

Associations between urinary phthalate monoesters and thyroid hormones in pregnant women

Po-Chin Huang¹, Pao-Lin Kuo², Yue-Liang Guo³, Pao-Chi Liao¹ and Ching-Chang Lee^{1,4}

¹Department of Environmental and Occupational Health, Medical College, National Cheng Kung University, Tainan, Taiwan, Republic of China; ²Department of Obstetrics and Gynecology, National Cheng Kung University Hospital, Tainan, Taiwan, Republic of China;

³Department of Environmental and Occupational Medicine, College of Medicine, National Taiwan University, Taiwan, Republic of China

⁴Correspondence address. Tel: +886-6-274-4412; Fax: +886-6-274-3748; E-mail: clee@mail.ncku.edu.tw

BACKGROUND: Maternal hypothyroidism during pregnancy can cause adverse effects in the fetus. Scientific evidence has shown that probable thyroid-like function of some phthalates *in vitro* and *in vivo*, and phthalates exposure, can begin in utero. This study investigated the association between phthalate exposure and thyroid hormones in pregnant women. **METHODS:** Serum and spot urine samples were collected from 76 Taiwanese pregnant women at second trimester. Thyroid hormones, including thyroid-stimulating hormone (TSH), triiodothyronine (T₃), thyroxine (T₄) and free T₄ (FT₄) were analysed in serum samples, and five urinary phthalate monoesters, including mono butyl phthalate (MBP), monoethyl phthalate (MEP) and mono ethylhexyl phthalate (MEHP), were measured. **RESULTS:** Urinary MBP, MEP and MEHP, the median levels of which were 81.8, 27.7 and 20.6 ng/ml, respectively, were the predominant substances in the urinary phthalate monoesters. Significant mild negative correlations were found between T₄ and urinary MBP ($R = -0.248$, $P < 0.05$), and between FT₄ and urinary MBP ($R = -0.368$, $P < 0.05$). After adjusting for age, BMI and gestation, urinary MBP levels showed negative associations with FT₄ and T₄ (FT₄: $\beta = -0.110$, $P < 0.001$; T₄: $\beta = -0.112$, $P = 0.003$). **CONCLUSIONS:** Exposure to di-*n*-butyl phthalate (DBP) may affect thyroid activity in pregnant women, but how DBP affects thyroid function is unclear. Further studies are needed to elucidate the mechanism of action and to investigate whether any other factors related to DBP exposure alter the thyroid function.

Keywords: pregnant women; urinary phthalate monoesters; thyroid hormones.

Introduction

Thyroid hormone is essential for fetal development of the brain, neurons, heart and other organs during critical points of gestation (Cunningham *et al.*, 2006). The prevalence of sub-clinical hypothyroidism is 2–5% in pregnant women. Maternal hypothyroidism during pregnancy causes preterm birth and low birth weight, and it impairs post-natal mental development in infants (Haddow *et al.*, 1999; Pop *et al.*, 1999; Poppe and Glinioer, 2003). Therefore, fetal thyroid function crucially depends on the supply of maternal thyroid hormones during pregnancy (Morreale de Escobar *et al.*, 2004).

Some environmental toxicants, such as perchlorate, polychlorinated biphenyls (PCBs) and inorganic mercury (Hg), potentially alter human thyroid function during pregnancy by inhibiting iodide transport, inhibiting thyroglobulin iodination, or competitively inhibiting thyroid hormone binding receptors (Wolff, 1998; Takser *et al.*, 2005; Wang *et al.*, 2005). Phthalates, including butyl benzyl phthalate (BBP), di-*n*-butyl phthalate (DBP), di-(2-ethylhexyl) phthalate (DEHP) and

di-ethyl phthalate (DEP) are reproductive and developmental toxicants in animal models (Api, 2001; Kavlock *et al.*, 2002a,b, 2006). DBP and DEHP are considered anti-androgenic endocrine disruptors because of their possible effect on animal gonads and reproduction (Harris *et al.*, 1997; Ema and Miyawaki, 2001). In addition, some studies (Hinton *et al.*, 1986; Price *et al.*, 1988; Poon *et al.*, 1997; Sugiyama *et al.*, 2005; Pereira *et al.*, 2007) have reported possible antagonistic effects of phthalates on the thyroid gland *in vivo* and thyroid tissue *in vitro*. However, little is known about this issue in humans.

Phthalates are added to plastics to make them soft and flexible, to cosmetics as a vehicle for fragrance, and many other daily products, such as building materials, paints, children's toys and medical devices (Api, 2001; ATSDR, 1995, 2001, 2002; Kavlock *et al.*, 2002a). Because phthalates are released from these products, humans are exposed to them by food consumption, inhalation and dermal absorption. Phthalates are metabolized to their monoesters within a few hours or

days (Dirven *et al.*, 1993; Koch *et al.*, 2004); therefore, they do not appear to accumulate in humans. Because phthalates are ubiquitous in daily life, the potential consequences of human exposure to phthalates have raised concerns and have been widely studied in the general population (Blount *et al.*, 2000; Koch *et al.*, 2003; Silva *et al.*, 2004a). Urinary phthalate monoesters are considered good biomarkers for assessing phthalate exposure in humans because of their low contamination rate in the laboratory and reliability for indicating an individual's phthalate exposure (Hauser *et al.*, 2004; Koch *et al.*, 2004). Different species of urinary phthalate metabolites, such as mono butyl phthalate (MBP), monobenzyl phthalate (MBzP), mono-(2-ethylhexyl) phthalate (MEHP), monoethyl phthalate (MEP) and monomethyl phthalate (MMP), represent individual phthalate exposures from different sources.

A notable environmental burden of DBP and DEHP has been reported in several of Taiwan's rivers (Yuan *et al.*, 2002). Because phthalates can penetrate the placenta in rat and humans, high phthalate exposure levels in general, and in pregnant women in particular, in recent studies have raise the urgent issue of prenatal exposure to phthalate (Blount *et al.*, 2000; Adibi *et al.*, 2003; Latini *et al.*, 2003; Silva *et al.*, 2004b; Calafat *et al.*, 2006). The aim of this study was to investigate the association between phthalate exposure and thyroid hormone during pregnancy.

