Presence of parabens, phenols and phthalates in paired maternal serum, urine and amniotic fluid

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Abstract

Objective: To examine whether selected endocrine disrupting chemicals were present in pregnant women and passed through the placental barrier to amniotic fluid, potentially exposing the developing fetus.

Methods: Paired samples of maternal serum, urine and amniotic fluid were concurrently collected (<1 h) from 200 pregnant women (age >18 years) with a singleton pregnancy and undergoing amniocentesis between gestational weeks 12 – 36. The concentration of six different parabens, seven phenols, 31 metabolites from 15 phthalate diesters and the polychlorinated substance triclocarban were analyzed by isotope diluted TurboFlow liquid chromatography-tandem mass spectrometry.

Results: Concentrations of all included compounds were highest in maternal urine followed by serum, and lowest in amniotic fluid. Of the six parabens measured in amniotic fluid,
methylparaben (MeP) and ethylparaben (EtP) were detectable most often (87% and 33% of the samples, respectively). Of the seven phenols measured, three (2,4-dichlorophenol, 2,5-dichlorophenol, 2-propylphenol) were detectable in the range of 14–21% of the amniotic fluid samples, at low concentrations (<0.12 ng/ml). Two secondary phthalates metabolites, mono-(2-carboxymethyl-hexyl) phthalate and mono-carboxy-iso-octyl phthalate were each present in ≤5% of the amniotic fluid samples at concentrations 2–5 times lower than in maternal serum and 20–100 times lower than in maternal urine. A modest statistically significant correlation between the levels of MeP and EtP was detected in paired maternal urine-amniotic fluid samples was detected (Spearman $r_{MeP}$: 0.246; $r_{EtP}$: 0.364). Likewise, the concentration of mono-ethyl phthalate (MEP) in paired maternal urine and amniotic fluid samples indicated a modest statistically significant correlation (Spearman $r_{MEP}$: 0.264), driven by detectable levels of MEP in only 3% of the amniotic fluid samples.

**Conclusions:** In general, the included parabens, phenols and phthalates were effectively metabolized and excreted via the urine, which was the matrix that reflected the highest detectable levels. The detectable levels of several included parabens and phthalates in human amniotic fluid calls for further investigations of the toxicokinetic and potential endocrine disrupting properties of individual and multiple endocrine disruptors in order to better assess the risk to the developing fetus.

**Keywords**
Endocrine disrupting chemicals; Fetal exposure; Amniotic fluid; Parabens; Phenols; Phthalates

1. **Introduction**

Pregnancy is a unique and critical period during which mother and fetus are more susceptible to harmful health effects. As pioneered by Barker in 1998 (Barker, 1998), intrauterine environmental exposures have been suggested to affect the fetus’ development during sensitive periods and the subsequent health of the offspring. The hypothesis has since been extensively investigated in numerous epidemiological studies. This has been reviewed and evidence has linked environmental exposures during fetal life (maternal lifestyle, nutrition, air pollution, heavy metals, psychological stress, prescription medicines etc.) with a range of chronic conditions in the child later in life including but not limited to adverse reproductive function, asthma, cardiovascular disease, obesity and type 2 diabetes (Calkins and Devaskar, 2011; Skogen and Øverland, n.d.).

Pregnant women are ubiquitously exposed to a multitude of environmental chemicals. Among these are xenobiotic endocrine disrupting chemicals (EDCs) that may impact on fetal development. EDCs comprise a large group of chemicals that can *alter the functions of the endocrine system and consequently cause adverse health effects in an intact organism or its progeny* (European workshop on the impact of endocrine disrupters on human health and wildlife. Report of proceedings from a workshop held in Weybridge, UK, 2–4 December 1996., 1996). The developing fetus is considered to be of particular risk to effects of EDCs (WHO and UNEP, 2012), as correct hormonal stimuli is essential during fetal organ development and changes in normal hormonal levels in the uterus at critical developmental times have been linked to development failure (Moisiadis and Matthews, 2014a, 2014b; Soto
et al., 2013) and reproductive disorders in offspring (Skakkebaek et al., 2016; Welsh et al., 2008).

Several parabens, phenols, polychlorinated compounds and phthalates are known or suspected to have endocrine disrupting properties and have been used widely in food packaging, cosmetics, pharmaceuticals and building materials for nearly a century (Baird et al., 2010). Biomonitoring and epidemiological studies have shown widespread exposure to these specific EDCs in the general population, including pregnant women (Allmyr et al., 2006; Cantonwine et al., 2014; Casas et al., 2011; Fisher et al., 2021; Frederiksen et al., 2014, 2011; Gyllenhammar et al., 2012; Mendiola et al., 2010; Philippat et al., 2012, 2014, 2013; Silva et al., 2004; White et al., 2011; Wolff et al., 2008; Ye et al., 2009). However, the toxicokinetics of these chemicals is complex and the degree to which these chemicals reach the unborn human fetus is currently largely unknown. Previous studies have considered concentrations of EDCs in amniotic fluid and have reported levels of some EDCs including bisphenol A (BPA) and specific phthalate metabolites, organochlorines, flame retardant groups and benzophenones used as UV filters (Bradman et al., 2003; Chen et al., 2011; Edlow et al., 2012; Engel et al., 2006; Foster et al., 2000; Jensen et al., 2015, 2012; Krause et al., 2018; Luzardo et al., 2009; Miller et al., 2012; Philippat et al., 2013; Pinney et al., 2017; Yamada et al., 2002). But only one of these studies measuring benzophenones (Krause et al., 2018) have measured the EDCs in paired maternal serum, urine and amniotic fluid samples collected concurrently. In this study, we aimed to examine whether selected EDCs were present in serum and urine in pregnant women and passed through the placental barrier to amniotic fluid.

2. Methods

2.1. Study design

A biomonitoring study was performed within an established mother–child cohort to investigate levels of selected parabens, phenols, a single polychlorinated compound and phthalates in paired maternal serum, urine and amniotic fluid.

2.2. Study population and biological samples

The study population has previously been described in detail (Krause et al., 2018). In brief, we recruited 200 women (age >18 years) with a singleton pregnancy who underwent amniocentesis in the period September 2012 to August 2014 at Rigshospitalet, Copenhagen University or at Copenhagen University Hospital Hvidovre, Denmark. Parabens were quantified first. Thus, of the total study population, 75 women were included for analyses of parabens; and 107 women were included for analyses of phenols, the polychlorinated substance triclocarban (TCC) and phthalates. All women were included in the analyses of UV filters, which have already been reported (Krause et al., 2018).

