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REGULAR ARTICLE

Prenatal maternal blood triglyceride and fatty acid levels in relation to exposure to di(2-ethylhexyl)phthalate: a cross-sectional study

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Abstract
Objectives The hypolipidemic effects of di(2-ethylhexyl)phthalate (DEHP) exposure in humans have not been investigated. And the influences of maternal prenatal DEHP exposure on birth outcomes are not well-known. We aimed to estimate prenatal DEHP exposure in maternal blood, and evaluate its relationships to maternal blood triglyceride (TG) and fatty acid (FA) levels and to birth outcomes.
Methods We studied 318 mother–newborn pairs residing in Sapporo, Japan. Blood was taken one time during pregnancy for each mother. Maternal and infant characteristics were obtained from medical records and questionnaire survey. We measured DEHP metabolite, mono(2-ethylhexyl) phthalate (MEHP), along with TG and 9 FAs using maternal blood, and analyzed associations of MEHP level with maternal blood TG/FA levels and infant birth dimensions.
Results Maternal blood TG and palmitoleic/oleic acid levels were higher, but stearic/docosahexaenoic acids and MEHP were lower during late pregnancy. Maternal blood MEHP levels inversely correlated with TG and palmitic/palmitoleic/oleic/linoleic/α-linolenic acids. After adjustment for confounders, we found that a tenfold increase in blood MEHP levels correlated with a decrease in TG of 25.1 mg/dl [95 % confidence interval (CI) 4.8–45.3 mg/dl], and similar relations in palmitic ($\beta = -581.8$; 95 % CI −906.5, −257.0), oleic ($\beta = -304.2$; 95 % CI −518.0, −90.5), linoleic ($\beta = -348.6$; 95 % CI −510.6, −186.6), and α-linolenic ($\beta = -6.3$; 95 % CI −9.5, −3.0) acids. However, we observed no correlations between maternal blood MEHP levels and infant birth weight, length, chest circumference, or head circumference.
Conclusions Ambient DEHP exposure during pregnancy inversely correlated with maternal blood TG and 4 FA levels, but not birth outcomes.

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Keywords Di(2-ethylhexyl)phthalate · Prenatal exposure · Triglycerides · Fatty acids · Infant birth outcomes

Introduction

Di(2-ethylhexyl)phthalate (DEHP) is the most widely used plasticizer of polyvinylchloride in the manufacture of a wide variety of consumer goods, such as food packaging, building products, clothing, car products, medical devices and children's products (but not in toys intended for mouthing) [1, 2], although the use of DEHP in the latter two goods has been decreasing recently due to several government restrictions [3, 4]. DEHP is not chemically bound to polyvinylchloride and leaks from polyvinylchloride items with time and use. Consequently, it is a ubiquitous environmental contaminant [1, 2]. Ambient exposure to DEHP in the general adult population may be in the range of 3–30 μg/kg body weight/day [5], while that in Japanese pregnant women has been estimated to be 3.45–41.6 μg/kg/day [6]: no difference in the exposure levels between the former and the latter. Fujimaki et al. [6] identified that the maximum estimated intake level per body weight reached the old Tolerable Daily Intake level of 40–140 μg/kg/day set by Japanese Ministry of Health, Labour and Welfare. Importantly, the intake level was more than the latest Tolerable Daily Intake level of 30 μg/kg/day set by Food Safety Commission of Japan [7]. Therefore, ambient DEHP exposure and potential adverse effects in Japanese warrants close concerns. After entering the human body, DEHP is first metabolized to the monoester, mono(2-ethylhexyl) phthalate (MEHP), which can be oxidized further to oxidative metabolites [8].

DEHP is a known reproductive and developmental toxicant in animals [1, 5, 8]. The growing relevant toxicity reports from experimental animals, together with widespread human exposure, raise serious concerns over the potential risks from human exposure to DEHP. There have been emerging studies conducted of human health outcomes in relation to prenatal DEHP exposure in recent years. Prenatal DEHP exposure has been associated with shorter gestational age at birth [9, 10], lower mental and psychomotor development indices [11], and lower birth weight [12]. Moreover, prenatal DEHP exposure was reported to correlate with shorter anogenital distance, reduced penile size and incomplete testicular descent [13, 14], more non-optimal reflexes [15] and reduced masculine play behavior [16] in male infants, suggesting that possible sex difference exists in DEHP toxicity. Additionally, our group recently reported that maternal exposure to DEHP decreased plasma levels of triglycerides (TG) [1] and four fatty acid (FA) components [17] in prepartum mice, including palmitic acid, oleic acid, linoleic acid (LA), and α-linolenic acid (ALA), which was suspected to correlate with adverse effects of DEHP. However, no information regarding humans is available.

To date, the potential health hazards from exposure to DEHP and/or its main metabolite MEHP in humans at risk, such as pregnant women and infants, have not been well-documented and warrant extensive investigation. Thus, this study aimed to estimate MEHP levels in the blood of pregnant women as a biomarker of ambient DEHP exposure, and to evaluate potential associations with blood levels of TG and FAs in pregnant women and term birth outcomes in infants.

Materials and methods

Study population

This study was part of the "Hokkaido Study on Environment and Children's Health" [18–20]. Briefly, from July 2002 to October 2005, we approached pregnant women who were at 23–35 weeks of gestation and had no serious illnesses and medical complications to register with a hospital-based prospective cohort study at the Sapporo Toho Hospital in Sapporo, Hokkaido, Japan. The following were the exclusion criteria for study subjects: women with incomplete partner's information, women who had decided to enroll in the Japanese cord blood bank, or women who had decided to deliver the baby at another hospital. Some of the women we approached did not express interest in our study, and some were unable or unwilling to participate in the study. Ultimately, 514 pregnant women (30 % of those approached) were enrolled in this study by providing written informed consent. Maternal and infant medical information were obtained from medical records of antenatal and perinatal examinations at the hospital. A self-administered questionnaire survey was completed after the second trimester to collect potential confounders, as described in detail elsewhere [18–20]. This study was approved by the Institutional Ethical Board for Epidemiologic studies of Hokkaido University Graduate School of Medicine, and Ethics Review Committee of Nagoya University Graduate School of Medicine.

Blood sampling

A blood sample of approximately 40 ml was taken from the maternal peripheral vein at the time of the next prenatal hospital examination after recruitment. If the blood could not be taken during pregnancy due to maternal anemia, it was obtained during hospitalization within a week after delivery. As blood was obtained one time for each woman, the analyses using blood samples were cross-sectional in

nature. Consistent with published reports of "Hokkaido Study on Environment and Children's Health" [19], the blood sampling period was categorized into four groups: 23–31 weeks of gestation, 32–34 weeks of gestation, 35–41 weeks of gestation, within a week after delivery. All samples were stored at −80 °C until analysis.

MEHP level in maternal blood

Blood samples (30 μl) were mixed with 120 μl 1 N HCl, 350 μl saturated saline solution and 50 μl of 10 μM MEHP-d as an internal standard. MEHP was then extracted twice with 500 μl ethyl acetate after shaking for 15 min. The ethyl acetate layer was evaporated, and the sediments were dissolved into 40 μl ethyl acetate. After adding 20 μl *N*-methyl-*N*-(tert-butyldimethylsilyl) trifluoroacetamide, the tube was left at room temperature for 60 min, and the MEHP tert-butyldimethylsilyl derivative concentration formed was measured by gas chromatography–mass spectrometry (GC/MS) (6890N, 5973N; Agilent Technologies, CA, USA) under the analytical conditions mentioned previously by Ito et al. [21]. For each sample, duplicate analysis was performed. Ultimately, MEHP levels were available from 493 maternal blood samples. The detection limit of MEHP was 1 pmol/ml. Coefficient of variation (CV) of MEHP measurements within a day was 2.0–7.8 % for 6 days, and CV of day to day for 6 days was 6.2 % at 5 pmol/ml of concentration.

TG concentration in maternal blood

The TG level in blood was measured using TG-IE kits (Wako, Osaka, Japan) after extracting lipids as described by Folch et al. [22].

FA profiles in maternal blood

FA levels in maternal blood were determined duplicately by GC–MS as described in detail in our earlier study [17] after extracting lipids according to the method of Folch et al. [22]. Nine FA species targeted for measurement included palmitic and stearic acids of saturated FAs, palmitoleic and oleic acids of monounsaturated FAs, LA and arachidonic acid (AA) of the n-6 family, and ALA, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) of the n-3 family. Under the experimental conditions, the detection limits were 2.4 μg/ml for palmitic acid, 1.3 μg/ml for stearic acid, 0.69 μg/ml for palmitoleic acid, 3.6 μg/ml for oleic acid, and 2.0 μg/ml for the others.

Data analysis

Ten registered women were excluded due to miscarriage, stillbirth, relocation, or voluntary withdrawal from the study before follow-up. The following subjects were excluded from analysis: those with maternal pregnancy-induced hypertension ($n = 11$), diabetes mellitus ($n = 1$), fetal heart failure ($n = 1$), or multiple births ($n = 7$). We also excluded premature births ($n = 23$)—defined as birth at less than 37 weeks of gestation—from the data analysis to keep the focus on fetal growth [19, 23], and excluded postnatal blood samplings ($n = 134$) from the analysis to focus on prenatal subjects, resulting in a sample size of 327. Ultimately, the sample size available for analysis from the 327 was 318 subjects who completed the measurements of MEHP, TG and FAs.