Materials and Methods

Participants

Our participants were pregnant women for whom abnormal blood biochemical levels of alpha fetal protein and free β -hCG or advanced maternal age (>35 years old) suggested the need to undergo amniocentesis by the clinical suggestion from gynaecologists. After they signed the informed consent for amniocentesis (for medical purposes), we interviewed them and explained the benefits and risks of participating in this longitudinal project during 2005–2006. After signing the inform consent form for this study, 76 (86.3%) of the women initially recruited were followed up in their second trimester. There were 12 women excluded either because they were carrying a fetus with abnormal genetic defects or because they miscarried. The protocol and informed consent form were approved by the Institutional Review Board of National Cheng Kung University Hospital.

Sample collection

We drew 8-ml blood samples via venipuncture into chemically clean glass tubes containing no anti-coagulant. After the blood had been centrifuged, serum samples were obtained for thyroid hormone analysis. Urine samples were collected in 250-ml glass vessels and immediately transferred into 12-ml amber glass bottles for phthalate monoester analysis. To prevent possible contamination of the urine samples, all the glassware was washed in methanol (MeOH), acetonitrile (ACN) and acetone, and then sealed with aluminum foil. All the serum and urine samples were collected at the same time and stored at -70 and -20°C , respectively, until they were analysed.

Urinary phthalate monoester analysis

We used a slightly modified version of a previously published method of measuring urinary phthalate monoester levels using high performance liquid chromatography electrospray ionization tandem mass spectrometry (HPLC-ESI-MS/MS), offline solid-phase extraction

(SPE) and isotope dilution (Blount *et al.*, 2000; Silva *et al.*, 2004c). MBP, MBzP, MEHP, MEP, MMP (all $>99.9\%$) and their ^{13}C -labelled internal standard ($>99.9\%$) were purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA). Ammonium acetate ($>98\%$) was purchased from Sigma Aldrich Laboratories, Inc. (St. Louis, MO, USA). Ammonium hydroxide (30%) and ethyl acetate ($>99.9\%$) were purchased from J. T. Baker (Phillipsburg, NJ, USA). MeOH, ACN, formic acid (all $>98\%$) and water (HPLC-grade) were purchased from Merck (Darmstadt, Germany). β -glucuronidase (*Escherichia coli*-K12) was purchased from Roche Biomedical (Mannheim, Germany). The Mightysil RP-18 GP (L) (100 mm \times 2.0 mm, 5 μm) analytical column and Mightysil RP-18 GP (5 mm \times 2.0 mm, 5 μm) guard column were purchased from Kanto Chemical Industries (Tokyo, Japan).

Each urine sample (1 ml) was thawed, sonicated for 6 min and poured into a glass culture tube (75 \times 125 mm) (Kimax borosilicate glass tubes; Kimble/Kontes, Vineland, NJ, USA). Samples were then buffered with ammonium acetate [250 μl , 1M (pH 6.5)] and then spiked with a mixture of isotope phthalate monoester standards (100 ng, 0.1 ng/ μl) and β -glucuronidase enzyme (5 μl , 200 U/ml). The sample was incubated at 37°C for 90 min to deconjugate the glucuronidated phthalate metabolites. After the sample was incubated, it was loaded into a SPE cartridge (Nexus; Varian, Inc., Palo Alto, CA, USA). Aliquots of 1 ml each of formic acid and H_2O were eluted to remove hydrophilic compounds. Then, 2 ml of acetonitrile and 2 ml of ethyl acetate were added to collect phthalate monoesters. The extract was dried with nitrogen gas and reconstituted with 1 ml of HPLC-grade H_2O in a 2-ml glass vial. One blank and one quality control (QC) sample were included in each batch of samples analysed. The QC sample was spiked in pooled urine with a mixture of phthalate monoester standards (100 ng/ml). The Mightysil RP-18 GP (L) analytical column with a 5-mm guard column was used for chromatographic fractionation. The chromatographic fractionation was done using a linear gradient program with an organic solvent (acetonitrile and 0.1% formic acid) and an aqueous solvent [$\text{H}_2\text{O}/\text{MeOH}$ (9:1, v/v)] at a flow rate of 0.6 ml/min. The effluent was then directly analysed using tandem mass spectrometry (API 365; Applied Biosystems, Foster City, CA, USA) with electro-spray ionization. The limits of detection (LOD) for five urinary phthalate monoesters were 5.0 ng/ml (MBP), 1.8 ng/ml (MBzP), 0.9 ng/ml (MEHP), 1.4 ng/ml (MEP) and 1.8 ng/ml (MMP). The calibration ranges of urinary MBP and other urinary phthalate monoesters were 10–1000 and 5–1000 ppb, respectively. The correlation coefficient (R^2) and relative SD (RSD) of the calibration curve should be >0.995 and $<15\%$, respectively. The recoveries of $^{13}\text{C}_{12}$ -labelled internal-standard and native-standard of each phthalate monoester in samples should be >50 and 75% , respectively. The phthalate monoesters level of the blank sample should be lower than twice of the minimum detectable limit in each batch. The SPE recoveries of five phthalate monoesters ranged from 74 to 88%, and the RSD of spiked QCs ranged from 9 to 23%.

Creatinine and thyroid hormones examination

Creatinine in urine and thyroid hormones in serum samples was measured by our hospital's pathology department. Samples of 8 ml of urine that had been stored at -20°C were analysed using combined clinical chemistry and immunoassay tests (Modular Analytics Serum Work Area; Roche Diagnostics). Samples of 2 ml of serum were analysed for estradiol (E_2), FSH, progesterone (PG), triiodothyronine (T_3), thyroxin (T_4), free T_4 (FT_4) and thyroid-stimulating hormone (TSH) using an electrochemoluminescence immunoassay (ECLIA) (Elecsys 2010 and Modular Analytics E170; Roche Diagnostics).

When the level of creatinine exceeded the reference range, it was analysed again for confirmation.

Questionnaire

An interview questionnaire was designed to obtain information about the general phthalate-exposure scenarios of pregnant women. Participants were asked to provide information about personal characteristics (age, height, weight, education, occupational history, medical care status, pregnancy history, etc.) and life-style habits (alcohol intake and tobacco use) to adjust for other confounding factors. Trained interviewers administered the questionnaires according to standard operating procedures prepared in advance.

