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Urinary phthalate metabolite concentrations in girls with premature thelarche

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ABSTRACT

In girls, breast development before eight years of age is called "premature thelarche (PT)". There are few studies in literature that show the interaction between PT and phthalate exposure. The aim of this study was to determine the urinary levels of di-(2-ethylhexyl) phthalate (DEHP) metabolites and other phthalate metabolites in girls with PT. PT group consisted of 29 newly diagnosed subjects. Control group comprised of healthy age-matched girls (n = 25). Urinary phthalate metabolite concentrations were measured by liquid chromatography/tandem mass spectroscopy (LC–MS/MS). The urinary concentrations of mono-(2-ethyl-hexyl)phthalate (MEHP) in the PT group (33.96 ± 6.88 μg/g creatinine) were found to be significantly higher compared to control group (11.54 ± 1.39 μg/g creatinine, p = 0.002). In PT group, %MEHP was also markedly higher vs. control (17.84 ± 3.31 vs. 6.44 ± 1.13, p = 0.001). Our results suggest that DEHP is more efficiently converted to MEHP in girls with PT, the importance of which needs to be further elucidated.

1. Introduction

Premature thelarche (PT) is defined as "early breast development in girls before the age of eight" (Codner and Román, 2008; Chiabotto et al., 2006). Several factors, including endocrine disrupting chemicals (EDC), are suggested to play roles its etiology (Mouritsen et al., 2010; Roy et al., 2009). It should be taken into account that the metabolic clearance rate of estradiol is low in pre-pubertal children. Therefore, exposure particularly to estrogen-like chemicals might have a significant biological impact on pre-pubertal breast development (Codner and Román, 2008; Chiabotto et al., 2006).

Phthalates are di-esters of phthalic acid. They are used as plasticizers to impart flexibility to plastic products (ATSDR, 1993; EPA, 2016). In humans, phthalates are metabolized to monoester metabolites upon ingestion. Though human body tends to detoxify the ingested substances, sometimes bioactivation occurs instead, as in the case of phthalates and more active mono-ester phthalates are unfortunately produced by Phase I metabolism (Frederiksen et al., 2007). Short-branched phthalates are mainly excreted as monoester phthalates, while the long-branched phthalates undergo further biotransformation processes, by hydroxylation, oxidation and conjugation before excretion (Frederiksen et al., 2007; Heindel and Powell, 1992; Koch et al., 2005).

It is inevitable to prevent release of plasticizers into the environment as they are not covalently bound to plastics (Hauser and Calafat, 2005). Phthalates are suggested to have EDC-properties (Balbuena et al., 2013; Christiansen et al., 2010; Erkekoglu et al., 2011; Kortenkamp and Faust, 2010; Svechnikov et al., 2008). Moreover, these chemicals are associated with different reproductive and endocrine diseases, like precocious puberty (PP), diabetes, gynecomastia and obesity (Balci et al., 2016; Buluş et al., 2016; Dong et al., 2017; Durmaz et al., 2010, 2014; Kondolot et al., 2016; Stojanoska et al., 2017).

Di-(2-ethylhexyl)phthalate is suggested to have anti-androgenic potential and a widely used phthalate in flexible polyvinylchloride (PVC) products (ATSDR, 1993; EPA, 2016). DEHP is mainly metabolized to mono-(2-ethylhexyl) phthalate (MEHP) (Frederiksen et al., 2007; Heindel and Powell, 1992; Koch et al., 2005). MEHP is further metabolized to oxidative metabolites, mono(2-ethyl-5-oxohexyl) phthalate (MEOHP), mono(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP), and mono(2-ethyl-5-carboxypentyl) phthalate (MECPP) have been unequivocally identified in humans (Albro et al., 1981; Albro and Lavenhar, 1989; Albro and Moore, 1974; Frederiksen et al., 2007;

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Kavlock et al., 2002a; Koch et al., 2005, 2005; Preuss et al., 2005). MEOHP and MEHHP were examined as potential DEHP exposure biomarkers (Barr et al., 2003).

Diisononyl phthalate (DINP) is used in food-contact materials. DINP is hydrolyzed to minor urinary metabolite monoisononyl phthalate (MiNP), which is then oxidized to secondary oxidative metabolites with hydroxyl, oxo and carboxyl functional groups [mono(carboxyisooctyl) phthalate (MCiOP), mono(oxoisononyl) phthalate (MOiNP), and mono (hydroxyisononyl) phthalate (MHiNP)] (Calafat et al., 2011; Kato et al., 2007; Silva et al., 2007).

Diethyl phthalate (DEP) is mainly used in cosmetics. DEP is hydrolyzed to monoethyl phthalate (MEP) after ingestion (Api, 2001). Di-*n*-butyl phthalate (DnBP) is a widely used phthalate derivative and its main metabolite is mono-butyl phthalate (MnBP) (ECB, 2004; Kavlock et al., 2002b). Benzylbutyl phthalate (BBzP) is used as a plasticizer in PVC production and its major metabolite is monobenzyl phthalate (MBzP) (Toft et al., 2012). Diisobutyl phthalate (DiBP) is another butylphthalate derivative. Tts major metabolite is mono-isobutyl phthalate (MiBP) (CDC, 2016; ECHA, 2017). Di-*n*-octyl phthalate (DOP) is another phthalic acid derivative. Its urinary metabolites are mono-(3-carboxypropyl) phthalate (MCPP) and mono-*n*-octyl phthalate (MOP) (ATSDR, 1997; CDC, 2016; Kavlock et al., 2002c).

Based on this background and taking into account the high frequency of phthalate exposure in humans, particularly in young children, this study was designed to investigate the urinary phthalate metabolite concentrations in girls with PT.

2. Materials and methods

2.1. Subjects

The study was approved by Akdeniz University's Human Ethics Committee. All of the girls participated in the study voluntarily and a written consent was obtained from their parents. Subjects recruited in this study were grouped as follows:

a **PT group** consisted of 29 newly diagnosed, non-obese girls living in Antalya city. The subjects were included in the study according to the following criteria: (a) PT is observed as an isolated breast development before the age of 8; (b) ages of the girls were between 4–8 years; (c) girls who had no increase in bone age over one year of their chronological age were chosen; (d) girls were followed up regularly by a pediatrician for at least one year and girls who had breast development alone (without other progression of PP were included to the group) were recruited to the study. Pelvic USG findings were evaluated in all PT group to exclude pathological conditions, like ovarian cysts. Gonadotropin releasing hormone (GnRH) stimulation test (Gonadorelin acetate, Ferring®) was performed on girls with the diagnosis of PT to exclude the central PP.

b **Control group** consisted of 25 healthy, non-obese girls of comparable age (4–8 years), with no history of PT, any other endocrine disorder and no secondary sexual characteristics in their physical exam. Control girls were also born and were living in Antalya city. Control group were re-monitored 12 months later in order to evaluate their pubertal development and girls who had PT, premature pubarche, PP or any other pubertal signs in this second evaluation were excluded.

All study subjects were examined by the same clinical pediatrician. Height and body weight of the subjects were measured and body mass index [BMI] was calculated for each child. Obesity was defined as BMI > 95th percentile according to national standards (Bundak et al., 2006).

Table 1
Limit of detections for the phthalate metabolites.

	LOD (ng/ml)	Number of samples above LOD
MEHP	0,14	100
MEOHP	0,67	100
MECPP	0,55	100
MEHHP	0,91	100
MEP	0,53	100
MiBP	1,43	100
MnBP	1,1	100
MBzP	1,14	79,3
MiNP	0,61	24,1
MHiNP	0,26	93,1
MOiNP	0,25	82,8
MCiOP	0,53	100

LODs: Limit of detections.
MEHP: mono-(2-ethylhexyl)phthalate; MEP: monoethyl phthalate; MECCP: mono(2-ethyl-5-carboxypentyl) phthalate; MEHHP: mono(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP); MEOHP: mono(2-ethyl-5-oxohexyl) phthalate; MiBP: mono-isobutyl phthalate; MnBP: mono-butyl phthalate; BBzP: benzylbutylphthalate; MBzP: monobenzyl phthalate; MCPP: mono-(3-carboxypropyl) phthalate; MiNP: monoisononyl phthalate; MHiNP: mono(hydroxyisononyl) phthalate; MOiNP: mono(oxoisononyl) phthalate; MCiOP: mono(carboxyisooctyl) phthalate.

