Article

Anti-Müllerian hormone: clinical insights into a promising biomarker of ovarian follicular status



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Abstract

In contrast to most hormonal biomarkers of the follicular status, anti-Müllerian hormone (AMH) is exclusively produced by the granulosa cells of a wide range of follicles, from the large primary to the early antral stages, presumably FSHindependently and with little susceptibility to disorders of antral follicle growth during the luteal–follicular transition. This paper summarizes clinical research on the role of AMH as a marker of ovarian functioning. It shows that the relationship between antral follicle counts and serum AMH concentrations is stronger than that observed with FSH, inhibin B and oestradiol on day 3, and that intercycle reproducibility of AMH measurements is better than the latter parameters. In addition, peripheral AMH concentrations decline during ovarian stimulation, thus confirming that maturing follicles loose progressively their ability to produce AMH. Indeed, follicular fluid (FF) AMH concentrations in small antral follicles are 3-fold as high as AMH in preovulatory follicles. Further, human chorionic gonadotrophin-driven luteinization additionally curtails follicular AMH production. Finally, AMH production measured in FF from individual follicles is increased in women having normal follicular counts and responsiveness to ovarian stimulation. Together, these data reinforce the soundness of AMH measurements as a quantitative and possibly qualitative marker of granulosa cell activity and health.

Keywords: anti-Müllerian hormone, follicular fluid, intercycle variability, luteal phase, Müllerian-inhibiting substance, ovarian reserve

Introduction

Anti-Müllerian hormone (AMH) is a glycoprotein that belongs to the transforming growth factor β superfamily (Cate *et al.*, 1986; Pepinsky *et al.*, 1988). This molecule is chiefly expressed by the Sertoli cells in the fetal testis, where it is involved in the differentiation of the mammalian reproductive tract (Lee and Donahoe, 1993), and by the granulosa cells of ovarian follicles (Vigier *et al.*, 1984). Yet, the precise physiological role of AMH in adult women remains poorly understood. Studies in rodents have suggested that AMH might be involved in both the inhibition of primordial to primary follicle growth (Durlinger *et al.*, 2002) and the follicular responsiveness to FSH (Durlinger *et al.*, 2001). Moreover, previous experiments conducted in animals have suggested that AMH, probably via its specific type II receptors expressed in granulosa and theca cells, may reduce both aromatase activity and the number of LH receptors in FSH-stimulated granulosa cells (di Clemente *et al.*, 1992; Josso *et al.*, 1998) and inhibit testosterone production by theca cells (Teixeira *et al.*, 2001).

Ovarian AMH production is probably modulated by the degree of gonadic development, as it increases from barely detectable concentrations at birth (Rajpert-De Meyts *et al.*, 1999) to augmented, yet subtle, concentrations after puberty (Hudson *et al.*, 1990). Also, the stage of follicular growth is likely to influence AMH expression, with predominant production during pre-antral and early antral follicular stages. Indeed, AMH expression is detected in follicles at several stages of folliculogenesis (Durlinger *et al.*, 2002; Weenen *et al.*, 2004), and seems to decline during the final follicular maturation process and luteinization (Baarends *et al.*, 1995).



Data from ovarian physiology indicate that AMH exhibits at least three biological characteristics that are not shared by the conventional hormonal predictors of follicular status and that are clinically worthy. First, it is expressed in the granulosa cells of a wide variety of follicles (from large primary to early antral stages: Baarends et al., 1995; Durlinger et al., 2002; Weenen et al., 2004). Secondly, follicles that grow beyond the early antral follicle stage progressively lose their capacity to express AMH (Baarends et al., 1995), which relatively preserves AMH measurements from bias linked to early follicular development that are particularly frequent in ovarian-aged women (Klein et al., 1996). Thirdly, although the mechanisms involved in the promotion or the inhibition of AMH production by granulosa cells remain undetermined, recent clinical studies have provided support to the hypothesis that AMH production by ovarian follicles is probably FSH independent (Bath et al., 2003; Eldar-Geva et al., 2005). Therefore, peripheral AMH measurements may provide information on the activity of a larger span of follicles under little or no influence of the hormonal-follicular dynamics at the luteal-follicular transition, which is likely to spare AMH from noticeable intra- and inter-cycle variations.

