



Phthalate metabolites concentrations in amniotic fluid and maternal urine: Cumulative exposure and risk assessment

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ABSTRACT

Phthalates are used in industry as plasticizers or additives in everyday products and they have been considered as endocrine disrupting chemicals. Maternal exposure during pregnancy has been associated with neonatal exposure, preterm birth and impacts in the reproductive and respiratory systems. The aim of this study is to determine six phthalate metabolites (mono isobutyl 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-oxohexyl-phthalate, mEOHP) in amniotic fluid and urine from 100 pregnant women. Participants answered questionnaires for the use of plastics and cosmetics, dietary habits, health effects, pregnancy problems, health and infant development. Positive amniotic fluid samples ranged from 1% to 21% and urine from 27% to 54%. The median levels for amniotic fluid were 2.3 µg/L - 10.7 µg/L and for urine 4.9 µg/L - 46.7 µg/L. The major results include significant correlations between urinary phthalates indicating their common sources of exposure, the frequent use of deodorant was significantly associated with higher urinary miBP ($p = 0.050$) and mnBP ($p = 0.028$) and a weak inverse association was found for the use of make-up products with mBzP ($p = 0.053$). The frequent use of plastic food containers was significantly associated with urinary mEHP ($p = 0.026$), and a positive trend was noticed for mEHP in amniotic fluid ($p = 0.093$). An association although weak was found between urinary mEHP and lower birth length ($r_s = 0.396$, $p = 0.062$). No other associations were found for infant health problems or development. The daily intake of the total phthalates was calculated 5.4 µg/kg body weight/day which corresponds to hazard index 0.10 and exposure follows the declining trend that has been observed the last decades.

1. Introduction

Phthalate esters or phthalates are widely used plasticizers and additives which are found in various everyday mainly plastic products including plastic food containers, plastic bottles, floor and wall coverings, medical devices, adhesives, inks and paints, enteric-coated tablets, pre-packed coffee products, cosmetics, toilet tissue papers and personal care products [1–8]. Humans are exposed to phthalates through the use

of contaminated products and the compounds enter human body via ingestion, derma absorption and inhalation [9–11]. Parent compounds are firstly hydrolyzed to primary monoesters which are further metabolized to secondary products through oxidation and hydroxylation reactions. The second metabolic pathway includes conjugation with glucuronic acid and urinary excretion [12,13].

Phthalates are characterized as endocrine disrupting chemicals (EDCs) and several studies associate human exposure with male and

Abbreviations: BBzP, benzyl butyl phthalate; DnBP, di n-butyl phthalate; DiBP, di iso-butyl phthalate; DEHP, di 2-ethylhexyl phthalate; DiNP, di isononyl phthalate; EDCs, endocrine disrupting chemicals; EDI, estimated daily intake HQ, hazard quotient; HI, hazard index; LC-APCI-MS, liquid chromatography-atmospheric pressure chemical ionization-mass spectrometry; mBzP, mono benzyl phthalate; mECPP for 5cx-mEPP, mono 2-ethyl-5-carboxypentyl phthalate; 2cx-mMHP, mono 2-carboxymethyl-hexyl phthalate; mEHHP or 5OH-mEHP, mono 2-ethyl-5-hydroxyhexyl phthalate; mEHP, mono ethylhexyl phthalate; mEOHP or 5oxo-mEHP, mono 2-ethyl-5-oxohexyl-phthalate; mEP, mono ethyl phthalate; miBP, mono iso-butyl phthalate; mmp, mono methyl phthalate; mnBP, mono n-butyl phthalate

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Table 1
Phthalate metabolites and their respective parent compounds that were analyzed in the present study.

Phthalate diesters	Primary metabolites	Secondary metabolites
DnBP (di-n-butyl phthalate)	mnBP (mono-n-butyl phthalate)	3OH-mnBP † mHBP (mono 3-hydroxybutyl phthalate)* mCPP (mono 3- carboxypropyl phthalate)*
DiBP (di-iso-butyl phthalate)	miBP(mono-iso-butyl phthalate)	2OH-miBP (mono 2-hydroxy-isobutyl phthalate)*
BBzP (butyl benzyl phthalate)	mBzP (mono benzyl phthalate)	
DEHP (di 2-ethylhexyl phthalate)	mEHP (mono ethylhexyl phthalate)	mEHHP † 5OH-mEHP (mono 2-ethyl-5-hydroxyhexyl phthalate) mEOHP† 5oxo-mEHP (mono 2-ethyl-5-oxohexyl-phthalate) mECP † 5cx-mEPP (mono 2-ethyl-5-carboxypentyl phthalate)* mCMHP † 2cx-mMHP (mono 2-carboxymethylhexyl phthalate)*

*metabolites that were not investigated in the present study.

female infertility, genital malformations and reproductive abnormalities [8,14,15], impaired fetal/infant development [16,17], preterm birth [18,19], effects in the cardiovascular system [14], respiratory diseases and allergic outcomes [20,21]. Early-life exposure to hormone disruptors is crucial as hormones during the last stages of development have significant role in cell differentiation and organ formation. The lungs, the central nervous system, the immune and reproductive systems continue developing until puberty which means that exposure to EDCs during gestation, infancy or childhood may have severe health outcomes which are visible during puberty or later in life [22]. According to a study conducted in rats, exposure to different doses of di-n-butyl phthalate (DnBP) during fetal and lactational periods increased the incidence of inflammation and prostatic lesions in adulthood [23].

The scientific interest for maternal exposure to phthalates during pregnancy and the associated outcomes can be found in many studies which are focused on the biomonitoring of the compounds in maternal urine ([20,24–28]) and less to amniotic fluid [29,30–35]. The present study is a part of a research that aims to estimate the exposure of pregnant women to several EDCs [36,37]. In this study, urine and amniotic fluid were analyzed for six phthalate metabolites (Table 1) to estimate fetal and maternal exposures, biomonitoring data were statistically associated with information on exposure and health problems from questionnaires and finally, the daily intake of the parent compounds was calculated to assess the risk that cumulative exposure poses for maternal health. Although studies in literature are enough about phthalates in maternal urine, there are limited data about amniotic fluid, associations between biomonitoring and parameters from questionnaires and risk assessment.

