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Mediating role of oxidative/nitrosative stress biomarkers in the associations between phthalate exposure and thyroid function in Taiwanese adults

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ABSTRACT

Phthalate exposure was shown to alter thyroid function, however it is unclear, whether oxidative and nitrosative stress explains the intermediate biological mechanism. This study aimed to investigate the associations between phthalate exposure, oxidative/nitrosative stress, and thyroid function in adults, and to examine the mediating role of oxidative/nitrosative stress in the associations between phthalate exposure and thyroid function. Levels of eleven urinary phthalate metabolites, three urinary biomarkers of oxidative/nitrosative stress (malondialdehyde [MDA], 8-OHdG, and 8-NO_2Gua) and five serum thyroid hormones (thyroxine [T_4], free T_4, triiodothyronine, thyroid-stimulating hormone, and thyroxine-binding globulin) were measured in 266 Taiwanese adults. Cross-sectional associations between phthalate metabolites, biomarkers of oxidative/ nitrosative stress and thyroid hormones were analyzed using multivariate regression models. Mediation analysis was conducted to assess the role of oxidative/nitrosative stress in the associations between phthalate metabolites and thyroid hormone levels. Sum of di-(2-ethylhexyl) phthalate (DEHP) metabolites was positively associated with MDA ($\beta_{T1\text{-}T2}$ = 0.253, 95%CI [0.060, 0.447]; $\beta_{\geq T2}$ = 0.317, 95% CI [0.098, 0.536]; P_{trend} = 0.005) and 8-NO_2Gua ($\beta_{T1\text{-}T2}$ = −0.010, 95%CI [−0.138, 0.118]; $\beta_{\geq T2}$ = 0.144, 95% CI [−0.001, 0.289]; P_{trend} = 0.045). Mono-n-butyl phthalate (MnBP) was positively associated with 8-NO_2Gua ($\beta_{T1\text{-}T2}$ = 0.201, 95% CI [0.078, −0.324]; $\beta_{\geq T2}$ = 0.161, 95% CI [0.031, −0.292]; P_{trend} = 0.018). T_4 was negatively associated with MDA (β_{T1-T2} = −0.027, 95% CI [−0.088, 0.0034]; $\beta_{\geq T2}$ = −0.094, 95% CI [−0.161, −0.028]; P_{trend} = 0.005) and 8-NO_2Gua (β_{T1-T2} = −0.068, 95% CI [−0.127, −0.010]; $\beta_{\geq T2}$ = −0.125, 95% CI [−0.184, −0.066]; P_{trend} < 0.001). Free T_4 was positively associated with MDA (P_{trend} = 0.047) and with 8-NO_2Gua (P_{trend} < 0.001). 8-NO_2Gua mediated 11% of the association between the sum of DEHP metabolites and T_4, and 17% of the association between MnBP and free T_4. These results suggest that phthalate exposure may influence thyroid hormone levels through induced oxidative/nitrosative stress.

Abbreviations: 8-NO_2Gua, 8-nitroguanine; 8-OHdG, 8-hydroxy-2′-deoxyguanosine; BBzP, butyl benzyl phthalate; DBP, dibutyl phthalate; DEHP, di (2-ethylhexyl) phthalate; DEP, diethyl phthalate; DiBP, diisobutyl phthalate; DiNP, diisononyl phthalate; DMP, dimethyl phthalate; DnBP, di-n-butyl phthalate; MBzP, mono-benzyl phthalate; MCMHP, mono (2-carboxymethylhexyl) phthalate; MDA, malondialdehyde; MECPP, mono (2-ethyl-5-carboxypentyl) phthalate; MEHHP, mono (2-ethyl-5-hydroxyhexyl) phthalate; MEHP, mono (2-ethylhexyl) phthalate; MEOHP, mono (2-ethyl-5-oxo-hexyl) phthalate; MEP, mono-ethyl phthalate; MiBP, mono-iso-butyl phthalate; MiNP, mono-iso-nonyl phthalate; MMP, mono-methyl phthalate; MnBP, mono-n-butyl phthalate; RNS, reactive nitrogen species; ROS, reactive oxygen species; T_4, thyroxine; T_3, triiodothyronine; TSH, thyroid-stimulating hormone; TBG, thyroid-binding globulin

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1. Introduction

Phthalates are a group of industrial chemicals, ubiquitously used in the production of cosmetics, food containers, medical tools, pharmaceuticals, plastics, and building materials (Hauser and Calafat, 2005; Koch and Calafat, 2009). Because phthalates are not chemically bound to the product, their continuous leaching during the product's use and even after its disposal contributes to pervasive exposure of the general population through digestion, dermal contact, and inhalation (Heudorf et al., 2007).

Aggregated evidence suggests that phthalate exposure is detrimental to the endocrine system (Katsikantami et al., 2016; Mathieu-Denoncourt et al., 2015). Urinary phthalate metabolites were associated with thyroid hormone homeostasis (H. B. Huang et al., 2017; Y. F. Huang et al., 2017; Meeker and Ferguson, 2011), which is crucial to many physiological processes of the cardiovascular and reproductive systems, including fetal and child development (Diamanti-Kandarakis et al., 2009; Mathieu-Denoncourt et al., 2015; Miller et al., 2009). Epidemiologic studies in multiple countries have shown that di-2-ethylhexyl phthalate (DEHP) metabolites were negatively correlated with thyroxine (T_4) and triiodothyronine (T_3) and positively correlated with thyroid stimulating hormone (TSH) in the general population (H. B. Huang et al., 2017; Y. F. Huang et al., 2017; Meeker and Ferguson, 2011; Park et al., 2017), in children (Boas et al., 2010), and in pregnant women (Romano et al., 2018). However, the mechanism of phthalate exposure's effect on thyroid hormone regulation through specific mediators remains largely unknown.

Oxidative and nitrosative stress results from an imbalance between antioxidant defenses and the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS), which are crucial pathogenesis factors in various diseases and conditions (Halliwell and Gutteridge, 2015). Malondialdehyde (MDA) and 8-hydroxy-2′-deoxyguanosine (8-OHdG) are among the most commonly measured oxidative stress biomarkers (OSB) in human studies to assess lipid peroxidation and DNA damage, respectively (Il'yasova et al., 2012). Positive associations between specific phthalate metabolites and these OSB were found in different ethnicities and age groups, such as Korean and Brazilian children (Kim et al., 2014; Rocha et al., 2017), American and Saudi adults (Guo et al., 2014; Li et al., 2019), American pregnant women (Ferguson et al., 2014), Taiwanese adolescents (Lin et al., 2017), and elderly Chinese patients with diabetes (Dong et al., 2018). However, these results were not consistent for different phthalates and OSB, likely due to the diversity of study populations and methods.