Taken together, these characteristics single AMH out as a promising biomarker of ovarian follicular status. Indeed, controversies have been raised with regard to the predictability of the classical hormone triad represented by FSH (te Velde et al., 1998), inhibin B (Hall et al., 1999; Corson et al., 1999) and oestradiol concentrations (Vazquez et al., 1998) measured on day 3 of the menstrual cycle. The rationale for these hormone measurements is essentially based on the ability of early antral follicles to produce inhibin B and oestradiol in response to FSH and on the modulating action of both hormones on the FSH secretion during the luteal-follicular transition in the menstrual cycle. By definition, these hormonal tests disregard the status of other follicles that are barely or not sensitive to FSH and/or have not yet reached the antral stage, but which contribute to the functioning of subsequent menstrual cycles and to women's fertility potential (Gougeon, 1996). In addition, they are reportedly biased by confounding variables linked to the status of follicular growth and to size discrepancies of early antral follicles during the early follicular phase (Klein et al., 1996). It is conceivable that these limitations of traditional hormonal tests explain, at least in part, the noticeable variability of their results from one cycle to another (Scott et al., 1990; Brown et al., 1995; Scheffer et al., 1999; Hansen et al., 2003; Jain et al., 2003; Kwee et al., 2004), a phenomenon that contrasts with the overall short-term steadiness of ovarian follicular reserve and fertility potential (Gougeon, 1996).

Recent clinical evidence is in keeping with the hypothesis that AMH might be a more sensitive predictive parameter of ovarian status than the usual markers. Some investigators (de Vet *et al.*, 2002; van Rooij *et al.*, 2004) have demonstrated that serum AMH concentrations on cycle day 3 decrease progressively along with age and become undetectable after menopause. This suggests that peripheral AMH concentrations are a valuable parameter to monitor the relative follicular exhaustion due to ovarian ageing. Consistently, clinical data have indicated that peripheral AMH concentration, during the early follicular phase of the menstrual cycle, is a useful reflector of the number of oocytes retrieved in subsequent ovarian stimulation cycles (Seifer *et al.*, 2002). In line with this, late reports have shown that day 3 serum AMH concentrations were positively related

to pregnancy rate in IVF and embryo transfer cycles (Hazout *et al.*, 2004; Eldar-Geva *et al.*, 2005).

The present article summarizes clinical investigations that aimed at expanding the current knowledge on ovarian AMH and focuses on the following points: (i) relationship between serum AMH concentrations and early antral follicle count on day 3 (Fanchin *et al.*, 2003a); (ii) cycle-to-cycle reproducibility of serum AMH measurements (Fanchin *et al.*, 2005a); (iii) consequences of final follicle maturation (Fanchin *et al.*, 2003b), luteinization and corpus luteum formation (Fanchin *et al.*, 2005b) on serum AMH concentrations; and (iv) modulation of AMH secretion by the extent of follicular development and luteinization in a single follicle and its relationship with ovarian follicular status (Fanchin *et al.*, 2005c).

Relationship between serum AMH concentrations and early antral follicle count on day 3

The first investigation (Fanchin et al., 2003a) sought to investigate the relationship between early antral follicles and AMH concentrations against that between early antral follicles and other putative markers of ovarian function and fertility potential (serum concentrations of inhibin B, oestradiol, FSH and LH) on cycle day 3. In that study, 75 infertile normoovulating women, 25-40 years of age, were analysed. On day 3 of the menstrual cycle, all of them underwent blood sampling for serum AMH, inhibin B, oestradiol, FSH and LH measurements and ultrasound scans of their ovaries. Ultrasound scans were performed using a 4.5-7.2 MHz multifrequency transvaginal probe (Siemens Elegra; Siemens S.A.S., Saint-Denis, France) by a single operator, who was blinded to the results of the hormone assays. The objective of the ultrasound examination was to evaluate the number and sizes of early antral follicles and to calculate the mean ovarian volume. In an attempt to optimize the reliability of ovarian follicular assessment, the ultrasound scanner used was equipped with a tissue harmonic imaging system (Thomas and Rubin, 1998) that allowed improved image resolution and adequate recognition of follicular borders. Serum AMH concentrations were determined using 'first generation' ultrasensitive enzyme-linked immunosorbent assay (ELISA) (Beckman-Coulter, Villepinte, France) as described previously (Long et al., 2000). Serum inhibin B, oestradiol, FSH, and LH concentrations were determined using standard assays. Relationships were assessed by simple regression calculation.

The results showed that median day 3 serum concentrations of AMH were slightly and negatively related to age (r = -0.22, P < 0.04), but this did not apply to serum concentrations of inhibin B, oestradiol, FSH or LH (r = 0.06, r = 0.09, r = -0.13 and r = -0.02 respectively). Unlike serum concentrations of oestradiol and LH (r = -0.08 and r = 0.05), those of AMH, inhibin B and FSH were significantly related to the number of early antral follicles on cycle day 3. It is noteworthy that the relationship between serum AMH concentrations and the number of early antral follicles (r = 0.74, P < 0.0001) was significantly stronger (P < 0.001) than those between serum concentrations if inhibin B and FSH and the same parameter



(r = 0.29, P < 0.001; r = 0.29, P < 0.001 respectively). In agreement with this, serum AMH concentrations showed a stronger relationship (P < 0.001) with ovarian volume (r = 0.43, P < 0.0001) than did those of inhibin B (r = 0.11, not significant), oestradiol (r = 0.13, not significant), FSH (r = -0.27, P < 0.02) and LH (r = 0.02, not significant). Incidentally, serum AMH concentrations were related with those of inhibin B (r = 0.26, P < 0.02) and FSH (r = -0.27, P < 0.02), but not with those of oestradiol and LH.

