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Use of stored serum in the study of time trends and geographical differences in exposure of pregnant women to phthalates

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A B S T R A C T

Background: Exposure to some phthalate diesters has been associated with adverse reproductive health outcomes in both rodents and humans indicative of anti-androgenic effects. Exposure during sensitive periods of development, such as prenatally, is of particular concern.
Objectives: We wished to investigate whether phthalate metabolites measured in maternal serum samples from historical birth cohorts can be used to assess prenatal exposure. Further, we aimed to study temporal and geographical trends in phthalate exposure across three different birth cohorts.
Methods: We compared phthalate metabolite levels in maternal serum samples from an Australian (1989–91) and a Danish (1997–2001) birth cohort with levels in serum and urine samples from a recent Danish birth cohort (2012–14). Samples were analysed for 32 phthalate metabolites from 15 phthalate diesters by isotope-diluted liquid chromatography-tandem mass spectrometry (LC-MS/MS). Correlations between metabolites were tested by Spearman rank correlation test, and differences between the cohorts were tested by Mann-Whitney *U* test.
Results: Overall, we observed large variations in serum phthalate metabolite levels between individuals. Secondary metabolites of di-(2-ethyl-hexyl) phthalate (DEHP) and di-iso-nonyl phthalate (DiNP) in serum were weakly to moderately and positively correlated to the levels measured in urine, and secondary metabolites of DEHP were also moderately to strongly and significantly correlated in serum. Correlations with mono-(2-ethyl-hexyl) phthalate (MEHP) and mono-iso-nonyl phthalate (MiNP), the two primary metabolites of DEHP and DiNP, were inconsistent, and we found indications of sample contamination. We observed some significant differences in phthalate metabolite levels between the three cohorts with generally higher levels in the older birth cohorts.
Conclusion: Based on comparison across two older birth cohorts and a recent cohort, our results support the concept that historical biobanked serum samples may be used for assessment of prenatal exposure to phthalates when using serum levels of the monoesters of the low-molecular weight (LMW) phthalates and the secondary metabolites of the high-molecular weight (HMW) phthalates. Serum phthalate measurements are, however, not suitable for human biomonitoring and should only be used to exploit historical samples from cohorts, where urine samples were not collected. Our findings suggest that phthalate exposure may have decreased over time from the early 1990s to the 2010s.

1. Introduction

Phthalate diesters (phthalates) are used as softeners in plastics such as polyvinyl chloride (PVC) that came onto the market nearly a century ago (Baird et al., 2010). Today, phthalates are found in various consumer products such as building materials, clothing, packaging (including food packaging), personal care products (such as cosmetics and lotions) and medical devices (Heudorf et al., 2007).

As phthalates are not covalently bound to PVC, they can leach, migrate or evaporate, and humans are exposed through ingestion, inhalation and dermal routes (Heudorf et al., 2007). In adults, food is one of the main source of exposure to di-(2-ethyl-hexyl) phthalate (DEHP), one of the most commonly used phthalate plasticisers, and other high-molecular weight (HMW) phthalates (Koch and Calafat, 2009; Wittassek et al., 2011). Use of consumer products and different indoor sources dominate the exposure to the low-molecular weight (LMW) phthalates (Wormuth et al., 2006).

Exposure to phthalates also seems to vary by geographical region.

Abbreviations: AGD, anogenital distance; LC-MS/MS, isotope-diluted liquid chromatography-tandem mass spectrometry; LOD, limit of detection; GA, gestational age

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Studies have found differences in exposure between populations in rural and urban areas (Larsson et al., 2014); a study on phthalates in breast milk samples from Finnish and Danish mothers found between-country differences in concentrations (Main et al., 2006a). During hospitals visits, both children (Calafat et al., 2004; Frederiksen et al., 2014b) and adults (Dine et al., 2000; Sampson and de Korte, 2011; Takahashi et al., 2008) may be exposed to different phthalates via medical equipment.

Some phthalates, such as di-*n*-butyl phthalate (DnBP) DEHP, have anti-androgenic effects both *in vitro* and *in vivo*. In rodents, prenatal exposure to DnBP, di-iso-nonyl phthalate (DiNP), butylbenzyl phthalate (BBzP) and DEHP has been associated with testicular dysgenesis with Leydig cell clusters, immature Sertoli cells, congenital cryptorchidism and hypospadias, increased nipple retention, reduced anogenital distance (AGD) and reduced adult testis volume, sperm count and hypogonadism (Foster, 2006; Gray et al., 2000; van den Driesche et al., 2017). Exposure during so-called sensitive windows of development is of particular concern as this may cause irreversible damage to the reproductive system (Johansson et al., 2017; van den Driesche et al., 2017).

Although data are still limited, similar associations between anti-androgenic impact on reproductive endpoints and exposure to phthalates have been found in humans (Hauser and Calafat, 2005; Mariana et al., 2016; Matsumoto et al., 2008). Main adverse effects on male reproductive development comprise hypospadias, attenuated endogenous reproductive hormone levels in infant boys (Main et al., 2006b; Ormond et al., 2009; Sathyanarayana et al., 2016) and subtle changes in sperm quality and reproductive hormones in adult men (Joensen et al., 2012; Thurston et al., 2016; Wang et al., 2016). Adverse health effects in vulnerable populations such as pregnant women have given particular rise to concern. Studies have found an inverse relationship between AGD in male infants, an androgen-dependent trait that develops *in utero*, and prenatal levels of di-ethyl phthalate (DEP), DnBP, BBzP and mono-iso-butyl phthalate (MiBP) (Bornehag et al., 2015; Swan et al., 2015, 2005). A recent study in a less exposed Danish cohort, however, found no associations between shorter AGD and prenatal phthalate exposure (Jensen et al., 2016).

Congruent with the findings in rodents, a common fetal origin of male reproductive disorders was proposed in 2001 with the testicular dysgenesis syndrome hypothesis, and it was suggested that adverse environmental effects play a role in the development of this entity (Skakkebaek, 2001).

Published data on female reproductive endpoints associated with exposures to phthalates include delayed development of pubic hair associated with increased urinary phthalate levels in puberty (Frederiksen et al., 2012; Wolff et al., 2014) and a potential association with early onset of puberty and premature thelarche after prenatal exposure (Hart et al., 2014; Jurewicz and Hanke, 2011).

Exploring correlations between prenatal exposure and adult reproductive health in humans is challenging due to the long generation time. Existing samples from older birth cohorts, where the offspring has reached adulthood, are therefore highly valuable. As urine samples were not routinely collected when these historical cohorts were established, only serum samples may be available to assess prenatal exposure.

Measuring phthalates in serum, however, has limitations, and it has been regularly debated whether serum measurements are appropriate to assess phthalate exposure (Calafat et al., 2015, 2013; Hauser and Calafat, 2005; Högberg et al., 2008; Koch and Calafat, 2009). This is due to two main reasons:

First, phthalate diesters are quickly metabolized to their respective metabolites and have relatively short half-lives *in vivo* (Johns et al., 2015). As a result, concentrations in serum are many times lower than in urine, especially of the secondary oxidized monoesters (Frederiksen et al., 2010; Koch et al., 2005; Silva et al., 2003).

Second, due to their extensive use, phthalates are ubiquitously present in our environment, and contamination with phthalate diesters may occur through various routes. This is particularly challenging when measuring serum levels, as samples are collected with medical equipment potentially containing phthalates. Furthermore, serum contains esterases that can catalyse the conversion of diesters to the primary hydroxylated monoesters (Albro and Thomas, 1973; Inoue et al., 2005; Kato et al., 2003). As a result, concentrations of both phthalate diesters and hydroxylated monoesters may be elevated in serum due to an unknown contribution from contamination (Calafat and Needham, 2008). This contribution may be substantial relative to the low endogenous levels (Koch and Calafat, 2009). Although esterase activity can be inhibited by addition of acid, this may not have been done at the time of collection for historical cohort samples.

Thus, serum measurements of especially hydrolytic monoesters should be interpreted with caution. In contrast, oxidation of the hydroxylated monoesters only happens *in vivo*, hence contamination with these metabolites is not an issue.

Given the concern about effects on reproductive health, the use of certain phthalates has been restricted. In the European Union (EU), four phthalates (DEHP, DnBP, di-iso-butyl phthalate (DiBP) and BBzP) have been restricted in products that cause exposure through skin or by inhalation and other articles moulded or coated with plastic (European Chemicals Agency (ECHA), 2017), including food contact materials (European Commission, 2007). In the United States (US) and several European countries, recent studies indicate that restrictions of phthalate use have contributed to reductions in exposure of the populations (Frederiksen et al., 2020, 2014a; Gyllenhammar et al., 2017; Koch et al., 2017; Larsson et al., 2017; Schoeters et al., 2017; Zota et al., 2014). In the large biomonitoring programmes conducted in both Germany and the US, no data are collected on fetal exposures (Koch and Calafat, 2009).

In this study, we compared phthalate metabolites measured in prenatal maternal serum samples derived from two older cohorts, 1) the Western Australian Pregnancy Cohort (Raine) Study (1989–91) and 2) the Copenhagen Mother-Child cohort (Cop1, 1997–2001), with urine and serum phthalate measurements in a recent cohort from Copenhagen (Cop2, 2012–14). First, we aimed to investigate whether phthalate metabolites measured in historical serum samples can be used to assess maternal exposure when urine samples are not available. Second, we wanted to explore temporal and geographical trends in phthalate exposure across three different birth cohorts.

2. Materials and methods

2.1. Study populations and sampling

This study includes pregnant women from three different prospective population-based mother-child cohorts. Phthalate measurements have previously partly been published for the Raine Study (Hart et al., 2014, 2018) and the Cop1 cohort (Assens et al., 2019) while phthalate data from the Cop2 cohort have not been published before.

2.1.1. The Raine Study cohort

The Western Australian Pregnancy Cohort (Raine) Study (www.rainestudy.org.au) was established in 1989–91 (Newnham et al., 1993). Pregnant women ('Generation 1') were enrolled around 18 weeks of gestation, and singleton pregnancies were randomized to an intensive investigation group (n = 1415) and a regular group (n = 1419) (Newnham et al., 1993). In the intensive investigation group, maternal blood samples were collected at 18 weeks and 34–36 weeks and stored in aliquots at −80 °C (Hickey et al., 2009).

For each woman, 200 μL from thawed aliquots of the 18- and 34-/36-weeks samples were pooled and couriered frozen to Copenhagen, Denmark (Hart et al., 2014, 2018). Serum samples from a total of 986 women were analysed for phthalates and included in this study.

2.1.2. The Copenhagen mother-child cohort (Cop1)

The Copenhagen Mother-Child Cohort (Cop1) (http://edmarc.net/mother-child-cohort.html) is an ongoing population-based longitudinal birth cohort study established 1997–2001. Pregnant women were recruited consecutively from three university hospitals during the first trimester if they were healthy and both parents as well as grandparents of the unborn child were born and raised in Denmark (Boisen et al., 2004; Chellakooty et al., 2004; Wohlfahrt-Veje et al., 2014). The women belonged to the hospitals' primary geographic referral area. 2788 women entered the study.

