Phthalate Metabolites in Maternal and Cord Plasma and Their Relations to Other Selected Endocrine Disruptors and Steroids

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Summary

Endocrine disruptors (EDs) are known to have harmful effects on the human endocrine system; special effort is actually given to the exposure during pregnancy. Humans are usually exposed to a mixture of EDs, which may potentiate or antagonize each other, and the combined effect may be difficult to estimate. The main phthalate monoesters monoethyl-, mono-*n*-butyl-, monoisobutyl-, monobenzyl-, mono-(2-ethylhexyl)-, mono-(2-ethyl-5-hydroxyhexyl)- and mono-(2-ethyl-5-oxohexyl) phthalate were determined in 18 maternal (37th week of pregnancy) and cord plasma samples using liquid chromatography-tandem mass spectrometry. Previously determined levels of selected bisphenols, parabens and steroids were also considered in this study. In cord blood, there were significantly higher mono-*n*-butyl phthalate levels than in maternal blood (p=0.043). The results of multiple regression models showed that maternal plasma phthalates were negatively associated with cord plasma androstenedione, testosterone and dehydroepiandrosterone and positively associated with estradiol and estriol. For estriol, a cumulative association was also observed for Σbisphenols. To the best of our knowledge, this is the first pilot study evaluating the effect of prenatal exposure by multiple EDs on newborn steroidogenesis. Our results confirmed phthalate accumulation in the fetal area and disruption of fetal steroidogenesis. This preliminary study highlights the negative impacts of in utero EDs exposure on fetal steroidogenesis.

Key words

Endocrine disruptor • Pregnancy • Phthalate • Bisphenol • Steroid

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Introduction

The constant process of industrial development is closely related to environmental pollution and the presence of numerous chemicals in the water, soil and air. It is known that many chemicals may interfere with the human and animal endocrine systems and affect hormonal homeostasis; these substances have been termed endocrine disruptors (EDs) (Bergman *et al.* 2013, Diamanti-Kandarakis *et al.* 2009). Of the anthropogenic EDs, phthalates, bisphenols and parabens are among the most widespread. Various epidemiologic studies have associated ED exposure with adverse health outcomes in humans, predominantly disturbing the reproductive system in both men and women (Gore *et al.* 2015, Sifakis *et al.* 2017). Due to their structural similarity with estrogens, they interact with estrogen or androgen receptors and possess estrogenic or anti-androgenic effects (Kabir *et al.* 2015).

Phthalates are structurally diesters of phthalic acid (1,2-benzenedicarboxylic acid), a group of widely distributed industrial chemicals used in the manufacture of plastics, especially those made of polyvinyl chloride

(PVC). They give plastics elasticity, with their content being directly proportional to the plasticity of products (Benjamin *et al.* 2017, Giulivo *et al.* 2016, Johns *et al.* 2015). Phthalates are only bound physically within the plastic polymer structure, and therefore changes in the environment (temperature, pH, sunlight, pressure etc.) or contact with lipids or solvents can accelerate phthalate leaching or vaporizing from the plastic materials (Benjamin *et al.* 2017). In addition, low molecular weight phthalates (ester side-chain lengths, one to four carbons) such as diethylphthalate (DEP), di-n-butylphthalate (DnBP) and di-iso-butylphthalate (DiBP) are commonly used in cosmetics and personal care products, especially shampoo, soaps, nail polishes, perfumes and insect repellents, in pharmaceuticals as a component of tablet coatings (Bowman and Choudhury 2016, Hernandez-Diaz *et al.* 2009, Koo and Lee 2004), as well as in adhesives and industrial solvents (Phthalates 2008). High molecular weight phthalates (ester side-chain lengths, five or more carbons) such as di-(2-ethylhexyl) phthalate (DEHP) and benzyl-butyl-phthalate (BzBP) are usually used in PVC plastics employed in various consumer products, out of which they are leaching to food, water, air and soil (Benjamin *et al.* 2017, Bowman and Choudhury 2016, Giulivo *et al.* 2016, Johns *et al.* 2015, Phthalates 2008). Phthalates have been categorized as EDs and have been linked to various adverse health effects, particularly in relation to early life exposure (Bowman and Choudhury 2016, Katsikantami *et al.* 2016, Serrano *et al.* 2014). These substances are known to cross the human placenta (Mose *et al.* 2007), and multiple phthalates have been measured in human amniotic fluid and meconium (Arbuckle *et al.* 2016, Wittassek *et al.* 2009).

