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ARTICLE

Normal serum concentrations of anti-Müllerian hormone in women with regular menstrual cycles

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Abstract Anti-Müllerian hormone (AMH) has become the 'molecule of the moment' in the field of reproductive endocrinology. Indeed, it is valuable as a means of increasing understanding of ovarian pathophysiology and for guiding clinical management across a broad range of conditions. However, no normative values have been established for circulating AMH in healthy women. In this cross-sectional study, 277 healthy females (aged 18–50 years) were included. AMH was measured by commercial enzyme-linked immunosorbent assay. Serum AMH concentrations show a progressive decline with female ageing. The age-related changes in AMH were best fitted by a polynomial function. Mean AMH concentrations were not modified by past use of oral contraceptive and were independent of parity of women. Age-specific normative values for circulating AMH concentration were established. AMH concentrations seem to be independent of the reproductive history of the patient.

KEYWORDS: AMH, healthy women, normal values, ovarian reserve, reproductive period

Introduction

Anti-Müllerian hormone (AMH) is a dimeric glycoprotein member of the transforming growth factor-beta superfamily and is expressed in the growing follicles in the ovary until they have reached the size and differentiation state at which they may be selected for dominance. In the mouse, this occurs at the early antral stage in small growing follicles (Durlinger et al., 2002), whereas in the human it is evident in antral follicles 4–6 mm in diameter (Weenen et al., 2004). Thus, AMH is expressed in follicles that have undergone recruitment from the primordial follicle pool but have not been selected for dominance. AMH is not expressed in atretic follicles or theca cells (Munsterberg and Lovell-Badge, 1991). The main physiological role of AMH in the mouse ovary appears to be limited to the inhibition of

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the early stages of follicular development (Themmen, 2005; Visser and Themmen, 2005), since both in-vivo and in-vitro experiments have indicated that the transition from primordial into growing follicles becomes enhanced in the absence of AMH, leading to early exhaustion of the primordial follicle pool (Durlinger et al., 2001).

AMH is secreted by the ovary into the circulation, hence AMH is measurable in serum (La Marca and Volpe, 2006). The fact that circulating AMH appears to be solely of ovarian origin, comes from a study in which AMH was undetectable 3–5 days following bilateral ovariectomy (La Marca et al., 2005a). As serum AMH concentrations essentially reflect the ovarian follicular pool, reduction in the number of small growing follicles may be followed by a reduction in circulating AMH (La Marca and Volpe, 2006). In women, AMH concentrations are almost undetectable at birth, reach the highest values during late puberty and then show a progressive decline during reproductive life as the follicular reserve decreases, becoming undetectable after menopause (La Marca and Volpe, 2006; La Marca et al., 2005a; Van Rooij et al., 2004). AMH may constitute a unique endocrine parameter for the investigation of ovarian function, since several studies have demonstrated that, in contrast to sex steroids, gonadotrophins and peptides such as inhibin B, AMH serum concentrations do not significantly change throughout the menstrual cycle (Hehenkamp et al., 2006; La Marca et al., 2006a).

The observed relationship between the follicular ovarian pool and serum AMH concentrations indicates that serum concentrations could provide additional information (linked to the follicle dynamics) during the diagnostic evaluation of ovarian dysfunction as hypogonadotrophic and hypergonadotrophic hypogonadism (Knauff et al., 2008; La Marca et al., 2006a, 2009a; Méduri et al., 2007; Van Elburg et al., 2007) and polycystic ovary syndrome (PCOS; Fallat et al., 1997; La Marca et al., 2004; Laven et al., 2004; Pigny et al., 2003). Most importantly, AMH has been evaluated by several groups as a potential novel clinical marker of ovarian reserve and response to gonadotrophins permitting the identification of both the poor and the hyper-response to gonadotrophin administration (Fanchin et al., 2003; La Marca et al., 2007, 2009b; Muttukrishna et al., 2004; Seifer et al., 2002; Van Rooij et al., 2002).

Therefore, considering the wide use of AMH measurement in daily clinical practice and the large number of conditions in which it may be used, it is essential to establish the normal values in the healthy female population. The aim of the present study was to establish normative values for AMH in normal menstruating women and whether a relationship exists between AMH concentrations and the female reproductive characteristics.

