Longitudinal Assessment of Prenatal Phthalate Exposure on Serum and Cord Thyroid Hormones Homeostasis During Pregnancy- Tainan Birth Cohort Study (TBCS)

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Abstract

Increasing number of studies revealed that phthalate exposure alters thyroid hormone homeostasis in the general population, but insufficient evidence of longitudinal maternal phthalate exposure on maternal and fetal thyroid hormone during pregnancy. We aimed to longitudinally assess of prenatal phthalate exposure in pregnant women on cord and maternal thyroid hormone at three trimesters during pregnancy. We recruited 98 pregnant women, and collected their urine and blood samples at three trimesters from an obstetrics clinic in Southern Taiwan from 2013 to 2014. We analyzed the concentrations of 11 urinary phthalate metabolites, including monoethylhexyl phthalate (MEHP), mono-(2-ethyl-5-oxo-hexyl) phthalate (MEOHP), mono-(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP), mono-(2-ethyl-5-carboxypentyl) phthalate (MECPP), mono-n-butyl phthalate (MnBP), monoisobutyl phthalate (MiBP), monoethyl phthalate (MEP), using online liquid chromatography–tandem mass spectrometry. The cord and maternal serum levels of thyroxine (T_4), free T_4, triiodothyronine (T_3), thyroid-stimulating hormone (TSH), and thyroxine-binding globulin were measured using an electrochemiluminescence immunoassay. A mixed-model was utilized to assess longitudinal phthalate exposure on thyroid hormone and adjusted for significant covariant. We found that urinary MiBP ($\beta = -0.065$, 95%CI: −0.124, −0.005), MEOHP ($\beta = -0.083$, 95% CI: −0.157, −0.009), and were significant negatively associated with serum TSH. Urinary MECPP was inversely related to serum T_3 ($\beta = -0.027$, 95% CI: −0.047, −0.006). Urinary MEP ($\beta = 0.014$, 95% CI: −0.001, 0.028) and MiBP ($\beta = 0.033$, 95% CI: 0.018, 0.049) were positively related to free T_4. We found cord serum T_3 ($\beta = 0.067$, 95% CI: 0.003, 0.131) and free T_4 ($\beta = 0.031$, 95% CI: 0.001, 0.062) levels had significant positive association with maternal ΣDBPm levels at the second trimester. We concluded that different exposure windows of phthalates during gestation may alter cord and serum thyroid hormone homoeostasis.

1. Introduction

Phthalates are a family of chemicals whose structure only varies in the length (1 to > 10 carbons) of their linear or branched ortho-positioned hydrocarbon chain arms (Supplementary information; Table S1). They are widely used as plasticizers and softeners in various commercial and industrial products (Koch and Calafat 2009). Urinary phthalate metabolites can be considered biomarkers of phthalate exposure in humans (Calafat and McKee 2006; Koch and Calafat 2009; Wittassek et al. 2007). Because of their extensive use in such products, phthalate metabolites have been detected in humans worldwide, including pregnant women (Cantonwine et al. 2014; Chang et al. 2017; Silva et al. 2004). Accumulating evidence suggests that phthalates interfere with normal thyroid function (Hartoft-Nielsen et al. 2011; Schug et al. 2011; Huang et al. 2016).

Maintaining maternal thyroid homeostasis during pregnancy is important for fetal growth and development, particularly fetal neurodevelopment (Hartoft-Nielsen et al. 2011). Human studies have reported that hyperthyroidism and hypothyroidism in pregnant women may be associated with adverse birth outcomes such as preterm birth and low birth weight (Aggarawal et al. 2014; Chen et al. 2014). Even subtle alterations of the thyroid function in pregnant women can have detrimental effects on fetal health (Henrichs et al. 2010; Li et al. 2010).

Several epidemiological studies have examined the associations between phthalate exposure and thyroid function in adults, adolescents, and children (Boas et al. 2010; Huang et al. 2017; Meeker et al. 2007; Meeker and Ferguson 2011; de Cock et al., 2014; Park et al. 2017). These studies have reported that phthalate exposure is associated with one or more thyroid hormone parameters, but the results are still inconsistent. It remains unclear which phthalates may influence thyroid function in other susceptible populations, including pregnant women. Furthermore, although prior studies have assessed the relations between

exposure to phthalates and thyroid hormone levels in pregnant women (Huang et al. 2007; Kuo et al. 2015; Yao et al. 2016), few studies have investigated the associations between phthalate exposure and thyroid function during pregnancy, particularly in Asian pregnant women; therefore, we collected samples at three time points per participant and examined these associations in pregnant women. Moreover, we explored potential vulnerable window of exposure to phthalate on the basis of the sample data collected during the visits.

2. Materials and Methods

2.1 Study Population

All pregnant women clinically suggested to undergo amniocentesis by gynecologists at National Cheng Kung University Hospital were enrolled between 2013 and 2014 (Huang et al., 2016). We excluded pregnant women with preeclampsia and abnormal chromosomal disease. An examination of chromosomes in the amniotic fluid samples of the participants revealed that all fetuses were healthy. A total of 98 participants were recruited in the present study. The study protocol was approved by the Research Ethics Committee of the National Health Research Institutes (No. EC1020302) and the Institutional Review Board of National Cheng Kung University Hospital (No. A-ER-102-104) in Taiwan. Informed consent was obtained from each participant before study enrollment. At the initial study visit (median: 18 gestational weeks), the participants provided sociodemographic information (age, education, occupational history, and social economic status.), pregnancy history (gestational age, menarche age, and parity), lifestyle habits (tobacco use, passive smoking, and alcohol consumption), and exposure history (exposure to di-(2-ethylhexyl) phthalate [DEHP]-contaminated products before the DEHP episode and nutritional supplement consumption) as well as urine and blood samples for biomarker analysis. Urine and blood samples were also collected at visits 2 and 3 (median: 26 and 39 gestational weeks,

118 respectively), and additional cord blood sample was obtained at delivery.

