Ovarian Reserve and Anti-Mullerian Hormone (AMH) in Mothers of Dizygotic Twins

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This study aimed to explore if natural dizygotic (DZ) twinning is associated with earlier menopause and lower anti-Mullerian hormone (AMH) values. We investigated if advanced biological reproductive aging, which can be responsible for the multiple follicle growth in familial twinning, is similar to mechanisms that occur in normal ovarian aging, reflected by earlier menopause in mothers of DZ twins and lower levels of AMH. A total of 16 mothers of DZ twins enrolled with the Netherlands Twin Register (average age at first assessment: 35.9 ± 3.0 years) and 14 control mothers (35.1 ± 3 years) took part in a prospective study. Fifteen years after entry into the study, which included follicle-stimulating hormone (FSH) assessment, AMH was measured in stored serum samples and menopause status was evaluated. Average AMH levels were not significantly different between DZ twin mothers and controls (2.1 ± 2.4 µg/L vs. 1.9 ± 1.9 µg/L). Among the 16 mothers of twins, 7 had an elevated (FSH) value over 10 U/L at first assessment. Their AMH levels were lower than the nine twin mothers with normal FSH values: 0.6 ± 0.4 versus 3.4 ± 2.6 µg/L (p = .01). Of the mothers of twins, eight mothers had entered menopause at the second assessment compared with only one control mother (p = .07). Thus, slightly more DZ mothers were in menopause than the control mothers, although this difference was not significant. The subgroup of DZ twin mothers who had an increased FSH concentration 15 years ago had a limited ovarian reserve as reflected by lower AMH levels. These data indicate that advanced ovarian aging can be a feature in familial DZ twinning, particularly with elevated early follicular phase FSH.

∎ **Keywords:** familial dizygotic twinning, menopause, ovarian reserve, AMH

Dizygotic (DZ) twinning is a consequence of multiple follicle growth (Martin et al., 1991a), subsequent multiple ovulation, and fertilization of multiple oocytes. Known risk factors for natural dizygotic twinning include heredity, genetic predisposition, advanced maternal age, and maternal characteristics such as increased height and body mass index (BMI; Hoekstra et al., 2008, 2010; Lichtenstein et al., 1996; Parisi et al., 1983) In singleton pregnancies, one dominant follicle is selected when the follicle-stimulating hormone (FSH) level reaches a certain threshold (Schoemaker et al., 1993). However, if FSH levels exceed this threshold for too long, multiple follicles can be selected, thereby creating a risk for multiple pregnancies. Multiple follicular development in several cycles has been shown in mothers of dizygotic twins (Martin et al., 1991a). High FSH levels in the early follicular phase can also indicate a decreased ovarian reserve (Lambalk et al., 2009) associated with reproductive aging. Advanced maternal age is associated with more natural DZ twinning probably due to these increased FSH levels (Beemsterboer et al., 2006; Bulmer, 1979). In mothers of DZ twins who come from families with multiple twins, elevated FSH in the early follicular phase is also a characteristic feature (Lambalk et al., 1998b; Martin et al., 1991b; Thomas et al., 1998). In general, with familial dizygotic twinning, no clear indication for advanced ovarian aging has been found (Gilfillan et al., 1996), although it has been hypothesized that familial twinning may occur via a hereditary mechanism similar to that of advanced ovarian aging (te Velde et al., 1998), which could imply that DZ twinning is

associated with earlier menopause. So advanced biological aging may be responsible for the multiple follicle growth in familial twinning along mechanisms identical to that in normally aging women (Lambalk et al., 2009).

In a previous study, we found increased levels and pulsatility of FSH in mothers with familial dizygotic twinning but no clear signs of limited ovarian feedback (Lambalk et al., 1998a, 1998b). Recently, another hormone secreted by small non-developing follicles has been indicated as a good and subtler estimate for ovarian aging, namely anti-Mullerian hormone (AMH). Low or undetectable levels are associated with biologically ovaries of advanced age and menopause (Broer et al., 2011; Tehrani et al., 2009; van Disseldorp et al., 2008; Visser et al., 2006). Fifteen years after the first study, we aimed to measure AMH in the stored blood samples from the original participants (Lambalk et al., 1998b). Second, the occurrence of early menopause in the original cohort of mothers of DZ twins and their aged-matched controls was assessed by self-report, in order to test the hypothesis that having had a DZ pregnancy is associated with a lower ovarian reserve reflected by lower AMH levels and the occurrence of menopause at an earlier age.

