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Anti-Müllerian Hormone, Inhibin B, and Antral Follicle Count in Young Women with Ovarian Failure

Erik A. H. Knauff, Marinus J. C. Eijkemans, Cornelius B. Lambalk, Marianne J. ten Kate-Booij, Annemieke Hoek, Catharina C. M. Beerendonk, Joop S. E. Laven, Angelique J. Goverde, Frank J. M. Broekmans, Axel P. N. Themmen, Frank H. de Jong, and Bart C. J. M. Fauser, on behalf of the Dutch Premature Ovarian Failure Consortium

Department of Reproductive Medicine and Gynecology (E.A.H.K., M.J.C.E., A.J.G., F.J.M.B., B.C.J.M.F.), University Medical Center, 3584 CX Utrecht, The Netherlands; Department of Public Health (M.J.C.E.), Division of Reproductive Medicine (J.S.E.L.), Department of Obstetrics and Gynecology, and Department of Internal Medicine (A.P.N.T., F.H.d.J.), Erasmus Medical Center, 3000-CB Rotterdam, The Netherlands; Division of Reproductive Medicine (C.B.L.), Department of Obstetrics and Gynecology, Vrije Universiteit Medical Center, 1007 MB Amsterdam, The Netherlands; Department of Obstetrics and Gynecology (M.J.K.-B.), Amphia Hospital Langendijk, 4800 RL Breda, The Netherlands; Section of Reproductive Medicine (A.H.), Department of Obstetrics and Gynecology, University Medical Center Groningen, 9700 RB Groningen, The Netherlands; Department of Obstetrics and Gynecology (C.C.M.B.), University Medical Center St. Radboud, 6500 HB Nijmegen, The Netherlands

Context: Ovarian dysfunction is classically categorized on the basis of cycle history, FSH, and estradiol levels. Novel ovarian markers may provide a more direct insight into follicular quantity in hypergonadotropic women.

Objective: The objective of the study was to investigate the distribution of novel ovarian markers in young hypergonadotropic women as compared with normogonadotropic regularly menstruating women.

Design: This was a nationwide prospective cohort study.

Setting: The study was conducted at 10 hospitals in The Netherlands.

Patients: Women below age 40 yr with regular menses and normal FSH (controls; $n = 83$), regular menstrual cycles and elevated FSH [incipient ovarian failure (IOF); $n = 68$]; oligomenorrhea and elevated FSH [referred to as transitional ovarian failure (TOF); $n = 79$]; or at least 4 months amenorrhea together with FSH levels exceeding 40 IU/liter [premature ovarian failure (POF); $n = 112$].

Main Outcome Measures: Serum levels of anti-Müllerian hormone (AMH), inhibin B, and antral follicle count (AFC) was measured.

Results: All POF patients showed AMH levels below the fifth percentile (p_5) of normoovulatory women. Normal AMH levels ($>p_5$) could be identified in 75% of IOF, 33% of TOF patients, and 98% of controls. AFC and AMH levels changed with increasing age ($P < 0.0001$), whereas inhibin B did not ($P = 0.26$). AMH levels were significantly different between TOF and IOF over the entire age range, whereas AFC became similar for TOF and IOF at higher ages.

Conclusions: Compared with inhibin B and AFC, AMH was more consistently correlated with the clinical degree of follicle pool depletion in young women presenting with elevated FSH levels. AMH may provide a more accurate assessment of the follicle pool in young hypergonadotropic patients, especially in the clinically challenging subgroups of patients with elevated FSH and regular menses (*i.e.* IOF) and in hypergonadotropic women with cycle disturbances not fulfilling the POF diagnostic criteria (*i.e.* TOF). (*J Clin Endocrinol Metab* 94: 786–792, 2009)

Abnormal ovarian function is classified into three different subgroups, according to the World Health Organization (WHO). This classification is primarily based on serum levels of FSH and estradiol (1). FSH levels are regulated through negative feedback actions of inhibin and estradiol, produced by the ovarian follicles (2). A hypogonadotropic condition (WHO I) indicates disturbance at the hypothalamic-pituitary level, whereas a normogonadotropic oligo- or amenorrheic state (WHO II) is associated with a pituitary-ovarian dysbalance (3). In contrast, a hypergonadotropic status (WHO III) coincides with ovarian dysfunction due to follicle pool exhaustion (4).