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120 **2.2 Phthalate Metabolite Measurements**

121 As described by Huang et al. (2016), we analyzed the concentrations of 11 urinary phthalate
122 metabolites, namely monoethylhexyl phthalate (MEHP), mono-(2-ethyl-5-oxo-hexyl)
123 phthalate (MEOHP), mono-(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP),
124 mono-(2-ethyl-5-carboxypentyl) phthalate (MECPP), mono-(2-carboxymethylhexyl)
125 phthalate (MCMHP), mono-n-butyl phthalate (MnBP), monoisobutyl phthalate (MiBP),
126 monoethyl phthalate (MEP), monoisononyl phthalate (MiNP), monobenzyl phthalate (MBzP),
127 and monomethyl phthalate (MMP), in the urine samples. The levels of the metabolites were
128 determined through online liquid chromatography–tandem mass spectrometry (Agilent
129 1200/API 4000, Applied Biosystems, Foster City, CA, USA). The limits of detection (LOD)
130 for MEHP, MEOHP, MEHHP, MECPP, MCMHP, MnBP, MiBP, MEP, MiNP, MBzP, and
131 MMP were 0.7, 0.3, 0.3, 0.3, 0.1, 1, 1, 0.3, 0.1, 0.3, and 0.3 ng/mL, respectively. Phthalate
132 metabolite concentrations less than the LOD were assigned as half the LOD value. A blank,
133 repeated quality control (QC) sample, was included in each batch of the analyzed samples.
134 The concentrations of the QC samples were to be less than 2 times the method detection limit.
135 The QC sample for each sample was spiked in pooled urine samples with a mixture of
136 phthalate metabolite standards (20–50 ng/mL). The relative percent difference for the QC
137 sample as well as its recovery were to be less than ±30%.

138

139 **2.3 Serum Thyroid Hormones and Urinary Creatinine Analysis**

140 Serum thyroid function and urinary creatinine levels were measured by a Taiwan
141 Accreditation Foundation-certified laboratory (Nos. 1447 and 1673), recognized by the
142 International Laboratory Accreditation Cooperation Mutual Recognition Arrangement

(Huang et al. 2017). Urine samples that had been stored at −20°C were analyzed using combined clinical chemistry and immunoassay tests (Modular Analytics Serum Work Area; Roche Diagnostics). Thyroid function was analyzed as the serum concentrations of thyroxine (T_4), free T_4, triiodothyronine (T_3), thyroid-stimulating hormone (TSH), and T_4-binding globulin (TBG) by using an electrochemiluminescence immunoassay (Elecsys 2010 and Modular Analytics E170; Roche Diagnostics). All analyses were conducted by a blinded technician and in a random order.

2.4 Statistical Analysis

Descriptive statistics on participant demographics were tabulated with the distribution of phthalate metabolites concentrations and thyroid hormones. The levels of hormones and phthalate metabolites were transformed using the natural logarithm (ln) to meet the normality assumption. The mixed models were applied to compare these differences in urinary phthalate metabolite levels or thyroid hormones from different study visit. The associations between urinary phthalate metabolites and thyroid hormone levels were assessed using a mixed-model repeated measures analysis after adjustment for fixed covariates, such as maternal age at enrollment (continuous variable), gestational age at sample collection (categorical variable), urinary creatinine (continuous variable), and plasma TBG (continuous variable). These covariates were selected on the basis of previous studies (Boas et al. 2010, Meeker and Ferguson 2011) (such as maternal age at enrollment, gestational age at sample collection and urinary creatinine) and a 10% change-in-estimated criterion (Rothman et al. 2008) and Akaike's information criterion (AIC) (such as plasma TBG). These models considered the participants as random effects, as well as maternal age at enrollment, gestational age at sample collection, urinary creatinine, and plasma TBG as fixed effect and the first-order autoregressive and variance components were constructed as covariance

structures. The models were selected on the basis of Akaike's information criterion (AIC). Residual and influence analyses were conducted (Figure S1). The associations between urinary phthalate metabolites levels and thyroid function were not simultaneously adjusted for other phthalate metabolites. In our subsequent analyses, we examined the associations between urinary phthalate metabolites and thyroid hormone levels at visits 1–3 by using linear regression models. These models were adjusted for maternal age at enrollment, urinary creatinine, and plasma TBG levels. A two-sided $P < 0.05$ was considered statistically significant. All statistical analyses were performed using SAS (Version 9.1.3; SAS Institute Inc., Cary, NC, USA).

3. Results

3.1 Participant Characteristics

The mean age of all participants was 35 years (standard deviation: 3.5); they had high education levels (**Table 1**). Most participants had no history of smoking or alcohol consumption before pregnancy. Few participants had a history of consuming DEHP-contaminated products. All participants had no family or personal history of thyroid disease.

3.2 Distributions of Urinary Phthalate Metabolites and Serum Thyroid Hormones

Table 2 presents the urinary phthalate metabolite levels and thyroid function in pregnant women, stratified by study visit. Compared with the concentrations of urinary phthalate metabolites at visit 1, those of urinary MMP, MiBP, MEHHP, MEOHP, MECCP, MCMHP, and molar sum of DEHP (ΣDEHPm) were significantly higher, whereas those of urinary MBzP and MiNP were significantly lower at visit 2. Similarly, urinary MMP, MEP, MiBP, MnBP, MEHHP, MEOHP, MECCP, ΣDBPm, and DEHPm levels were significantly higher

and urinary MiNP levels at visit 3 were significantly lower than those at visit 1. Urinary MBzP and MiNP levels were not included in the subsequent analysis because of the low average detection rate (<30%). Concentrations of serum T_3 and free T_4 at visits 2 and 3 were significantly lower than those at visit 1, but TBG levels were significantly higher. In addition, the serum levels of TSH at visit 3 were significantly higher than those at visit 1. In addition, Most of urinary phthalate metabolites levels were significantly correlated within the same study visit in pregnant women. However, few of urinary phthalate metabolites levels from different study visit among pregnant women were significantly correlated (Table S2).

3.3 Associations between Urinary Phthalate Metabolites and Thyroid Function

The associations of thyroid hormones with urinary phthalate metabolites in pregnant women, as determined using linear mixed models, are shown in **Table 3**. We found that urinary MiBP levels were negatively associated with serum TSH levels (β = −0.065, 95% confidence interval [CI]: −0.124, −0.005, p=0.033) and positively associated with free T_4 levels (β = 0.033, 95% CI: 0.018, 0.049). Negative associations were observed between urinary MEOHP levels and TSH levels (β = −0.083, 95% CI: −0.157, −0.009, p=0.028), urinary MECPP and T_3 levels (β = −0.027, 95% CI: −0.047, −0.006, p=0.012), and MCMHP and T_3 levels (β = −0.018, 95% CI: −0.034, −0.002, p=0.032). In addition, the serum levels of TSH and T_3 and urinary ΣDEHPm levels of were marginally associated with TSH (β = −0.074, 95% CI: −0.161, 0.013, p=0.095) and T_3 (β = −0.022, 95% CI: −0.046, 0.003, p=0.086). Marginal and positive associations were observed between urinary MEP and free T_4 levels (β = 0.014, 95% CI: −0.001, 0.028, p=0.063). We have assessed these associations as well while considering the other phthalate metabolites levels in the models, indicating that these results were similar to those without adjusting for other phthalate metabolites levels (Table S3).

