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Prenatal di-2-ethylhexyl phthalate exposure and cord blood adipokine levels and birth size: The Hokkaido Study on Environment and Children's Health

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Abstract

Di-2-ethylhexyl phthalate (DEHP) is one of the most widely used phthalates. Metabolites of DEHP are detectable in majority of the population. Findings on adverse health outcomes, particularly birth weight in association with prenatal exposure to DEHP remain equivocal. Besides, there is insufficient evidence to address influence on metabolic function from epidemiological studies. Thus, our objective was to investigate cord blood adipokine levels and birth size in association with prenatal DEHP exposure in prospective birth cohort study.

Mono-2-methylhexyl phthalate (MEHP), primary metabolite of DEHP was determined as exposure by using maternal blood sample of 3rd trimester. Leptin and adiponectin levels in cord blood were measured as markers of metabolic function. Birth weight and length were obtained from birth record. Association between maternal MEHP levels and cord blood adiponectin and leptin levels, birth weight and ponderal index (PI) were examined for 167 mother-child pairs who had both MEHP and cord blood adipokine measurements.

The median MEHP level was 8.81 ng/ml and the detection rate was 100%. There was no sex difference in MEHP levels. Both leptin and adiponectin levels were higher in girls than in boys. MEHP level was positively associated with adiponectin level among boys and was negatively associated with leptin level among girls. MEHP level were negatively associated with PI only in girls and this could be due to decreased leptin level.

This study suggested that prenatal DEHP exposure may be associated with cord blood adipokine and birth size. The influence potentially be sex-specific and could be more significant in girls.

Keywords: phthalate, birth weight, ponderal index, adiponectin, leptin

Abbreviations

EDCs, Endocrine disrupting chemicals; DEHP, di-2-ethylhexyl phthalate; MEHP, mono-2-ethylhexyl phthalate; PPARγ, peroxisome proliferator-activated receptor gamma; GC/MS, gas chromatography-mass spectrometry; LOD, Limit of detection; CV, Coefficient of variation; HMW, High molecular weight; ELISA, Enzyme Linked Immuno Sorbent Assay; RIA, Radioimmunoassay; BMI, Body mass index; PI, Ponderal index; IQR, Interquartile range; GM, Geometric mean; CI, Confidence Interval; MEHHP, mono-2-ethyl-5-hydroxyhexyl phthalate; ORs, Odds ratio;

1. Introduction

Di-2-ethylhexyl phthalate (DEHP) is one of the most widely used phthalates in variety of consumer products and is ubiquitous in the environment. DEHP can be inhaled through contaminated air or dust, ingested through food and dermally absorbed through personal care products (Hernandez-Diaz et al., 2009). DEHP is rapidly hydrolyzed to the corresponding monoester, mono-2-ethylhexyl phthalate (MEHP) (Fredericksen, 2007) and MEHP is considered the biologically active metabolite (Hauser, 2005). MEHP has been detected in majority of population (Suzuki et al., 2009; Woodruff et al., 2011; Casas et al., 2011; Romero-Franco et al., 2011).

Experimental data has shown that DEHP exposure alters lipid metabolism and adipogenesis (Grün and Blumberg, 2009). Phthalate exposure may potentially promote weight gain by binding to peroxisome proliferator-activated receptor gamma (PPARγ), which regulates fatty acid storage and glucose metabolism (Desvergne et al., 2009). We previously reported inverse correlation between maternal MEHP levels and maternal triglyceride (TG) and fatty acid (FA) levels (Jia et al., 2015). Yet, the role of prenatal exposure to DEHP on pregnancy outcomes such as birth weight and fetal metabolic related biomarkers were poorly understood and

inconsistent (Lenters et al., 2016; Zhang et al., 2009). Adverse birth outcomes possibly due to prenatal exposure to DEHP remain a public health concern. Particularly, developmental fetal exposure to DEHP is of special concern as the fetal time period is crucial window for adipocyte development (McMillen et al., 2005; Newbold 2010; Hatch et al., 2010).

