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分担研究報告書

Prenatal MEHP, BPA exposure and cord blood adipokine levels

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研究要旨

There is a growing interest in the possibility of endocrine disrupting chemicals (EDCs) such as bisphenol A (BPA) and phthalates may contribute to obesity. However, there has been insufficient research addressing the obesogenic potential of prenatal exposure to EDCs in epidemiological studies. Thus, ours objective was to investigate fetal adipokine levels, birth weight in association with prenatal exposure to DEHP and BPA in prospective birth cohort study. MEHP levels in maternal blood in late pregnancy and BPA levels in cord blood were measured. Leptin and adiponectin levels in cord blood were measured as markers of metabolic function. Association between MEHP and BPA levels and fetal leptin and adiponectin levels, birth weight, were examined. Leptin and adiponectin levels were significantly higher among girls than boys. HEHP level was positively associated with adiponectin levels among boys and was negatively associated with leptin level among girls. This study suggested that prenatal DEHP exposure may have adverse influence on fetal adipokine levels but not on birth weight and the influence may potentially be sex-specific.

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A．研究目的

Obesity is known to closely link to physical activity and diet, however, recent research suggests that other factors can contribute to obesity etiology [1]. In 2006, the term “obesogen” was coined by Grün and Blumberg [2] and defined as “molecules that inappropriately regulate lipid metabolism and adipogenesis to promote obesity”. From growing number of in vivo and in vitro studies, endocrine disrupting chemicals (EDCs) have been considered as obesogens [3, 4]. EDCs including bisphenol A (BPA) and phthalates are ubiquitous in the environment and have been detected in majority of population [5-8]. Experimental data have shown that phthalate and BPA exposure alters lipid metabolism and adipogenesis (Grun, 2009 #109).

BPA is used in the manufacture of plastics and resins including food and drink containers, and as an additive in thermal paper, dental sealant, medical equipment and flame retardant [9, 10]. The predominant source of BPA exposure for general adult population is diet [11]. The effects of BPA on metabolic function by inhibiting adiponectin release from human adipose tissue have been reported [12].

Phthalates are group of chemicals widely

used in consumer products including personal care products as well as in industry for plasticizers [13]. In particular, the metabolite of di(2-ethylhexyl) phthalate (DEHP), one of the most commonly used plasticizer, mono(2-ethylhexyl) phthalate (MEHP), were widely detected in human urine and blood samples [14]. Phthalate exposure may potentially promote weight gain by binding to peroxisome proliferator-activated receptor gamma (PPAR-γ), which regulates fatty acid storage and glucose metabolism [15].

Although adult exposure to EDCs is important, developmental fetal exposure to EDCs is of particular concern [16-18] as the fetal time period is particularly crucial window for adipocyte development [19].

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 [20, 21]. For fetus, leptin signals that existing fat depots are sufficient [22]. Studies have suggested that both too much and too little leptin in fetus results in non-optimal fetal growth phenotypes that subsequently increase long term obesity risk [23]. Chemical exposures during fetal period may change growth and weight gain trajectory and may influence on 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 [18]. It has known that high cord blood leptin levels have been positively associated with birth weight [24] whereas low levels of cord blood leptin have been associated with small for gestational age [25]. Cord blood adiponectin levels were positively associated with birth weight [26].

There are only a few studies regarding prenatal BPA and phthalates exposure and cord blood adipokines [27-29]. Thus investigation of health effects of fetal BPA and phthalates exposure on metabolic function is warrant.

Thus, the objective of this study was to assess the association between maternal MEHP and cord blood BPA levels and fetal adipokines levels and birth weight.

B．研究方法

Study population

This prospective birth cohort study was based on the Sapporo Cohort, Hokkaido Study on Environment and Children's Health [30, 31]. 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. All subjects were resident in Sapporo City or surrounding areas. The participants completed the self-administered questionnaire survey after the second trimester 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. The prenatal information of the mothers and their neonates was collected from their medical records. This study was conducted with the informed consent of all participants in written forms. 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.