Humans are exposed to phthalates through ingestion, inhalation and dermal contact throughout their life including intrauterine development (Heudorf *et al.* 2007). Food is generally regarded as the major source of phthalate exposure. The variability of food contamination depends on the manufacturing process, food packaging, transportation, storage and many other factors (Giulivo *et al.* 2016). Following oral exposure, phthalate diesters are hydrolyzed by esterases to monoesters in the saliva or intestine. Monoesters may be absorbed in the body and cause harmful effects; they are the main detected metabolites of low molecular weight phthalates. The high molecular weight phthalate monoesters may be also further oxidized. Both phthalates are then conjugated as glucuronides and to a small extent sulfates, and excreted *via* the urine (Phthalates 2008). Systemic absorption of phthalates has also been observed after the dermal application of personal care products (Janjua *et al.* 2007, Witorsch and Thomas 2010) including various anti-stretch mark creams and ultrasound gel products (Messerlian *et al.* 2017). Some phthalate monoesters are products from more than one parent compound. The detailed metabolic degradation has been published by other authors (DeFlorio-Barker and Turyk 2016, Vrbík 2016). Human studies investigating the prenatal phthalate exposure to the reproductive effects have mostly been based on measurements of phthalate content in maternal urine. They have confirmed associations with various disorders such as decreased anogenital distance, testicular descent impairment (Swan *et al.* 2005), premature breast development in young girls (Colon *et al.* 2000), and disruption of folliculogenesis by altering ovarian and oocyte development (for review see Mallozzi *et al.* 2016).

Due to their leaching from plastics while chewing or sucking, the use of phthalates was restricted by the European Union (EU), and their use in foodstuffs was banned by Commission regulation (EU) 72/2002. Another important Commission regulation (EU) 84/2005 regulates the marketing and use of the phthalates DEHP, DBP and BBP in toys and childcare articles. DBP, BBP, DEHP and DiBP were classified as very dangerous substances in REACH – the Regulation on Registration, Evaluation, Authorisation and Restriction of Chemicals as part of the framework legislation on chemicals in the EU (Regulation (EC) No 1907/2006) (Ventrice *et al.* 2013).

Phthalates are not the only group of EDs known to leach from plastics. The estrogen active bisphenol A (BPA) is widely known and best studied of the bisphenols. It is used while processing the polycarbonate plastic materials and epoxy resins, and contained in consumer products like food and drink packaging, dental fillings, thermal receipts and others. Many recent studies have investigated BPA exposure during pregnancy, with BPA quantified in numerous maternal body fluids such as urine, milk and amniotic fluid, as well as in neonates and young children (Braun *et al.* 2011, Deceuninck *et al.* 2015, Ferguson *et al.* 2015, Shekhar *et al.* 2017). Though the European Commission is still tightening the regulations for BPA usage, it is often being fully or partly replaced by alternative bisphenols like bisphenol S (BPS), bisphenol F (BPF), bisphenol AF (BPAF) and others (Kolatorova *et al.* 2017, Kolatorova Sosvorova *et al.* 2017, Sartain and Hunt 2016), for which so far no

regulation limiting their usage exists. The total bisphenol exposure may be therefore higher than when BPA is used alone (Sartain and Hunt 2016). Another group of ubiquitous EDs are parabens, antimicrobial preservatives used in the food, cosmetics and pharmaceutical industries. The most commonly-employed are methylparaben (MP) and propylparaben (PP); however ethyl- (EP), butyl- (BP) and benzylparaben (BenzylP) are also used, especially in combination (Karpuzoglu *et al.* 2013, Nowak *et al.* 2018). The increasing number of studies documenting the harmful activities of parabens led to various regulations issued by European Commission (Kolatorova *et al.* 2018, Nowak *et al.* 2018). Currently, the European Chemical Agency (ECHA 2017) is performing reproduction tests with the most widely used paraben – PP – and the results should be known in June 2019.

During pregnancy, the fetus is exposed to many factors from the environment due to the placental transfer of lipophilic substances (Barry and Anthony 2008). Throughout the life, women are exposed to various lipophilic EDs, which may accumulate in their fat stores. Among others, pregnancy leads to the redistribution of mother fat reserves, out of which EDs may be released to the circulation. They can subsequently pass through the placenta to the fetal compartment in quite high concentrations (Modena and Fieni 2004). Moreover, the immature fetal organism has only limited liver first-pass effect that makes EDs difficult to metabolize (Matsumoto *et al.* 2002). The allowed limits of EDs therefore do not fulfill their role as such during the intrauterine development and in addition the effects of various EDs may interfere and/or multiply their effects (Mantovani 2016, Matsumoto *et al.* 2002, Shekhar *et al.* 2017).

The present work is continuation of previously published study, where we examined the exposure of bisphenols and parabens during pregnancy and their relations to steroid changes (Kolatorova *et al.* 2018). In this study we focused mainly on the analytical measurements of phthalate metabolite levels in cord and maternal plasma, their transplacental transport and the influence of phthalates and other previously determined EDs on the levels of steroids within and between cord and maternal plasma.

Material and Methods

Chemicals and reagents

Chemicals and reagents used for the analysis of steroid hormones, bisphenols and parabens were described in detail in our previous work (Kolatorova *et al.* 2018).

Standards of monoethyl phthalate (MEP), monoisobutyl phthalate (MiBP), monobenzyl phthalate (MBzP), mono-(2-ethylhexyl) phthalate (MEHP) were from AccuStandard (New Haven, USA). Standards of mono-*n*-butyl phthalate (MnBP), mono-(2-ethyl-5-hydroxyhexyl) phthalate (5-OH-MEHP), mono-(2-ethyl-5-oxohexyl) phthalate (5-oxo-MEHP) were purchased from Sigma-Aldrich (St. Louis, MO, USA), as were acetonitrile, ammonium acetate, formic acid and acetic acid. The deuterated phthalates (D4-MEP, D4-MnBP, D4-MiBP, D4-MBzP, D4-MEHP, D4-5-OH-MEHP, D4-5-oxo-MEHP) were synthesized by Clearsynth (Budapest, Hungary). LC-MS grade methanol, water for chromatography and β-glucuronidase-aryl sulphatase were from Merck AG (Darmstadt, Germany).