Correlations between MEHP concentration or TG and FA levels in maternal blood and characteristics of subjects were analyzed by Spearman's rank correlation test, Mann–Whitney *U* test and Kruskal–Wallis test. Finally, unadjusted and multivariable-adjusted linear regression analyses were performed to evaluate the association between MEHP levels and concentrations of TG and FAs in maternal blood. TG and FA components were dependent variables; MEHP level was independent variable. In the multivariable-adjusted models, maternal age [24], smoking and alcohol intake during pregnancy [25], inshore fish and deep-sea fish intake during pregnancy [24], and the blood sampling period (based on the correlation analyses) were included as potential confounders. We also performed unadjusted and multivariable-adjusted linear regression analyses to evaluate the association between maternal blood MEHP levels and birth outcome measures. Birth weight, birth length, chest circumference or head circumference were dependent variables; maternal blood MEHP was independent variable. In the multivariable-adjusted models, maternal age, height and pre-pregnancy weight, parity, smoking and alcohol intake during pregnancy, socioeconomic status (annual household income), gestational age, infant sex [19, 20, 23], and the blood sampling period were included as potential confounders. For head circumference, the adjusted model also included delivery type [19]. The linear regression analyses were also stratified by infant gender to clarify the interaction with infant gender. Because of the skewed distribution, we treated maternal blood MEHP level as a continuous variable on a $\log_{10}$ scale. All statistical analyses were performed using SPSS software. Results were statistically significant if $p < 0.05$.

Results

Maternal and infant characteristics and their association with maternal blood MEHP level

We included 318 mother-infant pairs in the study (Table 1). The women aged less than 30 years accounted for 48.1 %. Approximately, 51.9 % of the women were primiparous, and 37.4 % of them did not conceive before.

Table 1 Maternal and infant characteristics, and their association with maternal blood MEHP level ($n = 318$)

Characteristics	n (%)	MEHP (nmol/ml)[a]	p value
Maternal characteristics			
Age (years)			
<30	153 (48.1)	0.040	0.959
≥30	165 (51.9)	0.038	
Height (cm)			
<158.0	138 (43.4)	0.037	0.164
≥158.0	180 (56.6)	0.041	
Pre-pregnancy body weight (kg)[b]			
<52	152 (47.8)	0.041	0.726
≥52	163 (51.3)	0.037	
Pre-pregnancy BMI (kg/m^2)[b]			
<20.6	163 (51.3)	0.039	0.990
≥20.6	152 (47.8)	0.037	
Parity (times)[b]			
0	165 (51.9)	0.038	0.479
≥1	152 (47.8)	0.040	
Past conception (times)			
0	119 (37.4)	0.038	0.710
1	102 (32.1)	0.042	
≥2	97 (30.5)	0.038	
Educational level (years)			
≤12	142 (44.7)	0.038	0.590
≥13	176 (55.3)	0.040	
Annual household income (million yen)[b]			
<3	68 (21.4)	0.040	0.332
3–5	153 (48.1)	0.033	
5–7	63 (19.8)	0.042	
≥7	32 (10.1)	0.045	
History of smoking			
Yes	185 (58.2)	0.038	0.657
No	133 (41.8)	0.041	
Smoking during pregnancy			
Yes	68 (21.4)	0.035	0.438
No	250 (78.6)	0.039	
Alcohol intake during pregnancy			
Yes	106 (33.3)	0.039	0.797
No	212 (66.7)	0.038	
Alcohol intake among drinkers during pregnancy (g/day)			
<1.5	53 (50.0)	0.038	0.284
≥1.5	53 (50.0)	0.042	
Caffeine intake during pregnancy (mg/day)			
<116.5	159 (50.0)	0.036	0.062
≥116.5	159 (50.0)	0.041	
Fish intake during pregnancy			
Inshore fish			
≤1–2 times/month	174 (54.7)	0.037	0.513
≥1–2 times/week	144 (45.3)	0.042	
Deep-sea fish			
≤1–2 times/month	155 (48.7)	0.037	0.355
≥1–2 times/week	163 (51.3)	0.041	
Blood sampling period (gestational weeks)			
23–31	135 (42.5)	0.043	0.002[c]
32–34	81 (25.5)	0.046	
35–41	102 (32.1)	0.028	
Type of delivery			
Vaginal	273 (85.8)	0.039	0.916
Cesarean section	45 (14.2)	0.045	
Infant characteristics			
Sex			
Male	151 (47.5)	0.038	0.374
Female	167 (52.5)	0.041	
Gestational age (weeks)			
≤39	171 (53.8)	0.040	0.990
≥40	147 (46.2)	0.038	
Birth weight (g)			
<3098.0	157 (49.4)	0.041	0.588
≥3098.0	161 (50.6)	0.037	
Birth length (cm)			
<48.3	156 (49.1)	0.038	0.458
≥48.3	162 (50.9)	0.039	
Chest circumference (cm)			
<31.5	121 (38.1)	0.040	0.534
≥31.5	197 (61.9)	0.038	

Table 1 continued

Characteristics	*n* (%)	MEHP (nmol/ml)[a]	*p* value
Head circumference (cm)			
<33.2	157 (49.4)	0.039	0.934
≥33.2	161 (50.6)	0.039	

BMI body mass index, *MEHP* mono(2-ethylhexyl) phthalate, *SD* standard deviation

[a] Median

[b] Missing data: pre-pregnancy body weight (3), pre-pregnancy BMI (3), parity (1), annual household income (2)

[c] *p* value <0.05, indicating statistically significant correlation

Mothers had more than 13 years of education (55.3 %), and 3–5 million yen of annual household income (48.1 %). Mothers who smoked during pregnancy were 21.4, and 33.3 % had alcohol intake during pregnancy. The number of women who ate inshore fish and deep-sea fish at least 1–2 times/week was 144 (45.3 %) and 163 (51.3 %), respectively. Blood sampling was conducted during 23–31 gestational weeks for 42.5 % of women. Two-hundred seventy-three women had vaginal births (85.8 %). One hundred and fifty-one infants (47.5 %) were male, and the gestational age of 53.8 % pregnancy was less than 40 weeks. The number of infants with birth weight, birth length, chest circumference and head circumference less than 3098.0 g, 48.3, 31.5 and 33.2 cm, respectively, was 157 (49.4 %), 156 (49.1 %), 121 (38.1 %), and 157 (49.4 %), respectively. Additionally, we observed statistically significant differences in medians of MEHP levels by blood sampling period ($p = 0.002$), but did not find significant correlations with other characteristics. Thus, we adjusted the blood sampling period in the multivariate regression models.

MEHP level in blood of pregnant women

The mean (± SD) MEHP level was 0.049 ± 0.040 nmol/ml, ranging from 0.007 to 0.316 nmol/ml with a median of 0.039 nmol/ml.

TG and FA levels in maternal blood

Mean (± SD) TG was 94.1 ± 53.7 mg/dl (Table 2). Mean palmitic and stearic acids were 2052.4 (±853.4) and 569.1 (±206.3) μg/ml, respectively. Mean palmitoleic and oleic acids were 122.9 (±75.8) and 1215.5 (±562.7) μg/ml, respectively. Mean LA and AA were 735.6 (±426.6) and 71.4 (±42.6) μg/ml, respectively. Mean ALA, EPA, and DHA were 11.0 (±8.5), 10.0 (±8.4), and 30.2 (±21.8) μg/ml, respectively. Altogether, the mean (± SD) total FAs was 4818.2 ± 1982.0 μg/ml. Highly abundant and common FAs from the total FA pool were palmitic acid (42.8 %), oleic acid (25.0 %), LA (14.8 %), and stearic acid (12.4 %).

Table 2 Concentrations of TG and FA components in maternal blood ($n = 318$)

Parameters	Mean ± SD
TG (mg/dl)	94.1 ± 53.7
FA components (μg/ml)	
Palmitic acid	2052.4 ± 853.4 (42.8)[b]
Stearic acid	569.1 ± 206.3 (12.4)[b]
Palmitoleic acid	122.9 ± 75.8 (2.5)[b]
Oleic acid	1215.5 ± 562.7 (25.0)[b]
LA	735.6 ± 426.6 (14.8)[b]
AA	71.4 ± 42.6 (1.5)[b]
ALA	11.0 ± 8.5 (0.2)[b]
EPA	10.0 ± 8.4 (0.2)[b]
DHA	30.2 ± 21.8 (0.6)[b]
Total FAs (μg/ml)[a]	4818.2 ± 1982.0

AA arachidonic acid, *ALA* α-linolenic acid, *DHA* docosahexaenoic acid, *EPA* eicosapentaenoic acid, *FA* fatty acid, *LA* linoleic acid, *SD* standard deviation, *TG* triglycerides

[a] Total FAs correspond to the sum of all FA measured

[b] Values in parentheses denote the percentage of each FA in total FAs

TG and FA relationships to subject characteristics

The relationships between TG and FA levels and subject characteristics, which were potential confounders of TG and FAs, were checked (data not shown). We found significant differences in the ALA level by maternal age ($p = 0.044$), in the EPA level by inshore fish intake ($p = 0.001$), and in TG ($p < 0.001$), stearic acid ($p = 0.002$), palmitoleic acid ($p = 0.012$), oleic acid ($p = 0.003$), and DHA ($p = 0.020$) levels by the blood sampling period. Specially, except for stearic acid and DHA, the levels of TG, palmitoleic and oleic acids increased in the late gestational age when blood sampling.