Materials and methods

Subjects

This was a cross-sectional study aimed to evaluate normal serum AMH concentrations in normal menstruating women. Blood samples were obtained from Caucasian women volunteering for an ongoing study on the genetic determinants of the age at menopause (n = 389) and from women participating in the national programme of cervical cancer and breast cancer screening (n = 101 and 40, respectively). Blood samples from 530 women were available; however only 277 patients fulfilled all the inclusion criteria. Much of the data (n = 136) were also used in previously published studies as control groups (La Marca et al., 2005a,b, 2006a,b, 2007, 2009).

In order to be recruited for the study, the following information was essential: height and weight measurements, reproductive history, regularity of menstrual cycle, parity and current and previous contraceptive methods used. The study selected all women aged between 18 and 50 years at the time of the blood sample who had normal menstrual cycles (length 25–35 days). At the time of the blood sample, the women were not pregnant or using hormones or drugs that interfere with the menstrual cycle, had no history of hysterectomy, myomectomy, oophorectomy or any other surgery on their ovaries at the time of blood sampling. Patients included in the study had no known chronic, systemic, metabolic and endocrine disease. In particular, they did not have signs of hyperandrogenism (acne and hirsutism) nor galactorrhoea. To be included in the study, past oral contraceptive users should have stopped oral contraceptive use for at least 6 months before blood sampling. All women gave their written informed consent before the blood sampling.

AMH assay

The blood sample for AMH determination was performed on the day in which patients were recruited, independently of the last menstrual cycle. After a 12-h fasting state, blood samples were taken between 8 and 9 a.m. from the cubital vein. The blood was centrifuged at 2000g for 10 min and the serum was stored in polypropylene tubes at −80°C.

Serum AMH was measured by enzyme-linked immunosorbent assay using an AMH ELISA kit (Immunotech version; Beckman Coulter, Chaska, MN, USA). AMH values are presented in ng/ml (conversion factor to pmol/l = ng/ml × 7.143). The assay is a double-antibody sandwich type assay. In the first step, the AMH is captured by a monoclonal antibody bound to the wells of a microtitre plate. In the second step, a biotinylated antibody is added together with streptavidin-peroxidase. The biotinylated antibody binds to the solid phase antibody–antigen complex and in turn, binds the conjugate. The wells are washed and the antigen complex bound to the well detected by addition of a chromogenic substrate. The intensity of the colouration is proportional to the AMH concentration in the sample. The detection limit of the assay was 0.14 ng/ml; intra- and inter-assay coefficients of variation were 12.3% and 14.2%, respectively. The immunoassay is specific for AMH. No cross-reaction was observed with transforming growth factor-beta.

Statistical analysis

Data were analysed using the software Stata 10 (StataCorp, Texas, USA). The D'Agostino and Pearson test was used to test data for normality of distribution. When no normality of distribution was found, data were log-transformed with consequent normalisation. Regression analysis was

performed to determine whether age-related changes in AMH concentrations were best fitted by a linear, exponential or polynomial function. Where more than one function was significant, the one with the highest R^2 value was considered the best-fitting model.

Calculations of reference intervals were performed fulfilling the recommendations of the International Federation of Clinical Chemistry on the statistical treatment of reference values (IFCC-EPTRV, 1987). Briefly, log-transformation of the values to achieve normal distribution was performed. The distribution was checked against normal distribution to verify that the transformed material was normally distributed. When normal distribution of the material was achieved, subsets from this population were randomly selected and reference intervals were calculated. This calculation was performed 500 times and this forms the basis for the calculation of the reference intervals. Comparisons between the groups were performed using one-way ANOVA on log-transformed data and Student–Newman–Keuls test. $P < 0.05$ was considered statistically significant.

Results

AMH and age

AMH values were not normally distributed, hence these values were log-transformed before statistical analysis (**Figure 1**). Conversely age was normally distributed. AMH values were correlated with age and regression analysis revealed that age-related changes were best fitted by a polynomial function (log AMH = 1.0547 + (0.0546 age) − (0.0015 age^2); **Figure 2**).