Study group

The study involved 18 Czech healthy pregnant women with a physiological course of gravidity and single pregnancy, 15 gave birth spontaneously, 3 by caesarean section (November 2016 and January 2017), and all women were central-European origin. The mean age of pregnant women (n=18) was 34±3.7 years and mean pregnancy weight gain was 15.2±4.4 kg. Their mean BMI values before pregnancy and in the 37th week of pregnancy were 21.9±2.9 kg/m^2 and 26.8±2.6 kg/m^2, respectively. None of the women used medications affecting steroidogenesis, have gestational diabetes, thyreopathy, risk of premature birth, intrauterine growth restriction or other pregnancy complications. Our study followed the Declaration of Helsinki (2000) of the World Medical Association. The protocol of the study was approved by the Ethical Commission of the General University Hospital in Prague. Informed written consent with the use of biological materials for research was obtained from all women involved in the study.

Women were examined during the 37th week of pregnancy. They filled out an anonymous standardized questionnaire which included the data mentioned in Table 1. Thereafter, the cubital venous blood was collected (8-10 a.m.), the mixed cord blood was withdrawn at birth. Blood collected to K2EDTA tubes was immediately centrifuged (5 min, 2,000 g, 4 °C). To avoid contamination from collection devices, plasma was without delay transferred to the glass tubes and stored at -20 °C until analysis. The sample preparation during

Table 1. Characteristics of women (n=18) during the 37th week of pregnancy. The newborn sex and birth weight was noted after delivery.

Characteristics	n (%)
Maternal age (years)	
<30	2 (11.1)
30-35	10 (55.6)
>35	6 (33.3)
Mean (± SD)	34 (3.7)
Pre-pregnancy BMI (kg/m^2)	
<20	5 (27.8)
20-25	11 (61.1)
>25	2 (11.1)
Mean (± SD)	21.9 (2.9)
Parity	
1	5 (27.8)
2	8 (44.4)
3	4 (22.2)
4	1 (5.6)
Education	
High school	5 (27.8)
University	13 (72.2)
Monthly income (Czech crowns)	
10,000-25,000	9 (50.0)
>25,000	9 (50.0)
Week of delivery	
<39	0 (0.0)
39-40	17 (94.4)
>41	1 (5.6)
Mean (± SD)	39.8 (0.5)
Pregnancy weight gain (kg)	
<12	3 (16.7)
12-20	13 (72.2)
>20	2 (11.1)
Mean (± SD)	15.2 (4.4)
Birth weight (kg)	
<3	2 (11.1)
3-4	13 (72.2)
>4	3 (16.7)
Mean (± SD)	3.6 (0.4)
Marital status	
Married	11 (61.1)
Unmarried	7 (38.9)
Fetal sex	
Male	9 (50.0)
Female	9 (50.0)

the pre-analytical and analytical phases were performed with respect to the use of phthalate-, bisphenol- and paraben-free laboratory equipment. The way we have eliminated the contamination was described previously (Kolatorova Sosvorova *et al.* 2017). Possible false positive results of monoalkyl phthalates caused by contamination of the mobile phase were prevented by installing a chromatographic (trap) column between the HPLC mobile phase pump and the injector (see section Determination of analytes). A procedural blank sample was controlled with each batch of samples.

Determination of analytes

The plasma levels of unconjugated bisphenols (BPA, BPS, BPF, BPAF), parabens (MP, EP, PP, BP, BenzylP) and steroids (E1, E2, E3, cortisol, cortisone, DHEA, 7α-OH-DHEA, 7β-OH-DHEA, 7-oxo-DHEA, testosterone, androstenedione, pregnenolone, 17-hydroxy-pregnenolone and progesterone) were determined using two previously published validated liquid chromatography – tandem mass spectrometry (LC-MS/MS) methods. All the chromatography and mass spectrometry details can be found in the corresponding publications or their supplementaries (Kolatorova Sosvorova *et al.* 2017, Sosvorova *et al.* 2015, Vitku *et al.* 2016).

Phthalate metabolites (MEP, MnBP, MiBP, MBzP, MEHP, 5-OH-MEHP and 5-oxo-MEHP) were analyzed using enzymatic cleavage of glucuronide followed by ultra-high-performance liquid chromatography – electrospray ionization tandem mass spectrometry in a laboratory with external quality control: 500 µl of plasma was spiked with 10 µl of a solution containing isotopically labeled analyte analogs (5 µg/ml) and diluted with 100 µl of ammonium acetate solution (1 mol/l). 5 µl of β-glucuronidase-aryl sulphatase solution was added and samples were incublated for 90 min at 37 °C. After adding 500 µl of acetonitrile-acetic acid solution (50:1, v/v) followed by 300 µl of aqueous magnesium sulfate solution (25 %, w/w), the enzymatic reaction was stopped, the enzyme precipitated out and two liquid phases formed. The resulting mixture was centrifuged to separate the phases. 3 µl of the upper acetonitrile layer were injected into an ultra-high performance liquid chromatograph (UPLC) Infinity 1290 (Agilent, Santa Clara, CA, USA) connected to the Agilent 6490A triple stage quadrupole mass spectrometer with electrospray ionization (Agilent, Santa Clara, CA, USA). Chromatographic separation was performed using a Kinetex Phenyl-hexyl 100A 2.6 µm