Oxidative and nitrosative stress has been linked to thyroid function in both animal and human studies (Mancini et al., 2016). The potential mediating role of 8-OHdG was shown on the pathways between phthalate exposure to prostate enlargement in elderly Taiwanese men (Chang et al., 2019) and to semen quality in Chinese men (Liu et al., 2019), whereas animal studies have suggested the possible mediation of the phthalate effect on thyroid function through 8-OHdG (Wu et al., 2017). Positive associations between phthalates and MDA have been identified in both animal (Ye et al., 2017; Zhang et al., 2017) and human studies (Dong et al., 2018; Kim et al., 2014). While elevated levels of MDA were observed in patients with thyroid dysfunctions (Muzza et al., 2016), no mediation effect has been reported.

Only a few studies have investigated oxidative DNA damage and lipid peroxidation in the pathway between phthalate exposure and different outcomes, and none have assessed the mediation of the phthalate exposure effect on thyroid hormones through oxidative and nitrosative stress. Previously, we found that phthalates influenced thyroid hormone levels in the general population of Taiwanese adults and children (H. B. Huang et al., 2017; Y. F. Huang et al., 2017). In the present study, we explore the mediating roles of lipid peroxidation (MDA) and oxidative (8-OHdG) and nitrosative (8-NO_2Gua) DNA damage in the association between phthalate metabolites and thyroid functions in the general Taiwanese adult population.

2. Methods

2.1. Study participants and design

The data for this cross-sectional study were retrieved from the Taiwan Environmental Survey for Toxicants (TEST) conducted in 2013 (Chang et al., 2017; H. B. Huang et al., 2017; Y. F. Huang et al., 2017). Detailed description of the recruitment process was provided previously (Huang et al., 2015; Supplements). Briefly, subjects had to be Taiwanese ≥ 7 years old, and excluded pregnant and breast-feeding women, individuals with severe disease (e.g. cancer patients), and citizens in hospitals and jails. We interviewed a total of 500 subjects from seventeen townships of eleven cities/ counties in Taiwan from May to December 2013. A total of 394 subjects participated in the TEST. This study included participants aged 18 years or older from the TEST and excluded 49 participants without blood samples or with insufficient urine samples, resulting in a final sample size of 266 adults. Information on individual characteristics (age, sex, body-mass index, etc.), and life style exposures (use of cigarettes, alcohol, insecticides, etc.) were collected through an interviewer-administered questionnaire. However, we cannot obtain their medical record about their clinical disease history (i.e.: thyroid diseases or diabetes or heart diseases). The Research Ethics Committee of the National Health Research Institutes in Taiwan granted approval no. EC1020206 to this study, and each participant provided informed consent to participate in the survey.

2.2. Analytical method of oxidative/nitrosative stress biomarkers

Urinary levels of oxidative (8-OHdG) and nitrosative (8-NO_2Gua) stress biomarkers were measured using isotope dilution liquid chromatography–tandem mass spectrometry (LC–MS/MS) method, which was previously described in details (Wu et al., 2016). MDA levels in urine samples were assessed by measuring of thiobarbituric acid (TBA) reactive substances, resulting from the reaction between MDA and TBA at high temperatures of 90–100 °C. Urinary samples were assayed using a commercial kit (Cayman Chemicals No.10009055, MI, USA) according to the manufacturer's protocol. The formed MDA–TBA product was measured colorimetrically at 530–540 nm. The inter- and intra-assay coefficients of variation were 7.6% and 5.1%, respectively.

2.3. Analytical method of urinary phthalate metabolites

Description of the analytical method for 11 urinary phthalate metabolites have been published elsewhere (H. B. Huang et al., 2017; Huang et al., 2015; Y. F. Huang et al., 2017; Liao et al., 2018) (Supplements). Briefly, we used online LC-MS/MS method (API 4000; Applied Biosystems, Foster City, CA, USA) to assess levels of eleven phthalate metabolites, like mono (2-ethyl-5-carboxypentyl) phthalate (MECPP) and mono-n-butyl phthalate (MnBP), etc. The sums of molar concentrations were calculated for DEHP metabolites (ΣDEHPm) and DBP metabolites (ΣDBPm) divided by molecular weight individually (Supplements). The limit of detection (LOD) for MEHP, MEOHP, MEHHP, MECPP, MCMHP, MnBP, MiBP, MEP, MiNP, MBzP, and 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. Levels below the LOD, were analyzed as half of their LOD value (Hornung and Reed, 1990).

2.4. Analytical method of serum thyroid hormones

Serum levels of thyroid hormones (T_4, free T_4, T_3 and TSH) and thyroxine binding globulin (TBG) were analyzed in the morning blood samples that were instantly centrifuged for 20 min at 4 °C after collection and then stored at −80 °C until analysis. We used chemiluminescent microparticle immunoassay (Beckman Coulter Inc., Brea, CA, USA) and immuno enzymometric assay (Monobind Inc., Product Code 3525-300) to quantify the serum levels of thyroid hormones. Most of

thyroid hormone levels in our participants were within the normal ranges (Supplements). All analyses were conducted in a certified laboratory (H. B. Huang et al., 2017; Y. F. Huang et al., 2017) in random order and label-blinded for the technician.

2.5. Statistical analysis

We summarized the participants' demographic characteristics and their distributions of urinary phthalate metabolites and biomarkers of oxidative/nitrosative stress, and serum thyroid hormones. Analytes with detection rates of less than 50% were excluded from further analysis. Urinary phthalate metabolites levels, urinary MDA, 8-OHdG, 8-NO_2Gua, and serum thyroid hormones levels were transformed with natural logarithms (ln) to meet the assumption of the normality. Multivariate linear regressions were fitted to assess the associations between tertiles of phthalate metabolites and oxidative/nitrosative stress biomarkers, oxidative/nitrosative stress biomarkers and thyroid hormones, and phthalate metabolites and thyroid hormones. Trends across tertiles were assessed with p values after entering the tertiles of urinary phthalate metabolites or oxidative stress as continuous variables of ordinal integer values (i.e., 1–3) into multivariate regressions. All the regression models were adjusted for the following covariates: age (continuous), gender (categorical), body mass index (BMI, continuous), cigarette smoking (binary), urinary creatinine levels and serum TBG (continuous). We referred the previous study (Barr et al., 2005) which revealed that adjusting urinary creatinine could significantly bias the results. Urinary creatinine or specific gravity was used as the covariate in the models to minimize the intra-individual variability of spot urine sample measurements (Li et al., 2019; Ferguson et al. 2017; Chang et al., 2019; Liu et al., 2019). Therefore, we adopted their suggestion to use urinary creatinine levels as a covariate factor. Our previous studies (Huang et al., 2015) showed high (ranged from 0.83 to 0.96) correlation between levels of urinary phthalates with creatinine-adjusted and those without. The selection of covariates was based on earlier studies (Boas et al., 2010; Meeker and Ferguson, 2011) and on the criterion of at least 10% change in the estimated coefficient (Rothman et al., 2008).