Measurement of MEHP and BPA

The concentrations of MEHP were measured in maternal serum samples collected after the second trimester of their pregnancy. 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 chromatography-mass spectrometry (GC/MS) at Nagoya University under the analytical conditions mentioned previously [32]. 30 µl of blood samples were mixed with 120 µl of 1N HCl to deactivate the serum enzymes, 350 µl of saturated saline solution and 50 µl of 10 µM MEHP-d as an internal standard. Then MEHP was extracted two times with 500 µl of ethyl acetate after shaking for 15 minutes. No incubation process until extraction. The ethyl acetate layer was evaporated then the residue was dissolved into 40 µl of ethyl acetate. After addition of 20 µl of N-methyl-N-(tert-butyldimethylsilyl) trifluoroacetamide (GL Sciences, Tokyo, Japan), the reaction was left for 60 minutes at room temperature. The concentration of MEHP tertbutyldimethylsilyl derivative was measured by GC/MS (6890N, 5973N; Agilent Technologies, CA, USA). Two ions, m/z 227 as quantification ion and 339 for confirmation ion, were used to detect MEHP [33]. The limit of detection (LOD) was 0.278 ng/ml (1 pmol/ml). For each sample, duplicate analysis was performed. Ultimately, MEHP level was available from 493 samples. To determine background levels, MEHP levels in a tube containing the same medium as the reaction vial were measured. All glass wares were heated at 200℃ for 2 hours to exclude the possibility of environmental contamination. 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 [34].

The concentration of BPA in cord blood was measured by using isotope dilution-liquid chromatography-tandem mass spectrometry (ID-LC/MS/MS) at IDEA Consultants, Inc. Briefly cord blood was obtained at delivery and stored at -80℃ until analysis. 1.0 mL whole blood was spiked with Bisphenol A-d16 as an internal standard. After addition of 0.2 M acetate buffer (pH 5.0) and β-glucuronidase, the sample was held in an incubator at 37°C for 5 hours. The diluted sample was applied to a solid-phase extraction column (ISOLUTE multimode (500 mg/3 mL) cartridges) from Biotage (Biotage Japan, Tokyo, Japan). BPA was extracted using acetonitrile. Then, BPA-d4 was added to the extract as an internal standard. The organic extract was concentrated and the sample was analyzed by ID-LC/MS/MS (Agilent 1100 liquid chromatograph, API 4000 Q Trap mass spectrometer). Ultimately 285 cord blood samples for BPA measurements were available and the LOD was 0.048ng/ml.

Fetal adipokines

Total and high molecular weight (HMW) adiponectin and leptin levels in cord blood were measured in 264 and 257 neonates, respectively. Adiponectin analysis was done by ELISA using Human Adiponectin Assay kit from Sekisui Medical Co. Ltd (Tokyo, Japan). Leptin analysis was done 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. Analysis

was repeated for all samples with coefficients of variation (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.

Data analysis

To consider potential confounding variables, we used data from medical record at birth. We examined the following variables as potential cofounders based on previous literatures; child sex, parental BMIs, gestational age.

BMI were calculated from body weight and height obtained from questionnaire. As distribution of BPA and MEHP were skewed, concentrations were $\log_{10}$ transformed for statistical analysis. For MEHP levels, 493 samples were obtained, however, we excluded blood samples withdrawn after delivery as there might be MEHP exposure from medical devices during delivery, and for the final analysis, 335 samples were included. For BPA levels, we had 285 available data. Cord blood levels of adipokine were also $\log_{10}$ transformed for statistical analysis. Given evidences of sex differences in the relationship between BPA and dipokines [27], all the analyses were conducted for boys and girls combined as well as boys and girls separately.Concentrations below LOD were assigned the value of one-half of the LOD. Results were considered significant at $p < 0.05$. All analyses were conducted using SPSS (Version 22.0J; SPSS, Chicago, IL, USA).

C．研究結果

Initially the prospective birth cohort was consisted of 514 mothers. We excluded total 23 participants for dropping out before delivery (n=10), multiple birth (n=7) and pre-term birth (n=6). For the final analysis of this study, 491 subjects were included (Figure 1). Birth weight was significantly heavier among boys compared to girls, however, ponderal index (PI), which is calculated as weight divided by height raised to the power of 3 did not differ between boys and girls.

Median (IQR) MEHP and BPA levels (ng/ml) were 10.70 (6.30-17.05) and 0.051 (LOD-0.076), with the detection rate of 100% and 83.2%, respectively.