(150 x 2.1 mm) column (Phenomenex, Torrance, CA, USA) maintained at 40 °C. The Kinetex C18 100A 5 µm (50 x 2.1 mm) chromatographic column (Phenomenex, Torrance, CA, USA) was installed between the UPLC mobile phase pump and injector to avoid contamination from the mobile phase. The mobile phase gradient of demineralized water (A) and methanol (B) both containing 0.1 % of formic acid was as follows: 0 min, 20 % B, 0.45 ml/min; 1 min, 45 % B, 0.4 ml/min; 7 min, 55 % B, 0.4 ml/min; 9 min, 90 % B, 0.4 ml/min; 11 min, 90 % B, 0.5 ml/min. Using this gradient, MiBP and MnBP were baseline separated. Validation parameters are given in Table 2. Other chromatography and mass spectrometry details were presented in Vrbík *et al.* (2016). The laboratory is accredited according to EN ISO/IEC 17025:2005 and regularly participates on external G-EQUAS external quality assessment scheme for phthalate metabolites.

Table 2. Retention times and validation parameters of phthalate metabolites.

Phthalate	MEP	MiBP	MnBP	5-oxo-MEHP	5-OH-MEHP	MBzP	MEHP
Retention time (min)	3.7	6.7	7.0	7.6	7.7	8.2	10.1
LOQ (ng/ml)*	3.5	2.2	3	2.7	2.8	2.4	0.61
*Recoveries at 10 ng/ml (%)***	92	101	116	108	90	98	104
RSD at 10 ng/ml (%)	16	9.5	16	11	14	11	6.5

LOQ – limit of quantification, RSD – relative standard deviation, MEP – monoethyl phthalate, MiBP – monoisobutyl phthalate, MnBP – mono-*n*-butyl phthalate, 5-oxo-MEHP – mono-(2-ethyl-5-oxohexyl) phthalate, 5-OH-MEHP – mono-(2-ethyl-5-hydroxyhexyl) phthalate, MBzP – monobenzyl phthalate, MEHP – mono-(2-ethylhexyl) phthalate. * LOQs were determined as LOQ=10 s_a/b, where s_a is the standard deviation of the intercept and b is the average slope in the repeated calibration curve analyses (1 to 1000 ng/ml, n=6). ** Recoveries were not significantly different from 100 % (p>0.34).

Statistical analysis

According to Hornung and Reed (1990), data under the limit of detection were replaced by LOQ/√2. Afterwards, the Wilcoxon-Mann-Whitney test was used to evaluate differences in ED and steroid concentrations in cord plasma between male and female fetuses. Similarly, this test was used for comparison of the same set of analytes between group of fetuses born vaginally and group of fetuses born by caesarean section. ED and steroid concentrations in maternal plasma and cord plasma were compared by a sign test. Data were subsequently transformed by Box-Cox transformation and multiple linear regression analysis (backward stepwise selection) was performed to explore relations between each steroid as a dependent variable and EDs (phthalates, bisphenols, parabens) as predictors. Each model was adjusted for birth weight, maternal age, newborn gender and pregnancy weight gain. Birth weight, maternal age and pregnancy weight gain were modeled as continuous independent variables. The rest of covariates were modeled as dichotomous variables. All statistical testing was performed in Statgraphics Centurion XVI software from Statpoint Inc. (Warrenton, VA, USA).

Results

In maternal and cord plasma, the main conjugated phthalate monoesters were determined: monoethyl phthalate (MEP) – metabolite of DEP, mono-*n*-butyl phthalate (MnBP) – a metabolite of DnBP, monoisobutyl phthalate (MiBP) – a metabolite of DiBP, monobenzyl phthalate (MBzP) – a metabolite of BzBP, and mono-(2-ethylhexyl) phthalate (MEHP), mono-(2-ethyl-5-hydroxyhexyl) phthalate (5-OH-MEHP), and mono-(2-ethyl-5-oxohexyl) phthalate (5-oxo-MEHP), which are metabolites of DEHP. The other groups of unconjugated EDs as well as the levels of steroid hormones were determined as a part of our previous study (Kolatorova *et al.* 2018).

MiBP was detected in all maternal plasma samples and MnBP in 77.8 % of the samples. MEP and MEHP were detected in two samples, and MBzP and 5-OH-MeHP in one sample only. 5-oxo-MEHP was not detected. Phthalate metabolites MiBP and MnBP were quantified in all cord plasma samples, MEP in six samples (33.3 %) and MEHP in 3 samples. MBzP, 5-OH-MeHP and 5-oxo-MeHP were not detected.

