Prenatal paraben exposure and anogenital distance and reproductive hormones during mini-puberty: A study from the Odense Child Cohort

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HIGHLIGHTS

• Parabens are antimicrobial preservatives and suggested to have endocrine disrupting abilities.
• Among 536 Danish pregnant women paraben in urine were low, but 10.4% exceeded the threshold for adverse estrogenic effects.
• Higher maternal paraben exposure was associated with shorter anogenital distance (AGD) in boys and longer AGD in girls.
• Sex hormone concentrations were lower in girls with high prenatal paraben exposure.
• Parabens may affect humans at vulnerable time periods during development.

ABSTRACT

Background: Parabens are added to personal care products as antimicrobial preservatives. They have been suggested to have endocrine disrupting abilities. Prenatal exposure to parabens has been associated with reproductive endpoints including reduced male anogenital distance (AGD, distance from anus to genitals), which is sensitive to prenatal anti-androgenic exposure.

Objectives: To study the associations between maternal paraben concentrations in second trimester urine and AGD and reproductive hormone concentrations at 3 months of age in offspring.

Methods: Pregnant women from Odense, Denmark were included in early pregnancy from 2010 to 12, and their children are being followed up. Fasting spot urine samples from 536 pregnant women were analyzed for methylparaben (MeP), ethyl-paraben (EtP), iso-propylparaben (i-PrP), n-propylparaben (n-PrP), n-butylparaben (n-BuP) and benzylparaben (BzP) by liquid chromatography tandem mass spectrometry and thereafter osmolarity adjusted. Three months after expected date of birth, AGD was measured in 452 children, and serum concentrations of follicle stimulating hormone (FSH), luteinizing (LH), testosterone, dehydroepiandrosterone-sulphate (DHEAS), androstenedione and 17-hydroxyprogesterone (17-OHP) were measured in 198 children. Maternal paraben exposure was categorized into tertiles or below and above level.
of detection, and sex-stratified multiple linear regression analyses were performed with AGD or reproductive hormones as outcomes. Results: Most pregnant women had low concentrations of parabens in urine, but 10% exceeded the threshold for adverse estrogenic effects. Higher maternal paraben exposure was associated with shorter AGD in male offspring and longer AGD in girls, although only significant for MeP in boys. In addition, FSH, LH, DHEAS, 17-OHP concentrations were lower in girls with high prenatal paraben exposure, whereas no consistent pattern was found in boys. Discussion: The endocrine disrupting abilities of parabens may affect humans at vulnerable time periods during development, which may have long term impact on reproductive function. This is the first study to find these associations in girls and our findings need confirmation.

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1. Introduction

Parabens constitute a group of alkyl esters of p-hydroxybenzoic acid that are commonly added to personal care products, cosmetics, pharmaceuticals, beverage and food processed as antimicrobial preservatives (Sanchis et al., 2020). Widespread human exposure to parabens occurs through dermal contact, inhalation or ingestion. Biomonitoring studies have found that paraben exposure is common among different populations, with detectable concentrations in the urine of most of the people tested (CDC, 2019; Frederiksen et al., 2014; Moos et al., 2015). Parabens have previously been thought to have relatively low toxicity (Golden et al., 2005), but several recent animal studies have shown that parabens have multiple endocrine disrupting effects such as both estrogenic and anti-androgenic effects (Boberg et al., 2010; Boberg et al., 2016; Chen et al., 2007; Darbre and Harvey, 2008; Taxvig et al., 2008; Vo et al., 2010). Most evident were the effects of butylparaben in a recent study, where prenatal exposure of time-mated Wistar rats led to several adverse effects on endocrine-sensitive endpoints in the offspring; the anogenital distance of newborn male and female offspring was significantly reduced. In prepubertal females, ovary weights were reduced, and mammary gland outgrowth was increased, while in male offspring, sperm count was significantly reduced, testicular CYP19a1 aromatase and Nr5a1 expression were reduced in respectively prepubertal and adult testes. Finally, prostate histology was altered at prepuberty and adult prostate weight was reduced (Boberg et al., 2016).

Parabens cross the placental barrier, thus exposing the fetus (Towers et al., 2015) and accumulate in rat fetus (Frederiksen et al., 2008). Fetal life represents a vulnerable window for environmental influence on fetal development, including programming of the endocrine system (Skakkebaek et al., 2016). The anogenital distance (AGD; distance from anus to genitals) is sex-dimorphic in mammals, including humans (Thankamony et al., 2016). In rodents, AGD has been shown to reflect the amount of androgens to which a male fetus is exposed in early development; thus, males have longer AGD than females, and higher in utero androgen exposure results in longer AGD (Thankamony et al., 2016). Numerous both human and rodent studies have shown that prenatal exposure to anti-androgens e.g. phthalates shortens male AGD (Radke et al., 2018). To our knowledge only two human studies have addressed the association between in utero exposure to parabens and AGD in male offspring. A Japanese study among 111 boys found no association between gestational paraben and AGD (Shirai et al., 2013), whereas maternal serum n-propylparaben (n-PrP) was associated with shorter AGD in 334 boys from birth to 24 months of age in an English study (Fisher et al., 2020).

