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Human urinary excretion of non-persistent environmental chemicals: an overview of Danish data collected between 2006 and 2012

Hanne Frederiksen[1], Tina Kold Jensen[1,2], Niels Jørgensen[1], Henriette Boye Kyhl[3], Steffen Husby[3], Niels E Skakkebæk[1], Katharina M Main[1], Anders Juul[1] and Anna-Maria Andersson[1]

[1]*Department of Growth and Reproduction, GR 5064, Rigshospitalet, Faculty of Health and Medical Sciences, University of Copenhagen, Blegdamsvej 9, DK-2100 Copenhagen, Denmark,* [2]*Department of Environmental Medicine, Institute of Public Health, University of Southern Denmark, Odense, Denmark and* [3]*Hans Christian Andersen Children's Hospital, Odense University Hospital, Odense, Denmark*

Correspondence should be addressed to H Frederiksen; Email: hanne.frederiksen@regionh.dk

Abstract

Several non-persistent industrial chemicals have shown endocrine disrupting effects in animal studies and are suspected to be involved in human reproductive disorders. Among the non-persistent chemicals that have been discussed intensively during the past years are phthalates, bisphenol A (BPA), triclosan (TCS), and parabens because of their anti-androgenic and/or estrogenic effects. Phthalates are plasticizers used in numerous industrial products. Bisphenol A is the main component of polycarbonate plastics and epoxy resins. Parabens and TCS are antimicrobial preservatives and other phenols such as benzophenone-3 (BP-3) act as a UV-screener, while chlorophenols and phenyl phenols are used as pesticides and fungicides in agriculture. In spite of the widespread use of industrial chemicals, knowledge of exposure sources and human biomonitoring studies among different segments of the population is very limited. In Denmark, we have no survey programs for non-persistent environmental chemicals, unlike some countries such as the USA (NHANES) and Germany (GerES). However, we have analyzed the excretion of seven parabens, nine phenols, and the metabolites of eight different phthalates in urine samples collected over the past 6 years from four Danish cohorts. Here, we present biomonitoring data on more than 3600 Danish children, adolescents, young men, and pregnant women from the general population. Our study shows that nearly all Danes were exposed to the six most common phthalates, to BPA, TCS, and BP-3, and to at least two of the parabens. The exposure to other non-persistent chemicals was also widespread. Our data indicate decreasing excretion of two common phthalates (di-*n*-butyl phthalate and di-(2-ethylhexyl) phthalate) over time.

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Introduction

Due to their suspected endocrine-disrupting properties in human reproductive health, three groups of non-persistent environmental chemicals – phthalates, parabens, and phenols – have been in particular focus over the past decade.

Phthalates are widely used as plasticizers in industrial products such as toys, bags, shoes, cosmetics, food packaging, medical equipment, and building materials (Anderson *et al*. 2001, Wittassek *et al*. 2011). Bisphenol A (BPA) is used in the manufacture of polycarbonate used for plastic products such as drinking bottles, toys, and medical devices and in epoxy resins used to line food/beverage containers and electronic devices (Rubin 2011, Duty *et al*. 2013). Phenols such as triclosan (TCS) and triclocarban (TCC) are used as antibacterial agents in a range of personal care products such as soaps, toothpaste, deodorants, and disinfectants (Dann & Hontela 2011). Parabens are antimicrobial preservatives commonly used in a wide range of personal care products (CIR Expert Panel 2009). Two parabens

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(methylparaben (MeP) and ethylparaben (EtP)) are also used as preservatives in certain foods (Boberg *et al*. 2010). Benzophenone-3 (BP-3) is a sunscreen agent used in cosmetic sunscreen products and is also used in plastic films as a UV stabilizer for food packaging material and other consumer products (Calafat *et al*. 2008, Krause *et al*. 2012); 2,4-dichlorophenol (2,4-DCP), 2,5-dichlorophenol (2,5-DCP), and 2,4,5-trichlorophenol (2,4,5-TCP) are pesticides or intermediates from industrial production (Agency for Toxic Substances and Disease Registry (ATSDR) 1999). Further, 2,5-DCP is the major urinary metabolite of *p*-dichlorobenzene, which is used for disinfection and as a pesticide (Yoshida *et al*. 2002). In addition, 2,4-DCP can be used for the synthesis of TCS and is also a photo-degradation product of TCS (Latch *et al*. 2005). Finally, propylphenols (2-PP and 4-PP) are known fungicides (US Environmental Protection Agency (US EPA) 2006).

Based on The American National Health and Nutrition Examination Survey program (NHANES), the Germany Environmental Survey program (GerES), and the German Environmental Specimen Bank (ESB), human biomonitoring studies have shown that a majority of the population are more or less exposed to all these environmental chemicals (Schulz *et al*. 2011, CDC 2012, Kolossa-Gehring *et al*. 2012). Humans are primarily exposed to BPA via ingestion (Morgan *et al*. 2011, Rubin 2011); to phthalates and TCS through ingestion, inhalation, or dermal contact (Wittassek *et al*. 2011); and to parabens primarily through dermal contact or ingestion (CIR Expert Panel 2009).

