Maternal smoking during pregnancy and reproductive health of daughters: a follow-up study spanning two decades

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\textbf{STUDY QUESTION:} Does \textit{in utero} exposure to constituents of cigarette smoke have a programming effect on daughters’ age of menarche and markers of long-term reproductive health?

\textbf{SUMMARY ANSWER:} \textit{In utero} exposure to constituents of cigarette smoke was associated with earlier age of menarche and—to a lesser extent—changes in the testosterone profile of the young women.

\textbf{WHAT IS KNOWN ALREADY:} Studies observe potential effects of \textit{in utero} exposure to constituents of cigarette smoke on the intra-uterine formation of female gonads, but the consequences on long-term reproductive health in daughters remain unclear.

\textbf{STUDY DESIGN, SIZE AND DURATION:} A prospective cohort study was designed using data from 965 pregnant women enrolled prior to a routine 30th-week antenatal examination at a midwifery practice in Denmark from 1988 to 1989 and a follow-up of their 19–21-year-old daughters in 2008.

\textbf{PARTICIPANTS/MATERIALS, SETTING AND METHODS:} The pregnant women provided information on lifestyle factors during pregnancy, including the exact number of cigarettes smoked per day during the first and the second trimesters. A total of 438 eligible daughters were asked to complete a web-based questionnaire on reproductive health and subsequently invited to participate in a clinical examination during 2008. Of the 367 daughters (84%) who answered the questionnaire, 267 (61%) agreed to further examination. Information on menstrual pattern was provided at examination, blood samples were drawn to be analyzed for serum levels of reproductive hormones [FSH, LH, estradiol (E\textsubscript{2}), sex hormone-binding globulin, anti-Müllerian hormone, dehydroepiandrosterone-sulphate (DHEAS), free testosterone and free E\textsubscript{2}] and number of follicles (2–9 mm) were examined by transvaginal ultrasound.

The daughters were divided into three exposure groups according to the level of maternal smoking during first trimester [non-exposed (reference), low-exposed (mother smoking \(\leq 9\) cigarettes/day) and high-exposed (mother smoking \(\geq 10\) cigarettes/day)]. Data were analyzed by multiple regression analyses in which we adjusted for potential confounders. Both crude and adjusted test for trend were carried out using maternal smoking during the first trimester as a continuous variable.

\textbf{MAIN RESULTS AND THE ROLE OF CHANCE:} We observed an inverse association between \textit{in utero} exposure to constituents of cigarette smoke and age of menarche (\(P = 0.001\)). Daughters exposed to \(>0–9\) cigarettes/day debuted with –2.7 [95\% confidence interval (CI) \(-5.2\) to \(-0.1\)] percentage earlier age of menarche, whereas daughters exposed to \(\geq 10\) cigarettes/day had \(-4.1\) (95\% CI: \(-6.6\) to \(-1.5\)) percentage earlier age of menarche corresponding to 6.5 (95\% CI: \(-10.7\) to \(-2.2\)) months. There was a non-significant tendency towards lower levels of testosterone and DHEAS with increasing \textit{in utero} exposure to constituents of cigarette smoke but no associations with follicle number, cycle length or serum levels of the other reproductive hormones were observed.

\textbf{LIMITATIONS AND REASONS FOR CAUTION:} We collected information on age of menarche retrospectively but the recall time was relatively short (2–10 years) and the reported values were within the normal range of Caucasians. Analyses of reproductive hormones are presented only for the group of daughters who were non-users of hormonal contraceptives because users were excluded, leaving only a low number of daughters available for the analyses (\(n = 75\)), as reflected in the wide CIs. The analyses of hormones were

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Introduction

Current cigarette smoking is known to be associated with higher risk of female infertility and prolonged time to pregnancy (TTP) (Baird and Wilcox, 1985; Bolumar et al., 1996; Augood et al., 1998) as well as adverse pregnancy outcomes, including spontaneous abortion (Ness et al., 1999), preterm delivery (Kharrazi et al., 2004; Bruin et al., 2010) and low birthweight (England et al., 2001). In general, studies observe potential effects of cigarette smoke at several stages of female reproductive function (Shiverick and Salafia, 1999; Dechanet et al., 2011), but the influence of prenatal exposure on future reproductive health in daughters remains unclear.