Adipocyte-produced hormones including adiponectin and leptin have been used as biomarkers of fetal metabolic function. The roles of these hormones in metabolic homeostasis and regulation, recently have been recognized (Fiaschi et al., 2014; Farooqi et al., 2014). Studies have suggested that both too much and too little leptin in fetus result in non-optimal fetal growth phenotypes that subsequently increase long term obesity risk (Ornoy, 2011). Chemical exposures during fetal period may change growth and weight gain trajectory and may influence the risk of obesity in later life or may cause long lasting metabolic disorders because it is known that fetal period is a critical window of development of adipocyte (Hatch et al., 2010). It has been known that high cord blood leptin levels have been positively associated with birth weight (Karakosta et al., 2011) whereas low levels of cord blood leptin have been associated with small for gestational age (SGA) (Romano et al., 2014). It was reported that child adiponectin levels at birth and birth weight were unrelated

(Volberg et al., 2013), while the other reported that cord blood adiponectin levels were positively associated with birth weight (Mantzoros et al., 2009) and lower adiponectin was associated with preterm birth and SGA (Yeung et al., 2015). There was a progressively significant negative association between adiponectin and BMI at 2, 5, and 9 years of age (Volberg et al., 2013). In adults, low adiponectin levels are the implication of obesity, metabolic syndrome and type 2 diabetes (Mather and Goldberg, 2014).

Despite the importance of understanding influence of fetal exposure to phthalates on fetal metabolic outcomes, human data is lacking. To our knowledge, investigation of prenatal phthalates exposure and cord blood adipokine was still very limited and not investigated in association with birth size. Thus investigation of health effects of fetal phthalates exposure on birth size and metabolic function is warranted. The objective of this study was to assess the association between DEHP exposure and cord blood adipokine, birth weight and ponderal index (PI).

2. Material and methods

2.1 Study population

This prospective birth cohort study was based on the Sapporo Cohort, the Hokkaido Study on Environment and Children's Health (Kishi et al., 2011, 2013). The

Sapporo Cohort is an ongoing cohort study that began in 2002. Briefly we recruited pregnant women at 23-35 weeks of gestation between July 2002 and October 2005 from the Sapporo Toho Hospital in Hokkaido, Japan. 514 women agreed to participate in the cohort study. All subjects were residents in Sapporo City or surrounding areas. The participants completed the self-administered questionnaire during their pregnancy. The questionnaire contained baseline information including their dietary habits, exposure to chemical compounds in their daily life, smoking history, alcohol consumption, caffeine intake, family income, educational levels of themselves and partners. Maternal anthropometric measurement data and medical history were obtained from medical record and birth outcomes such as birth weight and length were collected from birth records. We used the following criteria to include the participants into the analyses; singleton baby born at term (37-42 weeks of gestation). Participants with no blood available for MEHP analysis (n=10) or those with blood collected during 2^{nd} trimester (n=31) or after delivery (n=130) were excluded because of the MEHP short half-life. The final regression analyses were conducted for 167 participants who had both MEHP and adipokine measurements (Figure 1). This study was conducted in accordance with the Declaration of Helsinki, and the protocol used in this study was approved by the Institutional ethical board

for epidemiological studies at the Hokkaido University Graduate School of Medicine and Hokkaido University Center for Environment and Health Sciences and Ethics Review Committee of Nagoya University Graduate School of Medicine. This study was conducted with the informed consent of all participants in written forms.

2.2 Measurement of MEHP

The concentrations of MEHP were measured in maternal serum samples collected at 3rd trimester of their pregnancy. The mean gestational age of blood sampling was 34.3 weeks (minimum 29.9 weeks and maximum 40.9 weeks). Approximately 40 mL of maternal blood samples were collected from each woman and samples were stored at -80℃ until the analysis. The measurement was carried out by using gas chromatography-mass spectrometry (GC/MS) at Nagoya University under the analytical conditions mentioned previously. The detailed method can be found elsewhere (Araki et al., 2014; Jia et al., 2015). The limit of detection (LOD) was 0.278 ng/ml (1 pmol/ml). All glass wares were heated at 200℃ for 2 hours to exclude the possibility of environmental contamination. Additionally, we confirmed that there was no background level MEHP detected from the equipment used for the GC/MS analysis. 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 (Jia et al., 2015).

