



ENVIRONMENTAL
HEALTH
PERSPECTIVES

<http://www.ehponline.org>

**A Longitudinal Study of Urinary Phthalate Excretion
in 58 Full-Term and 67 Preterm Infants from Birth
through 14 Months**

**Hanne Frederiksen, Tanja Kuiri-Hänninen, Katharina M. Main,
Leo Dunkel, and Ulla Sankilampi**

<http://dx.doi.org/10.1289/ehp.1307569>

Received: 29 August 2013

Accepted: 29 May 2014

Advance Publication: 30 May 2014

A Longitudinal Study of Urinary Phthalate Excretion in 58 Full-Term and 67 Preterm Infants from Birth through 14 Months

Hanne Frederiksen,¹ Tanja Kuiri-Hänninen,^{2,3} Katharina M. Main,¹ Leo Dunkel,^{3,4} and Ulla Sankilampi³

¹University Department of Growth and Reproduction, Rigshospitalet, Faculty of Health Sciences, Copenhagen, Denmark; ²Department of Pediatrics, School of Medicine, University of Eastern Finland, FI-70211 Kuopio, Finland; ³Department of Pediatrics, Kuopio University Hospital, FI-70029 KYS, Finland; ⁴William Harvey Research Institute, Barts and the London, Queen Mary University of London, London EC1M 6BQ, UK

Address correspondence to H. Frederiksen, Department of Growth and Reproduction, Copenhagen University Hospital (Rigshospitalet), Section 5064, Blegdamsvej 9, DK-2100 Copenhagen, Denmark. Telephone: +45 3545 6359. Fax: +45 3545 6054. E-mail: hanne.frederiksen@regionh.dk

Running title: Longitudinal study of urinary phthalates in infants

Acknowledgments: We are grateful to all the participating infants and their parents. Furthermore we thank Anneli Paloranta and Ole Nielsen for skilful assistance during data collection and chemical analysis. This study was financially supported by the Danish Agency for Science, Technology and Innovation 09-067180 and by Kuopio University Hospital, EVO funding, Finland. The instrumental equipment was financially supported by the Velux Foundation.

Disclaimer: The findings and conclusions in the present study are those of the authors and do not necessarily represent the views of the funding sources.

Competing financial interests: The authors declare they have no competing financial interests.

Abstract

Background: Some phthalates have shown anti-androgenic effects in rat offspring. Premature infants may be exposed to high amounts of specific phthalates during hospitalization and thus are potentially at risk.

Objective: We evaluated longitudinal phthalate exposure and metabolism in full term (FT) and preterm (PT) infants.

Methods: Fifty-eight FT and 67 PT (gestational age 24.7–36.6 weeks) infants were recruited at birth and followed until 14 months (nine times). Urinary concentrations of metabolites of diethyl phthalate (DEP), dibutyl phthalate isomers (DiBP and DnBP), butylbenzyl phthalate (BBzP), di-(2-ethylhexyl) phthalate (DEHP), and di-iso-nonyl phthalate (DiNP) were measured in 894 samples. Daily intake and a hazard index for antiandrogenic effects were estimated, and excretion patterns of DEHP and DiNP metabolites analyzed.

Results: Metabolites of BBzP, DiNP, and DEHP were 5-50 times higher at day 7 (D7) and month 1 (M1) in PT than FT infants. Thereafter, metabolite concentrations were similar between the two groups. The estimated hazard index for combined DiBP, DnBP, BBzP, and DEHP exposures seven days after birth exceeded the anti-androgenic threshold in more than 80% of PT and 30% of FT infants, and after M2, in 30% of all infants. The excretion pattern of DEHP and DiNP metabolites changed with age.

Conclusion: The majority of PT infants and approximately one third of healthy FT newborns were exposed to phthalates during early life at a potentially harmful level according to EFSA's recommended limits of daily exposure. Changes in the relative proportions of secondary phthalate metabolites over time were consistent with maturation of infant metabolic pathways

during the first year of life. Further research is needed on the health effects of phthalate exposures and the influence of changes in metabolic capacity in neonates and infants.

Introduction

Phthalates are widely used as plasticizers in e.g. toys, cosmetics, food packaging, medical equipment and building materials (Wittassek et al. 2011). In rats some of the most commonly used phthalates have been shown to cause outcomes consistent with anti-androgenic effects, such as impaired spermatogenesis, undescended testes (cryptorchidism), and reduced anogenital distance (Borch et al. 2006; Foster 2006; Howdeshell et al. 2007).

Associations of early phthalate exposures with allergic asthma and reproductive and behavioural outcomes suggest that the fetus, neonate, and infant may be particularly vulnerable to endocrine disruptive effects of these compounds (Bergman et al. 2012; Bornehag and Nanberg 2010; Main et al. 2006; Miodovnik et al. 2011; Swan et al. 2005; Swan et al. 2010; Whyatt et al. 2012). Despite these concerns, data on phthalate exposure levels during this period of life are sparse (Adibi et al. 2008; Enke et al. 2013; Sathyanarayana et al. 2008a; Sathyanarayana et al. 2008b). Some studies have reported extremely high exposure to DEHP during hospitalization in preterm children (Silva et al. 2006; Su et al. 2012; Weuve et al. 2006), suggesting a risk of adverse health effects in this group as gonadal development is not completed (Rey and Josso 2013) and detoxification processes are still immature (Coughtrie et al. 1988; Tan et al. 1990).

In the present longitudinal study, we investigated urinary phthalate exposure from birth to 14 months of age in a group of full term (FT) and preterm (PT) infants, covering periods of hospitalization and home stay. We also assessed whether these exposure levels exceeded European Food Safety Authorities (EFSA) guidelines for tolerable daily intakes (EFSA 2005a; EFSA 2005b; EFSA 2005c).

Materials and Methods

Study population

As part of the Finnish Minipuberty study, a total of 173 mothers either with a normal singleton pregnancy or with a threat of premature delivery before 37.0 gestation weeks were recruited in a consecutive order inside these cohorts between August 2006 and March 2008 at Kuopio University Hospital, Finland. After delivery, infants of these mothers were recruited for the study including clinical examinations and sample collection at the age of 1-3 days (D1-3), 7 days (D7) and 1, 2, 3, 4, 5 and 6 months (M1-M6), and finally, at the corrected age of 14 months (M14; 14 months from the expected date of delivery, approximately 100 weeks of postmenstrual (PM) age, where PM age was defined as sum of gestational age at birth plus postnatal age at examination to signify the ‘true’ biological age of the infant). All together 125 children of 113 mothers completed the follow-up, and these included 58 full term (FT, 29 boys) and 67 preterm (PT, 33 boys) infants (Table 1). All together 894 urine samples were obtained at 1067 visits. Two FT infants were twin sisters; nine PT boys and 11 PT girls were twins, three PT girls were triplets. FT infants stayed at the hospital 2-18 days (mean 4.3) while PT infants were hospitalized 3-156 days (mean 39.6), up to 38–42 PM weeks (Kuiri-Hanninen et al. 2011a; Kuiri-Hanninen et al. 2011b).

Ethics

Both parents gave informed consent at initial recruitment and at follow-up at M14. The study was approved by the Ethics Committee of the Northern-Savo Health Care District.

Sample collection

Spot urine samples were collected in polyethylene urine collection bags (Paediatric Urine Collector, Unomedical, Denmark, and U-Bag Pediatric, Mabis Healthcare, USA) or by clean catch into polystyrene plastic cups (Econo Plastic Container, Huhtamäki OyJ, Finland) at every visit, and transported in 10 ml polypropene vials (Linkoputki, Mekalasi Oy, Finland). Urinary creatinine was measured by an enzymatic method before storage at -70°C in 3 ml polypropene vials (Mekamini, Mekalasi Oy, Finland).