As the infant clears the placental hormones during the early postnatal days, the hypothalamic-pituitary-gonadal (HPG)-axis is briefly activated during the first six months of postnatal life, a period which has been referred to as “mini-puberty”, after which it remains dormant until (pre)puberty (Kuiri-Hanninen et al., 2014). Accordingly, increased concentrations of sex hormones are found in neonatal life during mini-puberty (Kuiri-Hanninen et al., 2014; Lanciotti et al., 2018). The long-term impact of these changes in hormones during this sensitive window has to our knowledge not been addressed, and no studies have investigated the effects of prenatal paraben exposure on reproductive hormone concentrations in mini-puberty. In the Odense Child Cohort, we therefore aimed to examine the association between paraben concentrations in maternal urine in second trimester and AGD and reproductive hormone concentrations in the offspring at three months of age.

2. Methods

2.1. Study settings and design

The study was based on data from the Odense Child Cohort (Kyhl et al., 2015). Briefly, newly pregnant women residing in Odense, Denmark, between 2010 and 2012 were recruited at Odense University Hospital at gestational age (GA) 8–16 weeks. Odense University Hospital is the only hospital in the municipality. Initially, 2874 were included, currently 2530 children are being followed up. Participants were better educated and more often of Danish origin than non-participants (Kyhl et al., 2015). Information regarding maternal education and health status, including smoking and body mass index (BMI) before pregnancy was obtained through questionnaires filled in during pregnancy. Data on birth characteristics was extracted from obstetric and pediatric hospital records. Last menstrual period was used to calculate the GA of all participants.

2.2. Paraben measurements

Fasting spot urine samples were collected at approximately GA 28 weeks (median 28.7, range 26.4–30.4 weeks) before 09.30 h for 1207 women and stored in freezers at the Open Patient data Explorative Network (OPEN). Samples were stored at −80 °C until chemical analyses. Paraben concentrations were due to financial constrains measured in a random subset of 536 women previously published in (Frederiksen et al., 2014; Tefre de Renzy-Martin et al., 2013). Urine samples were analyzed for the total (the sum of free and conjugated) content of methylparaben (MeP), ethylparaben (EtP), iso-propylparaben (i-PrP), n-PrP, n-butylparaben (n-BuP) and benzylparaben (BzP) by isotope diluted liquid chromatography tandem mass spectrometry (LC-MS/MS) with preceding enzymatic deconjugation followed by solid phase extraction as previously described (Frederiksen et al., 2011). In order to separate i-PrP from n-PrP, and n-BuP from possible content of iso-butylparaben, a longer solvent gradient for measurement by LC-MS/MS was used as previously described (Frederiksen et al., 2014). In brief samples were analyzed in 17 batches all including standards for calibration curves, two blanks, two un-spiked urine pool controls and two times two urine pool controls spiked with a mixture of native paraben standards in low (QC_low) and high (QC_high) concentration levels. The mean recovery was >95% for all parabens in both QC_low and QC_high. The relative standard deviations (RSD) ranged from 8 to 20% for QC_low and from 7 to 10% for QC_high.
2.3. Osmolality adjustment

To account for urinary dilution, all measured paraben concentrations were adjusted for osmolality. In contrast to urinary creatinine adjustment, urine osmolality is directly related to the number of particles in solution and is unaffected by the molecular weight and size of these particles (Middleton et al., 2016). Osmolality was measured by freezing point depression method with automatic cryoscopic osmometer (Osmomat® 030 from Gonotec, Berlin, Germany). All paraben concentrations were subsequently adjusted for osmolality by dividing the individual urine sample concentration with the sample osmolality and normalize by multiplying with the median osmolality (0.63 osm/kg) for all samples. Urinary paraben concentrations below the limit of detection (LOD) were not adjusted for osmolality but substituted by the paraben specific LOD divided by the square root of 2. The number of participants with concentrations below the LOD is shown in Table S1.

2.4. Estimated of daily intake, hazard quotient and hazard index

The daily intake (DI) (μg/kg/day) of each paraben was estimated based on osmolality-adjusted urinary excretion of parabens or summed parabens as follows:

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DI = \frac{U_{\text{concentration of the paraben}}}{U_{\text{volume}} \times FUE \times BW}\]

where \(U_{\text{osmol}}\) is the osmolality-adjusted urinary concentration of parabens and \(U_{\text{volume}}\) is the median diuresis per 24 h = 1.77 L/day (range = 0.253–4.51 L/day). The median diuresis per 24 h in pregnant women was based on a collection of 24h urine samples from 604 participants in this cohort. FUE is the estimated fraction of parabens excreted in urine, and BW is the individual body weight of each participant (self-reported weight before pregnancy). The following values of FUE's were based on a human kinetic study of adults after oral intake of deuterium-labelled parabens: 17.4% of MeP and 5.6% of n-ButP were excreted as respectively MeP or n-ButP and conjugated metabolites of these parabens (Moos et al., 2016). Based on the kinetics of MeP and n-ButP, Moos et al. (Moos et al., 2017) also extrapolated FUE’s for EtP (13.1%), i-PrP (10.2%) and n-PrP (9.7%). No FUE was estimated for BzP, so the FUE for n-ButP (5.6%) was used.