Because of low detection rate of several phthalates, the sum of phthalates (Σphthalates) was

calculated from all phthalates quantified. The medians of the most frequently detected phthalates together with previously quantified bisphenols and parabens as well as steroid hormone levels (Kolatorova *et al.* 2018) are shown in Table 3. Differences were observed in MnBP levels between maternal and cord plasma, with significantly higher MnBP levels in cord blood (p=0.043).

Table 3. Selected most frequently detected endocrine disruptors and steroid hormones in maternal and cord plasma (medians with lower and upper quartiles).

Analyte	Maternal plasma (ng/ml)	Mixed cord plasma (ng/ml)	P-value
MEP	under LOQ	3.50 (3.50, 4.36)	0.800
MiBP	4.45 (3.79, 8.50)	7.63 (5.02, 9.18)	0.112
MnBP	4.37 (2.87, 5.93)	5.15 (3.97, 6.90)	**0.043**
Σphthalates	12.9 (10.6, 18.4)	16.4 (12.2, 19.4)	0.191
BPA	0.049 (0.020, 0.088)	0.098 (0.013, 0.242)	0.093
Σbisphenols	0.069 (0.027, 0.090)	0.101 (0.020, 0.242)	0.145
MP	0.014 (0.014, 0.850)	0.059 (0.014, 0.603)	1.000
PP	0.023 (0.011, 0.123)	0.016 (0.011, 0.147)	0.816
Σparabens	0.060 (0.032, 1.080)	0.121 (0.026, 0.744)	0.862
E1	19.5 (11.7, 24.5)	34.6 (19.2, 75.4)	**<0.001**
E2	18.4 (12.6, 21.8)	5.51 (3.24, 8.86)	**<0.001**
E3	8.3 (7.57, 10.5)	71.9 (54.6, 95.7)	**<0.001**
Cortisol	283 (236, 298)	80 (36, 143)	**0.006**
Cortisone	84 (68, 104)	292 (220, 341)	**0.006**
DHEA	1.57 (0.95, 2.31)	2.3 (1.36, 3.86)	1.000
7α-OH-DHEA	0.24 (0.20, 0.26)	1.37 (1.00, 2.51)	**0.006**
7β-OH-DHEA	0.071 (0.058, 0.078)	0.069 (0.051, 0.079)	0.476
7-oxo-DHEA	0.033 (0.017, 0.038)	0.091 (0.006, 0.194)	**0.006**
Pregnenolone	21.1 (17.0, 28.2)	49.2 (36.6, 84.4)	**0.019**
17-OH-pregnenolone	0.105 (0.105, 0.105)	8.6 (6.47, 13.7)	**0.006**
Testosterone	0.50 (0.33, 0.70)	0.13 (0.08, 0.17)	**0.006**
Androstenedione	0.62 (0.30, 1.05)	0.44 (0.31, 0.75)	0.610
Progesterone	64 (49, 71)	260 (167, 430)	**0.006**

P-values indicate statistical significance, with levels less than 0.05 highlighted in bold. Bisphenols, parabens and steroids were determined in another study (Kolatorova *et al.* 2018). Σbisphenols was calculated from BPA, BPS, BPF and BPAF, Σparabens was calculated from MP, EP, PP, BP and BenzylP.

Nine newborn boys and nine newborn girls were included in the study. Significantly higher levels were found in cord plasma from male newborns as follows: E2 (p<0.0001), E3 (p=0.034), 7α-OH-DHEA (p=0.009). We also investigated the influence of EDs on birth weight but found no association. There were also no differences found between the steroid hormone levels in caesarean and vaginal deliveries; however, only 3 caesarean sections were present in our study group. The levels of EDs in relation to the mother's education level were also compared, but no differences between mothers with university degree and high school education were observed. Interestingly, there were differences in the levels of PP between mothers with monthly income 10,000-25,000 CZK and >25,000 CZK, with mothers earning more than 25,000 CZK per month having significantly higher levels of PP (p=0.037).

In order to investigate possible associations between phthalates and steroid hormones, multiple regression models were applied, but no associations with maternal phthalates and maternal steroids were observed. The cord blood MP and Σparabens were inversely

associated with cord blood testosterone levels. The adjusted regression coefficients (β) with 95 % confidence intervals (CI) for the change in cord plasma steroids associated with interquartile range changes in cord plasma endocrine disruptor concentrations were as follows: β: -0.004 CI: (-0.007; -0.001), p=0.012 for MP and β: -0.003 CI: (-0.005; -0.001), p=0.016 for Σparabens.

The multiple regression models investigating the possible impact of maternal EDs on fetal steroid hormones showed that maternal blood levels of phthalates were positively associated with cord blood estrogens. Specifically, E2 was positively associated with MnBp and E3 with MnBP and Σphthalates. Maternal phthalates were negatively associated with several androgens (DHEA, androstenedione and testosterone). Maternal phthalates (Σphthalates and MiBP) were also negatively associated with cord blood DHEA and androstenedione, while MnBP was negatively associated with testosterone.

