

# Maternal menopause as a predictor of anti-Müllerian hormone level and antral follicle count in daughters during reproductive age

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Submitted on November 30, 2011; resubmitted on July 23, 2012; accepted on September 5, 2012

**STUDY QUESTION:** Is the ovarian reserve in a woman at a given age associated with her mother's age at menopause?

**SUMMARY ANSWER:** We demonstrated a significant, positive association between age at maternal menopause and serum anti-Müllerian hormone (AMH) levels and antral follicle count (AFC) in daughters. The rate of decline in serum-AMH level and AFC is also associated with age at maternal menopause.

**WHAT IS KNOWN AND WHAT THIS PAPER ADDS:** The association between menopausal age in mothers and daughters has been established through several epidemiological studies. This paper shows that early maternal menopause is related to an advanced depletion of the ovarian reserve and that late maternal menopause is related to a delayed depletion.

**STUDY DESIGN AND SIZE:** Cross-sectional data were obtained from a prospective cohort study of 863 women. The study comprised 527 participants from this prospective cohort whose mothers' age at natural menopause was known.

**PARTICIPANTS, SETTING AND METHODS:** Participants were recruited from female health care workers aged 20–40 years employed at Copenhagen University Hospital, Rigshospitalet, and were enrolled in the study between September 2008 and February 2010. The response rate was 52.1%. Endocrine and ovarian parameters related to reproductive ageing (AMH and AFC) were assessed by serum AMH analyses and transvaginal ovarian sonography on cycle Day 2–5. Data on reproductive history, including age at natural maternal menopause, were obtained through an internet-based questionnaire. We used an analysis of covariance model with serum-AMH and AFC as outcomes, age as the quantitative predictor and onset of maternal menopause as the categorical predictor, with further adjustments for BMI, use of oral contraceptives, participants' smoking habits and prenatal smoking exposure.

**MAIN FINDINGS:** We found a significant effect of age at maternal menopause on both serum AMH levels ( $P < 0.001$ ) and AFC ( $P = 0.005$ ). Median serum-AMH concentration declined by 8.6% per year [95% confidence interval (CI): 6.4–10.8%,  $P < 0.001$ ] in the group with early maternal menopausal age ( $\leq 45$  years), by 6.8% per year (95% CI: 5.0–8.6%,  $P < 0.001$ ) in the group with normal maternal menopausal age (46–54 years) and by 4.2% per year (95% CI: 2.0–6.4%,  $P < 0.001$ ) in the group with late maternal menopausal age ( $\geq 55$  years). Median AFC declined by 5.8% per year (95% CI: 4.0–7.5%,  $P < 0.001$ ) in the group with early maternal menopausal age ( $\leq 45$  years), by 4.7% per year (95% CI: 3.3–6.1%,  $P < 0.001$ ) in the group with normal maternal menopausal age (46–54 years) and by 3.2% per year (95% CI: 1.4–4.9%,  $P < 0.001$ ) in the group with late maternal age ( $\geq 55$  years) at menopause.

**BIAS, LIMITATIONS AND GENERALIZABILITY:** Information on 'age at maternal menopause' was obtained retrospectively and may be prone to recall bias and digit preference. The study population consisted of health care workers, which implies a potential selection bias. Finally, the cross-sectional nature of the data limits the generalizability.

**STUDY FUNDING/POTENTIAL COMPETING INTERESTS:** This study was co-financed by PhD scholarships where funding was covered by the Danish Agency for Science, Technology and Innovation, Copenhagen Graduate School of Health Science (CGSHS) and the Fertility Clinic at Copenhagen University Hospital, Rigshospitalet. No competing interests are declared.

**Key words:** ovarian reserve / antral follicle count / anti-Müllerian hormone / maternal menopause

## Introduction

Over the last three decades, couples in Europe have postponed childbearing. Since 1970, the mean age at first delivery has increased by 4–5 years in several European countries (Eurostat, 2006), and in Denmark the mean age at first delivery has increased from 23.7 years in the early 1970 to 29.1 years in recent years (Statistics Denmark, 2010).

Fertility declines substantially with female age (Larsen and Yan, 2000; Baird et al., 2005; Schmidt et al., 2012). An increasing proportion of women may experience reproductive problems due to this delay in childbearing; accordingly, it is essential to develop models predicting the reproductive life span of an individual woman. The loss of natural fecundity and, ultimately, the age at menopause are related to a decrease in the pool of primordial follicles and a deterioration of oocyte quality. It has been suggested that fixed time intervals exist between the first decline in fertility, the end of fertility and the menopause (Vegetti et al., 2000; te Velde and Pearson, 2002; Broekmans et al., 2009; van Houten et al., 2010;). The time interval from the decline in fertility to the menopause has been estimated to be ~20 years (te Velde and Pearson, 2002; Nikolaou and Templeton, 2003). Thus, a woman entering menopause at the age of 45 years may already experience an age-related decline in fertility at the age of 25 years.

Menopause before the age of 45 years is experienced by ~5–14.3% of women in general (Jacobsen et al., 1999, 2004; Vegetti et al., 2000), whereas premature ovarian failure, defined as menopause before the age of 40 years is found in 1–2% of women (Coulam et al., 1986; Vegetti et al., 2000). In general, women do not experience any symptoms of an accelerated ovarian follicular depletion, except for a possible shortening of their cycle length during reproductive ageing and the occurrence of subfertility (Treloar et al., 1967).

