Environmental Pollution 287 (2021) 117606

Contents lists available at ScienceDirect

Environmental Pollution

journal homepage: www.elsevier.com/locate/envpol

Associations of serum phthalate metabolites with thyroid hormones in GraMo cohort, Southern Spain☆

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ARTICLE INFO

Keywords:
Phthalates
Thyroid hormones
Endocrine disrupting chemicals
Cross-sectional study
Cohort study
Environmental epidemiology
Biological samples

ABSTRACT

The general population is continuously exposed to phthalates via various consumer products. Epidemiological research relating phthalate exposure to thyroid function during non-developmental periods is limited. This study aimed to investigate the associations between specific serum phthalate metabolites and indicators of thyroid function in adults. We measured 10 serum phthalate metabolites and thyroid hormones – total triiodothyronine (TT3), free thyroxine (FT4) and thyroid stimulating hormone (TSH) – in a subsample of 207 adults from the GraMo cohort. This subsample was made up of men and women (in equal proportions) of middle age (49 ± 17 years) and from Southern Spain (province of Granada). Data on age, sex, body mass index, residence area, tobacco use, alcohol consumption and attained education were obtained from a questionnaire. Phthalate metabolites were log-transformed and categorized into tertiles. Cross-sectional associations of each metabolite with thyroid hormones were analyzed using multivariable-adjusted linear regression models. The mixture effect of metabolite phthalates was assessed using weighted quantile sum regression. After multivariable-adjustment, the following phthalate metabolites were significantly associated with TT3 in a dose-response manner: MMP (β = **0.90**: 95% confidence interval 0.68,1.12), MEP (β = **0.67**: 0.44, 0.90), MiBP (β = **0.49**: 0.21, 0.77), MiDP (β = **0.27**: 0.03, 0.52), MBzP (β = **0.51**: 0.28, 0.73), MEHP (β = −**0.59**: −0.82, −0.35) and MiNP (β = **-0.43**: −0.71, −0.14), when comparing highest vs. lowest exposed. The sum of all metabolites was also linked to FT4 levels. No significant associations were observed for TSH except for MiNP. Although phthalate metabolites with different molecular weight showed opposite associations, overall metabolite concentrations seem to associate with increased TT3 and FT4 serum levels. The cross-sectional nature of this analysis limits causal inference.

1. Introduction

Phthalates, diesters of phthalic acids, are extensively used and can be found in numerous commercial products, including food packaging, building materials, furniture, interior and exterior of vehicles, children's toys, medical devices, perfumes, air fresheners, cleaning products, clothing, pharmaceuticals (Hauser et al., 2004) and personal care product (Romero-Franco et al., 2011) among others supplies (Abadin et al., 2007; Heudorf et al., 2007). Phthalates are not chemically bound to the end products, being easily transferred to indoor dust, air, food, and water (Guo et al., 2012). Therefore, phthalates are widely distributed in the ecosystem and are frequently detected in general population

☆ This paper has been recommended for acceptance by Dr. Da Chen.

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https://doi.org/10.1016/j.envpol.2021.117606
Received 22 January 2021; Received in revised form 26 May 2021; Accepted 14 June 2021
Available online 18 June 2021

worldwide (Wang et al., 2019). Phthalates have a short half-life in human bodies (less than 24 h), since they are rapidly metabolized into monoesters of phthalates (Silva et al., 2007; Johns et al., 2015a), which is supposed to be the toxic form (Kavlock et al., 2002).

Previous research has suggested that the diet, particularly fatty food (e.g., dairy, fish, oils), may contribute importantly to total phthalate exposure followed by indoor air and household dust (Petersen and Breindahl, 2000; Butte and Heinzow, 2002; Martinez et al., 2018; Wormuth et al., 2006). Biomarker measurements in biological fluids are an aggregate of all external exposure pathways and indicators of internal dose. Specifically, phthalate metabolites are suggested as indicators of the biologically effective dose of phthalate exposures. The measurement of phthalate monoester metabolites in urine has been the most common in epidemiology studies with some advantages over the measurement in blood, principally because of the higher concentrations of the metabolites and reduced potential for contamination by the parent diester phthalate and subsequent formation of metabolites by enzymes present in blood (Centers for Disease Control and Prevention, 2009). However, studies conducted in general population have reported from moderate to strong correlations between urine and serum concentrations for phthalate metabolites and both biological matrices can be used as valid indicators for human phthalate exposure (Frederiksen et al., 2010; Hines et al., 2009; Hogberg et al., 2008; Kato et al., 2004).

Phthalates have long been an object of concern because of their potential effects on human health, especially in the reproductive system (19). Phthalates have also elicited thyroid hormone disrupting potential, through interference of their synthesis, transport, and metabolism (Zhai et al., 2014; Liu et al., 2015), including interference with thyroid hormone, binding proteins and with the function of the hypothalamic-pituitary-thyroid (HPT) axis (Boas et al., 2012; Ye et al., 2017).

Most of the epidemiological studies assessing the potential association between phthalate exposure and thyroid function have been conducted in sensitive subpopulation groups, like pregnant women or infants and children, and have displayed conflicting results (Kim et al., 2019). Among the limited studies conducted in adults, the metabolites associated, and the direction of association have differed (Wang et al., 2018; Przybyla et al., 2018; Park et al., 2017; Meeker and Ferguson, 2011; Meeker et al., 2007; Huang et al., 2017). The purpose of this work was, therefore, to gain more knowledge about the potential interference of phthalate exposure on thyroid hormones. To this end, we investigated the associations between several phthalate metabolites in serum and biomarkers of thyroid function in a sample of middle aged adults from Southern Spain.

2. Material and methods

2.1. Design and study population

This research work is part of a larger, ongoing prospective study (GraMo cohort) that aims to investigate the role of various environmental exposures, including persistent organic pollutants (Barrios-Rodríguez et al., 2021; Mustieles et al., 2017), non-persistent chemicals (Artacho-Cordon et al., 2019), and metallic/metalloid elements (Salcedo-Bellido et al., 2021; Freire et al., 2020), on the development of chronic health conditions. The study design, recruitment and biological sample collection have been extensively described elsewhere (Arrebola et al., 2010; Arrebola et al., 2009; Echeverria et al., 2019; Rodriguez-Perez et al., 2018).

Briefly, the cohort was recruited in 2003–2004 in two public hospitals in the province of Granada (Southern Spain), the San Cecilio University Hospital in Granada city (240,000 inhabitants, urban area) and Santa Ana Hospital in the town of Motril (50,000 habitants, semi-rural area). Participants were recruited from patients undergoing routine non-cancer-related surgery (hernias (41%), gallbladder disease (20%), varicose veins (11%), and other conditions (28%)). Eligible participants had to meet the following criteria: aged over 16 years, residence in the study areas for at least 10 years and absence of cancer and any hormonal disease related to hypothalamic axis.