2. Materials and methods

2.1. Chemicals and supplies

Phthalate metabolites, mEHP, mEHHP, mEOHP, mnBP, miBP and mBzP were purchased at 98% purity from Toronto Research Chemicals (TRC Inc). Most of them were solids except for mEHHP and mEOHP which were oils. Methanol, ethyl acetate and acetonitrile were Chromasolv grade for HPLC and purchased from Sigma–Aldrich (St Louis, MO, USA). Phenobarbital-d5, used as internal standard was obtained from Isotec Inc (Miamisburg OH, USA). Ultrapure water was produced by a Direct-Q 3UV water purification system (Merck, Germany). *Escherichia Coli* (*E. Coli*) β -glucuronidase K12 (140 units/mg, 5 ml), was purchased from Sigma-Aldrich. Amniotic fluid was collected with glass syringes 10 ml FORTUNA OPTIMA (Germany) and it was stored in amber glass bottles 5 ml (Sigma-Aldrich). SPE Cartridges C18 (100 mg) were obtained from Sigma-Aldrich.

2.2. Study population

One hundred pregnant women (mean age 35.4 \pm 5.5 years, range

22–44 years) who underwent amniocentesis at the beginning of the second trimester of pregnancy were recruited in the survey, at the private obstetric and gynecological clinic “MITERA” in Heraklion in Crete. Information about the sampling are described in the studies Katsikantami et al. [37] and Karzi et al. [36]. Briefly, a questionnaire was answered by all participants regarding demographic data, somatometric characteristics, lifestyle habits, health issues, dietary habits, use of certain products that are source of exposure to the compounds (including plastic containers, cosmetics and personal care products). Information about head circumference, birth weight and length were collected after childbirth and the health of the infants (respiratory problems, allergies, genital malformations). This study was approved by the Ethics Committee of the University of Crete (43/22.11.2018).

2.3. Sample preparation

The applied analytical protocols for the analysis of amniotic fluid and urine samples were based on the liquid-liquid extraction technique. An aliquot of 1 ml amniotic fluid was mixed with 100 ng internal standard, 10 μ L *E. Coli* and 250 μ L phosphate buffer (pH = 6.8) and enzymatic hydrolysis was carried out at 37° C for 90 min for the de-conjugation of the analytes. After incubation, 100 μ L hydrochloric acid 2 M were added and 2 ml ethyl acetate for the extraction which was repeated for three times. Organic phase (total 6 ml) was evaporated to dryness and reconstituted in methanol prior to instrumental analysis using liquid chromatography-atmospheric pressure chemical ionization-mass spectrometry (LC-APCI-MS). The procedure for the urine preparation was as described for amniotic fluid, with an extra clean-up step with solid phase extraction (SPE) following the liquid-liquid extraction. The dry residue was reconstituted in 1 ml buffer (pH = 2). The SPE cartridges were cleaned and activated with 1 ml acetonitrile, following 1 ml acetonitrile-water (1:1) and 2 ml water. Sample was loaded and washed with 2 ml water. The analytes were collected with 2 ml acetonitrile-ethyl acetate (1:1) and the organic phase was evaporated to dryness, reconstituted in 100 μ L methanol and analyzed with LC-APCI-MS.

2.4. Instrumental analysis

A Shimadzu LC–MS-2010EV (Kyoto, Japan) was used for the detection and quantification of the analytes after the separation of the analytes on a Supelco Discovery C18 column (25 cm x4.6 mm, 5 μ m) (Sigma-Aldrich). Mobile phase was water (solvent A) and acetonitrile (solvent B), both containing 0.1% formic acid at a flow rate 0.6 ml/min. Gradient elution was initiated with concentration 10% of solvent B (time: 0.0 min) followed by 80% (time: 14.0 min), 95% (time: 18.0 min), 100% (time: 20.0 min), 10% (time: 21.0). Injection volume was 10 μ L. The column was thermostated at 30°C during analysis. Retention times and selected ions *m/z* for each compound were as follows: IS: 12.40 min, 236.05 *m/z*, mEHHP: 14.73 min, 293.20,

339.25 *m/z*, mEOHP: 15.00 min, 291.20, 337.25 *m/z*, miBP: 15.23 min, 221.05, 267.15 *m/z*, mnBP: 15.33 min, 221.05, 267.15 *m/z*, mBzP: 15.50 min, 225.15, 301.15 *m/z* and mEHP: 19.37 min, 277.20, 323.20 *m/z*.

The mass spectrometer was coupled with an APCI ion source and the detection was achieved in selected ion monitoring (SIM) in negative mode. Detector voltage was set at 1.5 KV, drying gas at 0.02 MPa, interface temperature was 400°C, CDL temperature was 200°C, nebulizing gas flow was set at 2.5 L/min and heat block temperature was 200°C.

2.5. Statistical methods

The statistical analysis was conducted as described in Katsikantami et al. [37]. Continuous variables were expressed in the form of mean and standard deviation (SD) and descriptive measures such as median, range (minimum-maximum) and quadrants (P25, P50, P75) were used. The discrete variables have been expressed in the form of frequencies and percentage frequencies, the correlation of discrete variables was estimated using Pearson's χ^2 test and the correlation of continuous variables with the Pearson correlation coefficient or the corresponding non-parametric Spearman rho. Changes in paired measurements of discrete variables were done either by Mc Nemar test for 2×2 tables or by Mc Nemar-Bowker test for $n \times n$ tables.

Concentrations of the measured phthalate metabolites were expressed when dispersion was large on a logarithmic scale. Simple, grouped and stacked bar charts, scatter plots, and columns (Box and Whisker plots) were used to plot the results. Data entry was done in EXCEL 2017, while the statistical analysis was performed with IBM SPSS Statistics 24.0. The level of significance for accepting or rejecting statistical hypotheses was set at $p = 0.05$.

