REPRODUCTION
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Current exposure of 200 pregnant Danish women to phthalates, parabens and phenols

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(This work was carried out at Affiliations 1 and 2)

Abstract

Many phthalates, parabens and phenols are suspected to have endocrine-disrupting properties in humans. They are found in consumer products, including food wrapping, cosmetics and building materials. The foetus is particularly vulnerable and exposure to these chemicals therefore is of concern for pregnant women. We investigated current exposure to several commonly used phthalates, parabens and phenols in healthy, pregnant Danish women. A total of 200 spot urine samples were collected between 8 and 30 weeks of gestation and analysed for metabolites of ten phenols, seven parabens and 16 phthalate by liquid chromatography–tandem mass spectrometry representing 26 non-persistent compounds. The majority of analytes were present in the urine sample collected from most women who participated. Thus, in 174 of the 200 women, metabolites of more than 13 (>50%) of 26 compounds were detected simultaneously. The number of compounds detected per woman (either as the parent compound or its metabolite(s)) ranged from 7 to 21 with a median of 16. The majority of compounds correlated positively with each other within and between chemical groups, suggesting combined exposure sources. Estimated daily intakes (DIs) of phthalates and bisphenol A (BPA) were below their individual tolerable DI (TDI) and with hazard quotients below 1. In conclusion, we found detectable levels of phthalate metabolites, parabens and phenols in almost all pregnant women, suggesting combined multiple exposures. Although the estimated DI of phthalates and BPA for an individual was below TDI, our results still raise concern, as current toxicological risk assessments in humans do not take into account simultaneous exposure. The true cumulative risk for the foetus may therefore be underestimated.

Reproduction (2014) **147** 443–453

Introduction

During recent years, evidence that both humans and wildlife are exposed to a wide range of endocrine-disrupting chemicals (EDCs) has been mounting. Exposure of pregnant women is of particular concern because of the potential health impact on the vulnerable foetus, in which exposure may inflict life-long adverse health effects. Some of these chemicals have been shown to cross the placenta, enter the foetus and recycle via amniotic fluid (Bradman *et al.* 2003, Mose *et al.* 2007, Morck *et al.* 2010). Thus, a number of chemicals measured in maternal urine, serum and breast milk have also been found in amniotic fluid, cord blood and meconium (Ikezuki *et al.* 2002, Main *et al.* 2006, Barr *et al.* 2007, Jensen *et al.* 2012). A recent study has reported detectable levels of metabolites from two common phthalate diesters, di(2-ethylhexyl) (DEHP) and di-isononyl phthalate (DiNP), and perfluorooctanesulfonic acid in amniotic fluid collected from Danish women (Jensen *et al.* 2012).

This paper forms part of a special issue of *Reproduction* on Endocrine Disrupters. This article was presented at the 7th Copenhagen Workshop on Endocrine Disrupters, 28–31 May 2013. The meeting was supported by the Danish Ministry of the Environment – Environmental Protection Agency as an activity under the Danish Centre on Endocrine Disrupters. Publication of this special issue has been supported by the Society for Reproduction and Fertility. The opinions or views expressed in this special issue are those of the authors, and do not necessarily reflect the opinions or recommendations of the Danish Ministry of the Environment – Environmental Protection Agency or the Society for Reproduction and Fertility. The Guest Editors for this special issue were Anna-Maria Andersson, Hanne Frederiksen, Niels Erik Skakkebæk, Rigshospitalet, Denmark, Kenneth M Grigor, Western General Hospital, Edinburgh, UK and Jorma Toppari, University of Turku, Finland.

ISSN 1470–1626 (paper) 1741–7899 (online)
DOI: 10.1530/REP-13-0461
Online version via www.reproduction-online.org

Phthalates, phenols and parabens are non-persistent chemicals, to which humans are exposed life-long and ubiquitously due to their widespread application in many modern consumer products, i.e. in food, plastics including food packaging, fabrics, personal care items, cosmetics and building materials (Golden *et al*. 2005, Chapin *et al*. 2008, Wittassek *et al*. 2011, Jung *et al*. 2012, National Center for Biotechnology Information, www.ncbi.nlm.nih.gov/pmc/articles/PMC2453187/pdf/ehp0116-a0306a.pdf, last accessed Sep 2013).

Rodent and human data suggest adverse health effects of phthalates, in particular anti-androgenic effects on male reproductive development after prenatal exposure (Swan *et al*. 2005, Main *et al*. 2006, Desdoits-Lethimonier *et al*. 2012).

Among the group of phenols, bisphenol A (BPA) is the most intensively studied compound, which shows adverse effects on reproduction, thyroid gland and the immune system in both human and animals (Vandenberg *et al*. 2009). BPA is used in polycarbonate and epoxy resins (Chapin *et al*. 2008). Triclosan (TCS), benzophenone-3 (BP-3) (a u.v.-filter) and several other phenols also appear to have endocrine-disrupting activity with adverse effects on, e.g. thyroid and reproductive function (Zhang *et al*. 2008, Li *et al*. 2010, Rodriguez & Sanchez 2010, Jung *et al*. 2012, Krause *et al*. 2012, Henry & Fair 2013).