To derive individual hazard quotient (HQ)’s for each of the parabens, the estimated DIs were divided with their corresponding acceptable DI (ADI) or tolerable DI (TDI). For this approach a grouped ADI of 10 mg/kg/day (no-observed-adverse-effect levels (NOAELs) of 1000 mg/kg/day with an uncertainty factor of 100) for the sum of MeP and EtP was established based on adverse estrogenic effects by the European Food Safety Authorities ((EFSA), 2004; Oishi, 2004). No official ADI or TDI values are until now established for the other parabens, so for i-PrP, n-PrP and n-ButP, we used 20 μg/kg/day (Scientific Committee on Consumer Safety, 2013) as an approximate value for TDI as previously described (Moos et al., 2016). This approximate TDI value was derived from a conservative no-observed-effect level (NOEL) with an uncertainty factor of 100 for n-ButP based on reproductive toxicity studies in rodents (Fisher et al., 1999).

For the parabens with potential effects on the estrogenic activity, HQ estimates were combined to derive a single estimated Hazard Index (HI) for each participant, where HI = 1 is the threshold for effects on the reproductive system and a HI > 1 indicates a potential risk of effects on the reproductive system.

2.5. AGD measurements

Three months after expected date of birth (median age 3.3 months, range 2.1–6.7 months) the children were invited to a clinical examination including measurements of length, weight and AGD (Jensen et al., 2016). In 883 boys, a short AGD was measured with a Vernier caliper from the center of the anus to the posterior base of the scrotum (AGDas), and a long AGD was measured from the center of the anus to the cephalad insertion of the penis (AGDap). Penile width was also measured. Correspondingly, in 705 girls a short AGD was measured from the center of the anus to the posterior fourchette (AGDaf), and a long AGD from the center of the anus to the top of the clitoris (AGDac). The genital measures were performed blinded to maternal paraben exposure levels and repeated three times in each child without repositioning the child, and an arithmetic mean was calculated. All four technicians attended training sessions and supervision of AGD measuring and measurements for the first 46 girls were excluded due to low accuracy. Between-examiner variation in this study was also calculated based on measurements conducted with the child in the same position (Priskorn et al., 2018). The coefficients of variation (CV’s) for AGD were below 10% (i.e. 3% for AGDas, 2% for AGDap, 4% for AGDaf and 3% for AGDac) for all subsequent tripartite AGD measurements, except for AGDaf, in which two girls had CVs of 10% and 14%, respectively.

2.6. Hormone analyses

Non-fasting blood samples were drawn from an antecubital vein from 526 infants at the three-month clinical examination of whom 198 had parabens measured (Fig. 1). Subsequently, blood samples were clotted and centrifuged, and serum was stored at −20 °C for a maximum of five years before analysis. Serum concentrations of follicle stimulating hormone (FSH) and luteinizing hormone (LH) were analyzed with solid-phase, two-site chemiluminescent immunometric assay (Immulite®, Siemens Healthcare Diagnostic, Marburg, Germany) at The University Hospital of Southern Denmark, Esbjerg, Denmark. Serum concentrations of testosterone, dehydroepiandrosterone-sulphate (DHEAS), androstenedione (adione) and 17-hydroxyprogesterone (17-OHP) were analyzed by LC-MS/MS at the Chemical Laboratory at Department of Growth and Reproduction, Rigshospitalet, University of Copenhagen, Denmark (Soeborg et al., 2013). The inter-assay CV’s were 5% for DHEAS, 8% for androstenedione, 9% for 17-OHP, 9% for testosterone, 7% for LH, and 6% for FSH. Additionally, the testosterone/LH ratio and the FSH/LH ratio were calculated for boys. The hormone-specific limits of quantification (LOQ’s) and the percentages of samples below LOQ in girls and boys are presented in Table S4. Serum concentrations of reproductive hormones were measurable in most children although testosterone was measurable in 14% of the girls, and 42% of the girls had LH at LOQ (Table S2).

2.7. Ethical approval

The study was performed in accordance with the Helsinki Declaration II and approved by the Regional Scientific Ethical Review Committee for Southern Denmark (ProjectID S-0090130) and the Danish Data Protection Agency (J.No.18/33119). All participants received written and oral information and provided their written consent for participation in the study.

2.8. Statistical methods

The osmolality-adjusted parabens [ng/mL(osm)], MeP and n-PrP were divided into tertiles due to non-normal distributions. Respectively 50% and 67% of samples were below LOD for EtP and n-ButP, these were divided into two groups as concentration below and above LOD. Iso-PrP and BzP was only measured in 5 and 9%, respectively, of samples and therefore not included in further analysis. Measurements of AGD and penile width were left untransformed due to acceptable normal distributions. Differences in distributions of paraben concentrations (medians and 25–75 percentiles) according to population characteristics were assessed by the Mann-Whitney U test.

Multivariable linear regression analysis was used to analyze the associations between maternal second trimester urinary paraben
excretion dichotomized or in tertiles and the measurements of AGD and penile width in the offspring. AGD measurements vary with age and weight of the child (Swan, 2008), and because the clinical examination was scheduled to take place three months after expected date of birth, we constructed a measure of postconceptional age (in days) defined as the sum of gestational age at birth (days) and the age of the child at the AGD measurement (days). Analyses of associations between urinary paraben concentration and measurements of AGD and penile width were thus adjusted for the postconceptional age, and the individual weight-for-age standard deviation score (z-score). The latter was calculated using the total population data from the Odense Child cohort as a reference (Jensen et al., 2016). We additionally adjusted for maternal smoking, BMI, parity and education which did not change the directions of the findings, so due to small sample size these were not included in final analyses. Breastfeeding was considered both a confounder and an effect modifier but did not modify the associations significantly. As prenatal paraben exposure was associated with lower z-score for weight at the three months examination, we performed the analyses without this adjustment, as slow growth may be a mediator of the association between paraben exposure and AGD. We tested trends across tertiles of maternal paraben exposure by inserting ordinal categorical variable coded using integer values (1, 2, 3) in the regression.