Commonly used phthalates such as di-iso-butyl phthalate (DiBP), di-*n*-butyl phthalate (DnBP), butylbenzyl phthalate, and di-(2-ethylhexyl) phthalate (DEHP) have shown adverse health effects in rodents, in particular anti-androgenic effects on male reproductive development after prenatal exposure (Howdeshell *et al*. 2008, Welsh *et al*. 2008). Similar associations have been observed in human population studies (Swan *et al*. 2005, Main *et al*. 2006). Human phthalate exposure has also been associated with late pubarche in adolescent females and decreased semen quality in adult males (Hauser 2008, Frederiksen *et al*. 2012). On the other hand, not all phthalates have shown endocrine-disrupting effects; for instance, low molecular weight hydrophilic phthalates, such as diethyl phthalate (DEP) which is commonly used in cosmetic products, do not seem to have reproduction toxic effects in animals or humans.

BPA has been considered as a weak estrogen due to its affinity to the estrogen receptor *in vitro*, and a variety of adverse health effects, such as on the brain, behavior, obesity, and male reproductive development, have been shown in animal studies (Vandenberg *et al*. 2010, vom Saal *et al*. 2012, McCaffrey *et al*. 2013). In a recent study, we observed associations between BPA and reproductive hormones in a population of young men indicating anti-androgenic and/or anti-estrogenic effects on the hypothalamic–pituitary–gonadal hormone feedback system, possibly through a competitive inhibition at the receptor level (Lassen *et al*. 2014). Epidemiological studies have shown associations between BPA and diabetes, cardiovascular disease, and obesity (Wolff *et al*. 2007, Meeker *et al*. 2010, Melzer *et al*. 2012, Shankar *et al*. 2012).

Finally, in animal studies, some parabens have shown estrogenic properties (Boberg *et al*. 2010) and TCS, BP-3, chlorophenols, and propylphenols have shown potential estrogenic activity in the male reproductive system (Amer & Aly 2001, Calafat *et al*. 2008, Li *et al*. 2010, Dann & Hontela 2011, Krause *et al*. 2012).

Although many human studies have indicated associations between reproductive health and phthalates or BPA, the effects of low-level exposure to parabens and most of the other phenols on human health are unknown. Furthermore, the most basic information such as knowledge of the sources and levels of exposure to these compounds in different sub-populations is limited to only a few studies presenting data on human exposure in a few areas/countries. Therefore, in this biomonitoring study, we review our own results for non-persistent chemicals from three different cohorts of children, adolescents, and young men from the general Danish population (Boas *et al*. 2010, Frederiksen *et al*. 2011*a*, 2012, 2013*a*, Joensen *et al*. 2012, Mieritz *et al*. 2012, Lassen *et al*. 2014, Mouritsen *et al*. 2013). In addition, we present new data on parabens in Danish children and adolescents and on phthalates, phenols, and parabens in a newly initiated cohort of Danish pregnant women and their children (Odense Child Cohort. http://www.odense.dk/odensekohorten. 2013).

Materials and methods

Study populations and sample collection

Spot urine or first morning urine samples were collected from participants in four different cohort studies, which are described in detail on their respective home pages and/or in previous publications. Cohort 1 included samples from 4- to 9-year-old children from The Copenhagen Mother–Child Cohort (The Copenhagen Mother–Child Cohort. http://www.reproduction.dk/index-filer/Page5546.htm. 2006, Boas *et al*. 2010); cohort 2 included 5- to 20-year-old children and adolescents from The Copenhagen Puberty Study (The Copenhagen Puberty Study. http://www.reproduction.dk/index-filer/Page5552.htm. 2006, Aksglaede *et al*. 2009, Sorensen *et al*. 2010); cohort 3 included young men from the general population (Joensen *et al*. 2012, Jorgensen *et al*. 2012, Copenhagen study on male reproductive health. http://www.eu-deer.net/index-filer/Page487.htm. 2013); and finally samples from pregnant women from the recently established Odense Child Cohort (cohort 4) (Odense Child Cohort. http://www.odense.dk/odensekohorten. 2013). In addition, 24-h urine samples were collected in a subgroup of cohort 2 as described previously (Frederiksen *et al*. 2011*a*, 2013*a*).

An overview of cohort participants, collection years, and sample types is shown in Table 1.

Chemical analysis

All urine samples were deconjugated by enzymatic hydrolysis and then the total (free and deconjugated) content of 13–16 phthalate metabolites, nine phenols, and seven parabens were measured by different isotope dilution liquid chromatography–tandem mass spectrometry (LC–MS/MS) methods as described below. Table 2 shows all measured chemicals and their abbreviations used in this study.

Phthalates

The measurement of samples from cohorts 1, 2, and 3 (Boas *et al*. 2010, Frederiksen *et al*. 2011*a*, 2012, Joensen *et al*. 2012, Mieritz *et al*. 2012), and the method for preparation of samples, standard solutions and quality controls, instrumental analysis, and the general method for validation has previously been described in detail (Frederiksen *et al*. 2010). However, as it is the first time phthalate metabolites in cohort 4 are presented, more details on the method are given here. Our phthalate method was used without modifications (Frederiksen *et al*. 2010) and samples were analyzed in 14 batches during two periods; 200 samples were analyzed in autumn 2011 and 373 samples at the end of 2012. Each batch included standards for calibration curves, about 40 unknown samples, two blanks, two urine pool controls, and two urine pool controls spiked with phthalate standards at low level. The inter-day variation expressed as the relative S.D. (RSD) was <12% for all analytes except MiDP (15%) and the recovery of spiked samples was >90% for all analytes except MiNP (81%), MiDP (82%), and MPP (85%). There was no difference in control material between the two measuring periods.