Parabens are widely used as anti-microbial preservatives (Rastogi *et al*. 1995, European Food Safety Authority (EFSA) 2004, Golden *et al*. 2005, Soni *et al*. 2005), often as mixtures. Data on exposure levels and health effects in humans are sparse, but parabens have shown to exhibit estrogenic properties in animal studies (Boberg *et al*. 2010) and appear to accumulate in amniotic fluid (Frederiksen *et al*. 2008).

In this study, we aimed to describe the current exposure of pregnant Danish women to phthalates, parabens and phenols in order to evaluate whether there is reason for concern with respect to potential adverse effects on the foetus.

Materials and methods

Study population and sample collection

The Odense Child Cohort is a collaborative study between Odense Municipality, Odense University Hospital and the University of Southern Denmark. All pregnant women and their partners living in the catchment area of Odense Municipality were invited to participate from January 2010 to December 2012 at an information meeting about pregnancy in gestational weeks 10–16. The women were followed through pregnancy and the children are planned to be followed with repetitive examinations until the age of 18 years. Given the general demographics of Odense Municipality, the majority of the women are white Caucasian and 92.3% were born in Denmark. At enrolment a serum sample was collected and the women completed a comprehensive questionnaire about general health, lifestyle and social factors including age, pre-pregnancy height, weight (from which the BMI was calculated), parity, marital status and education (high school or less; high school +1–3 years; and high school +4 years or more). In gestational weeks 8^{+0}–30^{+0}, a spot urine sample was collected and the women responded to another questionnaire. The participation rate is 51%.

In this study, urine samples from 200 women, collected between February 1st and June 7th, 2011, have been analysed for the content of phthalates, phenols and parabens. To avoid sample contamination during collection and storage, urines were collected in polyethylene containers and stored at −20 °C as 10-ml aliquots in 20-ml glass scintillation vials with tops packed with aluminium foil.

The study was conducted in accordance with Helsinki declaration II and approved by the regional ethical committee (the Ethics Committee for Biomedical Research in The Region of Southern Denmark S-20090130). After having received written and oral information, all participants gave their written consent.

Chemical analyses

BPA and other phenols

The urinary content of total (free and conjugated) BPA, TCS, triclocarban (TCCB), BP-3, 2,4-dichlorophenol (2,4-DCP), 2,5-DCP, 2,4,5-trichlorophenol (2,4,5-TCP), 2-phenylphenol (2-PP), 4-PP and 4-tert-octylphenol (4-tOP) was analysed by a newly developed method for simultaneous quantitative determination using isotope dilution TurboFlow-liquid chromatography–tandem mass spectrometry (LC-MS/MS) with preceding enzymatic deconjugation (Frederiksen *et al*. 2013). The first 50 urine samples were analysed in two batches with few days in between. In these samples 2,4-DCP and 2,5-DCP were analysed as one analyte (ΣDCP). The next 150 samples were analysed 3 months later in four batches during a period of 8 days. In these samples, 2,4-DCP and 2,5-DCP were separated. In short, each batch included standards for calibration curves, 25–30 unknown samples, two blanks, two urine pool controls and two urine pool controls spiked with phenol standards at low and high level respectively. The inter-day variation, expressed as the relative standard deviation (RSD), was <11% for most analytes in both spiked samples except for TCCB (<16%), BP-3 (<21%) and 4-tOP (<25%). The recovery of spiked samples was >96% for all analytes except for TCS (82%), 2,5-DCP (91%) and 4-tOP (84%).

Parabens

The total content (free and conjugated) of methylparaben (MeP), ethylparaben (EtP), *n*-propylparaben (*n*-PrP), iso-propylparaben (i-PrP), *n*-butylparaben (*n*-BuP), iso-butylparaben (i-BuP) and benzylparaben (BzP) was simultaneously analysed by LC-MS/MS as previously described by Frederiksen *et al*. (2011) with the following modification in order to separate i-PrP and *n*-PrP, and i-BuP and *n*-BuP, respectively, the solvent gradient described in Frederiksen *et al*. (2010) for a LC-MS/MS method for phthalate metabolites was used. The retention time periods for the analytes were 6.2 min (MeP), 8.6 min (EtP), 12.0 min (i-PrP), 12.6 min (*n*-PrP), 17.0 min

(i-BuP), 17.4 min (*n*-BuP) and 18.7 min (BzP). In this study all urine samples were analysed in six batches during a period of 2 weeks. In short, each batch included standards for calibration curves, 35–40 unknown samples, two blanks, two urine pool controls and two urine pool controls spiked with paraben standards at low and high level respectively. The inter-day variation, expressed as the RSD, was <12% and the recovery of spiked samples was >90% for all parabens at low and high spike levels.