3. Results

3.1. Method validation

The method was validated for the target phthalate metabolites (mEHP, mEHHP, mEOHP, miBP, mnBP, mBzP) and the evaluated parameters included linearity, recovery, between day precision and accuracy (Table 2). Calibration curves from standard solutions at six concentrations 0, 25, 50, 100, 250 and 500 $\mu\text{g/L}$ were built to estimate the instrument linearity from the relation coefficient R^2 which ranged from 0.993 to 0.999 for all analytes. Spiked samples were prepared from amniotic fluid and urine matrix that were free from the target

Table 2
Validation parameters of phthalate metabolites in amniotic fluid and urine samples.

Phthalate metabolites	LOD ($\mu\text{g/L}$)	LOQ ($\mu\text{g/L}$)	Linearity R^2 (N = 3)	% Recovery (N = 3)	Between day precision %RSD (N = 3)	% Accuracy (N = 4)
Amniotic fluid						
mEHHP	0.02	0.07	0.999	101.1	9.9	108.5
mEOHP	0.02	0.06	0.999	82.0	4.4	92.9
miBP	0.2	0.8	0.995	72.0	13.4	108.7
mnBP	0.5	1.7	0.991	79.5	10.9	118.7
mBzP	0.5	1.6	0.998	78.6	12.9	101.7
mEHP	0.06	0.2	0.991	94.4	10.8	116.6
Urine						
mEHHP	2.0	6.8	0.992	90.3	5.9	93.1
mEOHP	0.6	2.0	0.990	77.2	4.9	89.8
miBP	4.4	14.8	0.999	71.8	14.0	93.0
mnBP	4.5	14.9	0.979	67.5	6.9	67.5
mBzP	1.5	4.8	0.991	101.4	5.5	115.5
mEHP	0.3	1.1	0.995	60.7	7.4	92.8

compounds, at concentrations 0, 2.5, 5, 10, 25 and 50 $\mu\text{g/L}$. The method linearity was evaluated from the analysis of the spiked samples at all levels for three times and the factor R^2 which ranged from 0.991 to 0.999 for amniotic fluid and from 0.979 to 0.999 for urine (Table 2). The recovery of the method was calculated for three replicates of spiked samples at all six concentrations and the mean recovery ranged from 72.0% to 101.1% for amniotic fluid and 60.7% to 101.4% for urine.

The method precision was evaluated from three replicates of spiked samples that were analyzed at different days and it was expressed with the factor percentage relative standard deviation (%RSD) which ranged from 4.4% to 13.4% for amniotic fluid and from 4.9% to 14.0% for urine. The mean accuracy was calculated from four replicates of spiked samples and it was from 92.9% to 116.6% for amniotic fluid and 67.5% to 115.5% for urine.

The limits of detection (LOD) and quantification (LOQ) were calculated from the signal-to-noise ratio (S/N) which are $S/N > 3$ and $S/N > 10$, respectively. The achieved LODs for amniotic fluid ranged from 0.02 to 0.5 $\mu\text{g/L}$ and for urine from 0.3 to 4.5 $\mu\text{g/L}$.

3.2. Distribution of phthalate metabolites in amniotic fluid

The phthalate metabolite that was detected at higher frequency was mEHP at 21%. MiBP, mnBP, mBzP and mEOHP were below 5% and mEHHP was not detected in any sample. The median concentrations for miBP, mnBP and mEHP were 10.0 $\mu\text{g/L}$, 10.7 $\mu\text{g/L}$ and 2.3 $\mu\text{g/L}$, respectively (Table 3). The mean levels of the compounds that were detected at frequencies greater than 2% are presented in Fig. 1. The maximum concentrations were 102.9 $\mu\text{g/L}$ for miBP, 24.5 $\mu\text{g/L}$ for mnBP, 20.2 $\mu\text{g/L}$ for mBzP and 7.0 $\mu\text{g/L}$ for mEHP. MnBP and miBP had the greater contribution to the total phthalate median concentration in amniotic fluid samples, 47% and 43% respectively. The statistical analysis was done only for mEHP because the rest of the metabolites were detected at very low frequencies and a weak association was found between the frequent use of plastics for food storage and the higher concentrations of mEHP in amniotic fluid ($p = 0.093$).

3.3. Distribution of phthalate metabolites in urine

The concentrations and the positive samples for urine were higher than amniotic fluid. At least one phthalate metabolite was detected at 75% of the samples and 41% were detected with 1 to 3 compounds. The most frequently detected metabolites were miBP, mEOHP, mEHHP and mnBP at 54%, 52%, 50% and 44%, respectively and the medians ranged from 4.9 $\mu\text{g/L}$ (mEOHP) to 46.7 $\mu\text{g/L}$ (mBzP) (Table 3). The profile of the urinary concentrations at log scale is presented in Fig. 2. The secondary metabolites of DEHP, mEOHP and mEHHP were detected at

Table 3
Descriptive statistics for phthalate metabolites in amniotic fluid (N = 100) and urine samples (N = 100).

Phthalate metabolites	Positive samples (%)	Mean \pm SD	Median	Min	Max
Amniotic Fluid ($\mu\text{g/L}$)					
miBP	3	39.3 \pm 55.1	10.0	5.1	102.9
mnBP	5	12.0 \pm 9.8	10.7	2.4	24.5
mBzP	2	15.9 \pm 6.1	-	11.6	20.2
mEHP	21	2.7 \pm 1.3	2.3	1.4	7.0
mEHHP	< LOQ				
mEOHP	1	0.9	< LOQ	< LOQ	0.9
Urine ($\mu\text{g/L}$)					
miBP	54	251.6 \pm 453.3	41.5	4.7	2255.6
mnBP	44	103.6 \pm 192.4	28.1	4.6	847.6
mBzP	31	80.6 \pm 97.3	46.7	4.3	455.4
mEHP	27	17.4 \pm 49.9	6.1	1.3	263.4
mEHHP	50	40.7 \pm 88.0	17.9	2.4	563.7
mEOHP	52	10.7 \pm 21.3	4.9	0.6	139.1