2.3. Sample collection

All samples were collected in gestational weeks 12 to 36 (median gestational week 18) during the amniocentesis procedure. A total of 17 ml of amniotic fluid was collected, of which 15 ml was used for karyotyping (and other analyses related to amniocenteses).
The remaining 2 ml of amniotic fluid was collected separately for chemical analyses to minimize the risk of chemical contamination from laboratory equipment. Immediately after amniocentesis (<1 h), a blood sample (35 ml) and a urine sample (30 ml) were also collected.

2.4. Sample quality

All materials and laboratory equipment used for sample collection, handling and storage were tested for contamination prior to initiation of the project, as parabens and phthalates are widely used in medical devices due to their respective plasticizing and antiseptic properties (Calafat et al., 2004). Five sets of randomly distributed ‘field blank’ samples consisting of Milli-Q water cleaned in a Millipore Synthesis A10 system (Billerica, MA, USA) were collected at each of the recruiting hospitals during the project period by mimicking the collection and storage of maternal serum, urine and amniotic fluid samples. Field blank samples were analyzed for contamination together with the human samples blinded to the laboratory staff.

2.5. Sample handling and storage

Blood samples were allowed to clot. Clotted blood samples and amniotic fluid were centrifuged at 4 °C at 3,000 rpm before the supernatants of respectively serum and cell free amniotic fluid were pipetted for storage until analysis. All samples were stored in glass bottles at −20 °C until the time of chemical analysis.

2.6. Chemical analyses

The concentrations of six different parabens, seven phenols, TCC and 31 metabolites of 15 phthalate diesters were quantified using isotope diluted TurboFlow LC-MS/MS technology for parabens (Frederiksen et al., 2011), phenols (Frederiksen et al., 2013) and phthalates (Frederiksen et al., 2020; Hart et al., 2018). A complete list of all included compounds measured, abbreviations and specific limits of detection (LOD) in serum, urine and amniotic fluid are provided in Tables 1 and 2. From here on, all substance abbreviations will refer to Table 1 and 2. All chemical analyses were performed at the Department of Growth and Reproduction, Rigshospitalet, Copenhagen.

2.7. Adjustment for urinary and amniotic fluid dilution

To account for urinary dilution, all concentrations measured in maternal urine were adjusted for the osmolality of the sample (Middleton et al., 2016). Osmolality was measured by the freezing point depression method with automatic cryoscopy osmometer (Osmomat® 030 from Gonotec, Berlin, Germany). Urinary osmolality ranged from 0.063 to 1.070 osm/kg, which is within the ranges seen for individuals with normal kidney function. For adjustment, all measured urinary concentrations of compounds above LOD were divided by the osmolality of the sample and multiplied by the median osmolality of all samples (0.376 osm/kg for urinary samples collected at the time of amniocentesis).

The osmolality of amniotic fluid was measured in twenty of the collected amniotic fluid samples. The samples were randomly selected and represented gestational age ranging from 16 to 34 gestational weeks, including one of two samples with oligohydramnios. The
variation in osmolality in these amniotic fluid samples was small (<10% ranging from 0.250 to 0.272 osm/kg, with a mean osmolality of 0.263 osm/kg).

We deemed that there was no need to adjust the concentrations measured in amniotic fluid for variation in osmolality. Measured concentrations below LOD were registered as <LOD irrespective of the osmolality of the sample.

2.8. Statistical analysis

Descriptive statistics were calculated for chemical concentrations and presented as selected 25–75<sup>th</sup> percentiles. Chemicals with <5 paired samples above LOD in both compared matrices were not further considered in correlation analyses. Graphical analyses were performed to visualize a simplified indication of pathways between biological matrices. These were only performed for the compounds, which were detectable in ≥10% of the samples in at least two of the matrices examined. For all statistical analyses, analyte concentrations below LOD were substituted by LOD/√2. For urine samples, osmolality adjusted concentrations were used. Concentrations were natural log transformed since none of the compounds were normally distributed in any of the matrices. Associations between individual compounds in different matrices were initially screened graphically and associations with a monotonic component were tested by Spearman’s rank correlation. For the compounds detected in n <30 of the biological samples, we also employed an alternative strategy with <sup>2</sup>-test for categorical variables (not detected (ND), detected). To allow for nonlinear associations, we subsequently investigated the associations by general linear models (GLM) with the independent variable included in the model also as a squared and a cubic term. The amniotic fluid concentration was always included as the dependent variable, and the urinary concentration was always included as the independent variable. In models of the association between serum and urinary concentrations, the serum concentration was entered in the model as the dependent variable.

Data analysis was performed using IBM SPSS Statistics v. 22 (IBM, New York, USA). We considered a p ≤0.05 as statistically significant. In the graphical presentation of the associations, the polynomial trend curves were plotted. All the graphical presentations were performed in the R software package (v3.6.1) (R Development Core Team, https://cran.r-project.org/).

2.9. Ethical considerations

All participating women signed a declaration of informed consent prior to inclusion. The study was approved by The Ethics Committee of the Capital Region of Denmark (Amniocentesis protocol no.: H-2–2012–76) and by The Danish Data Protection Agency (Amniocentesis journal nr.: 2012–58–0004). All women had the opportunity to withdraw from the study and request to have their samples destroyed at any point.

3. Results

There was no evidence of contamination of field blanks representing urine samples with any of the analyzed parabens, phenols, TCC or phthalate metabolites. In contrast, field blank samples representing serum and amniotic fluid sampling were contaminated with n-PrP and
BPA. n-PrP and BPA were detected in 100% of the serum samples and in respectively 90% and 100% of the amniotic fluid samples in relatively high concentrations. Due to the contamination of the field blank samples for these two chemicals, it was deemed likely that the measured concentrations of n-PrP and BPA in these two matrices were compromised by contamination of the samples during sampling using specific equipment in the field. Accordingly, measured concentrations of n-PrP and BPA in serum and amniotic fluid were not further evaluated. The measured levels of MEHP and MiNP and other phthalate monoesters may also reflect some degree of contamination of the serum samples during collection and handling in the laboratories. There was no evidence of contamination of field blanks of any of the amniotic fluid with any of the included phthalates.