Data on socio-demographic characteristics, lifestyle, and health status were gathered in face-to-face interviews conducted by trained personnel at the time of recruitment during the hospital stay. Body mass index (BMI) was expressed as weight/height squared (kg/m^2). A participant was considered a smoker or alcohol consumer with any level of daily tobacco ($\geq$1 cigarette/day) or weekly alcohol ($\geq$1 drink/week) consumption.

Out of 409 individuals who were contacted, 405 donated 12 h-fasting blood samples. Phthalate metabolites and hormonal biomarkers were measured in serum samples from 230 individuals. We also excluded those with missing data in any of the covariables involved in these analyses ($n = 23$), leaving a final sample of 207 participants. No substantial sociodemographic differences were observed between the 207 participants included in the analysis and those that provided blood samples but had no phthalate exposure or clinical data ($n = 197$), except for the place of residence (urban or semi-rural) (Supplemental Table S1).

All participants signed their informed consent to participation in the study, which was approved by the Ethics Committee of Granada (Comité de Ética de Investigación Clínica de Granada, 8/2016).

2.2. Laboratory analysis

2.2.1. Phthalate metabolite assessment

The concentration of 32 phthalate metabolites from 15 different phthalate diesters (phthalates) were analyzed by isotope diluted online-TurboFlow- LC-MS/MS with preceding enzymatic de-conjugation. A detailed method description, validation, limits of detections (LOD), linear range, matrix effects, intra-day, and inter-day accuracy and precision have been previously published (Hart et al., 2018). For this study, samples were analyzed randomly and blinded for the technician in 5 batches, each including calibration standards, about 40–45 unknown samples, three blanks, three serum pool controls, and three serum pool controls spiked with native phthalate metabolite standards at low or high level. The inter-day variation expressed as the relative standard deviation was <21% for all analytes spiked in serum at low level and <11% for all analytes spiked in serum at high level.

Of the 32 phthalate metabolites measured in blood, we excluded those which levels were below the limit of detection (LOD) in more than 75% of the samples. These 10 metabolites were as follows: mono-methyl phthalate (MMP), mono-ethyl phthalate (MEP), mono-iso-butyl phthalate (MiBP), mono-n-butyl phthalate (MnBP), mono-benzyl phthalate (MBzP), mono-(2-ethyl-hexyl) phthalate (MEHP), mono-(2-ethyl-5-carboxypentyl) phthalate (MECPP), mono-(2-carboxymethyl-hexyl) phthalate (MCMHP), mono-iso-nonyl phthalate (MiNP) and mono-iso-decyl phthalate (MiDP). Values < LOD, were substituted with LOD/square root of 2 (Supplemental Tables S2 and S3).

Phthalates can be classified between low molecular weight (LMW) (ester side-chain lengths, one to four carbons) and high molecular weight (HMW) (ester side-chain lengths, five or more carbons). LMW phthalates are mostly used in hygiene and personal care products, paints, adhesives, and medicine coatings, and corresponding metabolites are: MMP, MEP, MiBP, MnBP and MBzP. Metabolites of LMW phthalates are MEHP, MECPP, MCMHP, MiNP and MiDP; are used primarily in PVC polymers and plastisol applications, plastics, food packaging, and food processing materials, vinyl toys and vinyl floor coverings, and building products (Danish EPA TDEPA, 2012; Wittassek et al., 2011) (Supplemental Table S2).

2.3. Thyroid hormones assessment

For the quantitative determination in serum of total triiodothyronine (TT3, nmol/L), free thyroxine (FT4, pmol/L) and thyroid stimulating hormone (TSH, mIU/L), in vitro immunological tests were performed on

Table 1
Baseline main characteristics of the subsample adults from the GraMo cohort according to phthalate metabolite sum levels in tertiles ($N = 207$).

Characteristics	Phtalate metabolite sum[a]		
	1st terile (lowest levels)	2nd tertile	3rd tertile (highest levels)
n	72	75	60
Male [%, (n)]	51.4% (37)	44.0% (33)	51.7% (31)
Age, yrs	48.9 (18.4)	49.9 (18.1)	48.3 (15.5)
Body mass index, kg/m^2	26.9 (5.3)	27.7 (6.1)	27.8 (4.9)
Education level [%, (n)]			
Incomplete primary	25% (18)	30.7% (23)	26.7% (16)
Primary	38.9% (28)	40% (30)	46.7% (28)
Secondary or higher	36.1% (26)	29.3% (22)	26.7% (16)
Residence (%)			
Urban (Granada)	68.1% (49)	20% (15)	13.3% (8)
Semi-rural (Motril)	31.9% (23)	80% (60)	86.7% (52)
Regular alcohol consumption (%)	45.8% (33)	50.7% (38)	65% (39)
Tobacco use [%, (n)]			
Never smoked	45.8% (33)	42.7% (32)	36.7% (22)
Former smokers	22.2% (16)	21.3% (16)	25% (15)
Current smokers	31.9% (23)	36% (27)	38.3% (23)
Estradiol blood levels, pg/ml	41.6 (48.8)	38.6 (56.1)	42.4 (57.6)
Thyroid hormones levels			
Total triiodothyronine (TT3), nmol/L	2.52 (0.71)	2.23 (0.72)	2.69 (0.66)
Free thyroxine (FT4), pmol/L	18.7 (6.8)	18.9 (7.8)	19.5 (8.2)
Thyroid stimulating hormone (TSH), mIU/L	2.21 (1.95)	2.21 (1.69)	2.57 (1.45)
Phtalate metabolite serum levels, ng/mL			
MMP	5.17 (7.63)	3.39 (7.91)	7.27 (5.86)
MEP	11.8 (26.2)	15.7 (19.1)	44.3 (72.4)
MiBP	1.13 (1.73)	4.98 (9.93)	19.83 (18.6)
MnBP	0.96 (1.62)	5.07 (9.32)	20.93 (19.5)
MBzP	0.2 (0.07)	0.27 (0.26)	0.72 (0.66)
MEHP	1.98	4.09 (4.24)	3.19 (3.46)
MECPP	0.82 (1.03)	1.26 (0.96)	2.44 (4.18)
MCMHP	1.08 (1.45)	1.62 (1.89)	2.85 (4.91)
MiNP	1.09 (1.12)	1.9 (1.31)	1.87 (1.25)
MiDP	0.69 (0.49)	1.2 (0.96)	2.33 (2.14)

Continuous variables are given as mean (standard deviation (SD)) and categorical variables are given as percentage (n).
Abbreviations: MMP: Mono-methyl phthalate; MEP: Mono-ethyl phthalate; MiBP: Mono-iso-butyl phthalate; MnBP: Mono-n-butyl phthalate; MBzP: Mono-benzyl phthalate; MEHP: Mono-(2-ethyl-hexyl) phthalate; MECPP: Mono-(2-ethyl-5-carboxypentyl) phthalate; MCMHP: Mono-(2-carboxymethyl-hexyl) phthalate; MiNP: Mono-iso-nonyl phthalate; MiDP: Mono-iso-decyl phthalate.
[a] The phthalate metabolite sum is the sum of orders of all metabolites (each metabolite was categorized in tertiles and the category number of each compound was summed).

the Cobas e-411 bioanalyzer (from Roche), based on electrochemiluminescence technology (ECLIA).