Statistical analysis

Commercially available statistical software (JMP version 5.01; SAS Institute, Cary, NC, USA) was used for statistical analysis. Outcomes were evaluated for normal distribution and outliers. Levels of T₃, T₄ and FT₄ were normal, so these data did not need to be log transformed. On the contrary, the concentrations of E₂, FSH, PG and TSH were log transformed to approximate normal distribution. Age, BMI and length of gestation were evaluated as continuous variables, while the experience of pregnancy and delivery, smoking status, residence and workplace building characteristics and medical care history were evaluated as nominal variables. All the measured phthalate monoesters were log transformed to approximate normal distribution. We categorized all the participants into two groups by median, and the Wilcoxon rank sum test was used to evaluate differences between the degree of phthalate exposure and each nominal variable. Spearman correlation coefficients were used to assess the associations between age, BMI, gestation, each hormone level and each urinary monoester phthalate level. In addition, multiple linear regression analysis was used to adjust for significant covariates.

Results

Demographic characteristics of participants

The mean age of the participants was 33.6 ± 3.3 years (range: 26–43 years). The average duration of gestation when recruited and BMI were 27.9 ± 2.3 weeks and 20.9 ± 2.5 , respectively. The average number of pregnancies and child-births per participant were 1.9 ± 1.0 and 1.5 ± 0.6 , respectively. All our participants were non-smokers, but 14 participants had been exposed to passive smoke (18.4%). None of the participants were ‘alcohol drinkers’, which was defined as ‘someone who consumed any alcohol at all during pregnancy’. About 25% of them had been in a newly decorated home or workplace within one year before the study. Less than 5% of them had received medical care, such as a blood transfusion, within the previous three months (Table 1). We found no significant difference in the levels of the five urinary phthalate monoesters between different confounders, e.g. smoking habits, home or workplace characteristics, or medical care status, which indicated that these demographic factors may not have any effect on their levels. In addition, according to their questionnaire answers, none of the participants was working at a plastics- or cosmetics-related job, except for one who quit her job as a cosmetologist after learning she was pregnant.

Table 1: Demographic characteristics of study participants ($n = 76$)

Characteristics	Mean \pm SD
Age (years)	33.6 \pm 3.3
BMI	20.9 \pm 2.5
Duration of gestation (weeks)	27.9 \pm 2.3
Pregnancies and births	
Number of current pregnancy	1.9 \pm 1.0
Number of current birth	1.5 \pm 0.6
Smoking status ($n/\%$)	
Active smoker	0/0
Passive smoker	14/18.4
Non-smoker	62/81.6
Alcohol drinker ($n/\%$)	0/0
New decoration of living or working place during previous 1 year ($n/\%$) ^a	
Home	
Moving to a new house	7/9.2
Just decorated	4/5.3
Workplace	
Moving to a new workplace	2/2.6
Just decorated	6/7.9
Medical care during previous 3 months ($n/\%$)	
Blood transfusion	3/3.9
Intravenous drip	3/3.9
Oxygen mask	1/1.3

^a‘Moving to new house or workplace’ means moved into a new building. ‘Just decorated’ means the place lived or worked in was recently decorated, e.g. painted or the flooring was changed.

Urinary phthalate monoesters

The detectable rates of MBP, MEHP, MEP, MMP and MBzP in all urine samples were 96, 100, 100, 63 and 17%, respectively (Table 2). Median levels with (and without) creatinine adjustments for five urinary phthalate monoesters were- 195 (81.8 ng/ml) for MBP, 68.0 (27.7 ng/ml) for MEP, 60.8 (20.6 ng/ml) for MEHP, 10.8 (4.3 ng/ml) for MMP and 3.7 (0.9 ng/ml) for MBzP (Table 2). Levels of urinary MBP, MEP and MEHP were the highest of the five metabolites measured, which indicated that the participants were exposed predominantly to the phthalates DBP, DEP and DEHP. Although median urinary MBP levels were three times higher than median urinary MEP levels, the 95th percentile level of urinary MEP was seven times higher than that of urinary MBP, which showed the large variation of DEP exposure in pregnant women. The creatinine-adjusted levels of urinary phthalate monoesters were at least twice those which were not creatinine-adjusted (Table 2). Therefore, we used creatinine-unadjusted phthalate monoester levels for further analysis.

E₂ and thyroid-related hormone levels

There is no reference range for E₂ or thyroid hormones during pregnancy. However, our data still provided some clues by comparing participants’ levels with those of the general population in Taiwan. More than 90% of the levels of the thyroid hormones T₃, T₄ and TSH were within the reference values of the general population (Table 3). The median level (0.93 ng/dl) of FT₄, however, merely matched the lowest level for the general population, which indicated that half of our participants might have had a mild insufficiency of T₄ (i.e. hypothyroidism). In addition, the median creatinine level

Table 2: Unadjusted and adjusted-creatinine concentrations of urinary phthalate monoesters in our study and compared with other studies

Urinary phthalate monoesters ^a	Percentile								Median (range)	
	<i>n</i>	Min	5th	25th	50th	75th	95th	Max	US pregnant women ^c	US female population ^d
Creatinine unadjusted (ng/ml) ^b										
MBP	76	13.2	21.6	40.6	81.8	131.0	368.0	580.0	–	30.0 (5.8–167)
MBzP	76	0.9	0.9	0.9	0.9	0.9	33.4	35.3	–	16.0 (2.4–103)
MEP	76	0.7	2.20	13.1	27.7	52.4	2346.0	5466.0	–	174.0 (28–2230)
MEHP	76	5.85	7.21	13.1	20.6	38.6	273.0	381.0	–	3.0 (ND–21.6)
MMP	76	0.7	0.7	0.7	4.3	14.7	87.8	237.2	–	–
Creatinine-adjusted (µg/g creatinine)										
MBP	76	57.8	88.9	127.0	195.0	339.0	839.0	1901.0	42.6 (21.3–105)	28.6 (10.6–131)
MBzP	76	0.5	0.8	2.0	3.7	6.0	24.0	69.9	12.1 (5.6–120)	14.7 (4.84–80.0)
MEP	76	5.0	8.3	27.0	68.0	205.0	4414.0	13 299.0	236 (26.7–5520)	157 (42.7–1920)
MEHP	76	12.2	15.8	31.4	60.8	121.0	885.0	1251.0	4.6 (1.8–449)	3.33 (ND–16.3)
MMP	76	0.4	0.9	3.7	10.8	34.9	263.0	363.0	–	–

^aMBP, monobutyl phthalate; MBzP, monobenzyl phthalate; MEP, monoethyl phthalate; MEHP, mono-2-ethylhexyl phthalate and MMP, monomethyl phthalate.