The estimates from the multivariate linear regressions were used as indicators for further mediation analysis. If the phthalate metabolites were significantly associated with the biomarkers of oxidative stress and with thyroid hormones and the biomarkers of oxidative stress were also significantly associated with thyroid hormones, we performed mediation analysis to evaluate the direct and indirect effects and the proportion mediated using R mediation package (Tingley et al., 2014). Bootstrapping with 1000 simulations was applied. The direct effect of phthalate metabolites on thyroid hormones was derived from the linear regression models adjusted for oxidative stress biomarkers. The indirect effect was calculated from the linear regression models linking the phthalate metabolites with the oxidative stress biomarkers and the oxidative stress biomarkers with the thyroid hormones. The proportion meditated by oxidative stress was calculated as the ratio of indirect effect to total effect. Two-sided $p < 0.05$ was considered statistically significant. The statistical analyses were performed using SAS version 9.2 (SAS Institute Inc., Cary, NC, USA) and R version 3.5.1 (R Foundation for Statistical Computing) software.

3. Results

3.1. Study population

Our study population included 266 Taiwanese adults (≥18 years old), approximately half (53.4%) of whom were women. The mean age and BMI were 53.6 ± 17.0 years and 24.7 ± 4.4, respectively. Most of the participants were from northern (31.2%) or central (24.1%) Taiwan, were married (73.3%), and had an education of college level or higher (35.3%). Approximately half of our participants drank tea (58.3%) and coffee (40.6%), while much lower percentages of participants reported cigarette smoking (24.4%), alcohol consumption (13.2%), betel nut chewing (7.1%), and the use of insecticide at home (23.7%) (Table 1).

Table1
Demographic characteristics of the study population (N = 266).

Variables	Adults (≧18 years, N = 266)	
	N	%
Gender		
Male	124	46.6
Female	142	53.4
Age (years, Mean ± SD)	266	53.6 ± 17.0
BMI (Mean ± SD)	266	24.7 ± 4.37
Region		
Northern Taiwan	83	31.2
Central Taiwan	64	24.1
Southern Taiwan	47	17.7
Eastern Taiwan	45	16.9
Remote islands	27	10.2
Marital status		
Single	44	16.5
Married	195	73.3
Divorce/ widowed	27	10.2
Education		
≦Elementary school	74	27.8
Junior high school	39	14.7
Senior high school	59	22.2
≧College/ Graduates	94	35.3
Cigarette smoking[a]		
Yes	65	24.4
Alcohol consumption[b]		
Yes	35	13.2
Tea drinking [c]		
Yes	155	58.3
Coffee drinking[c]		
Yes	108	40.6
Betel nut chewing[d]		
Yes	19	7.1
Insecticide use at home		
Yes	63	23.7

[a] Subjects consuming at least one cigarette per day.
[b] Subject consuming at least one bottle of alcohol drink per week.
[c] Subjects consuming at least one cup of tea or coffee per week.
[d] Subject chewing at least one betel nut per week.

3.2. Distribution of phthalate metabolites, biomarkers of oxidative stress, and thyroid hormones

Table 2 summarizes the distributions of urinary phthalate metabolites and oxidative/nitrosative stress biomarkers, and serum thyroid hormones levels. All of the thyroid hormones and oxidative/nitrosative stress biomarkers had detection rates of 100%. Most phthalate metabolites had detection rates ranging from 63.5% to 98.1%, except for MBzP (21.8%) and MiNP (10.2%), which were excluded from further analysis. The highest medians and ranges of urinary phthalate metabolites concentrations (ng/mL) were observed in MMP (24.12, ND – 7117), MECPP (20.30, ND – 975.1), MEHHP (16.46, ND – 487.9), MnBP (15.37, ND – 6106) and MEP (11.51, ND – 3286). The sums of molar concentrations (ΣDEHPm) ranged within 0.018–6.865 nmole/mL for ΣDEHPm, and within 0.001–27.54 nmole/mL for ΣDBPm. Urinary oxidative/nitrosative stress biomarkers were distributed as follows: MDA levels (μmole/L) ranged within 0.660–103.0, median = 7.02; 8-OHdG levels (ng/mL) ranged within 1.020–47.44, median = 3.845; and 8–NO_2Gua levels (ng/mL) ranged within 1.190–11.67, median = 1.950. The following medians and ranges of serum thyroid hormones were observed in T_3 (103.5 ng/dL, 15.00–192), TBG (21.11 μg/mL, 11.80–34.20), TSH (1.520 μIU/mL, 0.007–15.52), T_4 (7.278 μg/dL, 3.410–14.05), and free T_4 (0.928 ng/dL, 0.530–1.600).

Table 2
Distribution of urinary phthalate metabolites (ng/ mL), biomarkers of oxidative stress, and thyroid hormone levels among Taiwanese adults.

Variables	Adults (≧18 years) (N = 266)						
	> LOD[a] %	GM (95% CI)	Minimum	P_{33}	P_{50}	P_{66}	Maximum
Phthalate metabolites							
MMP	96.2	24.55 (20.24, 29.77)	N.D.	13.62	24.12	38.99	7117
MEP	90.6	10.17 (8.154, 12.69)	N.D.	6.278	11.51	19.61	3286
MiBP	71.1	3.522 (2.715, 4.569)	N.D.	3.653	7.160	14.00	286.6
MnBP	86.5	9.690 (7.645, 12.28)	N.D.	8.280	15.37	22.82	6106
MBzP	21.8	0.308 (0.261, 0.364)	N.D.	N.D.	N.D.	N.D.	15.75
MEHP	77.8	3.689 (2.955, 4.604)	N.D.	3.927	6.777	10.00	138.9
MEHHP	98.1	15.90 (14.01, 18.04)	N.D.	11.35	16.46	24.57	487.9
MEOHP	93.2	8.346 (7.128, 9.772)	N.D.	6.993	10.28	13.63	289.6
MECPP	96.2	17.57 (15.11, 20.44)	N.D.	14.58	20.30	28.69	975.1
MCMHP	63.5	1.524 (1.219, 1.906)	N.D.	N.D.	3.168	5.204	203.7
MiNP	10.2	0.209 (0.185, 0.236)	N.D.	N.D.	N.D.	N.D.	15.01
ΣDEHPm (nmole/mL)[b]		0.199 (0.180, 0.219)	0.018	0.141	0.198	0.275	6.865
ΣDBPm (nmole/mL)[b]		0.095 (0.080, 0.114)	0.001	0.071	0.109	0.163	27.54
Biomarkers of oxidative stress							
MDA (μmole/ L)	100	7.400 (6.820, 8.029)	0.660	6.010	7.020	8.660	103.0
8-OHdG (ng/mL)	100	3.991 (3.688, 4.319)	1.020	2.830	3.845	5.040	47.44
8-NO2Gua (ng/mL)	100	2.138 (2.033, 2.489)	1.190	1.680	1.950	2.340	11.67
Hormones							
TSH (μIU/mL)	100	1.520 (1.385, 1.668)	0.007	1.201	1.551	1.997	15.52
T_3 (ng/dL)	100	103.5 (100.6, 106.5)	15.00	97.00	106.5	114.0	192.0
T_4 (μg/dL)	100	7.278 (7.082, 7.477)	3.410	6.670	7.465	8.100	14.05
Free T_4 (ng/dL)	100	0.928 (0.908, 0.949)	0.530	0.860	0.930	1.010	1.600
TBG (μg/mL)	100	21.11 (20.57, 21.66)	11.80	20.00	21.70	23.60	34.20