2.3 Markers of metabolic function in cord blood

Total and high molecular weight (HMW) adiponectin and leptin levels in cord blood were measured in 167 and 162 neonates, respectively. Adiponectin levels were determined by Enzyme Linked Immuno Sorbent Assay (ELISA) using Human Adiponectin Assay kit from Sekisui Medical Co. Ltd (Tokyo, Japan). Leptin levels were determined by Radioimmunoassay (RIA) using Human Leptin RIA kit from Linco Research Inc. (St. Charles, MO, USA). All the analyses were conducted at LSI Medience (Tokyo, Japan) according to the operation manual. Analyses were repeated for all samples with CV greater than 15 %. The LODs of adiponectin was 0.39 μg/ml and of leptin was 0.5 ng/ml. All samples were in the range of detection. Intra- and inter-assay CVs for total adiponectin were < 9.1% and <10.1%, for HMW adiponectin were <9.2% and <11.6% and for leptin were < 5.3% and < 8.1%, respectively.

2.4 Data analysis

To consider potential confounding variables, we used data from medical record at birth and questionnaires. Maternal MEHP levels did not distribute normally, thus MEHP levels were $\log_{10}$ transformed for linear regression analyses. Total and

HMW adiponectin levels distributed normally, thus the measured levels were used without any transformation for statistical analyses. As distribution of leptin was right skewed, concentrations were $\log_{10}$ transformed for statistical analysis. PI which was calculated as follows; PI(kg/m^3) = Birth weight (kg)/(Birth length (m))^3. The final linear regression model was adjusted for maternal body mass index (BMI), maternal blood sampling period (weeks), child sex, and gestational age (days).

All the analyses were conducted for boys and girls combined as well as boys and girls separately. Results were considered significant at $p < 0.05$. All analyses were conducted using SPSS Version 22.0J.

3. Results

Maternal characteristics were shown in Table 1. In addition, we obtained data of maternal smoking, alcohol and caffeine intake during pregnancy, and examined them in association with cord blood adipokine levels. These factors were not associated with either adiponectin or leptin levels.

Table 2 shows maternal MEHP levels, infant anthropometric measurement and cord blood adipokine levels at birth. The median level of MEHP was 8.81 ng/ml with 100% of detection rate. The median level of maternal MEHP was not significantly different between boys and girls. There was a weak trend of decreasing.

MEHP levels as blood sampling period got later, however, the trend was not significant (Spearman's ρ= -0.138, p=0.075).

The mean birth weight was 3162 g and PI was 27.6 kg/m^3. The mean gestational age was 279.3 days. Total and HMW adiponectin levels were positively correlated to PI with significance. Leptin level was positively correlated to both birth weight and PI. Both adiponectin and leptin levels were not significantly correlated to gestational age.

The median levels of total, HMW adiponectin and leptin were 19.2 μg/ml, 12.8 μg/ml and 6.0 ng/ml, respectively. The median levels of total, HMW adiponectin and leptin were significantly higher in girls than in boys ($p = 0.048$ for total adiponectin, $p = 0.028$ for HMW adiponectin, $p < 0.001$ for leptin, respectively).

The association between maternal MEHP levels and cord blood adipokine levels was shown in Table 3. After adjusting with covariates including maternal BMI, blood sampling period (weeks) and gestational age (days), MEHP levels were positively associated with total and HMW adiponectin levels among boys ($\beta = 4.63$, 95% Confidence Interval (CI); 0.77, 8.49, $\beta = 3.64$, 95% CI; 0.44, 6.84, respectively). MEHP levels were negatively associated with leptin levels among girls

(β = -0.31, 95% CI; -0.52, -0.10).

The association between maternal MEHP levels and birth weight and PI were shown in Table 4. Birth weight was not associated with MEHP levels. For PI, negative association was observed overall (β = -1.28, 95% CI; -2.43, -0.13) and after stratification by child sex, significance was observed only among girls (β = -1.88, 95% CI; -3.50, -0.26).

4. Discussion

In this study we found that MEHP level was positively associated with adiponectin levels among boys and was negatively associated with leptin level among girls. MEHP levels were negatively associated with PI and the association was significant only in girls and this could be due to decreased leptin level. The results indicated that prenatal DEHP exposure may be associated with cord blood adipokine and birth size and it may be sex-specific, and could be more significant in girls.

