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Maternal phthalate exposure during pregnancy and testis function of young adult sons

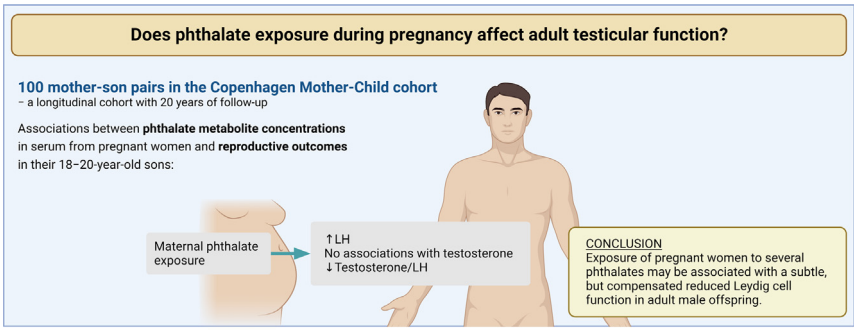
Louise Scheutz Henriksen [a,b,*], Hanne Frederiksen [a,b], Niels Jørgensen [a,b], Anders Juul [a,b,c], Niels E. Skakkebæk [a,b,c], Jorma Toppari [d,e,f], Jørgen Holm Petersen [a,g], Katharina M. Main [a,b,c]

[a] Department of Growth and Reproduction, Copenhagen University Hospital – Rigshospitalet, Copenhagen, Denmark
[b] International Center for Research & Training in Endocrine Disruption of Male Reproduction & Child Health (EDMaRC), Copenhagen University Hospital - Rigshospitalet, Copenhagen, Denmark
[c] Department of Clinical Medicine, University of Copenhagen, Copenhagen, Denmark
[d] Research Centre for Integrative Physiology and Pharmacology, Institute of Biomedicine, University of Turku, Turku, Finland
[e] Centre for Population Health Research, University of Turku and Turku University Hospital, Turku, Finland
[f] Department of Pediatrics, Turku University Hospital, Turku, Finland
[g] Section of Biostatistics, Department of Public Health, University of Copenhagen, Copenhagen, Denmark

HIGHLIGHTS

- Effects of human fetal phthalate exposure on reproduction remain largely unexplored.
- Maternal phthalate exposure during pregnancy in 100 mother-son pairs was assessed.
- Associations with reproductive data from their 20–year-old sons were examined.
- Higher exposure was associated with a compensated reduction in Leydig cell function.
- Thus, male phthalate exposure in utero may have long-term reproductive effects.

GRAPHICAL ABSTRACT



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ABSTRACT

Background: Phthalate exposure during fetal life may disrupt testicular development. Congruent with this, studies have found shorter anogenital distance, reduced penile size and altered hormone levels in infant boys whose mothers were exposed to higher levels of some phthalates during pregnancy. Few studies have explored if such adverse effects persist in adulthood. Thus, we aimed to explore if there is an association between fetal phthalate exposure and markers of testicular function in young adult men.

Methods: In a longitudinal mother-child cohort from Copenhagen, Denmark, we examined 100 young men whose mothers during pregnancy had serum drawn and analyzed for 34 phthalate metabolites. Examinations of the young men took place at 18–20 years of age and included measurements of adult markers of testicular function (reproductive hormones, penile size, anogenital distance (AGD), testis volume, semen quality) and growth factors. Associations between maternal serum concentrations of phthalate metabolites and reproductive measures in the young men were tested using multiple linear regression.

Results: Most consistently, higher maternal phthalate exposure was associated with higher luteinizing hormone (LH) but unchanged testosterone in adult sons. Congruently, higher maternal exposure was associated with lower total and free testosterone/LH ratios in adult sons. For example, twice as high maternal MiNP was associated with a 7.9 % (95 % CI 1.6–13.8) lower free testosterone/LH ratio. There was no consistent pattern of associations between

* Corresponding author at: Department of Growth and Reproduction, Copenhagen University Hospital – Rigshospitalet, Copenhagen, Denmark.
E-mail address: louise.scheutz.henriksen.01@regionh.dk (L.S. Henriksen).

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the different phthalate metabolites and other reproductive hormones, clinical outcomes, or semen quality. None of the tested associations was significant after multiplicity adjustment.
Conclusions: In this exploratory study, higher maternal exposure to some phthalates was associated with impaired testicular Leydig cell function evidenced by a lower total and free testosterone/LH ratio in adult sons. This unique 18–20-year follow-up study raises concern and suggests that exposure of pregnant women to phthalates may have long-term effects on adult reproductive health in male offspring.

1. Introduction

In recent years, increased concern has been raised about the possible role of environmental factors in the decline in male reproductive health observed over the past generations (Rodprasert et al., 2019; Skakkebæk et al., 2022). One particular focus area has been the potential harmful effects of phthalates. Phthalates are the most used plasticizers, and, as a result, human exposure is both vast and ubiquitous (Wittassek et al., 2011). Some are classified as toxic to reproduction, and their effects are generally considered to be anti-androgenic (Li and Spade, 2021). Exposure to endocrine-disrupting chemicals (EDCs) is believed to be most harmful during fetal life when the development of reproductive organs is regulated by sex hormones (Bigsby et al., 1999; Skakkebæk et al., 2015; Welsh et al., 2008), and harmful exposure may cause testicular disruption (Li and Spade, 2021). Thus, fetal exposure to some EDCs is suggested to be associated with shorter anogenital distance (AGD) and play a role in the development of male reproductive diseases such as cryptorchidism, poor semen quality and testicular cancer (for reviews see (Bräuner et al., 2021; Skakkebæk et al., 2015)). Similarly, the so-called 'phthalate syndrome' with shorter AGD, reduced genital size, altered levels of reproductive hormones, cryptorchism and nipple retention can be induced in male rodents by exposing pregnant dams to di-(2-ehtyl-hexyl) phthalate (DEHP), butylbenzyl phthalate (BBzP), di-n-butyl phthalate (DnBP) or di-isononyl phthalate (DiNP) (Boberg et al., 2011; Foster, 2006; Gray et al., 2000; Parks et al., 2000; van den Driesche et al., 2020, 2017). However, in experimental models where human fetal testes have been implanted in nude mice, phthalates did not inhibit steroidogenesis (for review see (Kilcoyne and Mitchell, 2019)), suggesting that human fetal testis tissue would be less susceptible to antiandrogenic effects than that of rodents.

Some studies have explored the associations between prenatal phthalate exposure and markers of testis function in infant boys. In accordance with the suspected anti-androgenic effects of some phthalates, an inverse association between AGD in infant boys and maternal urinary concentrations of DEHP and DnBP during gestation is the most consisting finding (for review see (Radke et al., 2018)), but also associations with reduced penile size (Bustamante-Montes et al., 2013; Swan, 2008), follicle-stimulating hormone (FSH), testosterone and testosterone/luteinizing hormone (LH) ratio (Muerköster et al., 2020) have been found. However, it remains unknown whether these effects persist in adulthood. Due to the long human maturation time, epidemiologic evidence on adult consequences of exposure to EDCs during fetal development is very limited (Bonde et al., 2016), but the existing evidence suggests that higher prenatal phthalate exposure may be associated with negative effects on adult reproductive outcomes (Axelsson et al., 2015; Hart et al., 2018). The current study adds to the limited existing body of evidence based on one study from Sweden (Axelsson et al., 2015) and one from Australia (Hart et al., 2018), representing two countries with different phthalate exposure patterns in the 1980'ies and 1990'ies. Our study is based on a more recent cohort of young men born around 10 years later and thus reflects the reduced exposure risk after several European initiatives for regulation of phthalate use in consumer products.