We additionally identified those participants with TT3 y/o FT4 levels above the range considered normal (Education.endocrine.org., 2020; Ross et al., 2016). Hence, we considered as potential subclinical hyperthyroidism if any of TT3 > 3.08 nmol/L and/or FT4 >23.17 pmol/L.

2.4. Statistical analysis

Pairwise correlations between metabolites and hormones were assessed using the Spearman rank correlation coefficient (rho) and graphed. Concentrations of each phthalate were natural log-transformed and evaluated as continuous and as categorical, which relax the linearity assumption. MEP and MEHP, with <8% observations <LOD, were categorized into tertiles of the distribution by the usual procedure. MBzP, with 75% observations below the LOD, were dichotomized into <LOD and ≥ LOD. For the rest of the metabolites, the first category (reference) comprised those participants with undetectable phthalate levels (<LOD), and the rest of the participants were dichotomized and constituted the second and third categories. A sum of all phthalate metabolites was also calculated by summing the orders of each metabolite after categorization (i.e., the category number of each compound – 1, 2 or 3 – was summed).

Associations of phthalate concentrations with thyroid hormones (continuous) and with hyperthyroidism (dichotomous) were assessed cross-sectionally using multivariable linear and logistic regression analyses, generating beta coefficients (β) and odds ratios (OR), respectively, with corresponding 95% confidence intervals (CI). The β (95% CI) stratifying by sex were also estimated.

Among covariates frequently reported in the existing bibliography, we based the selection of the covariates to be included as confounders to those that changed the estimation >5% or that were statistically significant. Thus, model 1 was adjusted for age and sex and model 2 was further adjusted for BMI (continuous), residence area (urban/semi-rural), smoking habits (never, former, current), alcohol consumption (regular consumer/non-consumer) and attained education (incomplete primary, primary, higher education). Phthalates bind to estrogen receptors, which may be part of the mechanism of action on the disruption of thyroid hormones. On the other hand, estrogen levels (due to physiological causes such as pregnancy, menstrual cycle, or other causes with iatrogenic/pathological origin) induce an increase in transporter globulin (TBG). Since 75–80% of thyroid hormone is bound to TBG, the increase in TBG determines an increase in protein-bound thyroid hormone with a consequent decrease in free thyroid hormones. Therefore, we further adjusted the models for estrogen levels, but the results were unchanged.

We further explored the association of phthalate metabolite concentrations with TT3 levels mutually adjusting each phthalate for the others, that is, including all phthalates in the same model. However, high correlation between chemicals might lead to result distortion

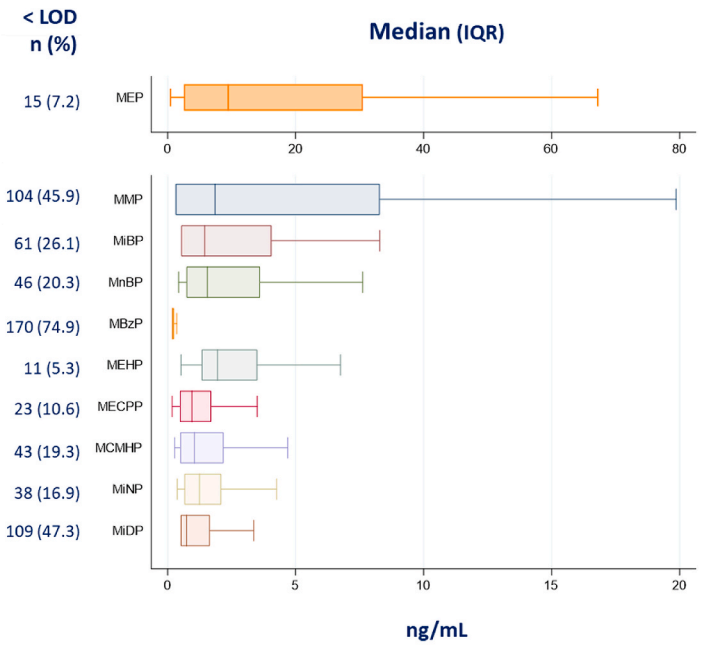


Fig. 1. Box plot of blood phthalate metabolite median levels (ng/mL) of total study population and percentages of phthalate metabolite below limit of detection. Descriptive statistics ($N = 207$).

(KAJAem, 2004). One strategy for evaluating environmental mixtures in observational studies is a focus on the concept of a mixture effect, where relevant environmental chemicals may be at exposures below an effect level, but joint action of the components may produce significant effects (Silva et al., 2002). Therefore, the potential mixture effect of the different phthalate metabolites on thyroid hormones was additionally assessed by means of weighted quantile sum (WQS) regression. WQS estimates a weighted index based on the combination of several exposures, considering their individual associations with the outcome. The mixture effect of phthalate concentrations on thyroid hormone levels was then explored by entering the index as the independent variable in a single linear regression with the levels of each hormone as the dependent variable and adjusting for the same covariates as the individual associations. The individual weight of each phthalate in the model was calculated in percentage. Considering that WQS regression needs to a priori set the expected direction of the association, we calculated two models for mixture effects (positive and negative) for each outcome.

Associations of each WQS index with TT3 and FT4 levels were further studied by using multivariable-adjusted linear regression, adjusting for the same covariates included in the previous models. All WQS analyses were performed with tertile-scored pollutant concentrations, using a training set defined as a 40% random sample of the dataset ($n = 83$), being the remaining 60% used for model validation ($n = 124$). The final weights were calculated using a total of 1000 bootstrap steps. The level of statistical significance was set at 0.05 and all tests were 2-tailed. The statistical software STATA/SE version 16.0 (Stata Corp LP, College Station, TX) was used to manage the database of the study and to perform statistical analyses. WQS analysis was performed by using R statistical computing environment v 3.4.0 (Team.RC, 2018), with gWQS package v2.0.1 (Stefano Renzetti et al., 2020).

3. Results

Table 1 summarizes the characteristics of the study population by tertiles of total phthalate concentrations sum. Participants with higher phthalate blood concentrations had lower attained education, were more likely to consume alcohol, to be current smokers and more likely to live in a semi-rural area (Motril).