4.1 DEHP exposure level

Compared to the study of serum MEHP measurements of pregnant women at 18 weeks (median=1.18 ng/ml), our result showed higher level (Hart et al., 2014). Generally, the levels of phthalate metabolites were considerably higher in urine samples (Frederiksen et al., 2010). MEHP level in maternal blood in this study

was lower compared to urinary MEHP levels of pregnant women in the U.S. (geometric mean=12.7 ng/ml specific gravity–corrected) (Cantonwine et al., 2016), however, was higher compared to urinary MEHP levels of pregnant women from other studies of Canada and Danish (Arbuckle et al., 2016; Jensen et al., 2016). Maternal dietary changes during pregnancy could be responsible since diet was major route of DEHP exposure (Schettler, 2006). In addition, difference in levels of DEHP production and usage between countries may explain the difference. In fact, DEHP intake in Japanese population was higher than that of most other studies (Ait Bamai et al., 2015).

4.2 Adipokine levels in cord blood

Adiponectin and leptin levels in this study were similar levels to those from Japanese study (Nakano et al., 2012). Also our observed levels of adipokine were close range to the reported levels in Taiwan and Korea (Chou et al., 2011; Kim et al., 2016). Leptin levels in our study were lower compared to the recently reported levels from Canadian study (Ashley-Martin et al., 2014). Adiponectin levels in cord blood from the previous studies showed relatively higher levels in Caucasian population (Brynhildsen et al., 2013; Luo et al., 2013; Lagiou et al., 2013) compared to Chinese population (Lagiou et al., 2013). As previously reported (West et al.,

2014), leptin and adiponectin levels vary among ethnicities, and in adult study Asian population showed lower adiponectin levels compared to those of European people (Mente et al., 2010). Relatively lower levels of adiponectin and leptin observed in our study of Japanese population was consistent with previously reported observations.

4.3 Adipokine and birth size in association with DEHP exposure

Positive association between maternal MEHP levels and adiponectin levels in boys was found in this study. Additionally, negative association between MEHP levels and leptin levels was found only in girls. There are limited previous studies regarding prenatal phthalate exposure and adipokine levels in cord blood. One prospective study found that maternal urinary MEHP level was not associated with fetal adipokine levels (Ashley-Martin et al., 2014). The other study showed only cross-sectional result of no significant association between ΣDEHP (sum of MEHHP and MEOHP) in newborn's urine and cord blood leptin levels (Kim et al., 2016). Previous prospective studies have shown no significant association between prenatal phthalate exposure and birth weight (Wolff et al., 2008; Suzuki et al., 2010; Philippat et al., 2012; Casas et al., 2015) and we have added the evidence of no association between maternal phthalate levels and birth weight. However, the influence of prenatal DEHP exposure on PI has not been investigated in previous

studies. PI is commonly used to examine body density and adiposity of infants thus it is ideal to examine PI as well as birth weight for deep understanding of birth size. Besides, none of these previous studies investigated association between prenatal phthalate exposures and metabolic function by using biomarkers.

In addition to our previous findings of reduced maternal FA/TG levels in association with maternal MEHP levels (Jia et al., 2015), this study showed association between maternal MEHP levels on cord blood adipokine. Yet relationship between and adverse influence on neonate adipokine levels and PI is still unknown.

We observed decreased leptin in association with maternal MEHP levels in girls. Sex-specific effects of DEHP exposure on metabolic biomarkers are largely unknown. Previously we reported associations of prenatal DEHP exposure with sex steroid hormone (Araki et al., 2014) and thyroid hormone levels (Minatoya et al., 2016) in sex-specific manner. This study added more evidence to support that prenatal DEHP exposure has influence as an endocrine disruptor on child health outcomes.

4.4 Limitations

First of all, using the data from one-time exposure measurement may not accurately reflect exposure of whole pregnancy period due to short half-life of DEHP.

Secondly, we used blood samples for exposure assessment though many researchers in this field advocated the use of urine samples for the assessment of phthalate exposure in humans as urine samples can avoid the influence of external contamination. Unfortunately, urine samples were not available in our study. In our study, blood samples were immediately stored at -80 ℃ and acid was added to the samples right after thawing to inhibit enzyme activity of converting external contamination to MEHP (Kato et al., 2003). Besides, we measured background levels of MEHP and confirmed that the influences of external contamination were null. Lastly, statistical analysis was conducted for those who had both of MEHP levels and cord blood adipokine levels, therefore, potential selection bias might exist. However, comparison between whole population (n=469) and participants with both MEHP and adipokine measurements (n=167) showed no differences in maternal characteristics. Thus, selection bias was not a major concern. Findings from this study may be applicable to whole cohort population. Yet, we were not able to eliminate the possibility of the influence of unmeasured co-exposures including other endocrine disrupting chemicals. Experimental studies have suggested that endocrine disrupting chemicals such as bisphenol A and polychlorinated biphenyl 95 may disrupt fetal thyroid-adipokine (Ahmed 2013, 2016). In our future work, other

environmental chemicals such as dioxins and perfluorinated compounds that we previously found negative association with birth weight (Konishi et al., 2009; Washino et al., 2009) should be investigated in association with neonate adipokine.