To date, the heritability of age at menopause has been addressed through population-based studies, showing strong associations in menopausal age between twins, siblings, and mothers and daughters, respectively (Cramer et al., 1995; Torgerson et al., 1997; Vegetti et al., 2000; van Asselt et al., 2004; Morris et al., 2011). In the general population, genetic factors are considered to account for ~31–87% of the individual variability in age at menopause (de Bruin et al., 2001; Voorhuis et al., 2011). Although several specific, well-described conditions such as autoimmune diseases (e.g. Addison disease and systemic lupus erythematosus) and genetic disorders such as X chromosome linked disorders (e.g. fragile X syndrome and Turner syndrome) have been associated with premature or early menopause, the majority of cases still remain unexplained.

While hereditary factors may influence age at menopause, it is, however, uncertain whether maternal factors also have an impact on a woman's fertility potential in terms of ovarian reserve. Accord-

ly, we hypothesized that age at maternal menopause is associated with ovarian reserve at a certain female age. We therefore compared maternal age at menopause with known parameters of ovarian reserve, i.e. anti-Müllerian hormone (AMH) and antral follicle count (AFC), in a large population of female health care workers.

## Materials and Methods

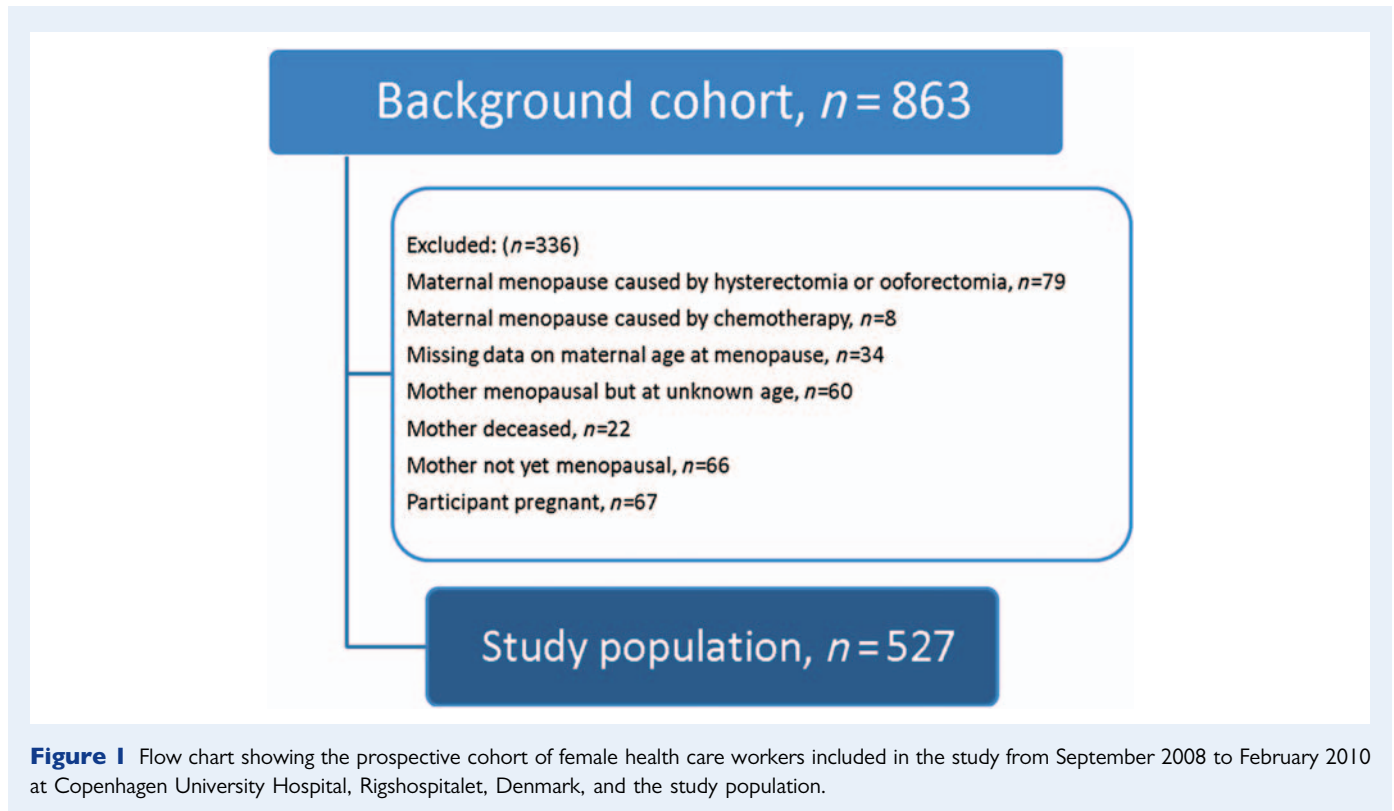
This study was part of a prospective cohort study of 863 healthy women aged 20–40 years employed as health care workers at Copenhagen University Hospital, Rigshospitalet. We assessed ovarian and endocrine parameters related to biological ageing within the normal reproductive period. In addition, a detailed reproductive history was obtained through an internet-based questionnaire completed by all participants before the physical examination. Questions included: gynecological and reproductive history, including menstrual cycle characteristics; use of contraceptives; pregnancies and deliveries; socio-economic characteristics; smoking habits, including prenatal smoking exposure; alcohol consumption; physical activities; self-reported body weight and height; hereditary and chronic diseases and mothers' age at menopause. With regard to records of age at maternal menopause, the participants were asked to obtain information about maternal age at menopause from their mothers. The definition of menopause was specified in the questionnaire as absence of menstrual bleeding for >12 months, and for this study natural menopause was defined as at least 12 months of amenorrhea that was not related to oophorectomy or chemotherapy. Mothers who had been using hormone replacement therapy or oral contraceptives up to the last menstrual bleeding, or had had a hysterectomy or ooforectomy were not classified as natural menopausal.