Abbreviations: 8-nitroguanine (8-NO_2Gua), 8-hydroxy-2′-deoxyguanosine (8-OHdG), geometric mean (GM), limit of detection (LOD), malondialdehyde (MDA), mono-methyl phthalate (MMP), mono-ethyl phthalate (MEP), mono-*iso*-butyl phthalate (MiBP), mono-n-butyl phthalate (MnBP), mono-benzyl phthalate (MBzP), mono-ethylhexyl phthalate (MEHP), mono-(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP), mono-(2-ethyl-5-oxo-hexyl) phthalate (MEOHP), mono-(2-ethyl-5-carboxypentyl) phthalate (MECPP), mono-(2-carboxymethylhexyl) phthalate (MCMHP), mono-*iso*-nonyl phthalate(MiNP), not detectable (ND), thyroxine (T_4), thyroid-binding globulin (TBG), thyroid-stimulating hormone (TSH), triiodothyronine (T_3).

[a] The limit of detection (LOD) for MEHP, MEOHP, MEHHP, MECPP, MCMHP, MnBP, MiBP, MEP, MiNP, MBzP, and 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.

[b] ΣDEHPm = sum molar concentrations of MEHP + MEHHP + MEOHP + MECPP + MCMHP; ΣDBPm = sum molar concentrations of MiBP + MnBP.

3.3. Associations between urinary phthalate metabolites and oxidative stress biomarkers

After adjustment for age, sex, BMI, cigarette smoking, and urinary creatinine levels, the log-transformed levels of oxidative/nitrosative stress biomarkers (MDA, 8-OHdG, and 8-NO_2Gua) were significantly associated with the tertiles of urinary concentration of several phthalate metabolites. Significantly higher estimates of log-transformed MDA compared with the first tertile were observed in the second and third tertiles of MEP ($\beta_{T1\text{-}T2}$ = 0.328, 95% CI [0.145, 0.511]; $\beta_{\geqq T2}$ = 0.377, 95%CI [0.185, 0.570]; P_{trend} < 0.001), MECPP ($\beta_{T1\text{-}T2}$ = 0.239, 95% CI [0.051, 0.426]; $\beta_{\geqq T2}$ = 0.405, 95% CI [0.207, 0.603]; P_{trend} < 0.001) and ΣDEHPm ($\beta_{T1\text{-}T2}$ = 0.253, 95%CI [0.060, 0.447]; $\beta_{\geqq T2}$ = 0.317, 95% CI [0.098, 0.536]; P_{trend} = 0.005). Significant trends of log-transformed MDA were also found for positive associations with MiBP ($\beta_{T1\text{-}T2}$ = 0.147, 95%CI [-0.036, 0.330]; $\beta_{\geqq T2}$ = 0.257, 95% CI [0.065, 0.449]; P_{trend} = 0.009) and MEHHP ($\beta_{T1\text{-}T2}$ = 0.117, 95%CI [-0.076, 0.310]; $\beta_{\geqq T2}$ = 0.326, 95% CI [0.109, 0.544]; P_{trend} = 0.003) across the tertiles, whereas only the third tertile showed significantly higher levels of log-transformed MDA compared with the first tertile (Fig. 1, supplementary Table S1).

Significantly lower estimates of log-transformed 8-NO_2Gua compared with the first tertile were observed in the second and third tertiles of MiBP ($\beta_{T1\text{-}T2}$ = −0.207, 95% CI [−0.327, −0.088]; $\beta_{\geqq T2}$ = −0.135, 95% CI [−0.261, −0.010]; P_{trend} = 0.036). However, those estimates were significantly higher for MnBP ($\beta_{T1\text{-}T2}$ = 0.201, 95% CI [0.078, −0.324]; $\beta_{\geqq T2}$ = 0.161, 95% CI [0.031, −0.292]; P_{trend} = 0.018). Significant trends of log-transformed 8-NO_2Gua were found for positive associations with MEHHP ($\beta_{T1\text{-}T2}$ = −0.056, 95%CI [−0.183, 0.071]; $\beta_{\geqq T2}$ = 0.145, 95% CI [0.002, 0.288]; P_{trend} = 0.043) and MEOHP ($\beta_{T1\text{-}T2}$ = 0.028, 95%CI [-0.097, 0.154]; $\beta_{\geqq T2}$ = 0.178, 95% CI [0.041, 0.315]; P_{trend} = 0.010) across the tertiles, whereas only the third tertile showed significantly higher levels of log-transformed 8-NO_2Gua in comparison to the first tertile. A marginally significant trend across the tertiles ($\beta_{T1\text{-}T2}$ = -0.010, 95%CI [-0.138, 0.118]; $\beta_{\geqq T2}$ = 0.144, 95% CI [-0.001, 0.289]; P_{trend} = 0.045) was also observed for ΣDEHPm. A significant trend was also observed in MMP tertiles for log-transformed 8-OHdG ($\beta_{T1\text{-}T2}$ = 0.078, 95%CI [-0.089, 0.245]; $\beta_{\geqq T2}$ = 0.195, 95% CI [0.018, 0.373]; P_{trend} = 0.031), whereas only the third tertile was significantly associated with log-transformed 8-OHdG compared with the first tertile (Fig. 1, supplementary Table S1).

3.4. Associations between oxidative stress biomarkers and thyroid hormones

Fig. 2 presents the relationships between the tertiles of the oxidative stress biomarkers and log-transformed serum thyroid hormones levels after adjustment for age, sex, BMI, cigarette smoking, TBG, and urinary creatinine levels in our participants. Significant associations with oxidative stress biomarkers were found for T_4 and free T_4, but not for T_3 or TSH.

The dose–response relationship was noted, as significantly lower estimates of log-transformed T_4 compared with the first tertile were associated with second and third tertiles of 8-NO_2Gua ($\beta_{T1\text{–}T2}$ = −0.068, 95% CI [−0.127, −0.010]; $\beta_{\geqq T2}$ = −0.125, 95% CI [−0.184, −0.066]; P_{trend} < 0.001). Significant trends of log-transformed T_4 were also found for negative associations with MDA ($\beta_{T1\text{-}T2}$ = −0.027, 95%CI [-0.088, 0.034]; $\beta_{\geqq T2}$ = −0.094, 95% CI [−0.161, −0.028]; P_{trend} = 0.005) across the tertiles, whereas only the third tertile showed significantly higher levels of log-transformed T_4 compared with the first tertile. No significant association was found between T_4 and 8-OHdG (Fig. 2, supplementary Table S2).