Chemical analysis

Twelve primary and secondary metabolites of six phthalate diesters [diethyl phthalate (DEP), di-iso-butyl phthalate (DiBP), di-n-butyl phthalate (DnBP), butylbenzyl phthalate (BBzP), di-(2-ethylhexyl) phthalate (DEHP), and di-iso-nonyl phthalate (DiNP)] were analyzed. The total (sum of free and conjugated) content of monoethyl phthalate (MEP), mono-iso-butyl phthalate (MiBP), mono-n-butyl phthalate (MnBP), monobenzyl phthalate (MBzP), mono(2-ethylhexyl) phthalate (MEHP), mono(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP), mono(2-ethyl-5-oxohexyl) phthalate (MEOHP), mono(2-ethyl-5-carboxypentyl) phthalate (MECPP), mono-iso-nonyl phthalate (MiNP), mono(hydroxy-iso-nonyl) phthalate (MHiNP), mono(oxo-iso-nonyl) phthalate (MOiNP) and mono(carboxy-iso-octyl) phthalate (MCiOP) was analyzed by isotope dilution liquid chromatography tandem mass spectrometry (LC-MS/MS) with preceding enzymatic deconjugation followed by automatic solid phase extraction. The method details have been described previously (Frederiksen et al. 2010). Samples were analyzed in 22 batches during 8 weeks. Each batch included standards, 40 samples, two blanks, two pooled urine control samples, and two pooled urine control samples spiked with standards to an added concentration on 10 ng/mL. The recovery was above 90% for all analytes and the inter-day variation, expressed

as relative standard deviation (RSD), was below 3% for all analytes except MiBP (7%) and MnBP (9%). Limits of detection (LOD) are shown in Table 2.

Statistics

We measured one hydroxylated monoester and three oxidized secondary metabolites of DEHP and DiNP, respectively. To simplify data analysis, we calculated the molar sum of DEHP metabolites (MEHP, MEHHP, MEOHP and MECPP) and DiNP metabolites (MiNP, MHiNP, MOiNP and MCiOP) by multiplying the molar sums with the molecular weights of DEHP and DiNP, respectively ($\sum\text{DEHPm}$ and $\sum\text{DiNPm}$ in ng/ml, respectively).

Medians, percentiles, and ranges (minimum-maximum) of all measured phthalate concentrations were computed. All urinary metabolite concentrations $<$ LOD were set to 0.0 except for the analyses of intraclass correlation coefficients (ICCs) or if ln-transformation was used; in these cases data $<$ LOD were set to $\text{LOD}/\sqrt{2}$.

We also provide descriptive statistics for samples after adjustment for urinary creatinine to account for dilution. However, because urinary creatinine 1-3 days after birth reflects maternal creatinine (Matos et al. 1998), we excluded samples collected on D1-3 from this analysis.

Within- and between-person variance as well as ICCs were calculated. The ICC takes a value between 0 and 1 and reflects the relationships between the within- and between-subject variance. ICCs were classified as weak ($<$ 0.4), moderate (0.4 - 0.6), or good ($>$ 0.6) (Philippat et al. 2013).

For each child i we estimated the daily intake (DI) ($\mu\text{g}/\text{kg}/\text{day}$) of each phthalate diester p at sampling time t as:

$$\text{DI}_{pit} = [\sum_{k=1}^n (\text{UE}_{kit} / \text{MW}_k)] \times \text{MW}_p \times \text{CE}_{smoothed} / (\text{FUE}_p \times \text{BW}_{it}), \quad [1]$$

where UE_{kit} represents the creatinine-adjusted urinary concentration ($\mu\text{g/g}$ creatinine) of metabolite k in child i at time t , MW_k is the molecular mass of metabolite k ($\mu\text{g}/\mu\text{mol}$), MW_p is the molecular mass of diester p ($\mu\text{g}/\mu\text{mol}$), $CE_{smoothed}$ is the estimated average 24-hour urinary creatinine excretion (g/day) based on a study of newborns at 28-42 weeks of gestation (Al-Dahhan et al. 1988). FUE_p is the estimated fraction of diester p excreted in urine, and BW_{it} is the body weight (kg) of child i at time t . We assumed the following values of FUE_p based on studies of adults after oral intake of deuterium labeled phthalate diesters: 84% of DnBP excreted as MnBP, 70.3% of DiBP as MiBP, 70% of BBzP as MBzP, 45.3% of DEHP as DEHP metabolites (MEHP, MEHHP, MEOHP and MECPP), and 30% of DiNP as DiNP metabolites (MiNP, MHiNP, MOiNP and MCIOP) (Anderson et al. 2001; Anderson et al. 2011; Koch et al. 2012). For diethyl phthalate (DEP) we assumed an FUE of 69% (excreted as MEP) based on previous estimate for DnBP excretion. This estimate has often been used, since a human kinetic study on DEP is missing (Koch and Calafat 2009). To obtain a single median and selected percentile estimates for each phthalate at each sampling time (D7 through M14) according to PT or FT birth, estimates of DI for each child at time t were calculated as centiles. DI was not estimated for infants at time point D1-3, because creatinine is known to be elevated due to maternal contamination (Matos et al. 1998)

We estimated a hazard quotient (HQ) for phthalate diesters based on tolerable DI (TDI) values established by the European Food Safety Authorities (EFSA) (EFSA 2005a; EFSA 2005b; EFSA 2005c). TDI values for DnBP ($10 \mu\text{g}/\text{kg}/\text{day}$ ~ lowest observed adverse effect level (LOAEL) with an uncertainty factor of 200), BBzP ($500 \mu\text{g}/\text{kg}/\text{day}$ ~ no observed adverse effect level (NOAEL) with an uncertainty factor of 100) and DEHP ($50 \mu\text{g}/\text{kg}/\text{day}$ ~ NOAEL with an uncertainty factor of 100) are based on experimental animal studies. Since also DiBP has shown

similar effects as DnBP (Borch et al. 2006), TDI for DnBP was used to calculate HQ for DiBP. We divided estimated DIs by their corresponding TDI to derive HQs for phthalate diesters with evidence of anti-androgenic effects, i.e., DiBP, DnBP, BBzP, and DEHP, and combined the HQ estimates for these compounds to derive a single estimated Hazard Index (HI) for each infant (Koch et al. 2011; Soeborg et al. 2012):

$$HI_t = \sum_{p=1}^n (DI_{pt} / TDI_p), \quad [2]$$

where DI_{pt} is the estimated average daily intake of diester p ($\mu\text{g}/\text{kg}/\text{day}$) at time t , and TDI_p is the EFSA guideline value ($\mu\text{g}/\text{kg}/\text{day}$) for diester p (as indicated above). $HI = 1$ is the anti-androgenic threshold and a level > 1 indicates a potential risk of anti-androgenic effects.

We used mixed models analysis for statistical testing to account for the correlation structure due to the repeated measurements and multiple births in the cohort. We derived between- and within-group comparisons of phthalate metabolite concentrations, and estimated differences according to sex, hospitalization at the time of sampling (yes/no), breastfeeding (none, partial, or full), and child's weight at sampling. Data were analyzed according to calendar age (i.e., time from birth) and, to account for the immaturity in the PT infants, according to postmenstrual (PM) age (defined as sum of gestational age at birth plus postnatal age at examination to signify the 'true' biological age of the infant) categorized into intervals (30.1–34.0, 34.1–38.0, 38.1–42.0, 42.1–46.0, 46.1–50.0, 50.1–54.0, 54.1–58.0, 58.1–62.0, 62.1–68.5 and 96-106 PM weeks) corresponding to sampling times at D1-3, D7, M1-M6 and the corrected age of M14, respectively. Birth group (PT or FT), time point (or PM age category), sex, breastfeeding, hospitalization, and weight were modeled as fixed effects, while subject and an indicator for multiple births were modeled as random effects. Associations between the concentrations of

different phthalate metabolites and estimated DI were also analysed using the mixed model. We report standardized coefficients of ln-transformed phthalate metabolite concentrations, which are interpreted as correlation coefficients. p -values < 0.05 were considered statistically significant. SPSS software version 19.0 (SPSS Inc., Chicago, IL) was used for statistical analyses.

Results

Phthalate concentrations

At least one metabolites of each of the six phthalates was detectable in virtually all urine samples of both FT and PT infants, indicating that all infants were exposed to DEP, BBzP and DEHP and almost all infants were exposed to DiBP, DnBP, and DiNP at all time points. Only in one single urine sample metabolites of DiBP or DnBP were not detectable, while five urine samples did not contain measurable concentrations of any of the DiNP metabolites (Table 2). \sum DEHPm had the highest median concentration in both groups, followed by MBzP, MiBP, MnBP, MEP, and \sum DiNPm. With the exception of MEP and MnBP, PT infants had significantly higher median concentrations than FT infants for all metabolites ($p < 0.05$ for MiBP, otherwise $p < 0.001$). Creatinine adjusted phthalate metabolite concentrations were about 10-times higher than measured concentrations, with similar differences between PT and FT infants as in the unadjusted values (Supplemental Material, Table S1).