The overall distribution of AMH values (median, interquartiles, 2.5th and 97.5th percentiles) in the whole population is reported in **Figure 3**. Women were also categorized in the following groups on the basis of their age: ≤30 ($n = 81$); 31–35 ($n = 58$); 36–40 ($n = 57$); 41–45 ($n = 49$); 46–50 ($n = 32$). In **Figure 4**, the corresponding lower and upper limits for AMH values according to the age groups are shown. To increase the usefulness of the

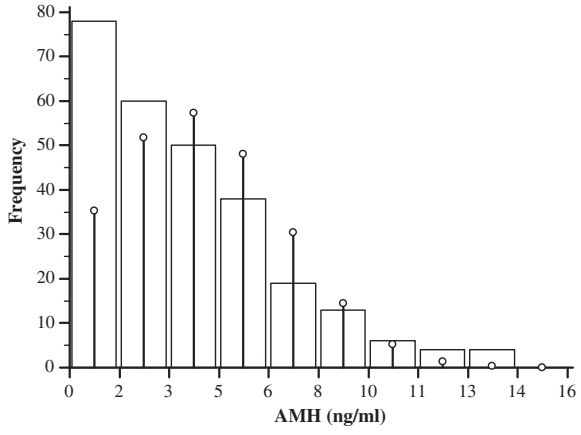

Figure 1 Distribution of serum anti-Müllerian hormone (AMH) concentrations in the study population. Data showed a non-normal distribution. Vertical lines = an ideal bell-shaped normal distribution curve.

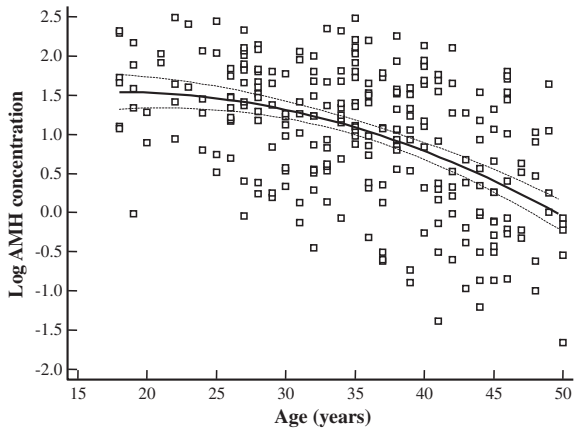

Figure 2 Correlation between log anti-Müllerian hormone (log AMH) concentration and age of women. Regression analysis revealed that age-related changes were best fitted by a polynomial function, log AMH = 1.0547 + (0.0546 age) − (0.0015 age^2). Median and 95% confidence interval are shown. $R^2 = 0.24$; $P < 0.001$.

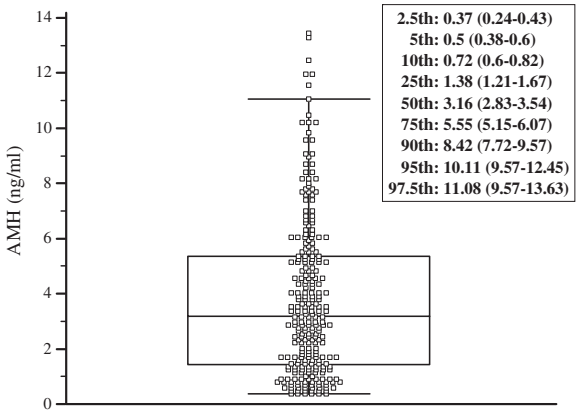

Figure 3 Serum anti-Müllerian hormone (AMH) concentrations in the whole population ($n = 277$). Bars indicate the 2.5th, 25th, 50th, 75th and 97.5th percentiles.

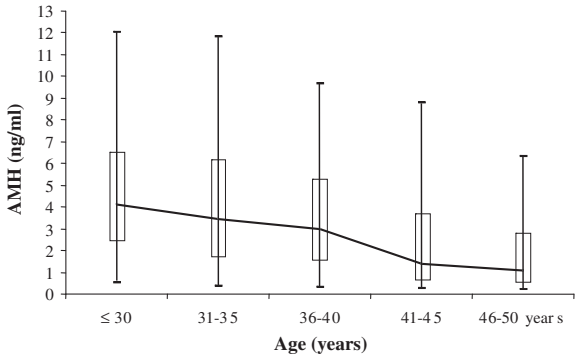

Figure 4 Serum anti-Müllerian hormone (AMH) concentrations throughout the reproductive period. Median, lower (2.5th percentile) and upper (97.5th percentile) limits and 25th and 75th percentiles are shown.

data as reference intervals, **Table 1** has been compiled with percentiles of AMH value distribution according to age and with respective 90% confidence intervals.