Participants were recruited from a list provided by the Human Resource Department, Copenhagen University Hospital, Rigshospitalet, Denmark, of employed female health care workers between 20 and 40 years of age. Invitations to participate in the investigation were sent by post from August 2008 to December 2009. The response rate was 52.1%. Participants were enrolled in the study from September 2008 to February 2010.

## Study population

The study included a subgroup of women ( $n = 527$ ) from the prospective cohort, whose mothers' age at natural menopause was known (Fig. 1). Maternal age at menopause was retrospectively recorded. As shown in Fig. 1, the study excluded women whose mothers' age at natural menopause was unknown because of the mother: undergoing hysterectomy or oophorectomy ( $n = 79$ ) or chemotherapy ( $n = 8$ ); being deceased ( $n = 22$ ); not yet experiencing menopause ( $n = 66$ ); or not knowing their age at onset of menopause ( $n = 60$ ) or data on age at maternal menopause being not available ( $n = 34$ ). Additionally, 67 women were excluded because of pregnancy at the time of inclusion.

Women were categorized into three groups: (i) early maternal age at menopause ( $\leq 45$  years,  $n = 68$ ), (ii) normal maternal age at menopause (46–54 years,  $n = 382$ ) and (iii) late maternal age at menopause ( $\geq 55$  years,  $n = 77$ ).



**Figure 1** Flow chart showing the prospective cohort of female health care workers included in the study from September 2008 to February 2010 at Copenhagen University Hospital, Rigshospitalet, Denmark, and the study population.

## Ovarian sonography

On menstrual cycle day 2–5, a transvaginal sonography was performed. Users of oral contraceptives were studied on menstrual cycle day 2–5 during the withdrawal bleeding. At sonography, the investigator used the transducer to scroll through the ovary in two planes, longitudinal and transverse. Antral follicles were counted and grouped according to three pre-defined size categories: 2.0–4.0, 5.0–7.0 and 8.0–10.0 mm. All echo-free structures in the ovaries were regarded as follicles. The ovarian volume was measured in three planes and was calculated using the formula for an ellipsoid  $V = D1 \times D2 \times D3 \times 0.523$  (Diameters: D1 = longitudinal, D2 = antero-posterior and D3 = transversal). The diameters D1 and D2 were obtained on the X- and Y-axes in one plane; the transducer was then rotated 90°, and D3 was obtained on the Z-axis, which then appeared as the X-axis in the new plane (Rosendahl *et al.*, 2010). The ovarian volume was calculated as the mean value of the left and right ovary volumes. All ultrasound scans were performed using the same equipment (BK pro focus ultrasound scanner, transducer 4-9 MHz BK Medical, Denmark) and all examinations were carried out by the same investigator (J.G.B.).

At clinical examination, the investigator was unaware of the participants' questionnaire answers and their hormonal profile.

## Endocrine parameters

Blood samples were taken for analysis of reproductive hormone concentrations on cycle Day 2–5, including serum AMH levels, FSH levels and estradiol ( $E_2$ ) levels, respectively. Fresh blood samples were centrifuged at 3000g for 12 min, and serum samples were stored in Nunc-tubes at  $-24^\circ\text{C}$ . Laboratory analyses were performed at the Department of Clinical Biochemistry, Copenhagen University Hospital, Rigshospitalet. Serum-AMH concentrations were measured by an enzyme-linked immunosorbent assay using the AMH/MIS kit (Immunotech, Beckman Coulter, Marseilles, France). The sensitivity was 0.7 pmol/l and the

intra- and inter-assay coefficients of variation were 12.3 and 14.2%, respectively. Serum concentrations of FSH levels and  $E_2$  levels were measured by an electrochemiluminescence immunoassay using the E170 kit (Roche, Mannheim, Germany). For serum-FSH, the sensitivity was  $<0.1$  IU/l, and the intra- and inter-assay coefficients of variation were 2.8 and 4.5%, respectively, and for serum- $E_2$ , the sensitivity was 0.02 nmol/l and the intra- and inter-assay coefficients of variation were 3.3 and 4.7%, respectively.

## Statistical analysis

Baseline characteristics were expressed as mean and standard deviations, medians and inter-quartile ranges (IQR) or exact numbers and percentages, whichever was the most appropriate.

Non-response analyses were carried out by either  $\chi^2$  test or Man-Whitney *U*-test.

Raw data were initially plotted in a scatter diagram showing the relationship between the age of participants' and AFC and serum-AMH, respectively. An approximately linear association was found between  $\log(\text{AMH})$  and  $\log(\text{AFC})$ , which we assessed by Pearson's correlation coefficient (*r*). Subsequently, data were analyzed in an analysis of covariance model (a general linear model) with serum-AMH and AFC as outcomes, age as the quantitative predictor and onset of maternal menopause (early:  $\leq 45$  years of age, normal: 46–54 years of age, late:  $\geq 55$  years of age) as the categorical predictor, and with further adjustments for BMI (underweight:  $< 18.5$ , normal: 18.5–25, overweight: 25–30, obesity  $> 30$ ), use of oral contraceptives (yes/no), participants' smoking habits (current, previous or non-smoker) and prenatal smoking exposure (yes/no). Both serum-AMH concentrations and AFC were found to have skewed distributions, and for this reason, logarithmic transformation was applied to satisfy the model assumption of normally distributed residuals with homogeneous variances. The transformation implied that the estimated levels of serum-AMH and AFC were expressed as medians, and estimated

differences between groups were expressed as relative (i.e. %-wise) differences. We imposed a non-inferiority assumption on the intercept of the model to compensate for the possible bias of non-randomly distributed missing data from the youngest participants with mothers experiencing normal to late onset of menopause.