3.4 Associations between Urinary Phthalate Metabolites and Thyroid Function, Stratified by Study Visit

To determine the possible window of exposure to phthalates on thyroid function in pregnant women, we further examined the associations between urinary phthalate metabolites and thyroid hormone levels at the study visits (**Figure 1**). Urinary MEOHP, MECCP, and DEHPm levels were negatively associated with TSH levels at visit 2. Urinary MMP levels at visit 1 and urinary MiBP, MnBP, and ΣDBPm levels at visit 2 were positively associated with T_3 levels. Moreover, negative associations were observed between urinary MEHHP, MEOHP, MECCP, and DEHPm levels and T_3 levels at visit 3. Urinary MnBP levels were negatively associated with T_4 levels at visit 1, and urinary MCMHP levels were positively associated with T_4 levels at visit 1. Urinary MEP and MiBP levels were positively associated with free T_4 levels at visit 1.

In addition, we found that maternal MnBP levels andΣDBPm levels at the visit 2 were positively associated with the T_3 levels in cord serum (β = 0.054, 95% CI: 0.008, 0.1 for MnBP; β = 0.067, 95% CI: 0.003, 0.131 for ΣDBPm) (Table 4). Positive relations between maternal ΣDBPm levels at the visit 2 and the free T_4 levels in cord serum were observed (β = 0.031, 95% CI: 0.001, 0.062). These effects in the models were still similar after adjusting maternal thyroid hormone levels (Table S4).

4. Discussion

Our repeated measurement analyses revealed that urinary MiBP and MEOHP levels were negatively associated with TSH levels, urinary MECPP and MCMHP levels were inversely associated with T_3 levels, and urinary MiBP levels were positively associated with free T_4 levels in pregnant women. In a cross-sectional analysis stratified by study visit, the magnitude and direction of these associations differed with the gestational trimester. Several metabolites, such as MEOHP, MECCP, and DEHPm, were inversely associated with TSH at visit 2 and with T_3 levels at visit 3. We observed that T_4 levels at visit 1 were associated negatively with urinary MnBP levels and positively with urinary MCMHP levels. At visit 1, urinary MEP and MiBP levels were positively associated with free T_4 levels. These results revealed that exposure to environmental phthalates can influence thyroid function in pregnant women. Furthermore, our findings indicate that different windows of exposure to phthalates during gestation can alter levels of thyroid hormones, and the timing of phthalate exposure may be an important determinant of susceptibility to thyroid disruption in pregnant women.

Johns et al. (2016) conducted a repeated measurement study to investigate the associations between urinary phthalate metabolites and thyroid hormones levels in 439 pregnant women during gestation. Similar to our results, they reported inverse associations between urinary MEHP and MiBP levels and TSH levels. However, in contrast to our observations, they did not observe negative associations between urinary MECCP levels and T_3 levels or positive associations between urinary MiBP levels and free T_4 levels. In an earlier study, no statistically significant associations were observed between urinary phthalate metabolites and serum TSH or free T_4 levels in a repeated measures analysis of two study visits in Puerto Rico (Johns et al. 2015). However, in a cross-sectional analysis, urinary MiBP levels were found to be significantly and positively associated with free T_4 levels at a median gestation

period of 18 weeks, and these results are in agreement with our own results in the present study. Johns et al. (2016) observed similar associations at a median gestation period of 18 weeks, but these were non-significant.

Several cross-sectional epidemiological studies have explored the potential effects of phthalates on thyroid function in pregnant women. We observed significant inverse associations between urinary MnBP and T_4 levels at visit 1, whereas Huang et al. (2007) observed similar associations between urinary MBP and T_4 levels at a mean gestation period of 27.9 weeks. Furthermore, we found that urinary MEOHP, MECCP, and DEHPm levels were negatively associated with TSH levels at visit 2 (median: 26 gestational weeks). Similarly, Johns et al. (2016) reported inverse associations between these phthalate metabolites and TSH levels at a median gestation period of 10 weeks, whereas Kuo et al. (2015) observed the same associations but in the third trimester. However, Yao et al. (2016) reported positive associations between urinary MEHP and MEHHP and TSH levels in 2521 pregnant women at <14 gestational weeks in China. These discrepancies among the studies can be because of differences in the population size, sample collection timing, phthalate exposure profiles, population demographic characteristics, and study design.

Compared with the results of the current study, previous cross-sectional studies involving adult men and nonpregnant women have reported conflicting observations of urinary DEHP metabolites and TSH levels. Meeker et al. (2007) observed no associations between urinary DEHP metabolites and TSH levels in 408 men recruited from a fertility clinic. Our previous studies did not find any associations in 279 Taiwanese adults (Huang et al. 2017). However, Park et al. (2017) reported positive associations between urinary MEOHP levels and TSH levels in 6003 adults in Korea. These differences in exposure levels and the physiological

state of participants may have contributed to the discrepancies between the results of these studies and the present study.

Biological mechanisms underlying the thyroid disruption effects of phthalate exposure have been examined in animal and in vitro studies. Studies have indicated thyroid alterations and lower plasma T_4 concentrations in rats fed with DEHP-contaminated products than in controls (Hinton et al. 1986; Howarth et al. 2001; Poon et al. 1997). Moreover, histopathological changes in rats' thyroid after DEHP exposure have been reported to correspond to thyroid hyperactivity (Howarth et al. 2001; Mitchell et al. 1985). In their vitro study, Wenzel et al. (2005) demonstrated that phthalates can alter the transcriptional activity of the sodium–iodide symporter and cause changes in the iodide uptake of thyroid follicular cells. Furthermore, phthalates can affect thyroid hormones not only through biosynthesis and biotransport but also through biotransformation and metabolism (Liu et al. 2015). Recent animal experiments have suggested that DEHP can influence thyroid hormones by disturbing the hypothalamus–pituitary–thyroid axis (Dong et al. 2017) and activating the Ras–Akt–thyrotropin-releasing hormone receptor pathway and inducing hepatic enzymes (Ye et al. 2017).