This first study not only confirmed the tight relationship between serum AMH concentrations and antral follicle counts on day 3 (de Vet *et al.*, 2002; Pigny *et al.*, 2003), but also showed that it is stronger than that observed with the usual hormonal markers of the ovarian follicular status. These results also indicate that early antral follicles (2–12 mm) are probably a major source of AMH in adult women. The results have been corroborated subsequently (Eldar-Geva *et al.*, 2005; Muttukrishna *et al.*, 2005). Yet, the contribution of smaller follicles is supposed also to influence serum AMH concentrations. If this hypothesis is true, AMH production by FSH-independent follicles could reduce the intercycle variability of peripheral AMH measurements.

High cycle-to-cycle reproducibility of serum AMH measurements

In an effort to challenge the hypothesis that peripheral AMH measurements provide information on a larger span of follicles with little or no influence of the hormonal–follicular dynamics at the luteal–follicular transition, a subsequent study was conducted (Fanchin *et al.*, 2005a). It analysed reproducibility of serum AMH measurements and compared it with that of conventional markers of follicular status such as serum inhibin B, oestradiol, FSH and early antral follicle counts on cycle day 3 during three consecutive menstrual cycles. For this, 47 other normo-ovulating infertile women who were undergoing hormone measurements and ultrasound scans of their ovaries on day 3 during three consecutive menstrual

cycles (I, II and III) were studied. Reproducibility of hormonal and ultrasonographic results was calculated by the intraclass correlation coefficient (ICC) and its 95% confidence intervals for each parameter. The study also evaluated for each parameter the number of repeated measurements needed to reach satisfactory reliability (ICC ≥ 0.80).

During the three cycles, serum AMH concentrations were positively related to early antral follicle counts (r = 0.80, P < 0.0001; r = 0.59, P < 0.0001; and r = 0.71, P < 0.0001respectively) and with inhibin B (r = 0.32, P < 0.03; r = 0.28, P < 0.05; and r = 0.45, P < 0.001 respectively) and tended to be negatively related to FSH (r = -0.28, P < 0.05; r = -0.28, not significant; and r = -0.46, P < 0.001 respectively). Serum oestradiol concentrations were not significantly related to any other variable during the three observation cycles. As expected, patient's ages were negatively related to serum AMH concentrations during the three consecutive menstrual cycles (r = -0.32, P < 0.03; r = -0.35, P < 0.02; and r = -0.30, P < 0.04 respectively), but did not show any significant relationship with the remaining hormonal and ultrasonographic parameters.

Data on ICC values and the number of repeated measurements to achieve satisfactory reliability of the mean are summarized in **Table 1**. As shown, ICC values for AMH were significantly higher than for inhibin B (P < 0.03), oestradiol (P < 0.0001), FSH (P < 0.01) and early antral follicle count (P < 0.001), and required only one measurement to achieve satisfactory reliability of the mean.

These findings indicated that the intercycle variability of AMH concentrations was lower compared with that of conventional markers of follicular status such as serum inhibin B, oestradiol, FSH and early follicle counts on cycle day 3. This phenomenon may be attributable to the reduced susceptibility of some AMH-producing follicles to cyclic changes, and stresses the cost-effectiveness of AMH measurements in the prediction of ovarian follicular status.

Table 1. Intraclass correlation coefficient (ICC) and number of repeated measurements needed to reach reliability of the mean of each marker of ovarian follicular status during three consecutive menstrual cycles.

| Parameter | ICC | No. of repeated measurements ^a |
|---|---|---|
| Serum AMH concentrations Serum inhibin B concentrations Serum oestradiol concentrations Serum FSH concentrations Follicle count (2–12 mm) | 0.89 (0.83–0.94) ^{b,c} 0.76 (0.66–0.86) 0.22 (0.03–0.41) 0.55 (0.39–0.71) 0.73 (0.62–0.84) | $ \begin{array}{c} 1 (0-1)^{b} \\ 1 (1-2) \\ 14 (6-120) \\ 3 (2-6) \\ 1 (1-2) \end{array} $ |

AMH = anti-Müllerian hormone.

^aTotal number of measurements theoretically required to achieve an ICC value >0.80. ^bValues in parentheses are 95% confidence intervals.