MEP was found at notably higher levels than the rest of the metabolites and MBzP concentrations were below the LOD in most of the subjects (Fig. 1). The concentrations were slightly lower in men than in women, except for MMP whose levels were the double in men than in women (Supplemental Table S2). Spearman correlation among phthalate metabolites were generally positive but lower than 0.5. Exceptions were for the pairs MiBP-MnBP and MECPP- MCMHP with stronger positive correlations (both rho: ~0.8) (Fig. 2 and Supplemental Figure S2).

The mean (standard deviation (SD)) of TT3, FT4 and TSH levels was 2.5 (0.7) nmol/L, 19.0 (7.6) pmol/L and 2.3 (1.7) mIU/L, respectively. Thus, the 31% of the participants ($n = 64$) had levels of TT3 or FT4 above the reference values (i.e., 3.08 nmol/L for TT3 and 23.17 pmol/L for FT4) (Education.endocrine.org., 2020; Ross et al., 2016).

After multivariable adjustment, all the LMW phthalate metabolites were individually associated with elevated TT3 blood levels in a dose-response manner, but no significant associations were observed for FT4 or TSH. Thus, when comparing the highest vs. the lowest category of phthalate metabolites levels, MMP, MEP, MiBP, MnBP and MBzP were associated with higher TT3 serum levels. By contrast, the HMW phthalate metabolites were inclined to be associated with lower TT3 and TSH levels, although only reaching statistical significance in the case of MEHP and TT3 and for MiNP and both TT3 and TSH. The exception was MiDP, which is an HMW phthalate, but still significantly associated with elevated TT3 levels. The overall sum of phthalate metabolites was associated with increased TT3 and FT4 blood levels: those in the highest category vs. those in the lowest category of the phthalate metabolite sum had, on average, 0.52 (95% CI 0.26, 0.78) nmol/L higher levels of TT3 and 4.36 (1.47, 7.25) pmol/L higher levels of FT4 (Table 2, Fig. 3, and Supplemental Table S4).

Although the associations of phthalate metabolites mutually adjusted for the others with TT3 levels decreased in magnitude, the trend remained the same (Supplemental Table S5). The mean variance inflation factor (VIF) for the overall resulting model was 3 (reaching 5

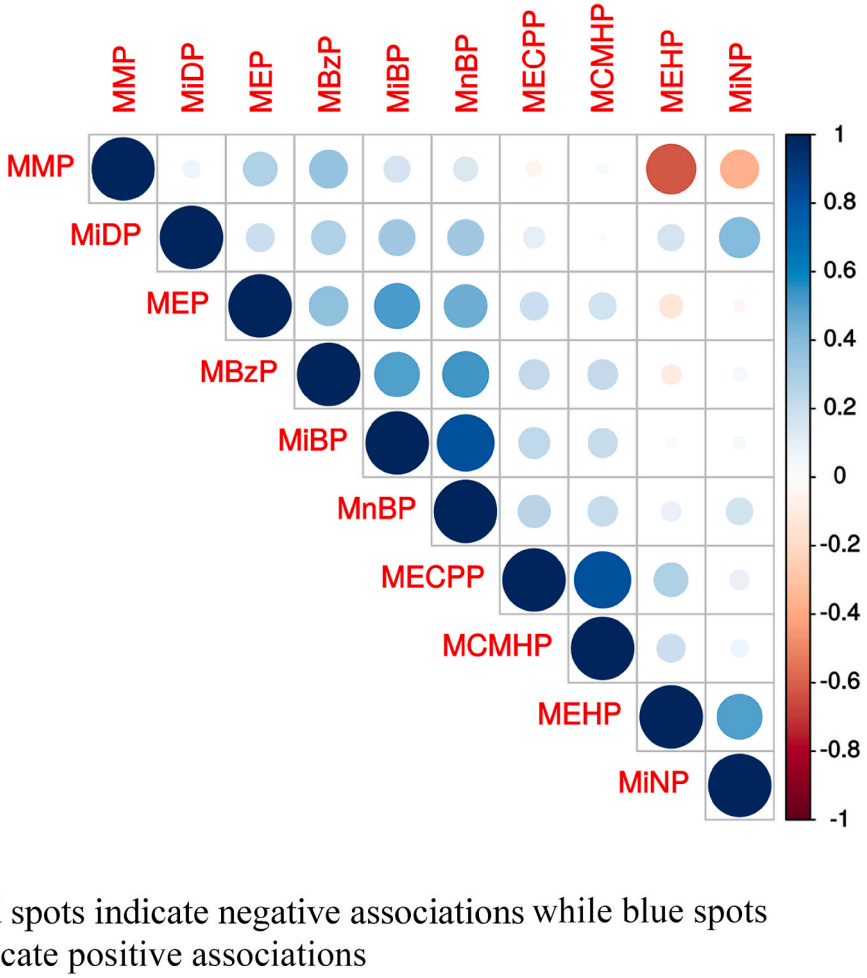


Fig. 2. Spearman correlations between phthalate metabolite levels.

for some individual metabolites), therefore, certain impact of multicollinearity in this approach cannot be ruled out. After sex-stratification, the associations were fully consistent in terms of direction of the association – although slightly more pronounced in women than men – and statistical significance in both women and men; (Supplemental Table S5).

When assessing the risk of being above the normal range in thyroid hormones, despite the of statistical power limitations, findings were consistent. In this approach, the strongest associations were found for MMP and MiDP; thus, those with highest levels of MMP and MiDP showed to have twice the risk of having a potential subclinical hyperthyroidism than those with undetectable levels (OR 2.41; 1.01, 5.73 and 2.36; 1.01, 5.50; respectively) (Table 3). Adjustment for estradiol levels did not change the associations found.

To account for the potential mixture effect of phthalate metabolites on thyroid hormone levels, we calculated a WQS index as a measure of the combined effect. In the multivariable linear regression model, the "positive" WQS index was positively and significantly associated with TT3 levels ($\beta = 0.362$; p value < 0.001), with MMP (32%), MnBP (23%), and MEP (21%) accounting for much of the index. In addition, the "negative" WQS index was negatively and significantly associated with TT3 levels ($\beta = -0.193$; p value 0.002), being mainly represented by MEHP (38%), followed by MINP (17%) and MBZP (16%) (Fig. 4). While the "positive" WQS index was not significantly associated with FT4 ($\beta = 0.142$; p value 0.116), the "negative" WQS index was negatively and significantly associated with FT4 levels ($\beta = -0267$, p value 0.007).