Concentrations of all metabolites were correlated, indicating that infants were simultaneously exposed to multiple phthalates. The weakest correlations were observed between MEP and MBzP, \sum DEHPm, and \sum DiNPm (parameter estimates from the standardized mixed model 0.41, 0.25, and 0.30, respectively); between MiBP and \sum DEHPm and \sum DiNPm (0.31 and 0.38, respectively), and MnBP and \sum DEHPm and \sum DiNPm (0.36 and 0.45, respectively). Correlations

among all other metabolites were stronger, with parameter estimates ranging from 0.52 (MiBP and MBzP) to 0.87 (MiBP and MnBP). From around M1 for FT and M2 for PT infants, each infant tended to stay within his/her trajectory of low or high exposure (Figure 1), although the ICC was low to moderate for most metabolites in both FT and PT infants (Supplemental Material, Table S2). The highest ICCs were in general observed from M1 to M6 in FT infants and from M2 to M6 in PT infants indicating consistent exposure in this period, but ICCs decreased if M14 was included.

Factors associated with urinary phthalate metabolite levels

Sex and breastfeeding were not significantly associated with urinary phthalate levels (Supplemental Material, Table S3). Therefore, these factors were not included in the subsequent models. MEP, MiBP and MnBP excreted from D1-3 to M14 showed a similar pattern for FT and PT infants except for MEP and MnBP at D7, for which PT infants had significantly higher excretion (Figure 2). Concentrations of other phthalate metabolites were significantly higher in PT infants compared to FT infants in D1-3, D7, and M1 samples for MBzP and \sum DEHPm, and in D7 and M1 samples for \sum DiNPm (Figure 2). Significantly higher concentrations of MBzP in M1 and M2 samples ($p < 0.01$), and of \sum DEHPm and \sum DiNPm in M1, M2, and M3 samples ($p < 0.01$) were estimated for hospitalized PT infants compared with PT infants at home (Supplemental Material, Figure S1). However, differences between FT and PT infants in MBzP and \sum DEHPm (at D1-3 and D7) and \sum DiNPm (at D7 and M1) concentrations remained statistically significant even after adjustment for hospitalization (Figure 2).

After discharge from hospital all metabolites except MBzP in FT infants increased significantly from D7 to M14 ($p < 0.05$, adjusted for weight). In discharged PT infants, a similar, significant

increase was observed in all metabolites except MEP from M1 to M14 ($p < 0.01$, adjusted for weight) (Supplemental Material, Figure S1).

Urinary phthalate metabolites by postmenstrual (PM) age

MBzP, Σ DEHPm and Σ DiNPm concentrations in PT infants were significantly higher in PM weeks 30.1-34.4 and 34.1-38.0 than concentrations in later PM weeks in both PT and FT infants ($p < 0.01$, adjusted for weight) (Supplemental Material, Figure S2). Furthermore concentrations of Σ DEHPm in PM weeks 46.1-50.0 and 54.1-58.0 samples and of Σ DiNPm in samples collected at PM weeks 42.1–46.0 through 54.1–58.0 were significantly higher in PT compared to FT infants (Supplemental Material, Figure S2).

Urinary phthalate excretion pattern

The proportion of monoester levels (MEHP and MiNP, respectively) relative to other DEHP and DiNP metabolites, respectively, was stable during the whole follow-up (Figure 3). A considerable change in the excretion pattern of secondary metabolites was observed by increasing maturation. The relative proportion of the carboxylated metabolites decreased significantly by PM age from on average of 66% to 35% MECPP in FT and PT infants and from 78% to 58% MCiOP in FT and from 71% to 55% MCiOP in PT infants. The proportions of other oxidized metabolites increased in the same period: hydroxylated metabolites from on average of 18% to 39% (MEHHP) and from 13% to 27% (MHiNP), and the keto-modified metabolites from 13% to 20% (MEOHP) and from 12% to 15% (MOiNP). In some samples, only carboxylated metabolites were detectable in concentrations above LODs. There was no statistically significant association of excretion patterns with estimated absolute exposure levels or gender (data not shown).

Daily intake (DI), hazard quotient (HQ), and hazard index (HI) estimates

Median estimated DIs of phthalate diesters from D7 to M14 are shown in Figure 4 (see Supplemental Material, Table S4 for corresponding numeric data). A constant estimated DI over time was observed for all phthalates except a significant decrease of exposure to BBzP, DEHP, and DiNP in PT infants from D7 to M2-3. The median DIs of DEP, DiBP, and DnBP did not differ significantly between FT and PT infants over time. In contrast, median DIs of BBzP, DEHP, and DiNP concentrations were significantly higher in PT than FT newborns from D7 to M2 (data not shown).

Median estimated HQs based on tolerable daily intake (TDI) values for DiBP, DnBP, BBzP, and DEHP are shown in Figure 4 and Supplemental Material, Table S4. At D7 more than 80% of the PT infants exceeded the anti-androgenic threshold ($HI > 1$), while approximately 30% of the FT infants (and the PT infants from M2) exceeded the threshold during the entire first year of life.

Discussion

To our knowledge, this is the first comprehensive longitudinal study of FT and PT infants from birth to 14 months of age, which documents a considerable exposure of all infants to multiple phthalates with an endocrine disrupting potential. Our findings regarding estimated daily intake and accumulated hazard index raise concern. Most PT children and approximately one third of FT children exceed the EFSA safety margin calculated for anti-androgenic effects, in particular shortly after birth. Such safety margins are estimated based on toxicological studies with rats defining exposure limits for either no observed effects or lowest dose with observed effect. Thus, our results raise concern as the observed phthalate exposures may put these infants at risk.

Epidemiological studies in humans have reported that phthalate exposure prenatally and in early life is associated with changes in infant sex hormones and decreased anogenital distance (AGD) (Main et al. 2006; Swan et al. 2005), allergic asthma (Bornehag and Nanberg 2010) and changes in gender specific behavioural patterns (Miodovnik et al. 2011; Swan et al. 2010; Whyatt et al. 2012). This is corroborated by numerous animal experiments showing decreased AGD and testicular testosterone production after in utero exposure (Borch et al. 2006; Foster 2006; Howdeshell et al. 2007).

It has previously been described that premature children are highly exposed to DEHP from medical equipment (Silva et al. 2006; Su et al. 2012; Weuve et al. 2006). It was assumed that this exposure was restricted to DEHP (Weuve et al. 2006). However, our study shows that higher phthalate exposure also occurred among hospitalized FT infants and was not restricted to DEHP. While concentrations of metabolites in urine suggest that the Finnish PT infants in our population were exposed to DEP, DiBP, and DnBP at levels similar to the FT infants at hospital and at home, they were significantly higher exposed to BBzP, DEHP and DiNP during hospitalization compared to PT infants after discharge from hospital, i.e. the neonatal intensive care unit was the likely exposure source. After discharge exposure patterns for BBzP, DEHP, and DiNP were similar for PT and FT infants. Regional differences may exist in the use of phthalate diesters in medical equipment. However, domestic exposure also needs to be addressed when planning preventive measures. Urinary phthalate metabolite concentrations increased with age but the estimated DI per kilo body weight remained constant over time. Diet has been pointed out as the main source of phthalate exposure, especially for DEHP (Wittassek et al. 2011). However, only low amounts of phthalate diesters or their metabolites have been quantified in human breast milk (Fromme et al. 2011; Main et al. 2006) but especially low

molecular phthalates, such as dimethyl phthalate, DEP and DiBP have been observed in baby care products (Sathyanarayana et al. 2008b).