Tests were regarded as statistically significant for a two-sided *P*-value of <0.05. Descriptive statistics were computed using the SPSS 18.0 software (SPSS Inc., Chicago, IL). Statistical analyses were performed with R version 2.13.0. R Development Core Team, 2011 (R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL <http://www.R-project.org>).

## Ethical approvals

The Ethics Committee of the Capital region of Denmark approved the study (No. H-B-2007-129), and verbal and written informed consent was obtained from all the participants prior to study inclusion. Approval was granted from the Danish Data Protection Agency (Journal number 2008-41-1881).

## Results

### Baseline characteristics

Characteristics of the study population are summarized in Table I. The study comprised 527 women, whose mean (SD) age was 32.7 (4.1) years. The median BMI of the participants was 22.0, and 409 (77.6%) participants had a normal BMI (18.5–25.0). Overall, 146 (27.7%) participants used oral contraceptives or a vaginal contraceptive ring. Current smoking was reported in 106 (20.3%) participants, of whom 13 (12.3%) smoked >10 cigarettes per day.

### Non-response analyses

Apart from the group of academics who represented 5.0% (*n* = 71) of the respondents and 2.9% (*n* = 37) of the non-respondents, no significant differences were found between respondents and non-respondents in relation to the profession (nurses, doctors, secretaries, midwives and laboratory technicians; *P* = 0.1). The median age between respondents and non-respondents was significantly different (*P* < 0.001). The median age (IQR) of respondents was 32.5 years (IQR: 29.3–35.7) compared with 33.4 years (IQR: 29.8–36.8) of non-respondents.

### Maternal menopause and maternal lifestyle during pregnancy

The mean (SD) age at maternal age at menopause was 50.2 (4.3) years. Prenatal exposure to maternal smoking was reported in 148 (28.1%) participants, non-exposure was reported in 362 (68.7%) participants and data were missing in 17 (3.2%) participants (Table I).

### Endocrine and sonographic parameters of ovarian reserve

Table II shows the endocrine and sonographical characteristics of the study population. Median serum-AMH concentration (pmol/l) was 20.0 (IQR: 11.1–33.0), and the mean AFC was 19.0 (13.0–28.0). Median serum-FSH concentration (IU/l) was 6.8 (IQR: 5.8–8.3), and median serum-E<sub>2</sub> concentration (nmol/l) was 0.16 (0.12–0.21). Median ovarian volume (ml) was 4.6 (3.3–6.2). An approximately

**Table I** Baseline characteristics of the study population.

| Characteristics                       | <i>n</i> | %      | Mean (SD) <sup>a</sup> or median (IQR) <sup>b</sup> |
|---------------------------------------|----------|--------|---|
| Age (years)                           |          |        |   |
| All                                   | 527      | (100)  | 32.7 (4.1) <sup>a</sup>                             |
| Use of oral contraceptives            |          |        |   |
| Yes                                   | 146      | (27.7) |   |
| No                                    | 377      | (71.5) |   |
| Missing                               | 4        | (0.8)  |   |
| Smoking habits                        |          |        |   |
| Current smoker Cigarettes per day:    | 106      | (20.1) |   |
| 1–10                                  | 93       | (87.7) |   |
| >10                                   | 13       | (12.3) |   |
| Former smoker                         | 143      | (27.1) |   |
| Never smoker                          | 278      | (52.8) |   |
| BMI (kg/m <sup>2</sup> )              |          |        |   |
| All                                   | 527      | (100)  | 22.0 (20.7–24.2) <sup>b</sup>                       |
| Underweight (<18.5)                   | 12       | (2.3)  |   |
| Normal range (18.5–25.0)              | 409      | (77.6) |   |
| Overweight (>25.0)                    | 80       | (15.2) |   |
| Obese (>30.0)                         | 26       | (4.3)  |   |
| Prenatal exposure to maternal smoking |          |        |   |
| Yes                                   | 148      | (28.1) |   |
| No                                    | 362      | (68.7) |   |
| Missing                               | 17       | (3.2)  |   |
| Maternal age at menopause             |          |        |   |
| All                                   | 527      | (100)  | 50.2 (4.3) <sup>a</sup>                             |
| Early (≤45 year)                      | 68       | (12.9) |   |
| Normal (46–54 years)                  | 382      | (72.5) |   |
| Late (≥55 years)                      | 77       | (14.6) |   |

IQR, interquartile range; SD, standard deviation; BMI, body mass index.

<sup>a</sup>Mean (SD).

<sup>b</sup>Median (IQR).

**Table II** Endocrine and sonographic characteristics of the study population.

| Parameters                                  | <i>n</i>         | Median (IQR)     |
|---|------------------|------------------|
| Serum-AMH concentration (pmol/l)            | 527              | 20.0 (11.1–33.0) |
| Serum-FSH concentration (IU/l)              | 527              | 6.8 (5.8–8.3)    |
| Serum-E <sub>2</sub> concentration (nmol/l) | 527              | 0.16 (0.12–0.21) |
| AFC (2–10 mm; number)                       | 527              | 19.0 (13.0–28.0) |
| Ovarian volume (ml) <sup>a</sup>            | 486 <sup>b</sup> | 4.6 (3.3–6.2)    |

IQR, interquartile range; AMH, anti-Müllerian hormone; FSH, follicle-stimulating hormone; AFC, antral follicle count.

<sup>a</sup>Ovarian volume = (left ovary volume + right ovary volume)/2.

<sup>b</sup>In 41 women, it was not possible to measure ovarian volume.

linear relation was found between  $\log(\text{AMH})$  and  $\log(\text{AFC})$  with correlation coefficient  $r = 0.82$  ( $P < 0.001$ ).