^cStatistically significant compared with inhibin B (P < 0.03), oestradiol (P < 0.001), FSH (P < 0.01) and early antral follicle count (P < 0.001).



Dynamics of serum AMH concentrations during the late follicular phase and during the luteal phase of ovarian stimulation

The second part of the research on ovarian AMH focused on the influence of final follicle maturation and luteinization on the ability of granulosa cells to produce AMH. Additional insights into these aspects could provide useful information on AMH regulation in adult women and give further ground to the use of this hormone in the assessment of ovarian function.

Consequences of final follicle maturation on serum AMH concentrations

Spurred by the relative lack of information on the dynamics of AMH secretion when the ovarian follicles grow beyond the early antral stage and enter the final maturation process, a third study (Fanchin *et al.*, 2003b) was conducted. For this, ovarian stimulation constituted an attractive model, since it is characterized by the thorough transformation of small antral follicles into maturing follicles as a result of exogenous FSH. Indeed, this process is not reproduced during the menstrual cycle, in which the early antral follicle cohort remains nearly intact throughout the follicular phase and only a single follicle reaches maturation. Also, given that large antral follicles may express AMH only weakly (Baarends *et al.*, 1995), the multiplicity of maturing follicles could be instrumental in exacerbating overall serum AMH concentrations and improving their detection.

Ninety-three infertile normo-ovulating women were studied, aged from 24 to 41 years, undergoing ovarian stimulation with a timed-release gonadotrophin-releasing hormone (GnRH) agonist/FSH protocol. On the day in which pituitary desensitization was confirmed (baseline), on days 6 and 8) of FSH treatment, and on the day of human chorionic gonadotrophin (HCG) administration (dHCG), women underwent measurement of serum concentrations of AMH, inhibin B, oestradiol, progesterone, testosterone and $\Delta 4$ androstenedione. At baseline, on day 8, and on the day of HCG, ovarian ultrasound scans were performed. The objective of ultrasound examinations was to evaluate the number and sizes of ovarian antral follicles. For the purposes of the study, the antral follicles were sorted into small (3-11 mm diameter) and large (12-22 mm diameter) sizes. The choice of this threshold for defining small and large follicles was arbitrary and based on the fact that, in the menstrual cycle, the sizes of non-dominant follicles remain <11 mm, whereas only the dominant follicle develops further and matures (Pache et al., 1990).

The results of that investigation showed that serum AMH concentrations display a remarkable, gradual decrease during ovarian stimulation (baseline, 1.21 ± 0.11 ng/ml; day $6, 0.91 \pm 0.09$ ng/ml; day $8, 0.77 \pm 0.08$ ng/ml; and day of HCG, 0.53 ± 0.06 ng/ml) (P < 0.001). It is noteworthy that this phenomenon was paralleled by a decrease in the number of small antral follicles (16.6 ± 0.6 , 10.8 ± 0.6 and 4.0 ± 0.4 follicles <12 mm in diameter on baseline, day 8 and day of HCG respectively).

Furthermore, a positive and steady correlation between serum AMH concentrations and number of early antral follicles was noted throughout ovarian stimulation (r = 0.73, P < 0.0001; r = 0.65, P < 0.0001 and r = 0.73, P < 0.0001 on baseline, day 8 and day of HCG respectively). In contrast, no correlation was observed between serum AMH concentrations and the number of growing follicles (≥ 12 mm diameter) at any observation day. As expected, the remaining ovarian hormone concentrations (inhibin B, oestradiol, progesterone, testosterone and A) increased progressively and significantly in response to exogenous FSH treatment (P < 0.001), which corresponded to the concomitant increase in the number of large antral follicles (≥ 12 mm diameter).

Further, no influence of clinical indications for IVF and embryo transfer on serum AMH concentrations was observed. Similarly, the duration and dose of FSH treatment were not related to AMH concentrations. In contrast, serum AMH concentrations were positively related to the total number of oocytes retrieved, in particular at baseline (r = 0.43, P < 0.0001). Afterwards, the strength of such a relationship declined progressively (r = 0.42, P < 0.0002 on day 6; r =0.27, P < 0.03 on day 8, and r = 0.22, not significant on day of HCG). In keeping with this, there was a positive, yet weaker, relationship between AMH concentrations and the number of mature oocytes. The numbers of available and transferred embryos were not associated with serum AMH concentrations. Consistently, baseline AMH concentrations were positively related to the number of pre-ovulatory follicles (r = 0.36, P < 0.002) and serum oestradiol concentrations (r = 0.25, P <0.04) on day of HCG. These findings indicate that serum AMH concentrations decline gradually during multiple follicular maturation, probably reflecting the dramatic reduction in the number of small antral follicles due to ovarian stimulation, and confirm the scarce AMH expression by preovulatory follicles.