Phthalates

Urine samples were analysed for the total content (free and glucuronidated) of 16 phthalate metabolites: monoethyl phthalate (MEP), mono-iso-butyl phthalate (MiBP), mono-*n*-butyl phthalate (MnBP), monobenzyl phthalate (MBzP), mono-*n*-pentyl phthalate (MPP), mono(2-ethylhexyl) phthalate (MEHP), mono(2-ethyl-5-hydroxyhexyl) (MEHHP), mono(2-ethyl-5-oxohexyl) phthalate (MEOHP), mono(2-ethyl-5-carboxypentyl) phthalate (MECPP), mono-*n*-octyl phthalate (MOP), mono(3-carboxypropyl) phthalate (MCPP), mono-iso-nonyl phthalate (MiNP), mono(hydroxy-iso-nonyl) phthalate (MHiNP), mono(oxo-iso-nonyl) phthalate (MOiNP), mono(carboxy-iso-octyl) phthalate (MCiOP) and mono-iso-decyl phthalate (MiDP) by LC-MS/MS with preceding enzymatic deglucuronidation followed by solid-phase extraction. The method for the preparation of samples, standard solutions and quality controls, as well as the instrumental analysis and general validation method, has previously been described in detail (Frederiksen *et al.* 2010). In this study, samples were analysed in six batches during a two-week period. In short, each batch included standards for calibration curves, about 35 unknown samples, two blanks, two urine pool controls and two urine pool controls spiked with phthalates standards at low level. The inter-day variation, expressed as the RSD, was <12% for all analytes except MiDP (14.5%) and the recovery of spiked samples was >90% for all analytes except MnBP (82%), MPP (85%) and MiDP (87%). Creatinine was determined in all urine samples by colorimetric enzymatic assay (Roche Diagnostics GmbH).

Statistical analysis

The measured concentrations (ng/ml) of each compound are presented as 25th, 50th and 95th percentiles, minimum and maximum levels. To adjust for variation in urinary dilution, all concentrations were creatinine-adjusted using the following equation:

$$UE_{crea}\left(\frac{\mu g}{g_{crea}}\right) = \frac{UC\left(\frac{\mu g}{L}\right) \times 1000\left(\frac{mg}{g}\right)}{UC_{crea}\left(\frac{mmol}{L}\right) \times MW_{crea}\left(\frac{mg}{mmol}\right)}$$

where UE_{crea} is the creatinine-adjusted urinary analyte (μg/g) and UC is the measured urinary concentration of the compounds. UC_{crea} and MW_{crea} are the urinary concentration and the molar mass (113.12 mg/mmol) of creatinine respectively.

In order to estimate the daily intake (DI) of BPA per kilo body weight per day, the following equation was used with the assumption that BPA was almost completely excreted in urine within 24 h (Volkel *et al.* 2002, Fisher *et al.* 2011):

$$DI(\mu g/kg \text{ per day}) = \frac{UE_{crea}\left(\frac{\mu g}{g_{crea}}\right) \times CE_{smoothed}\left(\frac{g}{day}\right)}{BW(kg)}$$

where $CE_{smoothed}$ is an average reference value (1.12 g/day) for urinary creatinine excretion for pregnant women in gestational weeks 25–28 (Gallery *et al.* 1996) and BW is self-reported pre-pregnancy body weight. The same equation was used for the parabens and the rest of the phenols in order to estimate the daily excretion with the assumption of an excretion factor of 100% because of lack of human pharmacokinetic studies.

Estimation of DI for phthalate diesters per kilo body weight per day was calculated as:

$$DI(\mu g/kg\,/\,day) = \frac{\left(\left(\frac{UE_{m1\ crea}\left(\frac{\mu g}{g_{crea}}\right)}{MW_{m1}\left(\frac{\mu g}{\mu mol}\right)}\right) + \left(\frac{UE_{m2\ crea}\left(\frac{\mu g}{g_{crea}}\right)}{MW_{m2}\left(\frac{\mu g}{\mu mol}\right)}\right) + \cdots\right) \times MW_{p}\left(\frac{\mu g}{\mu mol}\right) \times CE_{smoothed}\left(\frac{g}{day}\right)}{FUE \times BW(kg)}$$

where $UE_{m1\ crea}$, $UE_{m2\ crea}$ MW_{m1} and MW_{m2} are the creatinine-adjusted urinary concentrations of phthalate metabolites and their respective molar masses; MW_p is the molar mass of the specific phthalate diester and FUE is the fraction of the phthalate diester excreted in urine. The fractional urinary excretion (FUE) factors were based on previous human studies of urinary excretion after oral intake of deuterium-labelled phthalate diesters, where 69% of di-*n*-butyl phthalate (DnBP) was excreted as MnBP, 73% of butylbenzyl phthalate (BBzP) as MBzP, 45.3% of DEHP was excreted as common DEHP metabolites (MEHP, MEHHP, MEOHP and MECPP) and 30.5% of DiNP as common DiNP metabolites (MiNP, MHiNP, MOiNP and MCiOP) (Anderson *et al.* 2001, 2011, Wittassek *et al.* 2011). FUEs for di-ethyl phthalate (DEP) and di-iso-butyl phthalate (DiBP) were assumed to be the same as for DnBP (Koch & Calafat 2009).

Estimated DIs were divided by the tolerable DI (TDI) to produce the hazard quotient (HQ) for four of the phthalates: DnBP, BBzP, DEHP and DiNP. These HQs were then added to produce the hazard index (HI; Soeborg *et al.* 2012). The HQs and HIs are presented as 25th, 50th and 95th percentiles as well as the minimum and maximum levels.