Each organ system has a different developmental trajectory, and the sensitive window for exposure to cause toxicity varies during tissue development in pregnancy. Therefore, the effects of in utero exposure depend not only on the type and dose of the chemical but also on the exposure time (Schug et al. 2011). In early pregnancy (before 20 gestational weeks), the mother is the major source of thyroid hormones for the fetus, and in later pregnancy (after 20 gestational weeks), fetal thyroid function starts and maternal thyroid hormones are still relatively important (Obregon et al. 2007). Even subtle changes in thyroid function in

311 pregnant women can have important influences on fetal health. Higher maternal free T_4 levels
312 in early pregnancy was reported to be associated with lower birth weight and increased risk
313 of small-for-gestational age birth (Medici et al. 2012). In particular, in the present study, two
314 phthalate metabolites were positively associated with free T_4 levels at visit 1 (median: 18
315 gestational weeks).
316 In the present study, we found that the T_3 and free T_4 levels in cord serum were positively
317 associated with maternalΣDBPm levels at the visit 2 (median: 26 weeks gestation). However,
318 Kuo et al. (2015) observed an inverse association between urinary MBzP levels in 148
319 pregnant women at the third trimester and TSH levels in cord blood. Furthermore, Yao et al.
320 (2016) did not find any relations between urinary phthalate metabolites in 2521 pregnant
321 women at the first trimester and thyroid function in cord serum. Maternal thyroid hormone
322 plays the major role for the fetus in early pregnancy (before 20 gestational weeks) (Obregon
323 et al. 2007). As the thyroid gland of fetus growth with increased gestational age, fetus can
324 excrete sufficient thyroid hormone by themselves after the second trimester. Our findings
325 indicated that the effects of maternal urinary phthalate metabolites before second trimester
326 may be crucial to the homeostasis of fetal thyroid hormone. Because of the limited samples in
327 cord serum in the present study, future large-scale studies are needed to address these
328 associations.
329
330 The study has several limitations. First, we did not collect information on the iodine or
331 selenium status of our participants, which may be critical because deficiencies in these trace
332 elements can impair normal thyroid function (Zimmermann and Kohrle 2002). However, no
333 participant reported having any thyroid-related disease. Moreover, previous studies have
334 indicated that iodine excretion negligibly affects the significant associations between
335 phthalate metabolites and thyroid hormones among a representative sample of adult men and

women in the United States (Mendez and Eftim 2012). Second, we conducted numerous comparisons and determined that some observed associations could have been chance events. We did not perform adjustment for multiple comparisons because available methods (e.g., Bonferroni adjustment) are often too conservative because of the underlying assumptions of independence and increased probability of type 2 errors, thus potentially masking truly important differences (Perneger 1998). Finally, the use of chemiluminescent immunoassays for determining serum-free T_4 levels in the present study could have been influenced by binding protein concentrations. Because we examined the associations between urinary phthalate metabolite and free T_4 levels adjusted for TBG levels, the effects of binding protein concentrations could be finite. Despite these limitations, our study has many advantages. The repeated-measure design used in this study minimized the effects of the genetic background of the participants. The collection of measured biomarkers at multiple time points in pregnancy favored statistical modeling techniques to more accurately detect the associations among repeated measurements.

5. Conclusions

Our results provide evidence that exposure to environmental phthalates can disturb the homeostasis of thyroid hormones in pregnant women during gestation. Future studies must determine the direction of specific associations and explicate periods of susceptibility to phthalate exposure in pregnancy.

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Table 1. Demographic characteristics of the study population

Variable	Subjects (N=98) N (%)
Maternal age at enrollment (Mean±SD)	35.0±3.5
Education	
≤Junior high school	1 (1.0)
Senior high school	3 (3.1)
≥University	94 (95.9)
Annual family income [USD] [a]	
≤15,600	16 (16.3)
15,600-31,250	52 (53.1)
≥31,250	30 (30.6)
Active smoker before pregnancy [b]	
Yes	2 (2.0)
Drank alcohol Before pregnancy [c]	
Yes	1 (1.0)
Have ever consumed DEHP-tainted products [d]	
Yes	13 (13.3)
Have ever taken a vitamin complex [e]	
Yes	39 (39.8)
Have ever taken folic acid [e]	
Yes	36 (36.7)
Family or personal medical history of thyroid disease	
No	98 (100)

[a] Currency exchange rate of USD to new Taiwan dollar is 1:32.

[b] Active smoker was defined as someone who consumed a cigarette >1 time per day.

[c] Drank alcohol was defined as someone who consumed >100 ml of alcohol per week.

[d] Have ever consumed DEHP-tainted products means before a DEHP episode (May 2011)

[e] Have ever taken means ever consumed the following nutritional supplements during the past 1 month.

Table 2. Distributions of urinary and plasma biomarkers in pregnant women by study visit

Biomarkers	Visit 1 (median: 18 weeks gestation)			Visit 2 (median: 26 weeks gestation)			Visit 3 (median: 39 weeks gestation)		
	<LOD (%)	N	GM (95%CI)	<LOD (%)	N	GM (95%CI)	<LOD (%)	N	GM (95%CI)
Phthalate metabolites									
MMP (ng/mL)	31	98	1.82 (1.18, 2.81)	9	87	4.35 (3.22, 5.88) *	18	84	4.22 (2.74, 6.52) *
MEP (ng/mL)	12	98	8.56 (5.89, 12.46)	23	87	5.50 (3.41, 8.86)	2	84	19.92 (14.33, 27.70) *
MiBP (ng/mL)	35	98	2.33 (1.50, 3.60)	9	87	5.66 (4.20, 7.63) *	8	84	7.08 (5.10, 9.81) *
MnBP (ng/mL)	19	98	6.06 (3.99, 9.21)	25	87	4.87 (2.95, 8.04)	6	84	15.54 (11.14, 21.67) *
MBzP (ng/mL)	82	98	0.19 (0.14, 0.26)	91	87	0.12 (0.09, 0.15) *	73	84	0.28 (0.18, 0.42)
MEHP (ng/mL)	29	98	2.43 (1.67, 3.52)	18	87	3.45 (2.43, 4.91)	31	84	2.49 (1.60, 3.87)
MEHHP (ng/mL)	24	98	2.67 (1.75, 4.08)	11	87	5.33 (3.63, 7.82) *	4	84	9.69 (7.27, 12.91) *
MEOHP (ng/mL)	23	98	3.41 (2.45, 4.75)	8	87	5.36 (4.06, 7.08) *	2	84	8.38 (6.68,10.52) *
MECPP (ng/mL)	14	98	6.15 (4.37, 8.65)	1	87	9.89 (7.95, 12.30) *	0	84	12.46 (10.03, 15.50) *
MCMHP (ng/mL)	75	98	0.34 (0.25, 0.46)	51	87	0.92 (0.61, 1.37) *	83	84	0.33 (0.23, 0.49)
MiNP (ng/mL)	85	98	1.16 (1.00, 1.35)	100	87	0.15 (0.15, 0.15) *	99	84	0.84 (0.78, 0.90) *

ΣDBPm (ng/mL)	98	11.57 (8.04, 16.64)	87	14.65 (10.53, 20.37)	84	25.76 (19.64, 33.78) *
ΣDEHPm (ng/mL)	98	21.64 (16.44, 28.25)	87	30.68 (24.51, 38.39) *	84	39.34 (31.60, 48.97) *
Thyroid hormones						
TSH (μIU/mL)	97	0.99 (0.82, 1.20)	69	1.04 (0.88, 1.23)	60	2.36 (1.90, 2.94) *
T_3 (ng/dL)	97	122.29 (115.8, 129.2)	69	116.25 (110.8, 122.0) *	60	105.63 (99.30, 112.4) *
T_4 (μg/dL)	97	8.90 (8.59, 9.22)	69	8.74 (8.22, 9.30)	60	8.75 (8.41, 9.10)
Free T_4 (ng/dL)	97	0.81 (0.77, 0.85)	69	0.63 (0.61, 0.65) *	60	0.64 (0.62, 0.66) *
TBG (μg/mL)	97	34.75 (33.02, 36.56)	61	38.15 (35.88, 40.57) *	60	38.44 (36.21, 40.81) *

*Significant difference ($P<0.05$) in urinary phthalate metabolite levels or thyroid hormones compared to visit 1 (reference) using linear mixed models.