Idiopathic premature ovarian failure (POF) represents the most extreme phenotype of diminished ovarian reserve at young age. The most frequently applied definition of POF is the spontaneous absence of menses for at least 4 months in combination with FSH levels exceeding 40 IU/liter before age 40 yr. This condition occurs in approximately 1% of the female population (5). It is not clear, however, how women below the age of 40 yr with cycle disturbances and a hypergonadotropic hormonal status who do not fulfill the strict definition of POF should be counseled with regard to fertility treatment nor whether they have a similar ovarian follicular status as POF patients. In the current study, this intermediate group is referred to as transitional ovarian failure (TOF).

Incipient ovarian failure (IOF) or late reproductive aging (stage 3) according to the Stages of Reproductive Aging Workshop classification, represents another subgroup characterized by elevated follicular phase FSH levels along with a regular menstrual cycle (6). IOF precedes the onset of cycle irregularity and hence the menopausal transition by 3–10 yr and may be considered an early sign of advanced ovarian aging in young women (7). Thorough insight into the ovarian reserve profile of this heterogeneous and clinically important group is still lacking (8).

In Western society, women are delaying starting a family until later in life. As a result, the number of female patients presenting with elevated FSH levels suggestive of reduced ovarian reserve, with or without cycle abnormalities, is increasing (9). Therefore, a more thorough insight into the ovarian phenotype of these patients is warranted. Numerous studies indicate that FSH itself cannot be used as a predictive marker for deciding to start infertility treatment or for ovarian response prediction (10). Recently more direct ovarian markers, such as anti-Müllerian hormone (AMH), inhibin B, and antral follicle count (AFC) have become available.

AMH is a product of the granulosa cells that envelop the oocyte and continues to be expressed until the antral stage (11). Inhibin B is produced by the cohort of developing preantral and early antral follicles, and its circulating concentrations are maximal during the early to midfollicular phase (12). Early follicular inhibin B levels decrease during reproductive aging leading to increasing FSH concentrations (13). Similarly, AFC decreases during reproductive aging in line with the contention that the number of visible antral follicles reflects the size of the primordial follicle pool (14). In contrast to FSH, estradiol, inhibin B, and AFC, AMH levels do not appear to vary with cycle day (15). Moreover, AMH has a superior cycle-to-cycle reproducibility compared with inhibin B and FSH (16). AMH levels show a decreasing trend with age, remaining relatively stable until age 30 yr but declining more steeply thereafter (17, 18).

Scant information exists with regard to AMH levels in patients presenting with a hypergonadotropic hormonal status at a young age. An earlier study compared patients with secondary amenorrhea with controls and identified a high percentage of very low or undetectable AMH levels in POF patients (19). Another small study identified low AMH levels as marker of diminished ovarian reserve in IOF patients with consistently elevated FSH levels (20). The present study aimed to show the relationship between several direct markers of ovarian reserve and varying clinical degrees of ovarian failure based on FSH values and cycle disturbances in women under forty.

Subjects and Methods

Subjects

From October 2004 onward, a nationwide standardized systematic screening protocol was applied for women with suspected diminished ovarian reserve visiting the infertility outpatient clinics of 10 Dutch hospitals. This protocol was approved by all local institutional review boards, and written informed consent was obtained from all participating women for standardized screening. This screening included a questionnaire regarding fertility, family history, and climacteric complaints as well as transvaginal ultrasonography and blood withdrawal.