Relationships between MEHP levels and TG/FA concentrations in maternal blood

Table 3 showed univariate and multivariate regression model results for maternal blood TG and FAs on $\log_{10}$-transformed MEHP concentration. Multivariate models of TG and FAs were adjusted for confounders correlated with TG and FAs at *p* values <0.05, factors known to be related to TG and FAs from previous reports [24, 25] and the blood sampling period. In the crude model, we found significant negative correlations of MEHP exposure with TG, palmitic

Table 3 Regression coefficients (95 % confidence interval, CI) between $\log_{10}$-transformed MEHP level (nmol/ml) and TG/FA in maternal blood

Dependent variable	Crude model β^c (95 % CI)	Adjusted model β^c (95 % CI)
TG (mg/dl)[a]	−30.4 (−51.0, −9.8)	−25.1 (−45.3, −4.8)
Palmitic acid (μg/ml)[a]	−635.7 (−959.6, −311.7)	−581.8 (−906.5, −257.0)
Stearic acid (μg/ml)[a]	−33.3 (−113.3, 46.8)	−38.8 (−119.9, 42.3)
Palmitoleic acid (μg/ml)[a]	−34.1 (−63.3, −5.0)	−27.5 (−56.6, 1.6)
Oleic acid (μg/ml)[a]	−355.8 (−570.7, −140.8)	−304.2 (−518.0, −90.5)
LA (μg/ml)[a]	−363.0 (−523.8, −202.2)	−348.6 (−510.6, −186.6)
AA (μg/ml)[a]	−8.3 (−24.8, 8.2)	−9.8 (−26.5, 6.9)
ALA (μg/ml)[a]	−6.4 (−9.7, −3.2)	−6.3 (−9.5, −3.0)
EPA (μg/ml)[b]	0.8 (−2.5, 4.1)	0.4 (−2.9, 3.6)
DHA (μg/ml)[b]	4.9 (−3.6, 13.4)	3.3 (−5.2, 11.8)

AA arachidonic acid, *ALA* α-linolenic acid, *DHA* docosahexaenoic acid, *EPA* eicosapentaenoic acid, *FA* fatty acid, *LA* linoleic acid, *TG* triglycerides

[a] Adjusted for blood sampling period, maternal age, smoking and alcohol intake during pregnancy

[b] Adjusted for blood sampling period, maternal age, smoking and alcohol intake during pregnancy, inshore fish and deep-sea fish intake during pregnancy

[c] Partial regression coefficient represents the expected change in dependent variables as a result of a tenfold change in MEHP level, because MEHP level was $\log_{10}$-transformed

acid, palmitoleic acid, oleic acid, LA and ALA in maternal blood. After full adjustment for potential confounders, we found that a tenfold increase in MEHP levels correlated with a decrease in TG of 25.1 mg/dl [95 % confidence interval (CI), 4.8–45.3 mg/dl], and similar relations in palmitic acid ($\beta = -581.8$; 95 % CI −906.5, −257.0), oleic acid ($\beta = -304.2$; 95 % CI −518.0, −90.5), LA ($\beta = -348.6$; 95 % CI −510.6, −186.6), and ALA ($\beta = -6.3$; 95 % CI −9.5, −3.0). We also evaluated the relationships of MEHP with TG and FA levels in maternal blood by blood sampling periods (Table 4). Maternal blood MEHP level significantly and negatively associated with palmitic acid, oleic acid, LA and ALA levels at 23–31 weeks of gestation, and TG, palmitic acid, palmitoleic acid, oleic acid, LA and ALA levels at 32–34 weeks in univariate and multivariate regression models. MEHP level in maternal blood negatively associated with LA in univariate regression model at 35–41 weeks, which was insignificant after adjusting for confounders.

Relationships between maternal blood MEHP levels and infant birth outcomes

Table 5 showed the results of univariate and multivariate regression analyses for the association between maternal blood MEHP levels and birth weight or birth size of infants. Multivariate models were adjusted for known risk factors correlated with birth outcomes from previous reports [19, 20, 23] and the blood sampling period. No significant associations with birth weight, birth length, chest circumference, or head circumference were found for prenatal MEHP level in maternal blood in all infants, males or females.

Discussion

To our knowledge, this study was the first to focus on the potential effects of DEHP exposure on blood levels of TG and FA components in pregnant women, and to find significant inverse associations between DEHP principal metabolite MEHP levels, and TG, palmitic acid, oleic acid, LA and ALA levels in maternal blood after adjustment for confounders, using a Japanese pregnancy cohort. TG is an ester derived from glycerol and three FAs. Since palmitic acid, oleic acid and LA were predominant FA components, accounting for 82.6 % of the total FA pool, it was conceivable that these three FAs also similarly declined upon DEHP exposure, as TG decreased. These findings were quite similar to the effects of DEHP exposure on TG and FA levels in the plasma of prepartum mice in our earlier study [1, 17]. We also observed significant differences in maternal blood MEHP, TG, stearic/palmitoleic/oleic acid and DHA levels by blood sampling period (gestational weeks): MEHP, stearic acid and DHA were lower, whereas TG and palmitoleic/oleic acids were higher, in late gestation (35–41 weeks). Additionally, maternal plasma free FAs are an important source of essential FAs to the developing fetus [26]. Although MEHP exposure negatively correlated with TG and several FAs in maternal

Table 4 Regression coefficients (95 % confidence interval, CI) between $\log_{10}$-transformed MEHP level (nmol/ml) and TG/FA in maternal blood by blood sampling periods

Dependent variable	23–31 weeks ($n = 135$)		32–34 weeks ($n = 81$)		35–41 weeks ($n = 102$)	
	Crude model β^c (95 % CI)	Adjusted model β^c (95 % CI)	Crude model β^c (95 % CI)	Adjusted model β^c (95 % CI)	Crude model β^c (95 % CI)	Adjusted model β^c (95 % CI)
TG (mg/dl)[a]	−17.8 (−44.1, 8.5)	−19.9 (−46.3, 6.6)	−72.1 (−116.1, −28.0)	−71.3 (−115.0, −27.6)	0.7 (−38.7, 40.2)	5.0 (−34.9, 44.9)
Palmitic acid (μg/ml)[a]	−553.3 (−1033.4, −73.2)	−601.1 (−1080.5, −121.6)	−932.9 (−1482.1, −383.6)	−923.5 (−1488.1, −358.8)	−391.8 (−1062.3, 278.7)	−263.2 (−928.7, 402.3)
Stearic acid (μg/ml)[a]	−68.5 (−208.1, 71.0)	−75.7 (−216.69, 65.2)	−27.9 (−196.1, 140.2)	−26.0 (−199.0, 146.9)	−59.8 (−177.8, 58.2)	−44.2 (−163.0, 74.7)
Palmitoleic acid (μg/ml)[a]	−29.9 (−67.9, 8.1)	−32.2 (−70.6, 6.2)	−59.4 (−113.3, −5.4)	−58.9 (−114.2, −3.5)	−0.8 (−62.6, 61.0)	13.0 (−47.8, 73.8)
Oleic acid (μg/ml)[a]	−357.7 (−647.4, −67.9)	−385.1 (−675.3, −94.9)	−445.2 (−813.4, −77.1)	−450.0 (−827.2, −72.2)	−178.4 (−640.9, 284.1)	−101.1 (−564.1, 361.9)
LA (μg/ml)[a]	−385.2 (−630.9, −139.5)	−396.9 (−642.5, −151.4)	−370.5 (−661.7, −79.2)	−394.3 (−686.8, −101.8)	−323.8 (−645.8, −1.8)	−280.9 (−603.3, 41.6)
AA (μg/ml)[a]	−19.7 (−45.9, 6.4)	−19.5 (−45.3, 6.3)	−26.0 (−63.7, 11.6)	−26.7 (−65.3, 12.0)	4.4 (−21.5, 30.4)	6.6 (−19.6, 32.7)
ALA (μg/ml)[a]	−5.9 (−11.1, −0.7)	−6.1 (−11.3, −0.8)	−7.6 (−13.4, −1.9)	−7.8 (−13.6, −2.1)	−6.2 (−12.4, 0.0)	−4.8 (−10.8, 1.2)
EPA (μg/ml)[b]	−0.5 (−6.3, 5.2)	−1.0 (−6.7, 4.7)	−1.1 (−7.8, 5.7)	−1.1 (−7.8, 5.6)	2.9 (−2.2, 8.0)	3.0 (−2.3, 8.2)
DHA (μg/ml)[b]	1.0 (−14.1, 16.2)	1.0 (−14.0, 15.9)	−5.8 (−23.0, 11.4)	−6.7 (−24.8, 11.3)	10.8 (−1.7, 23.2)	11.9 (−0.9, 24.7)

AA arachidonic acid, *ALA* α-linolenic acid, *DHA* docosahexaenoic acid, *EPA* eicosapentaenoic acid, *FA* fatty acid, *LA* linoleic acid, *TG* triglycerides

[a] Adjusted for maternal age, smoking and alcohol intake during pregnancy

[b] Adjusted for maternal age, smoking and alcohol intake during pregnancy, inshore fish and deep-sea fish intake during pregnancy

[c] Partial regression coefficient represents the expected change in dependent variables as a result of a tenfold change in MEHP level, because MEHP level was $\log_{10}$-transformed

Table 5 Regression coefficients (95 % confidence interval, CI) between maternal blood $\log_{10}$-transformed MEHP concentration (nmol/ml) and infant birth outcomes