Table III provides data on the impact of age at maternal menopause on the primary parameters of ovarian reserve, serum-AMH and AFC. We analyzed data in an analysis of covariance model (a general linear model) with participants' serum-AMH and AFC as outcomes, participants' age as the quantitative predictor and the onset of maternal menopause as the categorical predictor. The model was adjusted for participants' use of oral contraceptives, BMI, smoking habits and prenatal exposure to smoking. In our analysis, no evidence was found for interactions between smoking status and age for either AMH level ( $P = 0.6$ ) or AFC ( $P = 0.7$ ). Similarly, no significant interactions were found between prenatal exposure to smoking and age for either AMH level ( $P = 0.2$ ) or AFC ( $P = 0.06$ ). A significant effect of age at maternal menopause on both AMH levels ( $P < 0.001$ ) and AFC ( $P = 0.005$ ) was observed.

Further, the effect of 'maternal age at menopause' as a continuous variable on AMH and AFC was highly significant on both markers ( $P < 0.001$  for both markers).

Figure 2 illustrates median serum-AMH concentration as a function of age for women whose mothers entered menopause at early, normal or late age. Owing to varying levels, it is not reasonable to display all the 527 participants in the one graph. Accordingly, the data shown in Fig. 2 are limited to participants fulfilling the criteria of non-use of oral contraceptives and no prenatal exposure to maternal smoking ( $n = 266$ ). An exponential decline of serum-AMH levels was observed as data were transformed back to the original scale. Figure 2 shows that serum-AMH regression lines of the three groups of maternal menopause were significantly different ( $P < 0.001$ ). When analyzing all women, median serum-AMH concentration declined by 8.6% per year [95% confidence interval (CI): 6.4–10.8%,  $P < 0.001$ ] in the group of early maternal age ( $\leq 45$  years) at menopause, by 6.8% per year (95% CI: 5.0–8.6%,  $P < 0.001$ ) in the group of normal (46–54 years) maternal age at menopause and by 4.2% per year (95% CI: 2.0–6.4%,  $P < 0.001$ ) in the group of late ( $\geq 55$  years) maternal age at menopause (summarized in Table III). The difference between the results shown graphically and the remaining results are a matter of difference of level.

Serum-AMH levels were significantly lower (27.3, 95% CI: 13.9–38.5%) in users of oral contraceptives compared with non-users of oral contraceptives (Table III). We found no significant association between BMI and serum-AMH levels ( $P = 0.53$ ), and neither the participants' smoking habits nor their prenatal exposure to maternal smoking had a significant influence on serum-AMH levels (Table III).

Figure 3 illustrates median AFC (2–10 mm) as a function of age for participants according to the age category at maternal menopause.

The regression lines of the three groups of maternal menopause were significantly different ( $P = 0.005$ ). Median AFC declined 5.8% per year (95% CI: 4.0–7.5%,  $P < 0.001$ ) in the group of early ( $\leq 45$  years) maternal age at menopause, by 4.7% per year (95% CI: 3.3–6.1%,  $P < 0.001$ ) in the group of normal (46–54 years) maternal age at menopause and by 3.2% per year (95% CI: 1.4%–4.9%,  $P < 0.001$ ) in the group of late ( $\geq 55$  years) maternal age at menopause (summarized in Table III).

The median AFC level was significantly lower (26.8, 95% CI: 16.6–35.6%,  $P < 0.001$ ) in users compared with non-users of oral contraceptives. The relation between AFC and BMI was similar to the

results for AMH. A significant, negative association between prenatal exposure to maternal smoking and AFC was seen. In participants prenatally exposed to maternal smoking, the median AFC was 11.1% lower (95% CI: 0.1–21.1%,  $P = 0.04$ ) than among those with no prenatal exposure to smoking. We observed no association between participants' current smoking habits and AFC.

Overall, the model of covariance explained 13.9% of the identified variation of serum AMH ( $R^2 = 0.14$ ) and 12.8% of the variation of AFC ( $R^2 = 0.13$ ).

We observed no significant differences in the age-adjusted median serum  $E_2$ , median serum FSH or median ovarian volume between the three groups of maternal age at menopause. In users of oral contraceptives compared with non-users of oral contraceptives, the median serum  $E_2$  concentration was 21.3% (95% CI: 10.7–30.8%,  $P < 0.001$ ) lower, the median serum FSH concentration was 11.7% (95% CI: 3.8–20.2%,  $P = 0.004$ ) higher and the median ovarian volume was 41.0% (95% CI: 35.3–46.2%,  $P < 0.001$ ) lower.

## Discussion

To our knowledge, this was the first study to demonstrate a significant association between age at maternal menopause and serum AMH levels in daughters. The age-related decline in serum-AMH level and AFC has been observed in several studies (de Vet *et al.*, 2002; van Rooij *et al.*, 2005; van Disseldorp *et al.*, 2008; La Marca *et al.*, 2010; Voorhuis *et al.*, 2011), and our study confirmed this association in a population of female health care workers aged 20–40 years. In line with the previous studies, we observed a large variability in the ovarian reserve markers, but despite this variability, we identified factors significantly related to the two primary markers of ovarian reserve: AMH and AFC. As such, this study indicates that the age category of maternal menopause is related to the rate of decline in ovarian reserve markers, as we found a more pronounced decline per year in serum-AMH concentration and in AFC for participants with early ( $\leq 45$  years) maternal age at menopause, compared with participants with late maternal age at menopause ( $\geq 55$  years). Additionally, our data suggest that maternal smoking during pregnancy may have a negative influence on daughters' ovarian reserve in terms of AFC.

The main strength of this study lies in the large number of women examined. Furthermore, the same investigator carried out all sonographic examinations using the same ultrasound scanner, thereby minimizing the inter-observer variation. The sonographer was not aware of the maternal age at menopause at the time of the sonographic examination or the participant's hormonal profile.