^bDetection limit (LOD) of phthalate monoesters were: MBP, 5; MBzP, 1.8; MEP, 1.4; MEHP, 0.9; MMP, 1.4 ng/ml, respectively. Half of LOD was calculated as the detected value below the LOD. ^cNew York pregnant women 18–35 years old (*n* = 25). ^dData from NHANES 1999–2000 included children age 6 and older (*n* = 1326, range: 10th–95th percentile).

in our participants was 39.4 mg/dl; however, ~50% of them were below the reference range.

Association between urinary phthalate monoesters and hormones

We found significantly positive Spearman correlations between levels of E₂ and PG ($R = 0.566$, $P < 0.05$), levels of T₃ and T₄ ($R = 0.709$, $P < 0.05$) and levels of T₄ and FT₄ ($R = 0.761$, $P < 0.05$) (Table 4). Significantly mild negative correlations were found between T₄ and urinary MBP ($R = -0.248$, $P < 0.05$) and FT₄ and urinary MBP ($R = -0.368$, $P < 0.05$). Increasing age was correlated with lower T₃ and T₄ levels, and increasing BMI was correlated with higher urinary MBP and MEP levels. In addition, gestational age was correlated with lower E₂ levels. Therefore, these parameters were considered continuous variables for further adjustment.

Regression analysis

Fig. 1 shows a scatter plot of T₄ and FT₄ levels, and log urinary MBP levels (creatinine-adjusted and not creatinine-adjusted) for each participant. Creatinine-adjustment of the phthalate

monoesters in pregnant women may have obscured the correlation. The original concentration of each phthalate monoester is suggested for creatinine-sensitive individuals such as pregnant women. To identify the major factors contributing to serum T₄ and FT₄ concentrations, we used a multivariate regression model to examine the association between thyroid hormone levels and urinary phthalate monoesters (Table 5). After adjusting for age, BMI, gestational age and other phthalate monoesters of the participants, urinary MBP levels showed a negative association with FT₄ and T₄ (FT₄: $\beta = -0.110$, $P < 0.001$; T₄: $\beta = -0.112$, $P = 0.003$).

Discussion

We found a correlation between high-exposure levels of certain phthalates and alterations of some thyroid hormones in the pregnant women in the present study. High urinary MBP exposure was associated with low serum T₄ and FT₄ in these women during the second trimester. Although our sample size was small, the association between the concentrations of urinary MBP and FT₄ existed after a multivariate analysis. Few toxicological data are available on phthalate exposure,

Table 3: Estrogenic and thyroid hormone distribution of pregnant women (*n* = 76)

Hormone	Percentile								Reference range ^a
	Min	5th	25th	50th	75th	95th	Max		
E ₂ (pg/ml)	4856.0	7549.0	11 748.0	16 670.0	22 145.0	34 820.0	47 820.0	5.1–4250.0	
FSH (mIU/ml)	0.1	0.1	0.1	0.1	0.11	0.14	0.16	0.11–198.0	
Progesterone (ng/ml)	45.0	49.0	81.0	115.0	160.0	309.0	441.9	0.035–59.0	
T ₃ (ng/dl)	72.6	86.3	114.0	132.0	152.0	209.0	246.0	84.6–202.0	
T ₄ (µg/dl)	4.39	5.31	7.53	8.85	9.97	11.2	13.6	5.13–14.1	
Free T ₄ (ng/dl)	0.46	0.69	0.80	0.93	1.04	1.25	1.35	0.93–1.7	
TSH (µIU/ml)	0.22	0.31	0.74	1.1	1.6	3.4	5.19	0.27–4.2	
Creatinine (mg/dl)	3.9	6.4	17.3	39.4	75.5	158.6	185.6	30.0–125.0	

^aReference range for the general population in Taiwan. The analytic sensitivities of T₃, T₄, free T₄ and TSH were 19.5 ng/dl, 0.42 µg/dl, 0.023 ng/dl and 0.014 µIU/ml, respectively; those of creatinine, E₂, FSH and progesterone were 0.05 mg/dl, 5.0 pg/ml, 0.1 mIU/ml and 0.03 ng/ml, respectively. The coefficient variations of T₃, T₄, free T₄ and TSH were 2.9, 4.2, 3.1 and 3.0%, respectively; those of creatinine, E₂, FSH and progesterone were 1.8, 4.1, 3.4 and 4.0%, respectively.

Table 4: Spearman correlation coefficients between estrogenic hormones, thyroid hormone, age, BMI, duration of gestation and urinary phthalate monoester levels ($n = 75$)^a

	FSH ^b	E2 ^b	PG ^b	TSH ^b	T3	T4	FT4	Age	BMI	Gestation	MBP	MEP	MEHP	MBzP	MMP
FSH	1.0														
E2	-0.192	1.0													
PG	-0.252*	0.566*	1.0												
TSH	0.287*	-0.171	-0.102	1.0											
T3	-0.051	-0.033	0.078	0.145	1.0										
T4	-0.006	-0.039	0.054	0.291*	0.709*	1.0									
FT4	-0.095	-0.007	0.061	0.172	0.299*	0.761*	1.0								
Age	0.077	-0.009	0.025	0.203	-0.307*	-0.218*	-0.008	1.0							
BMI	-0.120	-0.087	-0.174	-0.044	0.309	0.137	-0.010	-0.106	1.0						
Gestation	-0.302*	0.419*	0.524*	-0.106	-0.018	0.038	0.057	-0.029	-0.131	1.0					
MBP	0.204	0.034	-0.046	0.079	-0.234	-0.248*	-0.368*	0.082	0.081*	-0.098	1.0				
MEP	-0.016	-0.178	-0.132	-0.020	-0.212*	-0.292*	-0.191*	0.156	-0.133	-0.116		1.0			
MEHP	-0.055	0.207	0.065	-0.082	-0.019	-0.039	0.017	0.080	0.095*	0.199	0.183*	1.0			
	-0.056	0.217	0.109	-0.107	-0.008	-0.021	0.041	0.142	0.064	0.234	0.208*		1.0		
MEHP	-0.007	0.127	-0.015	-0.060	-0.067	-0.100	-0.090	0.166	-0.030	0.030*	0.070*	0.066	1.0		
	-0.031	0.067	0.043	-0.009	-0.007	-0.059	-0.056	0.156	-0.114*	0.026	0.194*	0.130)		1.0	
MBzP	-0.055	0.027	-0.075	-0.080	-0.084	0.034	-0.007	-0.064	-0.027	0.118	0.150*	-0.041	-0.010	1.0	
	-0.103	-0.025	0.042	-0.113	-0.075	0.040	0.083	-0.064	-0.151	0.114	0.206*	-0.063	0.012*		1.0
MMP	-0.013	-0.010	-0.161	-0.078	-0.259	-0.089	0.007	-0.038	-0.006	-0.004	0.190	-0.031	0.184	0.182	1.0
	-0.015	-0.027	-0.094	-0.181	-0.223	-0.199	-0.037	0.118	-0.129	0.034	0.387*	0.394*	0.082	0.060)	