Concentrations of all analyzed compounds except those with potential contamination issues listed above are presented in Tables 1 and 2. In general, concentrations were highest in maternal urine followed by serum, and lowest in amniotic fluid.

### 3.1. Levels of parabens, phenols and TCC in maternal serum, urine and amniotic fluid

Most pregnant women in the study population were simultaneously exposed to several different parabens, phenols and TCC (Table 1).

Of the 13 included parabens and phenols, a total of six compounds were detected above LOD >50% of the analyzed urine samples, but to a varying degree: MeP (98.7%), EtP (93.3%), n-PrP (88.0%), BPA (72.0%), TCS (63.6%) and 2-PP (57.9%). The remaining parabens and phenols were detected in <50% and TCC was detected in 5.6% of the spot urine samples.

The same parabens and phenols were similarly detected in maternal serum but at lower levels than urine (up to 20-fold lower) and with lower percentages of serum samples above LODs: MeP (47.7%), EtP (46.6%), TCS (96.1%) and 2-PP (89.3%). The remaining parabens and phenols were present but only detected in 1.4–45.6% of the serum samples and TCC in 22.3%, while i-PrP was not detected in any serum samples.

In amniotic fluid, MeP was detected at higher levels than in serum and with percentages of samples above LOD that were comparable to MeP in urine: MeP (86.7%). Amongst the remaining parabens and phenols that were highly detectable in urine and serum, these were also generally detected in amniotic fluid but at lower levels and with lower percentages of amniotic fluid samples above LOD: EtP (33.3%), TCS (5.6%) and 2-PP (20.6%). The remaining parabens, phenols and polychlorinated compound, n-BuP, BzP, TCC, 2,4-DCP, 2,5-DCP, 2,4,5-DCP and 4-PP were present in amniotic fluid but only detected in 3.0–14.0% of the samples, whilst i-PrP was not detected in any amniotic fluid samples (Table 1). The presence of chemicals (excluding n-PrP and BPA) in amniotic fluid for each individual woman ranged from undetectable values of all the investigated parabens and phenols to the simultaneous presence of up to five of the chemicals at levels above LOD in one amniotic fluid sample (data not shown).
3.2. Levels of phthalates in maternal serum, urine and amniotic fluid

The concentrations of the phthalate metabolites were generally much lower (up to 20-fold) in maternal serum than in maternal urine, except for the primary metabolites of DEHP and DiNP (namely MEHP and MiNP, potentially due to contamination) (Table 2). Of the 31 included metabolites, the metabolites of the following diester phthalates were most widespread in urine (highest proportions detected above LOD): DEP (95.3%), DiBP (98.1%), DnBP (92.5%), BBzP (60.4%), DEHP (69.2–97.1%), DHpP (13.0–71.0%), DOP (76.6%), DiNP (29.9–83.2%).

Phthalate metabolite levels were lowest in amniotic fluid and most amniotic fluid samples had levels below the LOD (Table 2). The secondary metabolites of DEHP and DiNP were detectable in respectively 13.0% and 15.0% of the analyzed amniotic fluid samples at concentrations that were 2–5 times lower than in maternal serum and 20–100 times lower than in maternal urine.

3.3. Correlation of individual chemicals between paired matrices: Spearman rank correlations and X²-test

All correlations are presented in Table 3 (Spearman’s rank) and depicted graphically in Fig. 1 (a–k). P-values from the X²-test performed on compounds detected >LOD in n<30 biological paired samples are presented in Table 4.

3.3.1. Correlation of parabens measured in different matrices—For the two parabens, MeP and EtP, which were the most detectable of the six included parabens in amniotic fluid samples (87.0% and 33.0%, respectively), a modest statistically significant correlation between the levels measured in paired maternal urine-amniotic fluid samples was detected (Spearman rMeP: 0.246; rEtP: 0.364). The linear graphical depiction confirmed this (Fig. 1a and b) along with the results of the X²-test testing (P <0.001), Table 4. Likewise, a modest, statistically significant correlation in paired serum and urine samples, Spearman r ≥ 0.475 was detected for both MeP and EtP; also confirmed by the X²-test (P <0.001). This association was linear for MeP (Fig. 1c), but the association leveled off at higher concentrations for EtP (Fig. 1d). Considering paired serum and amniotic fluid samples, only MeP was significantly correlated in paired maternal samples, rMeP: 0.284 confirmed by the linear association (Fig. 1e).

3.3.2. Spearman rank correlation of phenols measured in different matrices—Phenols were largely undetected in amniotic fluid and the significant correlation of 2,4,5-TCP levels in paired serum and amniotic fluid (Spearman r₂,₄,₅-TCP: 0.291) was driven by detectable levels of 2,5-DCP in 7.5% of amniotic fluid samples. The X²-test did not confirm this and indicated a non-statistically significant correlation. Although 2,4,5-TCP, 2-PP and 4-PP were all detected in a large proportion of both serum and urine samples, only TCS was significantly correlated in paired maternal serum and urine samples (Spearman r₉TCS: 0.268).

3.3.3. Correlation of phthalates measured in different matrices—The concentrations of MCiOP in paired maternal urine and amniotic fluid samples were
modestly correlated (Spearman $r_{\text{MCiOP}}$: 0.340) with statistical significance, but levels were almost constant in amniotic fluid regardless of levels in urine (Fig. 1f). Likewise, a modest, statistically significant correlation in paired serum and urine samples, Spearman $r \geq 0.475$ was detected for MCiOP. The linear graphical depiction confirmed this (Fig. 1g) along with the results of the $X^2$-test ($P < 0.001$). The concentration of MEP in paired maternal urine and amniotic fluid samples indicated a modest, statistically significant correlation (Spearman $r_{\text{MEP}}$: 0.264). This was driven by detectable levels of MEP in only 3.0% of the amniotic fluid samples and was not fully confirmed in the $X^2$-test ($P = 0.075$). Considering paired serum and amniotic fluid samples, MEP, MCMHP, MCPP and MCiOP were all significantly correlated in the two matrices, Spearman $r \geq 0.263$ (Table 3 and Figures h-k). Likewise, when considering paired serum and urine samples, MEP, MECPP, MCPeP, MCHxP, MCPP and MCiOP were all significantly correlated, Spearman $r \geq 0.219$, this was confirmed in the $X^2$-test for all but MCPeP. MCiOP, a carboxylated metabolite of DiNP, detectable in 15.0% of the amniotic fluid samples, also correlated significantly with DiNP metabolites in maternal urine (Spearman $r_{\text{MCiOP}}$: 0.340, data not shown). In contrast, MCMHP, one of the two carboxylated metabolites of DEHP, detectable in 13.0% of the amniotic fluid samples did not correlate with any of the DEHP metabolites measured in maternal serum (data not shown).