Subjects and Methods

Subjects

The study, conducted in 1998, included 16 mothers of DZ twins with a family history of twinning and 14 control mothers (Lambalk et al., 1998b). Mothers of DZ twins were recruited through the annual newsletter of the Netherlands Twin Register (Boomsma et al., 2006; Lambalk et al., 1998b). Inclusion criteria were spontaneous birth of DZ twins of unequal sex (to establish zygosity) before age of 36, at least one other DZ twin pair in a first or second degree female relative, good general health, no medical treatment at the time of the study, and no hormonal treatment 6 months prior to the study advent. Inclusion criteria for the singleton control mothers were good general health, no medical treatment at the time of the study, no hormonal treatment 6 months preceding the study, having given birth to at least two singletons before the age of 36, and no history of familial twinning. Fifteen years later we approached the study population with a small survey consisting of items about general medical history and obstetric/gynaecological history (de Boer et al., 2002). BMI was based on self-reported height and weight. Women who had undergone a hysterectomy ($N = 4$) or who were currently using oral hormonal contraception or a Mirena intrauterine device ($N = 2$) were excluded from follow-up analysis as menopausal status could not be determined. Informed consent was provided by all participants and the study was approved by the institutional scientific review board and ethical committee.

Blood Sampling and Storage

For the first assessment, all women provided blood samples on cycle day 3. They kept a basal body temperature chart to verify time of ovulation. FSH and luteinizing hormone (LH) sampling was done between 8:00 and 9:00 in the morning. Blood samples were stored at −20°C (Lambalk et al., 1998b). In 2009, AMH was measured in these samples.

Laboratory Essays

In 1998, LH and FSH were measured by means of a double first monoclonal and second polyclonal antibody immunoradiometric assay (IRMA-mat BYK-Sangtec Diagnostica GmBH & Co., Dietzenbach, Germany) with a lower detection limit of 0.2 IU/L. References for expression of LH and FSH were first international reference preparations 68/40 and second international reference preparations 78/549, respectively. LH intra-assay coefficients of variation (CV) were 4.3% and 2.4% at the levels of # U/L and 23 U/L, respectively. FSH intra-assay CVs were 3% and 1.8% at levels 7 and 74 U/L, respectively. Inter-assay CVs were below 8% for both gonadotropins. For each individual, both hormone values were measured in the same assay.

Inhibin A and B were measured in duplicate with two-site, enzyme-linked immunoassays (Serotec, Oxford, UK; Groome et al., 1994, 1996). CVs were less than 5% within a plate and less than 7% between plates of both assays. The sensitivity was 3 pg/mL for Inhibin A and 15 pg/mL for Inhibin B.

Estradiol (E2) was measured in the previous study via radioimmunoassay (Sorin Biomedical, Silage, Italy) with a lower limit detection of 18 pmol/L and CVs of less than 5%.

For the current report, AMH was measured once given its small intercycle variation. Serum AMH levels were measured by an ultrasensitive enzyme-linked immunosorbent assay (Immunotech-Coulter, Marseilles, France), as described elsewhere (Long et al., 2000). The limit of detection (defined as negative control +3 SD of the negative control) was 0.05 μg/L. For positive quality control, high and low serum samples from separate assays were used. For negative quality control, samples from postmenopausal women were used. Intra- and inter-assay coefficients of variation were <5% and 8%, respectively.

Statistical Analysis

Maternal menopausal age, subject's menopausal status and menopausal age (if applicable), tobacco use, parity, data on gynecological surgery, menstrual cycle characteristics (past and present), and current and past hormonal treatment were compared between DZ twin mothers (cases) and age-matched singleton mothers (controls) using Student's *t* tests for continuous data and χ^2 tests for dichotomous outcome variables. For AMH levels, a cut-off of 0.5 μg/L was used (cut-off value for 95% of all values measured in controls). Mean hormone concentrations were compared using a Student's *t* test. Pearson's correlations were calculated between AMH and FSH. Data were analyzed in the Statistical Package for the Social Sciences (SPSS) 15.