A possible limitation is that our study population consisted of health care workers, which implies a potential selection bias, as health care workers in general might be more aware of healthy living than the general population. Additionally, social class and environment may be more homogeneous among health care workers than in the general population. However, the percentage of current smokers corresponds well with data on current smoking in the general population of Danish women. In our study population, the proportion of current smokers was 20.3% with 12.3% of the participants smoking 10 cigarettes or more per day. In the general population of Danish women, the proportion of current smokers is 23.1% with 9% smoking  $> 15$



**Table III** Impact of various factors on the primary parameters of ovarian reserve, serum-AMH and AFC.

| Effect (%)                   | AMH                     |         |                          |         |   |         | AFC                     |         |                          |         |   |         |
|------------------------------|-------------------------|---------|--------------------------|---------|---|---------|-------------------------|---------|--------------------------|---------|---|---------|
|                              | Univariate <sup>a</sup> |         | Bivariate <sup>b</sup>   |         | Multivariate <sup>c</sup> (R <sup>2</sup> = 0.14) |         | Univariate <sup>a</sup> |         | Bivariate <sup>b</sup>   |         | Multivariate <sup>c</sup> (R <sup>2</sup> = 0.13) |         |
|                              | 95% CI                  | P-value | 95% CI                   | P-value | 95% CI  | P-value | 95% CI                  | P-value | 95% CI                   | P-value | 95% CI  | P-value |
| Oral contraception (yes)     | -7.0 (-20.8 to 9.3)     | 0.4     | -28.1 [-39.1 to (-15.1)] | <0.001  | -27.3 [-38.5 to (-13.9)]                          | <0.001  | -14.6 [-24.4 to (-3.5)] | 0.01    | -28.7 [-37.2 to (-19.1)] | <0.001  | -26.8 [-35.6 to (-16.6)]                          | <0.001  |
| BMI <18.5                    | -3.1 (-5.8 to 11.3)     | 0.1     | -30.6 (-56.4 to 10.3)    | 0.1     | -28.9 (-55.0 to 12.4)                             | 0.1     | -11.5 (-38.7 to 27.9)   | 0.5     | -10.9 (-37.7 to 27.6)    | 0.5     | -10.8 (-37.3 to 26.8)                             | 0.5     |
| BMI >25                      | -8.5 (-25.2 to 12.0)    | 0.4     | -4.2 (-21.1 to 16.3)     | 0.7     | 1.2 (-17.4 to 24.1)                               | 0.9     | -12.2 (-24 to 72.3)     | 0.1     | -9.8 (-22.4 to 4.8)      | 0.2     | -6.1 (-19.7 to 9.8)                               | 0.4     |
| BMI >30                      | -12.5 (-37.4 to 22.2)   | 0.4     | -3.6 (-30.1 to 32.9)     | 0.8     | -2.7 (-30.1 to 35.3)                              | 0.9     | -13.4 (-32.8 to 11.7)   | 0.3     | -8.2 (-28.4 to 17.6)     | 0.5     | -8.0 (-28.6 to 18.6)                              | 0.5     |
| Smoking (previous)           | -9.6 (-23.8 to 7.1)     | 0.2     | -0.8 (-16.0 to 17.0)     | 0.9     | -5.5 (-20.0 to 11.7)                              | 0.5     | -2.3 (-14.2 to 11.2)    | 0.7     | 3.4 (-9.0 to 17.5)       | 0.6     | -0.7 (-12.6 to 13.0)                              | 0.9     |
| Smoking (current)            | 2.1 (-15.4 to 23.3)     | 0.8     | 0.8 (-15.9 to 20.9)      | 0.9     | -1.9 (-18.2 to 17.7)                              | 0.8     | 6.1 (-8.1 to 22.5)      | 0.4     | 5.3 (-8.5 to 21.1)       | 0.5     | 3.4 (-10.1 to 18.9)                               | 0.6     |
| Prenatal exposure to smoking | -13.0 (-25.9 to 2.2)    | 0.1     | -11.4 (-24.1 to 3.4)     | 0.1     | -6.8 (-20.2 to 8.8)                               | 0.4     | -15.7 [-25.4 to (-4.8)] | 0.006   | -14.8 [-24.4 to (-4.0)]  | 0.009   | -11.1 (-21.1 to 0.14)                             | 0.04    |
| Maternal menopause (early)   | 20.0 (-2.3 to 47.3)     | 0.08    | -7.7 [-9.7 to (-5.7)]    | <0.001  | -8.6 [-10.8 to (-6.4)]                            | <0.001  | 10.8 (-5.3 to 29.5)     | 0.2     | -4.7 [-6.3 to (-3.1)]    | <0.001  | -5.8 [-7.5 to (-4.0)]                             | <0.001  |
| Maternal menopause (normal)  | 0                       | NA      | -6.0 [-7.6 to (-4.3)]    | <0.001  | -6.8 [-8.6 to (-5.0)]                             | <0.001  | 0                       | NA      | -3.7 (-4.9 to (-2.4)]    | <0.001  | -4.7 [-6.1 to (-3.3)]                             | <0.001  |
| Maternal menopause (late)    | -20.1 [-35.6 to (-0.8)] | 0.04    | -3.3 [-5.3 to (-1.2)]    | 0.002   | -4.2 [-6.4 to (-2.0)]                             | <0.001  | -13.6 (-26.7 to 1.9)    | 0.08    | -2.0 [-3.6 to (40.4)]    | 0.002   | -3.2 [-4.9 to (-1.4)]                             | <0.001  |