At two hospitals, a serum sample was collected during pregnancy at a median gestational week of 23 (range 8–39), and a subgroup (selected based on offspring sex being female) was analysed for phthalates and included in this study (n = 114). At the third hospital, participants had blood samples taken repetitively throughout pregnancy (Chellakooty et al., 2004). Serum was stored in aliquots at −20 °C. Samples were analysed for phthalates and a sample as close to week 20 of pregnancy as possible was included in the present study (n = 99). Thus, a total of 213 serum samples were included in this study.

2.1.3. The Copenhagen mother-child cohort (Cop 2)

A new mother-child cohort (Cop2) (Krause et al., 2018) was established 2012–14. Pregnant women were recruited from two university hospitals. Women with singleton pregnancies who had amniocentesis performed, either on maternal request (n = 53) or medical indication (n = 147), were included in the study (n = 200). 78 women had either malformations on ultrasound scans (n = 61) and/or abnormal karyotype of the fetus (n = 17) (Krause et al., 2018). Aliquots of serum and urine samples collected at the same examination at median gestational week 18 (range 12–36) were stored in glass bottles at −20 °C (Krause et al., 2018). Immediately after collection, acid was added to serum samples. 103 serum samples and 107 urine samples were analysed for phthalates. 102 women provided both a serum and urine sample.

2.2. Measurement of phthalates

All serum and urine samples were analysed in the same laboratory at the Department of Growth and Reproduction, Rigshospitalet, Copenhagen. Samples were analysed for 32 phthalate metabolites from 15 phthalate diesters in both serum and urine (Table 1) by isotope-diluted liquid chromatography-tandem mass spectrometry (LC-MS/MS) preceded by enzymatic deconjugation as previously described in detail for 13 metabolites (Frederiksen et al., 2010). This method was recently expanded to include 32 metabolites and further modified by using online TurboFlow LC-MS/MS technology equipped with a probe for heated electrospray ionization (HESI) running in negative mode (Frederiksen et al., 2020; Hart et al., 2018).

In brief, samples were analysed in batches including standards for calibration curves, 30–40 participant samples, three blanks, three serum pool controls and three serum pool controls spiked with native phthalate metabolite standards at low and high level.

2.3. Adjustment for urinary dilution

Osmolality was measured by the freezing point depression method with automatic cryoscopic osmometer (Osmomat® 030 from Gonotec, Berlin, Germany). Urinary osmolality ranged from 0.006 to 1.070 Osm/

Table 1
|Phthalate diesters and their respective metabolites detected in maternal serum and urine and sums of metabolites used for statistical analysis.

Phthalate diester		Human serum and urine metabolite	
Di-methyl phthalate	DMP	Mono-methyl phthalate	MMP
Di-ethyl phthalate	DEP	Mono-ethyl phthalate	MEP
Di-iso-propyl phthalate	DiPrP	Mono-(4-oxopentyl) phthalate	MiPrP
	DPrP	Mono-propyl phthalate	MPrP
Di-iso-butyl phthalate	DiBP	Mono-iso-butyl phthalate	MiBP
Di-*n*-butyl phthalate	DnBP	Mono-*n*-butyl phthalate	MnBP
		Mono-(3-hydroxybutyl) phthalate	MHBP
Butylbenzyl phthalate	BBzP	Mono-benzyl phthalate	MBzP
Di-*n*-pentyl phthalate	DPP	Mono-*n*-pentyl phthalate	MPP
		Mono-(4-hydroxypentyl) phthalate	MHPP
Di-(2-ethyl-hexyl) phthalate	DEHP	Mono-(2-ethyl-hexyl) phthalate	MEHP
		Mono-(2-ethyl-5-hydroxyhexyl) phthalate	MEHHP
		Mono-(2-ethyl-5-oxohexyl) phthalate	MEOHP
		Mono-(2-ethyl-5-carboxypentyl) phthalate	MECPP
		Mono-(2-carboxymethyl-hexyl) phthalate	MCMHP
Di-*n*-hexyl phthalate	DHxP	Mono-*n*-hexyl phthalate	MHxP
		Mono-(5-hydroxyhexyl) phthalate	MHHxP
		Mono-(5-carboxypentyl) phthalate	MCPeP
Di-cyclohexyl phthalate	DCHP	Mono-cyclohexyl phthalate	MCHP
Di-*n*-heptyl phthalate	DHpP	Mono-*n*-heptyl phthalate	MHepP
		Mono-(6-hydroxyheptyl) phthalate	MHHpP
		Mono-(6-carboxyhexyl) phthalate	MCHxP
Di-octyl phthalate	DOP	Mono-octyl phthalate	MOP
		Mono-3-carboxypropyl phthalate	MCPP [a]
Di-iso-nonyl phthalate	DiNP	Mono-iso-nonyl phthalate	MiNP
		Mono-hydroxy-iso-nonyl phthalate	MHiNP
		Mono-oxo-iso-nonyl phthalate	MOiNP
		Mono-carboxy-iso-octyl phthalate	MCiOP
Di-iso-decylphthalate	DiDP	Mono-iso-decyl phthalate	MiDP
		Mono-(9-hydroxydecyl) phthalate	MHiDP
		Mono-(9-oxodecyl) phthalate	MOiDP
		Mono-(9-carboxynonyl) phthalate	MCiNP
ΣDEHPm	Molar sum of MCMHP and MECPP expressed as DEHP in ng/mL		
ΣLMW phth.m	Molar sum of MEP, MiBP, MnBP and MHBP expressed as MEP in ng/mL		
ΣHMW phth.m	Molar sum of MBzP, MECPP, MCMHP, MCPP and MCiOP expressed as MECPP in g/mL		
Σall phth.m	Molar sum of MEP, MiBP, MnBP, MHBP, MBzP, MECPP, MCMHP, MCPP and MCiOP expressed as MECPP in ng/mL		

[a] Major metabolite of DOP, not specific for DOP.

kg with a median value of 0.437 Osm/kg for all 107 urine samples.

To account for urinary dilution, phthalate concentrations were adjusted for individual urinary osmolality normalised to the median osmolality of all samples according to a previously described method (Lassen et al., 2013):

$$\text{Osmolality adjusted phthalate conc.} \left(\frac{ng}{mL}\right) = \frac{\text{Phthalate conc.} \left(\frac{ng}{mL}\right) \times 0.437 \left(\frac{Osm}{kg}\right)}{\text{Sample osmolality} \left(\frac{Osm}{kg}\right)}$$

This was done for all urinary phthalate concentrations ≥ limit of detection (LOD) while samples with phthalate concentrations < LOD were registered as < LOD regardless of the urine osmolality.

2.4. Covariates

Information on maternal age, parity, pre-pregnancy weight and height, fetal sex, smoking during pregnancy and educational status was obtained from all three cohorts (Table S1).

In the Raine cohort, all information was collected from a questionnaire completed at the time of enrolment with assistance from one of three research midwifes (Newnham et al., 1993).

In Cop1, questionnaires were administered once during pregnancy (Damgaard et al., 2008), and information on maternal age, parity, prepregnancy weight and height, smoking status and education was collected from these. Information on fetal sex and gestational age (GA) was collected from birth records.

In Cop2, information on GA at sampling, maternal age, parity, fetal sex and measurements of pre-pregnancy weight and height were obtained from medical records (Krause et al., 2018). Furthermore, all participants answered a questionnaire including questions on their educational status and smoking habits (Krause et al., 2018).

2.5. Statistical analyses

Selected percentiles (25, 50, 75, 95) as well as minimum and maximum concentrations were calculated for all 32 metabolites and four sums of phthalate metabolites. This was also done for all 32 metabolites in urine samples from Cop2.

According to a previously described method (Hart et al., 2014; Wolff et al., 2010), phthalate metabolites were divided into groups that represent similar sources of exposure: metabolites of DEHP (mono-(2-carboxymethyl-hexyl) phthalate (MCMHP), mono-(2-ethyl-5-carboxypentyl) phthalate (MECPP)) were combined into ΣDEHPm, the LMW phthalate metabolites (mono-ethyl phthalate (MEP), MiBP, mono-*n*-butyl phthalate (MnBP), mono-(3-hydroxybutyl) phthalate (MHBP)) were combined into ΣLMW phth.m, and HMW phthalate metabolites (mono-benzyl phthalate (MBzP), MECPP, MCMHP, mono-3-carboxypropyl phthalate (MCPP), mono-carboxy-iso-octyl phthalate (MCiOP)) were combined into ΣHMW phth.m. Finally, all phthalate metabolites that were detected in ≥ 40% of samples in at least one of the three cohorts were combined into Σall phth.m (MEP, MiBP, MnBP, MHBP, MBzP, MECPP, MCMHP, MCPP, MCiOP). Due to suspected contamination with mono-iso-nonyl phthalate (MiNP) and mono-(2-ethylhexyl) phthalate (MEHP) in Cop2 serum samples (further described in the discussion, section 4), three metabolites (MiNP, mono-iso-decyl phthalate (MiDP), MEHP) were excluded from all sums for all three cohorts.

Single analytes were reported in ng/mL. To combine phthalate metabolites into groups, molar concentrations (nmol/mL) of the phthalate metabolites were summed and multiplied by the molecular weight (ng/nmol) of either the corresponding phthalate diester (i.e. DEHP in ΣDEHPm) or the metabolite measured in the highest concentration (MEP for ΣLMW phth.m, and MCMHP for both ΣHMW phth.m and Σall phth.m). This was done to convert units for sums to ng/mL.

Differences between the demographic variables from the three cohorts were tested by Student's t-test for parametric data, Mann-Whitney *U* test for non-parametric data and Chi-squared test for categorical data.

Correlations between primary and secondary phthalate metabolites in each cohort as well as between phthalate metabolites in urine and serum in Cop2 were tested by Spearman rank correlation test. Serum phthalate levels were compared between the three cohorts by Mann-Whitney *U* test. To explore whether differences in serum phthalate metabolite levels between the cohorts could be explained by differences in demographics, we conducted sensitivity analyses where demographic variables that differed between the cohorts (maternal age, maternal BMI, parity (nullipara versus primi- and multipara), offspring gender, smoking, educational level and GA at sampling (only available for Cop1 and Cop2)) were included one by one in a linear regression model. For these analyses, metabolite levels were logarithmically transformed.

These analyses were performed for metabolites that were detected in ≥ 40% of samples in at least one of the three cohorts. However, MCPP was excluded from correlation tests as it is not specific to DOP. Due to suspected contamination with MiNP and MEHP, these two metabolites were excluded from the Mann-Whitney *U* tests.

$P < 0.05$ was considered statistically significant, while $P < 0.1$ was considered borderline significant. Concentrations below the LOD were assigned the value 0. LODs for sums were defined as the LOD for the most frequently detected metabolite in the sum. In Fig. 2 and for sensitivity analyses, serum metabolite levels below LOD were substituted with LOD/√2.