4. Discussion

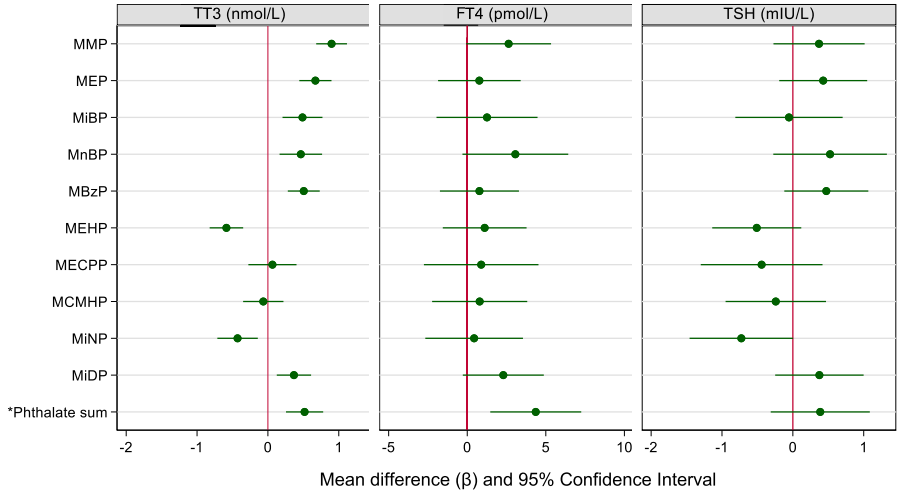
To the best of our knowledge, this is the first study evidencing associations between circulating serum phthalate metabolites and levels of thyroid hormones in an adult population. While LMW phthalate metabolites were individually associated with higher levels of TT3 in both sexes, HMW phthalate metabolites mostly associated with lower levels of serum TT3. The sum of all phthalate metabolites was associated with increases in TT3 and FT4 levels and this association was slightly more pronounced in women than men. No apparent associations between phthalate metabolites and TSH were found. Phthalate metabolites have similarities in their chemical structures and potential mechanisms of action and, therefore, there is increasing evidence of the potential additive/synergic effect of the exposure to their mixtures (Baralic et al., 2020). We addressed this issue by using two approaches. On the one hand, the sum of orders of all phthalate metabolites (LMW and HMW together) was associated with a significant increase in total TT3 levels, which indeed supports a potential additive mixture effect. However, this approach is based on chemical concentrations and does not take into account the possible distinct toxic potential that different phthalates may present. On the other hand, our analyses using WQS also supported a potential positive mixture effect of LMW phthalate metabolites on TT3 levels, which was mainly accounted for by MMP, MnBP, and MEP in this order; as well as a potential negative mixture effect of HMW phthalate metabolites on TT3 levels, being mainly represented mostly by MEHP, followed by MINP and MCMHP.

Phthalates have elicited endocrine-disrupting potential that can be mediated via interference with steroid hormones, including the sex

Table 2
Linear association between serum phthalate metabolite and thyroid hormone levels. Multivariable linear regression models (β and 95% CI per unit increase).

Phthalate metabolites (ng/mL) [c]	TT3 (nmol/L)		FT4 (pmol/L)		TSH (mIU/L)	
	Model 1	Model 2	Model 1	Model 2	Model 1	Model 2
	β (95% CI) [a]	β (95% CI) [b]	β (95% CI) [a]	β (95% CI) [b]	β (95% CI) [a]	β (95% CI) [b]
MMP	0.24 (0.19, 0.29)	0.24 (0.18, 0.30)	0.62 (0.00, 1.24)	0.45 (−0.25, 1.15)	0.13 (−0.01, 0.27)	0.11 (−0.05, 0.28)
MEP	0.13 (0.07, 0.19)	0.17 (0.11, 0.23)	−0.13 (−0.79, 0.54)	0.20 (−0.48, 0.88)	0.18 (0.03, 0.33)	0.18 (0.02, 0.34)
MiBP	0.11 (0.05, 0.18)	0.26 (0.19, 0.32)	−0.26 (−0.97, 0.44)	0.37 (−0.44, 1.17)	0.10 (−0.06, 0.26)	0.14 (−0.05, 0.33)
MnBP	0.10 (0.04, 0.16)	0.25 (0.19, 0.32)	−0.15 (−0.84, 0.53)	0.60 (−0.20, 1.39)	0.12 (−0.04, 0.28)	0.17 (−0.01, 0.36)
MBzP	0.31 (0.18, 0.44)	0.43 (0.31, 0.56)	0.22 (−1.24, 1.68)	1.32 (−0.19, 2.83)	0.32 (−0.02, 0.65)	0.30 (−0.06, 0.65)
MEHP	−0.39 (−0.50, −0.27)	−0.33 (−0.45, −0.20)	−0.08 (−1.41, 1.25)	0.55 (−0.85, 1.95)	−0.16 (−0.46, 0.15)	−0.12 (−0.45, 0.21)
MECPP	−0.05 (−0.16, 0.06)	0.02 (−0.09, 0.13)	−0.15 (−1.28, 0.97)	0.43 (−0.73, 1.60)	0.01 (−0.25, 0.27)	0.06 (−0.22, 0.33)
MCMHP	−0.04 (−0.14, 0.06)	0.00 (−0.10, 0.11)	−0.50 (−1.57, 0.57)	−0.07 (−1.15, 1.01)	0.00 (−0.24, 0.25)	0.03 (−0.23, 0.29)
MiNP	−0.28 (−0.41, −0.16)	−0.22 (−0.35, −0.09)	−0.38 (−1.73, 0.97)	0.49 (−0.93, 1.91)	−0.30 (−0.61, 0.00)	−0.37 (−0.70, −0.03)
MiDP	0.06 (−0.07, 0.19)	0.17 (0.03, 0.30)	0.09 (−1.29, 1.46)	1.09 (−0.33, 2.51)	0.16 (−0.01, 0.02)	0.14 (−0.20, 0.48)
Phthalate metabolite sum [d]	0.01 (−0.01, 0.04)	0.06 (0.03, 0.09)	−0.01 (−0.28, 0.27)	0.39 (0.07, 0.71)	0.02 (−0.05, 0.08)	0.02 (−0.06, 0.10)

Abbreviations: TT3, total triiodothyronine; FT4, free thyroxine; TSH, thyroid stimulating hormone; CI, confidence interval.
[a] Multivariable linear regression model adjusted for age and sex.
[b] Multivariable linear regression model adjusted for age, sex, body mass index, residence area, tobacco use, alcohol consumption, attained education and estradiol levels.
[c] Metabolites are log-transformed.
[d] The phthalate metabolite sum is the sum of orders of all metabolites (each metabolite was categorized in tertiles and the category number of each compound was summed).



