modification by fetus sex and reported that interaction between MEHP and sex was insignificant for glucocorticoid (Araki et al., 2017). Our study has shown that maternal urinary MEHP in 3rd trimester was positively associated with cord blood cortisol, and the association was more pronounced among female infants. Possible explanations for these inconsistent associations between prenatal phthalate exposure and cortisol levels might be the variations in the sample and the use of one-spot sample for exposure assessment (Braun et al., 2012). Maternal urinary phthalate metabolites have been widely used to evaluate phthalate exposure (Marie et al., 2015). Compared to blood and amniotic fluid, maternal urine was easy sampling and non-invasive, thus becoming the most suitable matrix which can be performed repeatedly to assess exposure throughout pregnancy.

We have found that urinary phthalate metabolites in 1st and 3rd trimester had the associations with cord blood glucocorticoid. Although possible mechanisms for these associations are not fully elucidated, several potential mechanisms that might explain the associations. First,

phthalates have the ability to bind to glucocorticoid receptors, making plausible direct stimulation of the hypothalamic–pituitary–adrenal (HPA) axis (Sarath Josh et al., 2016). Previous animal and vitro studies have also provided evidence that phthalates might inhibit the expression of 11 $\beta$ -HSD2, which was highly expressed in human placental to convert maternal cortisol to cortisone (Hong et al., 2009; Zhao et al., 2010; Fowden et al., 2016). This change of placental metabolism would potentially decrease the metabolism of maternal cortisol and allow increased access of maternal cortisol to the fetus. Second, phthalate such as MBzP might inhibit trophoblast invasion in early pregnancy via peroxisome proliferator-activated receptor protein gamma in early pregnancy (Adibi et al., 2017; Red-Horse et al., 2004). Inadequate trophoblast invasion in early pregnancy might have an adverse effect on placenta, and then the fetus glucocorticoids were disrupted in circumstances of placental dysfunction (Moisiadis and Matthews, 2014). Third, the fetal adrenal gland began to produce cortisol in the 3rd trimester. There appeared to be a surge in the levels of glucocorticoids in the fetal circulation in late pregnancy that paralleled the increased maturity of fetal organs (Murphy, 1982). Exposure to phthalate at this time might alter the timing of adrenarche through disruption in fetal adrenal development (Xing et al., 2015).

We also found the sex-specific associations of phthalate disruption effects on glucocorticoid levels. To the best of our knowledge, no previous studies have reported the sex difference on the association between phthalates and glucocorticoid. Phthalate exposure affected fetus sex hormone concentrations, which might also disrupt the development of the HPA axis. It was well documented that phthalate was an estrogenic endocrine disruptor and had anti-androgenic and weak estrogenic effects (ATSDR, 2002). Estrogen played a critical role in HPA axis

regulation by regulating of HPA axis feedback to suppress adrenocorticotrophic hormone and cortisol secretion (Weiser and Handa, 2009). It was plausible that the direction of up/downregulation of HPA axis would be different between sex following estrogen disruption. Therefore, further researches into the mechanisms underlying these associations are warranted.

In this study, we examined a wide range of phthalate metabolite levels across pregnancy, which enabled us to look at exposure across time within the same mother-infant pair rather than at a single-point. In addition, we assessed glucocorticoid levels using liquid chromatography mass spectrometry, which has higher sensitivity and specificity than other methods, such as the immunoassay. However, this study also bears potential limitations. First, we only included live infants, which might have a possibility of selection bias and underestimate of the effects of phthalates in our study. Second, the phthalate metabolites were moderately to highly correlated with each other (Spearman's coefficients: 0.3–0.9,  $P < 0.01$ ). Thus, we were unable to clarify the effect of individual phthalate metabolites when including multiple phthalate metabolites in the same model, which might lead to biased coefficient estimation and a loss of power (Chatterjee et al., 2000). Third, we used the product-to-substrate ratio rather than a strict measurement of 11 $\beta$ -HSD2 activity, and the maternal psychosocial status, such as depression and anxiety, might have effects on the fetus glucocorticoid. Further studies with the measurement of 11 $\beta$ -HSD2 activity and the information on maternal psychosocial status are then required to confirm the results.

## 5. Conclusions

Our findings suggest that maternal exposure to phthalates in early and late trimester is associated with fetal steroidogenesis, and these associations may be modified by fetal sex. To the best of our knowledge, the present study is the first one to assess phthalate exposure at three trimesters during pregnancy in relation to infant glucocorticoids. However, additional human and animal studies are required to elucidate the biological mechanisms involved in these relationships.

## Acknowledgments

This work was supported by the National Natural Science Foundation of China [grant numbers: 21677056, 81372959, 21437002, 81402649, and 91643207], the National Key Research and Development Plan [grant numbers: 2016YFC0206700, 2016YFC0206203], the Fundamental Research Funds for the Central Universities, HUST [grant numbers: 2016YXZD043, 2015ZDTD047], and General Research Fund from Research Grants Council of Hong Kong [grant number: 12304117].

## Declarations of interest

None.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.envint.2018.09.037>.

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