Using serum samples collected from pregnant women in a prospective birth cohort established 1997–2001, we aimed to assess the potential long-term effects of fetal exposure to phthalates on male reproductive health. We investigated associations between maternal serum concentrations of common phthalate metabolites and biochemical as well as clinical reproductive outcomes in their 18–20-year-old sons.

2. Materials and methods

2.1. Study design

This study included mothers and sons of a prospective population-based birth cohort study. The cohort was established 1997–2001 with inclusion of pregnant women in their first trimester (Boisen et al., 2004), and a serum sample was drawn at least once during pregnancy. In total, 1270 live-born boys were included in the study at birth and seen at repetitive examinations during infancy, childhood and puberty. At 18–20 years of age, they were invited to participate in an adult follow-up. Inclusion criteria for the current study were participation in the adult follow-up and an available maternal serum sample from pregnancy. This was the case for 101 young men, including one pair of twins. One young man was excluded due to suspected underlying reproductive pathology (Fig. 1). Measurements of penile width and maternal phthalate concentrations were not previously published. All other reproductive outcomes in the population of young men included in this study have previously been published, albeit not in relation to fetal phthalate exposure (Henriksen et al., 2022; Holmboe et al., 2022).

The study was performed following the Declaration of Helsinki II and approved by the Regional Committee on Health Research Ethics ((KF) 01-030/97 and H-17011468) and the Danish Data Protection Agency (1997-1200-074 and VD-2018-118/i-Suite 6358). All participants gave written informed consent.

2.2. Study setup

2.2.1. Maternal data

Information on maternal age at delivery and smoking habits during pregnancy was obtained from hospital records (Chellakooty et al., 2003). Gestational age (GA) at maternal serum sampling was determined based on routine ultrasound in pregnancy week 18–20 if available; if not, information on the last menstrual period was used (Boisen et al., 2004).

At two hospitals, only one maternal serum sample was collected during pregnancy. At the third hospital, participants had blood samples taken repetitively throughout pregnancy (Assens et al., 2019; Chellakooty et al., 2004), and multiple samples were thus available for these women. All samples were stored in aliquots at −20 °C from sampling (1997–2001) until phthalate analysis (2019).

For the subgroup of women who had multiple serum samples drawn during pregnancy, we selected the sample drawn at the earliest timepoint in pregnancy for this study, i.e., only one serum sample per woman was analyzed for phthalate metabolites (median GA 15.6 weeks, IQR 12.9–17.5). For all other women, who were only sampled once, this serum sample was included (median GA 22 weeks, IQR 20.1–31.9).

A total of 422 maternal serum samples were identified of which 364 samples contained enough serum to be analyzed for phthalate metabolites. Of those, 100 belonged to a mother whose son was included in the current study, including one pair of twins (Fig. 1).

2.2.2. Phthalate analyses

All maternal serum samples were analyzed at the Department of Growth and Reproduction at Rigshospitalet, Copenhagen, which serves as an international reference laboratory (European Human Biomonitoring Initiative, HBM4EU). Samples were analyzed for 34 phthalate metabolites from 16 phthalate diesters as shown in Supplementary Table 1. This was done by

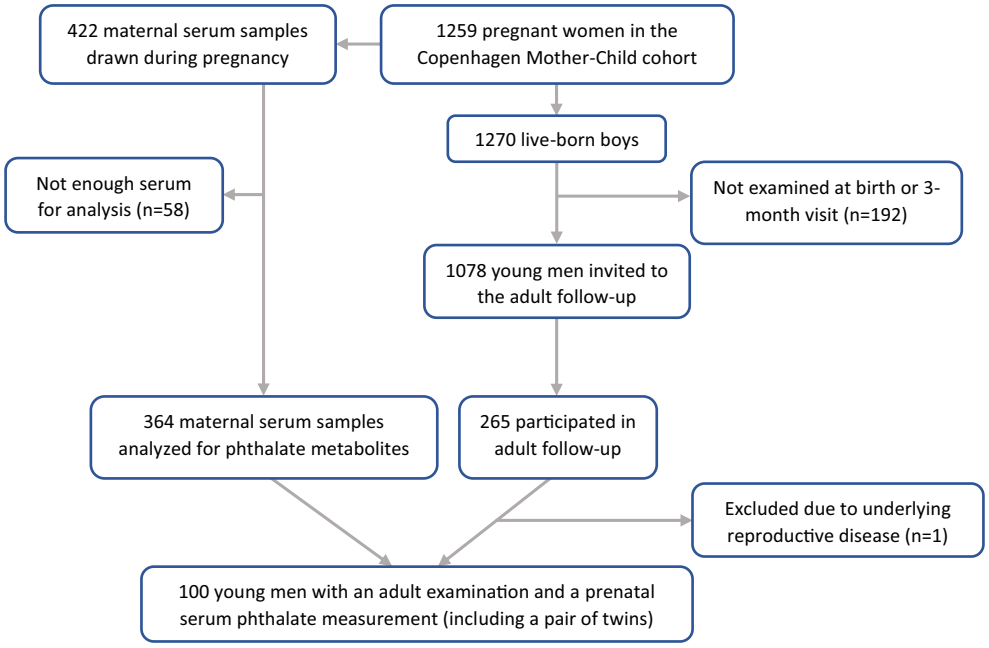


Fig. 1. Flow diagram of participant inclusion. The figure illustrates the inclusion of the 100 young men in our study population: Of 422 serum samples from pregnant women in the Copenhagen Mother-Child cohort, 364 were analyzed for phthalate metabolites. A total of 1259 women in the cohort gave birth to 1270 live-born boys, and 1078 of these were invited for an adult follow-up examination in which 265 participated. Of these, 100 young men had a prenatal phthalate measurement and were included in the current study.

isotope-diluted liquid chromatography-tandem mass spectrometry (LC-MS/MS) preceded by enzymatic deconjugation as previously described in detail for 13 metabolites (Frederiksen et al., 2010). This method was recently expanded to include 34 metabolites and further modified by using online-TurboFlow-LC-MS/MS technology equipped with a probe for heated electrospray ionization (HESI) running in negative mode (Frederiksen et al., 2019; Hart et al., 2018). As previously described (Henriksen et al., 2020), samples were analyzed in batches that included standards for calibration curves, 30–40 participant samples, three blanks, three serum pool controls, and three serum pool controls spiked with native phthalate metabolite standards at both low and high levels. Coefficients of variations (CVs) and limits of detection (LODs) for the individual metabolites measured in serum can be found in (Hart et al., 2018) and in Supplementary Table 1, respectively.