Consequences of follicule luteinization and corpus luteum formation on serum AMH concentrations

Beyond the pre-ovulatory stage, the possible effects of granulosa cell luteinization and corpus luteum formation on AMH production by the granulosa cells in women remained to be documented. Basic research studies conducted in rats indicated that isolated corpora lutea express much less AMH than small and large antral follicles (Baarends *et al.*, 1995). Yet, AMH concentrations measured in follicular fluids from women undergoing ovarian stimulation for IVF and embryo transfer remained detectable 32–34 h after HCG administration (Seifer *et al.*, 1993; Fallat *et al.*, 1997).

Hence, it was decided to investigate the dynamics of serum AMH concentrations during the early to mid-luteal phase in pituitary-desensitized ovarian stimulation cycles (Fanchin *et al.*, 2005b). In this work, ovarian stimulation was also used as an experimental model because it is characterized by an extensive, HCG-driven transformation of maturing follicles into multiple corpora lutea. This supraphysiological process was instrumental in amplifying the possible consequences of follicle luteinization and



corpora lutea activity on peripheral AMH concentrations. Thirty-four infertile normo-ovulating women were studied, aged 24-39 years treated with a long GnRH agonist protocol. On the day of HCG administration (day of HCG), 4 days later (HCG +4), and 7 days later (HCG +7), women underwent serum AMH, oestradiol, progesterone and HCG measurements. Serum AMH concentrations were determined using a 'second generation' enzyme-linked immunosorbent (reference A16507; Immunotech Beckman assav Coulter Laboratories, Paris, France). On the day of HCG administration, ovarian ultrasound scans were performed to evaluate the number and sizes of ovarian antral follicles. For the purposes of that study, antral follicles were sorted into three size classes: small (3-11 mm in diameter), intermediate (12-15 mm in diameter), and large (16-22 mm in diameter) follicles.

It was observed that serum AMH concentrations decreased by -64±3% from day of HCG to HCG +4. Thereafter, AMH concentrations increased by $82 \pm 28\%$ from HCG +4 to HCG +7. Serum oestradiol dynamics followed a pattern similar to that of AMH, with an initial decrease of -58 \pm 2% and secondary increase of 97 \pm 12%. As expected, serum progesterone concentrations increased rapidly from day of HCG to HCG +4, presumably due to a massive follicle luteinization. A further increase in progesterone concentrations occurred between HCG +4 and HCG +7, probablybecause of the combined effect of corpora lutea activity and exogenous progesterone administration used for luteal support of ovarian stimulation. Primary and secondary increases in progesterone concentrations were 9993 ± 1095 and 106 ± 14% respectively. Finally, following HCG administration (10,000 IU), serum HCG concentrations became detectable on HCG +4 but declined significantly on HCG +7. Primary increase and secondary decrease of HCG concentrations were 2732 \pm 318 and -78 \pm 2% respectively. Serum AMH and progesterone concentrations failed to show any relationship on HCG +4 and HCG +7. Furthermore, serum AMH and oestradiol concentrations showed a correlation only on HCG +4 (r = 0.41, P < 0.02). The magnitudes of serum oestradiol and progesterone changes from HCG +4 and HCG +7 were positively correlated with each other (r = 0.55, P < 0.001) but not with AMH concentrations.

Associations between the number of follicles in each size class and AMH concentrations on day of HCG, HCG +4 and HCG +7 are shown in Table 2. The strength of the association between AMH concentrations and follicle counts on day of HCG decreased progressively as follicle sizes increased. It is noteworthy that absolute AMH concentrations on HCG +7 and their percentage of increase from HCG +4 to HCG +7 were positively related to the number of small follicles on day of HCG (r = 0.71, P < 0.0001, Table 2; and r = -0.67, P < 0.0001 respectively), but not with the number of intermediate and large follicles. Incidentally, as expected, serum oestradiol concentrations were positively related to the total number of follicles (r = 0.44, P < 0.009) on the day of HCG administration, but the strength of such a relationship tended to decrease as the follicle size increased (small follicles, r = 0.35, P < 0.04; intermediate follicles, r = 0.32, P < 0.05; large follicles, r =0.21, not significant).

AMH secretion is modulated by the extent of follicular development and luteinization and may reflect qualitatively the ovarian follicular status

In an effort to challenge the results of the preceding study, it was decided to investigate the possible changes that occur in intrafollicle AMH concentrations along with the follicular maturation process. In addition, it still remained unclear whether increased peripheral AMH concentrations reflect exclusively the number of early antral follicles or were also due to increased per-follicle AMH secretion. Insights into this sensitive issue could help to clarify the role of AMH not only as a quantitative, but also as a qualitative indicator of ovarian follicular status.