Maternal age and pre-pregnancy BMI were categorised into three groups (see Table 1). We used Spearman's rank correlation coefficients for the analysis of associations between different chemicals, and to test associations between maternal characteristics (age and BMI) and exposure levels. To compare chemical levels across the groups (education and parity), non-parametric Mann–Whitney *U*-tests were used. A total of 95% of the women had creatinine levels within the normal range and the analyses were repeated among these women. All statistical tests were evaluated at a 5% significance level. SPSS (version 18.0, SPSS) was used for all analyses.

Results

Characteristics of the study population are shown in Table 1. Information on height, parity and pre-pregnancy

Table 1 Characteristics of 200 Danish pregnant women, 2011.

Characteristics	***n*** (%)	**Median** (range)
Maternal age (years)		30.2 (18.5–40.3)
<25	20 (10)	
25–35	147 (73)	
>35	33 (17)	
Maternal height (cm), $n=183$		168 (164–185)
Maternal pre-pregnancy weight (kg), $n=181$		67 (44–124)
Pre-pregnancy BMI (kg/m^2)		23.9 (17.8–41.4)
<18.5	4 (2)	
18.5–24.9	106 (59)	
>25	71 (39)	
Maternal education		
High school or less	49 (27)	
High school +1–3 years	109 (59)	
High school +4 years or more	25 (14)	
Marital status		
Married or living with a partner	178 (98)	
Single/divorced/widowed	3 (2)	
Parity ($n=182$)		
0	98 (54)	
≥1	84 (46)	

weight was not available for 17, 18 and 19 of the women, respectively, either because they did not fill out the prenatal questionnaire or because they joined the study after gestational week 26.

Urinary levels of chemicals

Unadjusted and creatinine-adjusted median urinary concentrations of the 33 analytes measured are presented in Table 2 along with selected percentiles, minimum and maximum levels, as well as percentage of samples greater than limit of detection (LOD) for each analyte. Molar median concentrations for the most prevalent analytes are shown in Fig. 1 for comparison.

The 33 analytes represent 26 non-persistent chemicals, as some of the analytes were phthalate metabolites of the same parent compound. The majority of analytes were present in urine collected from most women. In 174 of 200 women, metabolites of more than 13 (>50%) of the 26 parent compounds were detected simultaneously. The number of compounds per woman (either as the parent compound or its metabolite(s)) ranged from 7 to 21 with a median of 16.

Only two analytes (4-tOP and iBuP) were not detectable at all. Other analytes, which were only detectable in a limited number of samples, were 4-PP, iPrP, BzP, and the metabolites MPP, MOP and MiDP. These analytes were not further evaluated.

Associations between chemicals

For parabens, concentrations of *n*-PrP and MeP were highly correlated ($\rho=0.822$, $P<0.01$). All other parabens also correlated positively ($\rho=0.443$–0.562, $P<0.01$).

We observed several positive correlations between phenols ($\rho=0.146$–0.644, $P<0.05$), of which BPA, TCS, TCCB, BP-3, 2,4-DCP and 2,4,5-TCP also correlated positively with several parabens (MeP, EtP, *n*-PrP and *n*-BuP), ($\rho=0.143$–0.420, $P<0.05$).

The phthalates generally correlated positively with each other ($\rho=0.272$–0.971, $P<0.01$) as well as with parabens ($\rho=0.142$–0.373, $P<0.05$) and most phenols ($\rho=0.140$–0.519, $P<0.05$).

Associations between chemicals and maternal characteristics

Pre-pregnancy BMI was positively associated with 12 out of the 13 phthalate metabolites ($\rho=0.164$–0.317, $P<0.05$) and four phenols (BPA, 2,4-DCP, 2,4,5-TCP and 2-PP) ($\rho=0.153$–0.209, $P<0.05$). None of the analytes were consistently associated with maternal age, education or parity.

Estimates of DI and daily excretion

The estimated DI for BPA and six phthalate diesters and the daily excretion for parabens and other phenols are presented in Table 3 along with the EFSA TDI levels for DnBP, BBzP, DEHP, DiNP and BPA. None of the analytes were above the TDI. In Table 4, the HQs based on EFSA TDI values are given along with the corresponding HIs. None of the pregnant women exceeded the value of 1. Repeating the analyses among 95% of the women with creatinine levels within the normal range did not change the estimation.

Discussion

In recent years increasing attention has been given to volatile compounds such as phthalates and parabens for their potential endocrine-disrupting properties. In this contemporary study of urinary excretion of 26 non-persistent environmental chemicals in healthy Danish pregnant women, the average woman was simultaneously exposed to the majority of the studied chemicals. A high urinary concentration of one chemical in an individual was often associated with high concentrations of several other chemicals, suggesting a common exposure source or common exposures associated with specific lifestyles. Several chemicals are known or some are suggested EDCs that may target the same hormonal axis. The simultaneous exposure to many different EDCs is of concern, as animal studies have shown that dose-additive effects are to be expected from EDCs affecting the same hormone system (e.g. different phthalates; Gray *et al*. 2006, Christiansen *et al*. 2009, 2012).

Individual concentrations of some phenols, parabens and phthalates varied considerably between

Table 2 Concentration of phenols, parabens and phthalate metabolites in the urine samples collected from pregnant Danish women ($n=200$) at gestational weeks 8–30, given as μg/l and μg/g creatinine.