2.2.3. Adult examinations

All participants had a single blood sample drawn from an antecubital vein between 0730 h and 1200 h after at least 8 h of fasting. Penile length and midshaft-width (flaccid, vertically adjusted) were measured to the nearest millimeter. Testicular dimensions were determined three times using ultrasonography. Testicular volume was calculated using the formula height × width × length × (π / 6). The average of three calculations and the mean of left and right testicle were used. Finally, with the participant in the lithotomic position, both anopenile distance (APD, from the anterior base of the penis to the anterior wall of the anus) and anoscrotal distance (ASD, from the posterior base of the scrotum to the anterior wall of the anus) were measured three times to the nearest millimeter using a caliper (Mendiola et al., 2016), and means were calculated. All participants underwent a whole-body dual x-ray absorptiometry (DXA) scan (Lunar Prodigy, GE Healthcare, Madison, WI, using enCORE software, version 14.10.022) for assessment of total body fat percentage, wearing only light clothing. Participants received DKK 500 (≈€67) as compensation. Information on smoking habits was collected through online questionnaires.

2.2.4. Semen analyses

As previously described, the young men delivered a semen sample by masturbation in a designated room at the department (Henriksen et al., 2022). In brief, sexual abstinence of at least 48 h was recommended, and the period of abstinence was recorded. Sample analysis was initiated after twenty minutes of incubation at 37 °C and within one hour after ejaculation. Analyses were performed in accordance with the World Health Organization's 2010 guidelines (World Health Organization, 2010) as previously described (Priskorn et al., 2018). Semen volume was determined by weighing to the closest 0.1 mL. Sperm concentration (million/mL) was assessed using a NucleoCounter® NC-3000™ (ChemoMetec A/S, Alleroed, Denmark), and total sperm count was calculated as the product of semen volume and sperm concentration. To assess sperm motility, two drops of well-mixed semen were placed on a glass slide and examined with phase-contrast microscopy. Spermatozoa were classified as either progressively motile, non-progressively motile or immotile (Priskorn et al., 2018). Fixed and Papanicolaou stained morphology slides were evaluated according to stricter criteria (Menkveld et al., 1990) to determine the number of morphologically normal spermatozoa. All assessments were done in duplicates, and averages were used. The analyses were all accredited according to DS/EN ISO 15189.

2.2.5. Hormone analyses in young men

Hormone analyses were performed in our laboratory (Rigshospitalet, Copenhagen), also accredited according to DS/EN ISO 15189. Serum samples were centrifuged and stored at −20 °C until analysis. Serum concentrations of FSH and LH were measured using a two-sided time-resolved fluoroimmunometric assay (DELFIA, Wallac, Turku, Finland) with LODs of 0.05 U/L. The inter-assay CVs were <3 % and <5 % for FSH and LH, respectively. Serum inhibin B concentrations were measured by an enzyme-linked immunosorbent assay (ELISA; inhibin B gen II, Beckman Coulter, Brea, CA, United States) with an LOD of 3 pg/mL and an inter-assay CV <8 %. Serum estradiol, testosterone and SHBG concentrations were measured by a chemiluminescent enzyme immunoassay (Access2, Beckman Coulter, Brea, CA, United States). The LODs were 56 pmol/L for estradiol, 0.35 nmol/L for testosterone and 0.33 nmol/L for SHBG. Inter-assay CVs were <7 % for all three hormones. Serum IGF-1 and IGFBP-3 concentrations were measured by automated chemiluminescence immunoassays IDS-iSYS IGF-I and IDS-iSYS IGFBP-3 (Immunodiagnostic Systems, Boldon, United Kingdom) with LODs of 10 and 80 μg/L, respectively.

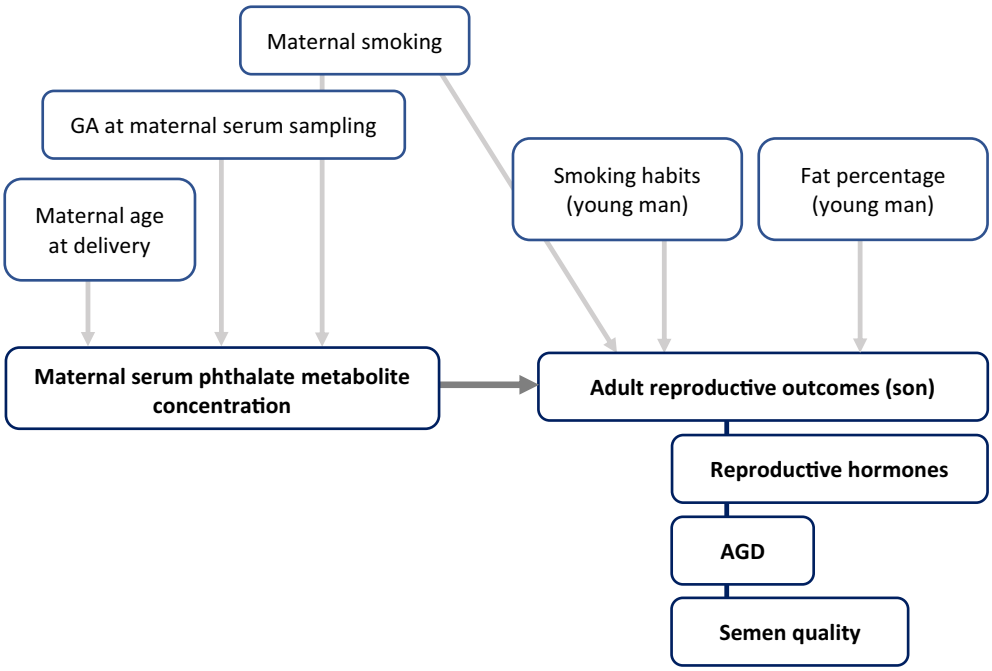


Fig. 2. Causal diagram for covariates included in the different regression models. Covariates were chosen a priori based on existing knowledge and availability of data. Abbreviations: GA, gestational age; AGD, anogenital distance.

Inter-assay CVs were <5 % for IGF-1 and <7 % for IGFBP-3. Serum INSL3 was determined using isotope diluted online TurboFlow-LC-MS/MS with an LOD of 0.03 μg/L (Albrethsen et al., 2020), and the inter-assay CV was <21 %. Free testosterone was calculated by Vermeulen's equation (Vermeulen et al., 1999). Ratios between testosterone/LH, free testosterone/LH, and inhibin B/FSH were calculated by division.

2.2.6. Statistical analysis

We generated descriptive statistics for phthalate metabolites and characteristics of both, mothers and sons. Maternal serum phthalate concentrations were compared in participants versus non-participants using the Mann-Whitney *U* test.

Phthalate metabolites were included in statistical analyses if they were ≥LOD in >50 % of serum samples. As a result, six primary (mono-ethyl phthalate (MEP), mono-isobutyl phthalate (MiBP), mono-n-butyl phthalate (MnBP), mono-(2-ethyl-hexyl) phthalate (MEHP), mono-isononyl phthalate (MiNP), mono-isodecyl phthalate (MiDP)) and five secondary (mono-(2-hydroxy-isobutyl) phthalate (2-OHMiBP), mono-(2-ethyl-5-carboxypentyl) phthalate (5cx-MEPP), mono-2-(carboxymethyl-hexyl) phthalate (2cx-MMHP), mono-carboxy-isooctyl phthalate (cx-MiOP), mono-(carboxy-isononyl) phthalate (cx-MiNP)) metabolites of six different phthalate diesters were included. All measurements <LOD were replaced with LOD/√2.