Animal and human studies provide evidence that in utero exposure to constituents of cigarette smoke, including nicotine, may affect developmental reproductive biology in females and suggest a general ovarian toxicity (MacKenzie and Angerv, 1981; Vahakangas et al., 1985; Matikainen et al., 2002; Smith et al., 2003; Holloway et al., 2006; Lutterodt et al., 2009; Mamsen et al., 2010). An early damaging effect on the reproductive system may have long-term consequences on traditional reproductive markers, such as age at menarche, reproductive hormonal status, fecundability measured as TTP, regularity of menstrual cycle and numbers of follicles in female offspring (Speroff and Fritz, 2004). However, epidemiological studies of the association between maternal smoking during pregnancy and fecundability in adult daughters have yielded inconsistent findings, with studies suggesting a prolonged TTP (Weinberg et al., 1989; Jensen et al., 1998, 2006; Ye et al., 2010), whereas other studies disagree (Baird and Wilcox, 1986; Joffe and Barnes, 2000). Information on maternal smoking was collected retrospectively in all studies, which may in part explain the contradictory results.

Only few studies have investigated the potential effect of in utero exposure to constituents of cigarette smoke on other reproductive markers in adult daughters. The studies of age of menarche have shown conflicting results, some indicating a tendency towards later age of menarche (Windham et al., 2008; Ferris et al., 2010) and others reporting a significantly earlier age of menarche, by 2–4 months (Windham et al., 2004; Rubin et al., 2009; Shrestha et al., 2011). One study associates maternal smoking during pregnancy with altered levels of anti-Müllerian hormone (AMH) in offspring as a sub-finding of their analyses (Kerkhof et al., 2010).

The aim of this study was to examine the associations between prenatal exposure to cigarette smoke and the traditional markers of reproductive health in adult daughters by use of prospectively collected data on prenatal exposure to cigarette smoke.

Materials and Methods

Study population

A Danish pregnancy cohort was established during 1988–1990 (Olsen et al., 1995). Among pregnant women scheduled to attend for a routine 30th-week examination at the main midwifery clinic in the city of Aarhus covering a well-defined geographical area, 965 (80%) were enrolled in the cohort. The pregnant women were Danish citizens and therefore most probably of Caucasian ethnicity. Less than 1% of Danish women aged 20–29 years were immigrants or descendants from immigrants during 1988 (Statistics Denmark, 2012). The participants filled out questionnaires on sociodemographics and lifestyle factors, including cigarette smoking during the first and the second trimesters and potential confounders, such as age, BMI, alcohol consumption and socioeconomic status. After birth, additional information on pregnancy outcomes was collected through hospital certificates.

Of 436 eligible daughters born to the pregnant women, 367 (84%) completed a web-based questionnaire and 267 (61%) participated in a clinical examination conducted throughout 2008–2009 (see Supplementary data).

Questions on age of menarche, use of hormonal contraceptives and lifestyle factors were part of the web-based questionnaire. At examination, the daughters were interviewed by a single physician on menstrual pattern, including maximum and minimum cycle length during the previous year and menstrual cycle day at the time of examination. Users of hormonal contraceptives (n = 178) were furthermore asked for maximum and minimum cycle length before initiation of use of hormonal contraceptives. All the participants were subsequently given a diary to register their current menstrual pattern on a daily basis for 3 months. An overnight fasting blood sample was drawn to analyze reproductive hormones. A total of 174 of the young women (40%) consented to determination of the number of follicles 2–9 mm in diameter by transvaginal ultrasound, as previously reported (Kristensen et al., 2010).

The BMI of daughters was calculated from height and weight measured in participants who attended clinical examination or from self-reported data in participants who answered only the web-based questionnaire. The study was approved by the local scientific ethical committee (reg. nr. M-20010157), and written informed consent was obtained from all the subjects before clinical examination.