Linear regression model adjusted for age, sex, body mass index, residence area, tobacco use, alcohol consumption and attained education. Phthalate metabolites are log-transformed. The first tertile comprises those subjects with metabolites levels <LOD except for MEP and MEHP (<8% observations < LOD) which have been categorized into tertiles by the usual procedure after imputing the LOD/square root of 2 to those with levels < LOD). For MBzP (>70 %observations <LOD) and was dichotomized in <LOD and ≥LOD.
* The phthalate metabolite sum is the sum of orders of all metabolites (each metabolite was categorized in tertiles and the category number of each compound was summed)

Fig. 3. Associations between serum phthalate metabolites and thyroid hormone levels. Multivariable linear regression models displaying mean differences (β) between 3rd and 1st tertiles of phthalate metabolites (ng/mL).

steroids, and thyroid hormone systems (Boas et al., 2012; Ghisari and Bonefeld-Jorgensen, 2009; Chebbi et al., 2020; Gore et al., 2015). DBP, MBP and DEHP have consistently exhibited an anti-androgenic as well as thyroid receptor (TR) antagonistic activity (Shi et al., 2011; Shen et al., 2009). DIDP, DINP and DEHP also showed their thyroid hormone disrupting potential by modulating sodium/iodide symporter (NIS)-mediated iodide uptake activity (Wenzel et al., 2005). DBP and MBP may also disrupt thyroid signaling by, e.g., inducing changes in the expression of thyroid hormone response genes or causing aberrant DNA methylation of TR (Shen et al., 2011). Further, DEHP reduces thyroid hormones via interacting with hormone synthesis-related proteins, deiodinases, transthyretin, receptors, and hepatic enzymes in rats (Liu et al., 2015; Dong et al., 2017; Erkekoglu et al., 2012). Likewise, acute exposure to MEHP alters whole-body contents of thyroid hormones in zebrafish embryos/larvae and changes the transcription of genes involved in the HPT axis, thus exerting thyroid endocrine toxicity (Zhai et al., 2014).

However, while over the past two decades there has been a substantial research effort to evaluate biological effects of phthalates, many uncertainties remain. Given the diversity and complexity of the endocrine system, additional pathways besides direct agonists or antagonists for hormone receptors need to be explored (Baken et al., 2019), especially at lower doses and during longer periods, to better reflect human exposure.

To our knowledge, a limited number of epidemiological studies looking at the potential association between phthalate exposure and

Table 3
Association between serum phthalate tertiles and prevalence of potential subclinical hyperthyroidism (TT3 y/o FT4 above the range considered normal).

Phthalate metabolites [c]	Cases	N	Prevalence of potential subclinical hyperthyroidism	
			Model 1	Model 2
			OR (95% CI)[a]	OR (95% CI)[b]
MMP				
1st Tertile	20	95	Ref.	Ref.
2nd Tertile	22	56	2.62 (1.25, 5.53)	2.03 (0.80, 5.11)
3rd Tertile	22	56	2.58 (1.22, 5.44)	2.41 (1.01, 5.73)
As continuous (ng/mL)			1.30 (1.08, 1.57)	1.24 (1.00, 1.55)
MEP†				
1st Tertile	20	69	Ref.	Ref.
2nd Tertile	22	69	1.13 (0.54, 2.35)	1.65 (0.73, 3.73)
3rd Tertile	22	69	1.18 (0.56, 2.46)	1.85 (0.80, 4.32)
Cont.			1.07 (0.88, 1.30)	1.19 (0.95, 1.48)
MiBP				
T1	20	54	Ref.	Ref.
T2	25	77	0.82 (0.39, 1.71)	1.38 (0.60, 3.20)
T3	19	76	0.52 (0.24, 1.12)	1.12 (0.41, 3.06)
Cont.			0.89 (0.72, 1.10)	1.09 (0.84, 1.40)
MnBP				
T1	13	42	Ref.	Ref.
T2	27	83	1.04 (0.46, 2.35)	2.19 (0.83, 5.74)
T3	24	82	0.83 (0.36, 1.90)	2.35 (0.80, 6.89)
Cont.			0.96 (0.79, 1.18)	1.23 (0.95, 1.59)
MBzP§				
< LOD	48	155	Ref.	Ref.
≥ LOD	16	52	0.99 (0.50, 1.98)	1.57 (0.71, 3.47)
Cont.			1.13 (0.75, 1.71)	1.46 (0.91, 2.35)
MEHP†				
T1	27	69	Ref.	Ref.
T2	16	69	0.45 (0.21, 0.96)	0.58 (0.26, 1.30)
T3	21	69	0.68 (0.33, 1.38)	0.97 (0.43, 2.18)
Cont.			0.74 (0.50, 1.11)	0.89 (0.57, 1.40)
MECPP				
T1	8	22	Ref.	Ref.
T2	31	93	0.92 (0.34, 2.45)	1.13 (0.39, 3.30)
T3	25	92	0.68 (0.25, 1.84)	0.94 (0.31, 2.84)
Cont.			0.72 (0.52, 1.01)	0.79 (0.55, 1.13)
MCMHP				
T1	18	40	Ref.	Ref.
T2	26	84	0.54 (0.24, 1.19)	0.72 (0.30, 1.75)
T3	20	83	0.41 (0.18, 0.92)	0.50 (0.20, 1.23)
Cont.			0.71 (0.51, 0.99)	0.75 (0.53, 1.06)
MiNP				
T1	14	35	Ref.	Ref.
T2	26	86	0.65 (0.28, 1.49)	0.92 (0.37, 2.27)
T3	24	86	0.59 (0.26, 1.37)	0.87 (0.34, 2.20)
Cont.			0.77 (0.52, 1,15)	0.89 (0.57, 1.38)
MiDP				
T1	27	98	Ref.	Ref.
T2	19	55	1.34 (0.65, 2.76)	1.76 (0.80, 3.85)
T3	18	54	1.24 (0.60,.2.56)	2.36 (1.01, 5.50)
Cont.			0.96 (0.65, 1.44)	1.30 (0.83, 2.06)
Phthalate sum				
T1	48	72	Ref.	Ref.
T2	21	75	0.76 (0.37, 1.54)	1.49 (0.63, 3.51)
T3	19	60	0.93 (0.44, 1.94)	2.15 (0.84, 5.50)
Cont.			0.98 (0.90, 1.06)	1.08 (0.97, 1.19)

4 The phthalate metabolite sum is the sum of orders of all metabolites (each metabolite was categorized in tertiles and the category number of each compound was summed).

Abbreviations: TT3, total triiodothyronine; FT4, free thyroxine; TSH, thyroid stimulating hormone; CI, confidence interval.

[a] Multivariable linear regression model adjusted for age and sex.

[b] Multivariable linear regression model adjusted for age, sex, body mass index, residence area, tobacco use, alcohol consumption and attained education.