We calculated two sums of metabolites (Hart et al., 2014): All six included primary metabolites (MEP, MiBP, MnBP, MEHP, MiNP, MiDP) were added to Σprimary, and the included five secondary metabolites (2OH-MiBP, 5cx-MEPP, 2cx-MMHP, cx-MiOP, cx-MiNP) were added to Σsecondary. To calculate these sums, the concentration of each phthalate metabolite (ng/mL) was divided by its own molecular weight (mol/L), and the resulting molar concentrations were summed. To convert units for sums to ng/mL, these sums were multiplied by the molecular weight (ng/nmol) of the metabolite measured in the highest concentration in the sum, i.e., MnBP for Σprimary and 2OH-MiBP for Σsecondary.

Associations between maternal serum phthalate concentrations and adult reproductive outcomes were explored in multiple linear regression models, both unadjusted and adjusted (Fig. 2). In fully adjusted models, we included maternal smoking during pregnancy [yes, no], maternal age at delivery, GA at maternal serum sampling, smoking habits [non-smoker, occasionally, daily] and total fat percentage of the young man. In models for hormones, we also adjusted for time of day for serum sampling. In models for semen quality outcomes, time passed between ejaculation and motility analysis (only for motility) and duration of sexual abstinence were included. In models for ASD and APD, height of the young man was also included.

Most reproductive outcomes were ln transformed to obtain normally distributed residuals in our models; only sperm motility, sperm morphology and INSL3 were included in models untransformed. Phthalate metabolite concentrations were log2 transformed to obtain linearity. To enable clinical interpretation of ln transformed outcomes, effect estimates (β) were back transformed using the formula $(e^{\beta} - 1) \times 100$. Resulting values represent the percentage change in the median value of the outcome when serum phthalate concentration is doubled.

Analyses were repeated after excluding one outlying observation of a key outcome measure, but as this did not substantially change the results, it was included in the final analyses. We adjusted for multiple comparisons using the Benjamini-Hochberg method (Benjamini and Hochberg, 1995) and validated all models using residual plots to test normality, linearity and homoscedasticity. All statistical analyses were conducted using RStudio (R Core Team, 2019), and plots were constructed using ggplot2 (Wickham, 2016). The 'stats' package was used to calculate multiplicity-adjusted *P* values. We considered *P* values <0.05 as statistically significant.

3. Results

Maternal blood sampling was performed at a median GA of 21.7 weeks. Median maternal age at delivery was 31 years, and 23 % of mothers smoked during pregnancy (Table 1). Median age and BMI of the young men at the adult follow-up were 19.2 years and 21.2 kg/m^2. While 11 % of the young men were daily cigarette smokers, 42 % smoked cigarettes occasionally (Table 1).

Unadjusted reproductive outcomes of the young men are presented in Supplementary Table 2. Nine of the 11 included phthalate metabolites were measured in >90 % of the serum samples, and MnBP had the highest median concentration of 31.2 ng/mL (Table 2). Overall, the median concentrations of the different phthalate metabolites in included samples were comparable to those of excluded ones which belonged to mothers

Table 1
Characteristics of the study population.

	n	Median (IQR); n (%)
Maternal characteristics		
Maternal age at delivery (years)	100	31.0 (28.3, 34.1)
Gestational age at maternal serum sampling	100	21.7 (19.4, 28.1)
Maternal parity, n (%)	100	
1		69 (69 %)
2		25 (25 %)
≥3		6 (6.0 %)
Maternal social class, n (%)	95	
1–2		50 (53 %)
3–4		24 (25 %)
5–7		21 (22 %)
Cigarette smoking during pregnancy, n (%)	100	23 (23 %)
Maternal pre-pregnancy BMI, n (%)	92	
18.5–24.9		75 (82 %)
<18.5		2 (2.2 %)
≥25		15 (16 %)
Maternal diabetes, n (%)	98	1 (1.0 %)
Birth characteristics		
Birth weight (kg)	100	3.6 (3.3, 3.9)
Gestational age at delivery, n (%)	100	
Full term		87 (87 %)
Preterm		4 (4.0 %)
Post term		9 (9.0 %)
Weight for gestational age, n (%)	100	
AGA		94 (94 %)
SGA		5 (5.0 %)
LGA		1 (1.0 %)
Cryptorchidism at birth[a], n (%)	98	0 (0 %)
Adult characteristics		
Age (years)	100	19.2 (18.7, 19.5)
Body height (cm)	100	184.6 (180.2, 189.6)
Body weight (kg)	100	72.5 (65.6, 81.1)
BMI (kg/m^2)	100	21.2 (19.7, 23.5)
Sexual abstinence (h)	98	62.1 (48.0, 85.0)
Cigarette smoking, n (%)	99	
None		46 (46 %)
Occasionally		42 (42 %)
Daily		11 (11 %)

Abbreviations: AGA, appropriate for gestational age; BMI, body mass index; IQR, interquartile range; LGA, large for gestational age; SGA, small for gestational age.

[a] Testis position was recorded after manipulation of the testis to the most distal position along the pathway of normal descent using firm but not forced traction (Boisen et al., 2004).

whose sons did not participate in the adult follow-up (Supplementary Table 3). Generally, when exploring associations between maternal serum concentrations of phthalate metabolites and adult reproductive outcomes, we reached the same conclusions with unadjusted and fully adjusted models.

3.1. Reproductive hormones and growth factors

Higher maternal serum concentrations of the primary metabolites MnBP, MEHP, MiNP and Σprimary were associated with increased LH in adult sons (Fig. 3, Supplementary Table 4). The strongest association was with Σprimary where LH increased by 13.0 % (95 % CI 5.4–21.1) when Σprimary doubled. Associations with MEP, MiBP, 2cx-MMHP, MiDP and cx-MiNP showed the same tendencies but were not significant. While MEHP was associated with higher testosterone (5.8 % (95 % CI 0.7–11.3) when maternal MEHP concentration doubled), no other phthalates were significantly associated with adult testosterone levels. Followingly, a doubling of maternal serum concentrations of MnBP, MiNP and Σprimary was associated with decreased testosterone/LH (−4.8 % (95 % CI −8.9–(−0.5)), −7.8 % (95 % CI −13.9–(−1.2)), and −11.1 % (95 % CI −17.5–(−4.3)), respectively) and free testosterone/LH ratios (−4.7 % (95 % CI −8.6–(−0.5)), −7.9 % (95 % CI −13.8–(−1.6)), and −10.6 % (95 % CI −16.8–(−3.9)), respectively). None of the phthalate metabolites were significantly associated with adult INSL3 or SHBG concentrations (Fig. 3, Supplementary Table 4).

There were no significant associations between maternal serum concentrations of any phthalate metabolite and FSH, inhibin B or inhibin B/FSH (Supplementary Fig. 1, Supplementary Table 5), but higher maternal MEHP was associated with increased estradiol (8.5 % (95 % CI 2.8–14.5) when maternal MEHP concentration was doubled; Supplementary Fig. 1, Supplementary Table 5). No other metabolites were significantly associated with estradiol concentrations. For growth factors, increased maternal concentrations of 2OH-MiBP was associated with decreased IGF-1 (−4.8 % (95 % CI −9.1–(−0.3)) when maternal 2OH-MiBP concentration was doubled) in the adult sons; reversely, increased maternal MiNP was associated with a higher concentration of IGFBP-3 (3.1 % (95 % CI 0.7–5.4) when maternal MiNP concentration was doubled; Supplementary Fig. 1, Supplementary Table 5).