2.2. Deplasticization of the glassware and check for phthalate contamination

To prevent the contact with plastic material throughout the study, all glassware (including glass beakers for the collection of urine samples) were kept in tetrahydrofuran:n-hexan (50:50, v/v) for 2 h and were dried in an incubator later (Nüve, Ankara, Turkey) for 2 h. In order to deplasticize vials and tubes, they were heated up to 400 °C for 4 h in a tube dry block heater (Stuart, Staffordshire, UK). In Denmark, all glassware used to analyze phthalate metabolites were rinsed and sonicated with methanol for deplasticization. All chemicals, solutions, and lab wares were checked for contamination with phthalate metabolites before use.

2.3. Collection of biological materials

Between September 2010 and February 2012, spot urine samples were collected into deplasticized glass beakers in Pediatric Endocrinology Unit of Akdeniz University. Later, samples were aliquoted in deplasticized glass vials (Agilent, Santa Clara, CA) and were kept at −20 °C. The urine samples were then transferred to Hacettepe University, Faculty of Pharmacy, Department of Toxicology and were kept at −80 °C before they were transferred to Department of Growth and Reproduction, University Hospital of Copenhagen, Copenhagen, Denmark from Hacettepe University on dry ice for the determination of urinary phthalate metabolites. The hormone levels were measured in Akdeniz University.

2.4. Chemicals and kits

MEP, MnBP, MiBP, MBzP, MEHP, MEHHP, MEOHP, MiNP, and their ^{13}C4-labeled internal standards (all > 98% purity) were purchased from Cambridge Isotope Laboratories (Andover, MA; distributed by Bie & Berntsen A/S, Rødovre, Denmark). MECPP, MHiNP, MOiNP, MCiOP, and the d4-labeled internal standards were purchased from IDM (Teltow, Germany).

Kits for follicle stimulating hormone (FSH) and luteinizing hormone (LH) were from Abcam (Cambridge, MA). Estradiol kit was from Roche (Mannheim, Germany). Thyroid stimulating hormone (TSH) and free T4 (fT4) kits were from Diasorin Liaison (Stillwater, MN).

All chemicals were of analytical or HPLC grade. Other chemicals were purchased either from Merck (Darmstadt, Germany, were

Table 2
Urinary MEHP, MEOHP, MECPP, and MEHHP levels and MEHP (%) in study groups.

		Control (n = 25) median (min-max) (μg/g creatinine)	PT (n = 29) median (min-max) (μg/g creatinine)	p
MEHP	Median	10.38	19.51	**0.002**
	Min-max	(1.68–30.04)	(1.68–176.66)	
	Mean ± SEM	11.54 ± 1.39	33.96 ± 6.88*	
MEOHP	Median	32.93	26.95	0.182
	Min-max	(12.25–216.15)	(6.87–143.47)	
	Mean ± SEM	46.13 ± 8.32	38.08 ± 6.19	
MECPP	Median	47.07	55.35	0.952
	Min-max	(23.37–249.54)	(15.09–210.17)	
	Mean ± SEM	64.35 ± 9.60	67.70 ± 10.10	
MEHHP	Median	66.15	52.20	0.109
	Min-max	(27.52–458.37)	(13.00–316.53)	
	Mean ± SEM	96.26 ± 17.79	75.35 ± 13.30	
DEHP metabolites	Median	18.46	11.84	0.700
	Min-max	(5.12–3024.58)	(3.12–67.85)	
	Mean ± SEM	191.12 ± 123.17	20.86 ± 3.53	
%MEHP	Median	5.49	14.12	**0.001**
	Min-max	(2.41–28.05)	(2.49–59.17)	
	Mean ± SEM	6.44 ± 1.13	17.84 ± 3.31*	

*$p < 0.05$ is considered as statistically significant.
MEHP: mono-(2-ethylhexyl)phthalate; MEOHP: mono(2-ethyl-5-oxohexyl) phthalate; MECCP: mono(2-ethyl-5-carboxypentyl) phthalate; MEHHP: mono(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP).
All metabolites were detectable in both of the study groups.
DEHP metabolites: sum of MEHP, MEOHP, MECPP and MEHHP expressed as DEHP.

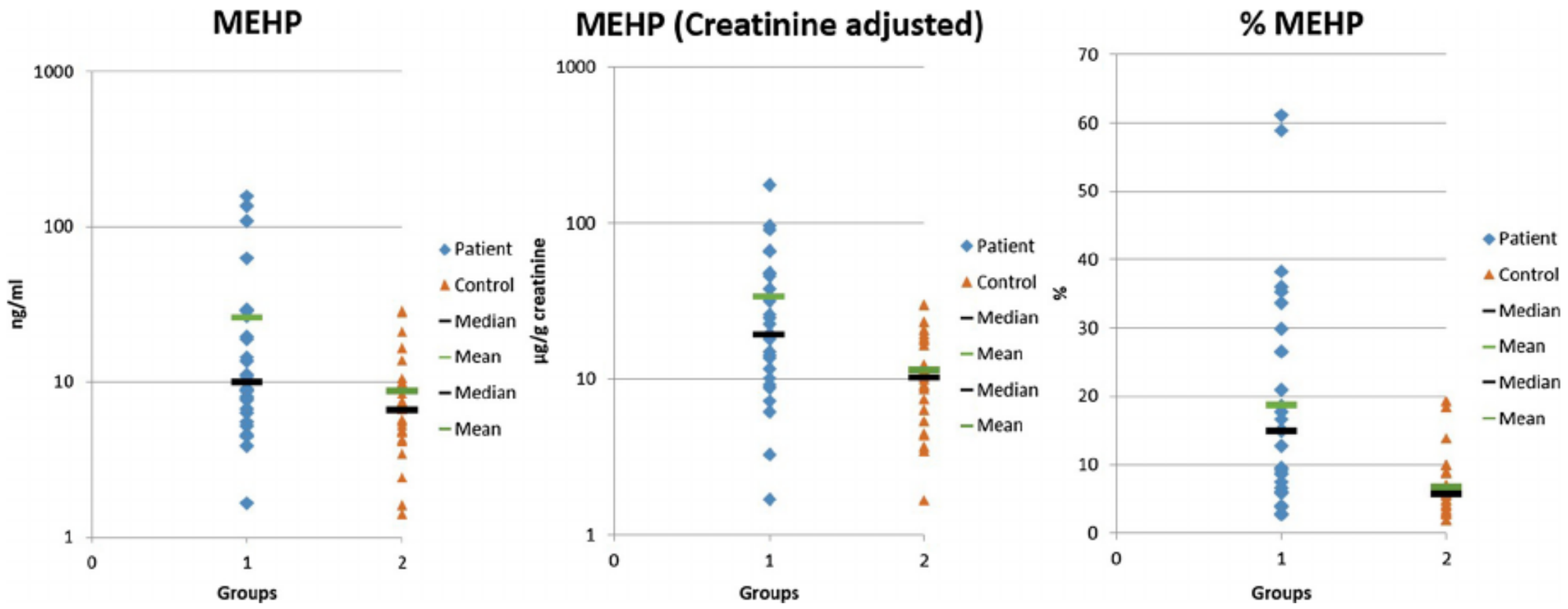

Fig. 1. Mono-(2-ethyl- hexyl)phthalate levels (in ng/ml and μg/g creatinine units) and mono-(2-ethyl- hexyl)phthalate %.
MEHP: mono-(2-ethylhexyl) phthalate.

distributed by Bie &Berntsen A/S) or from J.T. Baker (Phillipsburg, NJ, were distributed by Bie &Berntsen A/S), except ethyl acetate was from BDH Laboratories Supplies (Poole, England; distributed by VWR International, Rødovre, Denmark) and 4-methylumbelliferone and 4-methylumbelliferyl-β-D-glucuronide were from Sigma-Aldrich (Brøndby, Denmark). β-Glucuronidase (*Escherichia coli* K12) was obtained from Roche Diagnostics (Mannheim, Germany). Millipore Synthesis A10 system (Billerica, MA) was used to clean the Milli-Q. Solid-phase extraction (SPE) cartridges (Strata XL, 200 mg, 3 mL) were purchased from Phenomenex (Allerød, Denmark).