Serum concentrations of LH, DHEAS, androstenedione and 17-OHP, as well as the LH/FSH and testosterone/LH ratios were transformed by use of the natural logarithm, the FSH-measurements were transformed by cubic roots, and the testosterone-measurements were left untransformed in order to normalize residuals. In respectively 86% and 42% of girls, testosterone and LH concentrations were below LOQ. Testosterone was not analyzed further in girls, and a binary variable with the LH-concentration at LOQ and above LOQ was conducted (Table S4). DHEAS and adione concentrations below LOQ were replaced by LOQ/square root 2. Multiple linear regression analysis was then used to analyze the associations between maternal urinary paraben concentration dichotomized or in tertiles and reproductive hormone concentrations in the offspring during mini-puberty. Ln-transformed concentrations of reproductive hormones were back-transformed to produce the percentual change and if below zero estimate minus one. The association between maternal urinary paraben concentrations and serum concentrations of LH in girls was analyzed by multiple logistic regression. We adjusted for postconceptional age.

We evaluated the fit of the regression models by inspecting the residual plots for model assumption of homogeneity of variances. SPSS statistics v.21 was used and the results are presented with 95% confidence intervals (CIs); p-values < 0.05 were considered statistically significant.

3. Results

A total of 536 women had available paraben measurement; 452 mother-child pairs had available maternal urinary paraben concentrations and infant AGD measurements, whereas 198 out of the 526 with available infant serum reproductive hormone concentrations also had available maternal parabens (Fig. 1). The participants did not differ from the original cohort, although more were higher educated and
LOD: limit of detection of non-adjusted urinary concentration.

### Table 1
Osmolality-adjusted urinary-excreted concentrations of parabens (ng/ml) measured in 536 pregnant Danish women in gestational week 28.

| Parabens | LOD % > LOD | Mean | Percentiles | Maximum |
|----------|-------------|------|-------------|---------|
| MeP      | 0.26        | 85.8 | 79.7        | 2.8     |
| EiP      | 0.40        | 40.6 | 7.0         | <LOD    |
| i-PrP    | 0.18        | 5.2  | 0.4         | <LOD    |
| n-PrP    | 0.18        | 69.4 | 22.8        | <LOD    |
| n-BuP    | 0.07        | 32.6 | 2.5         | <LOD    |
| ∑(n-PrP + n-BuP) | 0.18 | 9.1  | 0.05        | <LOD    |
| ∑(PrP + BuP) | 25.6 | 6.6  | <LOD        | 4.2     |
| ∑parabens | 127.6 | 17.8 | 3.4         | 131.7   |

LOD: limit of detection of non-adjusted urinary concentration.

### Table 2
Median (25–75 percentile) maternal osmolality-adjusted urinary paraben concentrations (μmol/ml) according to maternal and child characteristics in 452 mother-child pairs.

| Maternal and child characteristics | N (%) | Osm MeP (ng/ml) | Osm nPrP (ng/ml) |
|-----------------------------------|-------|----------------|------------------|
| Maternal age (years)              |       |                |                  |
| <25                               | 39 (9)| 4.0 (1.2;18.8) | 0.9 (LOD;7.4)    |
| 25–34                             | 325 (72)| 11.1 (2.6;56.7)| 2.3 (LOD;14.9)  |
| >34                               | 88 (19)| 24.9 (5.2;93.4)| 2.3 (LOD;263)   |
| Pre-pregnancy BMI (kg/m²)         |       |                |                  |
| <20                               | 44 (10)| 6.6 (1.2;38.6)| 1.3 (LOD;7.3)   |
| 20–25                             | 241 (53)| 10.8 (2.4;56.4)| 18 (LOD;169)   |
| >25                               | 167 (37)| 17.8 (4.3;88.2)| 3.0 (LOD;163)  |
| Maternal education                |       |                |                  |
| High school or less               | 130 (29)| 14.4 (3.2;88.2)| 2.6 (LOD;171)  |
| High school + 1–4 years           | 225 (50)| 11.5 (2.9;65.8)| 2.3 (LOD;144)  |
| High school + > 4 years           | 95 (21)| 10.2 (2.1;57.8)| 1.2 (LOD;195)  |
| Parity                            |       |                |                  |
| Nulliparous                       | 249 (55)| 12.2 (3.8;64.6)| 2.4 (LOD;171)  |
| Multiparous                       | 203 (45)| 11.2 (2.6;55.7)| 1.9 (LOD;136)  |
| Maternal smoking during pregnancy |       |                |                  |
| No                                | 434 (96)| 11.8 (2.7;69.5)| 2.0 (LOD;154)  |
| Yes                               | 18 (4)| 30.8 (2.9;143.0)| 3.9 (LOD;378)  |
| Gestational days at birth         |       |                |                  |
| <281                              | 214 (48)| 10.4 (2.7;59.0)| 2.0 (LOD;146)  |
| ≥281                              | 236 (52)| 12.3 (2.7;76.7)| 2.2 (LOD;176)  |
| Birth weight (gram)               |       |                |                  |
| <3000                             | 65 (14)| 12.6 (2.8;90.7)| 3.7 (LOD;192)  |
| 3000–3999                         | 303 (67)| 11.5 (2.5;67.2)| 1.9 (LOD;119)  |
| ≥4000                             | 84 (19)| 13.3 (3.5;81.7)| 3.0 (LOD;251)  |
| Child sex                         |       |                |                  |
| Boy                               | 263 (58)| 11.9 (3.2;77.4)| 2.1 (LOD;195)  |
| Girl                              | 189 (42)| 11.9 (3.2;58.3)| 2.0 (LOD;130)  |
| Child Z-score for weight at 3-months examination | | | |
| <1                                | 71 (16)| 23.1 (2.6;70.7)| 5.2 (0.4;27.6)  |
| =1                                | 312 (69)| 10.4 (2.5;60.7)| 1.8 (LOD;116)  |
| >1                                | 68 (15)| 10.0 (2.7;43.1)| 2.0 (LOD;198)  |
| Child age at examination by 3-months examination + gestational age (days) | | | |
| <371                              | 135 (30)| 13.1 (2.2;87.5)| 3.3 (LOD;190)  |
| 371–399                           | 258 (57)| 12.3 (3.1;73.3)| 2.2 (LOD;140)  |
| ≥400                              | 58 (13)| 8.6 (2.5;46.8)| 1.1 (LOD;217)  |
| Breastfeeding                     |       |                |                  |
| Yes                               | 134 (33)| 12.3 (2.4;75.4)| 3.2 (LOD;220)  |
| No                                | 272 (67)| 11.1 (2.7;63.9)| 1.8 (LOD;142)  |