Inclusion criteria for screening were age between 25 and 40 yr, increased FSH serum levels (>10.2 IU/liter), a history of having experienced regular menstrual cycles (26–32 d), known last spontaneous menstruation date, no current use of hormone therapy, and no history of radiotherapy/chemotherapy or ovarian surgery. Women with regular cycles applying to these criteria were screened in the early follicular phase (cycle d 2–5), whereas women without a regular cycle were screened at random and progesterone levels were measured additionally. Serum was frozen in -20 C within 4 h for further analysis. Increased baseline FSH was defined as greater than 10.2 IU/liter, which is the upper 95% reference value of the FSH assay used in the UMC Utrecht (ADVIA Centaur/Bayer Corp., Tarrytown, NY). This arbitrary cutoff was chosen because this assay was used in the control cohort, and second, a Dutch study using another assay identified an upper value of 11.2 IU/liter in regularly menstruating women below age 35 yr (21); conversion to the ADVIA Centaur assay led to a cut-off of 10.4 IU/liter.

IOF was defined as regular cycles between 25 and 35 d with elevated FSH on cycle d 2–5. TOF patients had (a history of) transformation to irregular cycles (>35 d) with FSH levels exceeding 10.2 IU/liter without fulfilling the POF criteria. POF was defined as at least one episode of secondary amenorrhea for more than 120 d (4 months) in combination with FSH greater than 40 IU/liter.

Proven fertile, regularly menstruating women from an earlier described cohort served as controls (18). Women with early follicular FSH levels less than 10.2 IU/liter between 25 and 40 yr were selected for the current study.

Methods

All serum measurements in the hypergonadotropic patients were performed in the same laboratory using the same assays in a single run. FSH levels were measured using a chemoluminescence-based immunometric assay (Immulite 2000; Diagnostic Products Corp., Los Angeles, CA). The detection limit of the assay was 0.1 IU/liter, inter- and intraassay coefficients of variation were less than 3.0 and 5.6%. Progesterone was measured on an ADVIA Centaur immunoassay system (Bayer Corp., Tarrytown, NY). Interassay coefficients were 11, 6, and 5% at 6, 30, and 95 nmol/liter, respectively. Within-run variation for values greater than 10 nmol/liter was less than 3%, and at 5 nmol/liter it was 7%. Luteal progesterone was set at a value greater than 10 nmol/liter (which coincides with the 2.5th percentile in 84 normoovulatory subjects). Levels of inhibin B were measured using enzyme immunometric kits (Oxford

TABLE 1. Baseline characteristics (mean \pm SD or percentages) of control women and hypergonadotropic patients

	Controls (n = 83)	IOF (n = 68)	TOF (n = 79)	POF (n = 112)
Age (yr)	34.2 \pm 3.4	35.2 \pm 3.1	34.0 \pm 3.9	35.0 \pm 3.7
BMI (kg/m ²)	24.6 \pm 4.3	24.2 \pm 4.7	25.4 \pm 6.2	24.3 \pm 4.2
Pack-years of smoking	4.2 \pm 6.4	3.4 \pm 6.0	3.9 \pm 5.6	4.1 \pm 6.2
Age at menarche (yr)	13.2 \pm 1.6	12.9 \pm 1.4	12.8 \pm 1.7	13.1 \pm 1.4
Flushes (percent of patients) ^a	2	8	40	71
Night sweats (percent of patients) ^a	0	14	20	51
Cycle history characteristics at time of screening				
Last menses <35 d (percent of patients)	100	100	65	10
Last menses 35–120 d (percent of patients)	0	0	22	21
Last menses >120 d (percent of patients)	0	0	14	69

BMI, Body mass index.

^a Significantly ($P < 0.001$) different between all subgroups.

BioInnovation (Oxford, UK). Inter- and intraassay coefficients of variation were less than 7.0 and 14% at 240 ng/liter. The detection limit was 10 ng/liter. AMH levels were determined using the enzyme immuno-metric assay (Diagnostic Systems Laboratories, Webster, TX). Inter- and intraassay coefficients of variation were less than 5% at the level of 3 μ g/liter and less than 11% at the level of 13 μ g/liter. The detection limit of the assay was 0.026 μ g/liter. In controls AMH levels were measured using the Immunotech Coulter (Marseilles, France) enzyme immuno-metric assay and converted to Diagnostic Systems Laboratories assay values as described earlier (15).