2.5. Measurement of hormones

Serum estradiol levels were measured by electrochemiluminescence immunoassay (ECLIA) by using a commercial kit. Serum fT4 and TSH levels were measured by chemiluminescence microparticle immunoassay using “Liaison DiaSorin chemiluminescence immunoassay (CLIA) kits” on a Diasorin Liaison CLIA Analyzer (Stillwater, MN). Serum luteinizing hormone (LH) and follicle stimulating hormone (FSH) in plasma were measured by enzyme linked immunosorbent assay (ELISA).

2.6. Analysis of urinary monoester phthalate levels

Primary and secondary metabolites of seven phthalate diesters (DEP, DiBP, DnBP, BBzP, DEHP, DOP and DiNP) were analyzed. The total (sum of free and conjugated) content MEHP, MEOHP, MECPP, MEHHP, MEP, MiBP, MnBP, MBzP, MCPP, MiNP, MHiNP, MOiNP and MCiOP were analyzed by isotope dilution liquid chromatography tandem mass spectrometry (LC–MS/MS) with preceding enzymatic deconjugation followed by automatic solid phase extraction. The method details have been described previously (Frederiksen et al., 2010). Samples were analyzed in 2 batches. Each batch included standards, 27 samples, two blanks, two pooled urine control samples, and two pooled urine control samples spiked with standards to an added concentration on 10 ng/mL. The recovery was above 90% for all

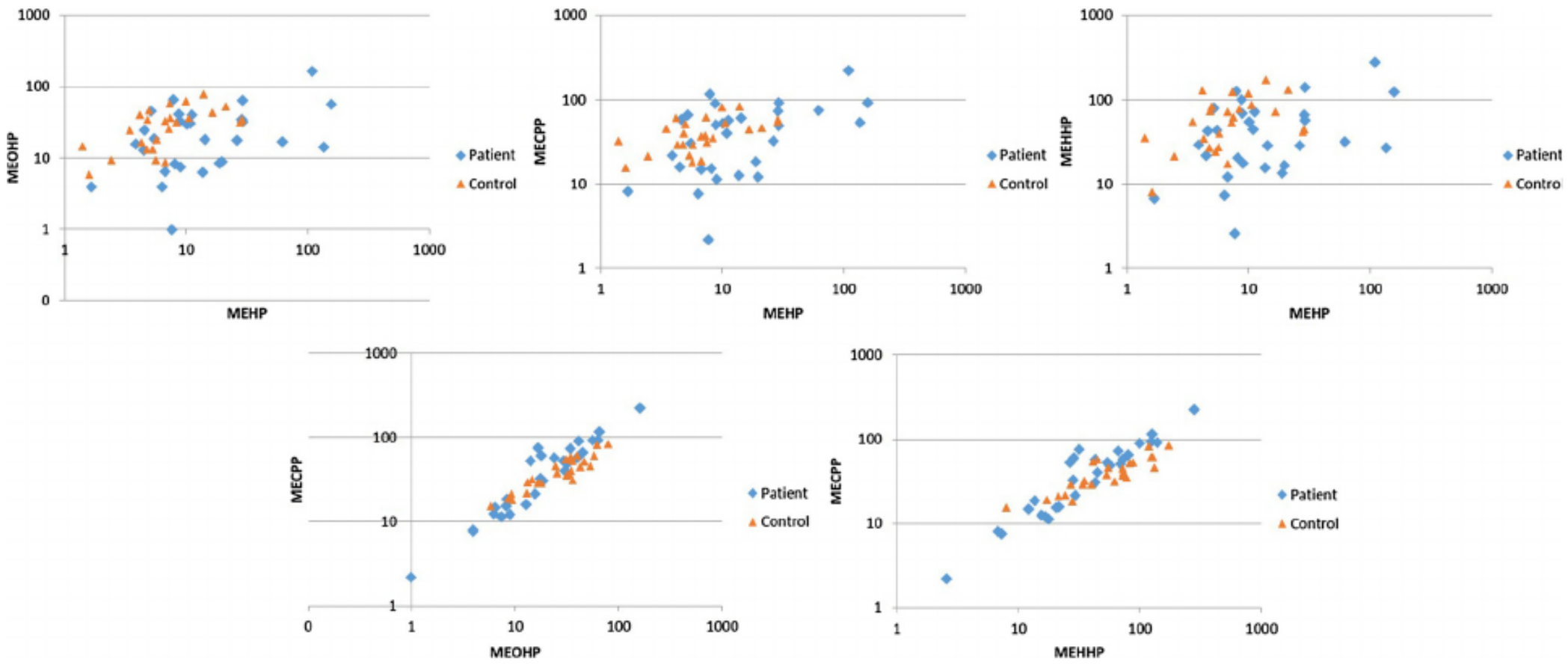

Fig. 2. Associations between different urinary di -(2-ethyl- hexyl)phthalate (DEHP) metabolites (MEHP vs. MEOHP, MEHP vs. MECPP, MEHP vs. MEHHP, MEOHP vs. MECPP and MEHHP vs. MECPP).
MECPP: mono(2-ethyl-5-carboxypentyl) phthalate.
MEHHP: mono(2-ethyl-5-hydroxyhexyl) phthalate.
MEHP: mono-(2-ethylhexyl) phthalate.
MEOHP: mono(2-ethyl-5-oxohexyl) phthalate.