^aSpearman correlation coefficients: * $P < 0.05$. One outlier was excluded because of hypothyroidism ($n = 75$). Correlation coefficient of using creatinine-adjusted urinary phthalate monoesters are given in parentheses.

especially to DBP and DEHP, in pregnant animals and its effects on thyroid hormones. After high doses of DEHP to male Wistar rats to assess the effects of DEHP on their thyroid hormone (Hinton *et al.*, 1986), serum T₄ concentrations decreased after 21 days, which is indicative of thyroid hyperactivity, but there was no significant effect on serum T₃ levels. A study using a thyroid hormone assay (Sugiyama *et al.*, 2005) evaluated possible T₃ antagonist activity in three phthalates, including BBzP and DBP. Both phthalates showed thyroid hormone antagonist activities *in vivo*. In addition, an investigation of the effects of six phthalates on the transcriptional activity of a sodium/iodide symporter (NIS) reported that DBP appeared to down-regulate the human NIS promoter (Breous *et al.*, 2005). Although this evidence is not strong enough to be considered definitive proof, it reveals that some phthalates, such as DBP and DEHP, may modulate the transcriptional activity of NIS to induce thyroid hyperactivity and decrease thyroxin concentrations.

The present study had some limitations. First, our study population was relatively small: only 76 women. Second, we collected only one serum sample and one-spot urine sample to measure thyroid hormone and phthalate monoester levels. Although the variation in serum thyroid hormone levels may underestimate the observed correlation, it can provide a reliable measurement of thyroid hormones in population-base studies (Hashimoto *et al.*, 2006). Third, while we took precautions to prevent contamination, measuring phthalate esters in the laboratory might be biased by contaminated plastic equipment and might overestimate the exposure level (Colón *et al.* 2000). Therefore, several studies have focused on using phthalate metabolites to measure the aggregate exposure of phthalate from various sources and pathways in epidemiological studies (Blount *et al.*, 2000; Koch *et al.*, 2003; Silva *et al.* 2004a). Fourth, although phthalate monoester levels vary

within days, it has been suggested (Hoppin *et al.* 2002) that a single urine sample is a good indicator for phthalate monoester measurement. Good sensitivity and specificity for predicting human exposure to urinary MEP, MBP, MBzP, MMP and MEHP has been reported to range between 0.56 and 0.74 and 0.83 and 0.9, respectively, (Hoppin *et al.* 2002). Therefore, temporal variability of phthalate metabolites may also reduce the correlation between thyroid hormone and urinary phthalate monoester levels. Fifth, using creatinine to adjust the phthalate monoester levels in pregnant women may be not appropriate. Creatinine is influenced by muscle mass, racial differences and dietary intake of meat. In addition, by the second trimester, the glomerular filtration rate and renal blood flow in pregnant women were ~50 and 70% higher, respectively, than in age-matched healthy women who were not pregnant (James *et al.*, 2005). The serum creatinine levels of pregnant women dropped about 10% in the second trimester and increased to normal values post-partum (Kuhlback Widholm, 1966). Therefore, urinary creatinine level may be unusually diluted or concentrated during pregnancy. Urinary MBP, MEP and MEHP levels in US pregnant women were ~1.5 times higher than in age-matched healthy US women who were not pregnant (Adibi *et al.*, 2003). Finally, although creatinine-adjusted phthalate monoester levels may be affected by using cosmetics or personal care products, they are ultimately determined by the physiological change of creatinine during pregnancy.

The highest urinary MEP level (5466 ppb) was found in a participant who was a cosmetologist and had worked for >10 years before she became pregnant. Urinary MEP is a major phthalate metabolite associated with people who use personal care products and with pregnant women exposed through inhalation (Adibi *et al.* 2003; Duty *et al.* 2005). Therefore, cosmetologists may be exposed to higher levels of DEP through direct skin contact and inhalation of DEP evaporation from