Prenatal cigarette smoke exposure

Information on maternal cigarette smoking during pregnancy was obtained by a self-administered questionnaire filled in prior to a meeting at a midwifery practice around the 30th week of gestation. The mothers were asked ‘Have you smoked during pregnancy?’ with following response options: ‘Yes’ or ‘No’. Those who reported smoking during pregnancy...
were further asked ‘How many cigarettes have you on average smoked per day during the first 3 months of pregnancy?’ and ‘How many cigarettes have you on average smoked per day during the next 3 months of pregnancy?’ On the basis of the findings of the few earlier studies of age of menarche and to have two approximately even-sized exposure groups, prenatal exposure to cigarette smoke was categorized into three strata from these answers: non-exposed, >0–9 cigarettes/day during first trimester (low-exposed) and ≥10 cigarettes/day during first trimester (high-exposed). Women who confirmed smoking during pregnancy but did not report quantity were in the main analyses placed in the low-exposed group (n = 7). First trimester was chosen as the exposure window because earlier studies suggest this period to be critical in ovarian formation (Mattison, 1982; Mattison et al., 1983; Bryskov, 1986) and, furthermore, only few mothers changed smoking status between the first and the second trimesters (n = 4). Information on maternal cigarette smoking was available for 362 daughters, who constituted the final study group. In the analyses of cycle length, follicle number and hormonal status, 264 of the 267 daughters who attended the clinical examination had information on in utero exposure.

Outcomes
Age of menarche was reported in response to ‘How old were you when you had your first menstral bleeding (years and months)?’ in the web-based questionnaire. A total of 349 daughters provided an age, 178 (51%) reporting both years and months and 171 (49%) reporting only years. The distribution of age in years was similar between the two groups of daughters and thus the same distribution of months was randomly imputed in those daughters who reported age only in years. Median cycle length was estimated as the median of responses to ‘What is the minimum length of your menstrual cycle during the last year?’ and ‘What is the maximum length of your menstrual cycle during the last year?’ asked at the clinical examination. Among users of hormonal contraceptives, median of responses to the maximum and the minimum length of menstrual cycle before initiation of use of hormonal contraceptives were used instead. Seven participants were excluded owing to oligomenorrhea and 13 participants did not provide sufficient information, restricting our study population to 244 in the analysis with cycle length as the outcome.

The number of follicles in each of the participants was calculated as the mean number of follicles in the left and the right ovary. Examination was not limited to a particular time of the menstrual cycle. When it was not possible to count follicles in both ovaries, the number of follicles from only one ovary was used in our analysis. One participant was excluded owing to pregnancy and insufficient image quality made it impossible to count follicles in at least one ovary in 19 women, leaving the study group for analysis of follicle number with 152 women. As a result of the possible effects of hormonal contraceptives on follicle number (Somun-kiran et al., 2007), statistical analyses were performed separately for users and non-users of hormonal contraceptives.

Users of hormonal contraceptives were not included in the analyses of reproductive hormones and, furthermore, 11 were excluded for different reasons [no blood sample (n = 1), pregnant/breast-feeding (n = 2) and key information missing (n = 8)]. A total number of 75 participants were accessible for our analyses (see Supplementary data). In the statistical analyses of reproductive hormones, menstrual phase (defined below) was taken into account because of the well-known variations of reproductive hormones during menstrual cycle.

Hormonal assay
After centrifugation, serum was stored in cryo-tubes at −80°C until analyses. Serum levels of testosterone, FSH, LH and estradiol (E2) were measured by immunoassay analyses (cobas 6000 e 601, Roche Diagnostics, Mannheim, Germany), whereas sex hormone-binding globulin (SHBG) and dehydroepiandrosterone-sulphate (DHEAS) were determined by immunometric assay (IMMULITE 2000, Siemens Healthcare, Gwynedd, UK). AMH concentrations were detected by enzyme-linked immunoassorbent assay kits according to the manufacturer’s instructions (DSL-10-14400; Diagnostic System Laboratories Inc., Webster, TX, USA). Two measurements of LH below the detection limit (0.3 nmol/l) were set to 0.15 nmol/l. Free testosterone and free E2 were calculated as (testosterone/SHBG) × 100 and (E2/SHBG) × 100, respectively (Carter et al., 1983; Wilke and Utley, 1987; Rinaldi et al., 2002).