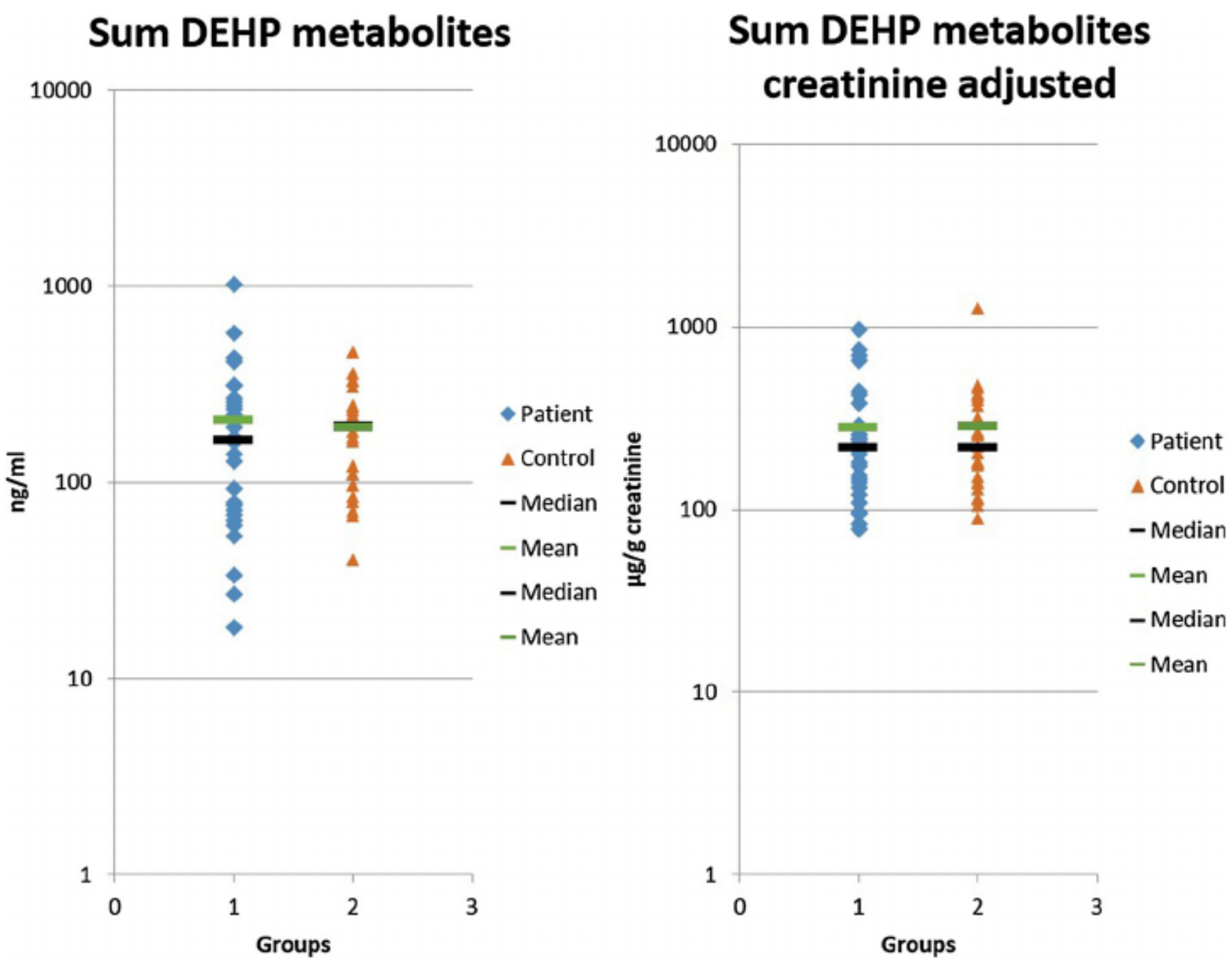

Fig. 3. Sum of DEHP metabolites (in ng/ml and µg/g creatinine units).

analytes (Frederiksen et al., 2010). Limit of detections (LODs) for the phthalate metabolites and number of samples (%) above LODs for the phthalate metabolites are shown in Table 1.

2.7. Urinary creatinine concentrations

Urinary creatinine concentrations were analyzed simultaneously by LC/MS/MS and the urinary phthalate monoester concentrations were adjusted by urinary creatinine concentrations.

2.8. Statistical analysis

Statistical analysis was conducted using PASW (Predictive Analytics SoftWare) Statistics, Release. Version 18.0 (SPSS, Inc., 2009, Chicago, IL). The comparison between 2 parametric values was performed by using Student's t test. For nonparametric values, Mann Whitney U test was used. The results were shown as mean ± standard deviation (SD). Besides, median (min-max) values were also given. Correlations between the selected parameters were evaluated with Spearman correlation coefficient (ρ). A p value of < 0.05 was accepted as significant.

Table 3
Urinary phthalate metabolites in study groups.

		Control (n = 25) (μg/g creatinine)		PT (n = 29) (μg/g creatinine)		p
	d/nd		d/nd			
DEP metabolite						
MEP	Median (min-max) Mean ± SEM	24/1	47.70 (7.66–624.80) 125.97 ± 33.37	26/3	31.12 (5.24–253.45) 44.60 ± 9.40*	0.051
DiBP metabolite						
MiBP	Median (min-max) Mean ± SEM	25/0	65.98 (16.96–1313,12) 161.81 ± 49.58	29/0	73.44 (21.26–264.03) 89.48 ± 11.08	0.091
DnBP metabolite						
MnBP	Median (min-max) Mean ± SEM	25/0	42.56 (8.93–376.73) 73.06 ± 15.25	29/0	39.94 (12.38–149.32) 49.73 ± 7.05	0.202
BBzP metabolite						
MBzP	Median (min-max) Mean ± SEM	23/2	6.21 (0.00-55.51) 13.08 ± 2.89	23/6	4.84 (0-00-310.84) 20.04 ± 10.71	0.202
DOP metabolite						
MCPP	Median (min-max) Mean ± SEM	24/1	47.07 (23.37–249.54) 64.35 ± 9.60	29/0	3.57 (0.55–19.64) 5.65 ± 1.05	0.527
DiNP metabolites						
MiNP	Median (min-max) Mean ± SEM	10/15	0.00 (0.00–19.73) 1.94 ± 1.02	7/22	0.00 (0.00–4.71) 0.51 ± 0.20	0.278
MHiNP	Median (min-max) Mean ± SEM	25/0	4.01 (0.72-408.66) 40.02 ± 20.20	27/2	3.39 (0.00–16.81) 4.97 ± 0.88	0.109
MOiNP	Median (min-max) Mean ± SEM	25/0	2.36 (0.29–239.02) 15.45 ± 9.80	24/5	1.01 (0.00–8.71) 2.18 ± 0.47*	**0.021**
MCiOP	Median (min-max) Mean ± SEM	23/2	7.33 (1.83–1628.46) 86.77 ± 65.48	29/0	4.94 (1.38–26.25) 8.00 ± 1.39*	**0.014**
DiNP metabolites	Median (min-max) Mean ± SEM	———	221.21 (88.92-1628.46) 287.39 ± 47.19	———	220.81 (78.08-970.11) 284.60 ± 41.35	0.051
low MW phthalate metabolites	Median (min-max) Mean ± SEM	———	179.49 (61.06–192.64) 331.214 ± 79.33	———	123.45 (42.23–614.67) 166.26 ± 30.81*	**0.022**
high MW phthalate metabolites	Median (min-max) Mean ± SEM	———	169.39 (69.67-271.96) 360.93 ± 111.67	———	165.32 (60.67–73.32) 222.66 ± 41.26	0.504

d: detectable; nd: non-detectable.
$p < 0.05$ is considered as statistically significant.
DEP: diethyl phthalate; MEP: monoethyl phthalate; DiBP: diisobutyl phthalate; MiBP: mono-isobutyl phthalate; DnBP: di-*n*-butyl phthalate; MnBP: mono-butyl phthalate; BBzP: benzylbutylphthalate; MBzP: monobenzyl phthalate; DOP: di-*n*-octyl phthalate; MCPP: mono-(3-carboxypropyl) phthalate; DiNP: diisononyl phthalate; MiNP: monoisononyl phthalate; MHiNP: mono(hydroxyisononyl) phthalate; MOiNP: mono(oxoisononyl) phthalate; MCiOP: mono(carboxyisooctyl) phthalate.
ΣDiNPmetabolites: sum of MiNP, MHiNP, MOiNP and MCIOP expressed as DiNP.
Σlow MW phth.metabolites: sum of MEP, MiBP and MnBP expressed as MEP in ng/ml.
Σhigh MW phth.metabolites: sum of MBzP, MEHP, MECPP, MiNP and MCiOP expressed as MEHP in ng/ml.

Table 4
Correlations between the urinary phthalate metabolites in PT group.