3.2. Clinical outcomes

Increased maternal concentrations of most phthalate metabolites appeared to be associated with slightly shorter penile length in adult sons, but this was only significant for MiBP where penile length decreased by 3.3 % (95 % CI 5.0–1.5) when maternal MiBP concentration doubled (Fig. 4, Supplementary Table 6). An exception was that a higher MiDP concentration was associated with both increased penile length and width. All other associations with penile width were negative and non-significant. A higher MEHP concentration was associated with larger testicular volume (5.7 % (95 % CI 0.6–11.1) when maternal MEHP concentration was doubled), while no other phthalate metabolites were significantly associated with this outcome (Fig. 4, Supplementary Table 6). No phthalate metabolites were associated with APD or ASD.

3.3. Semen quality outcomes

MiBP and MiNP were associated with a lower percentage of morphologically normal spermatozoa (Supplementary Fig. 2, Supplementary Table 7): Twice as high a maternal serum concentration of MiBP was associated with a change in morphologically normal spermatozoa of −1.1 percentage points (95 % CI −1.8–(−0.4)); for MiNP, the change in morphologically normal spermatozoa was −0.7 percentage points (95 % CI −1.4–(−0.03)). Further, associations for MnBP, MEHP, cx-MiOP, cx-MiNP and the two sums were also negative, but not significant. A higher maternal serum concentration of cx-MiNP was associated with a lower percentage of progressively motile spermatozoa (−1.8 % (95 % CI −3.3–(−0.4)) when maternal cx-MiNP concentration was doubled; Supplementary Fig. 2, Supplementary Table 7). There were no significant associations between maternal serum phthalate metabolite concentrations and semen volume, sperm concentration or total sperm count (Supplementary Fig. 2, Supplementary Table 7).

After multiplicity adjustment, none of the tested associations remained significant (data not shown).

4. Discussion

In this longitudinal population-based cohort study, we tested associations between maternal serum phthalate concentrations during pregnancy and reproductive outcomes in 100 healthy adult sons. Most consistently, we found an association between higher maternal serum levels of several phthalate metabolites and increased LH but unchanged testosterone levels. Consequently, higher maternal exposure was also associated with decreased testosterone/LH and free testosterone/LH ratios. Although some other associations were statistically significant, there was no consistent pattern of these which may indicate that they reflect chance findings. None of the associations between exposure measurements and adult reproductive parameters remained statistically significant after correction for multiple testing.

Thus, prenatal phthalate exposure was associated with a mild degree of compensated reduced Leydig cell function seen as unaltered testosterone levels on a background of simultaneously increased LH. This is a clinically

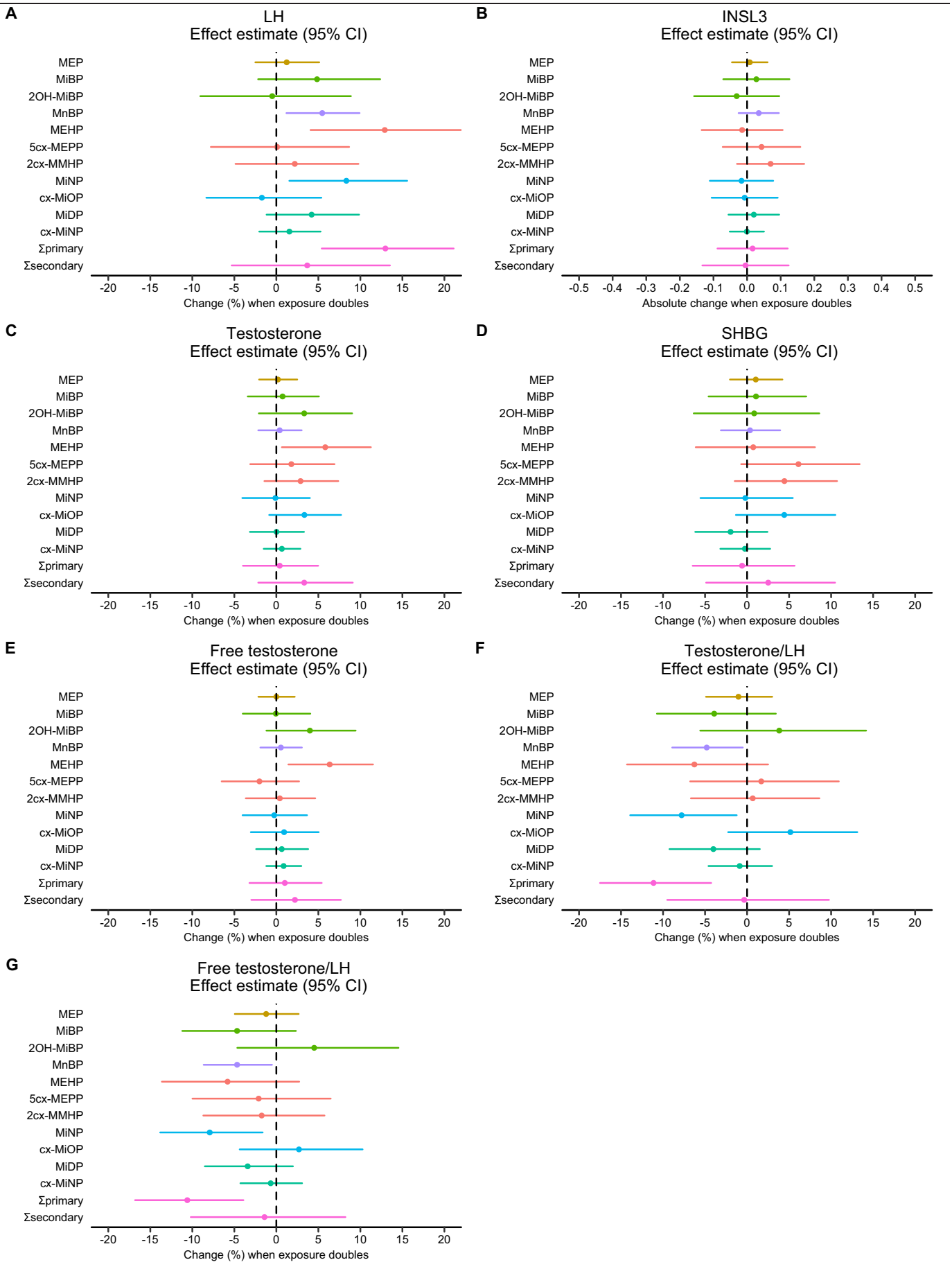
Table 2
Concentrations of phthalate metabolites (ng/mL) in 100[a] maternal serum samples drawn during pregnancy.