4. Discussion

In a unique analysis of multiple endocrine disrupting chemicals in paired amniotic fluid, maternal blood and urine sampled concurrently, we found that selected parabens and phthalates may cross the placental barrier to amniotic fluid, where they potentially interact with the intrauterine environment. All included substances were detected in highest concentrations in urine indicating substantial excretion. Placental transfer to amniotic fluid was generally low for most compounds and phenols were largely undetected.

In this study, parabens were detectable in a substantial number of samples in both maternal serum and urine and to a lesser extent in amniotic fluid. This is in line with a previous Danish study that found parabens detected in most urine samples from children, adolescents, young men and pregnant women indicating widespread exposure (Frederiksen et al., 2014) and most recently another study reporting similar detection levels in maternal serum collected from 279 pregnant women in the period 2001–2009 (Fisher et al., 2021).

Of the six included parabens in our present study, MeP and EtP were measurable most often in 87% and 33% of amniotic fluid respectively. Only one study has previously reported on levels of parabens in amniotic fluid and authors reported a lower presence of these compounds, namely that MeP was present in 42% of the samples, while EtP could not be detected in any of the amniotic fluid samples (Philippat et al., 2013), but this could potentially reflect differences in sensitivity in analytical methodology as that study was performed almost 10 years before our present study. In contrast to our present study, where we observed almost similar levels in serum and amniotic fluid, experimental data from rats suggest that parabens may be present in higher concentrations in amniotic fluid as compared to maternal plasma indicating different toxicokinetic mechanisms present in rodents than humans (Frederiksen et al., 2008).
In our study, the levels of both MeP and EtP were significantly correlated in paired maternal urine-amniotic fluid samples, whilst only MeP was significantly correlated in paired serum and amniotic fluid samples, potentially indicating different clearance rates and toxicokinetic pathways for these two parabens. Although low levels (≤0.63 ng/mL), the direct fetal paraben exposure indicated in our study is concerning and may lead to damage of the reproductive system in the offspring. In rodent studies, parabens have been linked to a lowered sperm count and motility (Boberg et al., 2016; Kang et al., 2002; Oishi, 2002; Zhang et al., 2014) and to shorter anogenital distance (AGD), implying anti-androgenic effects. In females, parabens may lead to longer AGD due to higher androgen levels caused by paraben aromatase inhibition (van Meeuwen et al., 2008). Although data in humans are limited, a recent study of Danish pregnant women from the Odense mother–child birth cohort reported that higher maternal MeP exposure was associated with shorter AGD in boys. That study furthermore showed that women with an EtP exposure above the median for the population gave birth to boys with narrower penile width, whilst in girls in-utero exposure was associated with a tendency toward longer AGD (Jensen et al., 2021).

The phenols TCS and 2-PP were detected in a substantial number of urine and serum samples and to a lesser extent in amniotic fluid. This supports two previous studies reporting TCS in up to 77% of urine samples. Most recently another study has reported similar detection levels for TCD and 2-PP in maternal serum from 279 women from a cohort established in 2001–2009 (Fisher et al., 2021). Most phenols were largely undetected in amniotic fluid (5.6–20.6% of samples had detectable levels). This is largely supported by the one previous study reporting that TCS, 2,4-DCP and 2,5-DCP were detected in 1–16% of the examined amniotic fluids samples (Philippat et al., 2013). Levels of quantification of BPA in amniotic fluid was high in our study, which is in line with a previous study that detected BPA in >50% of the amniotic fluid samples (Yamada et al., 2002). However, we cannot exclude contamination of our samples during collection and pre-storage and we acknowledge that the high levels detected in all samples were most likely falsely increased due to contamination (cf. limitations).

Phthalate metabolite levels were generally detectable in all samples, though lower in maternal serum than urine. Six previous studies have reported on phthalate metabolite levels in urine from pregnant women (Adibi et al., 2008; Cantonwine et al., 2014; Casas et al., 2011; Wolff et al., 2008; Ye et al., 2009) and one has reported on levels in maternal serum confirming the detection levels in urine (Fisher et al., 2021), all reporting similar detection and median levels for almost all phthalates. Noteworthy was that median levels of MEP were up to 20-fold lower potentially indicating different sources and levels of exposures to DEP in Denmark compared to Spain, Puerto Rico and USA. It was also noteworthy that metabolite levels of rather “new” phthalates being used at the time of this study were already emerging in the urine samples; namely the metabolites of DHpP and DHxP were detected in ~70.0% and ~50.0% of the maternal urine samples, respectively. In contrast to other metabolites, the primary metabolites of DEHP and DiNP (namely MEHP and MiNP) were detected at higher levels in serum than in urine. Blood samples contain active enzymes capable of hydrolyzing DEHP and DiNP into their respective primary metabolites, even ex vivo after sample collection (Kato et al., 2003; Silva et al., 2005), which is not the
case for the measured levels of the other metabolites of DEHP and DiNP as conversion to these secondary oxidized metabolites can occur only in vivo. Therefore, the measured serum concentrations of MEHP and MiNP are likely at risk of being falsely increased due to ex vivo transformation of DiNP and DEHP. However, the detection of the metabolites of DEHP and DiNP in the analyzed amniotic fluid samples (13.0% and 15.0%, respectively) is not suspected to be subject to contamination in our study as levels are normally distributed above and below the LOD as is expected for these compounds. Although these levels were low (≤0.29 ng/mL), it still implies exposure during the sensitive windows of fetal development (gestational weeks 12–36) and is of particular concern, as disruption in the hormonal milieu at this stage may cause irreversible damage to the reproductive system in offspring (Johansson et al., 2017; van den Driesche et al., 2017). This is supported by experimental studies that have previously reported an association between prenatal exposure to DiNP and DEHP (along with DnBP and BBzP) and male reproductive function in offspring (congenital cryptorchidism/hypospadias, increased nipple retention and reduced anogenital distance (AGD)/testis volume/sperm count) (Foster et al., 2006; Gray Jr. et al., 2000; van den Driesche et al., 2017). Although data on humans remain limited, similar associations between exposures to phthalates and antiandrogenic impact on reproductive endpoints have been found (Hauser and Calafat, 2005; Mariana et al., 2016; Matsumoto et al., 2008), in particular adverse effects on male reproductive development and hypospadias in infant boys (Main et al., 2006; Ormond et al., 2009; Sathyanarayana et al., 2016) and on neuro developmental health in child (Minatoya and Kishi, 2021). Published data on female reproductive endpoints associated with exposures to phthalates include delayed development of pubic hair following increased urinary phthalate levels in puberty (Frederiksen et al., 2012; Wolff et al., 2014) and a potential association between early onset of puberty and premature thelarche after prenatal exposure (Hart et al., 2014; Jurewicz and Hanke, 2011).