		MEP	MiBP	MnBP	MBzP	MEHP	MEHHP	MEOHP	MECPP	MCPP	MiNP	MHiNP	MOiNP	MCiOP	∑DEHP metabolites	∑DiNP metabolites
MEP	ρ	1	**0.467***	**0.433***	**0.439***	0.089	0.229	0.226	0.129	0.138	-0.006	0.051	0.174	-0.055	0.194	0.018
	p	.	**0.019**	**0.031**	**0.028**	0.671	0.270	0.277	0.538	0.509	0.977	0.810	0.405	0.793	0.353	0.933
MiBP	ρ	**0.467***	1	**0.925****	**0.547****	**0.423***	**0.531****	**0.573****	**0.559****	0.367	0.066	0.323	0.320	0.042	**0.581****	0.191
	p	**0.019**	.	**0.000**	**0.005**	**0.035**	**0.006**	**0.003**	**0.004**	0.071	0.754	0.115	0.119	0.841	**0.002**	0.361
MnBP	ρ	**0.433***	**0.925****	1	**0.597****	**0.415***	**0.462***	**0.509****	**0.549****	0.359	0.122	0.330	0.349	0.052	**0.535****	0.188
	p	**0.031**	**0.000**	.	**0.002**	**0.039**	**0.020**	**0.009**	**0.004**	0.078	0.563	0.107	0.087	0.807	**0.006**	0.367
MBzP	ρ	**0.439***	**0.547****	0.597**	1	0.234	0.309	**0.438***	0.352	0.210	0.189	0.257	0.318	0.063	0.364	0.190
	p	**0.028**	**0.005**	0.002	.	0.260	0.133	**0.028**	0.085	0.314	0.365	0.216	0.121	0.766	0.074	0.364
MEHP	ρ	0.089	**0.423***	**0.415***	0.234	1	**0.469***	**0.579****	**0.529****	0.175	**0.492***	**0.466***	**0.538****	0.220	**0.612****	**0.471***
	p	0.671	**0.035**	**0.039**	0.260	.	**0.018**	**0.002**	**0.007**	0.404	**0.013**	**0.019**	**0.006**	0.291	**0.001**	**0.018**
MEHHP	ρ	0.229	**0.531****	**0.462***	0.309	**0.469***	1	**0.946****	**0.842****	**0.453***	0.127	**0.548****	**0.524****	0.363	**0.945****	**0.504***
	p	0.270	**0.006**	**0.020**	0.133	**0.018**	.	**0.000**	**0.000**	**0.023**	0.546	**0.005**	**0.007**	0.074	**0.000**	**0.010**
MEOHP	ρ	0.226	**0.573****	**0.509****	**0.438***	**0.579****	**0.946****	1	**0.879****	**0.415***	0.275	**.555****	**.552****	0.382	**.974****	**0.528****
	p	0.277	**0.003**	**0.009**	**0.028**	**0.002**	**0.000**	.	**0.000**	**0.039**	0.183	**0.004**	**0.004**	0.060	**0.000**	**0.007**
MECPP	ρ	0.129	**0.559****	**0.549****	0.352	**0.529****	**0.842****	**0.879****	1	0.326	0.255	**0.455***	**0.482***	0.183	**0.937****	0.380
	p	0.538	**0.004**	**0.004**	0.085	**0.007**	**0.000**	**0.000**	.	0.112	0.218	**0.022**	**0.015**	0.381	**0.000**	0.061
MCPP	ρ	0.138	0.367	0.359	0.210	0.175	**0.453***	**0.415***	0.326	1	**0.429***	**0.728****	**0.746****	**0.616****	**0.429***	**0.708****
	p	0.509	0.071	0.078	0.314	0.404	**0.023**	**0.039**	0.112	.	**0.032**	**0.000**	**0.000**	**0.001**	**0.032**	**0.000**
MiNP	ρ	-0.006	0.066	0.122	0.189	**0.492***	0.127	0.275	0.255	**0.429***	1	**0.655****	**0.694****	**0.492***	0.295	**0.691****
	p	0.977	0.754	0.563	0.365	**0.013**	0.546	0.183	0.218	**0.032**	.	**0.000**	**0.000**	**0.013**	0.152	**0.000**
MHiNP	ρ	0.051	0.323	0.330	0.257	**0.466***	**0.548****	**0.555****	**0.455***	**0.728****	**0.655****	1	**0.915****	**0.715****	**0.558****	**0.954****
	p	0.810	0.115	0.107	0.216	**0.019**	**0.005**	**0.004**	**0.022**	**0.000**	**0.000**	.	**0.000**	**0.000**	**0.004**	**0.000**
MOiNP	ρ	0.174	0.320	0.349	0.318	**0.538****	**0.524****	**0.552****	**0.482***	**0.746****	**0.694****	**0.915****	1	**0.626****	**0.555****	**0.897****
	p	0.405	0.119	0.087	0.121	**0.006**	**0.007**	**0.004**	**0.015**	**0.000**	**0.000**	**0.000**	.	**0.001**	**0.004**	**0.000**
MCiOP	ρ	-0.055	0.042	0.052	0.063	0.220	0.363	0.382	0.183	**0.616****	**0.492***	**0.715****	**0.626****	1	0.332	**0.855****
	p	0.793	0.841	0.807	0.766	0.291	0.074	0.060	0.381	**0.001**	**0.013**	**0.000**	**0.001**	.	0.105	**0.000**
sDEHPm	ρ	0.194	**0.581****	**0.535****	0.364	**0.612****	**0.945****	**0.974****	**.937****	**0.429***	0.295	**0.558****	**0.555****	0.332	1	**0.506****
	p	0.353	**0.002**	**0.006**	0.074	**0.001**	**0.000**	**0.000**	**0.000**	**0.032**	0.152	**0.004**	**0.004**	0.105	.	**0.010**
sDiNPm	ρ	0.018	0.191	0.188	0.190	**0.471***	**0.504***	**0.528****	0.380	0.708**	0.691**	0.954**	0.897**	0.855**	0.506**	1
	p	0.933	0.361	0.367	0.364	**0.018**	**0.010**	**0.007**	0.061	0.000	0.000	0.000	0.000	0.000	0.010	.

*p < 0.05; **p < 0.01.

DEP: diethyl phthalate; MEP: monoethyl phthalate; DiBP: diisobutyl phthalate; MiBP: mono-isobutyl phthalate; DnBP: di-*n*-butyl phthalate; MnBP: mono-butyl phthalate; BBzP: benzylbutylphthalate; MBzP: monobenzyl phthalate; DOP: di-*n*-octyl phthalate; MCPP: mono-(3-carboxypropyl) phthalate; DiNP: diisononyl phthalate; MiNP: monoisononyl phthalate; MHiNP: mono(hydroxyisononyl) phthalate; MOiNP: mono(oxoisononyl) phthalate; MCiOP: mono(carboxyisooctyl) phthalate; MEHP: mono-(2-ethylhexyl)phthalate; MEOHP: mono(2-ethyl-5-oxohexyl) phthalate; MECCP: mono(2-ethyl-5-carboxypentyl) phthalate; MEHHP: mono (2-ethyl-5-hydroxyhexyl) phthalate (MEHHP).

3. Results

3.1. Subjects

There were no statistically significant differences between the ages (6.64 ± 0.88 in control vs. 6.83 ± 0.95 in PT group, p = 0.45) and BMIs (16.06 ± 2.07 in control vs. 16.3 ± 1.8 in PT group, p = 0.66) of the groups.

3.2. Hormone levels

The basal LH, basal FSH and basal estradiol levels in PT patients were 0.11 ± 0.03 IU/L, 1.72 ± 0.79 IU/L and 10.25 ± 6.58 pg/mL, respectively. After GnRH stimulation test, first peak LH levels were 3.01 ± 1.18 IU/L while second peak LH levels were 2.77 ± 1.07 IU/L. After GnRH stimulation test, first peak FSH levels were 11.15 ± 5.61 IU/L while second peak FSH levels were 10.86 ± 6.65 IU/L. In girls with PT, TSH levels were 2.34 ± 0.93 mIU/L while fT4 levels were 1.35 ± 0.15 ng/dL.

3.3. Urinary levels of di(2-ethylhexyl)phthalate metabolites

Creatinine adjusted urinary MEHP and %MEHP levels were significantly higher in the PT group vs. control group [For MEHP, median 19.51 (33.96 ± 6.88) μg/g creatinine in PT group and 10.38 (11.54 ± 1.39) μg/g creatinine in control group p = 0.002]. MEHP (%) was 5.49% (min-max: 2.41%–28.05%) in control group and 14.12% (2.49-59.17) in PT group (p = 0.001). Other DEHP metabolites (i.e. MEOHP, MECPP, and MEHHP) were not found to be markedly different in PT group vs. when compared to control (Table 2). MEHP levels (in ng/ml and μg/g creatinine units) and MEHP% values are given in Fig. 1. MEHP vs. MEOHP, MEHP vs. MECPP, MEHP vs. MEHHP, MEOHP vs. MECPP and MEHHP vs. MECPP are shown in Fig.2. Sum of DEHP metabolites (in ng/ml and μg/g creatinine units) are shown in Fig. 3.