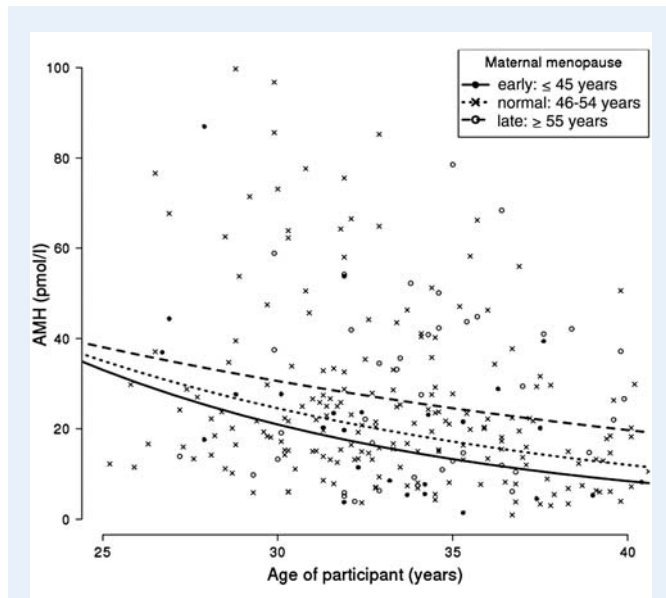
AMH, anti-Müllerian hormone; AFC, antral follicle count; 95% CI, 95% confidence interval; BMI, body mass index.

<sup>a</sup>Reference groups: non-users of oral contraceptives, BMI 18.5–25.0, smoking (never), no exposure to prenatal smoking and normal maternal menopause, respectively.

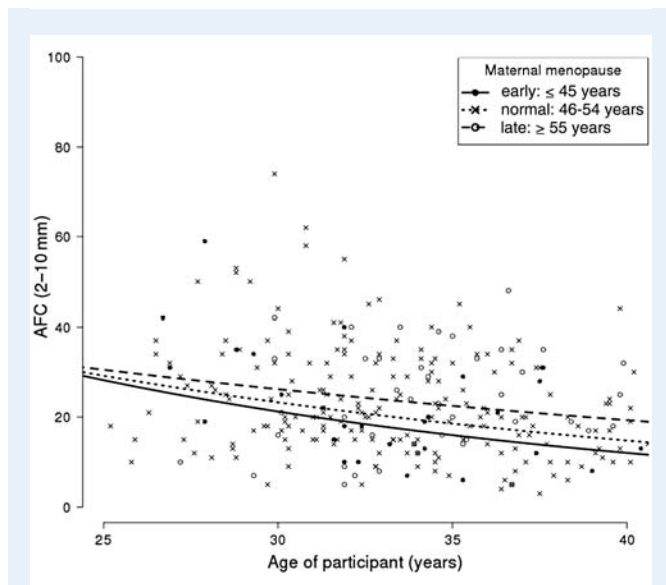
<sup>b</sup>Analyses adjusted for age.

<sup>c</sup>Adjusted analysis of covariance (all effects included).

<sup>d</sup>R<sup>2</sup>, variation explained by the adjusted ANCOVA model (all factors included).



**Figure 2** Relation between median serum levels of AMH (untransformed) and age in non-users of oral contraceptives and no prenatal exposure to maternal smoking. Linear regression lines are grouped by the category of age at maternal menopause (early, normal and late).



**Figure 3** Relationship between AFC (untransformed) and age in non-users of oral contraceptives and no prenatal exposure to maternal smoking. Linear regression lines are grouped by the category of age at maternal menopause (early, normal and late).

cigarettes per day (National Board of Health, Denmark & National Institute of Public Health, Denmark, 2010).

A further consideration is the potential risk of recall bias in the record of maternal age at menopause. To minimize this effect, we encouraged participants, prior to answering the questionnaire, to contact their mothers for information about the specific age at which they had had at least 1 year of amenorrhea. Since digit

preference could presumably lead to bias, we assumed that the variable 'maternal age at menopause' was probably best modeled as a discrete variable rather than as a continuous variable. By categorizing 'maternal age at menopause', we were able to test the effect of early maternal menopause at one end of the spectrum to late maternal menopause at the other end.

Despite a relatively low response rate, we did not suspect further selection bias as no significant differences were found between respondents and non-respondents in relation to the profession (nurses, doctors, secretaries, midwives and laboratory technicians), apart from the group of academics (5.0% of the respondents and 2.9% of the non-respondents). The median age of the respondents and non-respondents was significantly different, but the estimated difference of 0.9 years was considered clinically irrelevant.

One possible bias in our study is that non-random missing data occurred from unknown onset of maternal menopause among the youngest participants with mothers experiencing normal to late onset of menopause because their mothers were still premenopausal. To compensate for the possible bias of non-randomly distributed missing data, we imposed a non-inferiority assumption on the intercept of the model. When doing so, we assumed that the median serum-AMH concentration and AFC in women aged 22 years (the intercept) were similar among women whose mothers experienced late onset of menopause as among women whose mothers experienced early or normal onset of menopause. Furthermore, data from women beyond 40 years may have further improved the description of the age-related decrease in ovarian reserve markers.

Compared with our study, previous studies have explained more of the age-related variation in ovarian reserve markers (Rosen *et al.*, 2010; Almog *et al.*, 2011; Kelsey *et al.*, 2012; Seifer *et al.*, 2011), albeit the majority involved more homogeneous groups of infertile patients; we studied a population of female health care workers, which may be a more heterogeneous group. In our study, 8.4% of the variation in serum-AMH concentration and 5.1% of the variation in AFC were explained by age alone. However, the multivariate model accounting for maternal age at menopause and participants' age with further adjustments for BMI, use of oral contraceptives, participants' smoking habits and prenatal smoking exposure explained 13.9% of the variation in serum AMH level and 12.8% of the variation in AFC.