AFC was defined as the total number of visible round or oval, intra-ovarian transonic structures with diameter between 2 and 10 mm. Ultrasound examinations were performed by experienced fertility specialists in each of the participating centers. If one or both ovaries could not be visualized, the AFC was marked as not visible. Low AFC cutoff was set at less than five follicles because this number is associated with poor response and significantly lower rate of pregnancies in *in vitro* fertilization (IVF) (22). The menopausal threshold for serum parameters were set as inhibin B less than 10 ng/liter (23) and AMH less than 0.086 μ g/ml (24). Furthermore, lowered premenopausal AMH cutoffs were defined as less than the fifth percentile of the distribution within the normal population by age. This is for age 30 (0.3085 μ g/ml); 35 (0.2365 μ g/ml) and 40 [0.1036 μ g/ml, respectively; extracted from the original data of Ref. 24].

Statistics

Continuous variables were expressed as mean \pm SD and categorical variables as percentages. The ovarian reserve markers were logarithmically transformed in case of significant deviation from the normal distribution. This applied to AMH and inhibin B. Therefore, results for these ovarian markers were presented as medians and range. Between-group differences were tested with ANOVA for continuous parameters and χ^2 tests for categorical parameters, respectively. To assess a systematic change when moving from controls via IOF and TOF to POF, tests for linear trend were used. A separate analysis was performed to analyze the relationship between each ovarian reserve parameter and age: multiple linear regression was carried out with the ovarian reserve parameter as dependent and age and group as independent variable. Absolute differences between groups were assessed as well as in the interaction between group and age, defined by the slope of the regression line were tested.

Results

Up to January 2007, a total of 408 patients between 25 and 40 yr with idiopathic elevated FSH visited one of the participating clinics. For this study 62 current hormonal therapy users, 34 with unknown or unreliable last menses, and 25 patients with a regular cycle who were not screened in the early follicular phase

were excluded. Additionally, 28 samples were excluded because of insufficient amount or quality of serum for analysis. In total 342 women (68 IOF, 79 TOF, 112 POF patients and 83 controls) were included for the current study.

In Table 1 baseline and cycle characteristics at time of screening are outlined. No statistically significant differences existed in age ($P = 0.11$), body mass index ($P = 0.38$), age at menarche ($P = 0.36$), and pack-years of smoking ($P = 0.86$) between controls, IOF, TOF, and POF cases. Significant differences ($P < 0.001$) were identified in menopausal complaints (flushes and night sweats). By definition all control and IOF patients had experienced a spontaneous menstruation within the previous 35 d, and this was also the case in 65% of the TOF patients. Thirty-one percent of the POF patients had experienced a spontaneous bleed within the last 4 months. In 11% of POF patients, a second FSH measurement after diagnosis did not show an FSH level above 40 IU/liter (Fig. 1).

Endocrine screening (Table 2) identified luteal progesterone in 12 and 3% of TOF and POF patients, respectively. All measured mean or median ovarian reserve parameters (FSH, AMH, inhibin B, and AFC) differed between regular menstruating controls and the hypergonadotropic women ($P < 0.001$). AMH was detectable in 6% of the POF; all had AMH levels below the fifth percentile (p_5) for their age (Fig. 2). Median AMH value for IOF was 0.33 μ g/ml and 0.02 μ g/ml for TOF. Of the IOF patients 75% had AMH levels in the normal range ($>P_5$ for her age) compared with 33% in the TOF group.

When comparing the direct ovarian parameters inhibin B, AFC, and AMH (Fig. 3), the slope of the regression lines against age were significant ($P < 0.0001$) for AFC and AMH, indicating age dependency. In contrast, inhibin B levels were not significantly associated with age ($P = 0.26$) (Fig. 3). When comparing the regression lines by age for IOF and TOF, AFC failed to differentiate between these groups in the higher age groups, whereas discrimination between these two groups on the basis of AMH levels was possible at every age group: the difference became more pronounced at advanced age (Fig. 3).