All statistical analyses were conducted using R software (R Core Team (2014). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria) and IBM SPSS Statistics 22 (SPSS Inc., Chicago, IL). Figures were constructed in R using the ggplot2 package.

3. Results

3.1. Serum phthalate concentrations

Six phthalate metabolites (MEP, MnBP, MiNP, MEHP, MECPP and MCMHP) were detectable (≥ LOD) in ≥ 70% of both Raine and Cop1 serum samples. MiBP was detectable in 63% and 83% of the Raine and Cop1 serum samples respectively (Table 2). Five other metabolites (MHBP, MBzP, MCPP, MCiOP and MiDP) were detectable in ≥ 29% of the Raine serum samples, while three metabolites (MHBP, MCiOP and MiDP) were detectable in ≥ 17% of the Cop1 serum samples. In Cop2, only MEHP and MiNP were detectable in almost all serum samples (98% and 99%, respectively), while six other metabolites (MEP, MECPP, MCMHP, MCPP, MCiOP, MiDP) were measured in 17–38% of Cop2 serum samples.

All other metabolites were not detectable or only detectable in < 10% of serum samples in each of the three cohorts.

Potential post-collection contamination of samples was assessed through evaluation of inter-individual variation and calculation of correlations between primary and secondary phthalate metabolites. We observed large variations in serum metabolite levels between individuals. For instance, MEP concentrations ranged from < LOD to maximum levels of 2107, 67.2 and 17.7 ng/mL respectively in the three cohorts (Table 2).

Several significant correlations between metabolites of the same phthalate diester were found in serum from all three cohorts (Table 3). In serum samples from all three cohorts, there were moderate to strong correlations between MECPP and MCMHP ($r \geq 0.562$, $P < 0.001$). In the Raine cohort, MEHP was significantly correlated to MCMHP ($r = 0.153$, $P < 0.001$) but not to MECPP. In Cop1 serum, MEHP was

Table 2
|Serum levels of 32 phthalate metabolites (ng/mL) in pregnant women in three different prospective mother-child cohorts.

		Raine (n = 986)							Cop1 (n = 213)		
				Percentiles							Percentiles
	LOD	% > LOD	min.	25	50	75	95	max.	% > LOD	min.	25
MMP	0.44	6.2				< LOD	0.49	35.0	3.3		
MEP	0.65	79.8	< LOD	0.95	2.91	6.75	23.8	2107	77.5	< LOD	0.84
MiPrP	0.40	0.0						< LOD	4.7		
MPrP	0.23	0.0						< LOD	0.0		
MiBP	0.75	63.2		< LOD	1.19	2.22	7.01	74.0	82.6	< LOD	1.01
MnBP	0.61	89.7	< LOD	1.35	2.53	5.08	19.2	463	84.5	< LOD	0.85
MHBP	0.22	33.7			< LOD	0.29	0.58	2.53	67.1		< LOD
MBzP	0.26	42.5			< LOD	0.49	1.67	73.8	3.3		
MPP	0.27	0.0						< LOD	0.0		
MHPP	0.38	0.2					< LOD	0.46	10.3		
MEHP	0.74	99.9	< LOD	2.49	3.73	5.59	9.71	32.2	100.0	0.93	2.27
MEHHP	0.59	0.5					< LOD	1.73	0.0		
MEOHP	0.45	0.7					< LOD	1.2	0.0		
MECPP	0.25	92.9	< LOD	0.59	0.90	1.31	2.92	10.8	70.4		< LOD
MCMHP	0.39	98.1	< LOD	0.93	1.40	2.01	4.36	36.5	98.6	< LOD	0.80
MHxP	0.38	0.2					< LOD	1.33	0.5		
MHHxP	0.26	0.0						< LOD	0.5		
MCPeP	0.20	0.7					< LOD	0.54	0.0		
MCHP	0.27	0.1					< LOD	0.75	0.5		
MHepP	0.38	0.2					< LOD	0.42	0.0		
MHHpP	0.15	0.0						< LOD	0.0		
MCHxP	0.23	3.3					< LOD	2.19	8.9		
MOP	0.70	0.8					< LOD	1.55	2.8		
MCPP	0.19	41.9			< LOD	0.35	1.03	7.58	1.4		
MiNP	0.53	96.0	< LOD	2.03	3.78	5.74	8.12	53.7	79.8	< LOD	0.59
MHiNP	0.4	0.0						< LOD	0.0		
MOiNP	0.31	0.0						< LOD	0.0		
MCiOP	0.13	58.0		< LOD	0.15	0.28	0.81	15.3	16.9		
MiDP	0.72	28.9			< LOD	0.86	3.62	21.2	69.0		< LOD
MHiDP	0.31	0.0						< LOD	0.0		
MOiDP	0.31	0.0						< LOD	0.0		
MCiNP	0.32	0.0						< LOD	0.0		
ΣDEHPm	0.74	96.7	< LOD	1.99	2.91	4.27	9.08	52.7	96.7	< LOD	1.34
ΣLMW phth.m	0.61	94.5	< LOD	3.88	7.55	14.5	40.5	2573	100.0	0.62	5.39
ΣHMW phth.m	0.39	98.7	< LOD	2.08	3.13	4.88	9.97	93.33	98.6	< LOD	1.20
Σall phth.m	0.39	99.5	< LOD	9.44	15.7	27.8	71.9	4106	100.0	2.31	11.2

(*continued on next page*)

Table 2 (*continued*)

	Cop1 (n = 213)				Cop2 (n = 103)						
	Percentiles						Percentiles				
	50	75	95	max.	% > LOD	min.	25	50	75	95	max.
MMP			< LOD	0.85	1.0					< LOD	0.48
MEP	2.37	8.54	36.3	67.2	17.5				< LOD	7.71	17.7
MiPrP			< LOD	1.35	0.0						< LOD
MPrP				< LOD	0.0						< LOD
MiBP	2.12	4.07	10.7	134	2.9					< LOD	3.27
MnBP	2.47	20.3	90.4	213	1.0					< LOD	0.64
MHBP	0.64	1.03	1.73	4.91	0.0						< LOD
MBzP			< LOD	3.07	0.0						< LOD
MPP				< LOD	0.0						< LOD
MHPP		< LOD	0.49	2.16	0.0						< LOD
MEHP	3.69	6.85	40.7	172	98.1	< LOD	3.12	3.59	4.35	5.30	7.51
MEHHP				< LOD	0.0						< LOD
MEOHP				< LOD	0.0						< LOD
MECPP	0.33	0.53	0.98	1.77	25.2				< LOD	0.46	0.87
MCMHP	1.19	1.80	3.25	10.9	37.9			< LOD	0.54	1.13	2.61
MHxP			< LOD	1.88	0.0						< LOD
MHHxP			< LOD	0.28	0.0						< LOD
MCPeP				< LOD	1.0					< LOD	0.32
MCHP			< LOD	1.63	0.0						< LOD
MHepP				< LOD	0.0						< LOD
MHHpP				< LOD	0.0						< LOD
MCHxP		< LOD	0.26	0.49	3.9					< LOD	1.39
MOP			< LOD	1.62	1.9					< LOD	1.21
MCPP			< LOD	5.04	16.5				< LOD	0.75	7.44
MiNP	1.00	1.57	2.53	5.61	99.0	< LOD	4.24	5.87	7.59	10.4	12.4
MHiNP				< LOD	0.0						< LOD
MOiNP				< LOD	0.0						< LOD
MCiOP		< LOD	0.47	2.07	21.4				< LOD	1.03	12.2
MiDP	1.56	3.76	7.30	22.1	18.4				< LOD	1.95	2.74
MHiDP				< LOD	0.0						< LOD
MOiDP				< LOD	0.0						< LOD
MCiNP				< LOD	0.0						< LOD
ΣDEHPm	1.97	2.94	5.35	16.0	26.2			< LOD	0.84	1.91	4.32
ΣLMW phth.m	14.1	50.5	93.8	196	19.4				< LOD	7.71	17.7
ΣHMW phth.m	1.70	2.65	4.98	12.6	55.3		< LOD	0.43	1.10	2.20	14.3
Σall phth.m	25.3	82.7	150	313	60.2		< LOD	0.55	1.75	14.3	28.5

For abbreviations of phthalate metabolites and sums, please see Table 1. Bold font indicates metabolites selected for statistical analyses (measured in ≥ 40% of samples in one of the three cohorts). LOD, limit of detection. For sums, LOD was defined as the LOD of the most frequent metabolite in the sum.

Table 3
|Correlations (Spearman rho, *r*) between selected maternal serum phthalate metabolites in three prospective mother-child cohorts, Raine, Cop1 and Cop2.

Raine, n = 986	MEP	MiBP	MnBP	MHBP	MBzP	MEHP	MECPP	MCMHP	MiNP	MCiOP
MiBP	0.188***									
MnBP	0.273***	0.644***								
MHBP	0.087**	0.119***	0.245***							
MBzP	0.145***	0.358***	0.435***	0.110**						
MEHP	0.110**	0.007	0.222***	−0.102**	0.219***					
MECPP	0.180***	0.177***	0.230***	0.073*	0.135***	0.045				
MCMHP	0.117***	0.112**	0.169***	−0.031	0.102**	0.153***	0.710***			
MiNP	0.110**	0.116***	0.181***	0.295***	0.173***	0.038	0.226***	0.030		
MCiOP	0.141***	0.064*	0.099**	0.097**	0.100**	0.052	0.562***	0.344***	0.254***	
MiDP	−0.059	−0.050	−0.071*	−0.142***	−0.034	0.140***	−0.179***	−0.039	−0.101**	−0.164***

Cop1, n = 213	MEP	MiBP	MnBP	MHBP	MEHP	MECPP	MCMHP	MiNP	MCiOP
MiBP	0.014								
MnBP	−0.150*	0.647***							
MHBP	0.189**	0.069	−0.059						
MEHP	0.050	0.267***	0.377***	0.172*					
MECPP	−0.068	0.141	0.040	0.141*	0.106				
MCMHP	−0.025	−0.256***	−0.359***	0.085	−0.039	0.562***			
MiNP	0.088	−0.165*	−0.298***	0.133#	0.006	−0.067	0.185**		
MCiOP	−0.030	−0.185**	−0.152*	−0.046	−0.076	0.140*	0.231**	0.067	
MiDP	0.017	0.388***	0.488***	0.114#	0.288***	0.017	−0.295***	−0.103	−0.192***

Cop2, n = 103	MEHP	MECPP	MCMHP	MiNP	MCiOP
MECPP	0.208*				
MCMHP	0.168#	0.698***			
MiNP	0.052	0.153	0.182#		
MCiOP	−0.020	0.180#	0.276**	0.005	
MiDP	−0.125	−0.012	0.075	0.376***	0.097

Significant correlations: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, #$P < 0.1$.

not correlated to either MECPP or MCMHP. In Cop2 serum, MEHP was weakly correlated to MECPP ($r = 0.208$, $P < 0.05$) and weakly but only borderline significantly to MCMHP ($r = 0.168$, $P < 0.1$).