Menstrual phase
In the analyses of reproductive hormones, the menstrual cycle was divided into follicular phase (days before ovulation phase), ovulation phase (day of ovulation and one day before and after) and luteal phase (days after ovulation phase). The cycle day of ovulation was defined as cycle length minus 14 days (Speroff and Fritz, 2004). Each non-user of hormonal contraceptives (n = 75) was subsequently assigned to one of the phases according to their cycle day at the time of examination and cycle length. In order to have information specifically linked to menstrual cycle where blood samples were taken and analyzed, cycle length was in these analyses estimated as the number of days since the last menstrual bleeding, which was stated at the time of clinical examination, added to the number of days until the next menstrual bleeding seen in the dairies. In cases where dairies were not filled in completely, the median cycle length estimated from answers provided at clinical examination was used instead for defining the cycle length (n = 23).

Statistics
The mean (SD) outcome was calculated in the normally distributed variables (age of menarche, follicle number, FSH, testosterone, DHEAS, SHBG, AMH, free testosterone and free testosterone/free E2-ratio) and crude median 25- to 75-percentiles were calculated for the remaining non-normally distributed variables (cycle length, LH, E2, free E2). Multiple linear regression analyses were performed, using the three strata of prenatal exposure to cigarette smoke as categorical explanatory variables. All outcome variables were natural log transformed before multiple regression analyses were applied, and estimates are presented as adjusted percentage differences with 95% confidence intervals (CIs) on back-transformed scale with daughters of non-smokers as reference. Each regression model was evaluated by inspecting residuals and leverage plots. Maternal smoking during pregnancy was used as a continuous variable when testing for trend using Spearman’s rank correlation test on untransformed data and multiple linear regressions on transformed data. A sub-analyses was performed placing the daughters of smoking mothers with a missing quantity (n = 7) in the high-exposed group.

The following potential confounders were identified a priori: maternal age (continuous), maternal BMI (<25 kg/m², ≥25 kg/m²), maternal alcohol consumption during pregnancy (<2 g alcohol/day, ≥2 g alcohol/day), socioeconomic status (annual family income in 1988–1989: <21750 EUR, ≥21750 EUR), daughter’s age at examination (continuous), birthweight (continuous), daughter’s BMI (<18.5 kg/m², ≥18.5 kg/m²), daughter’s alcohol consumption (<3 episodes of alcohol intake/month, ≥3 episodes of alcohol intake/month), daughter’s smoking status (smoker, ex-smoker or never smoked). We did a stringent prioritization of the potential confounders with each outcome and included the strongest confounders (based on the literature) in the regression models. In the case of reproductive hormones, menstrual phase was taken into account.
Statistical analyses were performed using the Stata software v. 11.2 (Statacorp, College Station, TX, USA). A two-tailed $P$-value of $< 0.05$ was considered statistically significant.

**Results**

The characteristics of the 362 daughters and mothers according to the level of maternal smoking during the first trimester of pregnancy are presented in Table 1. The daughters were distributed as follows: non-exposed $= 226$ (62%), low-exposed $= 67$ (19%) and high-exposed $= 69$ (19%). Daughters prenatally exposed to cigarette smoke had younger mothers who reported a higher consumption of alcohol during pregnancy compared with non-exposed. Mother’s parity was equally distributed across exposure groups with 55 and 45% of all mothers being nulliparous and multiparous, respectively. The exposed daughters had a lower birthweight and were more often users of hormonal contraceptives. The group of low-exposed daughters tended to have a higher proportion of smokers in comparison with non- and high-exposed. The characteristics of the subgroup of daughters who attended the clinical examination ($n = 264$) were not different from the above (data not shown). The mean age of daughters in this subgroup was 20.1 years, ranging from 18.9 to 21.0 years.

Table 2 shows either mean (SD) or median (25- and 75-percentiles) for reproductive outcomes as well as adjusted percentage differences between exposed groups and the non-exposed reference group. We found an inverse association between prenatal exposure to cigarette smoke and age of menarche, before and after adjustment for confounders ($P$ for trend $= 0.001$). The crude difference (95% CI) in age of menarche between non-exposed and daughters exposed to $> 0–9$ and $\geq 10$ cigarettes/day during the first trimester of fetal life was $-0.4$ (95% CI: $-0.7$ to $-0.1$) years and $-0.6$ (95% CI: $-0.9$ to $-0.2$) years, respectively. After transformation and adjustment, daughters exposed to $> 0–9$ cigarettes/day debuted with $-2.7$

### Table 1: Characteristics of 362 daughters and mothers stratified by level of maternal smoking during the first trimester of pregnancy.