3.4. Urinary levels of other phthalate metabolites

Urinary levels of MEP, MiBP, MnBP, MBzP, MCPP, MiNP, MHiNP, MOiNP and MCiOP are given in Table 3. Besides, ΣDiNP metabolites, Σlow MW phthalate metabolites and Σhigh MW phthalate metabolites are also shown in Table 3. Interestingly, DiNP metabolites, namely MOiNP [1.01 (2.18 ± 0.47) μg/g creatinine in control vs. 2.36 (15.45 ± 9.80) μg/g creatinine in PT, p = 0.021] and MCiOP [7.33 (186.77 ± 65.48) μg/g creatinine in control vs. 4.94 (8.00 ± 1.39) μg/g creatinine in PT, p = 0.014] were significantly lower in PT group vs. control. Moreover, Σlow MW phthalate metabolites are markedly lower in PT group [179.49 (331.214 ± 79.33) μg/g creatinine] vs. control [123.45 (166.26 ± 30.81) μg/g creatinine] (p = 0.022).

3.5. Correlations between di erent urinary phthalate metabolites

Correlations between urinary phthalate metabolites for control group are given in Table 4 while correlations between urinary phthalate metabolites for PT group are given in Table 5. It can be stated that urinary MEHP levels is highly correlated with urinary MEHHP, MEOHP

Table 5
Correlations between the urinary phthalate metabolites in PT group.

		MEP	MiBP	MnBP	MBzP	MEHP	MEHHP	MEOHP	MECPP	%MEHP	MCPP	MiNP	MHiNP	MOiNP	MCiOP	∑DEHP metabolites	∑DiNP metabolites
MEP	ρ	1	**0.384***	**0.448***	0.200	0.172	0.319	00.337	0.364	0.091	**0.453***	0.055	**0.461***	0.335	0.363	0.301	**0.439***
	p	.	**0.040**	**0.015**	0.299	0.373	0.091	00.074	0.053	0,874	**0.014**	0.778	**0.012**	0.076	0.053	00.112	**0.017**
MiBP	ρ	**0.384***	1	**0.931****	**0.736****	0.350	**0.857****	**0.865****	**0.728****	**0.319^A**	**0.757****	**0.413***	**0.853****	**0.814****	**0.710****	**0.763****	**0.796****
	p	**0.040**	.	**0.000**	**0.000**	0.063	**0.000**	**0.000**	**0.000**	**0.045**	**0.000**	**0.026**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**
MnBP	ρ	**0.448***	**0.931****	1	**0.717****	0.278	**0.829****	**0.853****	**0.752****	0.154	**0.850****	0.280	**0.773****	**0.740****	**0.654****	**0.757****	**0.711****
	p	**0.015**	**0.000**	.	**0.000**	0.144	**0.000**	**0.000**	**0.000**	0.101	**0.000**	0.142	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**
MBzP	ρ	0.200	**0.736****	**00.717****	1	0.141	**0.764****	**0.735****	**0.564****	0.017	**0.642****	**00.422***	**0.682****	**0.757****	**0.549****	**0.637****	**0.643****
	p	0.299	**0.000**	**0.000**	.	0.466	**0.000**	**0.000**	**0.001**	0.717	**0.000**	**0.023**	**0.000**	**0.000**	**0.002**	**0.000**	**0.000**
MEHP	ρ	0.172	0.350	0.278	0.141	1	0.330	0.349	**0.454***	**0.645****	**0.465***	0.282	0.212	0.285	**0.390***	**00.552****	**0.332**
	p	0.373	0.063	0.144	0.466	.	0.080	0.064	**0.013**	**0.000**	**0.011**	0.139	0.270	0.134	**0.036**	**0.002**	**0.078**
MEHHP	ρ	0.319	**0.857****	**0.829****	**00.764****	0.330	1	**0.987****	**0.898****	**0.400***	**0.715****	**0.391***	**0.803****	**0.819****	**0.733****	**0.916****	**0.779****
	p	0.091	**0.000**	**0.000**	**0.000**	0.080	.	**0.000**	**0.000**	**0.014**	**0.000**	**0.036**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**
MEOHP	ρ	0.337	**0.865****	**0.853****	**0.735****	0.349	**0.987****	1	**0.908****	**-0,379**	**0.737****	0.355	**0.787****	**0.784****	**0.705****	**0.921****	**0.746****
	p	0.074	**0.000**	**0.000**	**0.000**	0.064	**0.000**	.	**0.000**	**0.015**	**0.000**	0.059	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**
MECPP	ρ	0.364	**0.728****	**0.752****	**0.564****	**0.454***	**0.898****	**0.908****	1	**0332***	**0.767****	0.279	**0.678****	**0.678****	**0.733****	**0.959****	**0.697****
	p	0.053	**0.000**	**0.000**	**0.001**	**0.013**	**0.000**	**0.000**	.	**0.042**	**0.000**	0.142	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**
MCPP	ρ	**0.453***	**0.757****	**0.850****	**0.642****	**0.465***	**0.715****	**0.737****	**0.767****	0.138	1	0.163	**0.680****	**0.638****	**0.686****	**0.782****	**0.672****
	p	**0.014**	**0.000**	**0.000**	**0.000**	**0.011**	**0.000**	**0.000**	**0.000**		.	0.399	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**
MiNP	ρ	0.055	**0.413***	0.280	**0.422***	0.282	**0.391***	0.355	0.279	**0.601****	0.163	1	**0.455***	**0.602****	**0.445***	0.339	**0.532****
	p	0.778	**0.026**	0.142	**0.023**	0.139	**0.036**	0.059	0.142	**0.000**	0.399	.	**0.013**	**0.001**	**0.016**	0.072	**0.003**
MHiNP	ρ	**0.461***	**0.853****	**0.773****	**0.682****	0.212	**0.803****	**0.787****	**0.678****	0.198	**0.680****	**0.455***	1	**0.884****	**0.860****	**0.682****	**0.952****
	p	**0.012**	**0.000**	**0.000**	**0.000**	0.270	**0.000**	**0.000**	**0.000**	0.059	**0.000**	**0.013**	.	**0.000**	**0.000**	**0.000**	**0.000**
MOiNP	ρ	0.335	**0.814****	**0.740****	**0.757****	0.285	**0.819****	**0.784****	**0.678****	**0.309***	**0.638****	**0.602****	**0.884****	1	**0.866****	**0.719****	**0.932****
	p	0.076	**0.000**	**0.000**	**0.000**	0.134	**0.000**	**0.000**	**0.000**	**0.041**	**0.000**	**0.001**	**0.000**	.	**0.000**	**0.000**	**0.000**
MCiOP	ρ	0.363	**0.710****	**0.654****	**0.549****	**0.390***	**0.733****	**0.705****	**0.733****	**0.295**	**0.686****	**0.445***	**0.860****	**0.866****	1	**0.767****	**0.957****
	p	0.053	**0.000**	**0.000**	**0.002**	**0.036**	**0.000**	**0.000**	**0.000**	0.047	**0.000**	**0.016**	**0.000**	**0.000**	.	**0.000**	**0.000**
sDEHPm	ρ	0.301	**0.763****	**0.757****	**0.637****	**0.552****	**0.916****	**0.921****	**0.959****	0.175	**0.782****	0.339	**0.682****	**0.719****	**0.767****	1	**0.731****
	p	0.112	**0.000**	**0.000**	**0.000**	**0.002**	**0.000**	**0.000**	**0.000**	0.081	**0.000**	0.072	**0.000**	**0.000**	**0.000**	.	**0.000**
sDiNPm	ρ	**0.439***	**0.796****	**0.711****	**0.643****	0.332	**0.779****	**0.746****	**0.697****	**0.323^A**	**0.672****	**0.532****	**0.952****	**0.932****	**0.957****	**0.731****	1
	p	**0.017**	**0.000**	**0.000**	**0.000**	0.078	**0.000**	**0.000**	**0.000**	**0.048**	**0.000**	**0.003**	**0.000**	**0.000**	**0.000**	**0.000**	.