Dependent variable	Overall[a] ($n = 318$)		Male[b] ($n = 151$)		Female[b] ($n = 167$)	
	Crude model β^c (95 % CI)	Adjusted model β^c (95 % CI)	Crude model β^c (95 % CI)	Adjusted model β^c (95 % CI)	Crude model β^c (95 % CI)	Adjusted model β^c (95 % CI)
Birth weight (g)	−77.7 (−212.3, 56.8)	−62.6 (−189.6, 64.4)	−28.1 (−249.1, 192.9)	−27.5 (−249.4, 194.4)	−101.2 (−272.5, 70.0)	−74.6 (−232.6, 83.5)
Birth length (cm)	−0.098 (−0.801, 0.605)	0.081 (−0.593, 0.755)	−0.022 (−1.311, 1.268)	−0.014 (−1.283, 1.256)	−0.046 (−0.815, 0.723)	0.072 (−0.673, 0.816)
Chest circumference (cm)	−0.181 (−0.746, 0.384)	−0.149 (−0.691, 0.393)	0.010 (−0.830, 0.850)	−0.162 (−1.004, 0.681)	−0.271 (−1.048, 0.506)	−0.075 (−0.819, 0.669)
Head circumference (cm)	0.079 (−0.392, 0.550)	0.117 (−0.343, 0.578)	0.402 (−0.368, 1.172)	0.244 (−0.558, 1.046)	−0.013 (−0.582, 0.557)	0.037 (−0.551, 0.626)

[a] Adjusted for maternal age, height, weight before pregnancy, parity, smoking and alcohol intake status during pregnancy, annual household income, gestational age, infant gender and blood sampling period in a multiple linear regression model. For head circumference, adjusted model also included delivery type

[b] Adjusted for maternal age, height, weight before pregnancy, parity, smoking and alcohol intake status during pregnancy, annual household income, gestational age and blood sampling period in a multiple linear regression model. For head circumference, adjusted model also included delivery type

[c] Partial regression coefficient represents the expected change in dependent variables as a result of a tenfold change in MEHP level, because MEHP level was $\log_{10}$-transformed

blood, no significant relationships of MEHP with birth weight and birth size of newborns were detected in the present study.

Maternal hypertriglyceridemia appears late in normal pregnancy [27]. DHA status steadily declines after a temporary increase until 18 weeks of gestation in maternal blood throughout normal pregnancy [28]. Here, maternal blood TG and palmitoleic/oleic acid levels were indeed significantly higher, whereas DHA was lower in women during late gestation (35–41 weeks) as the blood sampling period proceeded. Maternal physiological changes that occur normally in early pregnancy but are most pronounced in the third trimester have the potential to alter xenobiotic distribution and elimination [29]. The lower maternal blood MEHP level in late gestation most likely corresponded with increased renal blood flow and glomerular filtration rate, and unchanged renal tubular resorption, which enhanced the clearance of MEHP through the kidney. Additionally, the activity of hepatic DEHP metabolizing enzymes may have changed during pregnancy, which warrants further study. Taking the various alterations in maternal blood TG/FA concentrations during pregnancy into consideration, we did not consider that great influences by increased plasma volume and total body water would be likely.

The hypolipidemic effects of DEHP were first described in rats and mice by Reddy et al. [30]. Other studies confirmed the hypotriglyceridemic effects of DEHP in rats [31–33] and pregnant mice [1]. Most importantly, and in keeping with our finding of hypotriglyceridemic effects after DEHP exposure in pregnant mice [1], we further found that dietary DEHP exposure significantly reduced plasma levels of palmitic acid, oleic acid, LA and ALA in these pregnant mice with the same experimental protocol [17]. In humans, the maternal FA/lipid homeostasis environment may have dramatic changes upon exposure to DEHP during pregnancy, e.g. the composition and distribution of maternal FAs in the blood. However, no information is available, even though exposure to DEHP is common in the general population. In the present study, it was noteworthy that after adjustment for confounders including the blood sampling period, MEHP levels inversely correlated with TG, palmitic acid, oleic acid, LA and ALA levels in maternal blood. Moreover, maternal blood MEHP level inversely associated with palmitic acid, oleic acid, LA and ALA at 23–31 weeks of gestation, as well as TG, palmitic acid, palmitoleic acid, oleic acid, LA and ALA at 32–34 weeks in multivariate models. Short chain FAs with less than 20 carbon atoms may be susceptible to DEHP exposure in pregnant women. Among them, ALA may be more sensitive to DEHP, even though it accounted for only 0.2 % of total FAs. These findings were quite similar to those from pregnant mice [1, 17]. On the other

hand, at 35–41 weeks of gestation, no such relationships between maternal blood MEHP and TG/some FAs were observed. This may be due to significant low concentrations of MEHP in blood compared to those of 23–34 weeks of gestation, which mitigates the effects of DEHP on TG or FAs. Of course, the exact reason must be warranted. Therefore, we could not conclude whether effects of DEHP exposure on maternal blood TG/some FA levels are related to the physiological status of pregnancy or not. To answer this question, similar epidemiological study using non-pregnant women is also required.

The effects of DEHP on TG and FA levels were observed at lower exposure level than that of birth defects: DEHP exposure decreased ALA concentration in blood of wild-type mice by 50 % at a dose of 0.01 % [17], however, birth defects were observed at 0.05 % DEHP [1]. Plasma MEHP concentrations of mice at 0.01 % DEHP exposure were 0.98 ± 0.36 nmol/ml (unpublished data). The concentrations of MEHP in blood of pregnant women were 0.049 ± 0.040 nmol/ml (0.039 nmol/ml of median, ranging from 0.007 to 0.316 nmol/ml) in the present study. Lipase activity for DEHP was fivefold higher in mice than that of non-pregnant women [34]. Body burden in the pregnant women with highest MEHP level may be roughly estimated to be similar to that of mice exposed to 0.01 % DEHP. Therefore, it may be plausible that DEHP at the exposure levels influenced some FA levels in blood of pregnant women of current study. Of course, further study is warranted to clarify the exact exposure level of pregnant women. Ait Bamai et al. [35] compared DEHP level in floor dust among several countries and reported that the levels in Sapporo, Japan, where the current study was conducted, were higher than those in USA, Germany and Denmark. Although it is questionable that whether DEHP levels in the floor dust reflects the exact body burden in humans, the subjects in the current study may be exposed to relatively high level of DEHP. The present study not only for the first time observed the hypolipidemic effects of DEHP exposure in pregnant women, but got such findings at a mean level of MEHP exposure (0.049 ± 0.040 nmol/ml, that is 0.014 ± 0.011 μg/ml), lower than a mean MEHP concentration of 0.68 ± 0.85 μg/ml reported in the human maternal plasma at term in healthy subjects [36].

We further considered the possible mechanisms by which maternal MEHP level negatively correlated with blood levels of TG and four individual FAs, although the present study did not conduct mechanism study. It is well-understood that administration of DEHP to rodents in vivo and in vitro produces pleiotropic response in the liver, which is responsible for hypolipidemic effects [1, 37, 38], especially, a hallmark response of peroxisome proliferation to DEHP in the liver [30], the activation of peroxisome proliferator-activated receptor α (PPARα)-dependent hepatic FA catabolism by DEHP exposure [39], and a decrease in microsomal triglyceride transfer protein-mediated TG transport from liver to the blood after DEHP exposure [1]. Conversely, limited DEHP-specific human data are available. Previous study further found that distinct from wild-type mice expressing PPARα in several organs including liver, DEHP exposure did not influence the plasma levels of palmitic acid, oleic acid, LA and ALA in pregnant humanized PPARα and PPARα knockout mice [17]. Humanized PPARα mice over-expressed human PPARα only in liver, suggesting that PPARα in other organs but not in liver or species difference of PPARα function may be involved in the influence of DEHP on blood TG and FAs in the pregnant women. It may be difficult to infer the mechanism of DEHP influences on TG or FAs in pregnant women from our previous animal studies [1, 17]. Thus, studies aimed at elucidating the mechanism by which DEHP exposure inversely correlated to TG and FA levels in blood of pregnant women are required.

The growth and development of the fetus and its organs depend on a sufficient supply of nutrients including FAs and lipids crossing the placenta, and fetal growth determines the birth outcomes of newborns [40]. Given the significant negative correlations of DEHP exposure with maternal blood TG and four FA levels in this Japanese pregnancy cohort, we further evaluated the effects of maternal DEHP exposure on term birth outcomes of newborns. However, we did not find any significant relationships to birth weight, birth length, chest circumference, or head circumference in univariate and multivariate linear regression analyses. No abnormal birth outcomes were noted in newborns whose mothers had a relatively greater exposure to MEHP (mean ± SD, 0.68 ± 0.85 μg/ml in maternal plasma) during the prenatal period in Italy [36]. However, under a higher maternal MEHP exposure (e.g. median, 2.9 mg/L in maternal blood with low body weight infants), the cord blood MEHP level was associated with low birth weight in a nested case–control study of Chinese newborns [12], where a higher median MEHP (2.5 mg/l) level was found in cord blood with low birth weight infants. These varied findings do not necessarily support the consideration of race difference. The insignificant association in the present study was primarily related to lower MEHP exposure level, which further lowered during late pregnancy. MEHP effects on fetal growth and infant birth outcomes may occur at a much higher exposure level. On the other hand, maternal plasma levels of AA, EPA and DHA during pregnancy were reported to be associated with birth weight, birth length or head circumference [23, 41], while maternal blood MEHP level had no influences on maternal blood levels of these long-chain polyunsaturated FAs in the present study, as a result, MEHP did not influence birth outcomes.