		Unadjusted (μg/l)							**Creatinine adjusted** (μg/g)			
					Percentiles				Percentiles			
Compound		LOD	*n* (%) >LOD	Min.	25	50	95	Max.	25	50	95	Max.
Phenols												
BPA		0.12	179 (89.5)	<LOD	0.56	1.38	5.61	25.2	0.57	1.18	5.24	68.4
TCS		0.06	171 (85.5)	<LOD	0.21	0.70	438	1220	0.20	0.63	432	1435
TCCB		0.01	108 (54)		<LOD	0.02	0.42	0.68	<0.01	0.02	0.54	2.29
BP-3		0.07	194 (97)	<LOD	1.17	3.20	402	7753	1.27	2.99	944	6283
2,4-DCP[a]		0.07	124 (82.7)	<LOD	0.10	0.24	1.35	4.82	0.10	0.20	1.08	4.04
2,5-DCP[a]		0.07	96 (64)		<LOD	0.15	1.48	15.4	<0.01	0.13	1.07	13.2
ΣDCP[b]		0.07	31 (62)		<LOD	0.24	1.34	1.41	<0.01	0.16	1.85	2.60
2,4,5-TCP		0.06	99 (49.5)		<LOD	0.04	0.99	3.88		<0.01	0.82	3.76
2-PP		0.12	124 (62)		<LOD	0.19	0.87	3.03	<0.01	0.16	0.75	2.60
4-PP		0.13	27 (13.5)			<LOD	1.20	7.01		<0.01	0.95	4.44
4-tOP		0.87	0									
Parabens												
MeP		0.26	190 (95)	<LOD	4.09	20.7	474	3066	5.05	20.5	357	2950
EtP		0.40	120 (60)		<LOD	1.01	49.7	291	<0.01	0.88	40.3	156
i-PrP		0.18	15 (7.5)			<LOD	0.91	15.7		<0.01	1.33	13.3
n-PrP		0.18	166 (83)	<LOD	0.61	4.17	199	646	0.68	4.69	144	346
i-BuP		0.07	0					<LOD				
n-BuP		0.07	76 (38)			<LOD	13.0	87.5		<0.01	12.9	62.7
BzP		0.18	16 (8)			<LOD	0.30	2.35		<0.01	0.33	1.91
Phthalate metabolites												
Diester phthalate	Urinary metabolite											
DEP	MEP	0.53	199 (100)	<LOD	7.49	21.5	470	2710	9.04	18.9	355	2190
DiBP	MiBP	1.43	200 (100)	1.46	19.2	39.8	159	455	24.7	35.3	98.3	274
DnBP	MnBP	1.10	196 (98)	<LOD	7.24	16.0	58.8	184	9.09	13.9	37.5	124
BBzP	MBzP	1.14	154 (77)	<LOD	1.20	2.97	20.4	546	1.08	2.32	17.5	230
DPP	MPP	0.81	3 (1.5)				<LOD	5.48			<0.01	3.37
DEHP	MEHP	0.14	175 (88)	<LOD	0.43	1.15	5.80	61.1	0.45	1.12	3.82	25.8
	MEHHP	0.91	187 (94)	<LOD	3.34	6.25	20.3	121	3.54	5.70	13.4	51.0
	MEOHP	0.67	187 (94)	<LOD	2.30	4.32	13.6	106	2.41	3.72	9.22	44.9
	MECPP	0.55	192 (96)	<LOD	3.15	5.49	15.9	95.7	3.32	4.75	10.1	54.1
DOP	MOP	0.15	1 (0.5)				<LOD	1.40			<0.01	0.92
	MCPP	0.36	190 (95)	<LOD	1.34	2.62	9.88	346	1.41	2.16	9.75	195
DiNP	MiNP	0.61	22 (11)			<LOD	1.88	98.2		<0.01	1.26	55.5
	MHiNP	0.26	180 (90)	<LOD	0.79	1.71	20.7	800	0.75	1.47	11.5	453
	MOiNP	0.25	170 (85)	<LOD	0.53	1.21	11.0	472	0.45	1.02	8.90	267
	MCiOP	0.11	200 (100)	0.21	2.44	4.00	39.6	1332	2.37	3.65	34.9	753
DiDP	MiDP	0.69	1 (0.5)				<LOD	9.84			<0.01	5.56

LOD, limit of detection; *n*, number of samples.
[a]$n=150$. [b]Sum of 2,4-DCP and 2,5-DCP ($n=50$).

participants, indicating large differences in environmental exposure possibly due to personal housing conditions and life-style factors. For instance, TCS and BP-3 both showed a very wide range with a minimum less than LOD and a maximum of 1220 and 7753 μg/l respectively. This may indicate that it is possible to reduce the exposure to some of these chemicals by identifying exposure routes and changing habits.

Urinary levels measured in Danish women was compared with previously published studies (Tables 5 and 6). Although absolute values may differ due to types of sampling, national differences in regulations of industrial chemical production and application over time, and differences in study population, some patterns are nevertheless apparent.