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TABLE 1

Characteristics (Mean ± SD) of Premenopausal Mothers of Dizygotic Twins and Controls (12) at Baseline (in 1998)

Characteristics	Controls (n = 14)	Twin mothers (n = 16)	p
Age (year)	35.1 ± 3	35.9 ± 3.4	.5
Body mass index (kg/m²)	22.2 ± 3.9	23.0 ± 2.8	.511
Cycle length (days)	28.4 ± 2.7	26.4 ±2.2	.037
Smoking (packyears)	3.3 ± 5.6	7.0 ± 8.9	.051
Days from last delivery	1857 ± 1300	1615 ± 1153	.597
Parity	2.2 ± 0.4	1.9 ± 0.9	.351
Age at twin delivery	–	30.0 ± 5.2	–

TABLE 2

Reproductive Hormones on Cycle Day 3 in Familial DZ Mothers and Controls

	Controls (n = 14)	Twin mothers (n = 16)	p*
FSH (U/L)	6.0 ± 3.0	9.8 ± 5.5	.023
E2 (pmol/L)	140.0 ± 82.0	192.4 ± 170.4	.221
Inhibin A (pg/mL)	4.6 ± 3.7	3.8 ± 3.0	.549
Inhibin B (pg/mL)	77.8 ± 42.5	75.9 ± 45.8	.656
AMH (μg/L)	1.9 ± 1.9	2.1 ± 2.4	.252

Note: AMH = anti-Mullerian hormone; E2 = Estradiol; FSH = follicle-stimulating hormone.
*Presented data and the statistical evaluation of FSH, E2, and the inhibins were taken from the original publication (Lambalk et al., 1998b).

Results

Baseline characteristics of the cohort are summarized in Table 1 and AMH levels in Table 2. Mothers of DZ twins (cases) had a shorter menstrual cycle and tended to smoke more. On average there was no difference in AMH levels between cases and controls, whereas FSH was significantly higher in cases.

Table 2 also summarizes the early follicular phase values of FSH, E2, Inhibin A, and Inhibin B, of which only FSH was significantly higher. Seven out of the 16 twin mothers had an elevated FSH level (above 10 IU/L) in the early follicular phase versus 1 out of 14 controls ($p < .05$). Within the group of twin mothers, AMH was substantially lower in the mothers with elevated FSH levels. The twin mothers with normal FSH also had AMH levels in the normal range. In the twin mothers with elevated FSH levels, Inhibin A and Inhibin B tended to be lower ($p = .08$), whereas there were no differences in E2 levels (Table 3).

From the original group of 16 DZ twin mothers, 15 responded to the second assessment. From the 14 controls, 12 returned the survey. Results for menopause data in cases and controls are summarized in Table 4. The average age at time of response was around 50 years and was not significantly different between cases and controls ($50.9 ± 3.4$ for cases and $50.8 ± 3.36$ for controls $p = .9$). Two twin mothers could not provide usable information with regard to age at menopause; both had undergone a hysterectomy around the age of 40 for benign pathology. In the control group, five women could not provide usable data on menopause status.

TABLE 3

Cycle Day 3 Hormones in Mothers of Twins (n = 16) With Normal or Elevated FSH

	FSH < 10 U/L (n = 9)	FSH > 10 U/L (n = 7)	p
FSH (U/L)	6.1 ± 2.1	14.6 ± 5.0	.025
E2 (pmol/L)	194.0 ± 110.0	190.0 ± 237.0	1.0
Inhibin A (pg/mL)	5.0 ± 2.9	2.3 ± 2.7	.08
Inhibin B (pg/mL)	92.8 ± 49.0	54.3 ± 32.6	.08
AMH (μg/L)	3.4 ± 2.6	0.6 ± 0.4	.01

Note: AMH = anti-Mullerian hormone; E2 = Estradiol; FSH = follicle-stimulating hormone.

Two underwent a hysterectomy for benign pathology, two were undergoing hormonal treatment for menstrual cycle disturbances, and one mother had experienced menopause after having undergone chemotherapy for breast cancer. More than half of the twin mothers were postmenopausal versus only one mother in the control group ($p = .07$). Of the eight twin mothers for whom postmenopausal status could be determined, those who had an elevated basal FSH above 10 IU/L ($n = 4$) in the past, age of menopause was 48.25 years. Age of menopause for the other four twin mothers with normal basal FSH in the past was 50.25 years.