There are some limitations in this study. First, although this is a part of a prospective cohort study, the analyses of maternal blood MEHP, TG and FA levels are cross-sectional in nature. Second, confounders under consideration in multivariate linear regression analyses may not have been completed, e.g. second-hand smoke exposure was not assessed. Third, this study has a small sample size. Fourth, selection bias may have occurred because this cohort was based in one area hospital, which treated pregnant women in Sapporo and the surrounding areas. Fifth, this study measured MEHP alone which has a shorter half-life of elimination and tends to be influenced during sample collection and processing [8], but no oxidative metabolites of DEHP. Thus, the DEHP body burden in pregnant women might have been underestimated. In addition, pregnant women have several physiological changes: sex hormones may enhance TG/FA transport to muscles for oxidation; circulating TG is diverted to uptake by the mammary gland and by the placenta [27, 42]. Humans are exposed to a variety of environmental pollutants and chemicals which may elicit additive biological effects. These factors may cause an overestimated correlation between MEHP exposure and maternal blood TG/FA levels.

In conclusion, no adverse effects of maternal prenatal DEHP exposure on infant birth weight and birth size were observed. The hypotriglyceridemic effects of DEHP exposure in pregnant women were documented for the first time in this study, although at a lower exposure level. Furthermore, four individual FA levels in maternal blood inversely correlated with DEHP exposure. All these results raise concerns over the maternal blood FA/lipid homeostasis environment under ambient DEHP exposure during pregnancy, which warrants urgent investigation by epidemiological studies.

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Conflict of interest The authors declare that they have no competing interests.

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Association between Maternal Exposure to di(2-ethylhexyl) Phthalate and Reproductive Hormone Levels in Fetal Blood: The Hokkaido Study on Environment and Children's Health

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Abstract

Prenatal di(2-ethylhexyl) phthalate (DEHP) exposure can produce reproductive toxicity in animal models. Only limited data exist from human studies on maternal DEHP exposure and its effects on infants. We aimed to examine the associations between DEHP exposure *in utero* and reproductive hormone levels in cord blood. Between 2002 and 2005, 514 pregnant women agreed to participate in the Hokkaido Study Sapporo Cohort. Maternal blood samples were taken from 23–35 weeks of gestation and the concentration of the primary metabolite of DEHP, mono(2-ethylhexyl) phthalate (MEHP), was measured. Concentrations of infant reproductive hormones including estradiol (E2), total testosterone (T), and progesterone (P4), inhibin B, insulin-like factor 3 (INSL3), steroid hormone binding globulin, follicle-stimulating hormone, and luteinizing hormone were measured from cord blood. Two hundred and two samples with both MEHP and hormones' data were included in statistical analysis. The participants completed a self-administered questionnaire regarding information on maternal characteristics. Gestational age, birth weight and infant sex were obtained from birth records. In an adjusted linear regression analysis fit to all study participants, maternal MEHP levels were found to be associated with reduced levels of T/E2, P4, and inhibin B. For the stratified analyses for sex, inverse associations between maternal MEHP levels T/E2, P4, inhibin B, and INSL3 were statistically significant for males only. In addition, the MEHP quartile model showed a significant p-value trend for P4, inhibin B, and INSL3 decrease in males. Since inhibin B and INSL3 are major secretory products of Sertoli and Leydig cell, respectively, the results of this study suggest that DEHP exposure *in utero* may have adverse effects on both Sertoli and Leydig cell development in males, which agrees with the results obtained from animal studies. Comprehensive studies investigating phthalates' exposure in humans, as well as their long-term effects on reproductive development are needed.

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Data Availability: The authors confirm that, for approved reasons, some access restrictions apply to the data underlying the findings. Data are available from the Hokkaido University Center for Environmental Health and Sciences/Ethics Committee for researchers who meet the criteria for access to confidential data. Readers may contact Reiko Kishi, corresponding author and principle investigator of the study, to request the data.

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Introduction

Diesters of phthalic acid (phthalates) have been used as plasticizers for various plastic compounds, such as toys, food containers, furniture, personal care products, medical devices, and housing materials. Phthalates are not chemically bonded to polyvinyl chloride (PVC) in plastic products and, as a result, they can leach and migrate into the air, foodstuffs, and other materials. Consequently, humans are constantly exposed to phthalates and biomonitoring studies have shown the widespread exposure of the general population to these chemicals [1–4].

Di(2-ethylhexyl) phthalate (DEHP) is one of the major phthalate compounds and constitutes more than 50% of the phthalates used in production in Japan [5]. Phthalates are known to exert endocrine-disrupting effects, which have been the cause of some concern [6]. Animal studies have shown that fetal exposure to DEHP may induce abnormalities in the reproductive system,

reduce testosterone (T) and insulin-like factor 3 (INSL3) levels, and cause disruption to Leydig and Sertoli cell maturation [7–10].

In a study that looked at the effects of DEHP on humans, male study participants, who were mainly recruited from an infertility clinic and had elevated levels of phthalate metabolites in their urine, were found to exhibit lower sperm concentrations and T levels, higher levels of follicle-stimulating hormone (FSH), and have a higher incidence of damaged sperm DNA [11–16]. In female study participants the phthalate exposure level was associated with physical signs of puberty, such as breast enlargement, the growth of pubic and axillary hair, premature thelarche, and central precocious puberty [17–19].

However, epidemiological studies on the effects of DEHP exposure of infants *in utero* or in early life are limited. Swan et al. [20] indicated that maternal phthalate exposure was inversely related to the anogenital distance (AGI) of male infants and Huang et al. [21] reported that the levels of phthalates in amniotic fluid were inversely related to the AGI of female infants. Only two studies have examined the effects of phthalates on the reproductive hormone levels of infants. In one study maternal urinary metabolites were measured and the level of DEHP was found to be inversely correlated with free T (fT) and the fT/estradiol (E2) ratio in cord blood among female infants [22]. In another study reproductive hormone levels were measured from males with cryptorchidism and healthy control subjects at three months of age, as well as phthalate metabolites in breast milk. Phthalate metabolites (dimethyl phthalate (DMP), diethyl phthalate (DEP), and dibutyl phthalate (DBP)) positively correlated with the luteinizing hormone (LH)/fT ratio, and DEP and DBP showed positive correlations with steroid hormone-binding globulin (SHBG), while DBP was also found to inversely correlate with fT [23]. However, phthalate exposure has not been found to relate to cryptorchidism directly [23].

Although there is some evidence to suggest that fetal and neonatal phthalate exposure has an adverse effect on human reproductive development in both male and female infants, comprehensive studies are limited. Therefore, the aim of this study was to examine the associations between DEHP exposure *in utero* and reproductive hormone levels in cord blood in the general population in Japan.

Materials and Methods

Population

This prospective birth cohort study was based on the Sapporo Cohort, Hokkaido Study on Environment and Child Health [24,25]. Study details regarding the population, data collection, sampling of the biological specimens, and the contents of the questionnaire have been described elsewhere [24,25]. Briefly, native Japanese women living in Sapporo City or the surrounding areas were enrolled into the study at 23–35 weeks of gestation from July 2002 to October 2005 at the Sapporo Toho Hospital, which is an obstetrics and gynecology hospital in Sapporo, Hokkaido, Japan. Among the 1796 pregnant females approached, 25% were excluded as they were enrolled in the Japanese cord blood bank or delivered the baby at another hospital. Ultimately, 514 pregnant females were enrolled in this study (participation rate: 28.6%).

Assessment of exposure

Blood samples of approximately 40 mL were obtained from participants at the time of their hospital examination after recruitment. If the blood sample could not be taken during pregnancy due to maternal anemia, a blood sample was collected during hospitalization within a week after delivery. All samples were stored at −80°C until analysis. The concentration of mono(2-ehtylhexyl) phthalate (MEHP), which is the primary metabolite of DEHP, in the blood was determined. The method of Instrumental analysis, general method validation, and quality controls were previously described elsewhere with the following modifications of sample preparation in this study [26]. Blood samples (30 uL) were mixed with 120 uL 1N HCl, 350 uL saturated saline solution and 50 uL of 10 uM MEHP-d as an internal standard. MEHP was then extracted twice with 500 ul ethyl acetate after shaking for 15 min. The ethyl acetate layer was evaporated, and the sediments were dissolved into 40 uL ethyl acetate. After adding 20 uL N-methyl-N-(tert-butyldimethylsilyl) trifluoroacetamide (GL Sciences, Tokyo, Japan), the tube was left at room temperature for 60 min, and the MEHP tert-butyldimethylsilyl derivative concentration formed was measured by a GC-MS under the analytical conditions mentioned previously [26]. Under these conditions, the extraction recovery of MEHP was 95.6±1.9 (n = 6, mean±SD) [26]. Two ions, m/z 227 and 339 for quantification ion and confirmation ion, respectively, were used to detect MEHP [27]. The limit of detection (LODs) was 1 pmol/mL (0.278 ng/mL). MEHP levels in a tube containing the same medium as the reaction vial were measured to determine background levels. To exclude the possibility of environmental contamination of DEHP, all glassware used for MEHP measurements was heated at 200°C for 2 h. Ultimately, MEHP level was available in 493 maternal blood samples.