Median total and HMW adiponectin and leptin levels were significantly higher in girls than in boys ($p = 0.006$ for total adiponectin, $p = 0.005$ for HMW adiponectin, $p < 0.001$ for leptin, respectively, Table 2). The median leptin level was significantly higher in mothers with higher BMI ($p = 0.006$) and the total and HMW adiponectin levels were suggestively higher in mothers with higher BMI ($p < 0.10$). Birth weight was significantly heavier among neonates with higher maternal BMI. Although there was not statistically significant, neonates born from smokers had lower birth weight compared to nonsmokers. (Table S1).

Maternal MEHP and cord blood BPA levels according to maternal characteristics were shown in Table 3. Geometric mean (GM) of either MEHP or BPA levels did not differ among mothers of different ages, BMIs, educational levels, family income and smoking habit.

The association between maternal MEHP and cord blood BPA levels and adipokine levels were shown in Table 4. After

adjusting with covariates, MEHP level was positively associated with total and HMW adiponectin levels among boys (p = 0.009, p = 0.012, respectively). MEHP level was negatively associated with leptin levels with borderline significance over all (p = 0.063), and after stratification by child sex, the association was observed only among girls with statistical significance (p = 0.004). The association between maternal MEHP levels and cord blood adipokines were different between boys and girls. BPA level was not associated with any of the adipokine levels.

The association between maternal MEHP and cord blood BPA levels andbirth weight and ponderal index were shown in Table 5. Overall, maternal MEHP levels were not significantly associated with birth weight. The βs for birth weight in association with maternal MEHP levels showed opposite directions between boys and girls. Positive associations among boys, whereas negative associations among girls were observed. This indicated that prenatal DEHP exposure may influence on fetal adipokine levels and birth weight in sex specific manner. Cord blood BPA levels were not significantly associated with birth weight.

D．考察

In this prospective birth cohort study of Japanese women, we investigated the relationship between MEHP level in maternal blood and BPA level in cord blood and fetal adipokine levels, birth weight. The median concentration of MEHP levels was 10.70 ng/ml in this study. Compared to the study of serum MEHP measurements of pregnant women [35], the level was slightly higher. However, blood sampling periods were different between our study and previous study, which could explain the difference of maternal MEHP levels. When compared to 2 other studies of adult serum MEHP measurements from European countries[36, 37], MEHP levels in our study was higher, however, the production and use of DEHP varied among countries where studies were taken place, which could have caused differences in observed MEHP levels in blood. In fact, the levels of DEHP in house dust in Japan [38] were higher compared to studies from European countries [39-43], Asian countries [44, 45], and the USA [45, 46].

The cord blood BPA levels in this study was much lower compared to the previous reports [47-51].

We observed higher levels of leptin and total and HMW adiponectin levels in girls than in boys. Compared to previous studies, the leptin and adiponectin levels in this study is similar levels to those from Japanese study[52], however, they observed that male had higher adiponectin levels compared to female (18.8 μg/ml vs. 16.4 μg/ml). Also our observed levels of adipokines were close range to the reported levels in Taiwan [27]. Leptin levels in our study was lower compared to the recently reported levels from Canadian study [28] which showed higher leptin levels in female than in male (16.0 and 8.7 ng/ml, respectively). Several studies from western countries showed much higher levels of adiponectin [53] and leptin [54] compared to our results. Contrary, report from USA and China [55] showed relatively lower adiponectin levels compared to our results. As previously reported [56, 57], leptin and adiponectin levels vary among ethnicities and in adult study, Asian population showed lower adiponectin levels compared to those of European people [58].