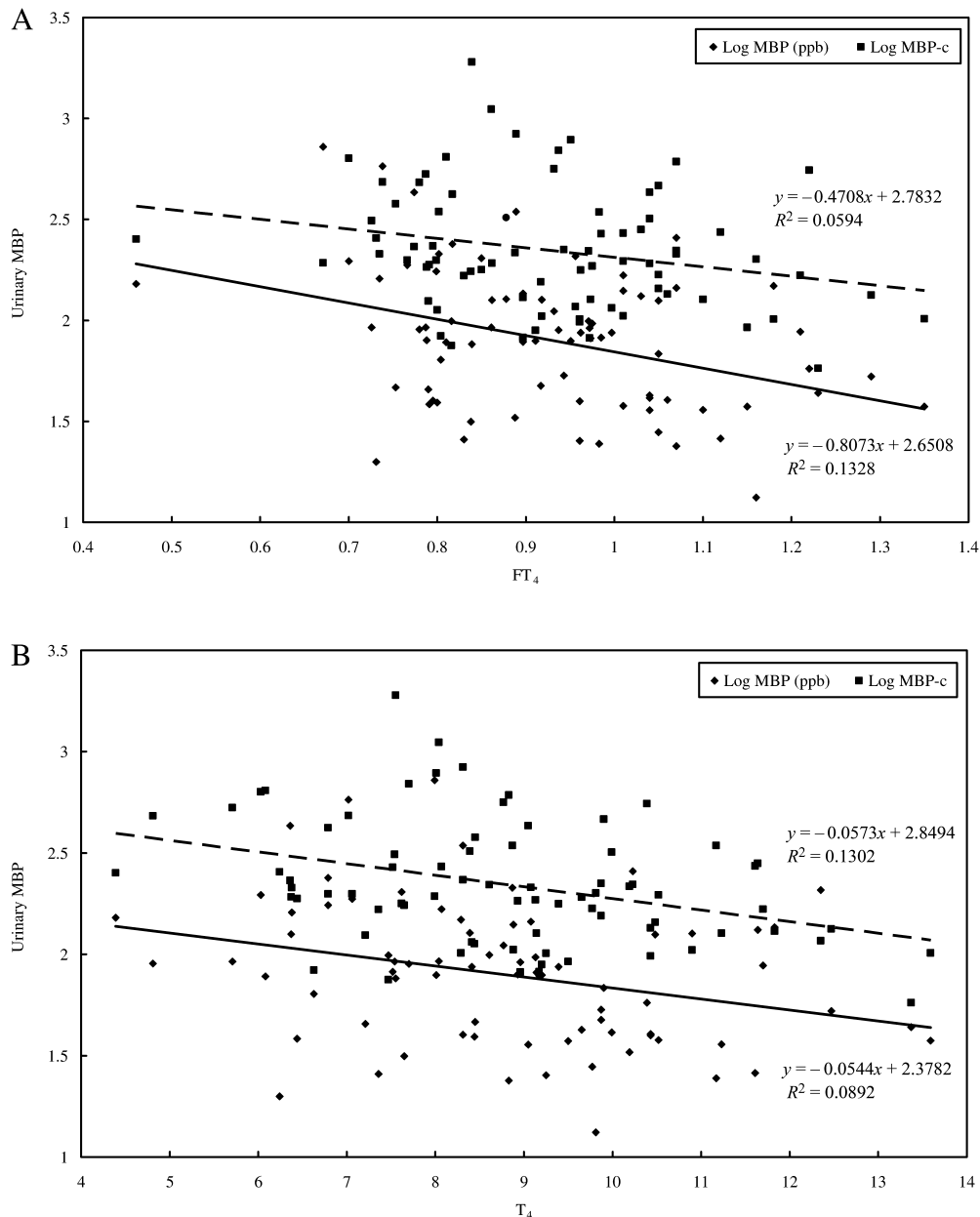


Figure 1: Linear regression of thyroid hormones and urinary MBP, creatinine-adjusted (squares, dashed line) and not creatinine-adjusted (diamonds, solid line)

cosmetic products. In addition, a recent study reported the synergistic effect of DEP and PCB on rat thyroids (Pereira *et al.*, 2007). We suggest that risk assessment of occupational phthalate exposure in cosmetologists, hairdressers, cosmetics saleswomen, etc. at reproductive age is worthy of further investigation.

This is the first report that shows an association between phthalate exposure and thyroid hormones in pregnant women. Although maternal thyroid hormone changes are linked to phthalate monoester levels in urine, we believe that our data may have missed the most critical period of fetal development. Fetal thyroid is not functional before the 12th week of gestation, which indicates that the fetus depends entirely on maternal thyroid hormones during the first trimester

(Morreale de Escobar and Rove, 2004; Zoeller *et al.*, 2004). However, a maternal surge of free T₄ concentrations occurs in the first trimester (between 6 and 12 weeks) in healthy pregnant women, and free T₄ concentrations gradually decrease and become steady after the middle of the second trimester, a suitable clinical point for determining the variation of thyroid hormones in pregnant women, and are maintained until term (Glinoe, 1997; Hume *et al.*, 2004; Morreale de Escobar *et al.*, 2004). Some longitudinal studies of large numbers of pregnant women without iodine deficiency reported that serum FT₄ levels were an average of 10–15% lower at delivery than in non-pregnant women. However, free hormone levels are maintained within the non-pregnant reference range in most pregnant women (Ball *et al.*, 1989;

Table 5: Multivariate linear regression between serum FT₄ and T₄ levels, and their corresponding urinary phthalate monoesters (FT₄: R² = 0.240; T₄: R² = 0.187)^a

Variables	FT ₄ (pmole/l)		T ₄ (nmole/l)	
	Estimate	Prob> t	Estimate	Prob> t
Intercept	1.270	0.013	2.362	0.001
Age	0.024	0.886	-0.321	0.177
BMI	0.088	0.579	0.348	0.120
Gestational age	-0.117	0.598	-0.066	0.831
MBP	-0.110	<0.001	-0.112	0.003
MEP	0.026	0.124	0.013	0.398
MEHP	-0.015	0.474	-0.007	0.814
MBzP	0.022	0.232	0.032	0.224
MMP	0.016	0.165	0.015	0.347

^aOne outlier was excluded because of hypothyroidism ($n = 75$). All the parameters were log transformed.

Burrow *et al.*, 1993; Sieiro Netto *et al.*, 2004). Based on the scientific evidence above, concentrations of urinary phthalate monoesters might be an alternation of maternal phthalates exposure in early pregnancy if the exposure profiles of phthalates in pregnant women were consistent. Since food consumption is the main exposure route of DBP in the general population (Chan and Meek, 1994), accounting for over 90% of DBP exposure. In addition, MBP has been detected in amniotic fluid, which revealed a possible indicator of thyroid function in the fetus (Silva *et al.* 2004b). Therefore, our data may be a proxy of maternal phthalate exposure in early pregnancy.

Conclusions

We found that levels of T₄ and FT₄ in pregnant women were significantly negatively associated with urinary MBP levels after adjusting for age, BMI and gestation time, whereas we found no significant association between E₂, FSH, or PG and urinary phthalate monoester levels. The fall in T₄ and FT₄ levels during pregnancy may be potentially harmful to fetal development. Hence, several questions require further investigation. Do phthalates affect thyroid hormones beginning in early pregnancy? What mechanisms do phthalates and their metabolites use to regulate thyroid hormones?