It is still debated whether urinary concentrations of phthalate metabolites should be adjusted for creatinine (Lorber et al. 2011) to correct for dilution. We did not use creatinine correction for the first 3 days as levels are elevated due to maternal contamination (Matos et al. 1998). Thus, in our study results from D1-3 were not included, when analysing data, which were creatinine corrected. Muscle mass in infants is extremely small, thus creatinine-adjusted levels are extremely high. This should be kept in mind when comparing exposure levels to older populations. During the first postnatal months urinary dilution will show less variation due to a more consistent eating and sleeping pattern in newborns compared to older children. Creatinine correction may therefore not be as appropriate as in older populations.

Since exposure through breastfeeding is believed to be low (Fromme et al. 2011) and our study did not reveal statistically significant differences in phthalate exposure levels between breast-fed and bottle-fed infants, it points to exposure sources other than diet. The relatively constant DI in our study indicates exposure sources such as personal care products and the general home environment. Phthalates are present in many consumer products (Buckley et al. 2012; Fromme et al. 2007; Sathyanarayana et al. 2008b), they sediment in house dust (Becker et al. 2004; Langer et al. 2013) and exposure occurs through ingestion, inhalation and dermally (Janjua et al. 2008; Langer et al. 2013; Wittassek et al. 2011). As infants share their mother's environment, exposure levels measured after birth may also reflect antenatal exposure (Sathyanarayana et al. 2008a; Wittassek et al. 2009). Both the prenatal and early postnatal periods are particularly vulnerable phases for reproductive development (Main et al. 2006; Swan et al. 2005).

As phthalates have a short half-life in humans (Anderson et al. 2001; Anderson et al. 2011; Koch et al. 2012), assessment of exposure levels is difficult. Concentrations of phthalate metabolites in urine are considered the best biomarker of exposure (Frederiksen et al. 2010). However, variation in urinary dilution and phthalate exposures (Sathyanarayana et al. 2008b) lead to variation in phthalate concentrations among spot urine samples collected from the same individual, which was also observed in our study. However, a given infant tended to stay within low, medium or high levels of exposures over time which was in accordance with previous observations (Mouritsen et al. 2013). Although we observed low to moderate ICCs for most phthalates, our data indicates that the within-subject variability was lower than the between-subject variability. These ICCs were similar to previous ICCs in pregnant women (Adibi et al. 2008; Frederiksen et al. 2013a). In young men ICCs were similar for MEP, MiBP, MnBP and BBzP but higher for Σ DEHPm and Σ DiNPm (Adibi et al. 2008; Frederiksen et al. 2013a). This suggests a more constant phthalate exposure in infants and pregnant women than in young men. Highest ICCs were observed from M1 to M6 in FT infants and from M2 to M6 in PT infants indicating a relatively consistent exposure during this period. Such consistency may be created by uniform diet (breast milk and/or formula) given regularly during the waking hours and a consistent home environment during the first months of life. As ICCs decreased slightly at 14 months of age this may indicate more variable sources of exposure during diet transition to more solid foods and to a more active and exploratory life style including mouthing of toys and objects.

Children with a relatively high exposure to one phthalate tended to also be highly exposed to the other five phthalates and exposures tended to be present at a relatively high level throughout infancy. Similar significant correlations were observed in several previous studies (Frederiksen

et al. 2012; Koch et al. 2011; Mieritz et al. 2012; Mouritsen et al. 2013). This may be of biological significance as high simultaneous exposure may exceed a safe threshold. Our estimates of daily phthalate intakes, hazard quotients, and a hazard index are rough estimates and should be interpreted with caution. Urinary excretion fractions used to estimate daily intakes were derived from adult studies because they are not available for infants (Anderson et al. 2001; Anderson et al. 2011; Koch et al. 2012). Likewise, we used estimates of the average excretion of creatinine based on 24 hours urine collections from 60 infants with gestational age 27-40 weeks and postnatal age ranging from 3-68 days (Al-Dahhan et al. 1988). Exposure measurements in the individual child will in addition vary over time. Thus, our data should only be taken as indicator of potential anti-androgenic effects. 30% of all infants exceed the anti-androgenic threshold and 80% of preterm babies during the first 2-3 months of life. The same children will simultaneously be exposed to other environmental chemicals (Kortenkamp and Faust 2010), which may act synergistically with phthalates. Thus, our observations raise concern.

Our study indicates a change in the detoxification metabolism of phthalates during the first year of life which is mostly likely linked to physiological maturation processes in newborn infants. Carboxylated metabolites decreased and oxidized metabolites increased during the first year of life. A high proportion of the carboxylated metabolite MECPP has been reported before in newborns (2-5 days after birth) and preterm infants (Enke et al. 2013; Silva et al. 2006). At 14 month of age the urinary metabolite distribution pattern for DEHP and DiNP was comparable with the pattern in older children and adults (Becker et al. 2004; CDC 2013; Frederiksen et al. 2011).

Phthalates follow a two-step metabolic pathway; a phase I biotransformation and phase II conjugation. In the first step all phthalate diesters are hydrolyzed into their respective hydrolytic

monoesters. The low molecular phthalates, such as DEP, DiBP, and DnBP are mainly excreted in urine as free or conjugated monoesters, while the high molecular monoesters such as MEHP and MiNP undergo further biotransformation to water-soluble hydroxy- or oxo-metabolites before conjugation (Koch and Calafat 2009; Rusyn et al. 2006). Phase II conjugation is mainly catalyzed by uridine 5'-diphosphoglucuronyl transferase (UGT) to form hydrophilic glucuronidated conjugates. An alternative pathway in high molecular phthalates is carboxylation of the monoesters. There are about 20 UGTs, some of which are involved in glucuronidation of androgens (Alcorn and McNamara 2002), i.e. UGT2B17 the expression of which increases significantly during the first weeks of life, whereas it is inactive in fetal liver cells or present in a low affinity form (Coughtrie et al. 1988; Tan et al. 1990).

It has been proposed that the phthalate metabolite excretion pattern of newborns could be a consequence of prematurity or *in utero* exposure (Enke et al. 2013). However, this metabolic pattern appeared to persist throughout the first year of life in our study population, consistent with an immature detoxification metabolism. Current risk assessments do not account for this. Since the carboxylated metabolite of DEHP has only recently become available previous studies on DEHP exposure of newborns may have systematically underestimated exposure levels (Su et al. 2012; Weuve et al. 2006).

Conclusions

All newborns and infants in our study population were exposed to phthalates, not only during hospitalization, but also at home. Most premature babies and up to one third of mature babies in our study population had estimated exposures that appeared to exceed current EFSA recommendations for daily intakes that were developed as guidelines to limit the risk of endocrine disruptive effects. Our findings support the need for additional research on exposures

and health effects of phthalates in infants, particularly with keeping in mind that phthalate metabolism may be limited during the first year of life.

References

- Adibi JJ, Whyatt RM, Williams PL, Calafat AM, Camann D, Herrick R, et al. 2008. Characterization of phthalate exposure among pregnant women assessed by repeat air and urine samples. *Environ Health Perspect* 116:467-473.
- Al-Dahhan J, Stimmler L, Chantler C, Haycock GB. 1988. Urinary creatinine excretion in the newborn. *Arch Dis Child* 63:398-402.
- Alcorn J, McNamara PJ. 2002. Ontogeny of hepatic and renal systemic clearance pathways in infants: part II. *Clin Pharmacokinet* 41:1077-1094.
- Anderson WA, Castle L, Hird S, Jeffery J, Scotter MJ. 2011. A twenty-volunteer study using deuterium labelling to determine the kinetics and fractional excretion of primary and secondary urinary metabolites of di-2-ethylhexylphthalate and di-iso-nonylphthalate. *Food Chem Toxicol* 49:2022-2029.
- Anderson WA, Castle L, Scotter MJ, Massey RC, Springall C. 2001. A biomarker approach to measuring human dietary exposure to certain phthalate diesters. *Food Addit Contam* 18:1068-1074.
- Becker K, Seiwert M, Angerer J, Heger W, Koch HM, Nagorka R, et al. 2004. DEHP metabolites in urine of children and DEHP in house dust. *Int J Hyg Environ Health* 207:409-417.
- Bergman A, Heindel J, Jobling S, Kidd K, Zoeller RT. 2012. State-of-the-science of endocrine disrupting chemicals, 2012. *Toxicology Letters* 211:S3.
- Borch J, Axelstad M, Vinggaard AM, Dalgaard M. 2006. Diisobutyl phthalate has comparable anti-androgenic effects to di-n-butyl phthalate in fetal rat testis. *Toxicol Lett* 163: 183-190.
- Bornehag CG, Nanberg E. 2010. Phthalate exposure and asthma in children. *Int J Androl* 33:333-345.
- Buckley JP, Palmieri RT, Matuszewski JM, Herring AH, Baird DD, Hartmann KE, et al. 2012. Consumer product exposures associated with urinary phthalate levels in pregnant women. *J Expo Sci Environ Epidemiol* 22:468-475.
- CDC. 2012. Fourth National report on Human Exposure to Environmental Chemicals, Updated tables, September 2013. Available: <http://www.cdc.gov/exposurereport> [assessed 6 May 2014].