Outcome measures

At the time of delivery, a blood sample (10–30 mL) was collected from the umbilical cord and stored at −80°C until analysis. Concentrations of E2, total T, and progesterone (P4) were measured using liquid chromatography–tandem mass spectrometry (LC–MS/MS) [28,29]. An immunoradiometric assay (IRMA) was used to measure the concentrations of LH (Spac-S LH Kit, TFB, Inc., Tokyo Japan), FSH (Spac-S FSH Kit, TFB, Inc., Tokyo Japan), SHBG (IRMA-Count SHBG, Siemens, Berlin, Germany), and prolactin (PRL) (Spac-S Prolactin kit, TFB, Inc., Tokyo, Japan). The concentration of inhibin B was measured by an enzyme-linked immunosorbent assay (ELISA) (inhibin B Gen II ELISA, Beckman Coulter, Inc., CA, USA). The concentration of INSL3 was measured using an enzyme immunoassay (EIA) (insulin-like 3 (INSL3)/RLF (human) EIA kit, Phoenix Pharmaceuticals, Inc., CA, USA). Inhibin B is a marker of Sertoli cell function [30], and INSL3 is a major Leydig cells product and an early marker of the testicular descent during fetal [31], and All reproductive hormone measurements were performed at Aska Pharma Medical Co., Ltd (Kanagawa, Japan).

Questionnaire and medical record

The participants completed a self-administered questionnaire regarding information on maternal age, educational level, household income, smoking status, alcohol intake, and medical history. Maternal alcohol intake was classified into two categories: no, who never intake alcohol since the first trimester, and yes, who still drink alcohol after the first trimester [25]. Maternal smoking status during pregnancy was classified into two categories: non-smokers, who never smoked or quit smoking during the first trimester, and smokers, who still smoked after the first trimester [25]. Medical records were obtained at delivery for information regarding pre-pregnancy body mass index (BMI), pregnancy complications, gestational age, infant gender, parity, congenital anomalies, including hypospadias and cryptorchidism, and infant weight.

Table 1. Maternal mono(2-ethylhexyl) phthalate (MEHP) concentrations in relation to the characteristics of mothers and infants.

Characteristics		*n* (%)	Mean ± SD	MEHP (ng/mL)	
				Med. (25^{th}–75^{th})	*p*-value
Maternal characteristics					
Age at delivery (years)		202	29.8±4.9	Spearman's ρ=0.035	0.624[a]
Pre-pregnancy BMI (kg/m^2)		202	21.1±3.1	Spearman's ρ=0.002	0.978[a]
Parity	Primiparous	110 (54.5)		10.4 (5.65–15.3)	0.672[b]
	Multiparous	92 (45.5)		10.4 (6.08–15.5)	
Annual household income (million yen per year)	<5	142 (71.0)		10.1 (5.56–15.2)	0.177[b]
	≥5	58 (29.0)		11.7 (6.40–15.7)	
Educational level (years)	≦12	91 (45.0)		10.4 (5.94–14.4)	0.960[b]
	≥13	111 (55.0)		10.5 (5.68–15.5)	
Smoking during pregnancy	Nonsmoker	158 (78.2)		10.5 (6.01–15.6)	0.158[b]
	Smoker	44 (21.8)		7.80 (4.99–14.4)	
Alcohol consumption during pregnancy	Nondrinker	132 (65.3)		10.5 (6.08–16.2)	0.386[b]
	Drinker	70 (34.7)		10.2 (5.34–14.7)	
Type of delivery	Vaginal	202 (100)			
	Caesarian section	0 (0.0)			
MEHP (ng/mL)		202		10.4 (5.88–15.3)	-
Infant characteristics					
Sex	Male	93 (46.0)		10.2 (6.30–14.3)	0.734[b]
	Female	109 (54.0)		10.4 (5.60–16.3)	
Birth weight (g)		202	3138.6±331.3	Spearman's ρ=−0.023	0.376[a]
Gestational age (weeks)		202	39.5±1.0	Spearman's ρ=0.002	0.959[a]

[a]*p*-values were calculated by the Spearman's *ρ* test,
[b]*p*-values were calculated by the Mann–Whitney U test.
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Statistical analyses

From 514 participants, ten were excluded from the study due to miscarriage, stillbirth, relocation, or voluntary withdrawal from the study before delivery. There were 493 available maternal blood samples for MEHP measurements. Maternal blood samples collected during hospitalization after delivery were excluded from analysis due to the relatively short biological half-life of DEHP. Two hundreds and ninety-five infant cord blood samples were available for reproductive hormone measurements. Finally, 202 samples were included in the statistical analysis, for which both MEHP levels and reproductive hormone levels had been assessed.

In preliminary data analysis the association between MEHP exposure and the characteristics of mothers and infants were calculated by a Spearman correlation test and a Mann–Whitney U test. Associations between maternal MEHP exposure and infant reproductive hormone levels were first calculated with a Spearman correlation test, and then multivariable linear regression analysis was performed. MEHP levels and the concentration of reproductive hormones were converted to a log10 scale as their data did not fall into a normal distribution. To evaluate whether the relationship between hormone levels and MEHP exposure differs based on sex, a multivariable linear regression model for all study participants was also constructed with the interaction term for hormone levels to sex and MEHP interaction. To improve interpretability, the interquartile range (IQR) for the MEHP concentration and the least squares means (LSM) of log-transformed hormone levels were calculated and back transformed. Linear trends of LSM were tested by modeling IQR as a continuous variable. The first quartile was also compared to the 2nd, 3rd and 4th quartile MEHP, and the P values were adjusted using Bonferroni's correction. The limit of detection (LOD) was determined and half LOD values were used when levels were below the LOD for individual hormones. Inclusion of covariates was based on biological considerations and included: maternal age (continuous), maternal smoking during pregnancy (yes or no), maternal alcohol consumption during pregnancy (yes or no), gestational age (continuous), and the blood sampling week of gestation (continuous). All statistical analyses were performed using JMP pro 10 (SAS Institute Inc., NC, USA).

Ethical approval

The study was approved by the institutional ethical board for epidemiological studies at Hokkaido University Graduate School of Medicine, Hokkaido University Center for Environmental and Health Sciences, and Nagoya University Graduate School of Medicine, in accordance of with principles of the Declaration of Helsinki. All participants provided written informed consent.

Results

The characteristics of the participants included in the present study with the corresponding median MEHP concentrations (n = 202) are shown Table 1. In the present study, there were no cases of cryptorchidism or hypospadias included, and all infants were born vaginally. MEHP was detected in 100% of samples and the median concentration was 10.4 ng/mL (IQR: 5.88–15.3 ng/

Table 2. Distribution of reproductive hormone concentrations.

	All participants				Males				Females			
	n	Med	(25th–75th)	>LOD (%)	n	Med	(25th–75th)	>LOD (%)	n	Med	(25th–75th)	>LOD (%)
T (pg/mL)	202	85.2	(59.7–113.9)	100	93	97.7	(77.3–124.4)	100	109	70.3	(51.8–102.7)	100
E2 (ng/mL)	202	5	(3.56–7.28)	100	93	5.37	(3.62–6.63)	100	109	4.75	(3.39–6.69)	100
T/E2	202	16.7	(12.2–22.3)	n.d.	93	17.7	(12.9–23.2)	n.d.	109	15.5	(12.0–20.9)	n.d.
P4 (ng/mL)	202	227.3	(182.9–278.8)	100	93	233.8	(186.4–302.6)	100	109	216.9	(175.0–271.8)	100
LH (mIU/mL)	198	<LOD	(<LOD-<LOD)	15.7	91	<LOD	(<LOD-0.81)	33	107	<LOD	(<LOD-<LOD)	1
LH/T		n.d		n.d.	91	0.003	(0.002–0.011)	n.d.	107	n.d.		
FSH (mIU/mL)	197	<LOD	(<LOD-<LOD)	20.3	91	<LOD	(<LOD-0.65)	44	106	<LOD	(<LOD-<LOD)	0
SHBG (nmol/L)	202	15.8	(13.5–18.7)	100	93	16.4	(13.6–19.3)	100	109	15.5	(13.2–18.3)	99.4
T/SHBG	202	5.18	(3.71–7.52)	n.d.	93	5.77	(3.94–8.10)	n.d.	109	4.51	(3.49–6.67)	n.d.
PRL (ng/mL)	199	85.8	(62.0–116.0)	99.5	91	82.9	(66.4–116)	100	109	86.9	(56.7–118.3)	99.1
Inhibin B (pg/mL)	202	22.2	(<LOD-44.0)	58.9	93	43.6	(35.4–58.3)	100	109	<LOD	(<LOD-<LOD)	23.9
INSL3	110	0.27	(0.23–0.31)	100	91	0.28	(0.24–0.32)	100	20	0.18	(0.17–0.23)	100

E2, estradiol; FSH, follicle stimulating hormone; INSL3, insulin like factor 3; LH, luteinizing hormone; LOD, limit of detection; n.d., not determined; P4, progesterone; PRL, prolactin; SHBG, steroid hormone binding globulin; T, testosterone.

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Table 3. Correlations between MEHP concentrations and hormone levels.