Osm: osmolality.
BMI: body-mass index.
LOD: limit of detection.
*p < 0.05 Mann-Whitney U test.

### Table 3
Estimated daily intake (DI) of parabens (μg/kg/day) in women (n = 531) participating in this study.

| Parabens | 50p (μg/kg/day) | 90p (μg/kg/day) | 95p (μg/kg/day) | Max (μg/kg/day) |
|----------|-----------------|-----------------|-----------------|----------------|
| MeP      | 1.83            | 26.4            | 56.5            | 273            |
| EtP      | 0.00            | 2.55            | 6.20            | 104            |
| i-PrP    | 0.00            | 0.00            | 0.10            | 33.1           |
| n-PrP    | 0.00            | 0.00            | 0.00            | 31.8           |
| n-BuP    | 0.00            | 2.15            | 5.69            | 35.1           |

For this estimation of DL 531 women had stated their body weight before pregnancy and a median value of 1.77 L/day were used for the calculation.

fewer smoked. The different parabens were detected in 5% to 86% of the urine samples (Table 1: osmolality-adjusted concentrations and Table S1: non-osmolality-adjusted concentrations), most below LOD for i-PrP and most above for MeP. MeP was observed in highest concentration (median 11.2 ng/mL) followed by n-PrP (Table 1). MeP were higher in older women, in women with high or low BMI, and in women who smoked. In addition, children with high prenatal paraben exposure had lower z-score for weight at examination (Table 2).

### 3.1. Estimated daily exposure of parabens

The estimated daily exposure were calculated for MeP, EtP, i-PrP, n-PrP and n-BuP based on urine excretion fractions of each of the parabens and a median value for urine excretion per day in participating women (Table 3). The median estimated exposure for MeP was 1.8 μg/kg/day followed by n-PrP (0.6 μg/kg/day). Characteristic for these estimated exposures were the large variation between median exposure levels and the highest exposed part of the women, where the 95th percentiles for MeP and n-PrP were about 30 and 50 times higher, respectively. HQ's for the parabens with established adverse daily intakes (ADI) or derived tolerable daily intakes (TDI) were calculated (Table 4) and based on similar potential endpoints, the individual HQ's were combined to a corresponding HI, where 10.4% of the women had a HI > 1 and thereby exceeding the threshold for adverse estrogenic effects. The maximum HI was 14 times higher than the threshold for potential adverse estrogenic effects.

### 3.2. AGD

Mean AGDas, AGDap, penile width, AGDaf and AGDac were respectively 36.7 (SD 5.5), 70.6 (6.5), 13.8 (1.2), 20.1 (3.6) and 37.8 (4.5) mm. Correlation coefficients between the different AGD measures were respectively r = 0.65 in boys and r = 0.59 in girls (p < 0.01).

AGD measures were not significantly associated to maternal characteristics but strongly associated to z-score for weight and age at the three-month examination (data not shown). There was a trend of shorter AGDas with higher prenatal paraben exposure, although only significant for MeP (Table 5). Boys in the highest tertile of MeP had −1.93 (95% CI −3.58; −0.29) mm shorter AGDas in the unadjusted analyses. The association was attenuated after adjustment for...
postconceptional age and weight z-score, mostly because paraben exposure was associated with lower weight z-score at examination. No clear association with AGDap was found, most associations were in fact positive. Women with EtP exposure above median gave birth to boys with −0.31 (95% CI −0.59; −0.03) mm narrower penile width, and generally paraben exposure in was associated with narrower penile width although not significant (Table 5). In girls higher in utero exposure to n-PrP was associated with a tendency to longer AGDas in a dose-response manner (p-trend = 0.10). No clear pattern between maternal paraben exposure and AGDap was found.