- Coughtrie MW, Burchell B, Leakey JE, Hume R. 1988. The inadequacy of perinatal glucuronidation: immunoblot analysis of the developmental expression of individual UDP-glucuronosyltransferase isoenzymes in rat and human liver microsomes. *Mol Pharmacol* 34:729-735.
- EFSA. 2005a. Opinion of the scientific panel in food additives, flavourings, processing aids and materials in contact with food (AFC) on a request from the Commission related to: Bis(2-ethylhexyl)phthalate (DEHP) for use in food contact materials. Question No EFSA-Q-2003-191. *EFSA J* 243:1-20.
- EFSA. 2005b. Opinion of the scientific panel in food additives, flavourings, processing aids and materials in contact with food (AFC) on a request from the Commission related to: Butylbenzylphthalate (BBP) for use in food contact materials. Question No EFSA-Q-2003-190. *EFSA J* 241:1-14.
- EFSA. 2005c. Opinion of the scientific panel in food additives, flavourings, processing aids and materials in contact with food (AFC) on a request from the Commission related to: di-Butylphthalate (DBP) for use in food contact materials. Question No EFSA-Q-2003-192. *EFSA J* 242: 1-18.
- Enke U, Schleussner E, Palmke C, Seyfarth L, Koch HM. 2013. Phthalate exposure in pregnant women and newborns - The urinary metabolite excretion pattern differs distinctly. *Int J Hyg Environ Health* 213:735-742.
- Foster PM. 2006. Disruption of reproductive development in male rat offspring following in utero exposure to phthalate esters. *Int J Androl* 29:140-147.
- Frederiksen H, Aksglaede L, Sorensen K, Skakkebaek NE, Juul A, Andersson AM. 2011. Urinary excretion of phthalate metabolites in 129 healthy Danish children and adolescents: estimation of daily phthalate intake. *Environ Res* 111:656-663.
- 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.
- Frederiksen H, Kranich SK, Jorgensen N, Taboureau O, Petersen JH, Andersson AM. 2013a. Temporal variability in urinary phthalate metabolite excretion based on spot, morning, and 24-h urine samples: considerations for epidemiological studies. *Environ Sci Technol* 47: 958-967.

- Frederiksen H, Sorensen K, Mouritsen A, Aksglaede L, Hagen CP, Petersen JH, et al. 2012. High urinary phthalate concentration associated with delayed pubarche in girls. *Int J Androl* 35:216-226.
- Fromme H, Gruber L, Schlummer M, Wolz G, Bohmer S, Angerer J, et al. 2007. Intake of phthalates and di(2-ethylhexyl)adipate: results of the Integrated Exposure Assessment Survey based on duplicate diet samples and biomonitoring data. *Environ Int* 33:1012-1020.
- Fromme H, Gruber L, Seckin E, Raab U, Zimmermann S, Kiranoglu M, et al. 2011. Phthalates and their metabolites in breast milk--results from the Bavarian Monitoring of Breast Milk (BAMBI). *Environ Int* 37:715-722.
- Howdeshell KL, Furr J, Lambright CR, Rider CV, Wilson VS, Gray LE, Jr. 2007. Cumulative effects of dibutyl phthalate and diethylhexyl phthalate on male rat reproductive tract development: altered fetal steroid hormones and genes. *Toxicol Sci* 99:190-202.
- Janjua NR, Frederiksen H, Skakkebaek NE, Wulf HC, Andersson AM. 2008. Urinary excretion of phthalates and paraben after repeated whole-body topical application in humans. *International Journal of Andrology* 31:118-129.
- Koch HM, Calafat AM. 2009. Human body burdens of chemicals used in plastic manufacture. *Philos Trans R Soc Lond B Biol Sci* 364:2063-2078.
- Koch HM, Christensen KL, Harth V, Lorber M, Bruning T. 2012. Di-n-butyl phthalate (DnBP) and diisobutyl phthalate (DiBP) metabolism in a human volunteer after single oral doses. *Arch Toxicol* 86:1829-1839.
- Koch HM, Wittassek M, Bruning T, Angerer J, Heudorf U. 2011. Exposure to phthalates in 5-6 years old primary school starters in Germany--a human biomonitoring study and a cumulative risk assessment. *Int J Hyg Environ Health* 214:188-195.
- Kortenkamp A, Faust M. 2010. Combined exposures to anti-androgenic chemicals: steps towards cumulative risk assessment. *Int J Androl* 33:463-474.
- Kuiri-Hanninen T, Kallio S, Seuri R, Tyrvaainen E, Liakka A, Tapanainen J, et al. 2011a. Postnatal developmental changes in the pituitary-ovarian axis in preterm and term infant girls. *J Clin Endocrinol Metab* 96:3432-3439.
- Kuiri-Hanninen T, Seuri R, Tyrvaainen E, Turpeinen U, Hamalainen E, Stenman UH, et al. 2011b. Increased activity of the hypothalamic-pituitary-testicular axis in infancy results in increased androgen action in premature boys. *J Clin Endocrinol Metab* 96:98-105.

- Langer S, Beko G, Weschler CJ, Brive LM, Toftum J, Callesen M, et al. 2014. Phthalate metabolites in urine samples from Danish children and correlations with phthalates in dust samples from their homes and daycare centers. *Int J Hyg Environ Health* 217:78-87
- Lorber M, Koch HM, Angerer J. 2011. A critical evaluation of the creatinine correction approach: can it underestimate intakes of phthalates? A case study with di-2-ethylhexyl phthalate. *J Expo Sci Environ Epidemiol* 21:576-586.
- Main KM, Mortensen GK, Kaleva MM, Boisen KA, Damgaard IN, Chellakooty M, et al. 2006. Human breast milk contamination with phthalates and alterations of endogenous reproductive hormones in infants three months of age. *Environ Health Perspect* 114:270-276.
- Matos P, Duarte-Silva M, Drukker A, Guignard JP. 1998. Creatinine reabsorption by the newborn rabbit kidney. *Pediatr Res* 44:639-641.
- Mieritz MG, Frederiksen H, Sorensen K, Aksglaede L, Mouritsen A, Hagen CP, et al. 2012. Urinary phthalate excretion in 555 healthy Danish boys with and without pubertal gynaecomastia. *Int J Androl* 35:227-235.
- Miodovnik A, Engel SM, Zhu C, Ye X, Soorya LV, Silva MJ, et al. 2011. Endocrine disruptors and childhood social impairment. *Neurotoxicology* 32:261-267.
- Mouritsen A, Frederiksen H, Sorensen K, Aksglaede L, Hagen C, Skakkebaek NE, et al. 2013. Urinary phthalates from 168 girls and boys measured twice a year during a 5-year period: associations with adrenal androgen levels and puberty. *J Clin Endocrinol Metab* 98:3755-3764.
- Philippat C, Wolff MS, Calafat AM, Ye X, Bausell R, Meadows M, et al. 2013. Prenatal exposure to environmental phenols: concentrations in amniotic fluid and variability in urinary concentrations during pregnancy. *Environ Health Perspect* 121:1225-1231.
- Rey R, Josso N. 2013. Sexual differentiation. Posted on 8 February 2013. Available: <http://www.endotext.org/chapter/sexual-differentiation> [assessed 6 May 2014].
- Rusyn I, Peters JM, Cunningham ML. 2006. Modes of action and species-specific effects of di-(2-ethylhexyl)phthalate in the liver. *Critical Reviews in Toxicology* 36:459-479.
- Sathyanarayana S, Calafat AM, Liu F, Swan SH. 2008a. Maternal and infant urinary phthalate metabolite concentrations: are they related? *Environ Res* 108: 413-418.