The primary and secondary metabolites of DiNP, MiNP and MCiOP, were weakly correlated in Raine serum samples ($r = 0.254$, $P < 0.001$). This did not reach significance in serum from Cop1 and Cop2 (Table 3). MnBP and MHBP were also weakly correlated in serum from the Raine cohort ($r = 0.245$, $P < 0.001$) but not in Cop1.

We also observed many positive correlations between serum metabolites originating from different phthalates, mainly in the Raine cohort where, e.g. secondary metabolites of DEHP and DiNP were weakly to moderately correlated ($r \geq 0.346$, $P < 0.001$) (Table 3).

3.2. Urinary phthalate concentrations in Cop2

Urinary levels of phthalates in Cop2 are presented adjusted for urine osmolality in Table 4 and unadjusted in Table S2.

Seven phthalate metabolites (MEP, MiBP, MnBP, mono-(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP), mono-(2-ethyl-5-oxohexyl) phthalate (MEOHP), MECPP, MCMHP and MCiOP) were detectable in ≥ 83.2% of urine samples. Seven phthalates (MBzP, MEHP, mono-(5-carboxypentyl) phthalate (MCPeP), mono-(6-carboxyhexyl) phthalate (MCHxP), MCPP, mono-hydroxy-iso-nonyl phthalate (MHiNP), mono-oxo-iso-nonyl phthalate (MOiNP)) were measured in between 45.8% and 71.0% of urine samples. Mono-methyl phthalate (MMP) was measured in 25.2% and MiNP in 29.9% of urine samples. All other metabolites were detected in ≤ 15.0% of urine samples. Compared to serum, detection frequencies (% ≥ LOD) of some metabolites were higher in urine. For instance, while MiBP was detectable in only 2.9% of serum samples in Cop2, it was measured in 98.1% of the urine samples.

There were large interindividual variations in urinary phthalate metabolite concentrations (Table 4). In urine, there were moderate to strong correlations between all five DEHP metabolites ($r = 0.731$–0.958, $P < 0.001$) and four DiNP metabolites ($r = 0.524$–0.800, $P < 0.001$) (results not shown).

Significant and weak to moderate correlations between concentrations of the same metabolite in serum and urine samples were observed for MEP ($r = 0.615$, $P < 0.01$), MECPP ($r = 0.533$, $P < 0.01$), MCMHP ($r = 0.496$, $P < 0.01$), and MCiOP ($r = 0.566$, $P < 0.01$) (Table 5).

The concentration of MEHP in serum was not significantly correlated to the concentration in urine (Table 5) but MECPP correlated weakly with urinary MEHP ($r = 0.419$, $P < 0.01$) and moderately with MCMHP ($r = 0.540$, $P < 0.01$). MCMHP in serum was weakly correlated to both MEHP ($r = 0.257$, $P < 0.01$) and MECPP ($r = 0.354$, $P < 0.01$) in urine.

Serum MiNP was not significantly correlated to urinary levels of MiNP ($r = 0.059$, $P = 0.56$) or MCiOP ($r = 0.095$, $P = 0.34$), but MCiOP in serum was weakly correlated to urinary MiNP ($r = 0.304$, $P < 0.01$).

3.3. Differences between the cohorts

Generally, in terms of maternal characteristics and lifestyle the two Danish cohorts were more alike than the Australian cohort (Table S1). Women in the Raine cohort were younger, shorter, weighed less and had lower BMIs ($P < 0.001$) than the two Danish cohorts. Mothers in the two older cohorts were more likely to smoke than mothers in the recent cohort ($P < 0.001$). Due to selection of samples for phthalate analyses belonging to mothers of girls in Cop1, the gender distribution was skewed in this cohort and thus significantly different from the two other cohorts ($P < 0.001$) where the male/female ratio was closer to 50/50. The educational level differed significantly between the Australian and the two Danish cohorts ($P < 0.001$) with 58% of Cop1 and 66% of Cop2 having a university degree while this number was only 10% in the Raine cohort (Table S1).

For most phthalates, we observed significant differences in serum

Table 4
|Urine levels of 32 phthalate metabolites (ng/mL, osmolality adjusted) in pregnant women in a recent prospective mother-child cohort, Cop2 (n = 107).

			Percentiles					
	LOD	% > LOD	min.	25	50	75	95	max.
MMP	0.53	25.2			< LOD	0.49	3.15	5.59
MEP	0.79	95.3	< LOD	5.16	13.0	35.4	590	1697
MiPrP	0.25	2.8					< LOD	3.41
MPrP	0.30	0.0						< LOD
MiBP	0.44	98.1	< LOD	7.05	12.5	23.8	51.6	219
MnBP	0.68	92.5	< LOD	4.72	8.31	14.5	32.0	46.7
MHBP	0.63	3.7					< LOD	83.3
MBzP	0.50	60.4		< LOD	1.14	2.45	11.4	21.4
MPP	0.28	1.9					< LOD	0.8
MHPP	0.35	5.6				< LOD	1.46	4.25
MEHP	0.42	69.2		< LOD	1.03	1.98	3.87	5.15
MEHHP	0.40	87.9	< LOD	1.69	3.54	5.91	12.9	18.5
MEOHP	0.46	84.1	< LOD	1.30	2.59	4.61	10.2	14.1
MECPP	0.28	98.1	< LOD	1.91	2.96	4.91	9.29	13.1
MCMHP	0.22	97.1	< LOD	2.50	4.34	7.11	12.7	29.4
MHxP	0.33	11.2				< LOD	0.83	2.33
MHHxP	0.21	5.6				< LOD	0.08	0.49
MCPeP	0.22	45.8			< LOD	1.15	2.43	5.80
MCHP	0.33	0.0						< LOD
MHepP	0.33	13.1				< LOD	0.65	4.52
MHHpP	0.19	15.0				< LOD	0.92	2.42
MCHxP	0.30	71.0		< LOD	0.95	1.64	4.32	9.51
MOP	0.32	2.8					< LOD	0.84
MCPP	2.32	54.2		< LOD	2.31	5.06	11.7	215
MiNP	0.39	29.9			< LOD	0.32	0.74	1.4
MHiNP	0.61	66.4		< LOD	1.35	2.78	6.05	20.1
MOiNP	0.57	52.3		< LOD	0.49	1.40	4.06	11.8
MCiOP	0.29	83.2	< LOD	0.67	1.57	3.06	9.55	35.9
MiDP	0.91	3.7						< LOD
MHiDP	0.27	0.9					< LOD	0.79
MOiDP	0.32	14.0				< LOD	0.50	1.52
MCiNP	0.27	0.0						< LOD

LOD, limit of detection. For abbreviations of phthalate metabolites, see Table 1.

metabolite levels between the three cohorts (Table 6, Figs. 1 and 2). Generally, serum metabolite levels were lower in the most recent Cop2 cohort than in the earlier samples. This was reflected in serum levels of ΣDEHPm, ΣLMW phth.m and Σall phth.m with median serum levels of Σall phth.m in Raine and Cop1 being almost 30- and 40-fold higher than the levels in Cop2 (Table 2, Fig. 2).

We found a decreasing level of secondary DEHP metabolites (MECPP, MCMHP) and ΣDEHPm from Raine to Cop1 and Cop2 (Figs. 1 and 2) in serum. A similar pattern was observed for MEP, MnBP and MCiOP (Fig. 1). The median serum levels of MiBP, MiDP and MHBP were significantly higher in Cop1 than in Raine which, in turn, was significantly higher than in Cop2 (Fig. 1, Table 6). Σall phth.m was higher in Cop1 than in Raine and Cop2 (Fig. 2, Table 6).

In sensitivity analyses, adjustment for demographic variables did not substantially change these findings (data not shown).

Table 6
|Differences between selected maternal serum phthalate metabolite levels in three prospective mother-child cohorts, Raine (n = 986), Cop1 (n = 213) and Cop2 (n = 103), tested by Mann-Whitney U test.

	Raine vs. Cop1	Raine vs. Cop2	Cop1 vs. Cop2
MEP	0.728	< **0.001**	< **0.001**
MiBP	< **0.001**	< **0.001**	< **0.001**
MnBP	0.284	< **0.001**	< **0.001**
MHBP	< **0.001**	< **0.001**	< **0.001**
MBzP	< **0.001**	< **0.001**	0.063
MECPP	< **0.001**	< **0.001**	< **0.001**
MCMHP	**0.005**	< **0.001**	< **0.001**
MCPP	< **0.001**	< **0.001**	< **0.001**
MCiOP	< **0.001**	< **0.001**	0.147
MiDP	< **0.001**	**0.026**	< **0.001**
Σall phth.m	< **0.001**	< **0.001**	< **0.001**

Depicted values are P values. Bold font indicates a significant difference between cohorts with P values < 0.05.

Table 5
|Correlations (Spearman rho, r) between selected maternal serum and urine phthalate metabolites in Cop2 (n = 102).

		Urine concentrations					
		MEP	MEHP	MECPP	MCMHP	MiNP	MCiOP
Serum concentrations	MEP	0.615***	0.150	0.063	0.099	−0.041	0.012
	MEHP	0.169#	0.177#	0.223*	0.174#	−0.003	0.050
	MECPP	0.244*	0.419***	0.533***	0.540***	0.091	0.238*
	MCMHP	0.171#	0.257**	0.354***	0.496***	−0.055	0.132
	MiNP	0.040	0.040	0.096	0.151	0.059	0.095
	MCiOP	0.136	0.154	0.217*	0.432***	0.304**	0.566***

Urine phthalate levels > LOD were adjusted for osmolality. Significant correlations: *P < 0.05, **P < 0.01, ***P < 0.001, #P < 0.1.

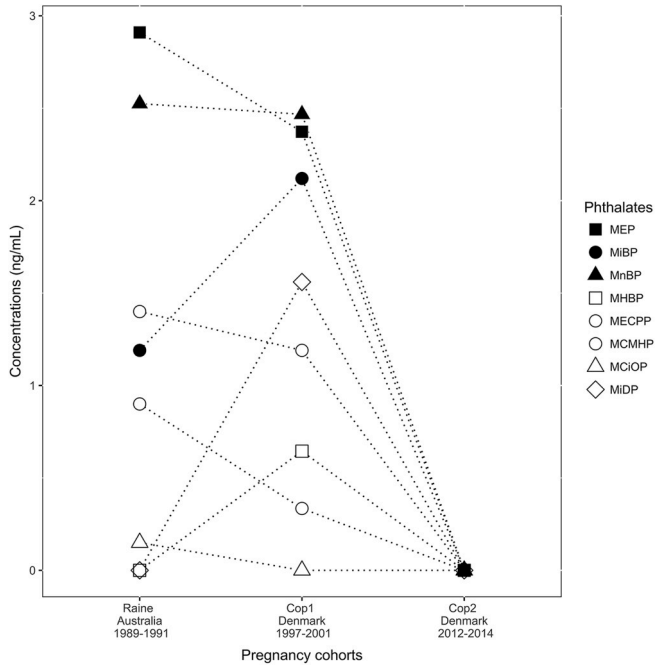

Fig. 1. |Median maternal serum concentrations of phthalate metabolites (ng/mL) in three mother-child cohorts. For abbreviations of phthalate metabolites, please see Table 1. (Single column fitting image).