### 3.3. Reproductive hormones

Infant reproductive hormone concentrations (Table 6) did not differ according to maternal characteristics but were highest among children examined at age three to four months of age (data not shown [Jensen et al., 2020]). Generally, girls with high prenatal exposure to parabens had lower sex hormones at 3 months of age (Table 7). Girls born to mothers in the highest tertile of MeP had −0.15 (−0.31; 0.00) lower FSH (cubit root transformed). Matrial n-PrP exposure was associated with lower FSH, DHEAS and 17-OHP concentrations in a dose dependent manner (p-trend from 0.08 to 0.04, Table 7) after adjustment for postconceptional age. No consistent pattern between maternal paraben exposure and reproductive hormone concentrations in boys were found, although boys born to mothers with the highest tertile of n-PrP exposure had significantly higher DHEAS concentrations (Table S2).

### 4. Discussion

In these young women, paraben exposure was relatively low, but nevertheless 10% exceeded the threshold for adverse estrogenic effects. We found a tendency to shorter AGDas, narrower penile width in boys and longer AGDas in girls at 3 months of age exposed to parabens in utero. Maternal exposure to parabens was associated with lower serum concentrations of FSH, LH, DHEAS and 17-OHP in girls at three months of age, whereas no similar pattern was found in boys. Interestingly, prenatal paraben exposure was associated with lower weight at three months of age which could not be explained by breastfeeding, but is in accordance with previous studies, suggesting that fetal and infant growth may be affected (Leppert et al., 2020; Wu et al., 2019; Zhong et al., 2020).

Pregnant women in this study had rather low median urinary concentrations of parabens compared to other human biomonitoring studies conducted in the same period e.g. the concentration of MeP and n-PrP in this study were about five times lower than median levels in German women (Moos et al., 2015) and about seven times lower than American women from the NHANES studies (CDC, 2019). However, previous populations did not include pregnant women. On the other hand, we observed a larger range between the median and the

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**Table 5** Changes in AGD (mm) and 95% confidence intervals in unadjusted and adjusted**a** linear regression models (β-coefficients) according to tertiles of osmolality-adjusted paraben concentrations in maternal urine.

| Osmolality-adjusted parabens | Boys N = 263 | | | Girls N = 189 | |
|-----------------------------|-------------|-------------|-------------|-------------|
| | AGDas (mm) | AGDap (mm) | Penile width | AGDas (mm) | AGDap (mm) |
| Unadjusted MeP | | | | | |
| 1th tertile (<4.7) | Reference | Reference | Reference | Reference | Reference |
| 2th tertile (4.7-39.0) | −0.73 (−2.37;0.91) | −0.25 (−2.19;1.70) | 0.16 (−0.19;0.51) | Reference | Reference |
| 3th tertile (>39.0) | −1.93 (−3.58;−0.29) | −0.78 (−2.74;1.18) | −0.24 (−0.59;0.11) | Reference | Reference |
| p-trend**b** | 0.02 | 0.43 | 0.18 | 0.97 (−0.29;2.24) | 0.96 (−0.62;2.53) |
| Adjusted MeP**a** | | | | | |
| 1th tertile (<4.7) | Reference | Reference | Reference | Reference | Reference |
| 2th tertile (4.7-39.0) | −0.66 (−2.25;0.94) | −0.06 (−1.71;1.60) | 0.16 (−0.18;0.51) | Reference | Reference |
| 3th tertile (>39.0) | −1.31(−2.94;0.32) | 0.84 (−0.86;2.54) | −0.15 (−0.50;0.20) | 0.09 (−0.23;2.20) | 0.36 (−0.84;1.57) |
| p-trend**b** | 0.10 | 0.33 | 0.40 | 0.53 | 0.64 |
| Unadjusted EtP | | | | | |
| LOD Above LOD | −1.16 (−2.25;0.16) | −0.60 (−2.19;0.98) | −0.31 (−0.59;−0.03) | −1.13 (−2.16;−0.10) | −0.73 (−2.01;0.56) |
| p-trend**b** | 0.37 | 0.24 | 0.28 | 0.02 | 0.16 |
| Adjusted EtP**a** | | | | | |
| LOD Above LOD | −0.65 (−1.98;0.68) | 0.85 (−0.53;2.23) | −0.26 (−0.55;0.03) | −0.89 (−1.88;0.11) | −0.33 (−1.56;0.90) |
| Unadjusted nPrP | | | | | |
| 1th tertile (<0.29) | Reference | Reference | Reference | Reference | Reference |
| 2th tertile (0.29-7.7) | −0.11 (−1.77;1.55) | 0.17 (−1.77;2.11) | −0.07 (−0.42;0.28) | Reference | Reference |
| p-trend**b** | 0.42 | 0.58 | 0.86 | 0.06 (−1.20;1.31) | 0.92 (−0.65;2.49) |
| Adjusted nPrP**a** | | | | | |
| 1th tertile (<0.29) | Reference | Reference | Reference | Reference | Reference |
| 2th tertile (0.29-7.7) | 0.06 (−1.55;1.67) | 0.50 (−1.15;2.14) | −0.03 (−0.37;0.32) | 0.25 (−0.96;1.45) | 0.68 (−0.80;2.16) |
| 3th tertile (>7.7) | −0.17 (−1.78;1.43) | 1.60 (−0.06;3.26) | 0.06 (−0.29;0.40) | 0.11 (−1.10;1.33) | 1.23 (0.25;2.72) |
| p-trend**b** | 0.83 | 0.06 | 0.75 | 0.84 | 0.10 |
| Unadjusted BuP | | | | | |
| LOD Above LOD | −0.51 (−1.95;0.93) | 0.02 (−1.68;1.73) | −0.07 (−0.37;0.24) | −0.93 (−2.05;0.20) | −0.15 (−1.55;1.25) |
| Adjusted BuP**a** | | | | | |
| LOD Above LOD | −0.19 (−1.58;1.21) | 0.83 (−0.63;2.28) | −0.03 (−0.33;0.27) | −0.68 (−1.77;0.39) | 0.22 (−1.11;1.55) |

**a** Adjusted for the postconceptional age (defined as the sum of gestational age at birth and the age of the child at the AGD measurements) and individual weight for age standard deviation score (z-score).