Our reported median concentration of urinary BPA is within the same order of magnitude as previously reported across Europe and USA, thus indicating a fairly consistent environmental exposure (Table 5). In contrast, TCS and BP-3 measurements from pregnant women differed considerably between countries, indicating regional differences in the use of, e.g. TCS as a disinfectant in personal care products (Table 5). Our samples collected between March and June show no discernible variation of BP-3 concentration over time (data not shown). Exposure during this period of the year may come from other sources than sunscreen, as BP-3 is also applied as u.v. stabiliser in plastics, food and household products to prevent photodegradation (Krause *et al.* 2012, National Center for Biotechnology Information, www.ncbi.nlm.nih.gov/pmc/articles/PMC2453187/pdf/

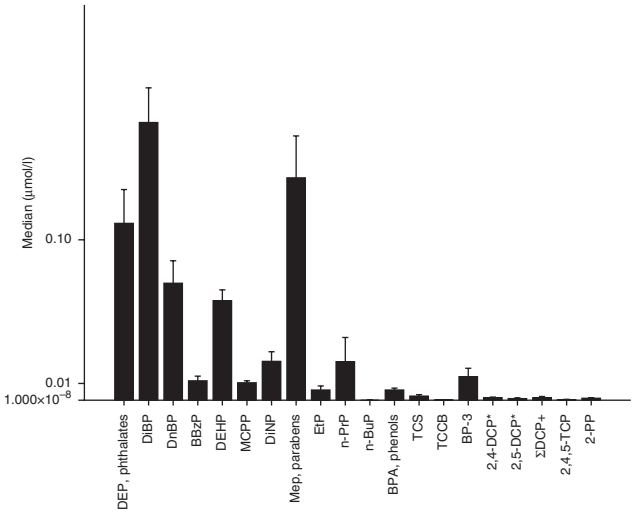

Figure 1 Median (95% error bars) concentrations (µmol/l) of phthalate metabolites, phenols and parabens detected in 200 pregnant Danish women (note the logarithmic *Y*-axis). **n*=150. $^{+}$Sum of 2,4-DCP and 2,5-DCP, *n*=50.

ehp0116-a0306a.pdf, last accessed Sep 2013). French and Spanish pregnant women and American women had higher paraben concentrations than the Danish women in our study (Calafat *et al*. 2006, Casas *et al*. 2011, Philippat *et al*. 2012). In another recent Danish study, young men had slightly lower paraben levels than the pregnant women in this study (Frederiksen *et al*. 2011). Americans have a slightly different phthalate excretion pattern than Danish and German women, with a higher excretion of MEP and lower MBP (Table 6). Such regional differences in excretion patterns may reflect national variations of industrial application or different consumer patterns of products containing phthalates. Phthalate metabolite levels measured in this study were generally lower than previously published studies performed among pregnant women in different countries (Table 6). This may be due to regional differences but could also reflect a more restricted use of phthalate over recent years, resulting in declining exposure.

In order to evaluate the potential risk of the observed exposures, including an evaluation of the risk of the combined exposures, we attempted to estimate the DI of individual chemicals based on creatinine-adjusted spot urine measures. This poses several challenges. Owing to an expected significant diurnal variability of urinary concentrations of non-persistent chemicals, e.g. BPA (Ye *et al*. 2011), this approach may lead to over-or underestimation of the true total 24-h urinary excretion, making extreme values less reliable. The estimation also relies on an average daily creatinine excretion, which may change throughout pregnancy due to changes in creatinine metabolism and excretion. However, these changes have been reported to be minimal (Gallery *et al*. 1996). In addition, exclusion of the 5% of women with creatinine levels above the normal values did not change the findings. Furthermore, the estimates of DI and excretion were based on self-reported pre-pregnancy weight, which is not an optimal parameter.

Table 3 Estimated DI of BPA and phthalate diesters (µg/kg BW per day) in Danish pregnant women in 2011, based on urinary excretion levels (*n*=181).

		Percentiles				
Compound	**Min.**	25	50	95	**Max.**	**TDI**[a]
Phthalate diester						
DEP		0.24	0.51	11.5	63.6	
DiBP	0.20	0.71	1.13	2.95	6.72	
DnBP		0.27	0.41	1.17	3.51	10
BBzP		0.04	0.07	0.55	4.45	500
DEHP		0.50	0.75	1.79	5.38	50
DiNP		0.30	0.47	4.52	85.1	150
BPA		0.01	0.02	0.10	0.75	50
Estimated (minimum) daily excretion of parabens and phenols, except BPA (µg/kg BW per day), *n*=181						
MeP		0.07	0.32	6.63	53.3	
EtP			0.01	0.65	2.46	
n-PrP		0.01	0.08	2.57	5.46	
n-BuP				0.19	1.08	
TCS		<0.01	0.01	7.65	27.7	
TCCB			<0.01	0.01	0.04	
BP-3		0.02	0.04	16.4	91.4	
2,4-DCP[b]		<0.01	<0.01	0.02	0.06	
2,5-DCP[b]			<0.01	0.02	0.22	
2,4,5-TCP			<0.01	0.01	0.07	
2-PP			<0.01	0.01	0.02	

BW, body weight; LOD, limit of detection; *n*, number of samples; Empty cells, not estimated because of urinary concentration <LOD.
[a]TDI, tolerable daily intake (µg/kg BW per day) (European Food Safety Authority (EFSA) 2005*a*, 2005*b*, 2005*c*, 2005*d*, 2010). [b]*n*=150.

Table 4 Hazard quotients (HQ) and hazard indexes (HI) based on EFSAs TDI values.