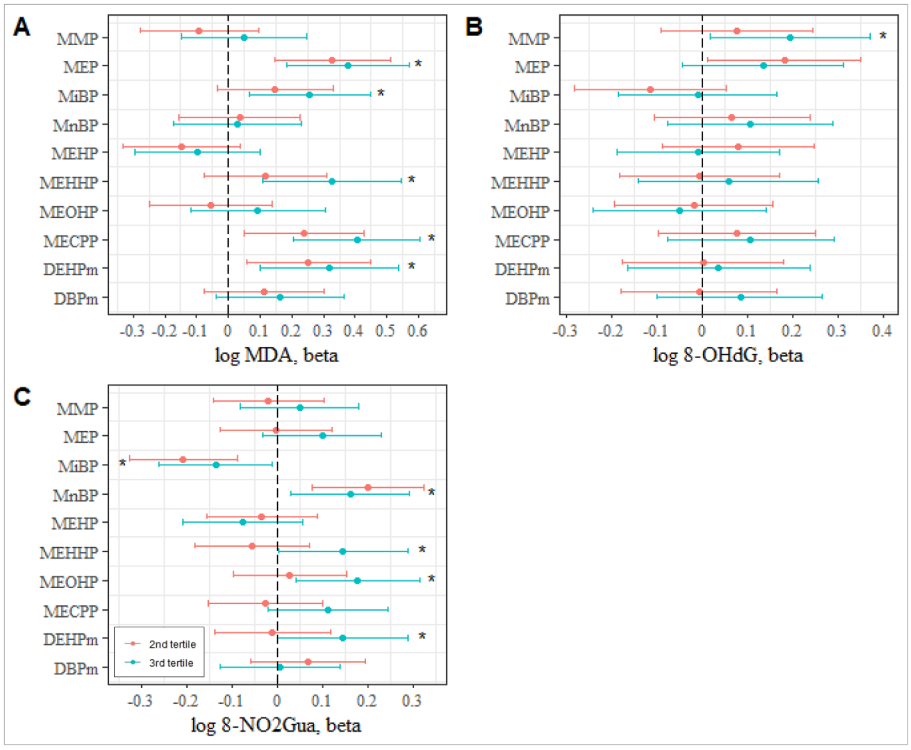

Fig. 1. Regression coefficients and 95% CI for changes in biomarkers of oxidative stress associated with tertiles of urinary phthalate metabolites. All regression models are adjusted for age, sex, BMI, cigarette smoking, and urinary creatinine levels. (DEHPm = sum molar concentrations of MEHP + MEHHP + MEOHP + MECPP + MCMHP; DBPm = sum molar concentrations of MiBP + MnBP; First tertile as reference; *: P for trend < 0.05.)

Significantly higher estimates of log-transformed free T_4 compared with the first tertile were observed in the second and third tertiles of 8-NO_2Gua (β_{T1-T2} = 0.061, 95% CI [0.009, 0.113]; $\beta_{\geqq T2}$ = 0.099, 95% CI [0.046, 0.151]; P_{trend} < 0.001), suggesting a dose–response relationship between 8-NO_2Gua and log-transformed free T_4. Significant trends of log-transformed free T_4 were also found for positive associations with MDA (β_{T1-T2} = 0.053, 95%CI [-0.002, 0.107]; $\beta_{\geqq T2}$ = 0.0.60, 95% CI [0.001, 0.119]; P_{trend} = 0.047) and 8-OHdG (β_{T1-T2} = 0.033, 95%CI [-0.021, 0.088]; $\beta_{\geqq T2}$ = 0.071, 95% CI [0.011, 0.130]; P_{trend} = 0.020) across the tertiles, whereas only the third tertile showed significantly higher levels of log-transformed T_4 compared with the first tertile (Fig. 2, supplementary Table S2).

3.5. Relationship between urinary phthalate metabolites and thyroid functions

Fig. 2 shows the relationships between log-transformed serum hormones levels and tertiles of urinary phthalate metabolites after

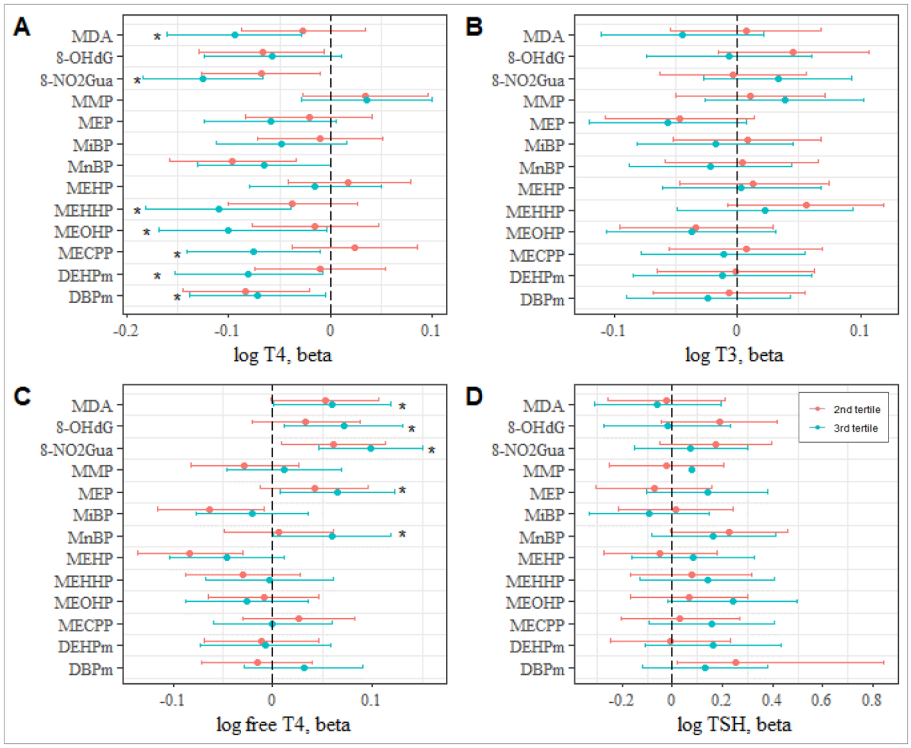

Fig. 2. Regression coefficients and 95% CI for changes in serum thyroid hormones associated with tertiles of oxidative stress biomarkers and urinary phthalate metabolites. All regression models are adjusted for age, sex, BMI, cigarette smoking, and urinary creatinine levels. (DEHPm = sum molar concentrations of MEHP + MEHHP + MEOHP + MECPP + MCMHP; DBPm = sum molar concentrations of MiBP + MnBP; First tertile as reference; *: P for trend < 0.05.)

adjustment for age, sex, BMI, cigarette smoking, urinary creatinine levels, and serum TBG levels in our participants. Significant associations were identified between log-transformed T_4 and MnBP, MEHHP, MEOHP, MECPP, ΣDEHPm, and ΣDBPm, but not with the other metabolites. Log-transformed free T_4 was only significantly associated with MEP and MnBP. No significant associations with phthalate metabolites were observed for T_3 and TSH.