4. Discussion

In this study we compared maternal serum phthalate metabolite levels in three different birth cohorts: the Australian Raine cohort (approximately 30 years old), the Danish Cop1 cohort (approximately 20 years old) and the Danish Cop2 cohort (6–8 years old).

As urine samples were not collected during pregnancy in the two oldest cohorts, only serum samples were available for phthalate metabolite measurements. In the recent Cop2 cohort, both serum and urine were collected for measurements of chemicals and clinical biomarkers.

To our knowledge, this is the first study to explore temporal and geographical trends in prenatal exposure to phthalates with all phthalate measurements performed in the same laboratory.

Urine is currently considered the best matrix to evaluate phthalate exposure as serum measurements have some limitations.

First, phthalate diesters are referred to as non-persistent chemicals, and several studies have shown that phthalate metabolite levels are 10–100 times higher in urine than in serum (Frederiksen et al., 2010; Koch et al., 2005; Silva et al., 2003) due to metabolism and elimination. Consequently, not all metabolites are present in detectable levels in circulating blood. This was also demonstrated in our study where the secondary oxidized metabolites of DEHP (MEHHP, MEOHP) and DiNP (MHiNP, MOiNP) in serum were below their respective LODs in all three cohorts. Generally, both concentrations and detection frequencies were higher in urine than in serum samples.

Second, as it has been shown that diester phthalates can be cleaved to monoesters by endogenous esterases present in blood, there is a potential risk that measured serum levels of phthalate monoesters may be falsely increased if post-collection contamination of the samples has occurred (Silva et al., 2005). To prevent this, it is possible to inhibit esterase activity by adding acid to the serum as soon as it has been separated from the blood cells. However, this best practice procedure was not established when the two early cohorts were initiated. It is possible to indirectly assess a potential contamination by evaluating the inter-individual variation in measured serum levels as a systematic source of contamination during sample handling would most likely lead to a systematic background level and less inter-individual variation. Correlations between different metabolites of the same diester can also be used to assess a potential contamination as only the initial cleavage of a diester to a monoester phthalate can occur in the samples after collection. Other metabolic reactions (e.g. phase one and phase two metabolism) is considered only to occur *in vivo*.

Our findings support this consideration that serum levels of the hydroxylated monoesters may reflect contamination during and after serum sampling. As opposed to most other phthalate metabolites, MEHP was detected in nearly all serum samples in Cop2 but only in around two thirds of the urine samples, and concentrations were similar to those in Raine and Cop1 with little interindividual variation. Thus, some contamination of Cop2 serum samples with DEHP is likely to have occurred although acid was added after collection, and MEHP was therefore excluded from all sums and analyses. However, the high levels of MEHP metabolites found in the urine samples of the same pregnant women indicate that there was an actual DEHP exposure as well.

In Cop2, the pregnant women were recruited at amniocentesis scheduled for reasons other than exposure biomonitoring. All three samples (urine, serum, amniotic fluid) were collected within an hour of the procedure (Krause et al., 2018). As an amniocentesis is performed with the aid of ultrasound guidance, it imposes a risk of procedure-related exposure, as found in a recent study (Messerlian et al., 2017). Previous studies have shown that exposure to phthalates, particularly DEHP, may be high during medical procedures (Heudorf et al., 2007) such as blood transfusions (Sampson and de Korte, 2011), haemodialysis (Dine et al., 2000) and cardiopulmonary bypass (Takahashi et al., 2008). While urinary levels of phthalate metabolites generally increase 7–12 h after exposure (Janjua et al., 2007; Messerlian et al., 2017), they may be detected in serum already 15 min after ingestion (Kessler et al., 2012). The unexpected high serum MEHP concentration in Cop2 may therefore be explained at least in part by the medical procedure. For all other phthalates, women in Cop2 were comparable to healthy Danish women's exposure levels (Frederiksen et al., 2014a).

Similarly, Cop2 serum samples showed high MiNP concentrations with little interindividual variation, and primary and secondary metabolites of DiNP were not correlated. MiNP was measured in 99% of the serum samples, but in only 29.9% of the urine samples. Further, median MiNP serum levels in Cop2 were approximately six times higher than in Cop1 and 10-fold higher than urine levels. Also, serum MiNP in Cop2 was not correlated to either urinary MiNP or urinary MCiOP. Thus, sample contamination with DiNP cannot be excluded in this recent cohort and MiNP was omitted from all sums and analyses. The significant albeit weak correlation between MiNP and MCiOP in serum from Raine indicates that the presence of MiNP in serum is caused by exposure to DiNP in this cohort.

In serum from all three cohorts, correlations between the two carboxylated metabolites of DEHP were highly significant and moderate to strong. In the most recent cohort, correlations between serum and urine levels of all investigated secondary metabolites were highly significant and of moderate strength. This supports the conclusion that secondary metabolites in serum seem more valid proxies for phthalate exposure to HMW phthalates than primary metabolites. This is in agreement previous studies (Calafat et al., 2015, 2013; Hauser and Calafat, 2005; Högberg et al., 2008; Koch and Calafat, 2009; Wittassek et al., 2009).

The primary and secondary metabolites of DnBP were significantly but only weakly correlated in the Raine cohort. In the recent cohort, most metabolites of the LMW phthalates, e.g. MiBP, MnBP and MHBP, were not detected in serum. MEP was detected in enough Cop2 serum samples and the correlation between serum and urine levels was significant and moderate, indicating that MEP levels in serum may reflect exposure to DEP. Serum levels of some metabolites originating from different phthalate diesters, e.g. MiBP and MnBP, were also significantly and moderately correlated. This has also been found in other studies (Frederiksen et al., 2020).

In summary, we were able to detect phthalate exposure in a substantial part of the pregnant women by using serum samples, especially

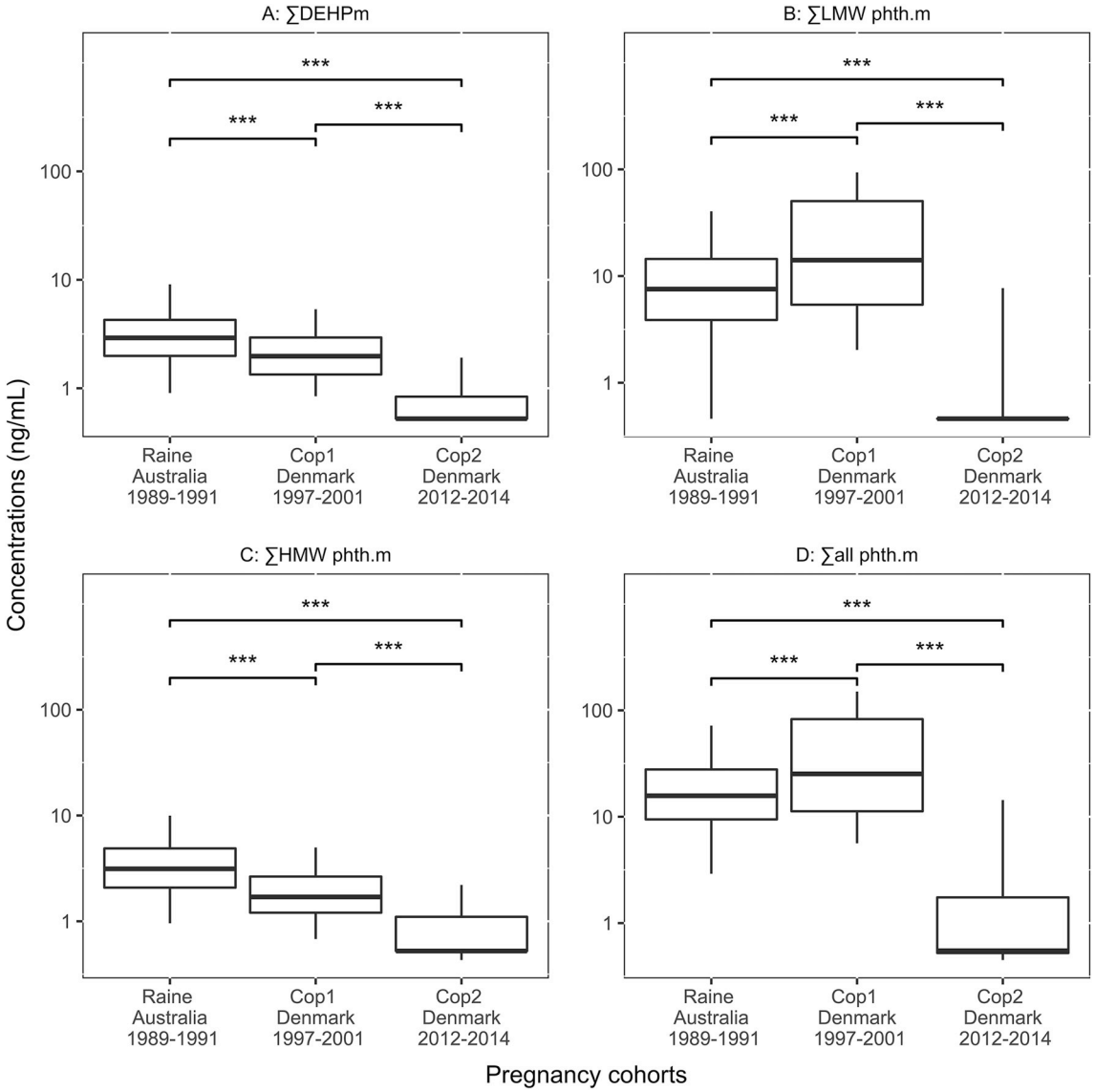

Fig. 2. |Sums are defined in Table 1. Percentiles displayed in the box plots are 5, 25, 50, 75 and 95. Differences between cohorts were tested by Mann-Whitney *U* test, and *P* values are displayed as following: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. Please note the log transformed Y axis.

in the two older cohorts and to a lesser extent in the most recent cohort. Levels of both primary and secondary metabolites were generally lower and had lower detection frequencies than in urine. Serum is thus an inappropriate matrix for phthalate exposure assessment in recent cohorts where phthalate exposure is low. Correlations between primary and secondary metabolites as well as between serum and urine levels were generally weak to moderate indicating substantial noise, and serum phthalate measurements are not appropriate for biomonitoring.

We suggest that serum may be used to assess maternal phthalate exposure only in historical cohorts established before regulations on phthalate use were enacted and when urine samples are not available. The correct precautions should be taken when evaluating and interpreting the data. The relevance of serum phthalate measurements is to exploit data from historical cohorts to assess potential long-term harmful effects of prenatal exposure to phthalates. Studies have shown considerable day-to-day variation in phthalate exposure. Thus, if associations between early phthalate exposure of historical cohorts and long-term health outcomes are found, they are likely true and deserve further corroboration. Reversely, failure to find an association could be due to noise in exposure classification. This is further supported by recent studies of our research group (Assens et al., 2019; Hart et al., 2014, 2018).