Discussion

The current study describes the direct ovarian reserve markers AMH, inhibin B, and AFC in young women presenting with

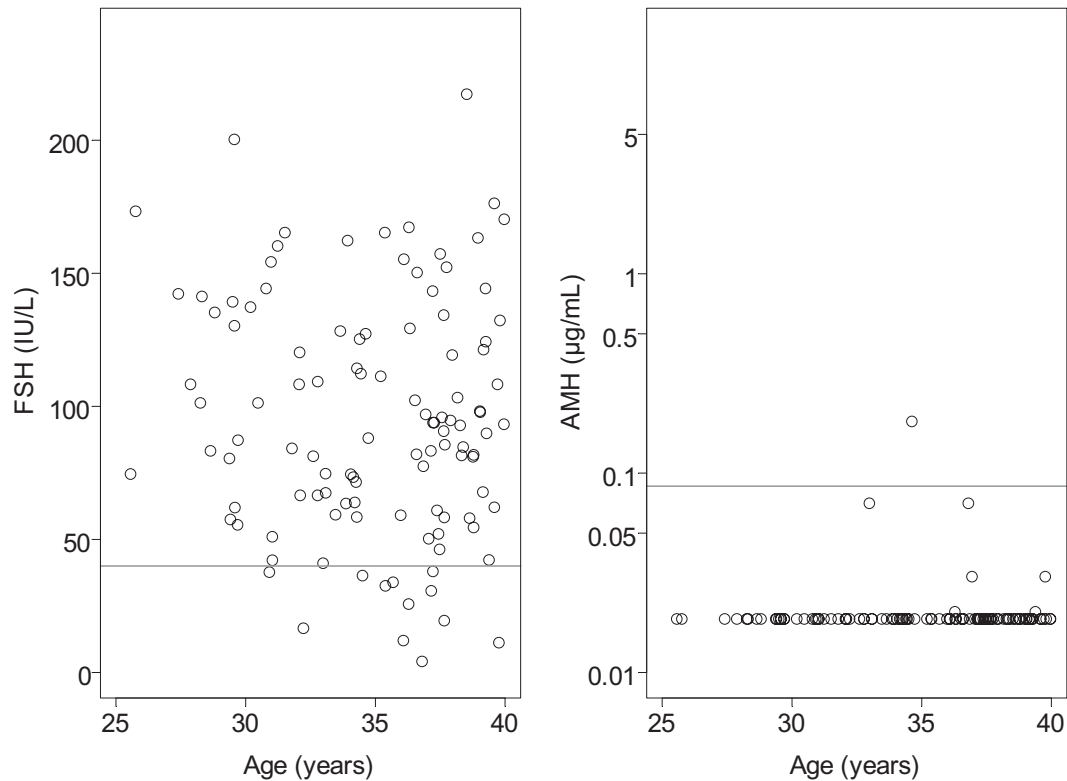


FIG. 1. FSH (second measurement after diagnosis) and AMH levels (log scale) in relation to age in 112 POF patients; the lines indicate the cutoff value of 40 IU/liter for FSH and the menopausal threshold (0.086 µg/ml) for AMH (24).

various degrees of hypergonadotropic ovarian failure. Although ovarian reserve is not well defined in the literature and no clinical end points such as successful IVF treatment and/or ongoing pregnancy are used in the current descriptive study, our data support

the application of AMH in estimating the extent of follicle pool depletion in young hypergonadotropic women.