		Percentiles (HQs)			
Compound	**Min. HQ**	25	50	95	**Max. HQ**
DnBP	0.01	0.03	0.04	0.12	0.35
BBzP	<0.01	<0.01	<0.01	<0.01	0.01
DEHP	<0.01	0.01	0.02	0.04	0.11
HI	0.01	0.04	0.06	0.16	0.37

Unfortunately, the study design did not permit to have a more accurate maternal weight at the precise time point of urine sampling.

For phthalate metabolites and BPA, human FUE factors exist, which can be used to estimate a DI based on an estimated daily urinary excretion. However, these FUEs are derived from small studies on non-pregnant adult volunteers (Anderson *et al*. 2001, 2011), and FUEs in pregnant women may be different dependent on gestational age. The DI estimates of phthalate diesters are in general lower than that found in a previous study on American pregnant women (Marsee *et al*. 2006). Owing to lack of human pharmacokinetic information for the remaining chemicals, we could not calculate an estimated DI and were restricted to estimate daily excretion instead. In contrast to BPA, which is mainly excreted as conjugated forms and urinary phthalate metabolites, which can be calculated back to the parent compounds; this is not possible for parabens. Several, *in vitro*, animal and a few human studies have shown that a major part of orally administered paraben is excreted in urine as the unspecific metabolite, *p*-hydroxybenzoic acid, while only a minor part is excreted as free paraben and its glucuronic and sulphuric acid conjugates (Soni *et al*. 2005, Ye *et al*. 2006, CIR Expert Panel 2008). Following skin application, a minor part of the parabens was excreted in urine as the parent paraben in free (unconjugated) and conjugated form (glucuronidated and sulphated) (Janjua *et al*. 2007). Hence, as our estimated daily excretion of parabens is based on the fraction that was excreted as the conjugated and unconjugated parent compound, it most likely only represents a minor fraction of the true exposure and as such is a very conservative estimate. Overall, we believe that our estimated DIs and daily excretions are conservative estimates.

The EFSA has provided estimated TDIs for DnBP, BBzP, DEHP, DiNP and BPA. These TDIs are based on no observed adverse effect levels in toxicological studies on animals, to which uncertainty factors are applied to adjust for potential species differences in susceptibility. None of the estimated DIs for the individual compounds exceeded their respective TDI. The estimated DI of DnBP was closest to the TDI, but the median DI was still 24 times and the 95 percentile DI nine times below the TDI. No TDI has been given by EFSA for DiBP, although this chemical has been shown to have similar anti-androgenic effects as DnBP in animal studies (Borch *et al*. 2006), which suggests that both forms of DBP can contribute to adverse effects.

We found that pregnant women from the general population are simultaneously exposed to the majority of the investigated chemicals. Animal experiments have shown that low dose exposures to compounds affecting the same hormone axis can cause dose additive effects (Christiansen *et al*. 2009, 2012). To quantitatively assess the human risk of chemicals, the ratio between the actual level of exposure (our estimated DI) and a tolerable level of exposure (TDI estimate) is traditionally used. This ratio is referred to as the HQ and a HQ with a value of more than one indicates a risk. For DnBP, BBzP and DEHP, the EFSA TDIs are all based on anti-androgenic effects during foetal development allowing us to assess the combined risk of these compounds expressed as the HI. Using this approach, the 95 percentile HI for the combined anti-androgenic effects was 0.16; still more

Table 5 Phenol and paraben studies reporting median/GM of phenols and parabens (μg/l) in spot urine samples of pregnant women.

				Phenols			**Parabens**			
Country (reference)	**Sampling years**	**Sample size** (*n*)		BPA	TCS	BP-3	MeP	EtP	*n*-PrP	*n*-BuP
Denmark (current study)	2011	200	Median	1.38	0.70	3.20	20.7	1.01	4.17	<LOD
The Netherlands (Ye *et al*. 2008)	2004–2006	100	Median	1.2						
USA (Braun *et al*. 2011)	2003–2006	370	Median	1.8						
Norway (Ye *et al*. 2009)	2004	Ten pooled samples with 11 individual samples in each pool	GM	2.81						
Spain (Casas *et al*. 2011)	2004–2008	120	Median	2.2	6.1	3.4	191	8.8	29.8[a]	2.4[a]
USA (Wolff *et al*. 2008)	1998–2002	367	Median	1.3	11.0	7.5				
USA (Woodruff *et al*. 2004)	2003–2004	86	Median	2.7	8.2	16.9				
France (Philippat *et al*. 2012)	2002–2006	191	Median	2.7	24.1	1.7	97.8	4.1	12.5[a]	1.7[a]

GM, geometrical mean.
[a]No information provided whether the normal or isoform of the parabens is reported for PrP and BuP.

Table 6 Phthalate studies reporting median/GM of phthalate metabolites (μg/l) in spot urine samples of pregnant women.