AMH and parity

Overall 137 (49%) patients were nulliparous, while 140 (51%) had at least one birth. AMH values were significantly lower in parous than nulliparous women (median 2.54 ng/ml, 95% CI 1.87–3.55 versus 3.49 ng/ml, 95% CI 3.04–4.07; $P = 0.02$); however, parous women were significantly older than nulliparous ones (median 41, 95% CI 39–42 versus 30, 95% CI 28–32; $P < 0.001$). This finding reflects the trend in Western countries, regarding the postponement of pregnancy to the late reproductive period. Hence analysis was limited to women older than 41, as this age has been considered as the mean for age-related female infertility (te Velde and Pearson, 2002). Parous women older than 41 ($n = 68$) had similar AMH values to nulliparous ($n = 13$) women of the same age (median 1.36 ng/ml, 95% CI 0.98–2.1 versus median 1.46, 95% CI 0.79–6.2). Moreover women over 41 and with AMH in the upper quartile for their age were compared with women with AMH in the lower quartile. No significant differences in the proportion of parous and nulliparous women and in the mean number of births were found (**Table 2**).

Table 2 Reproductive history of women over 41 years with serum anti-Müllerian hormone concentrations in the lower and upper quartile for their age.

Parameter	*AMH percentile*	
	<25	*>75*
n	18	26
Parous women (%)	16 (89)	21 (81)
No. of births/woman	1.43	1.47
Women with spontaneous abortions (%)	4 (22)	7 (27)

There were no statistically significant differences between the two groups.

AMH, oral contraceptive use and body mass index

Overall 67 (24.2%, mean age ± SD: 33 ± 9) patients had been past oral contraceptive users, while 210 (75.8%, mean age ± SD: 35 ± 8.1) women never used hormonal contraception. Serum AMH concentrations were not significantly different between oral contraceptive never and oral contraceptive past users (median 2.7 ng/ml, 95% CI 2.1–3.7 versus median 3.2 ng/ml, 95% CI 2.6–3.9, respectively). Also analysing this characteristic in the different age groups, statistical significance was not reached (data not shown).

AMH concentrations and body mass index (BMI) were significantly and negatively correlated (r −0.14; $P = 0.01$). Both variables were not normally distributed and log-transformed before analysis. Regression analysis revealed that the AMH–BMI relationship was best fitted by the following regression equation $\log \text{AMH} = 1.6387 - 0.8919 \log \text{BMI}$. However as BMI significantly increased with ageing, a stepwise multiple regression was performed, considering log AMH as a dependent variable and log BMI and age as independent variables. Analysis revealed that changes in AMH may be explained only by changes in age and BMI was not included in the model (data not shown). Hence the relationship between AMH and BMI seems to be secondary to the stronger relationships existing between age and BMI and age and AMH.

Discussion

It has been well established that with increasing age there is a decline in female reproductive function due to the reduction of the ovarian follicle pool (Macklon and Fauser, 2005). A reliable marker for the age at which subfertility will occur would have great potential value as a predictor of future reproductive lifespan. The ideal marker would show a significant change in concentrations from adolescence to the late reproductive period. Recent studies have indicated that AMH may constitute an important novel measure of ovarian reserve. Evidence for this comes from studies demonstrating that serum AMH concentrations fall throughout reproductive life (De Vet et al., 2002), with concentrations becoming undetectable after spontaneous menopause (La

Table 1 Serum anti-Müllerian hormone concentrations throughout the reproductive period.

Percentile	*Age (years)*				
	≤30	*31–35*	*36–40*	*41–45*	*46–50*
2.5	0.52 (0.11–0.96)	0.35 (0.11–0.79)	0.33 (0.26–0.51)	0.26 (0.25–0.39)	0.22 (0.18–0.31)
5	0.87 (0.5–1.27)	0.64 (0.11–1.12)	0.48 (0.26–0.6)	0.36 (0.25–0.51)	0.32 (0.2–0.43)
10	1.27 (0.96–1.68)	0.93 (0.64–1.66)	0.6 (0.48–1.37)	0.42 (0.38–0.6)	0.42 (0.35–0.5)
25	2.5 (1.7–3.26)	1.88 (1.63–2.69)	1.71 (1.25–2.42)	0.78 (0.6–0.99)	0.76 (0.53–0.91)
50	4.10 (3.61–4.98)	3.46 (2.87–4.08)	3 (2.65–4.46)	1.38 (1–1.75)	1.1 (0.8–1.41)
75	6.3 (5.45–7.58)	6.08 (4.65–7.65)	5.3 (4.59–6.67)	3.56 (1.96–5.39)	2.8 (2–3.6)
90	8.68 (7.88–10.89)	8.19 (7.63–9.97)	7.89 (6.67–13.9)	7.70 (4.68–12.29)	5.5 (3.2–9.7)
95	10.98 (8.92–12.09)	9.97 (8–34–12.44)	8.67 (7.97–12.95)	8.29 (6.55–12.61)	6.92 (4.98–8.7)
97.5	12.01 (10.27–13.08)	11.84 (10.14–12.44)	9.68 (8.45–12.75)	8.78 (7.2–12.6)	6.34 (3.9–7.5)