Phenols

All phenols were analyzed by a newly developed method for simultaneous quantitative determination using isotope dilution TurboFlow-LC–MS/MS (Frederiksen *et al*. 2013*a*). Details on measurements of samples from cohorts 2 and 3 are described elsewhere (Frederiksen *et al*. 2013*a*, Lassen *et al*. 2013, 2014). Regarding phenol measurements in pregnant women (cohort 4), samples were analyzed in 17 batches in two periods about a year apart; 200 samples were analyzed around New Year 2011–2012 and 373 samples at the end of 2012. Each batch included standards for calibration curves, about 35 unknown samples, two blanks, two urine pool controls, and two urine pool controls spiked with phenol standards at low and high levels. The inter-day variation, expressed as RSD, was ≤14% at both spiked levels except for TCC (<27%) and BP-3 (<18%). The recovery of spiked samples was >95% for all analytes except for TCS (>77%) and BP-3 (>87%). There was no difference in control material between the two measuring periods.

Parabens

In children (cohort 1), the content of parabens (MeP, EtP, $\Sigma PrP_{(i+n)}$, $\Sigma BuP_{(i+n)}$, and BzP) was simultaneously analyzed by LC–MS/MS as described previously (Frederiksen *et al*. 2011*b*).

Table 1 Study populations (in total 3625 subjects) and urine collection (3754 samples).

Cohorts	Subjects, *n*	Age in years, median (range)	Urine samples	Collection year	Compounds measured
1. Children (4–9 years)[a]					
Total	848	7.0 (4.2–9.7)	Spot	2006–07	Phthalates; parabens
Males	506	6.9 (4.2–9.7)			
Females	342	7.2 (4.2–9.6)			
2. Children and adolescents[b]					
Total	1311	10.5 (5.6–20.2)	First morning	2006–08	Phthalates
Males	561	10.7 (6.1–19.8)			
Females	750	10.4 (5.6–20.2)			
Age-group (years)					
5–9	556	8.5 (5.6–9.9)			
10–13	538	11.3 (10.0–13.9)			
14–20	217	17.1 (14.0–20.2)			
Total	129[c]	11.9 (6.2–20.4)	24 h	2007	Phthalates; phenols, parabens
Males	65	11.5 (6.4–19.8)			
Females	64	12.2 (6.2–20.4)			
Age-group (years)					
5–9	25	7.5 (6.2–9.9)			
10–13	73	11.5 (10.1–13.8)			
14–20	31	18.5 (14.2–20.4)			
3. Young men[d]	901[e]	19.3 (18.1–27.6)	Spot	2007–09	Phthalates; phenols
4. Pregnant women[f]	565	30.7 (18.5–41.8)	Spot	2011–12	Phthalates; phenols; parabens
Gestation week	553	28.7 (26.4–34.0)			

[a]The Copenhagen Mother-Child Cohort (http://www.reproduction.dk/index-filer/Page5546.htm). [b]The Copenhagen puberty study (http://www.reproduction.dk/index-filer/Page5552.htm). [c]24-h urine samples were collected from a minor subgroup of Cohort 2 in November 2007. [d]Jørgensen *et al*. (2012). [e]In Cohort 3, phthalate metabolites and phenols were measured in 901 and 310 urine samples, respectively. [f]Odense Child Cohort (http://www.odense.dk/odensekohorten).

Table 2 Non-persistent environmental chemicals measured in urine from four Danish populations.

Compound	Abbreviation	Human urine metabolite	Abbreviation
Phthalates[a]			
Diethyl phthalate	DEP	Monoethyl phthalate	MEP
Di-*n*-butyl phthalate	DnBP	Mono-*n*-butyl phthalate	MnBP
Di-iso-butyl phthalate	DiBP	Mono-iso-butyl phthalate	MiBP
		Sum of MBP isomers; MnBP and MiBP	$\Sigma MBP_{(i+n)}$
Butylbenzyl phthalate	BBzP	Monobenzyl phthalate	MBzP
Di-*n*-pentyl phthalate	DPP	Mono-*n*-pentyl phthalate	MPP
Di-(2-ethylhexyl) phthalate	DEHP	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
		Sum of DEHP metabolites	ΣDEHPm
Di-*n*-octyl phthalate	DOP	Mono-*n*-octyl phthalate	MOP
		Mono-3-carboxypropyl phthalate	MCPP
Di-iso-nonyl phthalate	DiNP	Mono-iso-nonyl phthalate	MiNP
		Mono-hydroxy-iso-nonyl phthalate	MHiNP
		Mono-oxo-iso-nonyl phthalate	MOiNP
		Mono-carboxy-iso-octyl phthalate	MCiOP
		Sum of DiNP metabolites	ΣDiNPm
Di-iso-decylphthalate	DiDP	Mono-iso-decyl phthalate	MiDP
Phenols			
Bisphenol A	BPA		
Triclosan	TCS		
Triclocarban	TCC		
Benzophenone-3	BP-3		
2,4-dichlorophenol	2,4-DCP		
2,5-dichlorophenol	2,5-DCP		
Sum of DCP isomers; 2,4-DCP and 2,5-DCP	ΣDCP_{isom}		
2,4,5-trichlorophenol	2,4,5-TCP		
2-phenylphenol	2-PP		
4-phenylphenol	4-PP		
Parabens			
Methyl paraben	MeP		
Ethyl paraben	EtP		
iso-propyl paraben	i-PrP		
n-propyl paraben	n-PrP		
Sum of PrP isomers; i-PrP and nPrP	$\Sigma PrP_{(i+n)}$		
iso-butyl paraben	i-BuP		
n-butyl paraben	n-BuP		
Sum of BuP isomers; i-BuP and n-BuP	$\Sigma BuP_{(i+n)}$		
Benzyl paraben	BzP		

[a]Phthalate diesters are excreted in urine as metabolites.