Discussion

We observed a trend that more mothers with familial DZ twins had reached menopause compared with controls, although this was not significantly different ($p = .07$) between the two groups in our small sample. However, AMH, a novel marker for ovarian aging, was not lower despite the prominently higher early follicular phase FSH values in the total group of twin mothers (Lambalk et al., 1998b). On the other hand, the subgroup of DZ twin mothers who had an increased FSH concentration 15 years ago shows a limited ovarian reserve as reflected by significantly lower AMH levels. These mothers tended to have menopause slightly earlier, namely at 48.25 years compared with the 50.25 years of those mothers with normal FSH. However, none of the twin mothers had premature menopause (<40 years) or early menopause (<45 years). None of the mothers who had AMH levels that were too low to be detected had menopause before the age of 48 years. Those twin mothers who did not have an elevated FSH level showed levels of AMH in the normal range. Together, our data point to the fact that advancing of ovarian aging can be a feature in a substantial number of situations of familial DZ twinning, in particular when elevated early follicular phase FSH is involved. But on the other hand, in many other twin mothers, no signs of ovarian aging such as higher FSH or lower AMH were present, indicating that the familial twinning trait remains multi-causal.

There is substantial evidence that age at menopause is genetically determined (Galloway et al., 2002; Montgomery et al., 2004; Moore et al., 2004; Palmer et al.,

TABLE 4

Menopause Data of Mothers of Dizygotic Twins and Controls

	Controls (*n* = 12)	Twin mothers (*n* = 15)		*p*
Age (year)	50.4 ± 3.0	51.1 ± 4.0		.57
Evaluable mothers	7	13		–
Postmenopausal status	1 (14%)	8 (67%)		.07
Age at menopause (if applicable, year)	50	Elevated FSH (*N* = 8)	48.2	–
		Normal FSH (*N* = 4)	50.2	
Not evaluable due to hysterectomy	2	2		–
Not evaluable due to use of hormonal treatment	2	0		–
Not evaluable due to chemotherapy	1	0		–

Note: Note: FSH = follicle-stimulating hormone.

2006; Stolk et al., 2012; Vink & Boomsma, 2005; Wyshak & White, 1965). Therefore, it is not unlikely that to some extent, genetic factors that are linked to female reproductive aging will be linked to familial DZ twinning. For example, follicular growth, maturation, and ovulation are tightly regulated via various pathways. One of these pathways is the transforming growth factor β (TGFβ) signaling pathway. The oocyte secretes various substances to control growth and ovulation rates, which include the growth differentiation factor-9 (GDF9) and bone morphogenetic protein-15 (BMP15). Mutations in BMP15 and GDF9 have been associated with increased DZ twinning and ovarian depletion in sheep (Galloway et al., 2002; Hanrahan et al., 2004). Remarkably, GDF9 mutation is also associated with increased twinning rates in humans (Montgomery et al., 2004; Moore et al., 2004; Palmer et al., 2006).

The mechanism by which the FSH is elevated in normal ovarian aging is seemingly also present in familial DZ twinning. When FSH is elevated, this is due to the presence of fewer follicles in the aging ovaries, leading to a reduced negative feedback via Inhibin B and estradiol (Lambalk et al., 1998b). Decreased inhibin levels cause a reduced suppression of FSH secretion (Lambalk et al., 2009). This allows multiple follicle growth, which is a prerequisite for DZ twinning. Older women indeed show spontaneous multiple follicle growth in up to 25% of cycles whereas this is only 5% in cycles of younger women (Beemsterboer et al., 2006). This seems paradoxical to their prominent decline in fertility. The risk of women over age 35 having DZ twins is twice that of women under 25. This overshoot of FSH may stimulate growth of multiple follicles. The risk factor for twinning probably lies in the sharp decrease in number of available follicles every month in older women and in the accompanying increase in chromosomal abnormalities in these oocytes, which are rendered non-viable. Overall, fewer pregnancies will occur, but occasionally two or more follicles with good quality oocytes are selected and so multiple pregnancy occurs (Lambalk, 2001). In summary, many mothers of DZ twins show subtle signs of limited ovarian reserve, as indicated by hormone values. Therefore, ovarian aging may play a role with familial DZ twinning and consequently genetic features responsible for ovarian aging and earlier ovarian aging could also attribute to the heredity of

DZ twinning in younger women. The fact that a number of DZ twin mothers showed no sign of limited ovarian reserve contributes to the notion that it is a trait of complex nature.

Acknowledgments

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