[c] Metabolites are log-transformed. The first tertile comprises those subjects with phthalate levels < LOD except for MEP and MEHP (<8% observations < LOD) which have been categorized into tertiles by the usual procedure after imputing the LOD/square root of 2 to those with levels < LOD). For MBzP (>70 %observations < LOD) and was dichotomized in <LOD and ≥LOD.

thyroid function in adult population have been conducted. Moreover, the metabolites associated and the direction of associations diverge among these studies (Wang et al., 2018; Przybyla et al., 2018; Park et al., 2017; Meeker and Ferguson, 2011; Meeker et al., 2007; Huang et al., 2017). Nevertheless, specifically DEHP and its metabolites – which have been analyzed in most of these studies conducted in different adult populations – have consistently linked to lowered total T3 and total or free T4 levels and increased TSH levels (Kim et al., 2019; Park et al., 2017; Meeker and Ferguson, 2011; Meeker et al., 2007; Huang et al., 2017). Our findings are somewhat consistent with this preceding evidence. We found a dose-dependent inverse association of MEHP with TT3 levels, although not with FT4 or TSH levels. The other DEHP's metabolites, i.e. MECPP and MCMHP, were present in much lower serum concentration than MEHP and no associations were evidenced with any thyroid hormone.

In relation to phthalates other than DEHP, even less has been studied; but when assessed, generally they were inversely associated with thyroid hormone levels (Wang et al., 2018; Przybyla et al., 2018; Park et al., 2017). The exception was a population-based study conducted with 279 Taiwanese adults, where urinary DEHP's metabolites were negatively associated with FT4 levels but MEP was positively associated (Huang et al., 2017), in line with our results. In the present study in GraMo cohort, LMW phthalates, including MEP but also MMP, MiBP, MnBP and MBzP, were significantly associated with higher levels of total TT3 levels. LMW phthalates have been poorly investigated in relation to thyroid hormone disruption.

Several factors could explain the discrepancy between our findings and previous studies, such as the phthalate exposure differences between countries and over time, the control for covariates that could bias the associations, the different biological matrix in which phthalates exposure was assessed or even genetic variations modulating the susceptibility of individuals to phthalates toxicity (Johns et al., 2015a).

Following exposure, phthalates are rapidly metabolized and excreted in urine and feces. Thus, because phthalates have relatively short half-lives (Calafat et al., 2015; Johns et al., 2015b), serum concentrations of phthalate metabolites may be substantially lower than in urine (Frederiksen et al., 2010; Koch et al., 2005; Silva et al., 2003). Also, urinary metabolite concentrations have reduced potential for contamination by the parent diester and subsequent formation of metabolites by enzymes present in blood (Johns et al., 2015a; Calafat et al., 2015; Koch and Calafat, 2009). Other advantages of measurement of phthalate metabolites in urine over in blood include ease of sample collection and larger sample volumes (Koch and Calafat, 2009). This justifies why previous studies of human phthalate exposure have largely been based on urinary concentrations of its metabolites (Wang et al., 2019). However, when assessing potential health effects of the exposure, serum phthalate concentrations may provide very valuable information since they lie closer to biologically effective dose.

The distribution pattern of metabolites in serum is suggested to be similar to that of urine and reasonable correlations between phthalate metabolite concentrations in urine and in serum have been reported (Frederiksen et al., 2010; Hines et al., 2009; Hogberg et al., 2008; Kato et al., 2004). Also, in most of these biomonitoring studies, whether based on urine or serum, metabolites of DEHP, DEP and DBP were the major compounds identified (Wang et al., 2019), which is in agreement with our findings. Finally, although for most phthalates the monoester metabolite is thought to be more biologically active than the parent diester Centers for Disease Control and Prevention (2009), some degree of random measurement error due to the lack of precise measures of the biologically effective dose at the target tissue for the entire duration of the relevant exposure cannot be ruled out. Thus, the measurement of metabolite concentrations in serum (and in urine) could serve as a valid approach to estimating exposure to phthalates (Henriksen et al., 2020), although careful consideration of the limitations of this approach is required when interpreting study results.