*p < 0.05; **p < 0.01.
ΣDiNPmetabolites: sum of MiNP, MHiNP, MOiNP and MCIOP expressed as DiNP.
DEHP metabolites: sum of MEHP,MEHHP, MEOHP and MECPP expressed as DEHP.
DEP: diethyl phthalate; MEP: monoethyl phthalate; DiBP: diisobutyl phthalate; MiBP: mono-isobutyl phthalate; DnBP: di-*n*-butyl phthalate; MnBP: mono-butyl phthalate; BBzP: benzylbutylphthalate; MBzP: monobenzyl phthalate; DOP: di-*n*-octyl phthalate; MCPP: mono-(3-carboxypropyl) phthalate; DiNP: diisononyl phthalate; MiNP: monoisononyl phthalate; MHiNP: mono(hydroxyisononyl) phthalate; MOiNP: mono(oxoisononyl) phthalate; MCiOP: mono(carboxyisooctyl) phthalate; MEHP: mono-(2-ethylhexyl)phthalate; MEOHP: mono(2-ethyl-5-oxohexyl) phthalate; MECCP: mono(2-ethyl-5-carboxypentyl) phthalate; MEHHP: mono (2-ethyl-5-hydroxyhexyl) phthalate (MEHHP).

and MECPP in both control and PT groups. Moreover, the oxidative metabolites of MEHP (i.e. MEHHP, MEOHP and MECPP) are also highly and significantly correlated with each other in both of the study groups. Significant correlations between other phthalate monoester metabolites were also observed in both of the study groups.

3.6. Correlations between sex hormones, ovary and uterus volumes, weight, BMI, thyroid parameters, pubic hair and urinary phthalate metabolites for PT group

Correlations between sex hormones, ovary and uterus volumes, weight, BMI and pubic hair and urinary phthalate metabolites for PT group were given Table 6.

We observed that basal FSH levels are positively and significantly correlated with MiBP ($\rho = 0.323$, $p = 0.045$), MEHP ($\rho = 0.315$, $p = 0.049$) and MCiOP ($\rho = 0.334$, $p = 0.048$).

Basal LH levels were only correlated with urinary MEHP levels ($\rho = 0.475$, $p = 0.041$). The weight of the girls with PT were positively correlated with MiBP, MEOHP, MiBP, MnBP, MEHHP, MECPP, MCPP, MiNP, MHiNP, MOiNP, MCiOP as well as ΣDEHP metabolites. BMIs of the PT group were also positively correlated with MiBP, MEOHP, MnBP, MEHHP, MECPP, MCPP, MiNP, MHiNP, MOiNP, MCiOP, ΣDEHP metabolites, ΣDiNP metabolites as well as with MBzP.

Uterus volume of girls with PT was negatively correlated with urinary MEHP levels. Serum TSH levels in PT group were negatively correlated with ΣDiNP metabolites ($\rho = -0.327$, $p = 0.048$). Free T_4 (fT4) levels were negatively correlated with urinary MiBP ($\rho = 0.385$, $p = 0.002$) and MCiOP ($\rho = 0.335$, $p = 0.041$) levels and ΣDEHP metabolites ($\rho = 0.356$, $p = 0.039$).

Pubic hair growth was significantly correlated with the urinary levels of MEHHP, ($\rho = -0.373$, $p = 0.041$), MEOHP ($\rho = -0.378$, $p = 0.002$), MECPP ($\rho = -0.407$, $p = 0.037$), MiNP ($\rho = 0.480$, $p = 0.000$) and MCiOP ($\rho = 0.440$, $p = 0.002$).

4. Discussion

Premature thelarche is defined as “early breast development in girls younger than 8 years” and it usually occurs without additional signs of sexual maturation. Breast development may be observed in one or two breasts with tenderness (Leung, 1989). PT may result from an “over-activation” of the hypothalamic-pituitary-gonadal axis in early childhood. In addition, the increased sensitivity of breast tissue to estrogens may be another reason in the development of PT. Moreover, exposure to EDCs (exogenous estrogenic compounds as well as to anti-androgenic substances) are also suggested to be underlying factors for PT. These compounds can be present in food, drugs, flame retardants, pesticides, detergents, toys, sun lotions and other cosmetics (Leung, 1989; Paris et al., 2013; Chiabotto et al., 2006).

There are not clear numbers for the prevalence of PT and PP in Europe and also in Turkey. A study conducted in Italy found the prevalence for both PT and PP were 6% (each) among 7-year-olds, and 11% and 9%, respectively, in the 8-year-olds (Danubio et al., 2004). In another study performed in Denmark, the researchers found that the occurrence of PT among girls younger than 8 years was 3% (Aksglaede et al., 2009). Herman-Giddens et al. (1997) reported that at the age of 7 years, 5% of white and 15.4% of black girls exhibited breast development, and pubic hair development was present in 2.8% of white and 17.7% of black girls in USA (Herman-Giddens et al., 1997). In Istanbul,

Table 6
Correlations between sex hormones, ovary and uterus volumes, weight, BMI, thyroid parameters, pubic hair and urinary phthalate metabolites for PT group.

		MEP	MiBP	MnBP	MBzP	MEHP	MEHHP	MEOHP	MECPP	%MEHP	MCPP	MiNP	MHiNP	MOiNP	MCiOP	∑DEHP metabolites	∑DiNP metabolites
Basal FSH	ρ	-0.187	**0.323***	0.038	-0.073	**0.315***	0.062	0.014	0.046	0.208	0.217	-0.167	0.228	0.113	**0.334***	0.223	0.114
	p	0.199	**0.045**	0.914	0.848	**0.049**	0.947	0.917	0.847	0.178	0.174	0.114	0.161	0.089	**0.048**	0.182	0.964
Basal LH	ρ	-0.104	-0.183	-0.212	-0.108	**0.475***	0.011	-0.020	-0.010	-0.104	-0.066	-0.068	0.106	0.097	0.013	-0.202	0.102
	p	0.075	0.111	0.158	0.891	**0.041**	0.851	0.917	0.981	0.788	0.847	0.921	0.085	0.091	0.899	0.187	0.951
Basal Estradiol	ρ	0.109	-0.076	-0.112	-0.155	-0.191	-0.075	-0.047	-0.028	0.255	-0.132	-0.009	0.025	0.148	0.185	-0.093	-0.093
	p	0.087	0.064	0.069	0.097	0.157	0.914	0.971	0.987	0.078	0.078	0.994	0.889	0.101	0.157	0.078	0.077
Right ovary	ρ	-0.046	-0.095	-0.127	-0.020	0.299	-0.195	-0.222	-0.210	**0.409^**	-0.185	0.038	-0.203	-0.118	-0.128	-0.111	-0.132
	p	0.885	0.894	0.089	0.901	0.058	0.154	0.145	0.151	**0.037**	0.164	0.897	0.142	0.089	0.091	0.089	0.099
Left ovary	ρ	0.054	-0.120	-0.166	-0.074	0.313	-0.218	-0.246	-0.247	0.173	-0.228	0.074	-0.242	-0.149	-0.154	-0.142	-0.156
	p	0.917	0.845	0.151	0.883	0.053	0.065	0.062	0.063	0.142	0.070	0.896	0.067	0.086	0.101	0.075	0.079
Weight	ρ	0.340	**0.742****	**0.550****	0.191	0.058	**0.450***	**0.468***	**0.389***	-0.127	**0.574****	**0.426***	**0.671****	**0.709****	**0.754****	**0.707****	**0.426***
	p	0.052	**0.000**	**0.000**	0.061	0.901	**0.014**	**0.013**	**0.031**	0.299	**0.001**	**0.025**	**0.000**	**0.000**	**0.000**	**0.000**	0.328
BMI	ρ	0.092	**0.574****	**0.611****	**0.375^**	0.215	**0.532****	**0.551****	**0.466***	0.046	**0.639*^**	**0.416***	**0.565****	**0.703****	**0.606****	**0.615****	**0.551****
	p	0.975	**0.002**	**0.000**	**0.041**	0.118	**0.000**	**0.000**	**0.001**	0.853	**0.000**	**0.022**	**0.000**	**0.000**	**0.000**	**0.000**	**0.003**
Uterus	ρ	0.097	-0.181	-0.153	0.151	**-0.339**	-0.096	-0.056	-0.150	**-0.307***	-0.121	-0.168	0.091	-0.112	-0.219	-0.180	-0.196
	p	0.847	0.128	0.111	0.118	**0.049**	0.878	0.841	0.081	**0.041**	0.074	0.084	0.070	0.075	0.064	0.071	0.074
TSH	ρ	0.143	-0.310	-0.177	-0.021	-0.182	-0.304	-0.294	-0.273	0.053	-0.067	-0.022	0.028	-0.218	-0.322	-0.273	**-0.327***
	p	0,095	0.055	0.121	0.941	0.154	0.056	0.061	0.059	0.741	0.845	0.911	0.902	0.067	0.055	0.059	**0.048**
fT4	ρ	-0.096	**-0.385****	-0.260	0.051	-0.052	-0.012	0.048	-0.035	0.014	-0.188	0.119	-0.282	-0.028	**-0.335***	**-0.356***	-0.021
	p	0.876	**0.002**	0.178	0.911	0.905	0.994	0.899	0.956	0.911	0.101	0.084	0.065	0.917	**0.041**	**0.039**	0.931
Pubic hair	ρ	0.192	0.096	-0.090	-0.062	0.047	**-0.373***	**-0.378****	**-0.407***	0.220	-0.293	**0.480****	0.126	0.184	**0.440***	0.026	-0.351
	p	0.078	0.847	0.799	0.905	0.923	**0.041**	**0.002**	**0.037**	0.117	0.056	**0.000**	0.084	0.101	**0.002**	0.965	0.052