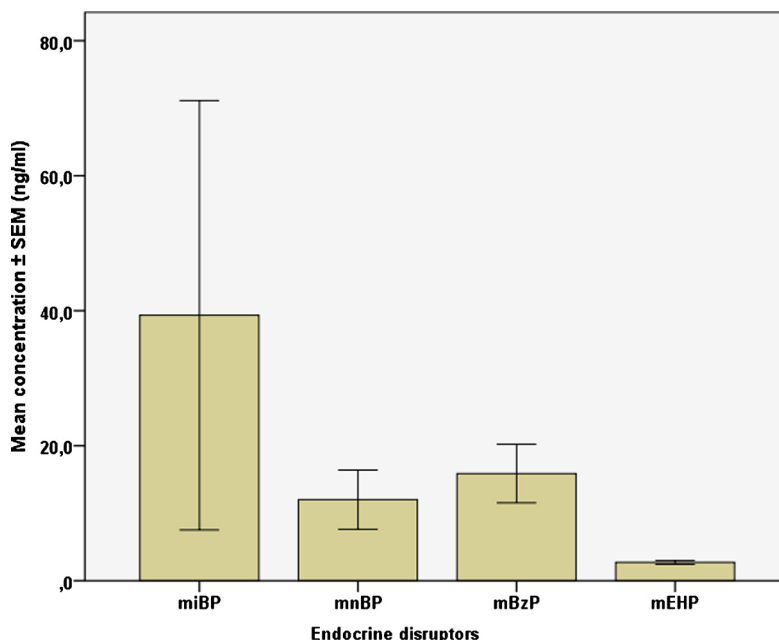


Fig. 1. Mean concentrations (± standard mean error) (µg/L) of phthalate metabolites that were detected in amniotic fluid at frequency above 2%.

similar percentages 52% and 50%, respectively and at higher frequency than the primary mEHP (27%). For mnBP and miBP, 41% of the samples were positive for both compounds (88% of these had higher burden for miBP) and 43% was not detected with any of the two metabolites.

3.4. Correlations between phthalate metabolites in urine samples

Significant correlations were found between phthalate metabolites in urine samples indicating their common sources of exposure or the common parent compound. More specifically, the pairs mEHHP and mEOHP (Spearman $r_s = 0.92$, $p < 0.001$), mEHP and mEHHP ($r_s = 0.41$, $p = 0.07$) showed a significant linear correlation (Fig. 3) which reflects their common parent compound. The correlations between mnBP and miBP ($r_s = 0.95$, $p < 0.001$), mBzP and miBP ($r_s = 0.70$, $p < 0.001$), mBzP and mnBP ($r_s = 0.63$, $p = 0.002$), mnBP and mEOHP ($r_s = 0.41$, $p = 0.02$) indicated the common sources of

exposure and the use of phthalates as mixtures in the products. Weak correlations were found between mEHP and miBP ($r_s = 0.39$, $p = 0.08$), mEHHP and mnBP ($r_s = 0.38$, $p = 0.05$) (Table 4).

3.5. Associations between phthalate metabolites in urine and results from questionnaires

The effect of maternal exposure on women’s health, pregnancy, development and infants’ health was investigated and the parameters that were examined were the occurrence of infants’ problems (allergies, respiratory diseases and malformations), birth weight and length, head circumference, maternal weight and height, health problems of pregnant women and progress of pregnancy. No significant associations came up except for a weak association between high levels of mEHP and lower birth length ($r_s = 0.396$, $p = 0.062$).

Regarding data on exposure from questionnaires and biomonitoring

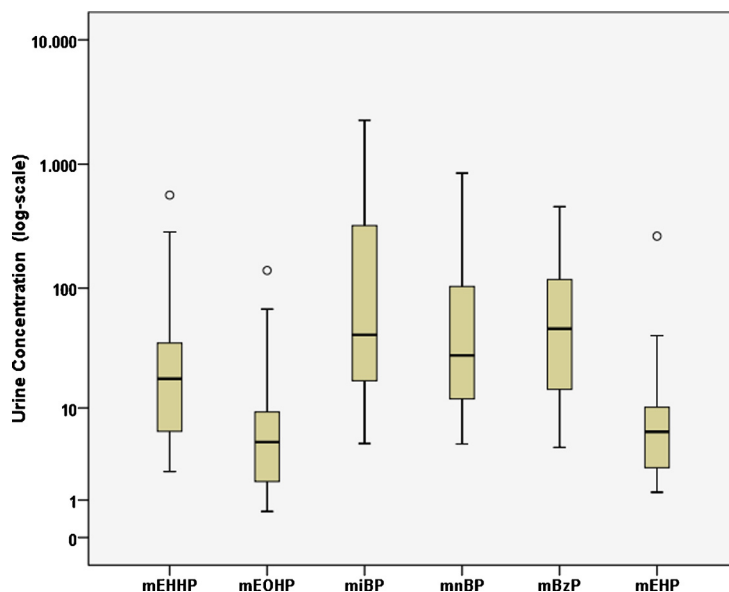


Fig. 2. Profiles of urinary phthalate metabolites (log scale) and standard deviation.