Conclusions

This study suggested that prenatal DEHP exposure may be associated with cord blood adipokine levels and birth size. The influence potentially be sex-specific and could be more significant in girls. Changes in cord blood adipokine levels and PI may relate to postnatal growth trajectory and may link to increase the risk of developing cardiovascular and metabolic diseases in adulthood.

Conflict of interest

The authors declare no conflict of interest.

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Table 1 Maternal characteristics of participants.

Characteristics	**% or mean (SD)**
Age at delivery (years)	30.0 (4.8)
Pre-pregnancy BMI (kg/m^2)	21.0 (3.1)
Smoking Never/quit before pregnancy	54.5%
Quit after finding pregnancy	26.9%
Current smoker	18.5%
Education ≦ High School	44.9%
≧ College	55.1%
Family income (yen) < 5M	72.5%
≧ 5M	26.9%

Table 2 Maternal MEHP levels, infant anthropometric measurement and cord blood adipokine levels at birth stratified by child sex.

		All		Boys		Girls	
	n	**Mean (SD) or median (IQR)**	**n**	**Mean (SD) or median (IQR)**	**n**	**Mean (SD) or median (IQR)**	**p-value[a)]**
MEHP (ng/ml)	167	8.81 (5.45-13.3)	81	8.49 (5.49-13.0)	86	10.1 (5.44-13.8)	0.682
Birth weight (g)	167	3162 (320)	81	3175 (306)	86	3150 (334)	0.608
Ponderal index (kg/m^3)	166	27.6 (2.2)	80	27.2 (2.0)	86	27.9 (2.3)	0.023
Gestational age (days)	167	279.3 (6.2)	81	278.7 (5.5)	86	279.8 (6.7)	0.258
Total adiponectin (μg/ml)	167	19.2 (15.5-22.6)	81	18.9 (14.8-22.2)	86	20.0 (16.4-23.5)	0.048
HMW adiponectin (μg/ml)	167	12.8 (9.8-15.4)	81	11.7 (9.6-14.6)	86	13.5 (10.3-16.7)	0.028
Leptin (ng/ml)	162	6.0 (3.7-10.2)	79	4.8 (3.4-6.9)	83	8.2 (4.6-13.1)	< 0.001

[a)] p-value was obtained by student's t-test or Mann-Whitney's U test. p-value between boys and girls.

HMW; high molecular weight

Table 3 Regression coefficients (95% CIs) between $\log_{10}$ transformed MEHP levels and the levels of adipokines in cord blood.

	All		Boys		Girls	
Outcomes	**β[a), c)]**	**95% CI**	**β[b), c)]**	**95% CI**	**β[b), c)]**	**95 % CI**
Total adiponectin (μg/ml)	0.32	-2.67, 3.30	4.63*	0.77, 8.49	-2.94	-7.42, 1.54
HMW adiponectin (μg/ml)	0.38	-2.05, 2.81	3.64*	0.44, 6.84	-2.05	-5.68, 1.57
Leptin (ng/ml)	-0.12	-0.27, 0.04	0.11	-0.10, 0.32	-0.31*	-0.52, -0.10

Leptin levels were $\log_{10}$ transformed.

[a)] Adjusted for maternal BMI, blood sampling period and child sex

[b)] Adjusted for maternal BMI and blood sampling period

[c)] For analysis of leptin, gestational age was additionally adjusted.

* $p < 0.05$.