Table 3. Results of thyroid hormones with urinary phthalate metabolites in pregnant women by linear mixed models (No=216)

Variable	Ln TSH[a,b]	Ln T_3[a,b]	Ln T_4[a,b]	Ln Free T_4[a,b]
	Beta (95% CI)	Beta (95% CI)	Beta (95% CI)	Beta (95% CI)
Phthalate metabolites				
Ln MMP (ng/mL)	-0.039 (-0.089, 0.012)	0.010 (-0.005, 0.024)	-0.002 (-0.012, 0.009)	0.008 (-0.005, 0.022)
Ln MEP (ng/mL)	-0.002 (-0.060, 0.056)	0.003 (-0.014, 0.019)	-0.003 (-0.014, 0.008)	0.014 (-0.001, 0.028) #
Ln MiBP (ng/mL)	-0.065 (-0.124, -0.005) *	0.010 (-0.007, 0.027)	0.001 (-0.012, 0.013)	0.033 (0.018, 0.049) **
Ln MnBP (ng/mL)	-0.003 (-0.052, 0.046)	0.002 (-0.012, 0.016)	-0.007 (-0.018, 0.003)	-0.002 (-0.015, 0.011)
Ln MEHP (ng/mL)	-0.006 (-0.059, 0.047)	-0.0001 (-0.016, 0.015)	-0.002 (-0.013, 0.009)	0.006 (-0.008, 0.020)
Ln MEHHP (ng/mL)	-0.018 (-0.072, 0.037)	-0.013 (-0.028, 0.002) #	-0.005 (-0.016, 0.007)	-0.008 (-0.023, 0.006)
Ln MEOHP (ng/mL)	-0.083 (-0.157, -0.009) *	-0.012 (-0.033, 0.010)	0.001 (-0.015, 0.016)	-0.011 (-0.031, 0.010)
Ln MECPP (ng/mL)	-0.051 (-0.124, 0.021)	-0.027 (-0.047, -0.006) *	0.004 (-0.011, 0.019)	-0.008 (-0.027, 0.011)
Ln MCMHP (ng/mL)	0.007 (-0.050, 0.064)	-0.018 (-0.034, -0.002) *	0.009 (-0.002, 0.021)	-0.012 (-0.027, 0.003)
Ln ΣDBPm (ng/mL)	-0.034 (-0.101, 0.033)	0.003 (-0.017, 0.022)	-0.010(-0.024, 0.004)	0.011 (-0.007, 0.029)
Ln ΣDEHPm (ng/mL)	-0.074 (-0.161, 0.013) #	-0.022 (-0.046, 0.003) #	0.003 (-0.015, 0.021)	0.007 (-0.017, 0.030)

[a]Adjusted for maternal age at enrollment, gestational age at time of sample collection, urinary creatinine, and serum TBG levels.

[b]All models were not simultaneously adjusted for other phthalate metabolites; #<0.1, *<0.05, **<0.01

Table 4. Adjusted regression coefficient and 95% CI for change in cord serum thyroid hormones in relation to unit-increased in Ln-phthalate metabolites (ng/mL) by study visit

Variable	Ln TSH[a,b]	Ln T_3[a,b]	Ln T_4[a,b]	Ln Free T_4[a,b,c]
	Beta (95% CI)	Beta (95% CI)	Beta (95% CI)	Beta (95% CI)
Visit 1 (N=50): median 18 weeks gestation				
Maternal phthalate metabolites				
Ln MMP (ng/mL)	-0.018 (-0.121, 0.085)	-0.011 (-0.063, 0.041)	-0.028 (-0.083, 0.027)	0.000 (-0.026, 0.024)
Ln MEP (ng/mL)	0.084 (-0.041, 0.208)	0.005 (-0.059, 0.068)	-0.026 (-0.093, 0.042)	0.007 (-0.024, 0.038)
Ln MiBP (ng/mL)	-0.063 (-0.174, 0.047)	-0.015 (-0.071, 0.041)	0.048 (-0.010, 0.106)	0.024 (-0.003, 0.050)#
Ln MnBP (ng/mL)	-0.018 (-0.124, 0.089)	0.009 (-0.044, 0.062)	-0.010 (-0.067, 0.047)	0.010 (-0.018, 0.037)
Ln MEHP (ng/mL)	-0.045 (-0.161, 0.071)	-0.032 (-0.090, 0.026)	-0.052 (-0.113, 0.009)	0.001 (-0.027, 0.029)
Ln MEHHP (ng/mL)	-0.023 (-0.128, 0.082)	0.016 (-0.037, 0.068)	-0.011 (-0.068, 0.045)	0.009 (-0.017, 0.034)
Ln MEOHP (ng/mL)	-0.007 (-0.172, 0.158)	0.027 (-0.056, 0.109)	-0.027 (-0.116, 0.061)	0.014 (-0.026, 0.054)
Ln MECPP (ng/mL)	-0.026 (-0.146, 0.095)	0.009 (-0.051, 0.069)	-0.028 (-0.093, 0.036)	-0.005 (-0.034, 0.024)
Ln MCMHP (ng/mL)	0.101 (-0.028, 0.231)	-0.005 (-0.072, 0.061)	-0.053 (-0.123, 0.016)	0.013 (-0.020, 0.045)
LnΣDBPm (ng/mL)	-0.087 (-0.219, 0.045)	-0.015 (-0.082, 0.052)	-0.011 (-0.084, 0.061)	0.018 (-0.014, 0.051)
LnΣDEHPm (ng/mL)	-0.017 (-0.183, 0.150)	-0.009 (-0.092, 0.075)	-0.047 (-0.135, 0.041)	0.008 (-0.032, 0.048)
Visit 2 (N=50): median 26 weeks gestation				
Maternal phthalate metabolites				
Ln MMP (ng/mL)	0.021 (-0.126, 0.167)	0.042 (-0.029, 0.112)	0.018 (-0.056, 0.093)	0.015 (-0.019, 0.049)
Ln MEP (ng/mL)	0.074 (-0.024,	0.037 (-0.011,	-0.008 (-0.059,	0.007 (-0.017,