However, in order to separate i-PrP from n-PrP, and i-BuP from n-BuP for measurement of cohort 2 and cohort 4 samples, the following modifications of the paraben method was introduced: the solvent gradient described by Frederiksen *et al.* (2010) for a LC–MS/MS method for phthalate metabolites was used. Retention times for the analytes were 6.16 min (MeP), 8.62 min (EtP), 11.98 min (i-PrP), 12.54 min (n-PrP), 16.97 min (i-BuP), 17.36 min (n-BuP), and 18.65 min (BzP). Parabens in cohort 4 were analyzed in 16 batches in two periods: 200 samples were analyzed in autumn 2011 and 373 samples at the end of 2012. Each batch included standards for calibration curves, about 35 unknown samples, two blanks, two urine pool controls, and two urine pool controls spiked with paraben standards at low and high levels. The inter-day variation, expressed as RSD, was $\leq$12% in both spike levels except for BzP ($<$15%). The recovery of spiked samples was $>$95% for all analytes except for i-BuP (83%). There was no difference in control material between the two measuring periods.

Statistical analysis

Mean, geometric mean (GM), 95% CI of GM (CI GM), selected percentiles, minimum and maximum concentrations of urinary phthalate metabolites, phenols, and parabens were calculated. For calculation of mean, GM, and CI GM values below limit of detection (LOD) were set to LOD/$\sqrt{2}$; no CI GM was given if GM and/or lower limit of CI GM was below LOD. To compare medians across groups, the two-tailed Mann–Whitney *U* test was used. The statistical analysis of association was only performed for compounds that were detected in levels above the individual LOD in more than 45% of urine samples. *P* values <0.05 were considered statistically significant. We used IBM SPSS Statistics 19 for statistical analysis. To simplify the presentation of phthalate metabolite excretion, the metabolites of DEHP and DiNP were expressed combined as the sum of DEHP metabolites (ΣDEHPm) and the sum of DiNP metabolites (ΣDiNPm). In order to combine the metabolites, the amount of each metabolite was converted into the

corresponding amount of its parent compound by correcting for the differences in molecular weight as described previously (Frederiksen *et al*. 2013*b*).

Results

Excretions of 24 non-persistent environmental chemicals in urine; phthalate metabolites, phenols, and parabens, were measured in up to 3625 samples from four Danish cohorts, representing the general Danish population (Table 1). Here, we present the major results and comments on the general trends. All results, mean, GM, CI GM, selected percentiles, minimum and maximum concentrations of the urinary phthalate metabolites, phenols, and parabens, are shown in the Supplementary Tables, see section on supplementary data given at the end of this article.

Urinary phthalate excretion

Thirteen to 16 different phthalate metabolites from eight different phthalate diesters were measured in spot urine (cohorts 1, 3, and 4) and first morning urine (cohort 2) samples. Phthalate metabolites in cohorts 1, 2, and 3 are reviewed here (Boas *et al*. 2010, Frederiksen *et al*. 2011*a*, 2012, Joensen *et al*. 2012, Mouritsen *et al*. 2013), while the original results of urinary phthalate metabolites in Danish pregnant women (cohort 4) are presented here and partly in Tefre de Renzy-Martin *et al*. (2014) for the first time. Metabolites of the phthalates DEP, DnBP, DiBP, DEHP, and DiNP were detected in levels above LOD in urine from more than 97% of all cohort participants and MBzP was detected in more than 68% of the cohort participants (Supplementary Tables). In general, the median urinary concentrations of these common phthalate metabolites were about 10–100 ng/ml in spot urine samples and both children and adults excreted the highest amounts of ΣDEHPm, followed by MiBP, MnBP, MEP, ΣDiNPm, and MBzP (Fig. 1). Urinary concentrations of these metabolites ranged from below LOD (for most of the metabolites <1 ng/ml) to several 1000-fold higher, which indicates large differences in individual exposure in addition to an overall wide exposure to phthalates in the general Danish population. When comparing cohorts 1, 3, and 4, a significant decrease in median levels with age and/or year of sample collection was observed for several of the metabolites in spot urine samples (Fig. 1A). Urine samples from pregnant women were collected 3–5 years later than samples in the other cohorts. Similar significant decrease with age was observed in first morning urine for most of the phthalates in cohort 2 (Fig. 1B).

In children (cohort 1) and children/adolescents (cohort 2), very few gender differences were observed; females in cohort 2 excreted significantly higher amounts of MiBP and MnBP than males (*P* level <0.005 for both) and males in cohort 1 excreted significantly higher amounts of MBzP (*P* level <0.005) and ΣDEHPm (*P* level <0.05) than females (data not shown). In general, first morning urine levels in the lowest age group children from cohort 2 (Fig. 1B) were higher than median spot urine levels in children from cohort 1 (Fig. 1A). This was also in accordance a previously reported tendency to excrete more concentrated first morning urine levels compared with spot urine and 24-h urine levels measured in a subgroup of cohort 2 (Frederiksen *et al*. 2011*a*).