HMW; high molecular weight

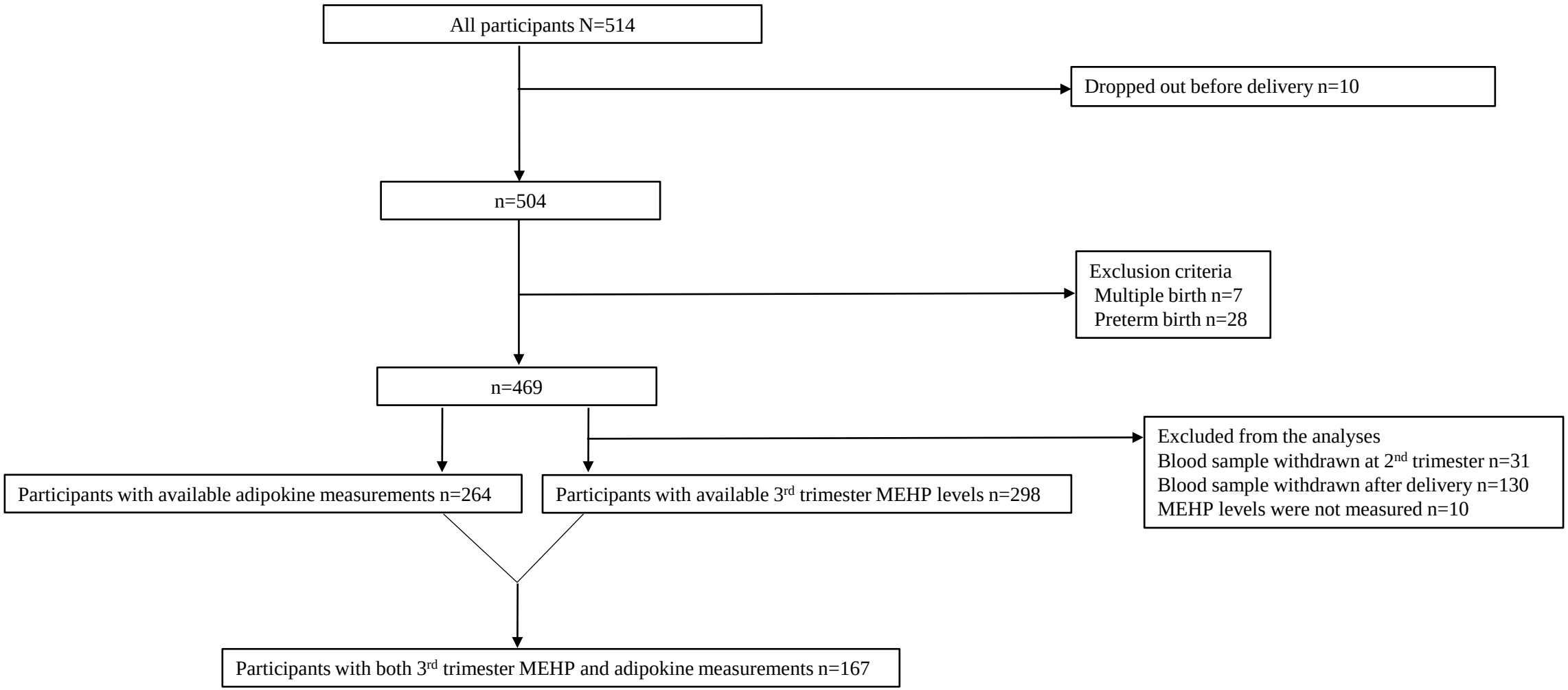
Table 4 Regression coefficients (95% CIs) between $\log_{10}$ transformed MEHP levels and birth weight and ponderal index.

	All		Boys		Girls	
Outcomes	**β[a)]**	**95%CI**	**β[b)]**	**95%CI**	**β[b)]**	**95%CI**
Birth weight (g)	-1	-171, 170	204	-49, 457	-153	-392, 87
Ponderal Index (kg/m^3)	-1.28*	-2.43, -0.13	-0.78	-2.47, 0.91	-1.88*	-3.50, -0.26

[a)] Adjusted for maternal BMI, blood sampling period, gestational age and child sex

[b)] Adjusted for maternal BMI, blood sampling period and gestational age

*$p < 0.05$.


All participants N=514
Dropped out before delivery n=10
n=504
Exclusion criteria
Multiple birth n=7
Preterm birth n=28
n=469
Excluded from the analyses
Blood sample withdrawn at 2nd trimester n=31
Blood sample withdrawn after delivery n=130
MEHP levels were not measured n=10
Participants with available adipokine measurements n=264
Participants with available 3rd trimester MEHP levels n=298
Participants with both 3rd trimester MEHP and adipokine measurements n=167