	0.172)	0.084)	0.043)	0.030)
Ln MiBP (ng/mL)	0.102 (-0.043, 0.248)	0.057 (-0.013, 0.128)	0.011 (-0.065, 0.087)	0.015 (-0.019, 0.049)
Ln MnBP (ng/mL)	0.036 (-0.064, 0.135)	0.054 (0.008, 0.100) *	-0.003 (-0.054, 0.049)	0.021 (-0.001, 0.044) #
Ln MEHP (ng/mL)	0.044 (-0.087, 0.176)	0.009 (-0.055, 0.073)	0.037 (-0.029, 0.104)	0.014 (-0.017, 0.044)
Ln MEHHP (ng/mL)	-0.056 (-0.177, 0.065)	0.043 (-0.015, 0.101)	0.027 (-0.035, 0.089)	0.023 (-0.004, 0.051)
Ln MEOHP (ng/mL)	0.033 (-0.175, 0.241)	0.039 (-0.062, 0.140)	0.069 (-0.035, 0.174)	0.019 (-0.029, 0.067)
Ln MECPP (ng/mL)	-0.187 (-0.473, 0.099)	0.012 (-0.131, 0.154)	0.102 (-0.044, 0.248)	0.027 (-0.041, 0.094)
Ln MCMHP (ng/mL)	0.004 (-0.158, 0.166)	-0.039 (-0.117, 0.039)	0.041 (-0.041, 0.122)	0.021 (-0.016, 0.058)
LnΣDBPm (ng/mL)	0.072 (-0.063, 0.207)	0.067 (0.003, 0.131) *	0.000 (-0.070, 0.070)	0.031 (0.001, 0.062) *
LnΣDEHPm (ng/mL)	-0.068 (-0.362, 0.227)	0.034 (-0.110, 0.178)	0.129 (-0.017, 0.275) #	0.053 (-0.014, 0.120)
Visit 3 (N=48): median 39 weeks gestation				
Maternal phthalate metabolites				
Ln MMP (ng/mL)	-0.005 (-0.108, 0.099)	-0.002 (-0.056, 0.052)	0.004 (-0.053, 0.061)	-0.008 (-0.033, 0.017)
Ln MEP (ng/mL)	-0.008 (-0.132, 0.115)	0.032 (-0.031, 0.096)	0.015 (-0.053, 0.083)	0.003 (-0.027, 0.033)
Ln MiBP (ng/mL)	-0.011 (-0.160, 0.138)	0.012 (-0.066, 0.089)	0.035 (-0.047, 0.117)	0.022 (-0.013, 0.057)
Ln MnBP (ng/mL)	-0.017 (-0.140, 0.106)	0.025 (-0.038, 0.089)	0.051 (-0.015, 0.117)	0.002 (-0.028, 0.031)
Ln MEHP (ng/mL)	-0.066 (-0.168, 0.035)	-0.024 (-0.077, 0.030)	-0.006 (-0.063, 0.051)	-0.019 (-0.043, 0.006)
Ln MEHHP (ng/mL)	-0.046 (-0.199, 0.106)	-0.024 (-0.103, 0.055)	0.030 (-0.055, 0.114)	-0.013 (-0.050, 0.023)
Ln MEOHP (ng/mL)	-0.121 (-0.331, 0.089)	-0.048 (-0.157, 0.061)	0.066 (-0.050, 0.182)	0.002 (-0.049, 0.053)

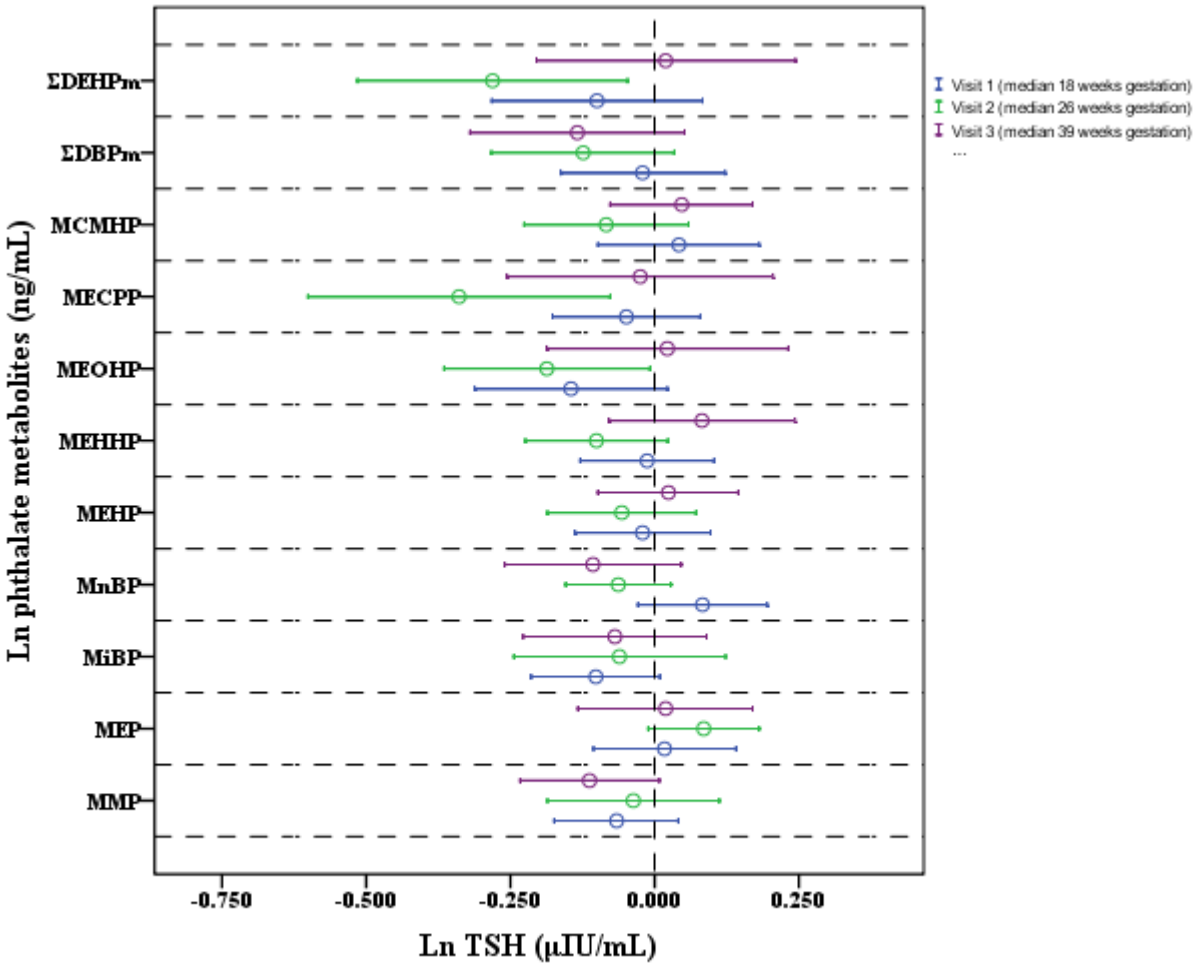
Ln MECPP (ng/mL)	-0.139 (-0.358, 0.080)	-0.071 (-0.185, 0.042)	0.051 (-0.071, 0.173)	0.002 (-0.051, 0.056)
Ln MCMHP (ng/mL)	-0.067 (-0.171, 0.036)	-0.030 (-0.084, 0.024)	0.028 (-0.029, 0.086)	0.002 (-0.024, 0.027)
LnΣDBPm (ng/mL)	-0.012 (-0.165, 0.141)	0.025 (-0.054, 0.104)	0.054 (-0.029, 0.137)	0.011 (-0.026, 0.048)
LnΣDEHPm (ng/mL)	-0.150 (-0.359, 0.059)	-0.077 (-0.185, 0.032)	0.048 (-0.069, 0.166)	-0.013 (-0.064, 0.038)