A negative dose–response was noted, as significantly lower estimates of log-transformed T_4 compared with the first tertile were observed in the second and third tertiles of ΣDBPm ($\beta_{T1-T2} = -0.083$, 95% CI [−0.145, −0.021; $\beta_{\geqq T2} = -0.072$, 95% CI [−0.138, −0.005]; $P_{trend} = 0.039$). Significant trends of log-transformed T_4 were found for negative associations with MEHPP ($\beta_{T1-T2} = -0.037$, 95%CI [−0.101, 0.026]; $\beta_{\geqq T2} = -0.110$, 95% CI [-0.182, −0.039]; $P_{trend} = 0.002$), MEOHP (β_{T1-T2} = -0.015, 95%CI [−0.077, 0.047]; $\beta_{\geqq T2} = -0.101$, 95% CI [−0.169, −0.003]; $P_{trend} = 0.004$), MECPP ($\beta_{T1-T2} = 0.024$, 95%CI [−0.038, 0.086]; $\beta_{\geqq T2} = -0.076$, 95% CI [−0.141, −0.010]; $P_{trend} = 0.022$), and ΣDEHPm (β_{T1-T2} = -0.010, 95%CI [-0.074, 0.054]; $\beta_{\geqq T2}$ = -0.081, 95% CI [-0.153, −0.008]; $P_{trend} = 0.027$) across the tertiles, whereas only the third tertile showed significantly higher levels of log-transformed T_4 compared with the first tertile (Fig. 2, supplementary Table S3).

Significant trends of log-transformed free T_4 were found for positive associations with MEP ($\beta_{T1-T2} = 0.042$, 95%CI [−0.013, 0.096]; $\beta_{\geqq T2} = 0.065$, 95% CI [0.008, 0.122]; $P_{trend} = 0.026$) and MnBP ($\beta_{T1-T2} = 0.006$, 95%CI [−0.049, 0.061]; $\beta_{\geqq T2} = 0.060$, 95% CI [0.001, 0.118]; $P_{trend} = 0.041$) across the tertiles, whereas only the third tertile showed significantly higher levels of log-transformed free T_4 compared with the first tertile (Fig. 2, supplementary Table S3).

3.6. Mediation of oxidative stress biomarkers in the relationship between phthalate metabolites and thyroid functions

Table 3 shows the results of the mediation analysis, which was performed only for those phthalate metabolites or their molar sums and oxidative/nitrosative stress biomarkers that were significantly associated with specific thyroid hormones. Our analysis showed that 17% of the association between MnBP and free T_4 was mediated by 8-NO_2Gua (indirect effect of 0.003, 95% CI [0.001, 0.01]). The association between ΣDEHPm and T_4 was also partially (11%) mediated by 8–NO2Gua. Borderline significant mediation (8%) was observed for the association between ΣDEHPm and T_4 by MDA. We also conducted a sensitivity analysis of using continuous data or creatinine-adjusted of biomarkers which revealed consistent results (Supplementary Table S4 to S9).

4. Discussion

This study is the first to explore the mediating role of oxidative/nitrosative stress in the association between phthalate exposure and thyroid function in Taiwanese adults. Our findings suggest that phthalates may influence thyroid hormones through lipid peroxidation and nitrosative DNA damage, as the inverse association between urinary metabolites of DEHP and serum T_4 was partially mediated by MDA (8%) and 8-NO_2Gua (11%), and the positive association between urinary MnBP and serum free T_4 was partially mediated by 8-NO_2Gua (17%) (Fig. 3). Our study provides an initial step in understanding the role of oxidative/nitrosative stress on the adverse outcome pathways of phthalate exposure to disrupt thyroid homeostasis in humans and the related reproductive and neurodevelopmental health outcomes (Baken et al., 2019).

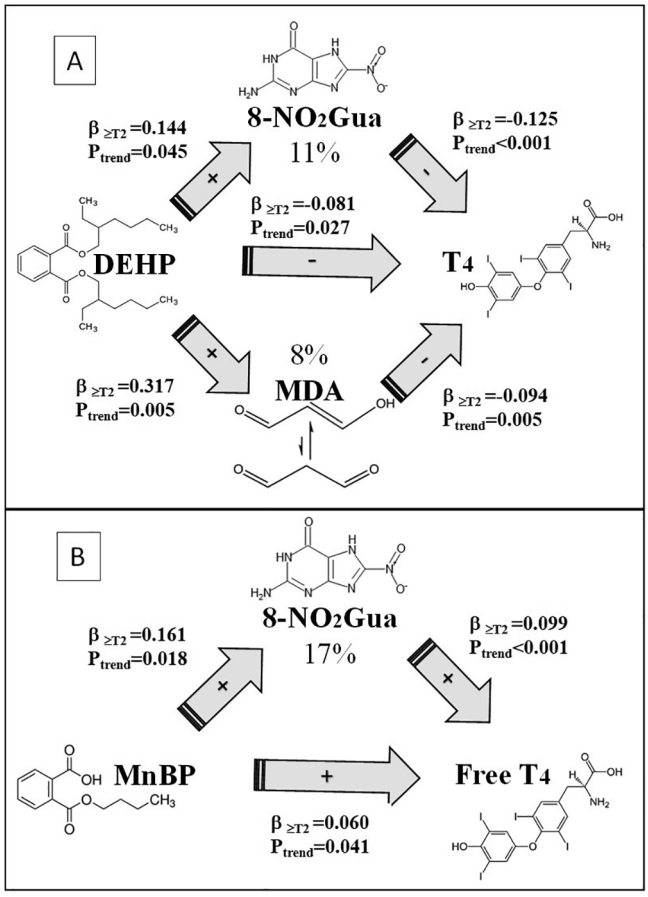

Fig. 3. Meditation of the association between urinary phthalate metabolites and serum thyroid hormones through biomarkers of oxidative/nitrosative stress (detailed information of β and confidence intervals are shown in the supplements; p-values are for trends in tertiles associated with the ln-transformed values; the signs in the arrows represent the direction of association; 8-NO_2Gua – 8-nitroguanine; DEHP – di (2-ethylhexyl) phthalate; MDA – malondialdehyde; MnBP – mono-n-butyl phthalate; T_4 – thyroxine).

Our observation of the inverse associations between DEHP metabolites and T_4 is consistent with the previously reported associations in 1346 American (Meeker and Ferguson, 2011) and 6003 Korean (Park et al., 2017) adults sampled from the general populations. The effect of DEHP on thyroid function through activated thyrotropin-receptor-mediated pathways was illuminated in mechanistic studies on human cells and Sprague–Dawley rats (Kim et al., 2019; Ye et al., 2017). Reduced release of thyroid hormones, including T_4, was accompanied by increased ROS production in thyroid follicular cells and MDA accumulation in rat livers (Ye et al., 2017). Oral exposure to DEHP also confirmed DNA damage in the thyroid tissue of rats (Kim et al., 2019).