**b** p-Values for trend across tertiles of paraben exposure inserting ordinal categorical variable.
10% highest exposed women compared to the other studies. It is of concern that 10% pregnant women in this study exceeded the threshold for potential estrogenic effects. However, it should be taken into account that these estimates were based on kinetic studies including intake of the respective parabens (Moos et al., 2017) instead of dermal uptake which might be an even more common pathway for paraben exposure. Furthermore, for the individual HQ’s for i-PrP, n-PrP and n-BuP and the accumulated HI were based on an approximated value for TDI derived from a conservative estimate of a NOEL as previously described (Fisher et al., 2019; Moos et al., 2016; Scientific Committee on Consumer Safety, 2013).

To our knowledge only two human studies have investigated impact of prenatal paraben exposure on AGD and penile length in boys (Fisher et al., 2020; Shirai et al., 2013). A Japanese study among 111 boys found no association between maternal urine paraben concentration measured throughout pregnancy and AGD, however, AGD was measured by 23 different examiners (Shirai et al., 2013). The Cambridge nested case-control study included mothers of 237 boys in whom parabens in serum at gestational age 10–17 weeks and AGD and penile length at age 0, 3, 12, 18, and 24 months (Fisher et al., 2020) were measured. Higher maternal serum n-PrP concentration was associated with shorter AGD as from birth to 24 months of age (Fisher et al., 2020) after adjustment for weight for age, whereas no clear association was found with penile length, which is in accordance to our findings. The exposure levels cannot be compared to ours, as they measured parabens in serum, which is not the optimal matrix due to the much lower concentrations in serum compared to urine, however parabens measured in serum and urine correlate positively (Frederiksen et al., 2011). Few studies have addressed adverse effects of paraben exposure in females. A cross-sectional study found no association between paraben exposure and age at menarche (Buttke et al., 2012), but earlier pubertal development in Latin children prenatally exposed to propylparaben has been reported (Harley et al., 2019).

Our findings are in accordance with animal studies, in which in utero exposure to especially long-branched parabens such as n-PrP and n-BuP affected male reproductive function e.g. AGD, sperm count and motility and sex hormones (Böberg et al., 2016; Kang et al., 2002; Oishi, 2002; Taxvig et al., 2008; Zhang et al., 2014). This suggests that prenatal paraben exposure may show lasting anti-androgenic effects in male humans which is confirmed by in vitro studies (Chen et al., 2007; Darbre and Harvey, 2008; Gomez et al., 2005; Ozdemir et al., 2018; Prusakiewicz et al., 2007; Taxvig et al., 2008; Vo et al., 2010). In addition, shorter AGD as from birth to 24 months of age (Fisher et al., 2020) after adjustment for weight for age, whereas no clear association was found with penile length, which is in accordance to our findings. The exposure levels cannot be compared to ours, as they measured parabens in serum, which is not the optimal matrix due to the much lower concentrations in serum compared to urine, however parabens measured in serum and urine correlate positively (Frederiksen et al., 2011). Few studies have addressed adverse effects of paraben exposure in females. A cross-sectional study found no association between paraben exposure and age at menarche (Buttke et al., 2012), but earlier pubertal development in Latin children prenatally exposed to propylparaben has been reported (Harley et al., 2019).

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parabens are aromatase inhibitors (van Meeuwen et al., 2008), which may result in higher androgen levels in girls and thereby a longer AGD. Accordingly, the European Chemicals Agency, has recognized BuP as having endocrine disrupting properties.

To our knowledge no studies have addressed the impact of maternal paraben exposure on sex hormone concentrations in three months old infants during the so called mini-puberty (Kuiri-Hanninen et al., 2014; Lanciotti et al., 2018). We previously found that maternal exposure to other endocrine disrupting chemicals e.g. phthalates and perfluoroalkyl substances were associated with respectively lower testosterone/LH ratio in male offspring (a measure of the Leydig cell function; a decreased T/LH ratio suggests that boys need of higher LH to maintain normal T), and a higher DHEAS in girls at three months of age in the same cohort (Jensen et al., 2020; Muerkötter et al., 2020), suggesting that these chemicals may affect mini-puberty. Interestingly, we found lower concentrations of reproductive hormones in girls with higher prenatal exposure to parabens, whereas no associations were found in boys. We can only speculate on the mechanisms behind these findings. Infant reproductive hormone levels during mini-puberty could possibly be affected by prenatal exposure if the setting of the pituitary-gonadal axis is programmed in utero, but also current exposures interfering with the hypothalamic-pituitary-gonadal hormone axis may play a role. Parabens have multiple endocrine disrupting effects. They are weak estrogens and at the same time inhibit the metabolism of endogenous estrogens (Boberg et al., 2016), which in turn deactivates the HPG-axis leading to lower FSH, LH and steroid concentrations. An in vitro-study suggested that butylparaben may interfere with the transport of cholesterol to the mitochondrion, thereby interfering with steroidogenesis and reducing LH and thereby DHEAS concentrations (Boberg et al., 2016). In addition, parabens are aromatase inhibitors (van Meeuwen et al., 2008) and have weak androgen receptor antagonists (Chen et al., 2007) effects, which may also affect the HPG-axis in girls. As boys have much lower endogenous estrogen and higher androgen production, the effects may not be seen in boys.