<table>
<thead>
<tr>
<th>Exposure to maternal smoking during the first trimester of pregnancy (cigarettes/day)</th>
<th>Non-exposed ($n = 226$)</th>
<th>$&gt;0–9$ ($n = 67$)</th>
<th>$\geq 10$ ($n = 69$)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Characteristics of mothers</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maternal age, years [mean (SD)]</td>
<td>29.2 (3.9)</td>
<td>29.0 (3.9)</td>
<td>27.9 (3.7)</td>
</tr>
<tr>
<td>Parity [no. (%)]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Nulliparous</td>
<td>130 (58)</td>
<td>37 (55)</td>
<td>35 (51)</td>
</tr>
<tr>
<td>- Multiparous</td>
<td>96 (42)</td>
<td>30 (45)</td>
<td>34 (49)</td>
</tr>
<tr>
<td>BMI, kg/m² [median (p25;75)]</td>
<td>20.9 (19.4–22.6)</td>
<td>20.6 (19.8–21.9)</td>
<td>21.1 (19.5–22.5)</td>
</tr>
<tr>
<td>Alcohol consumption during pregnancy, g/day [median (p25;75)]</td>
<td>1.7 (0.4–3.5)</td>
<td>2.5 (0.8–5.2)</td>
<td>2.5 (0.8–5.0)</td>
</tr>
<tr>
<td>Socioeconomic status [no. (%)]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Annual family income $&lt; 21.750$ EUR</td>
<td>80 (41)</td>
<td>19 (31)</td>
<td>27 (40)</td>
</tr>
<tr>
<td>Annual family income $\geq 21.750$ EUR</td>
<td>116 (59)</td>
<td>43 (69)</td>
<td>41 (60)</td>
</tr>
<tr>
<td><strong>Characteristics of daughters</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age, years [mean (SD)]</td>
<td>20.1 (0.4)</td>
<td>20.0 (0.3)</td>
<td>20.0 (0.3)</td>
</tr>
<tr>
<td>Birthweight, g [mean (SD)]</td>
<td>3510 (480)</td>
<td>3421 (522)</td>
<td>3343 (517)</td>
</tr>
<tr>
<td>BMI, kg/m² [median (p25;75)]</td>
<td>21.5 (19.6–23.4)</td>
<td>21.1 (19.7–23.9)</td>
<td>22.1 (19.4–24.9)</td>
</tr>
<tr>
<td>Use of hormonal contraception [no. (%)]</td>
<td>149 (67)</td>
<td>45 (70)</td>
<td>48 (72)</td>
</tr>
<tr>
<td>Smoking status [no. (%)]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Smoker</td>
<td>84 (38)</td>
<td>29 (45)</td>
<td>26 (38)</td>
</tr>
<tr>
<td>- Ex-smoker or never smoked</td>
<td>138 (62)</td>
<td>36 (55)</td>
<td>42 (62)</td>
</tr>
<tr>
<td>Alcohol intake [no. (%)]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\leq 3$ episodes of alcohol intake/month</td>
<td>147 (65)</td>
<td>41 (61)</td>
<td>46 (67)</td>
</tr>
<tr>
<td>$&gt; 3$ episodes of alcohol intake/month</td>
<td>79 (35)</td>
<td>26 (39)</td>
<td>23 (33)</td>
</tr>
<tr>
<td>Menstrual cycle [no. (%)]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Follicular phase</td>
<td>24 (65)</td>
<td>9 (64)</td>
<td>3 (38)</td>
</tr>
<tr>
<td>- Ovulation</td>
<td>4 (11)</td>
<td>2 (14)</td>
<td>1 (12)</td>
</tr>
<tr>
<td>- Luteal phase</td>
<td>9 (24)</td>
<td>3 (22)</td>
<td>4 (50)</td>
</tr>
</tbody>
</table>

$p$, percentile; no., number.

*The number of missing values across different characteristics: mothers’ BMI ($n = 14$), socioeconomic status ($n = 36$), daughter’s BMI ($n = 2$), use of hormonal contraception ($n = 8$), smoking status ($n = 7$) and menstrual cycle ($n = 16$).