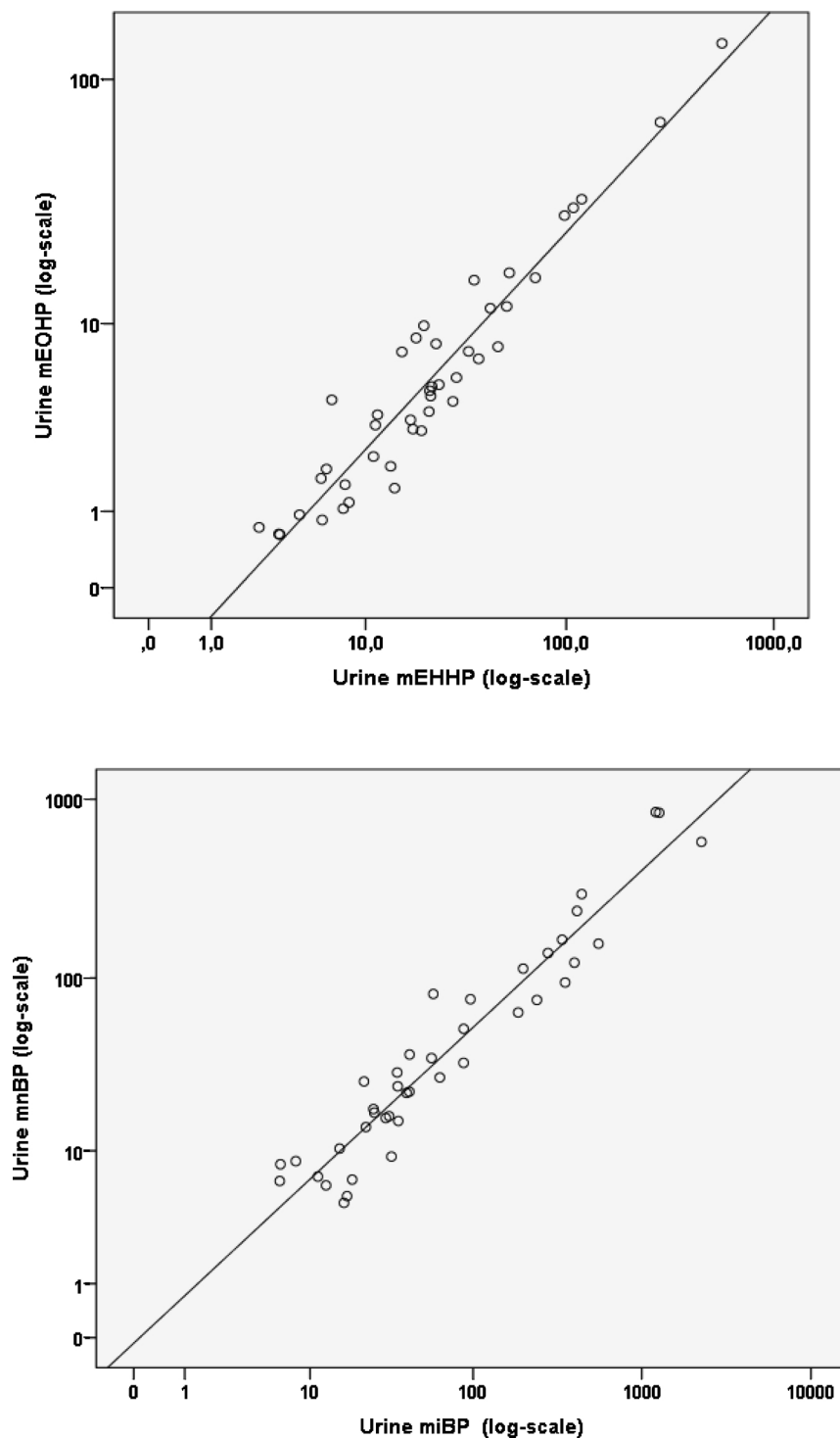


Fig. 3. Linear correlations in log-scale that were found for the pairs mEHHP-mEOHP ($r_s = 0.92$, $p < 0.001$), miBP-mnBP ($r_s = 0.95$, $p < 0.001$) and miBP-mBzP ($r_s = 0.70$, $p < 0.001$) in urine samples.

data, it was found that higher levels of mEHP were significantly associated with frequent use of plastic containers for food storage ($p = 0.026$), a weak inverse association was for the use of make-up products and mBzP ($p = 0.053$). The frequent use of deodorant before pregnancy was significantly associated with higher levels of miBP ($p = 0.050$), mnBP ($p = 0.028$) but the frequent use during the first trimester was inversely associated with mEHP ($p = 0.041$). The use of hair spray was also examined but no associations came up.

There were also other parameters that were examined (including occupation, consumption of beverages and canned food, drinking

bottled or tap water and the occurrence of plastic tanks and pipes in houses for the storage and transfer) but no associations came up with biomonitoring in urine.

3.6. Estimation of daily intake and risk assessment

Biomonitoring data from urine can be used for the estimation of the daily intake (Estimated Daily Intake, EDI) from a previously used [8,38–41] mathematical model (Eq. (1)) which converts the urinary concentrations of the metabolites to intake of the parent compound.

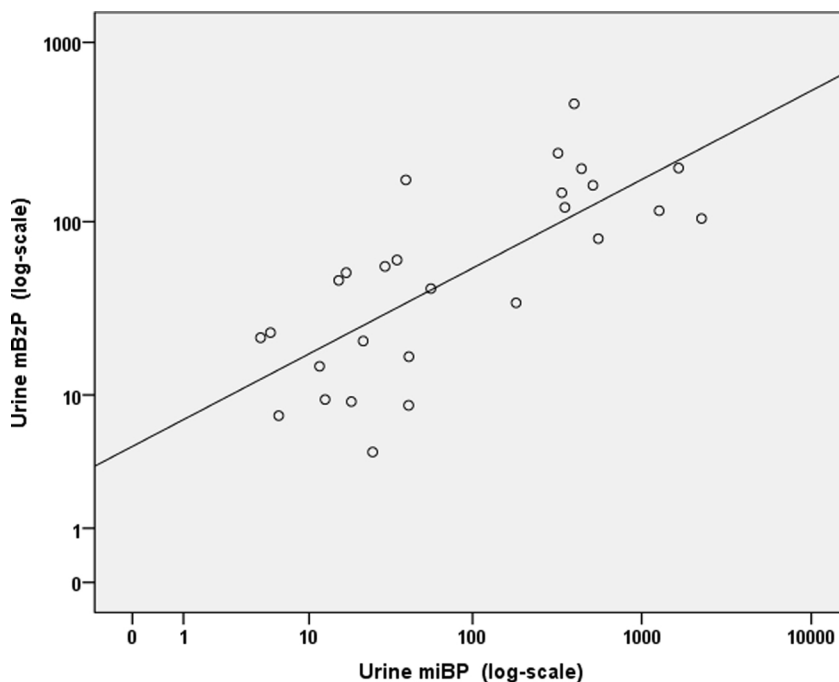


Fig. 3. (continued)

Table 4
Correlations (Spearman) between phthalate metabolites in urine samples.