Across many of the measured compounds, we experienced that urinary concentration levels in our study were notably lower than reported in previous studies assessing levels of the same non-persistent EDCs in pregnant women (Adibi et al., 2008; Birks et al., 2016; Cantonwine et al., 2014; Philippat et al., 2013, 2012; Wolff et al., 2008; Ye et al., 2009). In some instances, the variation may reflect issues of contamination that have not necessarily been taken into account in all other studies, or differences in sensitivity in analytical methodology. But the variation could also be due to changes in exposures and concentration levels over the years and between countries as we recently reported in a study assessing time trends and geographical differences in exposure of pregnant women to phthalates (Henriksen et al., 2020), also confirmed in other populations (CDC, 2019; Frederiksen et al., 2020). But available data on concentrations for specific non-persistent EDCs measured in maternal serum and amniotic fluid in pregnant women are limited and few previous studies have reported on this (Fisher et al., 2021; Philippat et al., 2014).

4.1. Perspectives

In most observational studies, investigating the effects of maternal exposures on subsequent health of the offspring, as well as in biomonitoring studies, quantification of EDCs or their metabolites is often performed in either maternal serum or urine as a proxy for fetal exposure. To our knowledge, only our own previous study on benzophenones used as UV
filters (Krause et al., 2018) has previously investigated the correlation of selected EDCs using paired samples in urine, serum and amniotic fluid that were sampled at the same time. The results of the present study can thus further support the postulate that measuring these compounds in serum and urine does reflect fetal exposure.

Furthermore, the ubiquitous and combined exposure to multiple EDCs at a critical window of human development detected in this study, albeit at low concentrations, implies co-fetal exposure to multiple individual EDCs. Although our results indicate that placental transfer appears to be low for some of the compounds studied, several parabens and phthalates were present in the amniotic fluid, where they can potentially interact with the fetus. Thus, further toxicokinetic studies are needed to better assess the risk to the developing fetus from exposure to both individual compounds (accounting for different actions and potencies of each compound) and multiple endocrine disruptor mixtures.

5. Strengths and limitations

This study is unique in its means of obtaining chemicals in amniotic fluid in addition to maternal matrices, providing an opportunity to better compare and understand the degree to which various compounds cross the placental barrier in humans.

However, some contamination of samples during collection/handling was unavoidable in our study. We tried to prevent sample contamination by substituting equipment and materials that were recognized to contaminate (e.g. phthalate tubing). Still, field blank samples collected on site showed that some of the pipettes used in handling of supernatant from amniotic fluid could not be replaced even though they seemed to leak some BPA. Furthermore, the ultrasound gel used in connection to ultrasound-guided amniocentesis contains n-PrP and represents a potential source of contamination of the amniotic fluid samples. It was not possible to find a paraben-free substitute for it. Thus, although this compound was measurable in 90.7% of amniotic fluid samples with a median level of 478 ng/mL, such a result should be interpreted with extreme caution and we did not consider these compounds further in this study.

Likewise, we were unable to conclude on the correlations of MEHP and MiNP in serum and the other matrices, as levels were unusually high and detected in 100% of all serum, and we suspect this to reflect some degree of contamination of the serum samples during collection and handling in the laboratories, giving a falsely increased impression of internal serum levels.

In our study, we included all pregnant women referred for amniocentesis. The indication for amniocentesis was divided into three pregnancy risk groups according to the severity of the indication for and outcome of the amniocentesis and the results of the ultrasound scan: risk group 1) women referred for amniocentesis because of maternal request and/or high maternal age and found to carry a fetus with normal karyotype and normal results of ultrasound scan; risk group 2) women with increased risk for neural tube defects (NTDs), intestinal atresia, trisomy 18 or 21 or unspecific echogenic bowl on ultrasound scan, but who were found to carry a fetus without any malformations and with a normal karyotype; and
risk group 3) women carrying a fetus with malformations found by ultrasound scan and/or with an abnormal karyotype. We are not aware of differences in homeostasis of pregnant women and because levels of EDCs did not vary significantly according to risk group (data not shown), we do not expect this to be a limitation, nevertheless, some caution should be exercised when extrapolating results to healthy pregnant populations.

Finally, power was low when performing correlation analyses of chemicals of paired compound analyses, as these were limited to the 75 women who had all compounds measured and this was exemplified by many compounds being below the limit of detection for our analytical methods.

6. Conclusions

Although placental transfer was generally low, several parabens and phthalates crossed the placental barrier and were present in amniotic fluid, where they potentially interact with the intrauterine environment. Further investigations are needed of the toxicokinetics and potential endocrine disrupting properties of individual and multiple endocrine disruptors to better assess the risk to the developing fetus.