*Age in the subgroup of daughters who attended clinical examination ($n = 264$).

*Menstrual phase was determined in non-users of hormonal contraception from clinical examination ($n = 75$).
Table 2: Age of menarche, number of follicles and level of reproductive hormones stratified by level of exposure to maternal smoking during the first trimester of pregnancy.

<table>
<thead>
<tr>
<th>Metric</th>
<th>Non-exposed</th>
<th>&gt;0–9 cig./day</th>
<th>≥10 cig./day</th>
<th>&gt;0–9 cig./day</th>
<th>≥10 cig./day</th>
<th>P-value from test for trend</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age of menarche (years)</td>
<td>349 (220, 64, 65)</td>
<td>13.5 (1.2)</td>
<td>13.1 (1.2)</td>
<td>12.9 (1.3)</td>
<td>-2.7 (-5.2 to -0.1)</td>
<td>-4.1 (-6.6 to -1.5)</td>
</tr>
<tr>
<td>Follicle number (No.)</td>
<td>152 (97, 29, 26)</td>
<td>14.1 (5.6)</td>
<td>12.8 (3.4)</td>
<td>13.9 (4.5)</td>
<td>-4.3 (-18.2 to 12.0)</td>
<td>7.2 (-9.4 to 26.7)</td>
</tr>
<tr>
<td>Cycle length (days) (median p25;75)</td>
<td>108 (66, 21, 21)</td>
<td>16.7 (6.0)</td>
<td>14.2 (3.1)</td>
<td>19.2 (5.9)</td>
<td>-12.4 (-37.6 to 23.1)</td>
<td>20.7 (-22.4 to 87.3)</td>
</tr>
<tr>
<td>Hormonal statusd</td>
<td>44 (31, 8, 5)</td>
<td>16.7 (6.0)</td>
<td>14.2 (3.1)</td>
<td>19.2 (5.9)</td>
<td>-12.4 (-37.6 to 23.1)</td>
<td>20.7 (-22.4 to 87.3)</td>
</tr>
<tr>
<td>FSH (IU/l)</td>
<td>75 (50, 15, 10)</td>
<td>5.2 (2.7)</td>
<td>6.0 (3.5)</td>
<td>4.0 (2.9)</td>
<td>16.1 (-9.6 to 49.1)</td>
<td>9.1 (-20.3 to 49.5)</td>
</tr>
<tr>
<td>LH (IU/l) (median p25;75)</td>
<td>-</td>
<td></td>
<td>-</td>
<td>6.6 (4.0 – 9.8)</td>
<td>7.2 (4.3 – 9.2)</td>
<td>7.1 (4.5 – 8.3)</td>
</tr>
<tr>
<td>Testosterone (nmol/l)</td>
<td>-</td>
<td></td>
<td>-</td>
<td>2.0 (0.9)</td>
<td>1.8 (0.7)</td>
<td>1.5 (0.3)</td>
</tr>
<tr>
<td>AMH (ng/ml)</td>
<td>-</td>
<td></td>
<td>-</td>
<td>3.1 (1.5)</td>
<td>3.4 (1.7)</td>
<td>3.4 (1.8)</td>
</tr>
<tr>
<td>Free testosterone (nmol/l)</td>
<td>-</td>
<td></td>
<td>-</td>
<td>5.0 (3.5)</td>
<td>3.7 (1.9)</td>
<td>3.8 (2.4)</td>
</tr>
<tr>
<td>Free testosterone/free E2-ratio</td>
<td>-</td>
<td></td>
<td>-</td>
<td>0.5 (0.3 – 0.8)</td>
<td>0.4 (0.3 – 0.8)</td>
<td>0.6 (0.3 – 1.6)</td>
</tr>
</tbody>
</table>

E2, estradiol; AMH, anti-Müllerian hormone; DHEAS, dehydroepiandrosterone-sulphate; SHBG, sex hormone-binding globulin; CI, confidence interval.

aOutcome variables not normally distributed (cycle length, LH, E2 and free E2) are presented as medians (25- and 75-percentiles).