To our knowledge, there have been two previous reports on prenatal BPA exposure

and cord blood adipokine levels and birth outcome [27, 28] Chou et al. showed that elevated prenatal BPA exposure increased the risk of adverse actions of adipokines, low adiponectin and high leptin in neonates, especially in male infants. In addition, they reported negative correlation between maternal serum BPA levels and birth weight (ρ = -0.24) and prenatal BPA exposure increased the risk of LBW and SGA. In their study, geometric mean (GM) concentration of cord blood BPA was 0.5 ng/ml, which was over 10 times higher than our result (GM = 0.045 ng/ml), thus the difference in exposure levels could be one of the reasons that we did not observe association between BPA levels and either adiponectin and leptin levels or birth weight. Ashley-Martin et al. observed an inverse, non-linear relationship between maternal urine BPA level and adiponectin level among males. The association between maternal urine BPA levels and birth size was not examined in their study. In our study, we found no association between BPA and adiponectin levels. In their study, maternal urinary samples were used for exposure assessment while we used cord blood samples, which made it difficult in comparison of the study results. Further, their study population was mainly Caucasian living in Canada whereas our population was Japanese, thus, genetic differences in metabolism among ethnic groups may explain the various finding. Associations between prenatal BPA exposure and birth size have been reported from several birth cohort studies [27, 51, 59-61], yet the results from those epidemiological studies were inconsistent. One study estimated prenatal BPA exposure only based on questionnaires with no bio monitoring data, thus comparison of results was difficult [61]. 2 birth cohort studies Korea, they reported that prenatal BPA exposure was associated with increased birth weight [51, 59]. However, cord blood levels of BPA was higher in Korean study [51], and this could be a reason that we did not find the same result. Experimental studies suggested that BPA increased gene expression of adipogenic transcription factors in 3T3-L1 preadipocytes [62] and perinatal BPA exposure was associated with the over-expression of adipocyte hypertrophy and of lipogenic genes in rats [63]. Although these studies provided some evidences that BPA exposure may alter adipokine secretion, we should consider that results from higher exposure levels in experimental setting may not be applicable to human exposure levels and thus examinations in lower exposure level in epidemiological studies still need to be conducted.

There have been one previous study regarding prenatal phthalate exposure and adipokine levels in cord blood [28]. They found that maternal urinary Mono-(3-carboxypropyl) phthalate (MCPP), metabolite of Di-n-octyl phthalate (DOP), level was associated with increased odds of high leptin among males (OR = 3.5, 95% CI: 1.1-11.6). They also have investigated other phthalate metabolites including MEHP, however, did not find any associations between these metabolites and fetal adipokine levels. In our study, we did not conduct exposure assessment of DOP, thus we were unable to compare our results with the previous study results, however, we found positive association between MEHP and adiponectin in boys and negative association between MEHP and leptin in girls. In the previous study [28], they only

have investigated the association between maternal urinary phthalate levels and fetal adipokines, but no birth outcomes such as birth weight were examined. Our study is the first study to investigate the association between prenatal phthalate exposure and fetal adipokines and birth outcome together. The results from our study could be interpreted that prenatal phthalate exposure may cause changes in fetal adipokine levels, but not adverse influence on birth size. Other previous studies have shown no significant association between prenatal phthalate exposure and birth size [64, 65]. Our result added an evidence that prenatal DEHP exposure did not have significant influence on birth weight. Yet influence of other phthalate exposure on fetus adipokines and birth size need to be investigated in the future work.

E．結論

This was the first study of investigating prenatal DEHP and BPA exposure on fetal adipokine levels along with investigating birth weight. This study suggested that prenatal BPA and DEHP exposure may have adverse influence on fetal metabolic function but not on birth size. Also these influences appeared to be potentially sex-specific. In the future, evaluation of postnatal exposure to these chemicals and examination of biomarkers of children is necessary to assess the association between EDCs exposure and childhood growth.