Compounds		Rs	P
mEHHP	mEOHP	0.92	< 0.001
	miBP	0.28	0.10
	mnBP	0.38	0.05
	mBzP	0.06	0.79
mEOHP	mEHP	0.41	0.07
	miBP	0.21	0.20
	mnBP	0.41	0.02
	mBzP	−0.01	0.97
miBP	mEHP	0.21	0.36
	mnBP	0.95	< 0.001
	mBzP	0.70	< 0.001
	mEHP	0.39	0.08
mnBP	mBzP	0.63	0.002
	mEHP	0.39	0.16
mBzP	mEHP	0.30	0.28

The values for the parameters of the equation have been previously described [8]. For the estimation of daily intake for DEHP the summed urinary concentrations of the three metabolites was used.

$$EDI \left(\frac{\mu\text{g}}{\text{kg bw}} \right) = \frac{C_U \left(\frac{\mu\text{g}}{\text{L}} \right) \times V_U \left(\frac{\text{L}}{\text{day}} \right) \times MW_P \left(\frac{\text{g}}{\text{mol}} \right)}{F_{UE} \times BW \text{ (kg)} \times MW_{PM} \left(\frac{\text{g}}{\text{mol}} \right)} \quad (1)$$

CU (μg/L) is the urinary concentration of the metabolite, VU (L) the daily urine volume, F_{UE} the 24 h urinary excretion factor for each compound, BW (kg) body weight, MW_P and MW_{PM} the molecular weights for the parent phthalate and the metabolite, respectively.

The EDI value can be further used to estimate the hazard risk that exposure poses for health which is the ratio between the EDI and the guideline value RfD (Reference Dose) from US EPA (Eq. (2)). The Hazard Quotient (HQ) refers to the risk from exposure to a single phthalate and Hazard Index (HI) to the cumulative exposure to the six parent phthalates that were examined.

$$HQ = \frac{EDI}{RfD}, \quad HI = \sum HQ_i \quad (2)$$

The results from the estimation of EDI are presented in Table 5. The

Table 5
Estimated Daily Intake (EDI) (μg/kg/day), Hazard Quotient (HQ) and Hazard Index (HI) values, for the exposure of pregnant women to phthalates.

Parent phthalate	Phthalate metabolites	Median urine concentration (μg/L)	EDI	EPA RfD	HQ
DiBP	miBP	41.5	1.6	100	0.016
DnBP	mnBP	28.1	0.9	100	0.009
BBzP	mBzP	46.7	1.7	200	0.008
DEHP	mEHP	6.1	1.2	20	0.062
	mEHHP	17.9			
	mEOHP	4.9			
Total Daily Intake			5.4	HI	0.10

studied population was exposed to 142 μg/L phthalates (median) and the daily intake for each compound was 1.6 μg/kg DiBP, 0.9 μg/kg DnBP, 1.7 μg/kg BBzP and 1.2 μg/kg DEHP. The total daily intake was 5.4 μg/kg which corresponds to HI 0.10 or 10%. According to Hannon and Flaws [42], the range for daily intake of DEHP is 3–30 μg/kg and the exposure of pregnant women in the present study is lower.

4. Discussion

4.1. Amniotic fluid

The concentrations of phthalate metabolites in amniotic fluid (medians: 2.3–10.7 μg/L) and the detection frequencies were lower than in urine (medians: 4.9–46.7 μg/L) and in literature it has been shown that the amniotic fluid concentrations can be either similar (22.1–85.5 μg/L amniotic fluid, 24.6–78.0 μg/L urine) or lower than urine (< LOD-7.8 μg/L amniotic fluid, 1.3–55.6 μg/L urine) [29,35]. This may indicate the difficulty of the compounds to cross the placenta tissue barrier. There are few studies for phthalates metabolites in amniotic fluid and the most recent ones are Li et al. [32] which was conducted in 80 pregnant women from China during 2015 and [30,31]) in samples from Danish biobank during 1980–1996 (Table 6).

In the present study, the oxidative metabolites of DEHP were not detected at levels above LOD except for one sample. Similar findings have been reported in the studies Li et al. [32] Silva et al. [33] and Wittassek et al. [35] in which mEHHP and mEOHP were either not

Table 6
Concentrations of phthalate metabolites in amniotic fluid ($\mu\text{g/L}$) from pregnant women in recent studies.