MPP, MOP, and MiDP are primary metabolites of DPP, DOP, and DiDP respectively and were measured in very

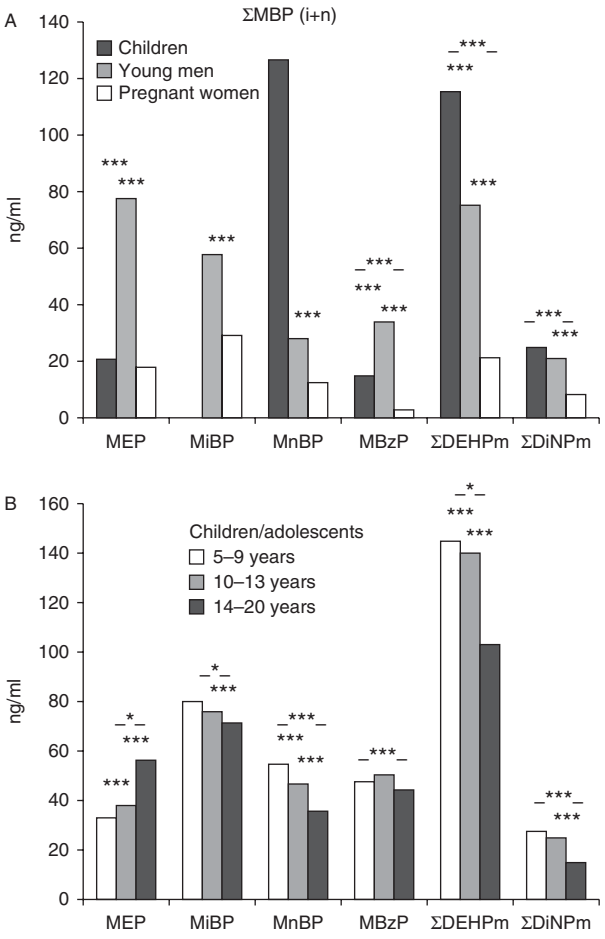

Figure 1 Median phthalate metabolite concentrations (ng/ml) in (A) spot urine sample from children (cohort 1, *n*=848), young men (cohort 3, *n*=901), and pregnant women (cohort 4, *n*=565) and (B) first morning urine sample from a total of 1311 children and adolescents (cohort 2) separated in age groups: 5- to 9-year-old children (*n*=556), 10- to 13-year-old children (*n*=538), and 14- to 20-year-old adolescents (*n*=217). In cohort 1, MiBP and MnBP were analyzed together as one ($\Sigma MBP_{(i+n)}$). ΣDEHPm=the sum of DEHP metabolites (MEHP, MEHHP, MEOHP, and MECPP) and ΣDiNPm=the sum of DiNP metabolites (MiNP, MHiNP, MOiNP, and MCiOP) adjusted for the molecular weights of the different metabolites (see Subjects and Methods). For further abbreviations, see Table 2. Significant differences (two-tailed Mann–Whitney *U* tests): *$P<0.05$ and ***$P<0.001$.

few samples and in low concentrations (Supplementary Tables). In children/adolescents (cohort 2) and in pregnant women (cohort 4), DPP was measured in 1.2 and 0.9% (maximum levels: 29.3 and 7.72 ng/ml) respectively while MiDP was measured in <1% of these two cohorts (maximum levels: 4.12 and 9.8 ng/ml respectively). MOP was measured in all four cohorts, with most detectable values and highest amounts in the two cohorts including children: 16% of the children in cohort 1 and 7.4% in cohort 2 (maximum levels: 11.0 and 15.3 ng/ml respectively). Only 2.7% (maximum = 0.89 ng/ml) and 1.6% (maximum = 1.40 ng/ml) of the young men and the pregnant women respectively excreted MOP. Almost all participants excreted MCPP (Supplementary Tables), which is an unspecific secondary metabolite of several phthalate diesters.

Urinary phenol excretion

The phenols have previously been measured in children, adolescents, and young men (cohorts 2 and 3) (Frederiksen *et al.* 2013*a*, Lassen *et al.* 2014), and now also in Danish pregnant women (cohort 4). All biomonitoring results are shown in Supplementary Tables. BPA, TCS, BP-3, 2,4-DCP, 2,5-DCP, 2-PP, and 4-PP were detected in 75% or more of all participants in all the three cohorts, except 2,4-DCP (70%), 2,5-DCP (42%), 2-PP (43%), and 4-PP (45%) in pregnant women and 4-PP (57%) in young men. TCC was measured in 54, 17, and 18% of the children/adolescents, young men, and pregnant women respectively and 2,4,5-TCP was measured in ≤44% of children/adolescents and pregnant women but in 91% of the young men. Highest amounts and widest ranges among the phenols were observed for urinary BP-3 (<LOD–30.9 μg/ml) and TCS (<LOD–2.61 μg/ml) in all groups of participants. Figure 2 shows median levels of the phenols, which in general were tenfold lower than phthalate metabolite median levels. In spot urine, young men had significantly higher levels of all phenols except BP-3 than pregnant women (Fig. 2A). In cohort 2, adolescents excreted significantly higher amounts of TCS, BP-3, and ΣDCP_{isom} than children, while the opposite pattern was observed for BPA (Fig. 2B). No gender differences in phenol levels were observed among the children and adolescents in cohort 2.