	All participants		Males		females	
	ρ	p-value	ρ	p-value	n	p-value
T (pg/mL)	−0.091	0.198	−0.089	0.398	−0.107	0.269
E2 (ng/mL)	0.015	0.830	0.101	0.334	−0.035	0.716
T/E2	−0.086	0.224	−0.147	0.160	−0.043	0.660
P4 (ng/mL)	**−0.202**	**0.004**	**−0.218**	**0.036**	−0.184	0.056
LH (mIU/mL)	n.d.		−0.024	0.822	n.d.	
LH/T	n.d.		0.075	0.478	n.d.	
FSH (mIU/mL)	n.d.		0.205	0.052	n.d.	
SHBG (nmol/L)	−0.047	0.508	−0.037	0.722	−0.050	0.606
T/SHBG	−0.055	0.436	−0.045	0.668	−0.070	0.470
PRL (ng/mL)	**−0.229**	**0.001**	−0.119	0.260	**−0.301**	**0.002**
Inhibin B (pg/mL)	**−0.235**	**0.001**	**−0.474**	**<0.001**	n.d.	
INSL3 (ng/mL)	n.d.		**−0.241**	**0.022**	n.d.	

E2, estradiol; FSH, follicle stimulating hormone; INSL3, insulin like factor 3; LH, luteinizing hormone; MEHP, mono(2-ehylhexyl) phthalate; n.d., not determined; P4, progesterone; PRL, prolactin; SHBG, steroid hormone binding globulin; T, testosterone.

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mL). The concentration of MEHP was not significantly associated with any of the maternal or infant characteristics.

Table 2 shows the levels of reproductive hormones among male and female infants. For females, the detected percentage of LH, FSH, and inhibin B was 1%, 0%, and 24.1%, respectively, and thus, these hormones were excluded from further analysis. In addition, INSL3 was measured in only 20 samples from female infants and was consequently also omitted from further analysis.

The correlations between the MEHP levels and the concentrations of reproductive hormones are shown in Table 3. There were significant negative correlations between the MEHP level and P4, PRL, and inhibin B concentrations for all participants, P4, inhibin B, and INSL3 concentrations among male infants, and PRL concentrations among female infants. The results of our linear regression are shown in Table 4. MEHP level was inversely associated with T/E2 ratio, P4, and inhibin B concentrations in the linear regression model fit to all study participants, and the relationship between MEHP levels and these hormones was not statistically significant between males and females ($P_{interaction} > 0.05$). In the stratified analyses, inverse associations were statistically significant between MEHP level and T/E2 ratio, P4, inhibin B, and INSL3 concentrations among males, but not females.

The associations between MEHP and the levels of reproductive hormones in infants were assessed for potential non-linear relationships. The MEHP concentration was divided into four sections and the LSM of each hormone in each MEHP quartile is shown in Figure 1 and Figure 2 for all participants and male infants, respectively. The adjusted LSM hormone levels in relation to the MEHP quartile showed a significant p-value trend for T/E2 and P4 in the model fit to all study participants. Sex did not modified the association of either MEHP and T/E2 or P4 ($P_{interaction} > 0.05$). When compared to the LSM of the 1st MEHP quartile, the 4th MEHP quartile of T/E2, P4 significantly decreased, whereas the 2nd quartile of T/E2 significantly increased. The 3rd and 4th MEHP quartile of inhibin B significantly decreased when compared to the LSM of the 1st quartile, and inhibin B levels differ in the 2nd quartile in the interaction term for MEHP and sex ($P_{interaction} = 0.008$). For sex stratification, the adjusted LSM hormone levels in relation to the MEHP quartile showed a significant p-value trend for P4, inhibin B, and INSL3 in males. In addition, when compared to the LSM of the 1st MEHP quartile, the 4th MEHP quartile of P4, inhibin B, and INSL3 significantly decreased.

Discussion

In the present study maternal MEHP levels were found to be associated with reduced levels of T/E2, P4, and inhibin B in an adjusted analysis when the model was fit to all study participants. For the stratified analyses for sex, inverse associations between maternal MEHP levels and T/E2, P4, inhibin B, and INSL3 were statistically significant for males. INLS3 is a major product secreted by Leydig cells. The testosterone produced by Leydig cells is regulated by a negative feedback loop, which is controlled by the hypothalamic–pituitary–gonadal axis, and is chronically influenced by the long-term differentiation status of the cells [32]. On the other hand, INSL3 is constitutively expressed by Leydig cells, and is thus more advantageous over testosterone as a marker of Leydig cell differentiation [32]. Fetal exposure to phthalates and their effect on Leydig cell development has been the subject of several review papers that have examined evidence from experiment *in vitro* cell studies and animal models [33,34]. The results obtained from the present study are consistent with previous research.

In the multivariable linear regression model fit to all study participants, MEHP level was inversely associated with T/E2 ratio, and there was no statistical significance between males and females. Similar in Taiwan, Lin et al. [22] found that the fT concentration and fT/E2 ratio in cord blood were inversely correlated with two DEHP metabolites among females. In a Danish–Finnish cohort study, Main and co-workers measured phthalate monoesters in breast milk and found an inverse association between the level of monoesters and the concentration of testosterone, as well as positive associations with the levels of SHBG and the LH/fT ratio in males [23]. An inverse association between T/E2 and MEHP was significant in males but not in females suggesting a more pronounced effect in males than in females in this study. Interestingly, an inverted J-shaped curve was

Table 4. Adjusted linear regression coefficients (β) of reproductive hormone levels in cord blood in relation to MEHP.

	All participants (n = 202)					Males (n = 93)				Females (n = 109)			
	β	(95%CI)		p-value[a]	p for interaction[b]	β	(95%CI)		p-value[c]	β	(95%CI)		p-value[c]
T (pg/mL)	−0.147	−0.301	0.006	0.059	0.799	−0.130	−0.352	0.093	0.250	−0.133	−0.339	0.073	0.204
E2 (ng/mL)	0.024	−0.107	0.155	0.718	0.703	0.065	−0.151	0.282	0.549	0.001	−0.160	0.161	0.993
T/E2	**−0.171**	**−0.295**	**−0.048**	**0.007**	0.473	**−0.195**	**−0.365**	**−0.025**	**0.025**	−0.134	−0.306	0.038	0.126
P4 (ng/mL)	**−0.237**	**−0.401**	**−0.074**	**0.005**	0.771	**−0.311**	**−0.528**	**−0.095**	**0.005**	−0.200	−0.430	0.029	0.086
LH (mIU/mL)	n.d.					−0.088	−0.353	0.177	0.510	n.d.			
LH/T	n.d.					0.054	−0.324	0.432	0.777	n.d.			
FSH (mIU/mL)	n.d.					0.171	−0.020	0.361	0.078	n.d.			
SHBG (nmol/L)	0.013	−0.056	0.082	0.709	0.525	0.026	−0.058	0.109	0.542	−0.006	−0.106	0.093	0.900
T/SHBG	−0.160	−0.336	0.015	0.073	0.637	−0.156	−0.384	0.073	0.179	−0.127	−0.376	0.123	0.317
PRL (ng/mL)	−0.080	−0.201	0.041	0.194	0.290	−0.041	−0.181	0.099	0.563	−0.132	−0.312	0.048	0.150
Inhibin B (pg/mL)	**−0.288**	**−0.405**	**−0.170**	**<0.001**	0.970	**−0.276**	**−0.404**	**−0.148**	**<0.001**	n.d.			
INSL3 (ng/mL)	n.d.					**−0.156**	**−0.258**	**−0.054**	**0.003**	n.d.			

Reproductive hormones levels and MEHP concentration were log10-transformed and included in the model separately.

[a] adjusted for maternal age, smoking during pregnancy, alcohol consumption during pregnancy, gestational age, blood sampling week, infant sex, and interaction of sex and MEHP.

[b] P for interaction of sex and MEHP.

[c] adjusted for maternal age, smoking during pregnancy, alcohol consumption during pregnancy, gestational age, blood sampling week.

E2, estradiol; FSH, follicle stimulating hormone; INSL3, insulin like factor 3; LH, luteinizing hormone; MEHP, mono(2-ehylhexyl) phthalate; n.d., not determined; P4, progesterone; PRL, prolactin; SHBG, steroid hormone binding globulin; T, testosterone.

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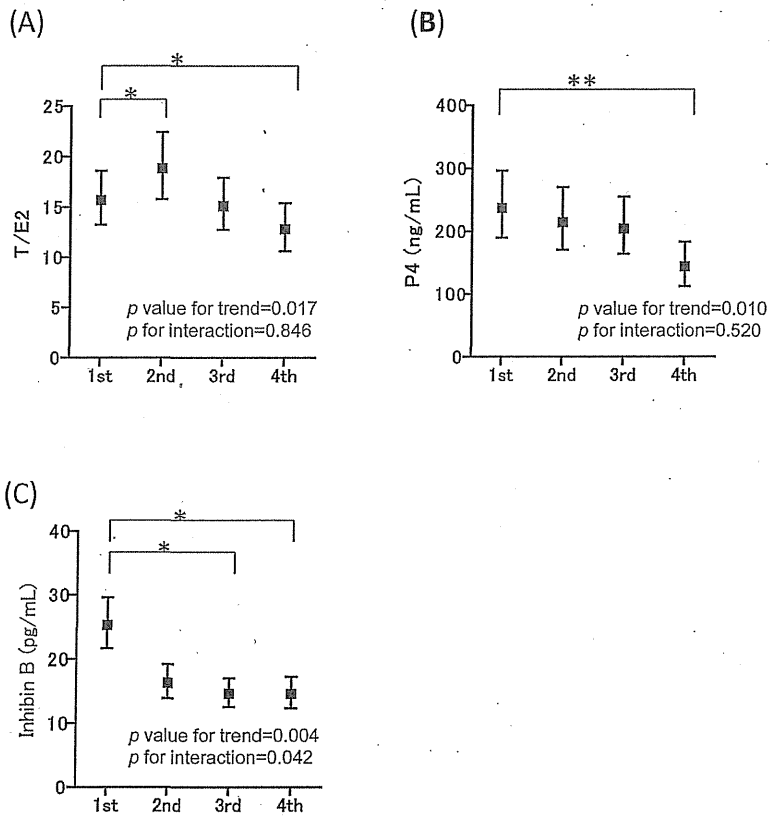