Values are mean concentrations of anti-Müllerian hormone in ng/ml (90% confidence intervals).

Marca and Volpe, 2006; La Marca et al., 2005a; Van Rooij et al., 2004).

As far as is known, this is the largest study aimed to investigate the changes in serum AMH concentrations throughout the reproductive period. Results clearly show that AMH concentrations fall with increasing age. AMH reduction with ageing is not linear. A trend for a reduction in AMH concentrations is also evident at a younger age.

Other than confirming the dynamic of AMH during the reproductive period, this study adds to the field the normal values for AMH for different age groups. This is particularly relevant for the clinician who until present has dealt with the interpretation of AMH measurement only on the basis of experience and on the basis of normal values reported in the AMH assay kit. Focusing on normal values for young women ($\leq$30 years), reported limits appear to be consistent with published studies (Knauff et al., 2008; La Marca et al., 2006a; Laven et al., 2004). The lower limit (2.5 percentile) is 0.52 ng/ml, under which a reduced ovarian reserve should be suspected. Reduced ovarian reserve is a condition particularly relevant in the infertility clinic. Indeed it is universally accepted that poor ovarian reserve is the main reason explaining the occurrence of poor ovarian response to FSH in the IVF setting. Patients with a poor response to FSH have an increased risk of early menopause in some years following IVF. These observations confirm the strong relationship existing between reduced ovarian reserve, reduced ovarian response to FSH and risk of early menopause (de Boer et al., 2003; Lawson et al., 2003). Several papers on AMH performance in the prediction of poor response in IVF reported values ranging between 0.3 and 0.75 ng/ml as clinically useful in the prediction of poor responders (La Marca et al., 2007, 2009b; Muttukrishna et al., 2004; Nelson et al., 2007; Van Rooij et al., 2002), hence confirming the reliability of the lower limit for AMH-assessed ovarian reserve calculated in this study. More important for the prediction of poor response *per se* is the possible application for AMH measurement in the individualization of ovarian stimulation regimens. Some authors have recently proposed adjusting the treatment strategy on the basis of AMH concentrations, which may result in reduced cycle cancellation and a trend towards increased clinical efficacy in women with low basal concentrations for this hormone (Nelson et al., 2007, 2009).

It may seem surprising to observe a small modification in the lowest AMH limit with ageing (0.35 ng/ml at age 31–35, 0.33 ng/ml at 36–40, 0.26 ng/ml at 41–45 and 0.22 at 46–50). However, this may be explained on the basis of the study design. Indeed, only women with regular menstrual cycles were included for analysis. Menstrual cycle irregularity may precede the menopausal transition by 3–10 years. In a recent study, serum concentrations of AMH in patients with elevated FSH and irregular cycles were below the 5th centile of normo-ovulatory women in 66% and were undetectable in 52% of patients (Knauff et al., 2008). Hence, excluding older women with irregular menstrual cycles from the study has prevented the lower limit reaching values close to zero when approaching the expected age of menopause.

Less clear is the clinical significance of the upper limit (97.5 percentile for women $\leq$30 years: 12.01 ng/ml). Several conditions are commonly considered to be associated with 'enhanced ovarian reserve'. A number of studies have shown serum AMH concentrations to be increased in women with PCOS compared with controls (Fallat et al., 1997; La Marca et al., 2004; Laven et al., 2004; Pigny et al., 2003). Mean serum AMH concentrations reported in the literature for PCOS patients range between 5.3 and 8.1 ng/ml (La Marca et al., 2004; Laven et al., 2004; Pigny et al., 2003), which are values to be considered normal on the basis of this study's nomogram even if they are in the upper AMH quartile.