Urinary paraben excretion

New biomonitoring data on parabens measured in spot urine from Danish children (cohort 1) and pregnant women (cohort 4) and in 24-h urine samples from children/adolescents (cohort 2) show that MeP and n-PrP were the highest amounts measured in children, adolescents, and pregnant women. They were detectable in spot urine in more than 86% (MeP) and 70% (n-PrP) and in 24-h urine in more than 95% (MeP) and

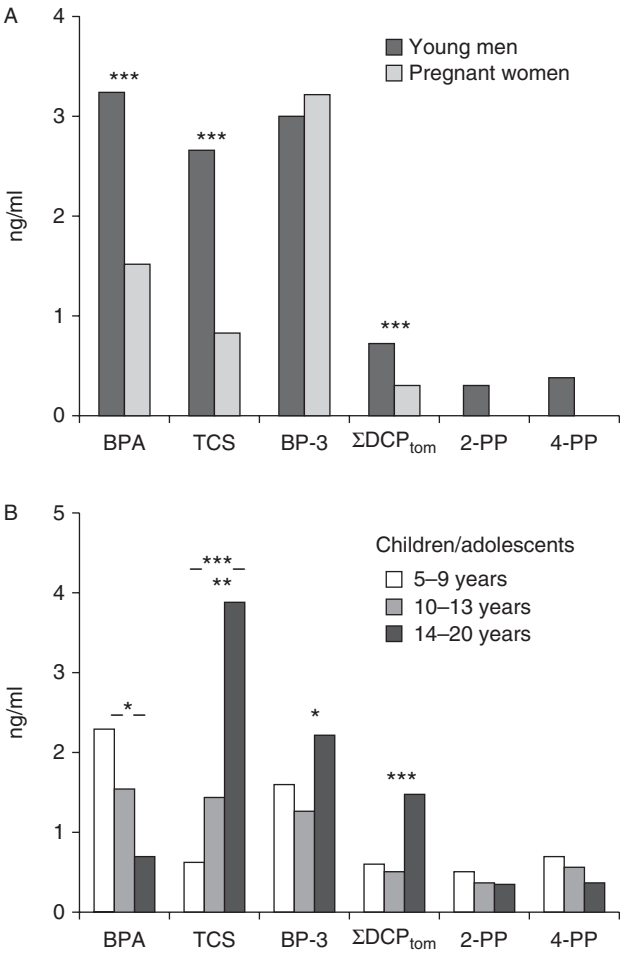

Figure 2 Median phenol concentrations (ng/ml) in (A) spot urine sample from young men (cohort 3, $n=901$) and pregnant women (cohort 4, $n=565$) and (B) 24-h urine sample from a total of 129 children and adolescents (cohort 2) separated in age groups: 5- to 9-year-old children ($n=25$), 10- to 13-year-old children ($n=73$), and 14- to 20-year-old adolescents ($n=31$). The two DCP isomers (2,4-DCP and 2,5-DCP) were measured together as one in cohort 2 (ΣDCP_{isom}) and measured separated but summed for this presentation in cohort 3 and 4. For further abbreviations, see Table 2. Significant differences (two-tailed Mann–Whitney *U* tests): $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$.

64% (n-PrP) of samples. All biomonitoring results are shown in Supplementary Tables. EtP was detectable in 49–60% of samples and in general in lower amounts than MeP and n-PrP. i-PrP was measured separately from n-PrP in pregnant women and in 24-h urine samples from children/adolescents and was only detectable in 5% of women and in two children (Supplementary Tables footnote). n-BuP was detectable in 50% of children (cohort 1), 14% of children/adolescents (cohort 2), and 33% of the pregnant women, while BzP was only detectable in <5% of children and <10% of the pregnant women. i-BuP was not detectable in any of the samples. For all the measured parabens, a very wide range was observed, with maximum levels of MeP at 4.6 and 2.4 μg/ml and of n-PrP at 2.2 μg/ml and 646 ng/ml in

children and pregnant women respectively. In general, the median paraben levels on average were relatively low (<1–12 ng/ml) (Fig. 3). Pregnant women excreted significantly higher amounts of $\Sigma PrP_{(i+n)}$ than children (cohort 1) and median levels for MeP also tended to be higher in women compared with children (Fig. 3A). In 24-h urine samples from cohort 2 (children/adolescents), a gender difference was observed: girls excreted significantly higher amounts of MeP and $\Sigma PrP_{(i+n)}$ than boys (Fig. 3B). Finally, the youngest children excreted significantly higher MeP than older children in cohort 2 (Fig. 3C). We did not observe any gender differences between children from cohort 1.

Discussion and concluding remarks

In Denmark, we have no surveillance programs for non-persistent environmental chemicals such as the American (NHANES) or German (GerES) survey programs (CDC 2012, Kolossa-Gehring *et al.* 2012). However, during recent years, we have measured non-persistent environmental chemicals in more than 3600 Danish children, adolescents, young men, and pregnant women from the general population. Our data clearly show that all segments of the Danish population are widely exposed to non-persistent environmental chemicals. This collection of Danish biomonitoring data, including our previously published data and new original data on parabens in the general population, and parabens, phenols, and phthalates in Danish pregnant women, is presented here in its entirety, along with some general comments regarding levels and trends.

Besides our own data, urinary levels of phthalate metabolites in Danes have been reported for 441 Danish day-care centre children (Langer *et al.* 2014), 145 Danish mother–child pairs collected in urban and rural areas (Frederiksen *et al.* 2013*b*), and for 128 Danish pregnant women (Toft *et al.* 2012). Comparison of the most common phthalate metabolites (MEP, MiBP, MnBP, and MBzP, ΣDEHPm, and ΣDiNPm) in Danish children showed very similar urinary levels (Boas *et al.* 2010, Frederiksen *et al.* 2011*a*, 2012, 2013*b*, Mieritz *et al.* 2012, Langer *et al.* 2014). In general, levels in Danish children were also comparable with levels and excretion patterns in German children of the same age groups (Becker *et al.* 2009, Koch *et al.* 2011, Kasper-Sonnenberg *et al.* 2012*a*), while levels of MEP, MBzP, and DEHPm were higher among children in Spain and the USA (Casas *et al.* 2011, CDC 2012) and MiBP was several fold lower in the USA than in Europe.