As concern the previously measured levels of bisphenols and parabens, we observed several relations. A positive association was found for maternal Σbisphenols and cord E3. Maternal BPA was also positively associated with 7β-OH-DHEA. Concerning parabens, opposite associations for MP and PP were observed – maternal MP was positively associated with cord blood E2 and testosterone, and maternal PP levels were conversely negatively associated with these steroids. For Σparabens, no associations for these mentioned steroids were found. MP was also positively associated with 7α-OH-DHEA, and PP positively associated with cortisone. Σparabens were found to have positive associations with cortisone and 7α-OH-DHEA. The results of the associations of maternal EDs and cord steroids are summarized in Table 4. The associations from all biological samples studied are schematically summarized in Table 5.

Discussion

Seven main phthalate metabolites, all known to act as EDs, were measured in maternal plasma at the 37th week of pregnancy, and in cord blood of their newborns. These metabolites belong to the most abundant in human plasma (Frederiksen *et al.* 2010, Vrbík 2016). The data were taken together with previously quantified bisphenols and parabens and compared with levels of previously measured major sex hormones, corticoids and selected neuro- and immunomodulatory steroids, in order to evaluate the combined effect of these disruptors on steroid profiles. The main effort was focused on phthalates, the effect of which together with other disruptors has not yet been studied.

So far most authors have measured phthalate levels in maternal urine along with cord serum from their offspring. Since urine is a water intake-dependent fluid influenced by various factors, it is difficult to interpret the mother-newborn relations from the urinary data (Kato *et al.* 2003). In this study, we determined the phthalate levels directly from plasma (maternal and cord). Unlike urine, plasma reflects the phthalate metabolism in the mother body and shows the phthalate metabolites, which may be transferred to the fetus *via* placental transport. The reported plasma levels are generally much lower than urinary. Therefore, the analytes which are known to be less frequent in urine were expected to have even lower or zero frequency in plasma and were not included. The detection of quantified phthalate metabolites in the cord plasma should be explained only by the placental transport, because there was no possible contamination by exposure to plastic materials from medical service in the maternity hospital.

Until now, only few papers have dealt with measurements of phthalate metabolites in human serum or plasma. Since 2003, several studies have appeared concerning the detection of conjugated MEP, MBP and MEHP. Later, MBzP and other (altogether nine) phthalates were also determined in human serum using LC-MS (Kato *et al.* 2003, Silva *et al.* 2003). The levels found were on the order tens of ng/ml, which is in accordance with our measurements. The authors found detectable concentrations of MEP, MBP and MEHP in most serum samples tested. In 2008, a Swedish research group investigated the levels of phthalates in human breast milk, serum, blood and urine of 42 women after normal delivery using GC-MS. They determined levels of phthalate di-esters and mono-esters with a LOD in the low ng/ml range, and detected the majority of phthalates of interest (Hogberg *et al.* 2008). In another study using LC-MS, only serum concentrations of oxidative phthalate metabolites were detected due to possible contamination by phthalate di- and mono-esters (Hines *et al.* 2009). In 2010 a Danish research group reported an LC-MS method for the determination of phthalate metabolites in urine, serum and seminal plasma (Frederiksen *et al.* 2010). They obtained similar results to ours, but they obtained better LODs (all below 1 ng/ml). In 2014 Araki *et al.* (2014) measured the levels of MEHP in maternal and cord blood using GC-MS. They reported a median

Table 4. Adjusted regression coefficients (β) with 95 % confidence intervals (CI) for the changes in cord plasma steroid levels associated with changes in maternal plasma endocrine disruptor concentrations.

	Monoisobutyl phthalate		**Mono-*n*-butyl phthalate**		**Σphthalates**	
	β (95 % CI)	**p-value**	**β (95 % CI)**	**p-value**	**β (95 % CI)**	**p-value**
Estradiol	-	-	11.296 (0.177; 22.416)	**0.047**	-	-
Estriol	-	-	111.95 (28.76; 195.13)	**0.012**	40.539 (13.798; 67.279)	**0.006**
Cortisone	-	-	-	-	-	-
DHEA	-3.253 (-5.865; -0.642)	**0.020**	-	-	-5.046 (-7.969; -2.123)	**0.003**
7α-OH-DHEA	-	-	-	-	-	-
7β-OH-DHEA	0.041 (0.008; 0.075)	**0.021**	-	-	-	-
Androstendione	-	-	-1.309 (-2.205; -0.413)	**0.011**	-0.544 (-0.876; -0.212)	**0.007**
Testosterone	-	-	-0.311 (-0.589; -0.032)	**0.036**	-	-

	Methylparaben		**Propylparaben**		**Σparabens**	
	β (95 % CI)	**p-value**	**β (95 % CI)**	**p-value**	**β (95 % CI)**	**p-value**
Estradiol	9.656 (5.151; 14.160)	**0.001**	-9.024 (-13.319; -4.729)	**0.001**	-	-
Estriol	-	-	-	-	-	-
Cortisone	-	-	137.49 (12.22; 262.76)	**0.035**	132.921 (2.912; 242.930)	**0.023**
DHEA	-	-	-	-	-	-
7α-OH-DHEA	1.580 (0.293; 2.868)	**0.021**	-	-	1.383 (0.292; 2.475)	**0.018**
7β-OH-DHEA	-	-	-	-	-	-
Androstendione	-	-	-	-	-	-
Testosterone	0.382 (0.224; 0.540)	**0.003**	-0.239 (-0.363; -0.114)	**0.006**	-	-