The mechanisms underlying the decline of follicles with age remain far from being fully understood. The association between early menopausal age and the lower ovarian reserve measured through AMH and AFC could be either due to impaired oogenesis or due to prenatal loss during the fetal-life period. Alternatively, there may be an accelerated decline of follicles during childhood or the reproductive-life period. In a recent study of infertile patients by Rosen *et al.* (2010), age at maternal menopause was found to be significantly associated with AFC. In contrast to our findings, Rosen *et al.* found a lower rate of decline in AFC with earlier maternal age at menopause, whereas we found a markedly higher rate of decline per year in AFC with earlier maternal age at menopause. We cannot explain this apparent contradiction, but it should be noted that the Rosen study included 124 infertile patients aged 25–48 years.

Earlier studies have reported a negative impact of current smoking on serum-AMH levels (Plante *et al.*, 2010; Waylen *et al.*, 2010). Although, we expected to find a negative effect of current smoking

on serum-AMH level and AFC, our data did not support that notion. Plante *et al.* studied women of late reproductive or perimenopausal age, in whom the exposure to smoking may have been more pronounced. However, we found an effect of prenatal exposure to maternal smoking on AFC but no effect on AMH level. Considering the high correlation between AMH level and AFC, the explanation for this finding remains unclear.

Estimates of the actual number of ovarian primordial follicles in women of different ages originate from cross-sectional histological analyses. Mathematical models have been proposed over the years to describe the relationship between non-growing follicles (NGFs) and age. Faddy *et al.* (1992) concluded that the rate of decline in oocytes followed a biphasic pattern with an accelerated rate of decline around the age of 37.5 years (Vegetti *et al.*, 2000). Subsequently, Faddy and Gosden (1996) produced a later publication in which the biphasic pattern was abandoned. Recently, Hansen *et al.* (2008, 2011) suggested a constantly increasing rate of decline in ovarian follicles with age. In a new study from Kelsey *et al.* (2012), it was demonstrated that serum-AMH levels are positively correlated with NGFs in women aged 24.5–51 years. The data analysis of Kelsey *et al.* was based upon a systematic data aggregation of 8 studies, in which the total population of NGFs was extrapolated from manual stereological counts of NGF from a small subset of ovarian tissue, and 25 studies reporting normative levels of AMH for cohorts with limited age ranges. Taken together, these data support that serum-AMH can be used as a reliable marker of the ovarian reserve. Additionally, our data indicate that the maternal menopause age category may predict the rate of decline in ovarian reserve.

Clearly, our data do not elucidate whether maternal age at menopause is a direct predictor of age at menopause of the offspring nor the chance of pregnancy. Nevertheless, from a biologically point of view, it may be reasonable to assume that a low ovarian reserve may have a long-term effect that will shorten the reproductive life span. We therefore assume that markers such as 'maternal age at menopause' in combination with AMH or AFC, and chronological age may represent a more complete picture when evaluating the ovarian reserve of the individual. This assumption awaits longitudinal studies before it can be put to test.

Undoubtedly, as long-term follow-up data become available, the patterns of age-related decline in ovarian reserve markers over time observed in our study can be more precisely addressed.

Environmental factors influencing age at menopause may change over time and thereby affect generations differently, which may be a potential bias in our study. A recent population-based study addressed both environmental effects and genetic effects on menopausal age (Morris *et al.*, 2011). This study included 2060 first-degree relatives aged 31–90 years. Menopause data were obtained through a self-administered questionnaire. Morris *et al.* found that 42% of the variation in age at natural menopause could be explained by an additive genetic effect and 14% was due to environmental factors shared by sisters, suggesting that earlier studies might have overestimated the genetic effect on age at menopause. Our findings support the notion that the ovarian reserve is influenced by hereditary factors although the cross-sectional design of our study restricts interpretation and longitudinal data are needed to confirm the findings. As such a 5-year follow-up is planned.

Additionally, we showed that the ovarian reserve markers were significantly lower in current users of oral contraceptives compared with non-users of oral contraceptives. To date, somewhat inconsistent results have been reported in studies of the relationship between hormonal contraceptives and serum-AMH level (Streuli *et al.*, 2008; van den Berg *et al.*, 2010). Our data included 146 users of oral contraceptives and suggest that the use of oral contraceptives induces a significant reduction in serum-AMH concentration and AFC. Certainly, further clarifying studies are needed to explore the impact of dose-response and duration of hormonal contraception on serum AMH concentration and AFC.

In a recent study of prediction of age at menopause based on follow-up data, Broer *et al.* (2011) developed a forecast table linking age and serum-AMH level to prediction of age at menopause; it was demonstrated that serum-AMH concentration may indeed be a good predictor of age at menopause.

In conclusion, the present prospective cohort study based on a large population of female health care workers showed that participants' serum AMH concentrations and AFC levels were significantly associated with their mothers' menopausal age category. To provide an estimate of an individual women's reproductive age, long-term follow-up studies are still required.

## Authors' roles

A.N.A., E.C.L., L.S. and J.G.B. were responsible for study design. J.G.B. was responsible for sample and data collection. T.H.J. and L.F.-H. contributed serum measurement data. J.L.F., A.P. and J.G.B. performed data analyses. J.G.B. drafted the paper. All the authors provided critical discussion and manuscript review.

## Funding

This study was co-financed by PhD scholarships where funding was covered by the Danish Agency for Science, Technology and Innovation, Copenhagen Graduate School of Health Science (CGSHS), and the Fertility Clinic at Copenhagen University Hospital, Rigshospitalet.

## Conflict of interest

None declared.

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