Table 3
Mediation effect of exposure to phthalates on thyroid hormones through biomarkers of oxidative stress. [a]

Exposure and outcome	Mediator	Estimate direct effect (95% CI)	Estimate Indirect effect (95% CI)	Estimated proportion mediated
MnBP and FreeT4	8-NO2Gua	0.014 (0.004, 0.020) **	0.003 (0.001, 0.010) **	17%
ΣDEHPm and T4	MDA	−0.046 (-0.093, 0.000) #	−0.004 (-0.013, 0.000) #	8%
ΣDEHPm and T4	8-NO2Gua	−0.044 (-0.091, −0.010) **	−0.006 (-0.013, −0.000) *	11%

[a] Adjusted for age, sex, BMI, cigarette smoking, TBG levels and urinary creatinine levels; # < 0.1, * < 0.05, ** < 0.01.

Table 4
Literature review of epidemiological and experimental studies on relationships between phthalate exposure and oxidative stress.

Study (year), country	Study design, population, N	Phthalates or metabolites	Oxidative stress biomarkers	Results	Mediation effect of oxidative stress
Lee et al. (2019), Saudi Arabia	Cross-sectional, children, 104	DMP, DEP, DiBP, DnBP, DPeP, DiPrP, DCHP, DHxP, DOP, DEHP, DiNP, DiDP	8-OHdG, MDA	Positive associations between 8-OHdG and 9 metabolites, between MDA and MMP, MEP, MnBP, MEOHP.	–
Li et al. (2019), Saudi Arabia	Case-control, adults, 52/40	MEHP, MEHHP, MEOHP, MECPP, MCMHP, MEP, MBP, MIBP, MCPP, MMP, PA, MBzP	8-OHdG, 8-isoPGF2α, MDA	Low-moderate correlations with all for 8-OHdG, 8-isoPGF2α; MDA only with MCMHP, MBP, MIBP, MMP, MBzP.	Type 2 diabetes
Chang et al. (2019), Taiwan	Cross-sectional, elderly men, 207	MMP, MEP, MiBP, MnBP, MBzP, MEHP, MEHHP, MEOHP, MECPP, MiNP, MiDP	8-OHdG, serum MDA	Positive dose–response between 8-OHdG and MEHHP, MEOHP, MECPP.	Prostate enlargement
Liu et al. (2019), China	Cross-sectional, men, 1034	MMP, MEP, MBP, MBzP, MEHP, MEHHP, MEOHP, MOP	8-OHdG, 8-isoPGF2α, 4-HNEMA	Positive dose–response.	Semen quality
van 't Erve et al. (2019), US	Cross-sectional, pregnant, 758	DEP, DBP, DiBP, DEHP, DEHTP, DOP, DNP, DDP	8-*iso*-PGF2α,	Positive dose–response.	–
Kim et al. (2018), Korea	Cross-sectional, children, 287	MEHHP, MEOHP, MiBP, MnBP	8-OHdG	Positive associations.	–
Dong et al. (2018), China	Cross-sectional, elderly diabetics, 300	MMP, MiBP, MnBP, MBzP, MEHP, MEHHP, MECPP, MCMHP	8-OHdG, MDA	Positive associations between 8-OHdG and MBzP, MEHP, MEHHP, MECPP, MCMHP, between MDA and MMP, MiBP, MBzP, MEHP, MCMHP.	–
Franken et al. (2017), Belgium	Cross-sectional, adolescents, 418	DEHP, MiBP, MEP, MnBP, MBzP	8-OHdG	Positive association.	–
Lin et al. (2017), Taiwan	Cross-sectional, adolescents and young adults, 751	DEHP, MMP, MiBP, MEP, MnBP, MBzP	8-OHdG, 8-isoPGF2α	Positive association between 8-OHdG and MMP and MiBP, 8-isoPGF2α and MMP and MBzP.	–
Rocha et al. (2017), Brazil	Cross-sectional, children, 300	DEP, DEHP, DEHP, DEHP, DEHP, DEHP, DIBP, DBP, DMP, DCHP, BBzP, DOP, DiNP, DIDP, DPeP, DIPeP, DPrP, DIPrP, DHxP, DHpP	8-OHdG	Significant, but low correlations.	–
Kataria et al. (2017), US	Cross-sectional, children, 41	DEHP DIDP DINP	8-OHdG	No significant association.	–
Duan et al. (2017), China	Cross-sectional, diabetics, 329	DEHP, MBzP, MCPP, MBP, MiBP, MEP, MMP	MDA	Positive association	–
Holland et al. (2016), US	Cohort, pregnant, 180	DEHP, MEP, MBP, MiBP, MBzP, MCPP, MCOP, MCNP	8-Isoprostane	Positive association with MCOP and MCNP at 13 weeks, and at 26 weeks with MEP, MBP, MiBP, MCPP, MCNP and sum of low molar weight metabolites	–
Guo et al. (2014), US	Cross-sectional, adults, 894	MMP, MEP, MCPP, MBP, MiBP, MBzP, DEHP	8-OHdG	moderate correlation with MEHP in males	–
Kim et al. (2014), Korea	Cross-sectional, children, 39	MiBP, MnBP, MEHP, MEOHP, MEP	MDA	Positive association with MiBP, MnBP, MEHP	–
Ferguson et al. (2014, 2017), US	Cohort, pregnant women, 482	DEHP, MEHP, MEOHP, MBzP, MBP, MiBP, MEP, MCPP	8-OHdG, 8-Isoprostane	Positive dose–response	Preterm birth
Ferguson et al. (2014), Puerto Rico	Cohort, pregnant women, 42	DEHP, MEHP, MEOHP, MBzP, MBP, MiBP, MEP, MCPP	8-OHdG, 8-Isoprostane	Positive dose–response between 8-OHdG and MEHP, MEHHP, MEOHP, MECPP, MBP, MEP; all with isoprostane	–
Kim et al. (2013), Korea	Cross-sectional, elderly, 560	DEHP	MDA	Positive association	Insulin resistance
Qin et al. (2018), China	Animal model	DIIDP	MDA	No significant difference	Asthma
Ye et al. (2017), China	Animal model	DEHP	MDA	Positive dose–response	Thyroid function
Wu et al. (2017), China	Animal model	DBP	8-OHdG	Positive dose–response	Thyroid function
Zhang et al. (2017), China	Animal model, in vitro	DEHP	MDA	Positive dose–response	Liver function, insulin resistance
Cho et al. (2015), Korea	in vitro	DEHP	ROS/NOS	Time and dose dependent relationships	–
Tetz et al. (2013), US	in vitro	MEHP	ROS, prostaglandins, oxidative DNA damage	Dose-response relationships	–

Activation of peroxisome proliferator-activated receptors was proposed as a potential mechanism for the oxidative lipid peroxidation and DNA damage following DEHP exposure in human endometrial stromal cells, human hepatocytes, and rats (Cho et al., 2015; Zhang et al., 2017). The suggested mechanism of oxidative/nitrosative stress involved in thyroid hormone regulation is complicated by the ability of thyroid hormones to act as oxidants and damage DNA (Mancini et al., 2016), which limited the interpretation of epidemiological findings. Further mechanistic and epidemiological research is recommended.