bBack-transformed percentage differences with non-exposed as reference group. The multiple regression analysis of age of menarche was adjusted for maternal age (continuous), maternal BMI (≥ 25 kg/m²), maternal alcohol consumption during pregnancy (<2 g alcohol/day), socioeconomic status based on family annual income in 1988–1989 (≥21750 EUR, ≥21750 EUR), birthweight (continuous) and daughter’s BMI (≥18.50, ≥25.00 kg/m²), while the analyses of cycle length and follicle number were adjusted for maternal BMI, socioeconomic status, birthweight, daughter’s BMI and daughter’s smoking status (smoker or ex-smoker and never smoked).

cMaternal smoking during pregnancy was used as a continuous variable when testing for trend with Spearman’s rank correlation test on crude data and multiple linear regression analysis on adjusted data.

dUsers of hormonal contraception were excluded in the analysis of hormonal status and regression models were adjusted for socioeconomic status, daughter’s BMI and menstrual phase at the time of examination.
reduce reproductive lifespan and cause earlier age of menopause, as daughters. A steady depletion of a smaller ovarian reserve may licles (2–9 mm in diameter) between exposed and non-exposed and in accordance with this we found similar numbers of growing fol-

(2002) suggest that the rate at which follicles grow to later and (or after stratification for use of hormonal contraceptives), cycle length, FSH, LH, E2, SHBG, AMH, free testosterone or free E2. We measured as maternal cigarette smoking during the second trimester (instead of first trimester) did not change the results essentially (data not shown). (ii) Dichotomizing the exposure into exposed (to maternal smoking in fetal life) and non-exposed did not bring to light any unexplored differences. Daughters born to a mother who smoked experienced −3.4 (95% CI: −5.4 to −1.4) adjusted percentage earlier age of menarche than non-exposed, and still did not differ significantly in other markers of reproductive health (data not shown). (iii) Shifting the daughters of mothers with missing quantity of cigarettes smoked per day (n = 7) from the low-exposed to the high-exposed group did not change the results (data not shown).

**Discussion**

To the best of our knowledge, this is the first population-based study to examine the associations between prenatal exposure to maternal cigarette smoking and several markers of reproductive health in females during early reproductive life. Maternal smoking during pregnancy was associated with earlier age of menarche, extending the findings of previous studies. Levels of testosterone and DHEAS tended to be lower with increasing in utero exposure to constituents of cigarette smoke, although statistically insignificant.

Studies have shown that in utero exposure to constituents of cigarette smoke interferes with the delicate and fine-tuned formation of ovaries but the mechanism by which these early influences may affect pubertal development and later reproductive function is still unclear. Polycyclic aromatic hydrocarbons, a major toxic component in cigarette smoke, induce apoptotic death of oogonia in murine embryonic ovaries (Matikainen et al., 2002), and in utero exposure of female mice to cigarette smoke and nicotine reduces the pool of primordial oocytes (MacKenzie and Angervine, 1981; Vahakangas et al., 1985) and interrupts ovarian steroidogenesis (Holloway et al., 2006). Two epidemiological studies have shown a reduction of both somatic cells and oogonia in gonads of fetuses aborted during the first trimester of women who smoked, supporting a decline in ovarian reserve at birth (Luttori et al., 2009; Mamsen et al., 2010). Matikainen et al. (2002) suggest that the rate at which follicles grow to later and larger stages remains unchanged after exposure to in utero smoke, and in accordance with this we found similar numbers of growing follicles (2–9 mm in diameter) between exposed and non-exposed daughters. A steady depletion of a smaller ovarian reserve may reduce reproductive lifespan and cause earlier age of menopause, as indicated in women whose mothers smoked during pregnancy (Srohoznitser et al., 2008). On the other hand, in the study, AMH was not associated with the level of maternal smoking during pregnancy. AMH has become an established marker of ovarian reserve, reflecting the remaining pool of follicles in the ovary (de Vet et al., 2002; van Rooij et al., 2002) and our results speak against the hypothesis that exposure to cigarette smoke during fetal life reduces ovarian reserve in the long term. A decline in ovarian reserve at birth may have been evened out by the time daughters reach 20 years of age, as indicated by the unchanged AMH levels across the exposure groups.