- Sathyanarayana S, Karr CJ, Lozano P, Brown E, Calafat AM, Liu F, et al. 2008b. Baby care products: possible sources of infant phthalate exposure. *Pediatrics* 121:e260-e268.
- Silva MJ, Reidy JA, Preau JL, Samandar E, Needham LL, Calafat AM. 2006. Measurement of eight urinary metabolites of di(2-ethylhexyl) phthalate as biomarkers for human exposure assessment. *Biomarkers* 11:1-13.
- Soeborg T, Frederiksen H, Andersson AM. 2012. Cumulative risk assessment of phthalate exposure of Danish children and adolescents using the hazard index approach. *Int J Androl* 35:245-252.
- Su PH, Chang YZ, Chang HP, Wang SL, Haung HI, Huang PC, et al. 2012. Exposure to di(2-ethylhexyl) phthalate in premature neonates in a neonatal intensive care unit in Taiwan. *Pediatr Crit Care Med* 13:671-677.
- Swan SH, Liu F, Hines M, Kruse RL, Wang C, Redmon JB, et al. 2010. Prenatal phthalate exposure and reduced masculine play in boys. *Int J Androl* 33:259-269.
- Swan SH, Main KM, Liu F, Stewart SL, Kruse RL, Calafat AM, et al. 2005. Decrease in anogenital distance among male infants with prenatal phthalate exposure. *Environ Health Perspect* 113:1056-1061.
- Tan TM, Sit KH, Wong KP. 1990. UDP-glucuronyltransferase activity toward harmol in human liver and human fetal liver cells in culture. *Anal Biochem* 185:44-50.
- Weuve J, Sanchez BN, Calafat AM, Schettler T, Green RA, Hu H, et al. 2006. Exposure to phthalates in neonatal intensive care unit infants: urinary concentrations of monoesters and oxidative metabolites. *Environ Health Perspect* 114:1424-1431.
- Whyatt RM, Liu X, Rauh VA, Calafat AM, Just AC, Hoepner L, et al. 2012. Maternal prenatal urinary phthalate metabolite concentrations and child mental, psychomotor, and behavioral development at 3 years of age. *Environ Health Perspect* 120:290-295.
- Wittassek M, Angerer J, Kolossa-Gehring M, Schafer SD, Klockenbusch W, Dobler L, et al. 2009. Fetal exposure to phthalates--a pilot study. *Int J Hyg Environ Health* 212:492-498.
- Wittassek M, Koch HM, Angerer J, Bruning T. 2011. Assessing exposure to phthalates - the human biomonitoring approach. *Mol Nutr Food Res* 55:7-31.

Table 1. Birth characteristics, n=125 [n (%) or mean \pm SD (range)].

	Full term infants	Preterm infants
Boys		
n (%)	29 (50.0)	33 (49.3)
Gestational weeks	39.8 \pm 1.37 (37.1-42.1)	31.8 \pm 3.26 (24.7-36.6)
Birth weight (g)	3274 \pm 741 (1910-4420)	1693 \pm 548 (550-2850)
Birth length (cm)	49.1 \pm 2.88 (42-53)	41.3 \pm 4.6 (30-48)
Days at hospital	4.9 \pm 4.16 (2-18)	42.9 \pm 32.0 (7-131)
Girls		
n (%)	29 (50.0)	34 (50.7)
Gestational weeks	39.5 \pm 1.33 (37.0-41.7)	32.9 \pm 3.03 (24.7-36.7)
Birth weight (g)	3373 \pm 630 (2070-4750)	1765 \pm 522 (530-2720)
Birth length (cm)	49.2 \pm 2.61 (44-54)	41.8 \pm 4.27 (29-47)
Days at hospital	3.8 \pm 2.26 (2-12)	36.4 \pm 31.4 (3-156)

Table 2. Urinary phthalate metabolite concentrations (ng/mL) in serial samples from birth to 14 months of age in 58 full term (FT, n = 432 samples) and 67 preterm (PT, n = 462 samples) infants.

Phthalate metabolites	LOD	FT n>LOD	FT %>LOD	FT Min.	FT 10th %ile	FT Median	FT 90th %ile	FT Max.	PT n>LOD	PT %>LOD	PT Min.	PT 10th %ile	PT Median	PT 90th %ile	PT Max.
MEP	0.53	432	100	0.82	3.46	10.7	49.1	486	462	100	0.71	3.51	11.9	44.0	190
MiBP	1.43	431	99.8	<LOD	7.45	21.0	62.3	337	462	100	3.21	8.40	23.3	92.4	5648
MnBP	1.10	431	99.8	<LOD	4.96	15.2	51.3	156	462	100	1.43	5.89	15.5	47.3	6533
MBzP	1.14	432	100	1.85	6.66	24.6	118	1985	462	100	2.13	8.66	43.1	244	1155
MEHP	0.14	329	76.2	<LOD	<LOD	0.44	2.11	849	396	85.7	<LOD	<LOD	0.99	27.4	1379
MEHHP	0.91	407	94.2	<LOD	1.27	5.01	26.0	480	451	97.6	<LOD	1.63	8.62	163	6934
MEOHP	0.67	414	95.8	<LOD	1.06	3.90	15.3	351	452	97.8	<LOD	1.50	6.12	86.4	4010
MECPP	0.55	432	100	0.75	3.74	10.7	40.2	1366	462	100	0.81	5.85	17.9	668	26011
ΣDEHPm					8.83	26.5	109	4032				12.3	45.3	1284	46488
MiNP	0.61	11	2.5	<LOD	<LOD	<LOD	0.18	2.88	49.0	10.6	<LOD	<LOD	<LOD	0.65	93.0
MHiNP	0.26	212	49.1	<LOD	<LOD	<LOD	2.16	27.1	312	67.5	<LOD	<LOD	0.61	6.46	135
MOiNP	0.25	200	46.3	<LOD	<LOD	<LOD	1.33	10.4	301	65.2	<LOD	<LOD	0.47	4.92	125
MCiOP	0.11	427	98.8	<LOD	0.29	1.32	5.40	58.2	458	99.1	<LOD	0.50	2.37	29.8	493
ΣDiNPm						2.36	11.6	107					4.74	62.7	1035

Abbreviations: LOD, limit of detection; ΣDEHPm, the sum of DEHP metabolites; ΣDiNPm, the sum of DiNP metabolites.

Figure legends

Figure 1. Within subject variation of urinary phthalate metabolite levels in 58 full term (FT) infants (307 samples) from M1-M6 and 67 preterm (PT) infants (290 samples) from M2-M6 for MEP, MiBP, MnBP, MBzP, \sum DEHPm, and \sum DiNPm. Vertical lines with dots represent individual samples of the same infant (Y-axis) versus the infant's mean phthalate metabolite level (X-axis). All concentrations were ln-transformed. Phthalate metabolite concentrations for PT infants at M1 were not included, because PT infants at M1 mostly were hospitalized, while only seven and five infants still were hospitalized at M2 and M3, respectively.

Figure 2. Median levels (error bars indicate the 25th – 75th percentiles) of urinary phthalate metabolites in 432 samples (D1-3, n=32; D7, n=50; M1, n=53; M2, n=54; M3, n=50; M4, n=51; M5, n=50; M6, n=49; M14, n=43) of full term (FT) and 462 samples (D1-3, n=41; D7, n=39; M1, n=48; M2, n=51; M3, n=59; M4, n=57; M5, n=61; M6, n=61; M14, n=45) of preterm (PT) infants according to age (day (D) and month (M)). The asterisks indicate statistical significance for group difference in the mixed-model analysis adjusted for weight (* $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$).

Figure 3. The proportion of individual DEHP or DiNP metabolites vs. the total sum of all measured DEHP metabolites (\sum DEHPm) or DiNP metabolites (\sum DiNPm) by postmenstrual age in 58 full term (FT) (~432 urine samples) and 67 preterm (PT) infants (~462 urine samples). In seven FT and three PT samples MECPP was the only metabolite of DEHP measured (MECPP/ \sum DEHPm = 100%) and in 201 FT and 135 PT samples MCiOP was the only metabolite of DiNP (MCiOP/ \sum DiNPm = 100%) measured.