	Bisphenol A		**Σbisphenols**	
	β (95 % CI)	**p-value**	**β (95 % CI)**	**p-value**
Estradiol	-	-	-	-
Estriol	-	-	50.838 (2.457; 99.219)	**0.041**
Cortisone	-	-	-	-
DHEA	-	-	-	-
7α-OH-DHEA	-	-	-	-
7β-OH-DHEA	0.0661 (0.014; 0.119)	**0.019**	-	-
Androstendione	-	-	-	-
Testosterone	-	-	-	-

Each model was adjusted for the same set of covariates (birth weight, mother's age, newborn gender, pregnancy weight gain, delivery type and smoking status). The models were performed using 19 maternal and 19 cord plasma samples. β is a measure of the strength between variables and p-value shows the statistical significance.

Table 5. The schematic results of multiple regression models showing the associations in different biological materials.

Plasma material	Association	Phthalates - steroids	Bisphenols - steroids	Parabens – steroids
Maternal - maternal	-	-	-	-
Maternal - cord	positive	Estradiol (MnBP) Estriol (MnBP, Σphthalates) 7β-OH-DHEA (MiBP)	7β-OH-DHEA BPA) Estriol (Σbisphenols)	Estradiol (MP) Cortisone (PP, Σparabens) 7α-OH-DHEA (MP, Σparabens) Testosterone (MP))
	negative	DHEA (MiBP, Σphthalates) A-dione (MnBP, phthalates) Testosterone (MnBP)	-	Estradiol (PP) Testosterone (PP)
Cord - cord	negative	-	-	Testosterone (MP, Σparabens)

A-dione – androstenedione.

maternal blood concentration of 10.4 ng/ml, which is much higher than in our study. These higher levels may have been caused by a much higher MEHP exposure in the Japanese population. Even higher results of DEHP and MEHP (LOD 50 ng/ml with LOQ 100 ng/ml) were found by other Japanese authors in plasma from healthy and autistic children by HPLC-UV (Kondolot *et al.* 2016). The plasma levels measured in children were much higher than the levels measured here in pregnant women and their offspring. This could have been caused by the high exposure of children to dangerous toys and other phthalate-releasing plastics.

The prenatal phase of human development is among the most active periods of life, and hormone signalization plays a significant role in fetal development (Hill *et al.* 2010, Paskova *et al.* 2014). All groups of EDs investigated in this study are capable of crossing the human placenta (Towers *et al.* 2015). The transfer of bisphenols and parabens was confirmed in our previous study (Kolatorova *et al.* 2018). The transplacental passage of phthalates in humans has also been documented by the finding of measurable concentrations of MEP, MBP and MEHP in human amniotic fluid (Silva *et al.* 2004) and by detectable levels of MBP, MEP and MEHP in umbilical cord plasma (Mose *et al.* 2007). Here we have also documented the accumulation of MnBP in the fetal compartment.

Brain development and fetal sexual differentiation are dependent on steroids produced in the fetal gonads. The levels of maternal estrogens are increased during pregnancy and therefore there is a mechanism protecting male fetuses from the maternal estrogen excess. The developing brain is protected from estrogens by alpha-fetoproteins that prevent estrogens from entering nerve cells. In male fetuses, the gonadal testosterone enters the cytoplasm where it is aromatized to E2, which binds to nuclear estrogen receptors and is responsible for the development of the male brain. During human gestation, estrogens are of maternal origin until about the 8^{th} week of pregnancy, when placenta production rises through the aromatization of fetal androgens (Adamcova *et al.* 2017). The significantly higher estrogen levels in the cord blood from male offspring found in our study may be caused by androgen aromatization by the placenta, similarly as in the study of Lin *et al.* (2011).

The estrogen and androgen balance is essential for the proper development of the fetus. Exposure to EDs with estrogenic and/or anti-androgenic activity may interfere with natural fetal development and cause various harmful effects (Kolatorova *et al.* 2017, Shekhar *et al.* 2017). Until recently, not many studies have focused on the complex effects of simultaneous prenatal exposures of the major groups of EDs on the fetal steroidogenesis. Many authors have investigated the effect of only one ED or group of structurally similar EDs (Araki *et al.* 2017, Araki *et al.* 2014, Liu *et al.* 2016). Several studies have examined phthalate exposure on androgen concentrations during human gestation and delivery, with the majority reporting a negative association between prenatal exposure to phthalates and free/total cord blood testosterone. This suggested the antivirilizing effect of phthalates, which persisted regardless of fetal sex (Lin *et al.* 2011, Main *et al.* 2006, Sathyanarayana *et al.* 2014,

Sathyanarayana *et al.* 2017). The negative association between maternal MnBP and cord testosterone was observed in our study as well, in accordance with other authors (Araki *et al.* 2014, Lin *et al.* 2011, Main *et al.* 2006, Sathyanarayana *et al.* 2014, Sathyanarayana *et al.* 2017, Wen *et al.* 2017).