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Fig. 1.
Graphical association of selected log-transformed parabens and phthalates between different matrices.
### Table 1

LOD and concentrations (ng/mL) of parabens, phenols and polychlorinated compounds in urine, serum and amniotic fluid collected at the time of amniocentesis in pregnant women. Median and percentiles were calculated based on the total samples.

| Parabens, n = 75 | Urine LOD % mean LOD (SD) (P25-P75) | Serum LOD % mean LOD (SD) (P25-P75) | Amniotic fluid LOD % mean LOD (SD) (P25-P75) |
|------------------|--------------------------------------|--------------------------------------|---------------------------------------------|
| methyl paraben   | MeP 0.08 98.7 38.4 (71.0) 5.64 (0.44–52.5) 0.38 47.7 1.79 (4.44) <0.38 (ND-1.08) 0.08 86.7 8.02 (15.2) 0.63 (0.27–5.72) |
| ethyl paraben    | EtP 0.04 93.3 12.2 (46.1) 0.41 (ND-2.25) 0.10 46.6 0.40 (2.09) <0.10 (ND-0.24) 0.04 33.3 0.08 (0.12) <0.13 (ND-0.17) |
| iso-propyl paraben | i-PrP 0.07 1.3 0.08 (0.00) <0.07 0.08 0.0 – – 0.07 0.0 – – |
| n-propyl paraben | n-PrP 0.05 88.0 10.8 (25.0) 2.51 (0.81–10.3) 0.10 a 0.05 a |
| n-butyl paraben  | n-BuP 0.05 42.7 2.10 (12.3) <0.05 0.07 1.4 <0.07 <0.07 0.05 2.7 <0.05 <0.05 |
| benzyl paraben   | BzP 0.05 45.3 0.13 (0.71) <0.05 0.12 1.4 <0.12 <0.12 0.05 2.7 <0.05 <0.05 |

| Phenols, n = 107 | Urine LOD % mean LOD (SD) (P25-P75) | Serum LOD % mean LOD (SD) (P25-P75) | Amniotic fluid LOD % mean LOD (SD) (P25-P75) |
|------------------|--------------------------------------|--------------------------------------|---------------------------------------------|
| bisphenol A      | BPA a 0.12 72.0 3.44 (13.6) 1.38 (ND-3.08) 0.10 a 0.12 a |
| triclosan        | TCS 0.06 63.6 15.2 (66.0) 0.39 (ND-1.40) 0.10 96.1 1.67 (2.92) 0.96 (0.64–1.5) 0.06 5.6 0.04 (0.19) <0.06 |
| 2,4-dichlorophenol | 2,4-DCP 0.07 40.2 0.40 (2.62) <0.07 (ND-0.16) 0.10 7.8 0.02 (0.07) <0.10 0.07 14.0 0.03 (0.09) <0.07 |
| 2,5-dichlorophenol | 2,5-DCP 0.07 20.6 14.3 (81.4) <0.07 0.10 8.7 0.06 (0.22) <0.10 0.07 14.0 0.04 (0.13) <0.07 |
| 2,4,5-trichlorophenol | 2,4,5-TCP 0.06 36.4 0.17 (0.50) <0.06 (ND-0.16) 0.10 21.4 0.05 (0.11) <0.10 0.06 7.5 0.02 (0.10) <0.06 |
| 2-propylphenol    | 2-PP 0.12 57.9 0.32 (0.82) 0.14 (ND-0.38) 0.10 89.3 0.35 (0.22) 0.33 (0.2–0.5) 0.12 20.6 0.09 (0.20) <0.12 |
| 4-propylphenol    | 4-PP 0.13 35.5 0.50 (0.75) <0.13 (ND-0.39) 0.10 45.6 0.15 (0.21) <0.10 (ND-0.27) 0.13 5.6 0.02 (0.11) <0.13 |

| Polychlorinated compounds, n = 107 | Urine LOD % mean LOD (SD) (P25-P75) | Serum LOD % mean LOD (SD) (P25-P75) | Amniotic fluid LOD % mean LOD (SD) (P25-P75) |
|-----------------------------------|--------------------------------------|--------------------------------------|---------------------------------------------|
| triclocarban                     | TCC 0.03 2.8 <0.03 <0.03 0.10 22.3 0.11 (0.25) <0.10 0.03 5.6 0.02 (0.10) <0.03 |

a Field blanks of serum and amniotic fluid were contaminated with n-PrP and BPA.

b Osmolality corrected.
Table 2

LOD (% ≥LOD) and concentrations (ng/mL) of phthalates (diesters and metabolites) in urine, serum and amniotic fluid collected at the time of amniocentesis in pregnant women. Median and percentiles were calculated based on the total samples.