[a]Adjusted for maternal age at enrollment, urinary creatinine.

[b]All models were not simultaneously adjusted for other phthalate metabolites; #<0.1, *<0.05, **<0.01.

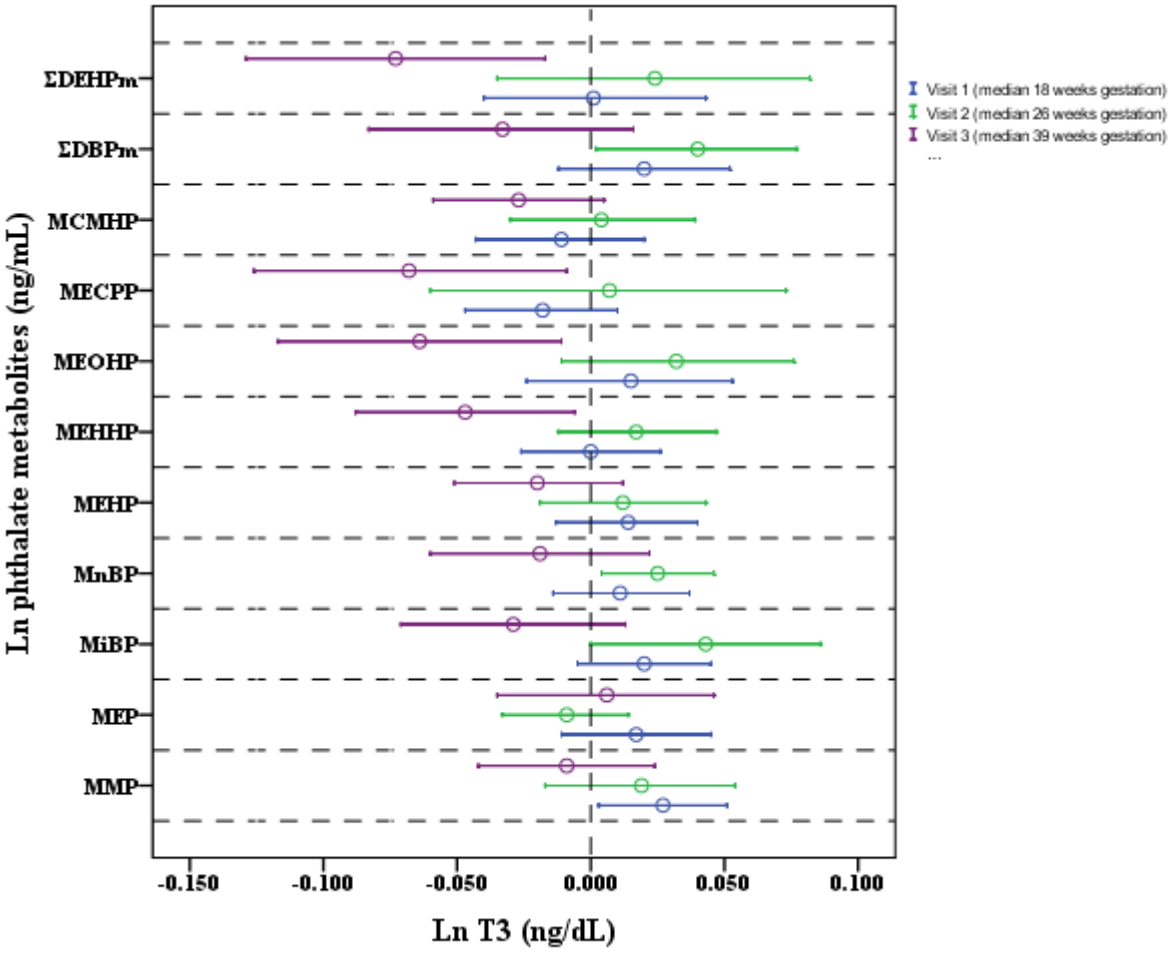
[c] The samples of cord serum for $FreeT_4$ were 49 at the visit 1, 49 at the visit 2 and 47 at the visit3, respectively.

(A)