Reference	N (Country, sampling year)	Metabolites	Mean \pm SD	Median	Range/Max	% Positive samples
Present study	100 (Greece, 2018)	miBP	39.3 \pm 55.1	10.0	5.1-102.9	3
		mnBP	12.0 \pm 9.8	10.7	2.4-24.5	5
		mBzP	< LOD	< LOD	11.6-20.2	2
		mEHP	2.7 \pm 1.3	2.3	1.4-7.0	21
		mEHHP	< LOD	< LOD	< LOD	0
		mEOHP	< LOD	< LOD	0.9	1
[32]	81 (China, 2015)	mnBP	4.2	3.7	0.9-45.1	100
		mEHP	0.8	0.7	< LOD-4.3	98.8
		mECPP	0.2	0.1	0.02-1.6	100
		mEP	0.5	0.2	< LOD-3.4	67
		mMP	3.1	2.7	< LOD-10.6	98.8
		mBzP	< LOD	< LOD	< LOD	0
		miBP	2.6	2.2	1.0-26.1	100
		mEOHP	< LOD	< LOD	< LOD	0
		mEHHP	< LOD	< LOD	< LOD	0
		mCMHP	1.1	0.9	0.1-5.8	100
		mCPP	< LOD	< LOD	< LOD	0
		[34]	70 (Europe)	mnBP	3.5 \pm 2.3	3.2
mEP	0.7 \pm 0.8			0.5	3.7	68.6
mBzP	0.2 \pm 0.1			0.1	0.5	78.6
mEHP	1.5 \pm 5.0			0.7	50.2	58.6
mEHHP	0.3 \pm 0.2			0.4	0.5	57.1
mMP	–			–	< LOD-2.9; < LOD	–
[29]	64 female; male (Taiwan, 2005-2006)	mEP	–	–	< LOD-6.5; < LOD-7.7	–
		mnBP	–	85.5;81.3	39.3-192.0;28.4-145.0	100
		mBzP	–	–	< LOD-233.0; < LOD-104.0	–
		mEHP	–	24.0;22.1	< LOD-148.0; < LOD-110.0	> 90
		miBP	9.1	7.8	18.7	100
		mBzP	10.0	4.2	35.7	100
[35]	11 (Germany)	mBzP	2.1	1.9	2.8	100
		mEHP	2.4	1.6	8.4	100
		mEHHP	< LOQ	< LOQ	0.31	72.7
		mEOHP	< LOD	< LOD	< LOQ	18.2
		mECPP	0.90	0.53	2.7	100
		mCMHP	0.60	0.64	0.92	100
		mHiNP	< LOD	< LOD	< LOQ	9.1
		mOiNP	< LOD	< LOD	< LOD	0
		mCiOP	0.51	< LOD	4.9	9.1
		mEP	–	< LOD	< LOD-9.0	92.6
		mnBP	–	5.8	< LOD-263.9	39
		mEHP	–	< LOD	< LOD-2.8	24

detected in any sample [32,33] or detected at very low concentrations [35]. On the other hand, the oxidative metabolites of DEHP (mEHHP, mEOHP, mECPP, mCMHP) can only be produced by metabolic processes and thus they are considered as reliable biomarkers of exposure [35]. DEHP can be metabolized from the fetal liver during the last step of development [43] and the measured levels of the metabolites in amniotic fluid come from the metabolic processes of the fetus.

No significant associations were found between the levels in amniotic fluid and information on exposure from the questionnaires maybe because the biomonitoring data were not enough. In literature, there is strong inverse correlation between mnBP and anogenital index adjusted by birth weight ($r = -0.32$, $p < 0.05$) [29] which indicated that prenatal exposure to DnBP may have anti-androgenic effects on the fetus. According to Jensen et al. [31], prenatal exposure to DEHP was not associated with genital malformations, however the high levels of the metabolite 5cx-mePP in amniotic fluid were related with 18% higher testosterone levels and 41% lower insulin-like factor 3. The time of amniocentesis has been positively associated with DEHP and di isononyl phthalate, DiNP [30] indicating that the levels of the highly lipophilic compounds are accumulated in amniotic fluid.

4.2. Urine

There are many studies that investigate the presence of phthalate metabolites in urine since it is the most common matrix for biological monitoring. Pregnant women from Crete in the present study had lower

body burden to the compounds and the positive samples ranged from 27% to 54% while in other similar studies the frequencies are above 80% (Table 7). Myridakis et al. [44] also examined the exposure of the Cretan pregnant women and the detected concentrations were similar between the two studies. However, the frequencies at this study were lower which may be because the achieved LODs ranged from 0.3 to 4.5 $\mu\text{g/L}$ while there are other studies with LODs from 0.2 to 0.8 $\mu\text{g/L}$ [27] or below 0.5 $\mu\text{g/L}$ [45].

The significant linear correlations that were found for urinary metabolites in pairs indicated the common source of exposure as they are used as mixtures in the products and also the common parent compound for the DEHP metabolites. Similar findings have also been previously reported for DEHP and DiNP metabolites urine and serum [48].

The effect of maternal exposure on infant health was examined but no associations came up between urinary concentrations and health problems or birth weight. A weak association was observed between high maternal exposure to mEHP and lower birth length and similar results for other phthalates are reported in literature [19,49,50].

Regarding the use of cosmetics and personal care products, it was found that women who reported frequent or daily use of deodorant had higher levels of miBP ($p = 0.050$) and mnBP ($p = 0.028$) in urine. In literature the use of leave-on skin cosmetics has been significantly associated with urinary mBzP and mono ethyl phthalate (mEP) in pregnant women [25]. The inverse association that was found in the present study for the use of cosmetics and urinary mBzP has also been reported in other studies [51,52].

Table 7
Concentrations of phthalate metabolites in urine ($\mu\text{g/L}$) from pregnant women in recent studies.