A general limitation in the epidemiological field is the cross-sectional

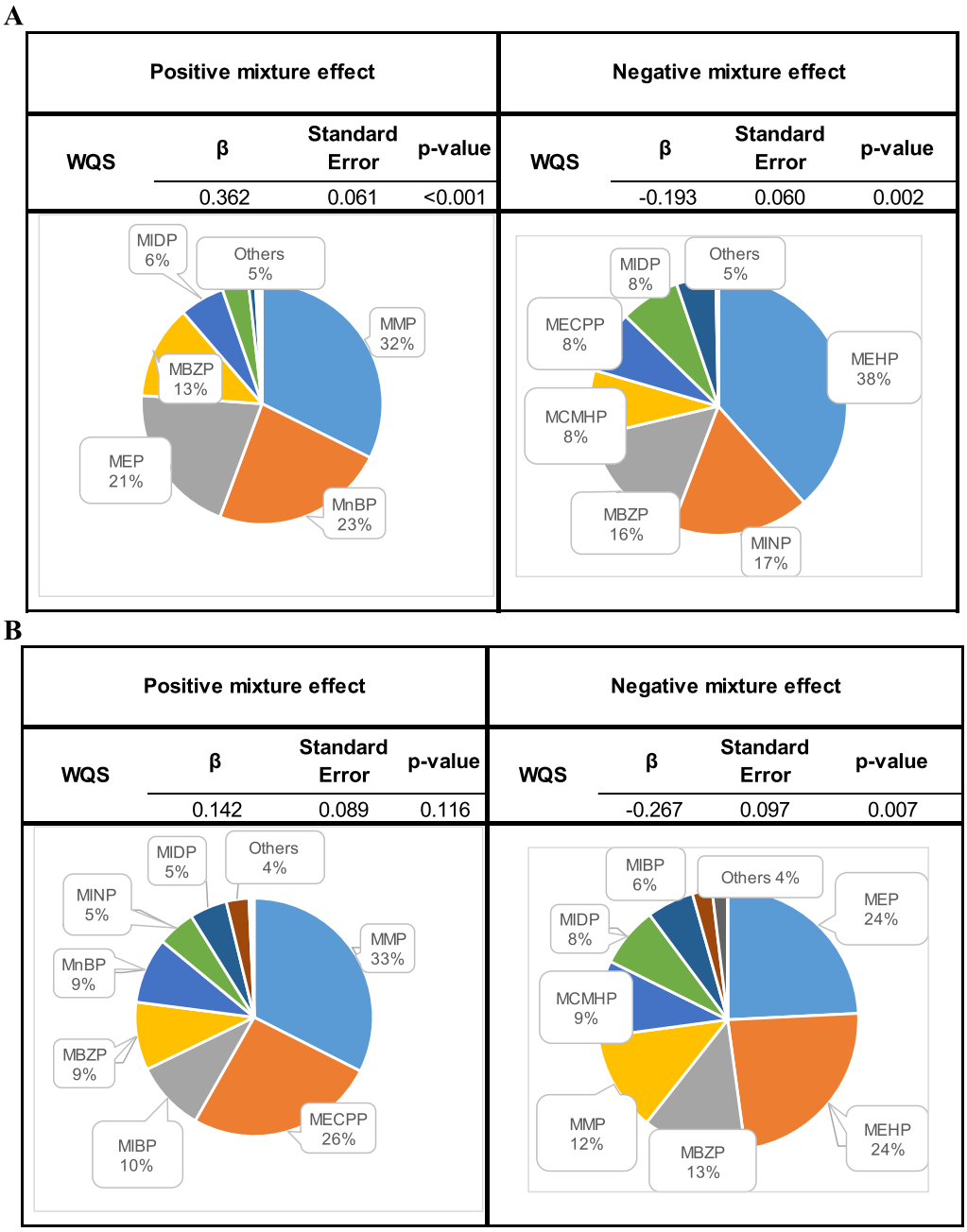


Fig. 4. Estimation of the mixture effect of phthalate metabolites on total triiodothyronine (A) and free thyroxine (B) levels. Weighted quantile sum regression (WQS) analyses.

nature of most studies, including the current work, which limits causal inference. However, it is highly unlikely – and there is not a known mechanism – that simultaneity or reverse causality occur in the thyroid hormone levels-phthalate metabolites relationship. No residual confusion can be guaranteed. Also, in our study, only a single serum sample per participant was used. Because phthalates are nonpersistent chemicals with short half-lives, detected phthalates in a single sample rather than reflecting a person's usual exposure over months to years, could reflect recent specific exposure.

WQS regression have become widely used to estimate the joint effect of all exposures in a mixture. One important limitation of WQS regression, however, is that the joint effects of chemicals with diverse effect directions cannot be assessed simultaneously. Also, it estimates the combined effect of these exposures under the assumption that associations are linear and additive and potential synergistic effects cannot be addressed (Zhang et al., 2019). Other different methodologies than WQS regression have recently developed to analyze the effects of chemical mixtures on health, such as the Bayesian kernel machine regression (Bobb et al., 2015) or the newest and promising method Quantile g-Computation (Schmidt, 2020). However, for the current data, we considered that the most suitable approach was probably the WQS regression. The WQS regression examines the joint action of the chemical exposures based on the weights empirically determined by bootstrap sampling. WQS regression performs well in characterizing chemicals of concern under multiple environmentally relevant simulation studies (Carrico et al., 2015; Hargarten and Wheeler, 2020; Bello, 2014). WQS

analyses are increasingly being performed in environmental epidemiology (Artacho-Cordon et al., 2019; Arrebola et al., 2019; Marks et al., 2021; Loftus et al., 2021; Wu et al., 2020). The advantages and limitations of this approach have been recently discussed elsewhere (Zhang et al., 2019; Gibson et al., 2019).

Although acid was added to serum samples immediately after centrifugation to inhibit enzyme activity, we cannot rule out that some conversion of diesters from sample contamination happens within the short period between drawing of blood, centrifugation, and final aliquoting (Calafat et al., 2015). While thyroid hormones were also assessed in one serum sample, human levels seems to be vary within relatively narrow limits over time (Andersen et al., 2002) and using of point measurements would yield to a non-differential error (random misclassification), that would bias measures of association toward the null value. The sample size available for these analyzes was limited and could compromise the statistical power in some analysis.

Importantly, despite we recruited a variety of individuals covering wide age, obesity and education ranges, our hospital-based cohort may not be entirely representative of the general population. However, levels of both serum phthalates and thyroid hormones are likely unrelated to the selection criteria (patients undergoing non-cancer-related surgery and free of any hormonal disease related to hypothalamic axis). In addition, we included a wide diversity of conditions, e.g., hernias in different locations, gallbladder disease, and varicose veins, among others. This might potentially represent a non-differential error and attenuate the associations; however, it is not likely to produce false positive associations. Therefore, we believe that the associations found in GraMo are relevant, deserve attention from the scientific community, and might be reproduced in the general population.

Regarding the biomarker selection, we acknowledge that urine concentrations might be more representative of the total phthalate exposure since these chemicals are more easily detected in urine. However, we used serum samples on the bases that they are closer to the biologically effective dose. Despite the mentioned limitations of serum phthalate concentrations, moderate to strong correlations have frequently been found (Frederiksen et al., 2010; Hines et al., 2009; Hogberg et al., 2008; Kato et al., 2004). Indeed, a review article concluded that despite these correlations might not always exist at an individual level, they can be relevant at a population level, in which people are continuously exposed to these chemicals (Johns et al., 2015b). Therefore, differences in serum vs urine would mainly affect exposure assessment to those metabolites found at lower concentrations.

As the main strength stands out the large number of phthalate metabolites assessed and consistency throughout the different approaches used. Likewise, phthalate metabolites in serum may be a better indicator of the biologically effective dose than other biomarker measurements, which can be indicators of phthalate internal dose but not necessarily the bioactive one.

This study represents a unique opportunity to assess potential early (sub)clinical damages in thyroid function in relation to the ubiquitous phthalate exposure in adult population. Early-life exposure to phthalates and other endocrine disrupting chemicals has been considered a major concern, meanwhile, exposures during non-developmental periods have not gained as much attention. Although there is no question that the developmental stage constitutes the most susceptible period to toxicity of environmental chemicals, the reality is that human exposure is continuous from conception to death. An integrated approach of further high-quality epidemiological studies including phthalate exposure over lifetime is warranted to provide a stronger basis for regulatory decisions.

5. Conclusions

Our results suggest that phthalate metabolites associate with serum levels of TT3 and FT4 in both sexes. The overall phthalate metabolite concentrations were related to higher serum TT3 and FT4 levels, slightly more pronounced in women. We also evidenced that phthalate metabolites with different molecular weight may be associated in the opposite way with thyroid hormones. Our study provides potentially relevant results for public health that warrant confirmation in further prospective epidemiological studies with larger sample size and using repeated measurements.

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.

Acknowledgments

These results would not have been achieved without the selfless collaboration of the staff from Santa Ana and San Cecilio Hospitals and the participants who took part in the study. Dr. JP Arrebola is under contract within the Ramón y Cajal Program (RYC-2016-20155, Ministerio de Economía, Industria y Competitividad, Spain) and Dr. C Donat-Vargas is under contract within the Atracción de Talento (from the community of Madrid, Spain). This study was supported by research grants from Instituto de Salud Carlos III (PI20/01568, PI16/01858).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2021.117606.

Research data for this article

The data from these analyzes will be made accessible upon request from other scientists for the purpose of replicating the results.

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