Comparing age groups in children and adolescents, urinary levels of the most common phthalate metabolites except MEP decreased with age in Denmark as well as in Germany and USA (Becker *et al.* 2009, Frederiksen *et al.* 2011*a*, 2012, CDC 2012, Mieritz *et al.* 2012).

Lower levels of, especially, MEP, MnBP, and ΣDEHPm were observed in the most recent Danish sample collection (Frederiksen *et al.* 2013*b*) and of MiBP, MnBP, MBzP, and ΣDEHPm in the most recent German sample collections (Koch *et al.* 2011, Kasper-Sonnenberg *et al.* 2012*a*). Similar decreasing time trends

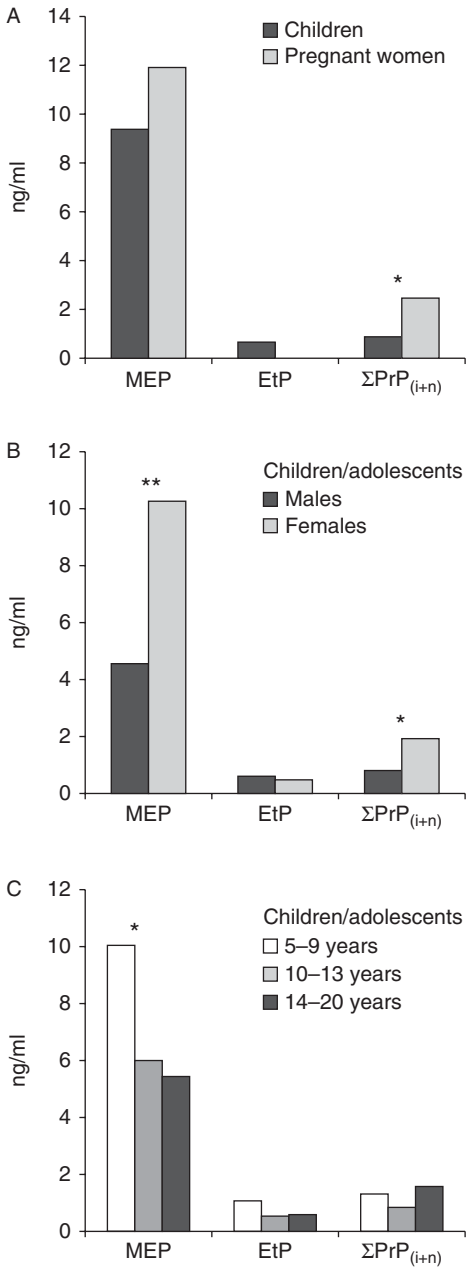

Figure 3 Median paraben concentrations (ng/ml) in (A) spot urine sample from children (cohort 1, $n=848$) and pregnant women (cohort 4, $n=565$), (B) 24-h urine sample from a total of 129 children and adolescents (cohort 2) separated in males ($n=65$) and females ($n=64$), and (C) 24-h urine sample from 129 children and adolescents (cohort 2) separated in age groups: 5- to 9-year-old children ($n=25$), 10- to 13-year-old children ($n=73$), and 14- to 20-year-old adolescents ($n=31$). The two PrP isomers (i-PrP and n-PrP) were measured separately but summed for this presentation $=\Sigma PrP_{(i+n)}$. For further abbreviations, see Table 2. Significant differences (two-tailed Mann–Whitney *U* tests): $^{*}P<0.05$ and $^{**}P<0.01$.

have also been shown for MnBP and DEHP metabolites among German students in the period 1988–2008 (Wittassek *et al*. 2007*a*, Goen *et al*. 2011) and in our study of young Danish men in the period 2007–2009 (Joensen *et al*. 2012). In contrast to the decreasing time trend for MnBP and ΣDEHPm, we observed higher levels of MiBP, MBzP, and DiNP metabolites in young Danish men compared with urinary levels in American men from the same period (CDC 2012) and in German students in the period 1988–2008 (Wittassek *et al*. 2007*a*, Goen *et al*. 2011). The observed change in phthalate exposure pattern could be the result of some regulatory restrictions on food contact materials and content in toys and childcare articles introduced by the European Commission (EU 2005, 2007).

Pregnant women in Denmark excreted MiBP and MnBP in a similar order of magnitude to pregnant women in other European countries such as Spain, France, and The Netherlands (Ye *et al*. 2008, Casas *et al*. 2011, Philippat *et al*. 2012, Tefre de Renzy-Martin *et al*. 2014). However, also in pregnant women there seems to be a weak tendency to increasing MiBP excretion over time, while MnBP excretion was decreasing. In general, levels of MiBP among pregnant women in Europe were higher than in countries outside Europe (Tefre de Renzy-Martin *et al*. 2014) including non-pregnant women in the USA (CDC 2012). All other common phthalate metabolites (MEP, MBzP, ΣDEHPm, and ΣDiNPm) were observed at lower levels in Danish pregnant women than in other biomonitoring studies of pregnant women (Braun *et al*. 2012, Tefre de Renzy-Martin *et al*. 2014). However, estimated daily exposure levels to the common phthalates and most of the other non-persistent chemicals in Danish pregnant women were similar to levels observed in non-pregnant Danish women (Frederiksen *et al*. 2013*b*, Tefre de Renzy-Martin *et al*. 2014).