The first temporary restrictions on the use of phthalates were enacted in December 1999 in the EU (Koch et al., 2017); these involved three phthalates (DEHP, BBzP, DnBP) and in addition the use of three other phthalates (DiNP, DiDP, DOP) specifically in toys and childcare articles. Since 2007, permanent restrictions on phthalate-use have been enacted by REACH (Koch et al., 2017). In 2015, four phthalates (DiBP, DnBP, BBzP, DEHP) were added to the so-called authorization list, i.e. Annex XIV of REACH (Koch et al., 2017). Certain medical equipment, where a high level of flexibility is necessary, may still contain phthalates, most commonly DEHP, as plasticisers (European Commission, 2019; Sampson and de Korte, 2011). From July 2020, the use of DnBP, DiBP, BBzP and DEHP will be further restricted on the European market (European Commission, 2018).

We observed a decline from Cop1 to Cop2 in metabolites of phthalates that have been subject to regulations, including MiBP, MnBP, MiDP and secondary metabolites of DEHP. MBzP and MCiOP were only measured in very few samples in both cohorts. Thus, regulations appear to have resulted in lower exposure which is in agreement with findings in European and American studies using urinary phthalate measurements (Centers for Disease Control and Prevention,

2019; Frederiksen et al., 2020, 2014a; Gyllenhammar et al., 2017; Koch et al., 2017; Larsson et al., 2017; Schoeters et al., 2017; Zota et al., 2014). When evaluating the sum of DEHP metabolites in our study, a significant decrease from Cop1 to Cop2 was observed. This is in agreement to the early restrictions on DEHP which has also been apparent in German time-trend studies (Koch et al., 2017).

In the US, DnBP, BBzP and DEHP were banned in any amount > 0.1% in child care articles in 2008, and at the same time a provisional restriction was placed on DiNP, DiDP and DOP in toys that can be put in the mouth (Zota et al., 2014). As a result, urinary metabolite concentrations of DnBP, BBzP and DEHP declined approximately 20–50% between 2001 and 2010 (Zota et al., 2014). In the same period, DiBP and DiNP increased by > 100%. This increase in phthalates substituting DEHP was not seen in this study but one German time-trend study also found an increase in urinary DiNP metabolites between 1988 and 2008 (Göen et al., 2011). In the US population, MnBP levels remained high in 2007–8, but decreased significantly by 2009–10 (Zota et al., 2014), i.e. some years later than in the current study, which is in agreement with a later regulation.

However, legislation alone does not explain our observed time trends. Although DEP remains unregulated in the EU, we found a significant decline in MEP levels from Cop1 to Cop2. A similar trend was reported by both European and American researchers (Centers for Disease Control and Prevention, 2019; Frederiksen et al., 2020; Gyllenhammar et al., 2017; Koch et al., 2017; Larsson et al., 2017; Zota et al., 2014). This most likely reflects non-regulated differences in the use and/or production of phthalate-containing products caused by an increasing awareness of the general population and the producers.

While we observed higher serum levels of MiBP, MiDP and MHBP in Cop1, serum levels of all other metabolites, including DEHP metabolites, were found in higher concentrations in Australian women. In Australia, the first temporary ban on DEHP was brought in 2010, made permanent in 2011 and included certain children's soft plastic toys and childcare articles (Australian Competition and Consumer Commission, 2010). As in the rest of the world, legislation on phthalate use in Australia was enacted only after the turn of the millennium. Differences between Raine and Cop1 may thus be explained by non-regulated national or consumer differences in phthalate use.

5. Conclusion

In this study on pregnant women, we observed weak to strong and significant correlations between some secondary phthalate metabolites in serum from early population-based cohorts and between serum and urinary levels of some phthalate metabolites in a recent cohort. These findings indicate that historical biobanked serum samples may be used for assessment of exposure to some but not all phthalates, when urine is not available. We found that the primary metabolites of the HMW phthalate diesters in serum should be handled cautiously whereas the carboxylated metabolites seem more valid. Serum phthalate measurements are not suitable for human biomonitoring and should only be used to exploit historical cohort biobanks to evaluate potential long-term effects of prenatal phthalate exposure.

We observed that prenatal phthalate exposure has decreased over time from the early 1990s to the 2010s, most likely due to regulations restricting the use of phthalates. National/local production and consumer patterns also seem to affect exposure.

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Ethics

Ethical approval for the Western Australian Pregnancy Cohort (Raine) Study was obtained from the University of Western Australia Human Research Ethics Committee, and all participants provided informed consent.

The Cop1 and Cop2 studies were approved by the Ethics Committee of the Capital Region of Denmark (KF 01–030/97, KF 02–125/95 and H-2-2012-76) as well as the Danish Data Protection Agency. All women received written information, and informed consent was obtained from all participants.

All three studies were carried out in accordance with the Declaration of Helsinki for experiments involving humans.

CRediT authorship contribution statement

Louise S. Henriksen: Writing - original draft, Writing - review & editing, Formal analysis. **Barbara K. Mathiesen:** Formal analysis, Visualization, Writing - review & editing. **Maria Assens:** Formal analysis, Writing - review & editing. **Marianna Krause:** Investigation, Writing - review & editing. **Niels Erik Skakkebæk:** Writing - review & editing. **Anders Juul:** Writing - review & editing, Conceptualization. **Anna-Maria Andersson:** Conceptualization, Project administration, Writing - review & editing. **Roger J. Hart:** Conceptualization, Funding acquisition, Project administration, Writing - review & editing. **John P. Newnham:** Resources, Writing - review & editing. **Jeffrey A. Keelan:** Resources, Writing - review & editing. **Craig Pennell:** Funding acquisition, Writing - review & editing. **Katharina M. Main:** Conceptualization, Resources, Funding acquisition, Project administration, Writing - review & editing. **Hanne Frederiksen:** Conceptualization, Methodology, Writing - original draft, Writing - review & editing, Visualization.

Declarations of competing interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envres.2020.109231.

References

Albro, P.W., Thomas, R.O., 1973. Enzymatic hydrolysis of di-(2-ethylhexyl) phthalate by lipases. Biochim. Biophys. Acta Lipids Lipid. Metabol. 306, 380–390. https://doi.org/10.1016/0005-2760(73)90176-8.

Assens, M., Frederiksen, H., Petersen, J.H., Larsen, T., Skakkebæk, N.E., Juul, A., Andersson, A., Main, K.M., 2019. Variations in repeated serum concentrations of UV filters, phthalates, phenols and parabens during pregnancy. Environ. Int. 123, 318–324. https://doi.org/10.1016/j.envint.2018.11.047.

Australian Competition and Consumer Commission, 2010. Children's Plastic Products with More than 1 Per Cent Diethylhexyl Phthalate (DEHP).

Baird, D.D., Saldana, T.M., Nepomnaschy, P.A., Hoppin, J.A., Longnecker, M.P., Weinberg, C.R., Wilcox, A.J., 2010. Within-person variability in urinary phthalate metabolite concentrations: measurements from specimens after long-term frozen storage. J. Expo. Sci. Environ. Epidemiol. 20, 169–175. https://doi.org/10.1038/jes.2009.17.

Boisen, K.A., Kaleva, M., Main, K.M., Virtanen, H.E., Haavisto, A.-M., Schmidt, I.M., Chellakooty, M., Damgaard, I.N., Mau, C., Reunanen, M., Skakkebaek, N.E., Toppari, J., 2004. Difference in prevalence of congenital cryptorchidism in infants between two Nordic countries. Lancet 363, 1264–1269. https://doi.org/10.1016/S0140-6736(04)15998-9.

Bornehag, C.G., Carlstedt, F., Jönsson, B.A., Lindh, C.H., Jensen, T.K., Bodin, A., Jonsson, C., Janson, S., Swan, S.H., 2015. Prenatal phthalate exposures and anogenital distance in Swedish boys. Environ. Health Perspect. 123, 101–107. https://doi.org/10.1289/ehp.1408163.

Calafat, A., Longnecker, M., Koch, H., Swan, S., Hauser, R., Goldman, L., Lanphear, B., Rudel, R., Engel, S., Teitelbaum, S., Whyatt, R., Wolff, M., 2015. Optimal exposure biomarkers for nonpersistent chemicals in environmental epidemiology. Environ. Health Perspect. 123, 166–168. https://doi.org/10.1289/ehp.1510041.

Calafat, A.M., Koch, H.M., Swan, S.H., Hauser, R., Goldman, L.R., Lanphear, B.P., Longnecker, M.P., Rudel, R.A., Teitelbaum, S.L., Whyatt, R.M., Wolff, M.S., 2013. Misuse of blood serum to assess exposure to bisphenol A and phthalates. Breast Cancer Res. 15, 1–2. https://doi.org/10.1186/bcr3494.

Calafat, A.M., Needham, L.L., 2008. Factors affecting the evaluation of biomonitoring data for human exposure assessment. Int. J. Androl. 31, 139–143. https://doi.org/10.1111/j.1365-2605.2007.00826.x.

Calafat, A.M., Needham, L.L., Silva, M.J., Lambert, G., 2004. Exposure to di-(2-ethylhexyl) phthalate among premature neonates in a neonatal intensive care unit. Pediatrics 113, e429–e434. https://doi.org/10.1542/peds.113.5.e429.

Centers for Disease Control and Prevention, 2019. Fourth National Report on Human Exposure to Environmental Chemicals. Updated Table, January 2019.

Chellakooty, M., Vangsgaard, K., Larsen, T., Scheike, T., Falck-Larsen, J., Legarth, J., Andersson, A.M., Main, K.M., Skakkebæk, N.E., Juul, A., 2004. A longitudinal study of intrauterine Growth and the placental Growth hormone (GH)-Insulin-Like Growth factor I Axis in maternal circulation: association between placental GH and fetal Growth. J. Clin. Endocrinol. Metab. 89, 384–391. https://doi.org/10.1210/jc.2003-030282.

Damgaard, I.N., Jensen, T.K., Petersen, J.H., Skakkebæk, N.E., Toppari, J., Main, K.M., Boisen, K.A., Chellakooty, M., Schmidt, I.M., Kaleva, M.M., Suomi, A.M., Virtanen, H.E., 2008. Risk factors for congenital cryptorchidism in a prospective birth cohort study. PLoS One 3. https://doi.org/10.1371/journal.pone.0003051.

Dine, T., Luyckx, M., Gressier, B., Brunet, C., Souhait, J., Nogarede, S., Vanpoucke, J., Courbon, F., Plusquellec, Y., Houin, G., 2000. A pharmacokinetic interpretation of increasing concentrations of DEHP in haemodialysed patients. Med. Eng. Phys. 22, 157–165. https://doi.org/10.1016/S1350-4533(00)00022-9.