Phthalate diester	Metabolites	% ≥ LOD	Minimum	Q1	Q2	Q3	Maximum
DMP	MMP[b]	11.0				<LOD	2.08
DEP	**MEP**[b]	98.0	<LOD	0.72	2.30	7.44	139
DiPrP	MiPrP[b]	29.0			<LOD	0.14	0.50
DnPrP	MnPrP[b]	2.0				<LOD	0.15
DiBP	**MiBP**[b]	100	0.4	1.80	3.18	6.87	17.5
	2OH-MiBP	94.0	<LOD	0.40	0.63	0.90	1.80
DnBP	**MnBP**[b]	99.0	<LOD	4.44	31.2	55.0	115
	3OH-MnBP	15.0				<LOD	0.61
BBzP	MBzP[b]	23.0				<LOD	1.11
DnPeP	MnPeP[b]	0.0					<LOD
	4OH-MnPeP/3OH-MiPeP[c]	22.0				<LOD	0.37
DiPeP	4OH-MiPeP	0.0					<LOD
DEHP	**MEHP**[b]	100	1.07	2.63	5.09	7.30	20.7
	5OH-MEHP	30.0			<LOD	0.13	0.55
	5oxo-MEHP	1.0				<LOD	0.13
	5cx-MEPP	100	0.09	0.31	0.46	0.69	4.00
	2cx-MMHP	93.0	<LOD	0.21	0.37	0.62	4.33
DnHxP	MnHxP[b]	0.0					<LOD
	5OH-MHxP	5.0				<LOD	0.23
	5cx-MPeP	40.0			<LOD	0.123	0.29
DCHP	MCHP[b]	7.0				<LOD	0.79
DnHpP	MnHpP[b]	1.0				<LOD	0.36
	6OH-MHpP	1.0				<LOD	0.05
	6cx-MHxP	22.0				<LOD	0.35
DnOP	MnOP[b]	12.0				<LOD	0.74
	MCPP[d]	24.0				<LOD	0.24
DiNP	**MiNP**[b]	95.0	<LOD	0.78	1.26	1.97	3.58
	OH-MiNP	2.0				<LOD	0.30
	oxo-MiNP	2.0				<LOD	0.16
	cx-MiOP	53.0		<LOD	0.085	0.17	1.03
DiDP	**MiDP**[b]	91.0	<LOD	1.69	3.53	5.45	15.7
	OH-MiDP	10.0				<LOD	0.09
	oxo-MiDP	0.0					<LOD
	cx-MiNP[b]	74.0		<LOD	0.18	0.50	11.2
Sums	Σprimary	100	6.70	19.3	49.1	79.5	190
	Σsecondary	100	0.49	1.17	1.66	2.28	12.0

Metabolites included in statistical analyses (measured in >50 % of samples) are highlighted in bold.
Σprimary is the molar sum of MEP, MiBP, MnBP, MEHP, MiNP, and MiDP expressed as MnBP; Σsecondary is the molar sum of 2OH-MiNP, 5cx-MEPP, 2cx-MMHP, cx-MiOP and cx-MiNP expressed as 2OH-MiNP.
Abbreviations: LOD, limit of detection; Q1, first quartile; Q2, second quartile; Q3, third quartile; Q4, fourth quartile. Phthalate metabolite abbreviations are shown in Supplementary Table 1.
[a] Includes one pair of twins who share the same maternal sample.
[b] Primary metabolites.
[c] These two metabolites cannot be analytically distinguished.
[d] MCPP is the major metabolite of DnOP but is not specific for DnOP.

relevant finding as a lower testosterone/LH ratio has been associated with lower semen quality (Jørgensen et al., 2016) and higher mortality (Holmboe et al., 2015) in population studies. Consistent with our findings, a retrospective Swedish study of 112 military conscripts born 1989–1992 found that higher maternal serum concentrations of DEHP and DiNP metabolites during pregnancy were associated with higher gonadotropins in the adult sons (Axelsson et al., 2015). While higher maternal cx-MiOP was associated with increased FSH in the young men in this study, only the two hydroxylated metabolites of DiNP (OH-MiOP and oxo-MiOP) were associated with LH. These metabolites were measured in <2 % of the samples in our study and thus not included in analyses. Similarly, an Australian cohort study of around 150 young men born 1989–1991 found that the sum of DEHP metabolites in maternal serum was positively correlated with LH in adult sons, while MEHP was negatively correlated with the testosterone/LH ratio (Hart et al., 2014). In line with the findings of the Swedish study (Axelsson et al., 2015), MiNP was positively correlated with FSH in the young Australian men. Congruent with our findings, a recent cohort study of 479 Danish infant boys also found that higher prenatal phthalate exposure, as measured in maternal urine samples, was associated with a decreased testosterone/LH ratio (Muerköster et al., 2020). This was found for the sum of MnBP and MiBP and for the sum of DiNP metabolites, which fits with our findings. However, as mechanisms of regulation of the hypothalamic-pituitary-gonadal axis may differ between infancy and adulthood, it is unknown if these findings in infants and adults reflect the same effect and if they are thus comparable. An association between phthalate metabolite levels in maternal breast milk samples and hormones related to Leydig cell function in early infancy was described in our cohort earlier (Main et al., 2006). In this study, an increased MiNP in breast milk was

Fig. 3. Maternal serum concentration of phthalate metabolites and reproductive hormone concentrations (LH/Leydig cell axis) in adult sons. The plots show effect estimates from linear regression analyses. Estimates are shown as the percent change in outcome when exposure doubles. For example, when maternal serum MEHP doubles, LH increases by 12.9 % (4.1–22.5). Please note that INSL3 concentrations were included in models untransformed and the shown effect estimates reflect the absolute change in INSL3 when exposure doubles. Abbreviations: LH, luteinizing hormone; INSL3, insulin-like factor 3; SHBG, sex hormone binding globulin. Abbreviations of phthalate metabolites are presented in Supplementary Table 1. Σprimary is the molar sum of MEP, MiBP, MnBP, MEHP, MiNP and MiDP expressed as MnBP; Σsecondary is the molar sum of 2OH-MiNP, 5cx-MEPP, 2cx-MMHP, cx-MiOP and cx-MiNP expressed as 2OH-MiNP.


A
LH
Effect estimate (95% CI)
B
INSL3
Effect estimate (95% CI)
C
Testosterone
Effect estimate (95% CI)
D
SHBG
Effect estimate (95% CI)
E
Free testosterone
Effect estimate (95% CI)
F
Testosterone/LH
Effect estimate (95% CI)
G
Free testosterone/LH
Effect estimate (95% CI)
MEP
MiBP
2OH-MiBP
MnBP
MEHP
5cx-MEPP
2cx-MMHP
MiNP
cx-MiOP
MiDP
cx-MiNP
Σprimary
Σsecondary
Change (%) when exposure doubles
Absolute change when exposure doubles

associated with an increase in LH. However, as breast milk is a different matrix and was collected after birth, these results may not be directly comparable.

These findings in human populations are in part supported by rodent experiments. Fetal exposure to DnBP during the male programming window could result in compensated Leydig cell failure with low to normal testosterone levels and elevated LH in adult male rats (Kilcoyne et al., 2014; van den Driesche et al., 2017). Effects of phthalates on steroidogenesis of human fetal testes have been studied in vitro (Hallmark et al., 2007; Lambrot et al., 2009) and in xenotransplants in mice (Mitchell et al., 2012; Spade et al., 2014) and marmoset monkeys (McKinnell et al., 2009). In none of these experimental models, phthalates affected testosterone production (for review see Kilcoyne and Mitchell, 2019). This is in line with the present results where testosterone levels were not associated with phthalate exposure. Gonadotropin levels were not measured in previous xenotransplantation studies, and experimental systems required human chorionic gonadotropin (hCG) stimulation in the rodent xenotransplantation studies. Therefore, we cannot know whether the hypothalamus-pituitary-testis axis was affected in those models. Basal steroidogenesis in human fetal testis was not affected by phthalates either, but neither was that of cultured rat fetal testis, making the findings inconclusive (Hallmark et al., 2007). Interestingly, in 2–7-day-old marmosets, treatment with a single dose of 500 mg/kg MnBP suppressed blood testosterone levels at 5 h significantly ($P = 0.019$). Similar treatment of newborn co-twin marmosets for two weeks resulted in increased Leydig cell volume per testis ($P = 0.011$) compared with co-twin controls; suggesting MnBP-induced inhibition of steroidogenesis followed by compensatory Leydig cell hyperplasia/hypertrophy (Hallmark et al., 2007).