In accordance with our results, Shrestha et al. (2011) and Windham et al. (2004) found early prenatal exposure to cigarette smoke at high doses to be associated with younger age of menarche. On the other hand, a later study by Windham et al. (2008) did not observe any association, whereas Ferris et al. (2010) reported a higher risk of late age of menarche in daughters prenatally exposed to tobacco smoking. Ferris et al. (2010) were, however, limited by a much longer recall time on AOM than the other studies and a low participation rate. Unfortunately, it was not possible to assess the effects of ethnicity on the changes in age of menarche as all of the mothers enrolled in the cohort were Caucasians, which may limit the generalizability of our results.

We did not observe any changes in the results after our sub-analyses, with maternal smoking during the second trimester as the exposure variable used in the statistical model instead of the first trimester smoking, as in our main analyses. The fact that only few mothers changed smoking status during pregnancy made it impossible to discriminate whether possible effects on reproductive health were established from exposure during the first or the second trimester. Neither did we find other associations when the exposure was dichotomized into non-exposed and exposed. A few earlier studies suggested that a trend towards earlier maturation in females may have undesirable effects on later reproductive function, primarily a higher risk of adverse pregnancy outcomes (Liestol, 1980; Martin et al., 1983; Wyshak, 1983; Sandler et al., 1984). We, therefore, attempted to see whether an earlier age of menarche in the daughters was associated with changes in the other measured markers of reproductive health. Our analysis showed that cycle length decreased significantly with earlier age of menarche but we did not detect any changes in the number of growing follicles or reproductive hormones. Whether this change in cycle length reflects or has any consequences for female fertility in the longer term (i.e. TTP, early pregnancy loss or earlier menopause) awaits a longer follow-up of this cohort or other studies.

The strengths of our study include use of prospectively collected data on maternal smoking during pregnancy with information on the number of cigarettes smoked per day during both the first and the second trimesters and a relatively large proportion of daughters with high exposure. On the other hand, information on maternal smoking was based on self-reports, which may cause misclassification, most likely an underestimation. However, we expect our data to be accurate as smoking during pregnancy was socially accepted in Denmark during the 1980s and as our analysis confirmed the well-known decrease in birthweight with increase in smoking status (Kramer, 1987; Wilcox, 1993); 167 g (95% CI: 34 to 299 g) lower mean birthweight among daughters of the high-exposure group compared with daughters of non-smokers. Studies validating cotinine
measurements and self-reported smoking habits in women from pregnancy cohorts established in the 1960 and 1990s indicate that women report their smoking accurately during pregnancy but conclude that serum cotinine concentration correlates more strongly with infant birthweight and may be a more reliable measure evaluating smoking habits during pregnancy (Klebanoff et al., 1998, 2001). Information on age of menarche was ascertained retrospectively but the time span between onset and age of reporting was only 2–10 years and the mean age of menarche in our exposure groups is within the range reported in previous studies, supporting the reliability of our data. Recent studies observed a poor to moderate agreement range reported in previous studies, supporting the reliability of our data.

The high participation rate of mothers in the pregnancy study (80%) (Olsen et al., 1995) was 20 years later, followed by a very high response rate to the web-based questionnaire (85%) among daughters and a somewhat lower participation rate in the clinical examination (61%). The risk of selection bias is believed to be minor since the majority of daughters were not aware of their reproductive health because of their young age. We were able to adjust for a number of potential confounding factors, such as maternal BMI and socioeconomic status, which may have lessened the extent of any confounding resulting from possible differential selection mechanisms; however, residual confounding cannot be excluded.

The inclusion of only non-users of hormonal contraceptives in the analyses of reproductive hormones reduced the number of observations in all exposure groups, resulting in wider CIs. At the same time, an exclusion of users of hormonal contraceptives leaves room for selection bias but no difference in in utero exposure to cigarette smoke was observed between users and non-users of hormonal contraceptives (data not shown). Our analyses of reproductive hormones were complicated by the timing of bio-specimen collection according to menstrual cycle (Speroff and Fritz, 2004) but the participants were divided into three groups of menstrual-cycle stage to account for this.

In conclusion, our study suggests that maternal smoking during pregnancy has a programming effect on reproductive health in daughters leading to an earlier age of menarche. Furthermore, prenatal exposure to cigarette smoke during fetal life, especially at high doses, may change the testosterone profile of young women.

Authors’ roles

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Conflict of interest
None declared.

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