Lipid peroxidation, as measured by urinary MDA, has been reported to be positively associated with DEHP exposure in Chinese patients with diabetes (Duan et al., 2017) and in elderly Korean people (Kim et al., 2013), similar to our results. However, only a few studies have investigated nitrosative DNA damage in association with phthalate exposure. A significant positive association between DEHP and inducible nitric oxide synthase was observed in elderly Taiwanese people (Chang et al., 2019), similar to the association observed between DEHP metabolites and 8-NO_2Gua in our study. Growing evidence suggests that 8-NO_2Gua may be a potential biomarker for nitrative DNA damage resulting from environmental exposure (Wu et al., 2016). Its urinary levels of were associated with nonylphenol exposure and inflammation in Taiwanese pregnant women (H. B. Huang et al., 2017; Y. F. Huang et al., 2017). In vitro study further supported the biological plausibility of phthalate-induced nitrative DNA damage, as a dose–response relationship was reported between DEHP and nitric oxide production in human endometrial stromal cells (Cho et al., 2015). Although we observed that 8-NO_2Gua partially mediated the association between MnBP and free T_4, animal and human studies have reported controversial findings on DBP exposure and thyroid hormones. Epidemiological studies have shown no significant associations between MnBP and thyroid hormones in American adults (Meeker and Ferguson, 2011), inverse association with T_3 in the general Korean population (Park et al., 2017), and inverse associations between DBP metabolites and serum levels of total and free T_4 in Taiwanese pregnant women (Huang et al., 2007). The associations of MnBP in the current study were negative for T_4 and positive for free T_4 with marginal significance, which could be attributed to insufficient sample size. Similar to our finding, a positive association between DBP and thyroid hormones was also reported in Wistar rats, with the proposed explanation that DBP could destroy thyroid follicular cells, allowing the thyroid hormones stored in the follicle to enter the blood and therefore increase levels of T_3 and T_4 (Duan et al., 2018). Nevertheless, DBP could induce ROS and DNA damage in rats (Duan et al., 2018; Wu et al., 2017), which is consistent with the positive association between MnBP and 8-NO_2Gua in our study.

The associations between phthalate exposure and oxidative stress have been assessed previously (Table 4) in multiple experimental (Cho et al., 2015; Hallgren et al., 2001; Kim et al., 2019; Liu et al., 2012; Qin et al., 2018; Tetz et al., 2013; Wu et al., 2017; Ye et al., 2017; Zhang et al., 2017) and epidemiological studies (Chang et al., 2019; Dong et al., 2018; Duan et al., 2017; Ferguson et al., 2017; Franken et al., 2017; Guo et al., 2014; Holland et al., 2016; Kataria et al., 2017; Kim et al., 2013, 2018; Lee et al., 2019; Lin et al., 2017; Liu et al., 2019; vanErve et al., 2019). Several studies proposed the mediation effect of oxidative stress on various health outcomes (Chang et al., 2019; Ferguson et al., 2017; Liu et al., 2019). However, only animal model research so far addressed the effect of phthalate on thyroid homeostasis via oxidative stress (Wu et al., 2017; Ye et al., 2017). Previous studies found that serum T_4, T_3 and TRH levels were reduced, whereas TSH level was not affected after DEHP exposure in animal models, indicating that DEHP could disrupt thyroid hormone homeostasis through activating the Ras/Akt/TRHr pathway which were modulated by oxidative stress, as well as inducing hepatic enzymes (Ye et al., 2017). The insensitivity of TSH as a marker of HPT axis and thyroid imbalance is consistent with findings on other endocrine disruptors. Previous studies (Table 4) have explored the associations between phthalates or other chemicals and RNOS, while only a few studies assessed the mediating effects of phthalates and relevant health outcomes through biomarkers of oxidative stress, and none did so for nitrative stress biomarkers. Thus, we suggest that exploration of more biomarkers of potential mediators are warranted for the complete establishment of adverse outcome pathways for different health outcomes in human.

The following limitations should be also considered while interpreting our study's results. First, although we observed partial mediation between phthalate exposure and thyroid function by lipid peroxidation and nitrosative DNA damage, other unmeasured factors could be involved in this pathway. Therefore, our results could underestimate the mediation by total oxidative/nitrosative stress. We performed mediation analysis with bootstrapping, which is considered a valid and powerful method; however, we could not ensure the absence of unmeasured confounding, which could alter the direct and indirect effects (VanderWeele, 2016). The adjustments for age, sex, BMI, cigarette smoking, TBG levels, and urinary creatinine may lower the chance of bias in our results. In addition, the cross-sectional design limits our ability to infer causality on the path from phthalate exposure to thyroid function through the proposed mediators. Second, the concentrations of phthalate metabolite and oxidative/nitrosative stress biomarkers were assessed from only one urine sample from each participant, which may not be descriptive of the mean body burden due to the short half-lives of these chemicals. Although previous studies have shown moderate representativeness of average long-term exposure for single spot urine samples of phthalate metabolites (Frederiksen et al., 2012; Teitelbaum et al., 2008) and low variations for intra- and inter-day measurements of oxidative/nitrosative stress biomarkers (Wu et al., 2016), the chance of misclassification between participants with low and high exposure still exists. Likewise, we obtained only one blood sample from each participant to assess serum thyroid functions, which could vary within individuals over time (Andersen et al., 2002). Sample collection of all the analytes in the same period (morning) could reduce their intra-individual variability. Lack of medical information about thyroid disease by a physician is another limitation. We cannot rule out the potential influence of other unmeasured chemicals with similar alteration on thyroid function which may underestimate our results.

5. Conclusion

Our findings suggest that nitrosative DNA damage and lipid peroxidation may play mediating roles in the effects of phthalate exposure on thyroid hormones in humans. Exploration of relevant mediators or chemicals with thyroid-like activity are warranted for elucidation the adverse outcome pathways. Mechanistic, prospective and large-scale epidemiological studies are necessary to confirm these associations.

CRediT authorship contribution statement

Po-Chin Huang: Conceptualization, Methodology, Software, Writing - original draft, Resources, Supervision. **Alexander Waits:** Visualization, Validation, Writing - review & editing. **Hsin-Chang Chen Chang:** Investigation, Methodology, Resources, Writing - review & editing. **Wan-Ting Chang:** Investigation. **Jouni J.K. Jaakkola:** Writing - review & editing, Supervision. **Han-Bin Huang:** Methodology, Software, Validation, Formal analysis, Data curation, Resources, Writing - review & editing, Supervision.

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Author Contributions

PCH conceived and designed the experiments, WTC and HCC performed the experiments, and HBH and AW analyzed the data. PCH and HCC contributed tools for reagents, materials, and analysis, and PCH wrote the paper. Specimen collection as well as sample arrangement and preparations were managed by WTC; HBH, JJKJ and PCH contributed to critical revision of the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2020.105751.

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