Acknowledgements

We are grateful for the pregnant women who participated in this study. We are also greatly in debt to our colleagues at the Research Center of Environmental Trace Toxic Substances, National Cheng Kung University, Tainan, Taiwan, for instrument support. This work was supported by grant (NSC 93-2621-Z-006-005) from the National Science Council in Taiwan.

References

Adibi JJ, Perera FP, Jedrychowski W, Camann D, Barr D, Jacek R, Whyatt RM. Prenatal exposures to phthalates among women in New York and Krakow, Poland. *Environ Health Perspect* 2003;**111**:1719–1722.

Api AM. Toxicological profile of diethyl phthalate: a vehicle for fragrance and cosmetic ingredients. *Food Chem Toxicol* 2001;**39**:97–108.

ATSDR. Toxicological Profile for Di(2-ethylhexyl) phthalate. Atlanta, GA: Agency for Toxic Substances and Disease Registry, 2002. <http://www.atsdr.cdc.gov/toxprofiles/tp9.html> (26 July 2004 date last accessed).

ATSDR. Toxicological Profile for Diethyl phthalate. Atlanta, GA: Agency for Toxic Substances and Disease Registry, 1995. <http://www.atsdr.cdc.gov/toxprofiles/tp73.html>. (26 July 2004, date last accessed).

ATSDR. Toxicological Profile for Di-n-butyl phthalate. Atlanta, GA: Agency for Toxic Substances and Disease Registry, 2001. <http://www.atsdr.cdc.gov/toxprofiles/tp135.html> (26 July 2004, date last accessed).

Ball R, Freedman DB, Holmes JC, Midgley JE, Sheehan P. Low-normal concentrations of free thyroxin in serum in late pregnancy: physiological fact, not technical artefact. *Clin Chem* 1989;**35**:1891–1896.

Blount BC, Silva MJ, Caudill SP, Needham LL, Pirkle JL, Sampson EJ, Lucier GW, Jackson RJ, Brock JW. Levels of seven urinary phthalate metabolites in a human reference population. *Environ Health Perspect* 2000;**108**:979–982.

Breous E, Wenzel A, Loos U. The promoter of the human sodium/iodide symporter responds to certain phthalate plasticizers. *Mol Cell Endocrinol* 2005;**244**:75–78.

Burrow GN. Thyroid function and hyperfunction during gestation. *Endocr Rev* 1993;**14**:194–202.

Calafat AM, Brock JW, Silva MJ, Gray LE, Reidy JA, Barr DB, Needham LL. Urinary and amniotic fluid levels of phthalate monoesters in rats after the oral administration of di(2-ethylhexyl) phthalate and di-n-butyl phthalate. *Toxicology* 2006;**217**:22–30.

Chan PK, Meek ME. Di-n-butyl phthalate: valuation of risks to health from environmental exposure in Canada. *J Environ Sci Health C Environ Carcinog Ecotoxicol Rev* 1994;**12**:257–268.

Colón I, Caro D, Bourdony CJ, Rosario O. Identification of phthalate esters in the serum of young Puerto Rican girls with premature breast development. *Environ Health Perspect* 2000;**108**:895–900.

Cunningham FG, Leveno KJ, Bloom SL, Hauth JC, Gilstrap LC, Wenstrom KD. *Williams Obstetrics*, 22nd edn. New York, NY: McGraw-Hill, 2006.

Dirven HA, van den Broek PH, Arends AM, Nordkamp HH, de Lepper AJ, Henderson PT, Jongeneelen FJ. Metabolites of the plasticizer di (2-ethylhexyl) phthalate in urine samples of workers in polyvinylchloride processing industries. *Int Arch Occup Environ Health* 1993;**64**:549–554.

Duty SM, Ackerman RM, Calafat AM, Hauser R. Personal care product use predicts urinary concentrations of some phthalate monoesters. *Environ Health Perspect* 2005;**113**:1530–1535.

Ema M, Miyawaki E. Adverse effects on development of the reproductive system in male offspring of rats given monobutyl phthalate, a metabolite of dibutyl phthalate, during late pregnancy. *Reprod Toxicol* 2001;**15**:189–194.

Glinoe D. The regulation of thyroid function in pregnancy: pathways of endocrine adaptation from physiology to pathology. *Endocr Rev* 1997;**18**:404–432.

Haddow JE, Palomaki GE, Allan WC, Williams JR, Knight GJ, Gagnon J, O'Heir CE, Mitchell ML, Hermos RJ, Waisbren SE *et al.* Maternal thyroid deficiency during pregnancy and subsequent neuropsychological development of the child. *N Engl J Med* 1999;**341**:549–555.

Harris CA, Henttu P, Parker MG, Sumpter JP. The estrogenic activity of phthalate esters *in vitro*. *Environ Health Perspect* 1997;**105**:802–811.

Hashimoto K, Yamakita N, Ikeda T, Matsuhisa T, Kuwayama A, Sano T, Hashimoto K, Yasuda K. Longitudinal study of patients with idiopathic isolated TSH deficiency: possible progression of pituitary dysfunction in lymphocytic adenohypophysitis. *Endocr J* 2006;**53**:593–601.

Hauser R, Meeker JD, Park S, Silva MJ, Calafat AM. Temporal variability of urinary phthalate metabolite levels in men of reproductive age. *Environ Health Perspect* 2004;**112**:1734–1740.

Hinton RH, Mitchell FE, Mann A, Chescoe D, Price SC, Nunn A, Grasso P, Bridges JW. Effects of phthalic-acid esters on liver and thyroid. *Environ Health Perspect* 1986;**70**:195–210.

Hoppin JA, Brock JW, Davis BJ, Baird DD. Reproducibility of urinary phthalate metabolites in first morning urine samples. *Environ Health Perspect* 2002;**110**:515–518.

Hume R, Simpson J, Delahunty C, van Toor H, Wu SY, Williams FL, Vissar TJ. Human fetal and cord serum thyroid hormones: developmental trends and interrelationships. *J Clin Endocrinol Metab* 2004;**89**:4097–4103.

James DK, Steer PJ, Weiner CP, Gonik B. *High Risk Pregnancy: Management Options*, 3rd edn. Philadelphia, PA: Elsevier-Saunders, 2005.