Figure 4. Median daily intake (DI), hazard quotient (HQ) and hazard index (HI) of phthalate diesters in full term (FT) (D7, n=49; M1, n=53; M2, n=54; M3, n=50; M4, n=51; M5, n=50; M6, n=49; M14, n=41) and preterm (PT) (D7, n=38; M1, n=47; M2, n=51; M3, n=58; M4, n=56; M5, n=61; M6, n=61; M14, n=45) infants according to age (day (D) and month (M)). Median DIs are shown for the phthalate diesters (upper figures) and median levels of HQ and HI (error bars indicate the 25th – 75th percentiles) are shown for DiBP, DnBP, BBzP and DEHP (lower figures). The blue dotted line indicates the anti-androgenic threshold value (HI = 1) and labels above error bars indicate the percentage of infants exceeding HI (HI was defined by summed ratios of estimated daily intakes to TDI based on EFSA recommendations). See Supplemental Material, Table S4 for corresponding numeric data.

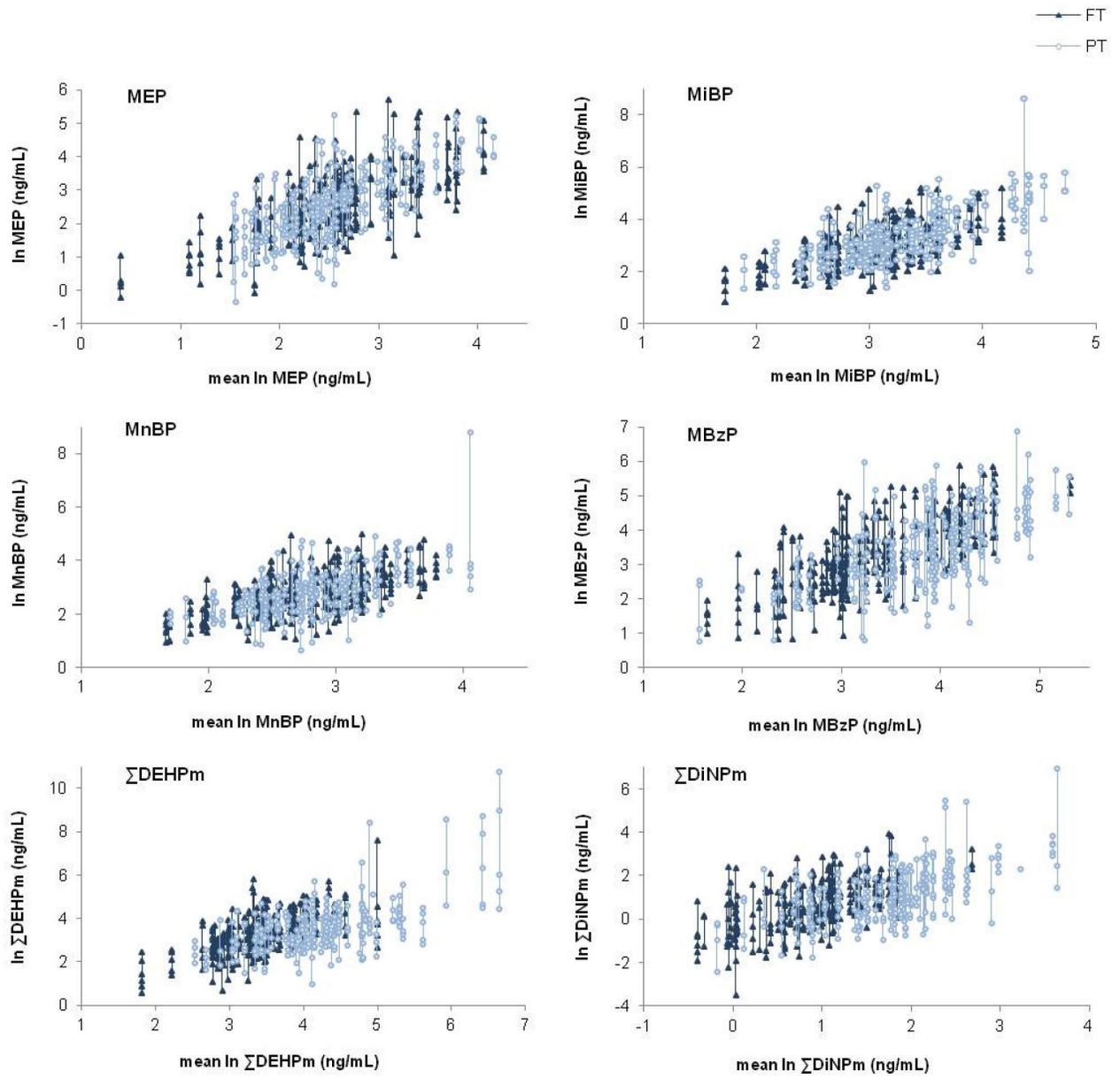


Figure 1

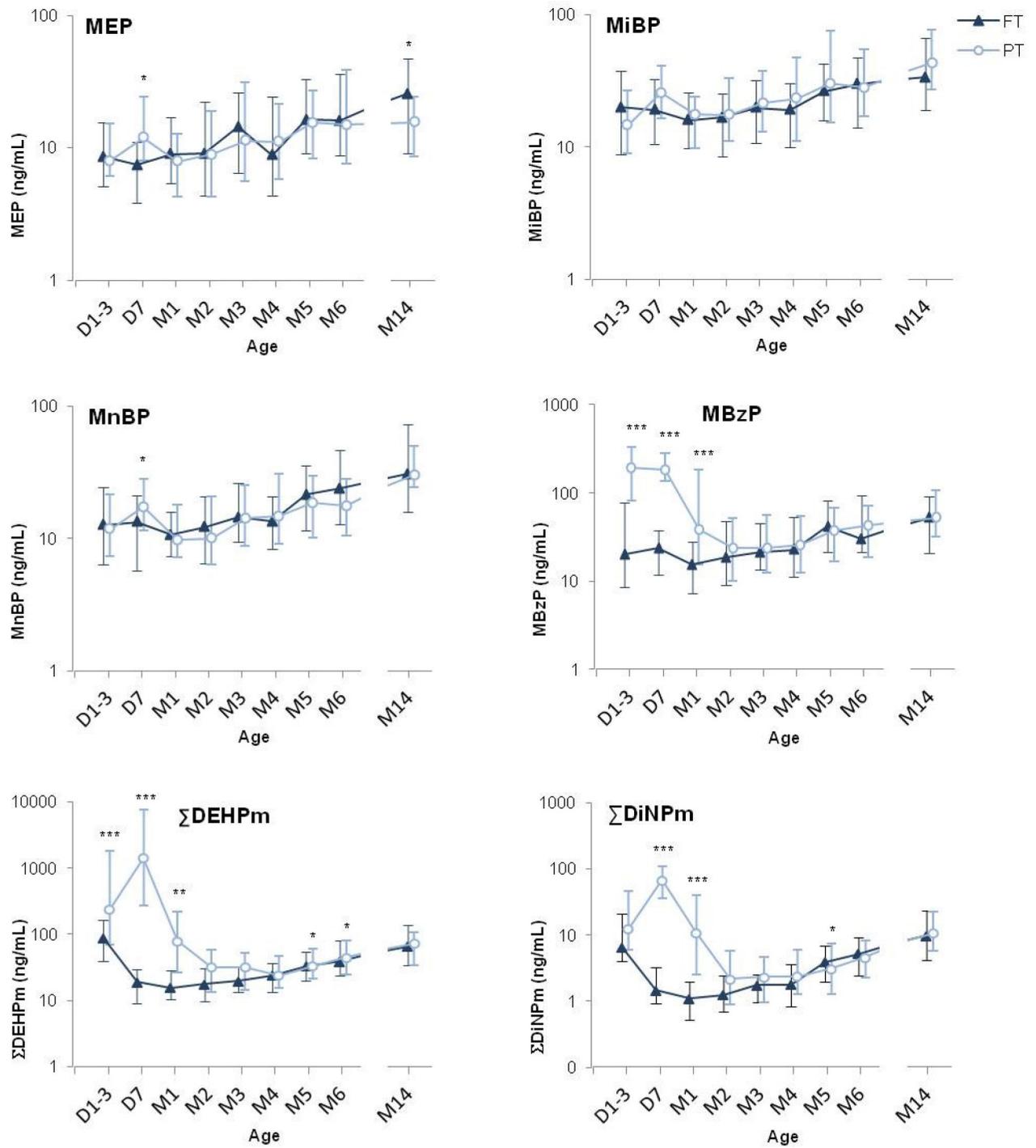


Figure 2

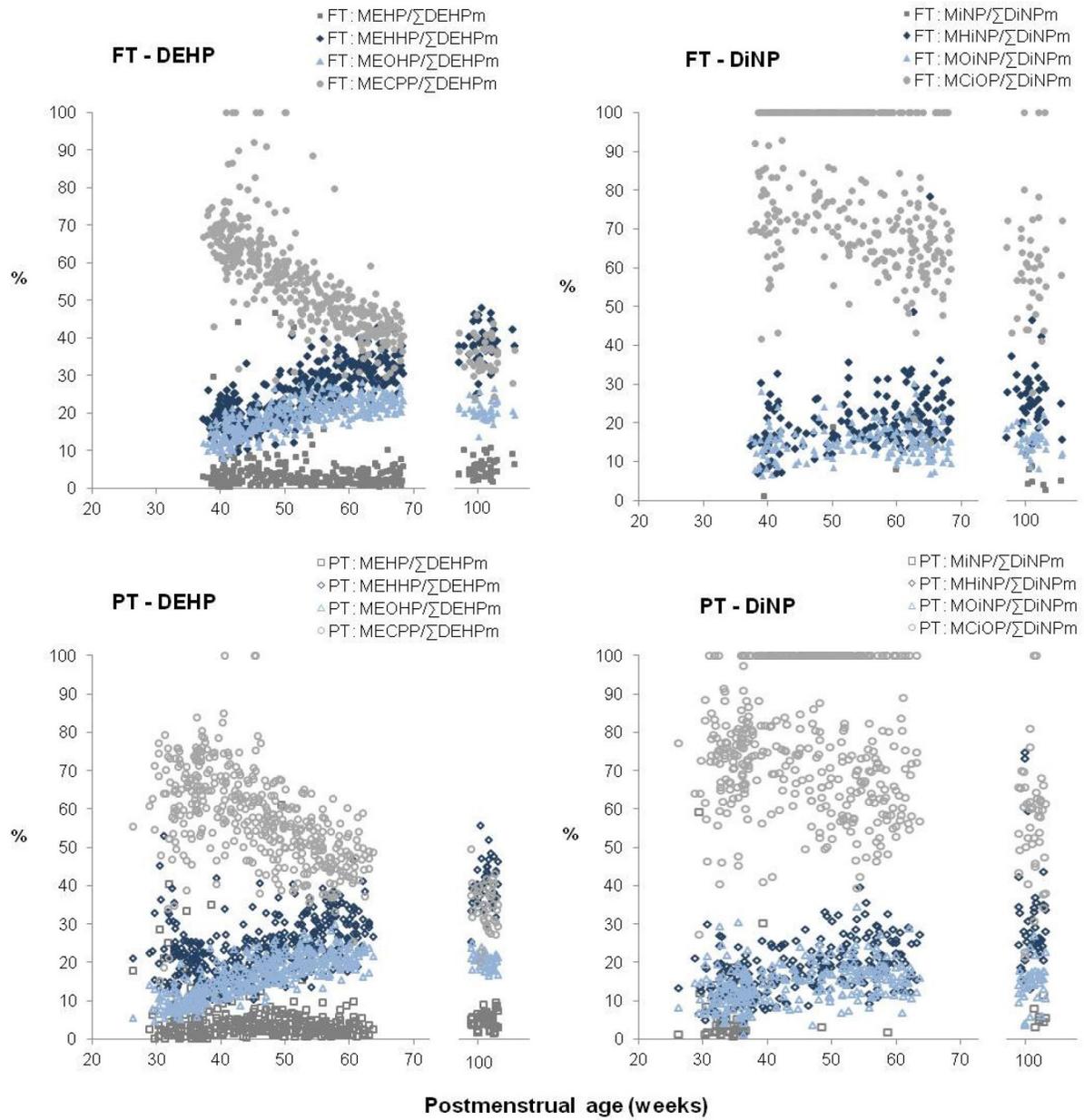


Figure 3

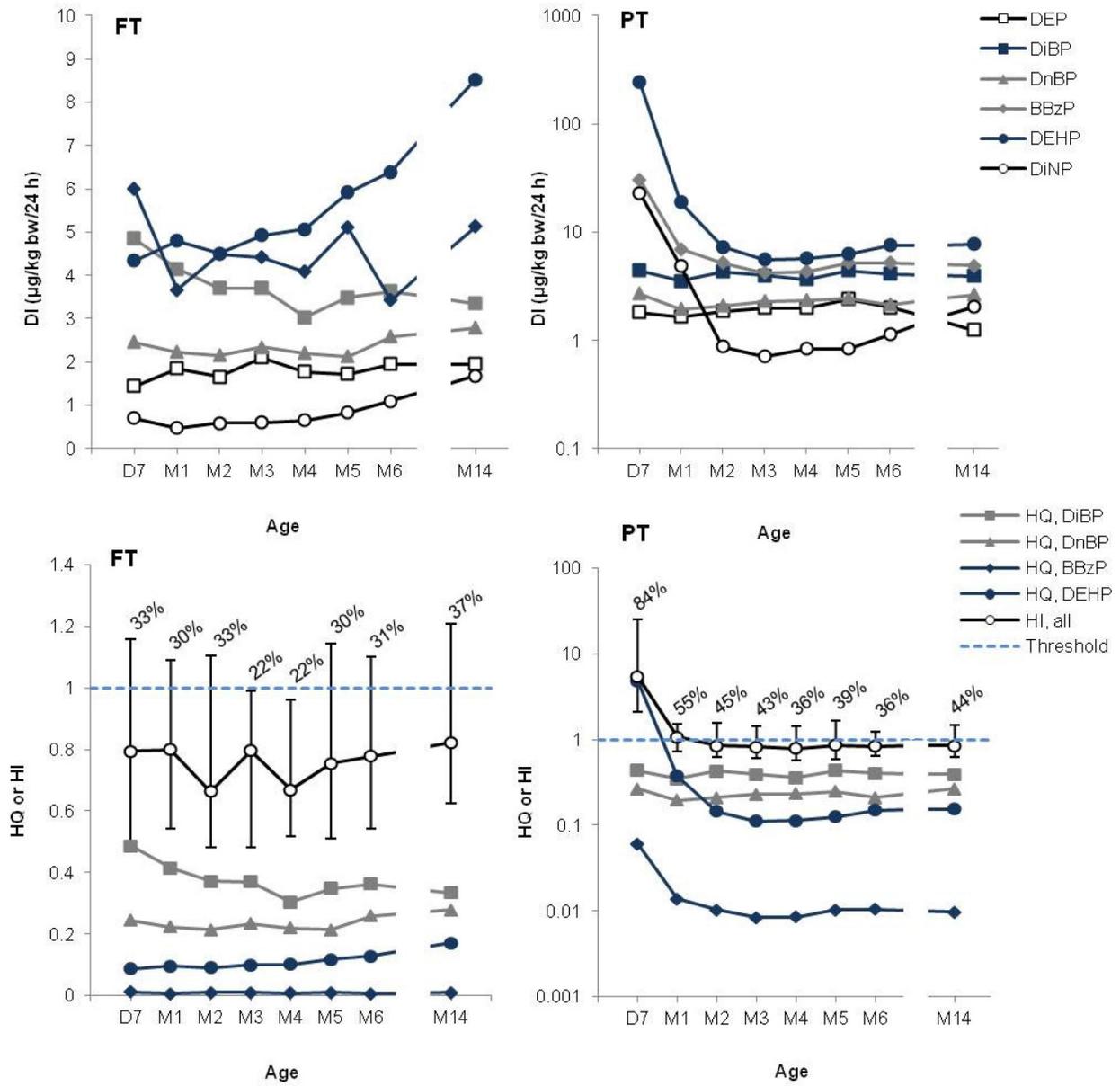


Figure 4