In the male fetus, the genitalia grow under androgen influence, and this may be affected by anti-androgenic exposures as seen in rodent studies (van den Driesche et al., 2017; Welsh et al., 2008). However, we found no consistent associations between maternal phthalate exposure and penile dimensions, testicular volume or AGD. Thus, we could not establish clear-cut clinical signs of reduced androgen action or increased gonadotropin drive. To our knowledge, no previous studies on prenatal exposure and long-term effects on male reproductive health have examined AGD or penile dimensions to allow comparisons. However, in newborn boys there is evidence of an inverse association between DEHP and DnBP exposure and AGD (Radke et al., 2018). Similarly, maternal MEHP has been associated with both reduced penile width (Swan, 2008) and length (Bustamante-Montes et al., 2013) in new-born boys, but not in all studies (Romao et al., 2020).

We also did not find any consistent pattern of associations with semen quality. This is in contrast to two previous longitudinal cohort studies which reported a reduced semen volume with increasing maternal phthalate exposure (Axelsson et al., 2015; Hart et al., 2018). One of these studies also found that a higher maternal cx-MiOP was associated with reduced sperm motility (Hart et al., 2018). In a multicenter cross-sectional study, maternal occupational exposure to phthalates assessed through questionnaires was associated with an almost doubling of the odds ratio for low semen volume and total sperm count in adult sons (Istvan et al., 2021). We could not corroborate these findings. One possible explanation may be population differences in phthalate exposure levels and in exposures to other EDCs that were not accounted for. Serum concentrations of secondary phthalate metabolites in the pregnant women in our study were lower than those measured in the Swedish study (Axelsson et al., 2015), which is consistent with samples being collected around 10 years later (Henriksen et al., 2020). Additionally, the LODs in our study were higher, so detection rates for secondary metabolites were correspondingly lower. In the Australian cohort, maternal serum levels were higher for some phthalate metabolites, but lower for others compared to those measured in our cohort (Hart et al., 2014). The slight inconsistency between studies demonstrates the considerable difficulties in human populations to unravel associations over a long period of time (Albert and Jégou, 2014). However, the overall pattern suggests an association between maternal phthalate exposure in pregnancy and a subtle, but compensated impairment of Leydig cell function in adult sons.

We conducted a considerable number of statistical tests, which increases the risk of type I error (false positive findings). In addition, we applied multiplicity adjustment to control the type I error but at the cost of disproportionately inflating the risk of type II error (false negative findings). No associations remained significant after adjusting for multiplicity. This has been taken into account in the interpretation of our results.

It should be considered that our study is one of the first in this area. It is based on a scientific hypothesis from animal exposure experiments and in vitro studies and was conducted as stringently as possible. In such a setting, it may not be appropriate to overinterpret results from multiplicity adjustments, as type I errors can self-correct over time when more human long-term cohort studies become available, while type II errors cannot (Rothman, 2014, 1990). We thus wish to stimulate further interest in this field, which we consider important from a human health perspective. In addition, our findings should be carefully evaluated based on human physiology, as hormonal values are interlinked through the negative feedback system within the pituitary-gonadal axis and physical parameters such as penile size or testis volume are consequences of these.

A major strength of our study was the longitudinal prospective setup and exposure assessments based on direct measurements in maternal serum rather than through questionnaires. However, as phthalates are non-persistent chemicals, their half-lives are short, and consequently, individual exposure levels may vary substantially over time (Johns et al., 2015). This imposes a risk of exposure misclassification, in particular when including only one sample per participant as in this study. In our previous study on 128 pregnant women in this mother-child cohort who had repetitive serum samples drawn, tracking during pregnancy was high for MEP, MiNP, and MiDP (ICC > 0.43, i.e., low within-person variability), but lower for MiBP, MnBP, MEHP, 5cx-MEPP, 2cx-MMHP, and cx-MiOP (ICC ≤ 0.12, i.e., high within-person variability) (Assens et al., 2019). Studies on the temporal stability of urinary concentrations have shown varying results, indicating that repeated samples may be necessary to avoid misclassification of exposure to some phthalates such as DEHP, while variability for metabolites of DEP, DnBP and DiBP may be lower (Adibi et al., 2008; Braun et al., 2012; Cantonwine et al., 2014). Additionally, there is a risk of post-collection contamination of biological samples with phthalates from the environment during sampling and/or handling before chemical analysis as serum contains enzymes that can convert diesters to their primary metabolites (Calafat et al., 2013; Johns et al., 2015). Congruently, secondary metabolites of the long-chained phthalates in serum are considered more reliable as these are only produced in vivo (Wittassek et al., 2011). In our recent study, we showed that primary metabolites of the short-chained phthalates (DEP, DiBP, DnBP) and secondary metabolites of the long-chained phthalates (DEHP, DiNP, DiDP) measured in serum seemed to reflect true exposure rather than contamination (Henriksen et al., 2020). Finally, metabolite concentrations are generally lower in serum than in urine which limits the rate of detection in serum (Hauser and Calafat, 2005; Johns et al., 2015). Overall, exposure misclassification tends to infer a risk of bias towards the null, i.e., there is a risk that we may have underestimated associations while we have no reason to suspect false positive findings. Maternal serum concentrations of phthalates in this study were similar to what we have previously reported in our cohort (Assens et al., 2019). Serum phthalate measurements in our cohort of pregnant women were higher than in a more recent Danish pregnancy cohort from 2012 to 2014 (Henriksen et al., 2020) in parallel with a general decline in phthalate exposure in Denmark over the past decade due to regulation (Frederiksen et al., 2019).

Our study population was small, and there is a risk that we may have underestimated true associations due to lack of power. As around 25 % of the invited young men participated in the adult follow-up, there is a risk of participation bias. However, participants did not differ from non-participants on available maternal, birth and postnatal data (Henriksen et al., 2022). Our population was generally comparable to a large group of young Danish men from the general population (Bang et al., 2021; Priskorn et al., 2018). Our participation rate was also similar to that of similar studies on semen quality in young men (Jørgensen et al., 2012). Additionally, serum concentrations of phthalate metabolites did not differ significantly between samples from mothers whose sons participated in the adult follow-up and those whose did not (Supplementary Table 3).