European Chemicals Agency (ECHA), 2017. Annex to a News Alert ECHA/NA/17/05. (Helsinki).

European Commission, 2019. Guidelines on the Benefit-Risk Assessment of the Presence of Phthalates in Certain Medical Devices.

European Commission, 2018. Commission regulation (EU) 2018/2005 of 17 december 2018 amending annex XVII to regulation (EC) No 1907/2006 of the European parliament and of the Council concerning the registration, evaluation, authorisation and restriction of chemicals (REACH) as regar. Off. J. Eur. Union 6 L 322/14-L 322/19.

European Commission, 2007. Commision Directive 2007/19/EC of 2 April 2007 Amending Directive 2002/72/EC Relating to Plastic Materials and Articles Intended to Come into Contact with Food and Council Directive 85/572/EEC Laying Down the List of Simulants to Be Used for Testing Migra.

Foster, P.M.D., 2006. Disruption of reproductive development in male rat offspring following in utero exposure to phthalate esters. Int. J. Androl. 29, 140–147. https://doi.org/10.1111/j.1365-2605.2005.00563.x.

Frederiksen, H., Jensen, T.K., Jørgensen, N., Kyhl, H.B., Husby, S., Skakkebæk, N.E., Main, K.M., Juul, A., Andersson, A.M., 2014a. Human urinary excretion of non-persistent environmental chemicals: an overview of Danish data collected between 2006 and 2012. Reproduction 147, 555–565. https://doi.org/10.1530/REP-13-0522.

Frederiksen, H., Jørgensen, N., Andersson, A.-M., 2010. Correlations between phthalate metabolites in urine, serum, and seminal plasma from young Danish men determined by isotope dilution liquid chromatography tandem mass spectrometry. J. Anal. Toxicol. 34, 400–410. https://doi.org/10.1038/jes.2010.6.

Frederiksen, H., Kuiri-hänninen, T., Main, K.M., Dunkel, L., Sankilampi, U., 2014b. A longitudinal study of urinary phthalate excretion in 58 full-term and 67 preterm infants from birth through 14 months. Environ. Health Perspect. 998, 998–1005.

Frederiksen, H., Nielsen, O., Koch, H.M., Skakkebaek, N.E., Juul, A., Andersson, A., 2020. Changes in urinary excretion of phthalates, phthalate substitutes, bisphenols and other polychlorinated and phenolic substances in young Danish men; 2009–2017. Int. J. Hyg Environ. Health 223 (1), 93–105. https://doi.org/10.1016/j.ijheh.2019.10.002.

Frederiksen, H., Soerensen, K., Mouritsen, A., Aksglaede, L., Hagen, C.P., Petersen, J.H., Skakkebaek, N.E., Andersson, A.M., Juul, A., 2012. High urinary phthalate concentration associated with delayed pubarche in girls. Int. J. Androl. 35, 216–226. https://doi.org/10.1111/j.1365-2605.2012.01260.x.

Gray, L.E., Ostby, J., Furr, J., Price, M., Veeramachaneni, D.N.R., Parks, L., 2000. Perinatal exposure to the phthalates DEHP, BBP, and DINP, but not DEP, DMP, or DOTP, alters sexual differentiation of the male rat. Toxicol. Sci. 58, 350–365. https://doi.org/10.1093/toxsci/58.2.350.

Gyllenhammar, I., Glynn, A., Jönsson, B.A.G., Lindh, C.H., Darnerud, P.O., Svensson, K., Lignell, S., 2017. Diverging temporal trends of human exposure to bisphenols and plastizisers, such as phthalates, caused by substitution of legacy EDCs? Environ. Res. 153, 48–54. https://doi.org/10.1016/j.envres.2016.11.012.

Göen, T., Dobler, L., Koschorreck, J., Müller, J., Wiesmüller, G.A., Drexler, H., Kolossa-Gehring, M., 2011. Trends of the internal phthalate exposure of young adults in Germany – Follow-up of a retrospective human biomonitoring study. Int. J. Hyg Environ. Health 215, 36–45. https://doi.org/10.1016/j.ijheh.2011.07.011.

Hart, R., Doherty, D.A., Frederiksen, H., Keelan, J.A., Hickey, M., Sloboda, D., Pennell, C.E., Newnham, J.P., Skakkebaek, N.E., Main, K.M., 2014. The influence of antenatal exposure to phthalates on subsequent female reproductive development in adolescence: a pilot study. Reproduction 147, 379–390. https://doi.org/10.1530/REP-13-0331.

Hart, R.J., Frederiksen, H., Doherty, D.A., Keelan, J.A., Skakkebaek, N.E., Minaee, N.S., McLachlan, R., Newnham, J.P., Dickinson, J.E., Pennell, C.E., Norman, R.J., Main, K.M., 2018. The Possible impact of antenatal exposure to Ubiquitous Phthalates upon Male reproductive Function at 20 Years of age. Front. Endocrinol. 9, 1–11. https://doi.org/10.3389/fendo.2018.00288.

Hauser, R., Calafat, A.M., 2005. Phthalates and human health. Occup. Environ. Med. 62, 806–818. https://doi.org/10.1136/oem.2004.017590.

Heudorf, U., Mersch-Sundermann, V., Angerer, J., 2007. Phthalates: toxicology and exposure. Int. J. Hyg Environ. Health 210, 623–634. https://doi.org/10.1016/j.ijheh.2007.07.011.

Hickey, M., Sloboda, D.M., Atkinson, H.C., Doherty, D. a, Franks, S., Norman, R.J., Newnham, J.P., Hart, R., 2009. The relationship between maternal and umbilical cord androgen levels and polycystic ovary syndrome in adolescence: a prospective cohort study. J. Clin. Endocrinol. Metab. 94, 3714–3720. https://doi.org/10.1210/jc.2009-0544.

Högberg, J., Hanberg, A., Berglund, M., Skerfving, S., Remberger, M., Calafat, A.M., Filipsson, A.F., Jansson, B., Johansson, N., Appelgren, M., Håkansson, H., 2008. Phthalate diesters and their metabolites in human breast milk, blood or serum, and urine as biomarkers of exposure in vulnerable populations. Environ. Health Perspect. 116, 334–339. https://doi.org/10.1289/ehp.10788.

Inoue, K., Kawaguchi, M., Yamanaka, R., Higuchi, T., Ito, R., Saito, K., Nakazawa, H., 2005. Evaluation and analysis of exposure levels of di(2-ethylhexyl) phthalate from blood bags. Clin. Chim. Acta 358, 159–166. https://doi.org/10.1016/j.cccn.2005.02.019.

Janjua, N.R., Mortensen, G.K., Andersson, A.M., Kongshoj, B., Skakkebæk, N.E., Wulf, H.C., 2007. Systemic uptake of diethyl phthalate, dibutyl phthalate, and butyl paraben following whole-body topical application and reproductive and thyroid hormone levels in humans. Environ. Sci. Technol. 41, 5564–5570. https://doi.org/10.1021/es0628755.

Jensen, T.K., Frederiksen, H., Kyhl, H.B., Lassen, T.H., Swan, S.H., Bornehag, C.G., Skakkebaek, N.E., Main, K.M., Lind, D.V., Husby, S., Andersson, A.M., 2016. Prenatal exposure to phthalates and anogenital distance in male infants from a low-exposed Danish cohort (2010-2012). Environ. Health Perspect. 124, 1107–1113. https://doi.org/10.1289/ehp.1509870.

Joensen, U.N., Frederiksen, H., Jensen, M.B., Lauritsen, M.P., Olesen, I.A., Lassen, T.H., Andersson, A.M., Jørgensen, N., 2012. Phthalate excretion pattern and testicular function: a study of 881 healthy Danish men. Environ. Health Perspect. 120, 1397–1403. https://doi.org/10.1289/ehp.1205113.

Johansson, H.K.L., Svingen, T., Fowler, P.A., Vinggaard, A.M., Boberg, J., 2017. Environmental influences on ovarian dysgenesis — developmental windows sensitive to chemical exposures. Nat. Rev. Endocrinol. 13, 400–414. https://doi.org/10.1038/nrendo.2017.36.

Johns, L.E., Cooper, G.S., Galizia, A., Meeker, J.D., 2015. Exposure assessment issues in epidemiology studies of phthalates. Environ. Int. 85, 27–39. https://doi.org/10.1016/j.envint.2015.08.005.

Jurewicz, J., Hanke, W., 2011. Exposure to phthalates: reproductive outcome and children health. A review of epidemiological studies. Int. J. Occup. Med. Environ. Health 24, 115–141. https://doi.org/10.2478/s13382-011-0022-2.

Kato, K., Silva, M.J., Brock, J.W., Reidy, J.A., Malekl, N.A., Hodge, C.C., Nakazawa, H., Needham, L.L., Barr, D.B., 2003. Quantitative detection of nine phthalate metabolites in human serum using reversed-phase high-performance liquid chromatography-electrospray ionization-tandem mass spectrometry. J. Anal. Toxicol. 27, 284–289. https://doi.org/10.1093/jat/27.5.284.

Kessler, W., Numtip, W., Völkel, W., Seckin, E., Csanády, G.A., Pütz, C., Klein, D., Fromme, H., Filser, J.G., 2012. Kinetics of di(2-ethylhexyl) phthalate (DEHP) and mono(2-ethylhexyl) phthalate in blood and of DEHP metabolites in urine of male volunteers after single ingestion of ring-deuterated DEHP. Toxicol. Appl. Pharmacol. 264, 284–291. https://doi.org/10.1016/j.taap.2012.08.009.

Koch, H.M., Bolt, H.M., Preuss, R., Angerer, J., 2005. New metabolites of di(2-ethylhexyl) phthalate (DEHP) in human urine and serum after single oral doses of deuterium-labelled DEHP. Arch. Toxicol. 79, 367–376. https://doi.org/10.1007/s00204-004-0642-4.

Koch, H.M., Calafat, A.M., 2009. Human body burdens of chemicals used in plastic manufacture. Philos. Trans. R. Soc. B Biol. Sci. 364, 2063–2078. https://doi.org/10.1098/rstb.2008.0208.

Koch, H.M., Rüther, M., Schütze, A., Conrad, A., Pälmke, C., Apel, P., Brüning, T., Kolossa-Gehring, M., 2017. Phthalate metabolites in 24-h urine samples of the German Environmental Specimen Bank (ESB) from 1988 to 2015 and a comparison with US NHANES data from 1999 to 2012. Int. J. Hyg Environ. Health 220, 130–141. https://doi.org/10.1016/j.ijheh.2016.11.003.

Krause, M., Frederiksen, H., Sundberg, K., Jørgensen, F.S., Jensen, L.N., Nørgaard, P., Jørgensen, C., Ertberg, P., Juul, A., Drzewiecki, K.T., Skakkebaek, N.E., Andersson, A.M., 2018. Presence of benzophenones commonly used as UV filters and absorbers in paired maternal and fetal samples. Environ. Int. 110, 51–60. https://doi.org/10.1016/j.envint.2017.10.005.