Our study has several strengths. It included 452 mother-child pairs and was population-based, however, only 42% of the eligible women participated in the OCC, and of these few had available urine paraben measurements, AGD and/or reproductive hormones, and participants were better educated than non-participants (Kyhl et al., 2015). As the women had no knowledge of their paraben exposure or the AGD and reproductive hormone measurements of their child at enrollment, these factors may unlikely have affected their participation. We adjusted for relevant confounders but cannot exclude the possibility of residual confounding by other factors associated with paraben exposure and growth measures, such as exposure to other environmental chemicals, lifestyle or health behavior. We performed many analyses, and some of our results may therefore be chance findings due to multiple testing. However, the trends in AGD in boys and reproductive hormones in girls were seen across most exposure categories.

Parabens are quickly metabolized with a urinary excretion half-life of less than 24 h (Moos et al., 2016). A single spot-urine sample collected around gestational week 28 may therefore not reflect fetal exposure in the sensitive developmental window in early gestational age or during mini-puberty. However, parabens are found in human breast milk, and as most infants were breastfed, prenatal exposure may be a good marker of exposure during mini-puberty (Dualde et al., 2020).

AGD measurements are well tolerated by all subjects and quiet to perform, with <5% CV representing good intra- and inter-examiner reliability, and currently few known factors need to be controlled for (age and body size). The CV was calculated without repositioning the child, and the within- and between-examiner variations are likely underestimated, and the reliability coefficients are overestimated. The short AGDs (AGDas and AGDaf) are less dependent on infant size and with easier identifiable margins and thus may be better biomarkers of anti-androgen exposure (Thankamony et al., 2014). However, in this study, the long AGD (AGDac) in girls was stronger associated with prenatal paraben exposure, which may be due to the fact that AGDas is shorter, and thus the same absolute measurement error is of relatively larger importance (Priskorn et al., 2018).

In male rodents, a shortened AGD persists into adulthood and predicts compromised reproductive function in the mature male (Thankamony et al., 2016). Compromised reproductive function has also been suggested by cross-sectional studies among adult men, which have found associations between adult AGD and semen quality (Eisenberg et al., 2011; Mendiola et al., 2011; Thankamony et al., 2014) and testosterone (Eisenberg et al., 2012). Less is known about AGD in females and their reproductive system characteristics. However, a study found positive associations between AGD and ovarian follicular number (Mendiola et al., 2012). The long-term effects of changes in reproductive hormone concentrations during mini-puberty have to our knowledge not been addressed and effects of both AGD and reproductive hormone concentrations in infancy on future reproductive health need to be addressed in future studies.

5. Conclusion

Even in this relatively low-exposed population, 10% of the pregnant women exceeded the threshold concentration for adverse estrogenic effects for parabens. Maternal paraben exposure was associated with shorter AGD in male offspring, and with longer AGD and decreased concentrations of reproductive hormones in females during pregnancy at three months of age. This suggests that parabens, which are believed to have low toxicity, may affect the human endocrine system at vulnerable time periods during development and thereby have long-term implications on reproductive function. Since this is the first study to report these associations our findings need confirmation.

**CRediT authorship contribution statement**

**Tina Kold Jensen:** Conceptualization, Software, Validation, Formal analysis, Methodology, Resources, Writing – original draft, Writing – review & editing, Funding acquisition. **Anna-Maria Andersson:** Conceptualization, Writing – original draft, Writing – review & editing, Funding acquisition. **Katharina M. Main:** Conceptualization, Writing – review & editing, Funding acquisition. **Trine Holm Johannsen:** Writing – review & editing. **Marianne S. Andersen:** Writing – review & editing. **Henriette Boye Kyhl:** Writing – review & editing. **Anders Juul:** Writing – review & editing. **Hanne Frederiksen:** Conceptualization, Validation, Formal analysis, Methodology, Resources, Writing – original draft, Writing – review & editing, Funding acquisition.

**Declaration of competing interest**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

**Acknowledgements**

The families in the Odense Child Cohort are acknowledged for their participation and commitment to the study. The health care professionals at the Hans Christian Andersen’s Children’s Hospital and the technicians at the Department Growth and Reproduction are acknowledged for their careful examination of the children and analysis of parabens and hormone concentrations in urine and serum samples. This work was supported by the Danish Council for Independent Research, Medical Sciences (4004-00352B and 8020-00123B), Odense University Hospital, the Region of Southern Denmark, the Municipality of Odense, the Danish Council for Strategic Research, Program Commission on Health, Food and Welfare (2101-08-0058), Novo Nordisk Foundation (NNF19OC0058266 and NNF17OC0029404), Odense University Hospital Research Foundation, Odense Patient data Exploratory Network.
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