| Phthalate diesters and their metabolites | Urine, n = 107 | Serum, n = 103 | Amniotic fluid, n = 107 |
|-----------------------------------------|----------------|----------------|------------------------|
|                                         | LOD % mean       | median | LOD % mean       | median | LOD % mean       | median |
|                                         | ≥LOD (SD)        | (P25-P75) | ≥LOD (SD)        | (P25-P75) | ≥LOD (SD)        | (P25-P75) |
| di-methyl phthalate                     | DMP              | MMP    | 0.53 25.2 0.78 (1.09) | <0.53 (ND-0.48) | 0.44 1.0 0.01 | <0.44 |
|                                         | DMP              | MMP    | 0.79 95.3 94.6 (196) | 14.1 (5.49–39.1) | 0.65 17.5 0.91 (2.83) | <0.65 |
| di-ethyl phthalate                      | DEP              | MEP    | 0.44 2.8 0.21 (0.60) | <0.25 | 0.40 0.0 – | – |
|                                         | DEP              | MEP    | 0.65 95.3 94.6 (196) | 14.1 (5.49–39.1) | 0.65 17.5 0.91 (2.83) | <0.65 |
| di-isopropyl phthalate                  | DiPrP            | MiPrP  | 0.25 2.8 0.21 (0.60) | <0.25 | 0.40 0.0 – | – |
|                                         | DiPrP            | MiPrP  | 0.25 2.8 0.21 (0.60) | <0.25 | 0.40 0.0 – | – |
| di-propyl phthalate                     | DiPrP            | MiPrP  | 0.25 2.8 0.21 (0.60) | <0.25 | 0.40 0.0 – | – |
|                                         | DiPrP            | MiPrP  | 0.25 2.8 0.21 (0.60) | <0.25 | 0.40 0.0 – | – |
| di-isobutyl phthalate                   | DiBP             | MiBP   | 0.44 98.1 18.4 (37.0) | 12.3 (6.94–23.3) | 0.75 2.9 0.06 (0.38) | <0.75 |
|                                         | DiBP             | MiBP   | 0.44 98.1 18.4 (37.0) | 12.3 (6.94–23.3) | 0.75 2.9 0.06 (0.38) | <0.75 |
| di-n-butyl phthalate                    | DnBP             | MnBP   | 0.68 92.5 10.7 (16.4) | 8.19 (4.64–14.2) | 0.61 1.0 0.01 (0.06) | <0.61 |
|                                         | DnBP             | MnBP   | 0.68 92.5 10.7 (16.4) | 8.19 (4.64–14.2) | 0.61 1.0 0.01 (0.06) | <0.61 |
| mono-(3-hydroxybutyl) phthalate         | MHBP             | 0.63 3.7 1.57 (10.4) | <0.63 | 0.22 0.0 – | – |
|                                         | MHBP             | 0.63 3.7 1.57 (10.4) | <0.63 | 0.22 0.0 – | – |
| butylbenzyl phthalate                   | BBzP             | MBzP   | 0.50 60.4 2.29 (6.74) | 1.12 (ND-2.41) | 0.26 0.0 – | – |
|                                         | BBzP             | MBzP   | 0.50 60.4 2.29 (6.74) | 1.12 (ND-2.41) | 0.26 0.0 – | – |
| di-n-pentyl phthalate                   | DPP              | MPP    | 0.28 1.9 0.21 (0.10) | <0.28 | 0.27 0.0 – | – |
|                                         | DPP              | MPP    | 0.28 1.9 0.21 (0.10) | <0.28 | 0.27 0.0 – | – |
| di-(2-ethylhexyl) phthalate             | DEHP             | MEHP   | 0.42 69.2 1.44 (1.74) | 1.09 (ND-2.10) | 0.74 98.1 3.73 (1.02) | 3.59 (3.09–4.35) |
|                                         | DEHP             | MEHP   | 0.42 69.2 1.44 (1.74) | 1.09 (ND-2.10) | 0.74 98.1 3.73 (1.02) | 3.59 (3.09–4.35) |
| di-(2-ethyl-5-hydroxyhexyl) phthalate   | DEHP             | MEHP   | 0.42 69.2 1.44 (1.74) | 1.09 (ND-2.10) | 0.74 98.1 3.73 (1.02) | 3.59 (3.09–4.35) |
|                                         | DEHP             | MEHP   | 0.42 69.2 1.44 (1.74) | 1.09 (ND-2.10) | 0.74 98.1 3.73 (1.02) | 3.59 (3.09–4.35) |
| di-(2-ethyl-5-carboxypentyl) phthalate  | DEHP             | MEHP   | 0.42 69.2 1.44 (1.74) | 1.09 (ND-2.10) | 0.74 98.1 3.73 (1.02) | 3.59 (3.09–4.35) |
|                                         | DEHP             | MEHP   | 0.42 69.2 1.44 (1.74) | 1.09 (ND-2.10) | 0.74 98.1 3.73 (1.02) | 3.59 (3.09–4.35) |
| di-(2-ethyl-5-carboxypentyl) phthalate  | DEHP             | MEHP   | 0.42 69.2 1.44 (1.74) | 1.09 (ND-2.10) | 0.74 98.1 3.73 (1.02) | 3.59 (3.09–4.35) |
|                                         | DEHP             | MEHP   | 0.42 69.2 1.44 (1.74) | 1.09 (ND-2.10) | 0.74 98.1 3.73 (1.02) | 3.59 (3.09–4.35) |
| di-(2-ethyl-5-carboxypentyl) phthalate  | DEHP             | MEHP   | 0.42 69.2 1.44 (1.74) | 1.09 (ND-2.10) | 0.74 98.1 3.73 (1.02) | 3.59 (3.09–4.35) |
|                                         | DEHP             | MEHP   | 0.42 69.2 1.44 (1.74) | 1.09 (ND-2.10) | 0.74 98.1 3.73 (1.02) | 3.59 (3.09–4.35) |
| di-(2-ethyl-5-carboxypentyl) phthalate  | DEHP             | MEHP   | 0.42 69.2 1.44 (1.74) | 1.09 (ND-2.10) | 0.74 98.1 3.73 (1.02) | 3.59 (3.09–4.35) |
|                                         | DEHP             | MEHP   | 0.42 69.2 1.44 (1.74) | 1.09 (ND-2.10) | 0.74 98.1 3.73 (1.02) | 3.59 (3.09–4.35) |
| di-(2-ethyl-5-carboxypentyl) phthalate  | DEHP             | MEHP   | 0.42 69.2 1.44 (1.74) | 1.09 (ND-2.10) | 0.74 98.1 3.73 (1.02) | 3.59 (3.09–4.35) |
|                                         | DEHP             | MEHP   | 0.42 69.2 1.44 (1.74) | 1.09 (ND-2.10) | 0.74 98.1 3.73 (1.02) | 3.59 (3.09–4.35) |
| Phthalate diesters and their metabolites | LOD % | mean $^a$ | median $^a$ | LOD % | mean | median | LOD % | mean | median |
|----------------------------------------|-------|------------|-------------|-------|------|--------|-------|------|--------|
|                                      | LOD   | (SD)       | (P25-P75)   | LOD   | (SD) | (P25-P75) | LOD   | (SD) | (P25-P75) |
| **Urine. n = 107**                    |       |            |             |       |      |         |       |      |         |
| mono-(2-carboxymethylhexyl) phthalate | MCMHP | 0.22       | 97.1 (6.41) | 4.62 (2.66–7.58) | 0.39 | 37.9 (0.48) | <0.39 | ND-0.54 | 0.22 | 13.1 (0.12) | <0.22 |
| di-n-hexyl phthalate                  | DHxP  | 0.33       | 11.2 (0.54) | <0.33 | 0.38 | 0.0 – – | 0.33 | 0.0 – – | 0.33 | 0.0 – – | <0.33 |
| mono-n-hexyl phthalate                | MHxP  | 0.21       | 5.6 (0.09)  | <0.21 | 0.26 | 1.0 (0.02) | <0.26 | 0.21 | 0.0 – – | <0.26 |
| mono-(5-hydroxyhexyl) phthalate       | MHxP  | 0.22       | 45.8 (1.60) | <0.22 | 0.20 | 6.5 (0.04) | <0.20 | 0.22 | 0.0 – – | <0.20 |
| di-cyclohexyl phthalate               | DCHP  | 0.33       | 0.0 – –     | 0.27 | 0.0 – – | 0.33 | 0.0 – – | 0.33 | 0.0 – – | <0.33 |
| di-n-heptyl phthalate                 | DHnP  | 0.33       | 13.1 (0.94) | <0.33 | 0.38 | 0.0 – – | 0.33 | 0.0 – – | 0.33 | 0.0 – – | <0.33 |
| mono-(6-carboxyhexyl) phthalate       | MHnP  | 0.19       | 15.0 (0.64) | <0.19 | 0.15 | 0.0 – – | 0.19 | 0.0 – – | 0.19 | 0.0 – – | <0.19 |
| mono-(6-hydroxyhexyl) phthalate       | MHnP  | 0.30       | 71.0 (2.41) | 0.93 | ND-1.62 | 0.23 | 3.9 (0.17) | <0.23 | 0.30 | 0.0 – – | <0.30 |
| di-octyl phthalate                    | DOP   | 0.32       | 2.8 (0.20)  | <0.32 | 0.70 | 1.9 (0.14) | <0.70 | 0.32 | 0.0 – – | <0.32 |
| mono-octyl phthalate                  | MOP   | 1.04       | 76.6 (45.6) | 2.46 (1.64–5.40) | 0.19 | 16.5 (0.76) | <0.19 | 1.04 | 0.9 (0.11) | <2.32 |
| mono-3-carboxypropyl phthalate        | MCOP  | 0.39       | 29.9 (0.39) | <0.39 | 0.86 | 99.0 (2.41) | 5.87 (4.24–7.60) | 0.39 | 4.7 (0.10) | <0.39 |
| di-iso-nonyl phthalate                | DiNP  | 0.61       | 66.4 (2.73) | 1.33 (ND-2.73) | 0.40 | 0.0 – – | 0.61 | 0.0 – – | 0.61 | 0.0 – – | <0.61 |
| mono-hydroxy-iso-nonyl phthalate      | MHNP  | 0.57       | 52.3 (2.15) | 0.48 (ND-1.38) | 0.31 | 0.0 – – | 0.57 | 0.0 – – | 0.57 | 0.0 – – | <0.57 |
| di-iso-decyl phthalate                | DiDP  | 0.29       | 83.2 (6.10) | 1.67 (0.71–3.26) | 0.43 | 21.4 (1.29) | <0.43 | 0.29 | 15.0 (0.26) | <0.29 |
| mono-iso-decyl phthalate              | MiDP  | 0.91       | 3.7 (0.12)  | <0.91 | 0.92 | 18.4 (0.70) | <0.92 | 0.91 | 1.9 (0.10) | <0.91 |
Phthalate diesters and their metabolites