Reference	N (Country, sampling year)	Metabolites	Mean	Median	Range/Max	% Positive samples		
Present study	100 (Greece, 2018)	miBP	251.6	41.5	4.7-2255.6	54.0		
		mnBP	103.6	28.1	4.6-847.6	44.0		
		mBzP	80.6	46.7	4.3-455.4	31.0		
		mEHP	17.4	6.1	1.3-263.4	27.0		
		mEHHP	40.7	17.9	2.4-563.7	50.0		
		mEOHP	10.7	4.9	0.6-139.1	52.0		
[25]	256 (Taiwan, 2012-2015)	mMP	5.3	–	–	95.0		
		mEP	16.1	–	–	92.4		
		mnBP	18.4	–	–	99.7		
		miBP	10.3	–	–	99.1		
		mBzP	0.5	–	–	56.7		
		miNP	–	–	–	0.9		
		mEHP	3.3	–	–	83.9		
		mEHHP	10.4	–	–	99.4		
		mEOHP	8.9	–	–	99.2		
		mECP	15.2	–	–	97.6		
		[27]	50 (Israel, 2015-2016)	mEP	–	56.7	–	100.0
mnBP	–			11.1	–	98.0		
mHBP	–			0.6	–	66.0		
miBP	–			12.5	–	100.0		
mHiBP	–			3.1	–	98.0		
mBzP	–			0.8	–	84.0		
mCPE	–			0.6	–	52.0		
mEHP	–			1.5	–	72.0		
mEHHP	–			6.2	–	100.0		
mEOHP	–			5.7	–	100.0		
mECP	–			9.9	–	100.0		
[46]	125 (Japan, 2009-2010)			mnBP	–	< LOD	< LOD-37	33.9
				miBP	–	47.3	< LOD-6946	95.3
		mBzP	–	11.6	< LOD-445	74.0		
		mEHP	–	28.6	< LOD-416	85.8		
		mEOHP	–	47.3	< LOD-199	98.4		
		mECP	–	7.5	< LOD-182	56.7		
		[44]	239 (Greece, 2009-2011)	mEP	141.9	133.9	2.6-4103.7	100.0
mnBP	32.1			36.1	< LOD-94670.7	95.9		
miBP	36.7			39.2	< LOD-616.1	98.0		
mBzP	6.9			6.0	< LOD-199.4	91.6		
mEHP	7.0			7.6	< LOD-3401.3	72.7		
mEHHP	22.1			25.7	< LOD-6267.3	96.4		
mEOHP	15.5			17.6	< LOD-3610.6	93.6		
[47]	139 (Puerto-Rico, 2010-2012)			mEP	102.2	–	12700	100.0
		mnBP	19.2	–	413	98.7		
		miBP	10.9	–	964	100.0		
		mBzP	3.9	–	305	98.4		
		mEHP	3.3	–	141	92.9		
		mEOHP	8.9	–	281	100.0		
		mEHHP	10.7	–	361	100.0		
		mECP	19.6	–	749	100.0		
		mCPE	2.3	–	109	98.9		
		mCNP	2.3	–	59.8	99.7		
		mCiOP	16.4	–	1230	100.0		

The frequent use of plastics containers for food storage was significantly associated with high urinary mEHP, similarly to the study Tranfo et al. [53] for mEHP and mEHHP. The presence of high concentrations of phthalates in food, food supplements and drinks in Taiwan [54,55] raised concerns and the government intervention. Other studies that were followed found also contaminated ice cream and frozen food. A study in Puerto Rico associated the metabolites mCPP, mCNP and mCOP in maternal urine with frequent consumption of ice cream and chicken and mEP with drinking bottled water [47] and in another study alcohol consumption, fish and packaged food were associated with high levels of DEHP metabolites, mnBP and mBzP [53]. However, in the present study no associations were found between maternal exposure and dietary habits. It has been found that the indoor removal of everyday products that are source of phthalates resulted in a significant decrease in the exposure of the family members to the compounds [56]. Plastic kitchen ware, toys and bathroom products were removed from the house and the family members avoided the consumption of packed food in plastics and after two months the family

body burden to certain phthalates was decreased.

In the present study, pregnant women had greater exposure to DiBP (1.6 $\mu\text{g/kg/day}$) than DnBP (0.9 $\mu\text{g/kg/day}$) which may indicate the partial substitution of DnBP from DiBP, due to the toxicological data on DnBP that show increased risk for human health and the application of strict regulation (199/815/EG, 2004/718/EG) for the use of the compound in certain products. The decrease in DnBP exposure and increase in DiBP body burden has been observed in the biomonitoring results [37,41,57] and the present study also follows this trend. Regarding the exposure to total phthalates as it was calculated from the EDI, the present study follows the annual drop of human exposure to DEHP and total phthalates since 1988 until 2013 [8,41,58].

Although there is an increasing scientific interest regarding exposure to phthalates, humans have daily contact with various chemicals such as pesticides, antibiotics and additives in cosmetics. This cumulative exposure may have additive and synergistic effects that will be clinically visible later in life [59–61]. Genotoxic, cytotoxic and cytopathological effects as well as altered haematological parameters have

been observed in rats after long term exposure to very low doses of a mixture of 13 chemicals, including bisphenol A [62–64]. Since risk assessment usually focuses on individual compounds, the current regulatory approach does not assess the overall risk of chemicals present in a mixture. It has been proposed that computational methods could contribute to assessing the potential health effects with lower cost and time compared with conventional in vivo and in vitro experiments [61]. Scientific community has recognized the need for assessing the health risk of combined exposures to toxicants and new risk assessment methodologies have already been employed towards this direction [65–67].

5. Conclusion

The present study focused on the investigation of exposure of pregnant women to phthalates through the biomonitoring of their metabolites in urine and amniotic fluid. Although there are many studies for phthalates in urine, there are less data about associations between biomonitoring and data from questionnaires and furthermore, recent data about phthalate metabolites in amniotic fluid are missing in literature. The present study presented the combination of biomonitoring, answering questionnaires about exposure and health problems and the evaluation of the hazard of exposure.

Higher levels and more compounds were detected in urine and the correlations that were found between the metabolites indicated the common parent compound and sources of exposure. The frequent use of plastic food containers was significantly associated with urinary mEHP ($p = 0.026$) and a trend was noticed for mEHP in amniotic fluid ($p = 0.093$), the frequent use of deodorant was significantly associated with higher urinary miBP ($p = 0.050$) and mnBP ($p = 0.028$). No other associations came up for infant health problems or development except for a trend between urinary mEHP and lower birth length ($p = 0.062$). The daily intake of the total phthalates was calculated 5.4 $\mu\text{g}/\text{kg}$ body weight/day which corresponds to hazard index 0.10 and exposure follows the declining trend that has been observed the last decades.

CRedit authorship contribution statement

Ioanna Katsikantami: Investigation, Formal analysis, Writing - review & editing. **Manolis N. Tzatzarakis:** Conceptualization, Supervision, Methodology. **Athanasios K. Alegakis:** Formal analysis, Data curation. **Vasiliki Karzi:** Investigation. **Eleftheria Hatzidaki:** Resources, Validation. **Athina Stavroulaki:** Investigation. **Elena Vakonaki:** Methodology. **Pelagia Xezonaki:** Resources. **Stavros Sifakis:** Resources, Validation. **Apostolos K. Rizos:** Conceptualization, Supervision. **Aristidis M. Tsatsakis:** Conceptualization, Supervision, Funding acquisition.

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.

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