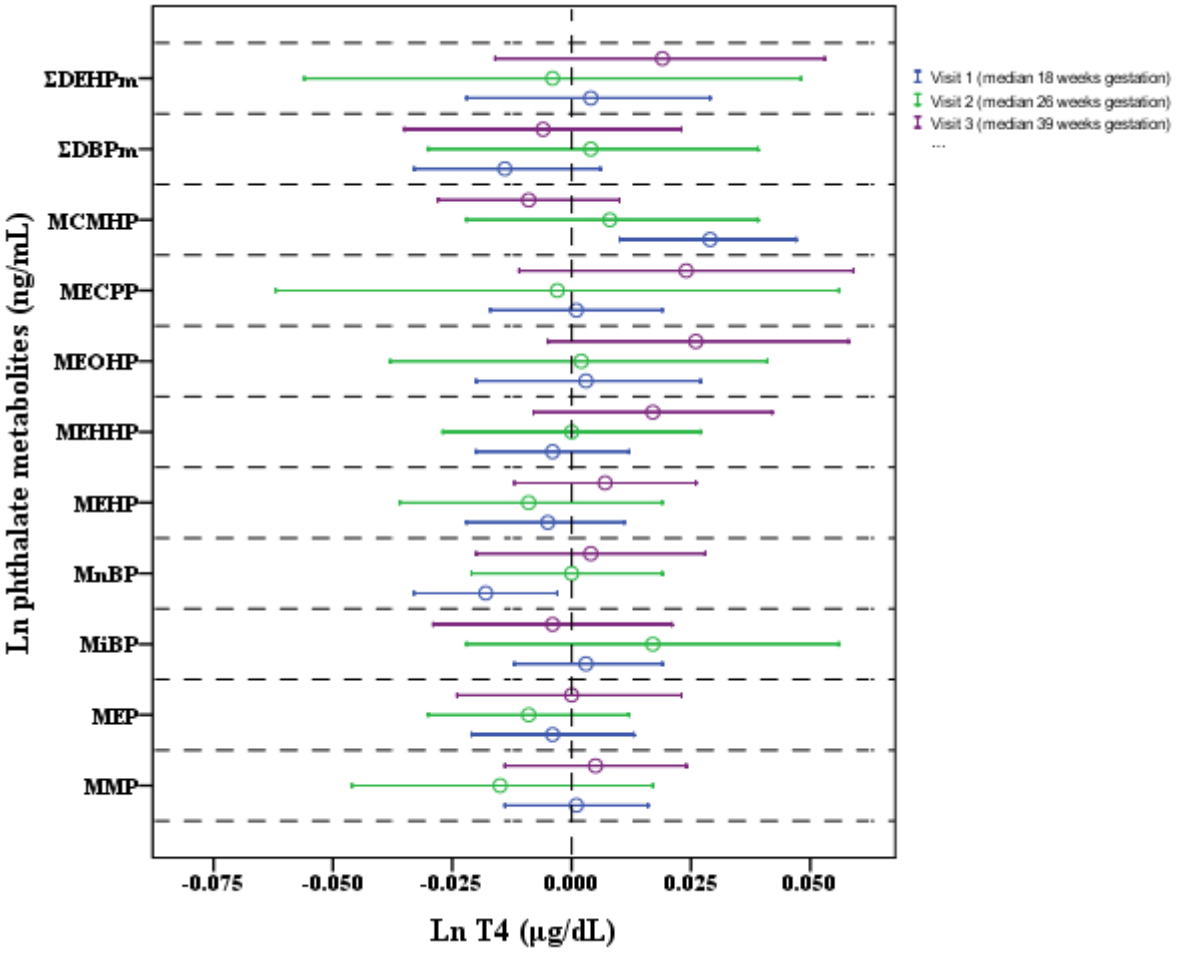
Adjusted regression coefficient (beta, 95%CI)

(B)



Adjusted regression coefficient (beta, 95%CI)

(C)



Adjusted regression coefficient (beta, 95%CI)

(D)

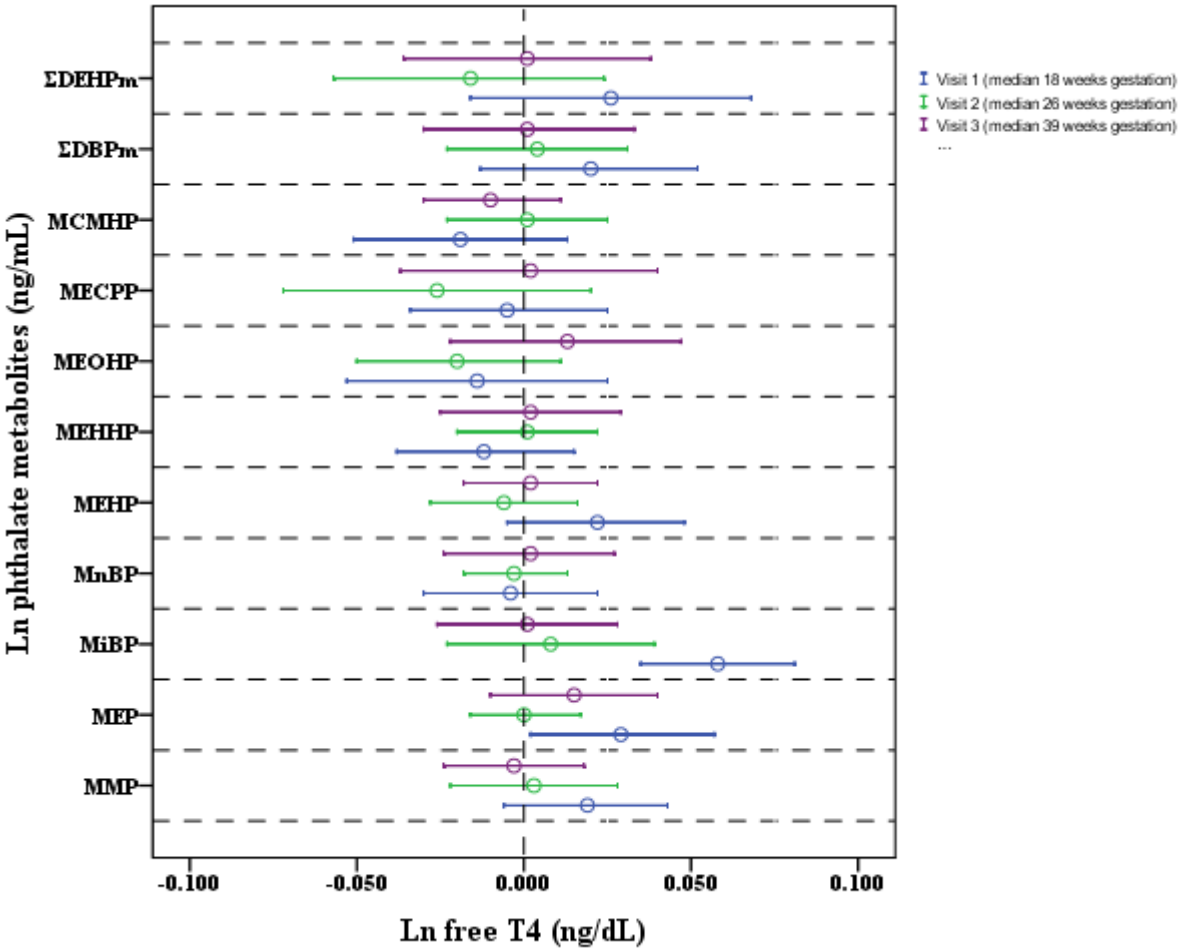


Figure 1. Cross-sectional analysis: adjusted regression coefficient and 95% CI for change in serum TSH (A), T_3 (B), T_4 (C), free T_4 (D) levels in relation to unit-increased in Ln-phthalate metabolites (ng/mL).