Figure 1. X-axis shows the MEHP quartiles, and Y-axis shows each hormone level. The adjusted LSMs (95% confident intervals) of each hormone level in cord blood in relation to the MEHP concentration quartile fit to all study participants are shown with *p*-value for trend, and *p* for interaction, respectively, for (**A**) T/E2 (0.017, 0.846), (**B**) P4 (0.010, 0.520), and (**C**) inhibin B (0.004, 0.042). First quartile (≦5.90 ng/mL) is also compared to the 2nd (5.91–10.39 ng/mL), 3rd (10.40–15.30 ng/mL) and 4th (15.31+ ng/mL) quartile MEHP. Statistical significance of the P value was $*p<0.017$, $**p<0.002$ based on Bonferroni's correction. When compared to the LSM of the 1st MEHP quartile, the 4th MEHP quartile of T/E2, P4, and the 3rd and 4th inhibin B significantly decreased, whereas the 2nd MEHP quartile of T/E2 significantly increased. LSMs were adjusted for maternal age, smoking during pregnancy, alcohol consumption during pregnancy, gestational age, and the blood sampling week, infant sex, and interaction of sex and MEHP.
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observed for T/E2 and MEHP quartile associations. Andrate et al. observed that DEHP exposure showed aromatase inhibition at low doses and stimulation at high doses in animal study [35]. However, the non-monotonic biological effect of DEHP exposure to aromatase activity within the range of MEHP quartiles in this study is questionable. In this study the MEHP level was found to be inversely related to P4 in infants. Previous *in vitro* research shown that MEHP can suppress steroidogenesis and down-regulate P4 production in MA-10 Leydig cell [36]. Reduced progesterone values in combination with normal testosterone values indicate that the steroidogenesis pathway from progesterone to testosterone is not affected in the DEHP exposure. Meanwhile, an association between MEHP concentration and testosterone level showed p values of 0.059 in all participants. Thus, insignificance of reducing testosterone maybe due to low statistical power, and thus, additional studies with larger sample size are needed.

Sertoli cells may also be the targets of reproductive toxicity induced by phthalate exposure *in utero* [37]. The MEHP level in maternal blood samples was inversely related to inhibin B in cord blood in males suggesting the fetal exposure of DEHP affected Sertoli cells in human infants, although in Main et al., the associations between phthalate monoesters and inhibin B were not clear [23]. These findings are in agreement with animal studies that have shown that neonatal exposure to DEHP reduces Sertoli cell numbers and proliferation in rodents [38,39]. The establishment of appropriate Sertoli cell numbers during development is critical for the production of sperm in adulthood [40]. Further study to evaluate the long-term effects of DEHP exposure *in utero* on testicular function should be considered. In this study, an inverse association between inhibin B and all participants was also observed, with the significant interaction term for MEHP and sex in quartile model. However, this significance may be due to the low detection rate of inhibin B in females. Therefore, more studies are needed to confirm these results.

The levels of MEHP detected in this study were slightly higher when compared to other populations, with the exception of one study carried out in Italy. For example, the median (IQR) MEHP levels in American adults (NHANES 1999–2000), elderly Swedish subjects, and pregnant women in Australia were 5.4 (3.4–8.9) ng/mL, 4.5 (2.0–15.5) ng/mL, and 1.18 (<LOD, 3.10) ng/mL, respectively [41–43]. One study performed with pregnant woman in Italy showed a mean MEHP concentration of 0.68±0.85 μg/mL [44], which is almost two orders of magnitude higher than

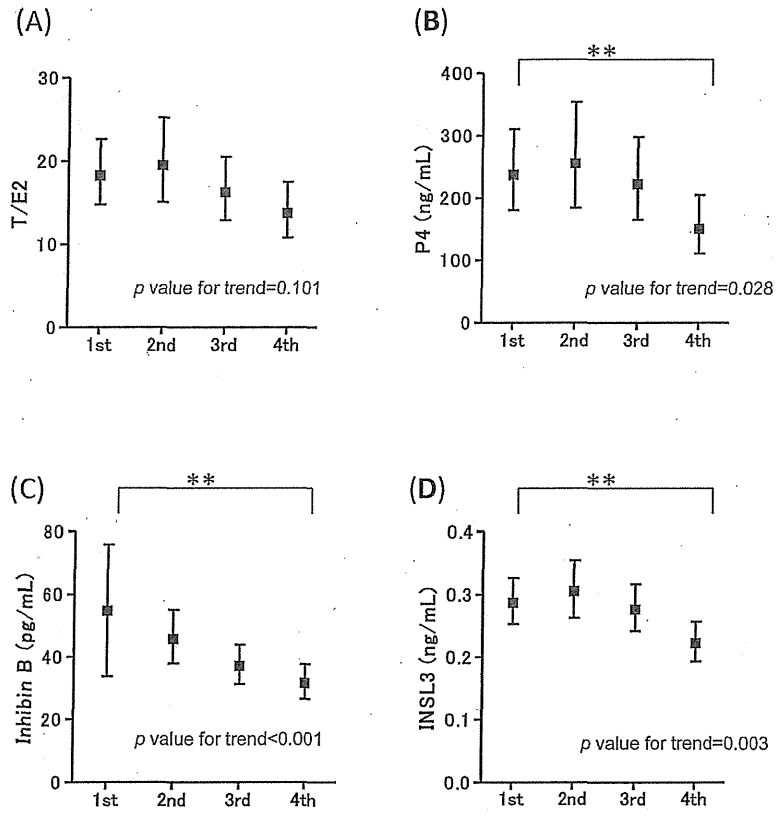

Figure 2. X-axis shows the MEHP quartiles, and Y-axis shows each hormone level. In males, the adjusted LSMs (95% confident intervals) of each hormone levels in cord blood in relation to the MEHP concentration quartile (*p*-value for trend) are (**A**) T/E2 (0.357), (**B**) P4 (0.028), (**C**) inhibin B (<0.001), (**D**) INSL3 (0.005). First quartile (≦6.36 ng/mL) is also compared to the 2nd (6.37–10.25 ng/mL), 3rd (10.25–14.28 ng/mL) and 4th (14.29+ ng/mL) quartile MEHP. Statistical significance of the P value was $*p<0.017$, $**p<0.002$ based on Bonferroni's correction. LSMs were adjusted for maternal age, smoking during pregnancy, alcohol consumption during pregnancy, gestational age, and the blood sampling week.
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what has been found in other studies, including the present work. However, the exposure levels of DEHP in this cohort are not externally comparative to previous studies due to the difference in measurement methods of each study. The majority of the recent studies assessed phthalate exposure based on urine samples. Unfortunately, urine samples were not available for the purposes of this cohort study.

It should be noted that, in the present study, only MEHP was measured, and this is a limitation to the study. MEHP is the primary metabolite of DEHP and there are several secondary metabolites, such as mono(2-ethyl-5-hydroxyhexyl) phthalate, mono(2-ethyl-5-oxohexyl) phthalate, mono(2-ethyl-5-carboxypentyl) phthalate, and mono(2-carboxymethylhexyl) phthalate [45–47]. In urine samples approximately 70% of detected phthalates are found in one of these four oxidized metabolite forms, whereas only 6% are found in the form of MEHP [47]. However, in blood samples, MEHP is detected in more than 80% of samples and the detection rates of the oxidized metabolites is less than 40% [48–50]. Therefore, the analysis of MEHP in blood does provide an indication of DEHP exposure. The associations between MEP and infant T and SHBG levels have been previously reported in three-month-old males, where MEP, monobutyl phthalate, and monobenzyl phthalate were found to be inversely related to the AGI [20,23]. Other phthalates, such as DEP, DBP, and butyl benzyl phthalate should also be considered in future studies.

It is also important to note that there may be potential contamination of blood samples with DEHP from medical devices, which are additional limitations of the present study. However, speed of diester to monoester conversion of DEHP is relatively longer [49] and the diester hydrolysis was not observed after one hour incubation at 37°C [51]. In this study, after blood samples were taken, they were handled at 4°C until storage at −80°C. In addition, all samples were collected at a single hospital so that any variation of medical devices used for withdraw blood would be low. Thus, although there is a possibility of DEHP contamination, the effect of *ex vivo* hydrolysis would have a low impact on the results. In addition, similar associations were found between the recorded levels of reproductive hormones and MEHP through the use of linear regression and quartile analysis, and this approach could be used in the future to minimize variations in MEHP exposure [14,41,52]. Consequently, the inverse associations observed between infant reproductive hormones and the concentration of MEHP in maternal blood should be considered relevant. Although it is unlikely to introduce a positive bias by sample contamination, future studies are needed to confirm the results.

Another limitation of this study is that the majority of maternal blood samples were taken during the third trimester of pregnancy. Therefore, the effects of fetal exposure to DEHP during the earlier stages of fetal development have not been directly assessed. In addition, the MEHP level was measured only once. However,