|                          | Phthalate diesters and their metabolites | Urine. n = 107 | Serum. n = 103 | Amniotic fluid. n = 107 |
|--------------------------|------------------------------------------|----------------|----------------|-------------------------|
|                          | LOD % mean a median a | LOD % mean | median | LOD % mean | median | LOD % mean | median |
|                          | LOD (SD) (P25-P75) | LOD (SD) (P25-P75) | LOD (SD) (P25-P75) | LOD (SD) (P25-P75) |
| mono-(9-hydroxydecyl) phthalate | MHiDP 0.27 3.7 0.20 (0.17) | <0.27 0.0 – – | 0.27 0.0 – – |
| mono-(9-oxodecyl) phthalate | MOiDP 0.32 14.0 0.26 (0.36) | <0.32 0.0 – – | 0.32 0.0 – – |
| mono-(9-carboxynonyl) phthalate | MCiNP 0.27 0.0 – – | 0.32 0.0 – – | 0.27 0.0 – – |

a Osmolality adjusted.
b Suspected to reflect some degree of contamination of the serum samples during collection and handling in the laboratories and therefore give a falsely increased impression of internal serum levels.
c Major metabolite of DnOP, not specific for DnOP.
Table 3
Correlation (Spearman’s rho) between individual detected urinary, serum and amniotic fluid concentrations detected in >5 paired samples.

|                | Urine   | Amniotic fluid |
|----------------|---------|----------------|
| **A. PARABENS**|         |                |
| Spearman’s rho |         |                |
| Serum          | 0.557*  | 0.284*         |
| Amniotic fluid | 0.246*  | 0.092          |

|                | Urine | Amniotic fluid |
|----------------|-------|----------------|
| **B. PHENOLS AND POLYCHLORINATED COMPOUND (TCC)** |       |                |
| Spearman’s rho |       |                |
| Serum          | 0.268* | 0.025          |
| Amniotic fluid | 0.120  | 0.018          |

|                | Urine | Amniotic fluid |
|----------------|-------|----------------|
| **C. PHTHALATE metabolites** |       |                |
| Spearman’s rho |       |                |
| Serum          | −0.059 | 0.264*         |

Shaded cells: in at least one of the compared matrices <10% of the samples had a concentration above the LOD for the respective compound.

Metabolites NOT DETECTED in >5 paired samples in the following matrices:
- Urine and serum matrices: MiPrP, MPrP, MHBP, MBzP, MPP, MEHP, MEHHP, MEOHP, MCMHP, MHxP, MHHxP, MCP, MCHP, MHHpP, MCHP, MHepP, MHHpP, MCHepP, MCiOP, MHiNP, MOiNP, MHiDP, MOiDP, MHiNP, MOiNP, MHiDP, MOiDP, MHiNP, MOiNP, MHiDP, MOiDP, MHiNP, MOiNP, MHiDP, MOiDP, MHiNP, MOiNP, MHiDP, MOiDP, MHiNP, MOiNP, MHiDP, MOiDP, MHiNP, MOiNP.

Shaded cells: in at least one of the compared matrices <10% of the samples had a concentration above the LOD for the respective compound.
Table 4

P-values derived from the X²-test performed as a sensitivity check on compounds detected >LOD in n<30 of the biological paired samples*.

|   | A. PARABENS |   |   |   |   |   |
|---|-------------|---|---|---|---|---|
|   | P<sub>Chi-sq test</sub> | Urine | Amniotic fluid | MeP | EtP |   |
|   | Serum       | <0.001 | <0.001 | <0.001 |   |   |
|   | Amniotic fluid | <0.001 | <0.001 |   |   |   |

|   | B. PHENOLS |   |   |
|---|-------------|---|---|
|   | P<sub>Chi-sq test</sub> | Amniotic fluid | TCS |   |
|   | Serum       | 0.100 |   |   |

|   | C. PHTHALATE metabolites |   |   |   |   |
|---|--------------------------|---|---|---|---|
|   | P<sub>Chi-sq test</sub> | Urine |   |   |   |   |
|   | Serum       | <0.001 | <0.001 | 0.893 | 0.0004 | <0.001 |
|   | Amniotic fluid | 0.075 |   |   |   |   |

*Used to determine the agreement of those not detected (ND) and detected in paired samples using a dichotomous variable (ND, detected).