It cannot be excluded that women with PCOS may be inadvertently included in the study, hence leading to the high upper limits for AMH at young age. However, only eumenorrhoeic women without signs of hyperandrogenism have been included in the study. The possibility that a woman with normal menstrual cycle and without hirsutism has PCOS is very remote (Azziz et al., 2009). A recent task force on the phenotype of the PCOS concluded that eumenorrhoea in the absence of dermatological features suggestive of hyperandrogenism could be used as a strong evidence of normal ovulation (Azziz et al., 2009). On this basis, the current study may conclude that there is a high probability that high AMH concentrations may also be found in normal-cycling women and thus should not be considered an exclusive marker of PCOS.

Another interesting consideration may be made on the relationship between high AMH concentrations and the risk of hyper-response and ovarian hyperstimulation syndrome (OHSS) in IVF. It seems that an 'enhanced ovarian reserve' may be associated with an increased risk of hyper-response to FSH administration. Indeed PCOS is a recognized risk factor for OHSS (Fauser et al., 2008). Similarly a high number of antral follicles (Fauser et al., 2008) and high AMH concentrations (La Marca et al., 2007; Lee et al., 2008; Nelson et al., 2007) have been associated with both hyper-response and OHSS. In a previous study (La Marca et al., 2007), all cases with ovarian hyper-response to ovarian stimulation were in the group of patients with basal AMH concentrations in the highest AMH quartile. The reported value was 7 ng/ml, which is very close to 6.3 ng/ml (75th percentile for women $\leq$30 years) found in the present study. With ageing, the proportion of women with AMH concentrations higher than 75th percentile for young women constantly diminishes and accordingly the incidence of OHSS dramatically drops with increasing age. Finally, serum AMH is a good marker for the extremes of the broad spectrum of the ovarian reserve, hence the adoption of AMH measurement to drive differential ovarian stimulation strategies seems to be appropriate (Nelson et al., 2007, 2009).

The relationship between serum AMH concentrations and reproductive history of women has been analysed. Due to the postponement of childbearing, analysis should be limited to women older than 41 (the mean for the age-related female infertility; te Velde and Pearson, 2002). The current study clearly showed that pregnancies and the number of offspring are distributed in an AMH unrelated pattern. In particular, the proportion of women with no pregnancies and the mean number of offspring were similar in the group of women with AMH concentrations in the upper and lower quartile (**Table 2**). Up to the present, it remains completely unknown whether AMH concentrations may enable age-independent prediction of an individual's fecundity in the general population. Also on the basis of these results, it may be concluded that the

application of the AMH measurement for spontaneous fertility assessment in the general population outwith the context of research studies is inappropriate.

AMH concentrations appear to be unmodified in conditions under which endogenous gonadotrophin release is substantially diminished, such as during pregnancy (La Marca et al., 2005b), under gonadotrophin-releasing hormone agonist pituitary down-regulation (Mohamed et al., 2006) and oral contraceptive administration (Arbo et al., 2007). The current results show similar AMH concentrations in oral contraceptive never and past users. The present study had a design that did not allow the identification of possible modifications in AMH concentrations during hormonal contraception. It may only be concluded that, as expected, oral contraceptive use should not be able to modify future ovarian reserve. Consistently, the age of menopause is reported to be similar in women who used or never used oral contraceptive (De Vries et al., 2001).

It has been reported that obese women show reduced concentrations of inhibin B and AMH (Gracia et al., 2005) suggesting that obesity may be associated with impaired ovarian reserve. A recent study (Su et al., 2008) examined the correlation of obesity with hormonal and ultrasound-derived markers of ovarian reserve and found that serum AMH concentrations are lower in obese women compared with age-matched women of normal weight, despite similar antral follicular count. This suggests that AMH concentrations in obese women may be lower for physiological reasons related to obesity itself and may not be necessarily indicative of impaired ovarian reserve (Su et al., 2008). In the present study, AMH concentrations in the general, normo-menstruating women decreased with increasing BMI. However, a careful analysis has highlighted an indirect relationship between AMH and BMI. Because mean BMI increased and AMH concentrations decreased with age, the relationship between BMI and AMH is secondary to their stronger relationship with age.

In conclusion, the present study established age-specific normative values for circulating AMH concentrations in the eumenorrhoeic female population. AMH measurement produces new information on ovarian pathophysiology and ovarian reserve. Whether this information is clinically more relevant than measurement of other known markers of ovarian function is still under investigation (La Marca et al., 2009c). However, the establishment of normative reference values for AMH is the first step to answer these questions in the near future.

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