Therefore, to investigate the possible influence of follicular maturation and luteinization on AMH secretion and the relationship between per-follicle AMH concentrations, ovarian follicular status, and responsiveness to ovarian stimulation, another study was conducted (Fanchin *et al.*, 2005c) involving 37 infertile normo-ovulating female volunteers, aged 23–41

Table 2. Associations between the number of small (3–11 mm in diameter), intermediate (12–15 mm in diameter), and large (16–22 mm in diameter) follicles on the day of human chorionic gonadotrophin (HCG) administration (dHCG) and serum anti-Müllerian hormone (AMH) concentrations on the 3 observation days (dHCG, HCG +4 and HCG +7) during the luteal phase of ovarian stimulation.

| No. of follicles | Serum AMH concentrat dHCG | tions HCG+4 | HCG+7 |
|------------------|------------------------------|----------------|-----------------------|
| Small | r = -0.63, P < 0.0001 | r = -0.02, NS | r = -0.71, P < 0.0001 |
| Intermediate | r = -0.37, P < 0.03 | r = -0.15, NS | r = -0.26, NS |
| Large | r = 0.25, NS | r = 0.10, NS | r = 0.16, NS |

NS = not significant.



years undergoing ovarian stimulation with a long GnRH agonist protocol. Within the 3 months preceding ovarian stimulation, all women underwent an ultrasonographic count of early antral follicles on day 3 of their menstrual cycles. At the end of the ovarian stimulation protocol on the day of HCG administration, the number of growing antral follicles, defined as those whose mean diameter equalled or surpassed 12 mm, was determined.

On the day of oocyte retrieval, they underwent blood samplings and follicular fluid collection from two distinct follicles whose mean diameters ranged from 8 to 12 mm (small follicles) and from two other distinct follicles whose mean diameter ranged from 16 to 20 mm (large follicles). Hormonal measurements were performed in serum and follicular fluids. Serum and follicular fluid AMH concentrations were determined using a 'second generation' enzyme-linked immunosorbent assay, as previously described (Fanchin *et al.*, 2005b). Serum and follicular fluid oestradiol and progesterone concentrations were determined using usual assays. To avoid possible bias due to follicular fluid volume variability, hormone concentrations in the follicular fluid were adjusted to its protein content (Franchimont *et al.*, 1989), and were expressed as ng/g of protein for AMH and as $\mu g/g$ of protein for oestradiol and progesterone.

Median AMH concentrations were almost 3 times as high in small follicles (111.0 ng/g of protein, range: 21.7–656.2 ng/g of protein) as in large follicles (40.6 ng/g of protein, range: 14.4–108.0 ng/g of protein; P < 0.0001). In contrast, small

follicles produced significantly less progesterone (0.11 µg/g of protein, range: 0.03–0.36 µg/g of protein; versus 0.20 µg/g of protein, range: 0.11–0.33 μ g/g of protein respectively; P <(0.0001) but secreted oestradiol concentrations (7742 µg/g of protein, range: 1186-18753 µg/g of protein; versus 8011 µg/g of protein, range: 4010–20,098 μ g/g of protein respectively) as compared with large follicles. Relationships between follicular fluid AMH, progesterone, and oestradiol concentrations are presented in Table 3. A negative correlation between follicular fluid progesterone and AMH concentrations in both small (r =-0.61, P < 0.0001) and large (r = -0.36, P < 0.03) follicles was observed. No correlation was observed between follicular fluid oestradiol and AMH concentrations in small or large follicles. Also, a positive correlation was found between follicular fluid progesterone and oestradiol concentrations in small (r = 0.34, P < 0.04) but not large follicles. In addition, a remarkable correlation between serum and follicular fluid AMH concentrations both in large (r = 0.89, P < 0.0001) and small (r = 0.48, P < 0.003) follicles was observed. In contrast, serum and follicular fluid concentrations of oestradiol and progesterone showed no correlation in either follicular group.

Relationships between follicular fluid AMH concentrations and baseline follicular status and ovarian response to ovarian stimulation are shown in **Table 4**. In both small and large follicles, follicular fluid AMH concentrations were positively related to the number of early antral follicles on cycle day 3 before ovarian stimulation (r = 0.37, P < 0.03; and r = 0.63,

Table 3. Relationships between follicular fluid anti-Müllerian hormone (AMH), progesterone, and oestradiol content in small (8–12 mm) and large (16–20 mm) follicles. Note that there is a negative and statistically significant relationship between follicular fluid AMH and progesterone concentrations.

| AMH | Progesterone | Oestradiol |
|-----------------|-----------------------|----------------|
| (ng/g protein) | (µg/g protein) | (µg/g protein) |
| Small follicles | r = -0.61; P < 0.0001 | r = -0.17; NS |
| Large follicles | r = -0.36; P < 0.03 | r = 0.01; NS |

NS = not significant.