AMH is already a proven ovarian marker with regard to reproduction in nonhypergonadotropic subjects (25). In regular

TABLE 2. Endocrine and ultrasonographic assessment at screening (means ± SD)

	Controls (n = 83)	IOF (n = 68)	TOF (n = 79)	POF (n = 112)
FSH in study batch (IU/liter)	N/A ^a	14.0 ± 7.8	29.0 ± 30.1	94.6 ± 44.7
Normal (≤10.2 IU/liter), %	N/A ^a	28	8	1
FSH >10.2 IU/liter, %	N/A ^a	72	92	99
FSH >40 IU/liter, %	N/A ^a	2	19	89
Luteal progesterone, %	N/A ^a	N/A	12	3
Ovarian reserve parameters				
AMH (µg/ml) ^b	3.51 (0.09–15.84)	0.33 (0.02–3.56)	0.02 (0.02–4.49)	0.02 (0.02–0.18)
Less than P ₅ for her age, %	2	25	66	100
Less than P ₅ at age 30 yr (0.3085), %	5	41	73	100
Less than P ₅ at age 35 yr (0.2365), %	4	24	66	100
Less than P ₅ at age 40 yr (0.1036), %	1	13	60	99
Less than mp threshold (0.086), %	0	13	58	99
Undetectable, % ^c	0	7	52	94
Inhibin B (ng/liter) ^b	93 (7–249)	85 (7–237)	17 (7–293)	11 (7–110)
Undetectable, % ^c	2	6	37	44
AFC (no. of follicles in two ovaries)	8.6 ± 5.7	4.4 ± 3.4	5.2 ± 6.8	1.2 ± 2.0
AFC <5, %	24	63	63	91
Zero follicles, %	1	7	21	37
Not visible, %	0	13	23	37

mp, Menopausal; N/A, not applicable.

^a In controls no second FSH measurement was performed because this group was selected on FSH level; mean FSH ± SD in controls was 6.3 ± 1.7 IU/liter.

^b AMH and inhibin B values were logarithmically transformed; therefore, medians and ranges are presented.

^c Undetectable levels are less than 0.026 µg/ml for AMH and less than 7 ng/liter for inhibin B.

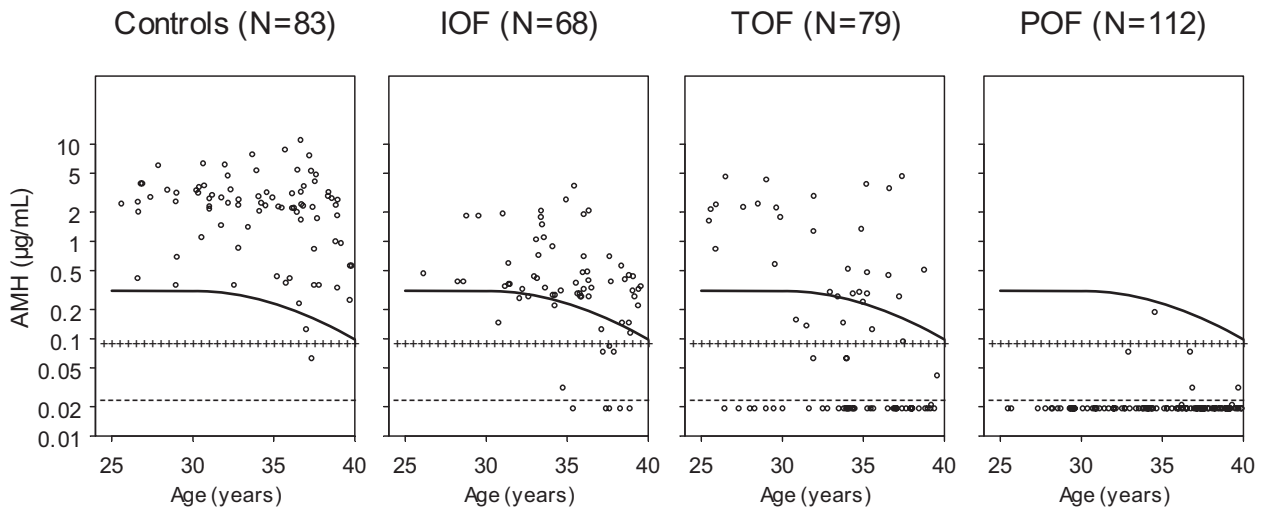


FIG. 2. Individual AMH levels (log scale) in relation to age in regularly menstruating controls and three subgroups of patients with elevated FSH levels. The *black lines* indicate the P_5 value for age. The + + + + + lines indicate the menopausal threshold value for AMH (0.086 $\mu\text{g/ml}$); the *dashed lines* indicate the lower level of detection of the AMH assay (0.023 $\mu\text{g/ml}$) (24).