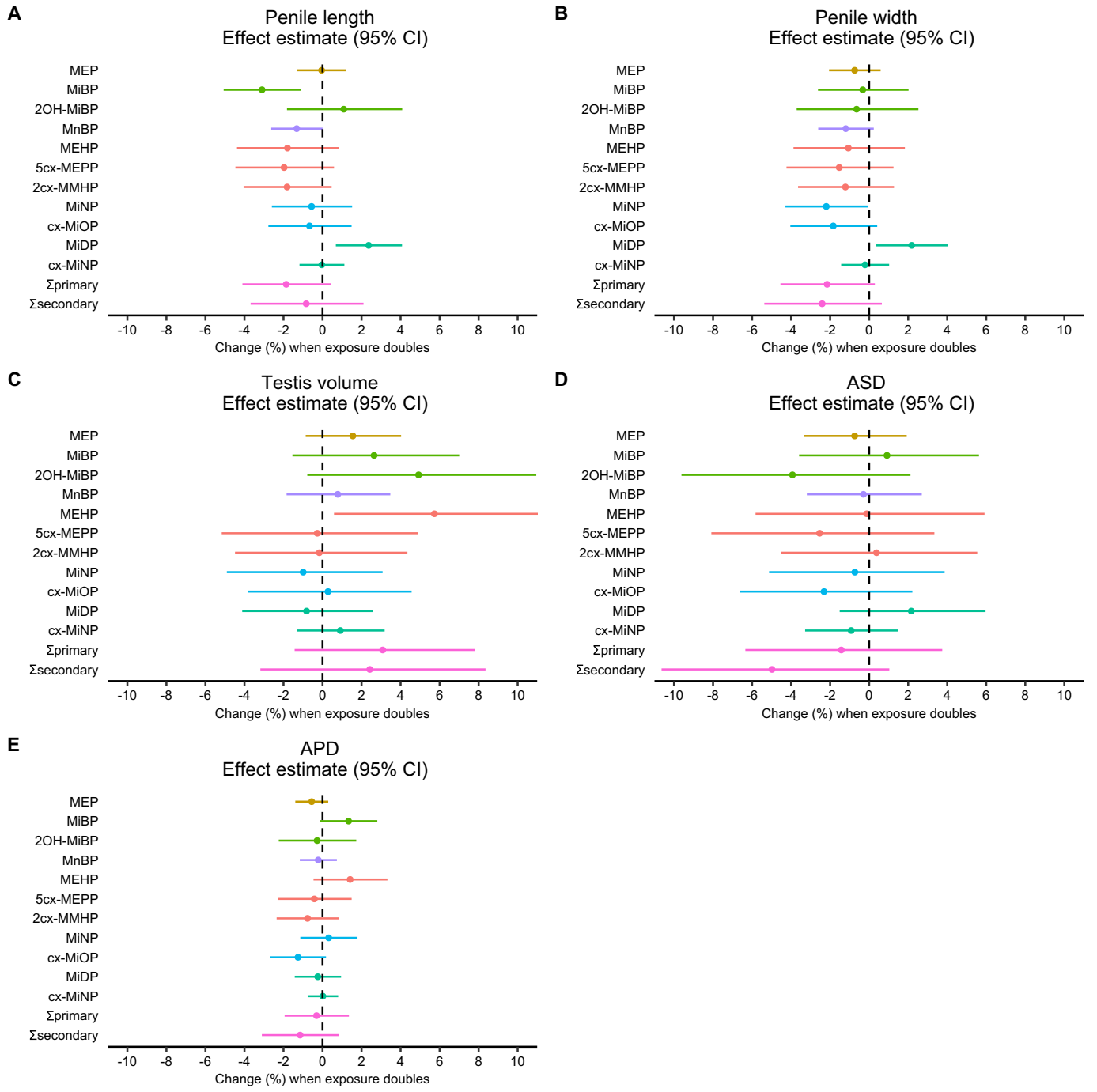


Fig. 4. Maternal serum concentration of phthalate metabolites and penile size, testis volume and anogenital distance in adult sons. The plots show effect estimates from linear regression analyses. Estimates are shown as percent change in outcome when exposure doubles. Abbreviations: ASD, anoscrotal distance; APD, anopenile distance. Abbreviations of phthalate metabolites are presented in Supplementary Table 1. Σprimary is the molar sum of MEP, MiBP, MnBP, MEHP, MiNP and MiDP expressed as MnBP; Σsecondary is the molar sum of 2OH-MiNP, 5cx-MEPP, 2cx-MMHP, cx-MiOP and cx-MiNP expressed as 2OH-MiNP.

We measured phthalates in one sample during pregnancy at a median GA of 22 weeks, but the range was wide. In humans, differentiation of the male gonads occurs during gestational weeks 7–15 (Rajpert-De Meyts, 2006; Skakkebæk et al., 2001), and exposure during this period may thus be most harmful for reproductive development (van den Driesche et al., 2017; Welsh et al., 2008). In support of this, an American study reported an association between higher maternal urine concentrations of DEHP metabolites and shorter AGD when urine samples were collected in the first trimester, but not in the second or third (Martino-Andrade et al., 2016). Thus, for some of our study participants sampling time during pregnancy may have been suboptimal.

In this study, the time passed between exposure and outcomes of interest was around 20 years which induces a high risk of introducing statistical 'noise' and not finding associations. Our study design was planned in 1995–1996 as one of the first human cohort studies to assess intergenerational effects of exposure based on animal studies which suggested that the fetal period may be particularly vulnerable to adverse effects, which may persist in adulthood. The study would have needed a much higher number of participants if all other known pre- and postnatal confounders and other factors influencing reproductive outcomes should be considered. Instead, we prioritized to have a detailed reproductive examination of each participant.

Multiple factors not included in our analyses are likely to have impacted reproductive outcomes in the young men. This includes adult exposure to phthalates or additional EDCs (for reviews see (Radke et al., 2018; Rodprasert et al., 2019)) which was not taken into consideration in this study. However, it was previously shown that concurrent urine concentrations of phthalate metabolites did not influence associations between prenatal exposure and adult outcomes (Axelsson et al., 2015).

We also did not consider simultaneous fetal exposure to other chemicals in this study, or exposure during childhood or adolescence. Animal studies have shown that mixed exposures of several phthalates (Howdeshell et al., 2008) or different chemical groups during fetal life may have additive effects on reproductive outcomes ((Conley et al., 2021), for review see (Howdeshell et al., 2017)). Thus, the observed modest effects in our study may be underestimated.

5. Conclusion

In conclusion, we showed that prenatal exposure to some phthalates was associated with a subtle, but compensated impairment of Leydig cell function in adulthood which supported our original hypothesis. However, none of the tested associations remained significant after adjusting for multiple testing. Intergenerational studies on adult consequences of prenatal exposure in humans are rare and difficult to perform. However, our results should stimulate further research into this area.

CRediT authorship contribution statement

Louise Scheutz Henriksen: Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing, Visualization, Project administration. **Hanne Frederiksen:** Investigation, Resources, Writing – review & editing, Supervision, Project administration. **Niels Jørgensen:** Methodology, Resources, Writing – review & editing, Supervision. **Anders Juul:** Conceptualization, Resources, Writing – review & editing, Funding acquisition. **Niels E. Skakkebæk:** Conceptualization, Writing – review & editing. **Jorma Toppari:** Conceptualization, Writing – review & editing. **Jørgen Holm Petersen:** Formal analysis, Writing – review & editing. **Katharina M. Main:** Conceptualization, Methodology, Validation, Investigation, Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Data availability

Some or all datasets generated and/or analyzed during the current study are not publicly available but are available from the corresponding author on reasonable request.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Niels Jørgensen reports a relationship with Gedeon Richter that includes: speaking and lecture fees. Anders Juul reports a relationship with Novo Nordisk that includes: speaking and lecture fees. Anders Juul reports a relationship with Ferring that includes: speaking and lecture fees. Anders Juul reports a relationship with Ipsen that includes: speaking and lecture fees. Jorma Toppari reports a relationship with Diagenode (Diamyd) that includes: board membership. Katharina M. Main reports a relationship with Novo Nordisk that includes: speaking and lecture fees.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2023.161914.

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