*p < 0.05; **p < 0.01.
ΣDiNPmetabolites: sum of MiNP, MHiNP, MOiNP and MCIOP expressed as DiNP.
DEHP metabolites: sum of MEHP,MEHHP, MEOHP and MECPP expressed as DEHP.
DEP: diethyl phthalate; MEP: monoethyl phthalate; DiBP: diisobutyl phthalate; MiBP: mono-isobutyl phthalate; DnBP: di-*n*-butyl phthalate; MnBP: mono-butyl phthalate; BBzP: benzylbutylphthalate; MBzP: monobenzyl phthalate; DOP: di-*n*-octyl phthalate; MCPP: mono-(3-carboxypropyl) phthalate; DiNP: diisononyl phthalate; MiNP: monoisononyl phthalate; MHiNP: mono(hydroxyisononyl) phthalate; MOiNP: mono(oxoisononyl) phthalate; MCiOP: mono(carboxyisooctyl) phthalate; MEHP: mono-(2-ethylhexyl)phthalate; MEOHP: mono(2-ethyl-5-oxohexyl) phthalate; MECCP: mono(2-ethyl-5-carboxypentyl) phthalate; MEHHP: mono (2-ethyl-5-hydroxyhexyl) phthalate (MEHHP).

the biggest province of Turkey, the prevalence of PT and PP were found to be 8.9% (73 of 820 girls) and 4.3% (35 of 810 girls), respectively and PT prevalence was in accordance with the results obtained from the study performed in Italy. However, the prevalence of PP in Turkey was found to be lower when compared to Italy (Atay et al., 2012).

EDC exposure starts during prenatal period and continues throughout life (Colborn et al., 1993; Cwiek-Ludwicka and Ludwicki, 2014; Harvey and Darbre, 2004; Maipas and Nicolopoulou-Stamati, 2015; Parent et al., 2016; Russ and Howard, 2016). Safety assessment for EDCs is complicated as some EDCs have significant low dose effects and these chemicals usually show non-monotonic dose-response curves. In addition, humans are exposed to these substances as mixtures in everyday life (Den and Schoeters, 2006; Erkekoglu and Giray, 2012; Vandenberg et al., 2012). Phthalates cause a broad range of birth defects and lifelong reproductive and organ impairments in animals after *in utero* and early postnatal exposures (Ema et al., 1998; Erkekoglu et al., 2011, 2012a; 2012b; Erkekoglu et al., 2014; Marsman, 1995; Mylchreest et al., 1998, 1999, 2000; Wine et al., 1997). Moreover, exposure to these compounds have been associated with a wide variety of endocrine diseases and several pathological conditions in humans (Durmaz et al., 2010, 2014; Franken et al., 2017; Heudorf et al., 2007; Kondolot et al., 2016; Parent et al., 2016).

In the present study, urinary phthalate monoester levels were determined in girls with PT. Our results indicate that the metabolism of DEHP is altered in PT girls, leading to significant increases in MEHP and MEHP(%). The urinary MEHP concentration of PT girls was ~2 times higher than the control group (p = 0.002). In control group, the percentage of MEHP among other oxidative phthalate metabolites was 663%, while in PT girls it was 1266% (p = 0.001). In a study by Joensen et al. (2012), the researchers observed that %MEHP and % MiNP were negatively associated with the ratio of testosterone/LH and testosterone/FSH. %MEHP was negatively associated with total testosterone, free testosterone, and ratio of testosterone/estradiol in men (n = 881). We did not observe significant correlations between sex hormones and %MEHP in PT girls, most probably due to the small number of subjects in the PT group.

Interestingly, the primary metabolite of DEP, namely MEP, is markedly higher in control group compared to PT group. Other than MEHP and MEP, other phthalate metabolites were not markedly different between PT and control groups. There are studies in literature concerning the relationship between phthalate exposure and breast development and early puberty in girls and gynecomastia in boys. However, these studies show conflicting results (Colón et al., 2000; Durmaz et al., 2010; Mieritz et al., 2012). In our recent studies, we have observed that gynecomastia patients had higher plasma DEHP and MEHP levels when compared to controls (p < 0.05) (Durmaz et al., 2010). Mieritz et al. (2012) studied a total of 555 healthy boys (age 6.07–19.83 years) as part of the COPENHAGEN Puberty Study. They did not observe any association between phthalate exposure and pubertal timing, testosterone levels or pubertal gynecomastia (Mieritz et al., 2012). However, in Puerto Rican girls, serum DEHP levels ranged from 187 to 2098 μg/L and high serum phthalate levels and early breast development were found to be correlated (Colón et al., 2000). We have also found that plasma DEHP levels were higher in central and peripheral PP patients vs. control while plasma MEHP levels were only higher in girls with central PP when compared to control group, indicating a lower biotransformation rate of DEHP to MEHP in peripheral PP patients (Buluş et al., 2016).

The significantly high correlations between phthalate monoester levels indicate that humans are exposed to these compounds as mixtures; however, there are no comprehensive studies in literature that evaluate the mixture toxicity of phthalates in humans. Combined effects of the phthalate monoester mixtures may also have different effects on endocrine system and may cause pathological alterations most of which are hard to predict. One of these effects may be early breast development in both genders.

We have observed most of the phthalate metabolites are positively and significantly correlated with weight and BMI of the girls in PT group. This finding also suggests that exposure to phthalates might lead to changes in body weight and adiposity. Teitelbaum et al. (2012)

observed that higher urinary MEP and low molecular weight phthalate levels were significantly associated with BMI and waist circumference among overweight children (n = 387, age 6–8). Stahlhut et al. (2007) found that higher urinary MBzP, MEHHP and MEOHP levels were significantly associated with increased waist circumference in males (n = 1451, age > 18). Smerieri et al. (2015) determined that mono-(2-ethyl-5-carboxypentyl) 1,2-benzenedicarboxylate (5-Cx-MEHP) levels were positively correlated with increased adipose tissue development in pre-pubertal obese children (n = 72, mean age 12).

In Turkey, DEHP was banned from baby bottles, nipples and other polycarbonate baby products in July 2012 by a notification which was included in Turkish Food Codex- Baby formulas and baby follow-up formulas- (TFC, 2012). However, there are still cheap baby bottles, toys or baby care products in the market containing different phthalates. On the other hand, throughout the globe, including Turkey, there is a trend towards consuming readily prepared or packaged food than homemade food, specifically among children and adolescents. This situation might be one of the underlying factors of increment of PT among girls and gynecomastia in boys.

In conclusion, the role of childhood phthalate exposure in early breast development requires further study. Although our study has some limitations (i.e. small number of subjects within the study groups), this might lead other researchers to design similar studies investigating a relationship between phthalates and PT with high subject numbers.

Con ict of interest

The authors declare no conflicts of interest.

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