Despite the decreasing time trend observed for, especially, excretion of DnBP and DEHP metabolites, the daily exposure estimated on subgroups from the presented cohorts showed that the highest exposed section of the Danish population was still highly exposed to DnBP and DEHP, near or above the tolerable daily intake (Frederiksen *et al*. 2011*a*, Soeborg *et al*. 2012, Kranich *et al*. 2014, Tefre de Renzy-Martin *et al*. 2014), and also sections of the German population were highly exposed to DnBP and DEHP (Wittassek *et al*. 2007*b*, Koch *et al*. 2011).

Primary metabolites of DPP, DOP, and DiDP were detected only in very few samples. It is possible that these primary metabolites may not the best biomarkers for human exposure to DPP, DOP, and DiDP, but they were included in the study as currently no secondary metabolites of these phthalates are commercially available. On the other hand, detectable levels of these primary metabolites indicate that at least a minor part of the Danish population has been exposed to their respective parent compounds. Furthermore, almost all participants excreted MCPP, which is an unspecific secondary metabolite of several phthalate diesters, and one of the major metabolites of DOP (Calafat *et al*. 2006).

Several studies have reported urinary levels of BPA (Becker *et al*. 2009, Casas *et al*. 2011, Braun *et al*. 2012, Kasper-Sonnenberg *et al*. 2012*b*, Koch *et al*. 2012, Philippat *et al*. 2012, Frederiksen *et al*. 2013*a*, 2013*b*, Lassen *et al*. 2013, 2014). Nearly all studies, including different subgroups of populations from both Europe and the USA, reported urinary BPA levels of a similar order of magnitude as observed in our studies. We observed a significant decrease with age (Frederiksen *et al*. 2013*a*) and a similar age-related trend was observed in German children (Becker *et al*. 2009). Furthermore, the BPA excretion level seems to be fairly constant over the years, despite a decrease in the industrial production of BPA (Koch *et al*. 2012, Kolossa-Gehring *et al*. 2012).

Regarding the other phenols and parabens, only few a European and American studies have reported biomonitoring data. In general, low levels of TCS, chlorophenols, phenylphenols, and the parabens MeP and n-PrP were measured in the Danish cohorts (Frederiksen *et al*. 2013*a*, 2013*b*, Lassen *et al*. 2013, 2014, Tefre de Renzy-Martin *et al*. 2014) compared with Spanish children and pregnant women, French pregnant women, and Americans from the general populations (Casas *et al*. 2011, CDC 2012, Philippat *et al*. 2012). BP-3 was measured in comparable levels in Denmark (Frederiksen *et al*. 2013*a*, 2013*b*, Lassen *et al*. 2013, 2014, Tefre de Renzy-Martin *et al*. 2014) to levels in French and Spanish children and pregnant women (Casas *et al*. 2011, Philippat *et al*. 2012), while the BP-3 level was about fivefold higher among Americans (CDC 2012).

In conclusion, our study shows that nearly all Danes were exposed to the six most common phthalates (DEP, DiBP, DnBP, BBzP, DEHP and DiNP), BPA, TCS, and BP-3 and to at least two of the parabens (MeP and n-PrP). The exposure to other non-persistent chemicals was also widespread. Our data indicates decreasing DnBP and DEHP excretion over time, which perhaps reflects a more restrictive use of these chemicals in ordinary consumer products.

Discussion from meeting

Russ Hauser (Boston, USA): Your trend data in relation to exposure to non-persistent chemicals is expressed as median values. However, different compounds might have the same median values but different mean values and different distribution curves. Trend data might better be expressed as variance, S.D., or 90th centile. The spread around the median might differ over time, and the extremes, especially at the upper end of the distribution, might differ markedly even if the median is the same. The variance of spread is an important value to consider, and the maximum value is not representative.

Hanne Frederiksen (Copenhagen, Denmark): You are right, but here we have focused on the small segment of the population that are highest exposed. We found that 5–10% of children in the Danish cohort had levels of phthalates above the hazard level for anti-androgens (Frederiksen *et al.* 2011*a*). It is important to focus in the 90–95% centile in relation to biological endpoints.

Laura Vandenberg (Medford, USA): Do you plan to follow and resample the women in the top 5% or top 10% exposure groups? They show concentration levels of 100 or 1000 ng/ml urine compared with the median/mean of about 1 ng/ml. Do they always give samples at the extreme levels, suggesting atypical exposure, or are the high levels only occasional, suggesting a low frequency of extremely high exposure? Also, patients with undetectable levels might be due to sampling if they happen to avoid exposure just before the test. Are they consistently at low levels?

Hanne Frederiksen: We do not plan to follow these women, but all their children will be followed in a longitudinal study including the high-exposed and low-exposed mothers. The pregnant women were asked many questions about behavior during pregnancy including diet. Participants in the puberty study also gave many answers on diet and behavior in their questionnaires. A longitudinal study in a subgroup of our Copenhagen puberty study showed a large intra-individual variation over a 5-year period but also that individuals tend to be either high exposed or low exposed in repeated samples (Mouritsen *et al.* 2013). Questionnaires and EDC excretion levels will be analyzed to identify possible associations between specific consumer behavior including diet and for instance the highest exposure levels.

Tina Kold Jensen (Odense, Denmark): You compared pregnant women with young adult males, but the metabolism and excretion pattern might be completely different during pregnancy.

Hanne Frederiksen: A paper by Braun *et al.* (2012) showed that urinary excretion levels of phthalates and BPA did not differ significantly before pregnancy and during pregnancy. However, the overall variability was compound specific.

Supplementary data

This is linked to the online version of the paper at http://dx.doi.org/10.1530/REP-13-0522.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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