Larsson, K., Lindh, C.H., Jönsson, B.A., Giovanoulis, G., Bibi, M., Bottai, M., Bergström, A., Berglund, M., 2017. Phthalates, non-phthalate plasticizers and bisphenols in Swedish preschool dust in relation to children's exposure. Environ. Int. 102, 114–124. https://doi.org/10.1016/j.envint.2017.02.006.

Larsson, K., Ljung Björklund, K., Palm, B., Wennberg, M., Kaj, L., Lindh, C.H., Jönsson, B.A.G., Berglund, M., 2014. Exposure determinants of phthalates, parabens, bisphenol A and triclosan in Swedish mothers and their children. Environ. Int. 73, 323–333. https://doi.org/10.1016/j.envint.2014.08.014.

Lassen, T.H., Frederiksen, H., Jensen, T.K., Petersen, J.H., Main, K.M., Skakkebæk, N.E., Jørgensen, N., Kranich, S.K., Andersson, A.M., 2013. Temporal variability in urinary excretion of bisphenol A and seven other phenols in spot, morning, and 24-h urine samples. Environ. Res. 126, 164–170. https://doi.org/10.1016/j.envres.2013.07.001.

Main, K.M., Mortensen, G.K., Kaleva, M.M., Boisen, K.A., Damgaard, I.N., Chellakooty, M., Schmidt, I.M., Suomi, A.M., Virtanen, H.E., Petersen, J.H., Andersson, A.-M., Toppari, J., Skakkebaek, N.E., 2006a. Human breast milk contamination with phthalates and alterations of endogenous reproductive hormones in infants three months of age. Environ. Health Perspect. 114, 270–276. https://doi.org/10.1289/ehp.8075.

Main, K.M., Mortensen, G.K., Kaleva, M.M., Boisen, K.A., Damgaard, I.N., Chellakooty, M., Schmidt, I.M., Suomi, A.M., Virtanen, H.E., Petersen, J.H., Andersson, A.M., Toppari, J., Skakkebæk, N.E., 2006b. Human breast milk contamination with phthalates and alterations of endogenous reproductive hormones in infants three months of age. Environ. Health Perspect. 114, 270–276. https://doi.org/10.1289/ehp.8075.

Mariana, M., Feiteiro, J., Verde, I., Cairrao, E., 2016. The effects of phthalates in the cardiovascular and reproductive systems: a review. Environ. Int. 94, 758–776. https://doi.org/10.1016/j.envint.2016.07.004.

Matsumoto, M., Hirata-Koizumi, M., Ema, M., 2008. Potential adverse effects of phthalic acid esters on human health: a review of recent studies on reproduction. Regul. Toxicol. Pharmacol. 50, 37–49. https://doi.org/10.1016/j.yrtph.2007.09.004.

Messerlian, C., Mustieles, V., Wylie, B.J., Ford, J.B., Keller, M., Ye, X., Calafat, A.M., Williams, P.L., Hauser, R., 2017. Ultrasound gel as an unrecognized source of exposure to phthalates and phenols among pregnant women undergoing routine scan. Int. J. Hyg Environ. Health 220, 1285–1294. https://doi.org/10.1016/j.ijheh.2017.08.003.

Newnham, J.P., Evans, S.F., Michael, C.A., Stanley, F.J., Landau, L.I., 1993. Effects of frequent ultrasound during pregnancy: a randomised controlled trial. Lancet 342, 887–891. https://doi.org/10.1016/0140-6736(93)91944-H.

Ormond, G., Nieuwenhuijsen, M.J., Nelson, P., Toledano, M.B., Iszatt, N., Geneletti, S., Elliott, P., 2009. Endocrine disruptors in the workplace, hair spray, folate supplementation, and risk of hypospadias: case-control study. Environ. Health Perspect. 117, 303–307. https://doi.org/10.1289/ehp.11933.

Sampson, J., de Korte, D., 2011. DEHP-plasticised PVC: relevance to blood services. Transfus. Med. 21, 73–83. https://doi.org/10.1111/j.1365-3148.2010.01056.x.

Sathyanarayana, S., Grady, R., Barrett, E.S., Redmon, B., Nguyen, R.H.N., Barthold, J.S., Bush, N.R., Swan, S.H., 2016. First trimester phthalate exposure and male newborn genital anomalies. Environ. Res. 151, 777–782. https://doi.org/10.1016/j.envres.2016.07.043.

Schoeters, G., Govarts, E., Bruckers, L., Den Hond, E., Nelen, V., De Henauw, S., Sioen, I., Nawrot, T.S., Plusquin, M., Vriens, A., Covaci, A., Loots, I., Morrens, B., Coertjens, D., Van Larebeke, N., De Craemer, S., Croes, K., Lambrechts, N., Colles, A., Baeyens, W., 2017. Three cycles of human biomonitoring in flanders − time trends observed in the flemish environment and health study. Int. J. Hyg Environ. Health 220, 36–45. https://doi.org/10.1016/j.ijheh.2016.11.006.

Silva, M.J., Barr, D.B., Reidy, J.A., Kato, K., Malek, N.A., Hodge, C.C., Hurtz, D., Calafat, A.M., Needham, L.L., Brock, J.W., 2003. Glucuronidation patterns of common urinary and serum monoester phthalate metabolites. Arch. Toxicol. 77, 561–567. https://doi.org/10.1007/s00204-003-0486-3.

Silva, M.J., Samandar, E., Preau, J.L.J., Reidy, J.A., Needham, L.L., Calafat, A., 2005. Automated solid-phase extraction and quantitative analysis of 14 phthalate metabolites in human serum using isotope dilution-high-performance liquid chromatography-tandem mass spectrometry. J. Anal. Toxicol. 29, 819–824.

Skakkebaek, N.E., 2001. Testicular dysgenesis syndrome: an increasingly common developmental disorder with environmental aspects. Hum. Reprod. 16, 972–978. https://doi.org/10.1093/humrep/16.5.972.

Swan, S.H., Main, K.M., Liu, F., Stewart, S.L., Kruse, R.L., Calafat, A.M., Mao, C.S., Redmon, J.B., Ternand, C.L., Sullivan, S., Teague, J.L., Drobnis, E.Z., Carter, B.S., Kelly, D., Simmons, T.M., Wang, C., Lumbreras, L., Villanueva, S., Diaz-Romero, M., Lomeli, M.B., Otero-Salazar, E., Hobel, C., Brock, B., Kwong, C., Muehlen, A., Sparks, A., Wolf, A., Whitham, J., Hatterman-Zogg, M., Maifeld, M., 2005. Decrease in anogenital distance among male infants with prenatal phthalate exposure. Environ. Health Perspect. 113, 1056–1061. https://doi.org/10.1289/ehp.8100.

Swan, S.H., Sathyanarayana, S., Barrett, E.S., Janssen, S., Liu, F., Nguyen, R.H.N., Redmon, J.B., 2015. First trimester phthalate exposure and anogenital distance in newborns. Hum. Reprod. 30, 963–972. https://doi.org/10.1093/humrep/deu363.

Takahashi, Y., Shibata, T., Sasaki, Y., Fujii, H., Bito, Y., Suehiro, S., 2008. Di(2-ethylhexyl) phthalate exposure during cardiopulmonary bypass. Asian Cardiovasc. Thorac. Ann. 16, 4–6.

Thurston, S.W., Mendiola, J., Bellamy, A.R., Levine, H., Wang, C., Sparks, A., Redmon, J.B., Drobnis, E.Z., Swan, S.H., 2016. Phthalate exposure and semen quality in fertile US men. Andrology 4, 632–638. https://doi.org/10.1111/andr.12124.

van den Driesche, S., Kilcoyne, K.R., Wagner, I., Rebourcet, D., Boyle, A., Mitchell, R., McKinnell, C., Macpherson, S., Donat, R., Shukla, C.J., Jorgensen, A., Meyts, E.R.-D., Skakkebaek, N.E., Sharpe, R.M., 2017. Experimentally induced testicular dysgenesis syndrome originates in the masculinization programming window. JCI insight 2, e91204. https://doi.org/10.1172/jci.insight.91204.

Wang, Y.X., Zeng, Q., Sun, Y., Yang, P., Wang, P., Li, J., Huang, Z., You, L., Huang, Y.H., Wang, C., Li, Y.F., Lu, W.Q., 2016. Semen phthalate metabolites, semen quality parameters and serum reproductive hormones: a cross-sectional study in China. Environ. Pollut. 211, 173–182. https://doi.org/10.1016/j.envpol.2015.12.052.

Wittassek, M., Koch, H.M., Angerer, J., Brüning, T., 2011. Assessing exposure to phthalates - the human biomonitoring approach. Mol. Nutr. Food Res. 55, 7–31. https://doi.org/10.1002/mnfr.201000121.

Wittassek, M., Angerer, J., Kolossa-Gehring, M., Schäfer, S.D., Klockenbusch, W., Dobler, L., Günsel, A.K., Müller, A., Wiesmüller, G.A., 2009. Fetal exposure to phthalates - a pilot study. Int. J. Hyg Environ. Health 212, 492–498. https://doi.org/10.1016/j.ijheh.2009.04.001.

Wohlfahrt-Veje, C., Tinggaard, J., Winther, K., Mouritsen, a, Hagen, C.P., Mieritz, M.G., de Renzy-Martin, K.T., Boas, M., Petersen, J.H., Main, K.M., 2014. Body fat throughout childhood in 2647 healthy Danish children: agreement of BMI, waist circumference, skinfolds with dual X-ray absorptiometry. Eur. J. Clin. Nutr. 68, 664–670. https://doi.org/10.1038/ejcn.2013.282.

Wolff, M.S., Teitelbaum, S.L., McGovern, K., Windham, G.C., Pinney, S.M., Galvez, M., Calafat, A.M., Kushi, L.H., Biro, F.M., 2014. Phthalate exposure and pubertal development in a longitudinal study of US girls. Hum. Reprod. 29, 1558–1566. https://doi.org/10.1093/humrep/deu081.

Wolff, M.S., Teitelbaum, S.L., Pinney, S.M., Windham, G., Liao, L., Biro, F., Kushi, L.H., Erdmann, C., Hiatt, R.A., Rybak, M.E., Calafat, A.M., 2010. Investigation of relationships between urinary biomarkers of phytoestrogens, phthalates, and phenols and pubertal stages in girls. Environ. Health Perspect. 118, 1039–1046. https://doi.org/10.1289/ehp.0901690.

Wormuth, M., Scheringer, M., Vollenweider, M., Hungerbühler, K., 2006. What are the sources of exposure to eight frequently used phthalic acid esters in Europeans? Risk Anal. 26, 803–824. https://doi.org/10.1111/j.1539-6924.2006.00770.x.

Zota, A.R., Calafat, A.M., Woodruff, T.J., 2014. Temporal trends in phthalate exposures: findings from the national health and nutrition examination survey, 2001-2010. Environ. Health Perspect. 122, 235–241. https://doi.org/10.1289/ehp.1306681.