F．研究発表

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G．知的財産権の出願・登録状況（予定を含む。）

該当なし

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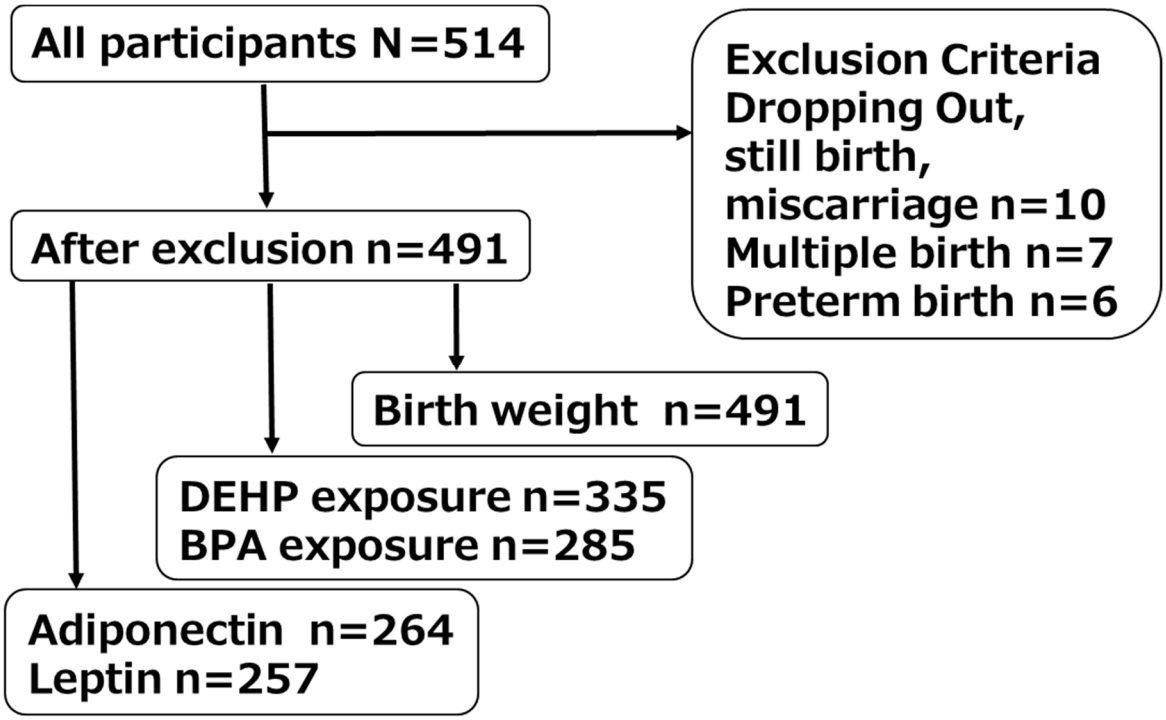

Figure 1. Participants' selection flow in this study.

Table 1 Characteristics of participants.

Characteristics	**All** **% or mean (SD)**	**Boys** **% or mean (SD)**	**Girls** **% or mean (SD)**
Maternal characteristics	N = 491		
Age at delivery (years)	30.7 (4.9)		
Pre-pregnancy BMI (kg/m^2)	21.2 (3.2)		
Parity-nulliparous	47.8%		
Smoking Never/quit before pregnancy	59.7%		
Quit after finding pregnancy	23.4%		
Current smoker	16.9%		
Education (years) ≦ 12	44.6%		
≧ 13	55.4%		
Family income (yen) < 5M	68.2%		
≧ 5M	31.2%		
Paternal characteristics	N = 491		
Age (years)	32.3 (5.7)		
BMI (kg/m^2)	23.2 (3.4)		
Education (years) ≦ 12	43.8%		
≧ 13	56.2%		
Child characteristics	N = 491	N=233	N=258
Gestational age (days)	275.6 (9.6)	275.0 (9.4)	276.0 (9.7)
Birth weight (g)	3064 (374)	3113 (378)	3020 (365)
Ponderal Index (kg/m^3)	27.6 (3.6)	27.6 (4.5)	27.6 (2.4)

Table 2 Measurements of MEHP, BAP and adipokines.

Biomarkers	**All**		**Boys**		**Girls**	
	N	**Median (IQR)**	**N**	**Median (IQR)**	**N**	**Median (IQR)**
MEHP (ng/ml)	335	10.70 (6.30-17.05)				
BPA (ng/ml)	285	0.051 (LOD-0.076)				
Total adiponectin (μg/ml)	264	19.1 (15.0-22.8)	127	18.3 (14.1-21.2)	137	19.7 (16.0-23.8)
HMW adiponectin (μg/ml)	264	12.7 (9.5-15.5)	127	11.4 (8.9-14.8)	137	13.1 (10.2-16.7)
Leptin (ng/ml)	257	5.9 (3.8-9.3)	125	5.0 (3.4-6.6)	132	7.4 (4.4-11.8)

Table 3 Distribution of maternal MEHP and cord blood BPA levels by maternal characteristics.

	MEHP (ng/ml) N=335		**BPA (ng/ml) N=285**	
Characteristics	**GM**	**p value**	**GM**	**p value**
Age (years) ≦ 24	11.21	0.823	0.051	0.714
25-29	11.20		0.047	
30-34	10.52		0.046	
≧ 35	10.84		0.046	
BMI (kg/m^2) < 18.5	11.44	0.819	0.048	0.123
18.5-24.9	10.88		0.046	
25.0-29.9	9.70		0.063	
≧ 30	10.76		0.063	
Parity 0	10.68	0.444	0.047	0.684
≧ 1	11.17		0.047	
Education (years) ≦ 12	10.94	0.885	0.046	0.967
≧ 13	10.84		0.048	
Family income (yen) < 5M	10.62	0.190	0.048	0.586
≧ 5M	11.42		0.046	
Smoking never/quit before pregnancy	10.94	0.802	0.046	0.794
quit after finding pregnancy	10.80		0.048	
current smoker	10.81		0.048	

p values were obtained from Mann-Whitney’s U test or Kruskal-Wallis test.