menstruating women, AMH appears to be more predictive of IVF outcome than other direct ovarian markers such as estradiol and inhibin B (26). Decreased AMH levels are clearly correlated to poor response in IVF, which is a functional outcome of diminished ovarian reserve (27). AMH has also been presented recently as a useful marker of ovarian dysfunction and prediction of outcomes of intervention in other clinical conditions such as normogonadotrophic anovulation (chiefly polycystic ovary syndrome) (28), anorexia nervosa (29), or chemotherapy-induced ovarian damage (30). Furthermore, recent studies suggest that AMH levels may predict age at menopause, and in women approaching menopause, extremely low AMH levels are observed (24, 31). However, it has not yet been established whether data from the normal menopausal transition may be applied to hypergonadotrophic ovarian failure at a much younger age.

Our data in POF patients show that AMH values are consequently below the menopausal threshold and in the vast majority even undetectable, despite fluctuations in FSH levels and inci-

dental vaginal bleedings. This finding provides further evidence for the notion that infertility treatment is of no benefit in these patients with the exception of oocyte donation. In contrast, in patients with elevated FSH levels and regular cycles (IOF) or oligo/amenorrhea (TOF), we found that AMH may still be normal, suggesting the presence of a fair amount of follicles. We therefore suggest that AMH may be applied to identify women with a less abnormal ovarian reserve (*i.e.* AMH levels above the fifth percentile) and thus possibly a better reproductive potential. Young IOF and TOF patients present more frequently with normal AMH levels for their age compared with older patients ($P = 0.07$ and $P = 0.007$, respectively). This observation supports the clinical finding of a normal response to ovarian stimulation in some women with elevated FSH levels (32). When using AMH rather than FSH to differentiate between normal or diminished ovarian reserve, only a quarter of all IOF and two thirds of TOF patients would be labeled as abnormal. In other words, in 75% of IOF and one third of TOF patients normal AMH concentra-