Table 4. Relationships between follicular fluid anti-Müllerian hormone (AMH) concentrations in small (8–12 mm) and large (16–20 mm) follices, ovarian follicular status, and ovarian stimulation parameters.

| | Follicle count (3–10 mm, day 3) | Follicle count (≤12 mm, day HCG) | FSH dose required for ovarian stimulation | No. of oocytes retrieved |
|---|------------------------------------|-------------------------------------|---|------------------------------------|
| AMH (ng/g of protein) in small follicles | r = 0.37; P < 0.03 | r = 0.32; P < 0.05 | r = -0.30; P < 0.05 | r = 0.31; P < 0.05 |
| AMH (ng/g of protein) in large follicles | <i>r</i> = 0.63; <i>P</i> < 0.0001 | r = 0.45; P < 0.005 | r = -0.48; P < 0.003 | <i>r</i> = 0.56; <i>P</i> < 0.0003 |

Day HCG = day of human chorionic gonadotrophin administration.



P <0.0001 respectively), growing follicles on the day of HCG administration (≥12 mm, *r* = 0.32, *P* < 0.05; and *r* = 0.45, *P* < 0.005 respectively) and oocytes retrieved (*r*=0.31, *P* < 0.05; and *r* = 0.56. *P* < 0.0003 respectively), but were negatively related to total recombinant FSH dose required for ovarian stimulation (*r* = -0.30, *P* < 0.05; and *r* = -0.48, *P* < 0.003 respectively). The strength of relationships between follicular fluid AMH and follicular status and ovarian stimulation parameters was not statistically significantly different between the two follicular classes. In contrast, follicular fluid progesterone and oestradiol concentrations were not statistically significantly related to the same parameters. No relationship was observed between AMH concentrations and the recombinant LH dose used for ovarian stimulation.

Further, as observed for follicular fluid AMH concentrations, a positive relationship was observed between serum AMH concentrations on the day of oocyte retrieval and the number of early antral follicles on cycle day 3 before ovarian stimulation (r = 0.67, P < 0.0001), the number of growing follicles on the day of HCG administration (≥ 12 mm; r = 0.62, P < 120.0001), and the number of oocytes retrieved (r = 0.68, P < 0.0001) 0.0001). This relationship was negatively related to total FSH requirement (r = -0.54, P < 0.0006). Incidentally, as expected, the number of early antral follicles on cycle day 3 before ovarian stimulation, the number of growing follicles on the day of HCG administration (≥12 mm), and the number of oocytes retrieved were positively and statistically significantly related to each other. These parameters were negatively related and statistically significantly with the total recombinant FSH dose required for ovarian stimulation.

The results showed that, in adult women, AMH concentrations are roughly 3 times higher in small as in large follicles. That study provided direct confirmation of the hypothesis that follicular maturation and luteinization interfere with the AMH production by granulosa cells in individual follicles. Also, they showed a relationship between AMH content in individual follicles and the surrounding follicular status, as represented by the number of early antral follicles on day 3 and their responsiveness to ovarian stimulation. This indicates that a possible association exists between AMH and follicle quality.

Discussion

The present paper summarizes five clinical studies conducted to refine understanding of AMH as a promising biomarker of the ovarian status. First, it was observed that serum AMH concentrations are tightly related to early antral follicle count, with a relationship that was significantly more intense than those obtained with serum concentrations of inhibin B, oestradiol, FSH and LH (Fanchin et al., 2003a). These results not only corroborate but also expand clinical data reported previously by other investigators (de Vet et al., 2002; Seifer et al., 2002). Also, in a subsequent study (Fanchin et al., 2005a), it was shown that serum AMH measurements are more reproducible from one cycle to another than the remaining parameters of the ovarian follicular status analysed. Both of these observations may be, at least in part, explained by the putative differences in the AMH regulation as compared with that of inhibin B, oestradiol and FSH during the luteal-follicular transition in the menstrual cycle.

The results of the third study (Fanchin et al., 2003b) indicated that, in parallel with the progressive reduction in the small antral follicle cohort and the increase in the number of larger follicles. a remarkable decline in peripheral AMH concentrations occurs. They suggest that AMH is preferentially and constantly secreted by small antral follicles during ovarian stimulation, and provide support to the hypothesis that differentiation of granulosa cells during follicular growth is likely to alter their ability of expressing AMH (Baarends et al., 1995). Follicular fluid measurements corroborate this hypothesis, since small antral follicles contain 3 times as much AMH as preovulatory follicles (Fanchin et al., 2005c). The physiological mechanisms implicated in the scarce expression of AMH by larger follicles and its possible consequences on the growth and differentiation of the follicle/oocyte complex during the late follicular phase remain unknown.