Recently, a further study has reported on the relation of urinary maternal phthalates to serum estrogens (E1, E2) in the first trimester of pregnancy, finding positive associations between estrogens (E1, E2) and phthalates (MBzP, MiBP, MEHP and MeOHP) and negative associations of free testosterone and several phthalates (Sathyanarayana *et al.* 2017). These results are in accordance with our findings, as well as with many animal and *in vitro* studies reporting phthalate estrogenicity. Of interest may be a persisting estrogenic effect of prenatal phthalate exposure (as measured in maternal urine) on the levels of plasma E2, testosterone and also progesterone, observed in children not only at birth, but even at 2, 5, 8 and 11 years of age (Wen *et al.* 2017).

Other authors have investigated associations between DEHP in maternal plasma (25-35 gestation week) and cord blood E2, testosterone and progesterone (Araki *et al.* 2014). They reported that increased maternal DEHP significantly decreased the progesterone and testosterone/E2 ratio in the cord blood. In 2017 these authors extended the number of steroids hormones measured to include DHEA, androstenedione, cortisol and cortisone (Araki *et al.* 2017), and found significant negative associations between maternal plasma DEHP and cord plasma androstenedione, cortisol and cortisone. In our study, however, we did not find any association of phthalates and either of the corticosteroids. Surprisingly, we found a positive association between cortisone and PP and Σparabens. The Japanese authors only determined the levels of DEHP, so our results and their measurements cannot be easily compared.

A few studies have also investigated the impact of phthalate exposure to DHEA or its sulfate in pubertal children of both sexes, but not in mother or their offspring; moreover, the results have been inconsistent (Ferguson *et al.* 2014, Mouritsen *et al.* 2013, Watkins *et al.* 2014). Our study and that of the Japanese group (Araki *et al.* 2017, Araki *et al.* 2014) were performed in the maternal and cord blood. The results revealed a negative association of phthalates and cord blood androstenedione, similar results were reported in the larger Japanese study (Araki *et al.* 2017). The negative associations of phthalates and offspring adrenal androgens are supported by the similar association with testosterone. These findings may confirm the anti-androgen effects of phthalates, which may be particularly important in male fetuses. We also studied associations between individual groups of EDs with 7-oxygenated metabolites of DHEA, believed to act as natural antiglucocorticoids with immunoprotective and neuromodulatory properties. Some positive associations were found between 7α-OH-DHEA and PP and Σparabens, and also between 7β-OH-DHEA with MiBP and BPA, respectively. These metabolites do not have androgenic activity, which may be reason for the opposite association as compared to DHEA. The altered levels of these metabolites may impair natural neurodevelopment, however no other information concerning this issue is currently available.

In our previous study we investigated the associations between bisphenols and parabens, and found no associations between maternal EDs and cord steroids (Kolatorova *et al.* 2018). In the current study we determined the levels of several phthalates, and we could thus assess the combined effect of multiple EDs. Investigating the phthalates, the multiple regression models taking into account the maternal EDs and cord steroids provided various associations. Overall, there were positive associations of maternal phthalates and cord estrogens (E2, E3) and negative associations between mother phthalates and cord androgens (androstenedione, testosterone, DHEA). These results are in accordance with the studies of Sathyanarayana *et al.* (2014, 2017). Prenatal exposure to phthalates has also been associated with adverse effects on neurodevelopment (Doherty *et al.* 2017). Because androgens play a crucial role in brain development, it is plausible that the anti-androgenic effect of phthalates and their capacity to affect fetal Leydig cell function and testosterone production and its conversion to E2 play an important role in the disruption of fetal neurodevelopment as well as sex organ development (Mallozzi *et al.* 2016). Concerning estrogens, in addition to phthalates we also found a positive association with bisphenols, which may emphasize the possible additive or synergic effect of these two groups of estrogen active EDs.

As concerns parabens, we confirmed our previously published negative associations with cord blood parabens and testosterone. On the other hand, different associations were found in the relations between

maternal MP and PP and cord blood E2 and testosterone. Anyway, our results indicate that parabens play a less important role in the prenatal phase and that the risk of exposure to bisphenols and especially phthalates is higher. Phthalates influenced steroidogenesis in the offspring more than any other group of measured EDs. In some cases (E3), we were able to observe a possible cocktail effect of exposure to multiple EDs. It is clear that in evaluating the effect of EDs it is important to take into account the possible combined impacts of the multiple EDs that humans are exposed to.

We are aware of the limitation of our study consisting in the sample number. However, to the best of our knowledge, this is the first preliminary study evaluating the effects of prenatal exposure to multiple EDs on newborn steroidogenesis (cord blood). The future validation study is of interest. In the subsequent study, we intend to enlarge the number of pregnant women and their offspring.

Conclusion

In this pilot study, we found negative associations between maternal phthalates and cord androgens (androstenedione, testosterone, DHEA) and positive associations between maternal phthalates and cord estrogens (E2, E3). For estrogens, a similar association was also observed for bisphenols, underlining the possible additive or synergic effect of these two groups of estrogen active EDs. All the associations point to the possibility of disruption to newborn steroidogenesis, which may influence their subsequent lives. We also found significantly higher levels of MnBP in the cord blood compared to the maternal exposure. This indicates a possible accumulation of EDs in the fetal compartment. Our findings should encourage the population as well as international authorities to reduce ED exposure because of possible impacts on future generations.

Conflict of Interest

There is no conflict of interest.

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