Table 4 Association between maternal MEHP and cord blood BPA levels and fetal adipokines.

	All			**Boys**			**Girls**		
	N	**β**	**95% CI**	**N**	**β**	**95% CI**	**N**	**β**	**95 % CI**
MEHP									
Total adiponectin (μg/ml)[a)]	187	0.02	-0.04, 0.09	91	0.12	0.03, 0.21**	96	-0.04	-0.13, 0.06
HMW adiponectin (μg/ml)[a)]	187	0.04	-0.05, 0.12	91	0.15	0.03, 0.27**	96	-0.03	-0.15, 0.09
Leptin (ng/ml)[b)]	181	-0.13	-0.27, 0.01†	89	0.08	-0.12, 0.28	92	-0.29	-0.49, -0.10**
BPA									
Total adiponectin (μg/ml)[a)]	251	0.03	-0.03, 0.09	118	0.04	-0.05, 0.13	133	0.03	-0.05, 0.10
HMW adiponectin (μg/ml)[a)]	251	0.04	-0.04, 0.11	118	0.04	-0.08, 0.16	133	0.04	-0.06, 0.13
Leptin (ng/ml)[b)]	245	0.07	-0.05, 0.19	117	0.05	-0.12, 0.21	128	0.09	-0.09, 0.27

[a)] Adjusted for child sex and maternal BMI

[b)] Adjusted for child sex, maternal BMI and gestational age

† $p < 0.10$, ** $p < 0.01$

Table 5 Association between maternal MEHP levels, cord blood BPA levels and birth weight.

	All			**Boys**			**Girls**		
	N	**β[a)]**	**95%CI**	**N**	**β[b)]**	**95%CI**	**N**	**β[b)]**	**95%CI**
MEHP	335			161			174		
Weight (g)		-36.5	-162.4, 89.4		33.5	-169.5, 236.5		-81.2	-243.0, 80.5
Ponderal Index (kg/m^3)		-0.57	-2.10, 0.96		-0.08	-3.21, 3.06		-0.85	-2.03, 0.33
BPA	285			127			158		
Weight (g)		54.1	-69.5, 177.7		63.4	-116.4, 243.2		46.8	-126.5, 220.2
Ponderal Index (kg/m^3)		0.44	-0.47, 1.36		1.10	-0.26, 2.45		-0.04	-1.29, 1.21

[a)] Adjusted for child sex, parental BMIs and gestational age.
[b)] Adjusted for parental BMI and gestational age.

Table S1 Distribution of adipokines and birth weight by maternal characteristics.

	Total adiponectin (μg/ml)	**HMW adiponectin (μg/ml)**	**Leptin (ng/ml)**	**Birth weight (g)**
Maternal characteristics	**median**	**median**	**median**	**mean (SD)**
Age (years) ≦ 24	18.7	12.2	8.2	3068 (367)
25-29	18.8	12.6	5.5	3077 (375)
30-34	20.3	13.4	6.3	3068 (376)
≧ 35	16.9	11.0	5.4	3039 (375)
BMI (kg/m^2) < 18.5	18.2†	11.4†	4.3*	2951 (371)*
18.5-24.9	18.8	12.6	6.0	3083 (371)
25.0-29.9	20.4	12.4	7.0	3059 (435)
≧ 30	22.9	16.1	8.6	3163 (160)
Education (years) ≦ 12	19.7	12.8	6.1	3044 (376)
≧ 13	18.6	12.4	5.7	3079 (372)
Family income (yen) < 5M	19.5	12.8	5.8	3076 (368)
≧5M	18.3	11.9	6.0	3042 (386)
Smoking never/quit before pregnancy	19.0	12.7	5.8	3062 (378)
quit after finding pregnancy	18.4	12.0	5.9	3097 (392)
current smoker	19.7	12.8	6.0	3022 (331)

p values were obtained from Mann-Whitney's U test or Kruskal-Wallis test.
* $P < 0.05$, † $p < 0.10$