In addition, mean serum AMH concentrations at the achievement of pituitary suppression were similar to those reported during the early follicular phase of the menstrual cycle (Cook *et al.*, 2000; de Vet *et al.*, 2002), thereby indicating that endogenous FSH suppression has little or no effect on AMH secretion by early antral follicles. Moreover, the observation that AMH concentrations measured after pituitary desensitization remain a useful predictor of ovarian response to ovarian stimulation opens new perspectives in the use of AMH measurements for the assessment of ovarian status.

The initial decrease in serum AMH concentrations observed after HCG administration is probably related to the putative adaptations that granulosa cells undergo during the follicle luteinization process (Fanchin et al., 2005b). The mechanisms involved in the subtle, yet significant, AMH increase observed from HCG +4 to HCG +7 are less evident. They are possibly linked to the hormonogenesis by corpora lutea and/or antral follicles during the luteal phase of ovarian stimulation. Yet, the first possibility is challenged by at least two facts. First, corpora lutea have been shown to express negligible amounts of AMH (Baarends et al., 1995). Second, the magnitude of AMH elevation was clearly dissociated from that of the putative markers of luteal activity, such as progesterone and oestradiol. Another possible explanation for the secondary increase in AMH concentrations is that it could result from the development of antral follicles during the luteal phase of ovarian stimulation. The positive relationship between the magnitude of AMH increase from HCG +4 to HCG +7 and the number of small follicles on day of HCG is in agreement with this hypothesis. Recent well designed studies have demonstrated that subtle waves of follicular growth sporadically occur during the luteal phase in the menstrual cycle despite the putative FSH suppression by luteal inhibin A and oestradiol secretion (Baerwald et al., 2003a,b).

The observed relationship between AMH content in individual follicles and the surrounding follicular status (Fanchin *et al.*, 2005c), as represented by the number of early antral follicles on day 3 and their responsiveness to ovarian stimulation, is remarkable. It indicates that peripheral AMH concentrations are not exclusively dependent on the number of follicles but also modulated by the ability of individual follicles to produce AMH. Hence, elevated peripheral AMH concentrations indicate not only that the number of antral follicles is increased, but also that each follicle probably produces more AMH individually.



This contributes to the understanding of the reported association between peripheral AMH concentrations and the ovarian fertility potential, and leads to speculation that serum AMH measurements could reflect not only quantitative but also qualitative ovarian responsiveness to ovarian stimulation.

In conclusion, the wealth of data presented in this review leads to the following assumptions: (i) the relationship between antral follicle counts and serum AMH concentrations is more reliable than that observed with FSH, inhibin B and oestradiol on cycle day 3; (ii) serum AMH measurements on cycle day 3 are more reproducible from one cycle to another than the remaining parameters; (iii) peripheral AMH concentrations decline during ovarian stimulation confirming that maturing follicles progressively lose their ability to produce AMH. This observation is corroborated by follicular fluid measurements that showed a higher AMH content in small versus preovulatory antral follicles; (iv) HCG-driven luteinization additionally curtails AMH production by granulosa cells; (v) per-follicle measurements indicate that AMH production is increased in follicles from women exhibiting a normal follicle count and responsiveness to ovarian stimulation. These data reinforce the clinical soundness of AMH measurements not only as quantitative but maybe a qualitative marker of granulosa cell activity and health. From a practical standpoint, predictability of AMH overcomes that of the usual markers (Fanchin et al., 2003a; Hazout et al., 2004; van Rooij et al., 2005). Yet, uncertainties persist with respect to the control of granulosa cell AMH production and its physiological role during the final maturation. The understanding of these key issues will be helpful to refine future clinical applications of AMH measurements in evaluating the fertility potential of women and monitoring infertility treatments. In addition, the results showing that AMH concentrations measured after pituitary suppression by GnRH agonists remain predictive of ovarian stimulation outcome may encourage further investigation on the value of AMH measurements during ovarian stimulation in the evaluation of ovarian responsiveness to exogenous gonadotrophins, follicular quality, and perhaps also embryo implantation outcome, although this latter event involves extra-ovarian mechanisms (Urman et al., 2005). Further studies, possibly looking at the fate of oocytes and embryos derived from individual follicles containing high or low AMH concentrations, are also required to verify the hypothesis that serum AMH measurements might provide not only quantitative but also qualitative information about ovarian follicular status.

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Paper based on contribution presented at the International Serono Symposium 'How to improve ART outcome by gamete selection' in Gubbio, Italy, October 7-8, 2005.

Received 19 December 2005; refereed 17 January 2006; accepted 1 March 2006.