Kavlock R, Boekelheide K, Chapin R, Cunningham M, Faustman E, Foster P, Golub M, Henderson R, Hinberg I, Little R *et al.* NTP Center for the Evaluation of Risks to Human Reproduction: phthalates expert panel

- report on the reproductive and developmental toxicity of butyl benzyl phthalate. *Reprod Toxicol* 2002a;**16**:453–487.
- Kavlock R, Boekelheide K, Chapin R, Cunningham M, Faustman E, Foster P, Golub M, Henderson R, Hinberg I, Little R *et al.* NTP Center for the Evaluation of Risks to Human Reproduction: phthalates expert panel report on the reproductive and developmental toxicity of di-*n*-butyl phthalate. *Reprod Toxicol* 2002b;**16**:489–527.
- Kavlock R, Barr D, Boekelheide K, Breslin W, Breyse P, Chapin R, Gaido K, Hodgson E, Marcus M, Shea K *et al.* NTP-CHRHR expert panel update on the reproductive and developmental toxicity of di(2-ethylhexyl) phthalate. National Toxicology Program, US Department of Health and Human Services. 2006.<http://cerhr.niehs.nih.gov/chemicals/dehp/dehp-eval.html> (13 December 2006, date last accessed)
- Koch HM, Drexler H, Angerer J. An estimation of the daily intake of di(2-ethylhexyl) phthalate (DEHP) and other phthalates in the general population. *Int J Hyg Environ Health* 2003;**206**:77–83.
- Koch HM, Bolt HM, Angerer J. Di(2-ethylhexyl)phthalate (DEHP) metabolites in human urine and serum after a single oral dose of deuterium-labelled DEHP. *Arch Toxicol* 2004;**78**:123–130.
- Kuhlback B, Widholm O. Plasma creatinine in normal pregnancy. *Scand J Clin Lab Invest* 1966;**18**:654–656.
- Latini G, De Felice C, Presta G, Del Vecchio A, Paris I, Ruggieri F, Mazzeo P. *In utero* exposure to di-(2-ethylhexyl) phthalate and duration of human pregnancy. *Environ Health Perspect* 2003;**111**:1783–1785.
- Morreale de Escobar G, Obregón MJ, Escobar del Rey F. Maternal thyroid hormones early in pregnancy and fetal brain development. *Best Pract Res Clin Endoc Metab* 2004;**18**:225–248.
- Pereira C, Mapuskar K, Rao CV. A two-generation chronic mixture toxicity study of Clophen A60 and diethyl phthalate on histology of adrenal cortex and thyroid of rats. *Acta Histochem* 2007;**109**:29–36.
- Poppe K, Glinoe D. Thyroid autoimmunity and hypothyroidism before and during pregnancy. *Hum Reprod Update* 2003;**9**:149–161.
- Pop VJ, Kuijpers JL, van Baar AL, Verkerk G, van Son MM, de Vijlder JJ, Vulsma T, Wiersinga WM, Drexhage HA, Vader HL. Low maternal free thyroxine concentrations during early pregnancy are associated with impaired psychomotor development in infancy. *Clin Endocrinol* 1999;**50**:149–155.
- Poon R, Lecavalier P, Mueller R, Valli VE, Procter BG, Chu I. Subchronic oral toxicity of di-*n*-octyl phthalate and di(2-ethylhexyl) phthalate in the rat. *Food Chem Toxicol* 1997;**35**:225–239.
- Price SC, Chescoe D, Grasso P, Wright M, Hinton RH. Alterations in the thyroids of rats treated for long periods with di-(2-ethylhexyl) phthalate or with hypolipidaemic agents. *Toxicol Lett* 1988;**40**:37–46.
- Sieiro Netto LS, Coeli CM, Micmacher E, Mamede SC, Nazar LO, Correa EK, Arrastia M, Galvão D, Buescu A, Vaisman H. Longitudinal study of pituitary-thyroid axis in pregnancy. *Arq Bras Endocrinol Metab* 2004;**48**:493–498.
- Silva MJ, Barr DB, Reidy JA, Malek NA, Hodge CC, Caudill SP, Brock JW, Needham LL, Calafat AM. Urinary level of seven phthalate metabolites in the U.S. population from the national health and nutrition examination survey (NANES) 1999–2000. *Environ Health Perspect* 2004a;**112**:331–338.
- Silva MJ, Reidy JA, Herbert AR, Preau JL Jr, Needham LL, Calafat AM. Detection of phthalate metabolites in human amniotic fluid. *Bull Environ Contam Toxicol* 2004b;**72**:1226–1231.
- Silva MJ, Slakman AR, Reidy JA, Preau JL, Herbert AR, Samandar E, Needham LL, Calafat AM. Analysis of human urine for fifteen phthalate metabolites using automated solid-phase extraction. *J Chromatogr B* 2004c;**805**:161–167.
- Sugiyama SI, Shimada N, Miyoshi H, Yamauchi K. Detection of thyroid system-disrupting chemicals using *in vitro* and *in vivo* screening assays in *Xenopus laevis*. *Toxicol Sci* 2005;**88**:367–374.
- Takser L, Mergler D, Baldwin M, de Grosbois S, Smargiassi A, Lafond J. Thyroid hormones in pregnancy in relation to environmental exposure to organochlorine compounds and mercury. *Environ Health Perspect* 2005;**113**:1039–1045.
- Wang SL, Su PH, Jong SB, Guo YL, Chou WL, Pöpke O. *In utero* exposure to dioxins and polychlorinated biphenyls and its relations to thyroid function and growth hormone in newborns. *Environ Health Perspect* 2005;**113**:1645–1650.
- Wolff J. Perchlorate and thyroid gland. *Pharmacol Rev* 1998;**50**:89–105.
- Yuan SY, Liu C, Liao CS, Chang BV. Occurrence and microbial degradation of phthalate esters in Taiwan river sediments. *Chemosphere* 2002;**49**:1295–1299.
- Zoeller RT, Rove J. Timing of thyroid hormone action in the developing brain: clinical observations and experimental findings. *Neuroendocrinology* 2004;**16**:809–818.

Submitted on March 10, 2007; resubmitted on June 3, 2007; accepted on June 13, 2007