Country (reference)	**Sampling years**	**Sample size** (*n*)		**MEP**	**MiBP**	**MnBP**	**MBzP**	**MEHP**	**MEHHP**	**MEOHP**	**MECPP**	**MCPP**	**MHiNP**	**MOiNP**	**MCiOP**
Denmark (current study)	2011	200	Median	21.5	39.8	16.0	2.97	1.15	6.25	4.32	5.49	2.62	1.71	1.21	4.00
Israel (Berman *et al.* 2009)	2006	19	Median	165	15.6	30.8	5.3	6.8	21.5	17.5	26.7	1.3			3.0
Norway (Ye *et al.* 2009)	2004	Ten pooled samples with 11 individual samples in each pool	GM	133	22.8	21.7	5.53	14.1	15.5	14.5	21.2	0.33			
The Netherlands (Ye *et al.* 2008)	2004–2006	100	Median	117	42.1	42.7	7.5	6.9	14.0	14.5	18.4	1.0			
USA (Ye *et al.* 2009)	2001–2002	111	GM	200	2.47	19.8	17.4	7.31	19.2	15.6		2.66			
Germany (Wittassek *et al.* 2009)	NA	11	Median		33.6	23.7	5.0		10.5	12.0	27.0		2.5	1.3	5.6
USA (Swan *et al.* 2005)	1999–2002	85	Median	128	2.5	13.5	8.3	3.3	11.4	11.1		2.1			
USA (Woodruff *et al.* 2004)	2003–2004	91	Median	266	4.4	17.1	17.8								
Peru (Irvin *et al.* 2010)	2004	79	GM	32.2	1.2	9.3	1.1	1.6	4.1	3.1	10.5	0.3			
Mexico (Meeker *et al.* 2009)	2001–2003	30	Median	108	2.0	33.4	2.85	3.0	17.1	13.6	38.2	1.25			0.80
USA (Adibi *et al.* 2008)	NA	246	Median	202	10.2	35.5	17.2	4.8	19.9	17.5	37.1	2.0			
USA (Wolff *et al.* 2008)	1998–2002	382	Median	380	6.2	36	22.0	6.0	20.0	17.0	35.0	3.2			
Taiwan (Lin *et al.* 2011)	2001–2002	100	Median		10.32	52.39	1.23	10.46	21.74	20.8	27.91		<LOD	<LOD	<LOD
Spain (Casas *et al.* 2011)	2004–2008	118	Median	324	29.9	27.5	10.5	4.4	17.3	15.7	32.2	1.5			4.0
France (Philippat *et al.* 2012)	2002–2006	287	Median	167	45.9	48.1	24.6	7.1	32.3	25.0	43.8	10.0			2.7

GM, geometrical mean.

than six times below 1. However, phthalates are not the only anti-androgenic compounds to which humans are exposed. For a thorough assessment of the cumulative risk of exposure to chemicals with anti-androgenic effects, other compounds should be included. BPA, TCS, several parabens, and various pesticides and biocides have shown also to possess anti-androgenic properties (Chen *et al*. 2007, Luccio-Camelo & Prins 2011). With 37% of the HI for anti-androgenic effects already taken up by only three phthalates in the most exposed woman (maximum), there is not much room left for additional exposure to anti-androgens.

A growing number of animal and epidemiological studies suggest adverse endocrine-disrupting effects on human health associated with exposure to many of the non-persistent EDCs measured in this study, encompassing effects on growth, reproductive development and thyroid function (Swan *et al*. 2005, Main *et al*. 2006, vom Saal *et al*. 2007, Chapin *et al*. 2008, Wolff *et al*. 2008, Boas *et al*. 2010, Chevrier *et al*. 2012, Philippat *et al*. 2012). In this context, it raises concern that we found detectable levels of phthalate metabolites, parabens and phenols in almost all pregnant women.

Conclusion

Our estimated DIs of different phthalates and BPA were below their individual TDI. However, a high individual exposure to one chemical was often associated with a high exposure to others and the possibility of combination effects of multiple simultaneous exposures cannot be excluded. Current toxicological risk assessments in humans do not account for combination effects of exposures, thus potentially underestimating the true cumulative risk for the developing foetus.

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.

Funding

This work was supported by the Danish Centre for Hormone Disrupting Chemicals, the Danish Foundation for Scientific Innovation and Technology (09-067180), Ronald McDonald Children Foundation, K A Rohde's and Wife's Foundation, Odense University Hospital and Region of Southern Denmark, Danielsen Foundation, The Danish Council for Strategic Research, Programme Commission on Health, Food and Welfare (2101-08-0058) and Odense Patient data Exploratory Network (OPEN). The LC-MS/MS equipment was financially supported by the Velux Foundation, Augustinus Foundation and Svend Andersen Foundation. This article is based on work presented at the 7th Copenhagen Workshop on Endocrine Disrupters, which was supported by the Danish Ministry of the Environment – Environmental Protection Agency. Publication of this special issue was supported by the Society for Reproduction and Fertility. K Tefre de Renzy-Martin, J S Christensen, H Boye Kyhl,T Barington and S Husby have no relationship with any sponsors of COW meeting, Danish Ministry of Environment and Environment Protection Agency.

Acknowledgements

The authors are grateful to all participants in the study. They appreciate the skilled technical assistance of Ole Nielsen, Department of Growth and Reproduction, Rigshospitalet, Denmark, in the analysis of chemical compounds and the Department of Clinical Biochemistry, Rigshospitalet, Denmark for analysing urinary creatinine.

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Received 18 September 2013
Accepted 26 November 2013