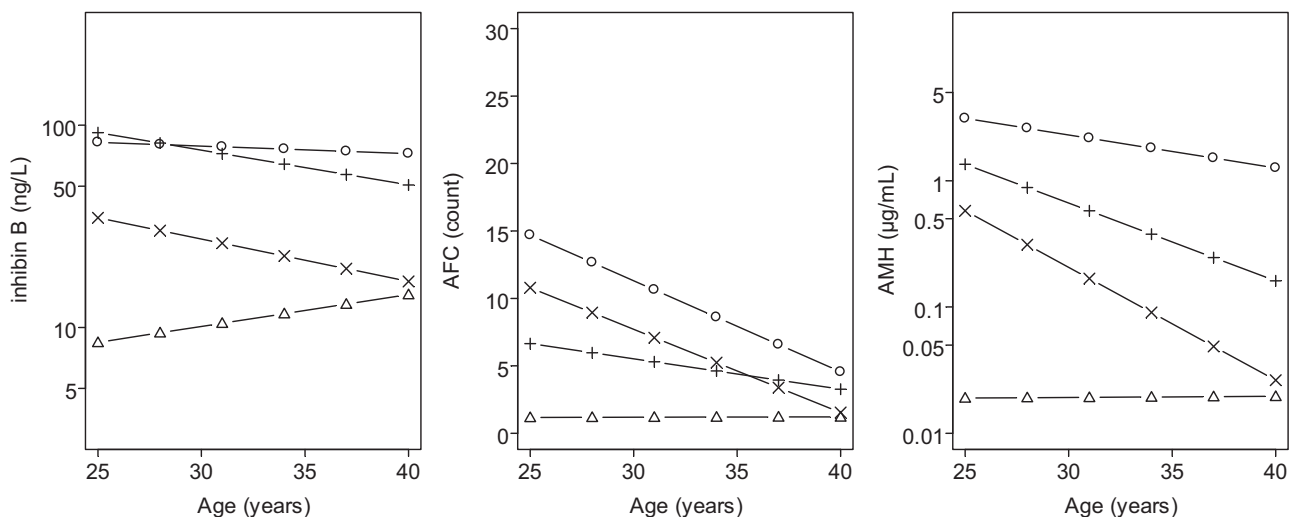


FIG. 3. Regression lines of inhibin B (log scale), AFC, and AMH (log scale) values by age for subgroups of young hypergonadotrophic women. ○-○-○, Controls; +-+-, IOF; x-x-x, TOF; Δ-Δ-Δ, POF.

tions were observed despite elevated FSH and cycle disturbances. However, before AMH can be applied clinically, longitudinal follow-up studies should prove the ability of AMH to predict clinical outcome in young hypergonadotropic patients.

Moreover, our data also suggest that AMH is more consistent than inhibin B or AFC as a measure to assess the extent of the follicle pool in these young hypergonadotropic patients, although the results of the regression analysis should be interpreted with caution given the transverse nature of the data. It is interesting, however, that AFC and AMH, which are both direct markers of ovarian reserve, differ, particularly between the IOF and TOF subjects. IOF subjects have lower (and more slowly falling) antral follicle counts than those with TOF. This finding may indicate that a milder follicle depletion pattern is present in IOF patients.

Although inhibin B is known to be decreased in older women (33) and is significantly decreased in TOF and POF patients, its capacity to differentiate between controls and IOF was absent in our cohort. Inhibin B is probably a mere marker of ovarian activity rather than of ovarian reserve. This may be due to its direct relationship with the cohort of growing small antral follicles after secondary follicle recruitment during the luteofollicular transition (12). A recent longitudinal study in a general population cohort demonstrated inhibin B was less predictive of menopause in the general population than AMH (34). Finally, inhibin B levels may also be affected by the waxing and waning of ovarian function often seen during ovarian aging as well as throughout the menstrual cycle (20).

Overall, the discriminative power of AFC to differentiate between various subgroups decreases significantly with increasing age. Earlier studies have shown strong correlations between AMH and early follicular phase AFC in the context of ovarian aging (18). Despite this correlation, AFC may reflect the active cohort of growing follicles (35) rather than the preantral follicle pool. Our current observations in hypergonadotropic patients indicate that ultrasonography is not conclusive in 26% of the patients because one or both ovaries were not visible. Moreover, AFC requires state-of-the-art ultrasound machines and experienced ultrasonographers (36).

The arbitrary cutoff value for FSH should also be considered. Different studies, using different outcomes, have used different FSH cutoff levels (10). A recent large Dutch multicenter trial (using different assays) using FSH as continuous variable identified fewer pregnancies with FSH levels exceeding 8 IU/liter (37). Moreover other factors may be involved in regulating absolute levels of FSH (21), including FSH receptor polymorphisms (38, 39). Hence, factors different from ovarian reserve may also impact on absolute FSH concentrations.

In conclusion, the current prospective, cross-sectional evaluation of ovarian reserve markers in young hypergonadotropic women indicates that AMH may represent a useful future marker to assess the extent of diminished ovarian reserve for a given patient. Moreover, our data further suggest that the classical role of serum FSH as the primary determinant for diagnosing premature follicle pool depletion, with POF as its extreme phenotype, may need to be revised. Before the widespread clinical application of AMH can be recommended, longitudinal fol-

low-up data for the general population are needed (34). It would be extremely useful to define age-dependent AMH cutoff levels. Furthermore, studies using AMH as an ovarian reserve marker for clear clinical outcome measures may improve its predictive value.

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Address all correspondence and requests for reprints to: E. A. H. Knauff, M.D., Department of Reproductive Medicine and Gynecology, University Medical Centre, Heidelberglaan 100, 3584 CX Utrecht, The Netherlands. E-mail: knauff@gmail.com.

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