Anti-mullerian hormone: clinical relevance in assisted reproductive therapy

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INTRODUCTION

In France, over 1 out of every 7 couples deals with infertility, while over 60,000 new couples are referred yearly for failure to conceive [23]. To investigate the reasons for infertility, couples generally undergo hormonal tests to assess the ovarian status of the woman amongst other medical examinations. The usual parameters are FSH, estradiol, LH, and inhibin B, but none of these supply reliable and early information on a possible alteration of the ovarian reserve [13, 45].

The anti-Mullerian hormone (AMH) is a member of the TGF-β family. AMH is a glycoprotein produced by the testicular Sertoli cells and the ovarian granulosa cells (GC). In the male fetus, AMH triggers the regression of Mullerian ducts at the beginning of sex differentiation. AMH and testosterone measures can be useful in boys to detect ectopic testis [25]: AMH serum levels are closely correlated with the presence of testicular tissues while undetectable levels strongly suggest anorchism. In females, AMH synthesis begins a few days after birth, progressively increases after puberty and decreases during adulthood, becoming undetectable by menopause [35, 47]. AMH is mainly expressed by GC of preantral follicles and small growing antral follicles that constitute the ovarian reserve. Therefore serum AMH levels might be a marker of ovarian reserve.

Anti-Müllerian Hormone (AMH) is a member of the transforming Growth Factor-β (TGF-β) family synthesized exclusively by the gonads of both sexes. Over the last four years, numerous studies have examined the clinical usefulness of serum AMH levels as a predictor of ovarian response and pregnancy in assisted reproductive technology cycles. Assessment of ovarian reserve in women undergoing assisted reproduction is useful in optimising the treatment protocol. Availability of a reliable measure of ovarian reserve is essential. Currently, serum AMH level seems to be more strongly related to the ovarian reserve and to be a more discriminatory marker of assisted reproductive technology outcome than follicle-stimulating hormone, inhibin B or estradiol, which are more commonly used markers. Our study involving 69 women undergoing a cycle of in vitro fertilisation (IVF) or intracytoplasmic sperm injection (ICSI) treatment, confirmed these results. We have shown in this study that AMH is significantly correlated with the number of eggs collected and is of great interest as a negative predictive value for the success of assisted reproductive technology (ART). Further studies are needed to determine AMH cut-off values.

Key words: Anti-Mullerian hormone, ovarian reserve.
reserve and of the ovary’s capacity to respond to hormonal stimulation. Recently, several teams studied the relationship between serum AMH and ovarian reserve. They showed that AMH is a better marker than the other commonly used serum markers [13, 39].

**GENERAL DATA**

**Structure and mechanism of action**

AMH is a 140,000-dalton dimeric glycoprotein that belongs to the TGF-β superfamily. The mature protein is made of 535 amino acids and contains a short C-terminal domain that carries the biological activity and a long N-terminal domain with no intrinsic activity. Only the C-terminal region bears sequence homology with other members of the TGF-β family [16, 20, 35]. The molecular action of AMH relies on 2 types of membrane receptors: AMHR1 and AMHR2. AMHR1 is common to all the members in a subfamily of TGF-β receptors called BMP (Bone Morphogenetic Proteins) receptors. The structure and function of this receptor have yet to be clearly identified [7, 8, 20]. AMHR2 is made of 573 amino acids and has a molecular size of 82 kDa in its mature form. Its extracellular domain is the binding site for the ligand. Its intracellular domain carries the serin-threonine kinase activity, but has no autophosphorylation activity [38]. A variety of genetic alterations of the gene coding for AMHR2 have been described. They result in approximately 40% of cases of persisting Mullerian ducts [18, 32]. This rare malformation that affects fetuses with a 46XY karyotype is due to the defective action of AMH, which fails to trigger the regression of Mullerian ducts. Clinically, patients suffer from pseudohermaphroditism with virilized external genitalia but a persisting uterus and fallopian tubes [18, 36]. In such cases, there are 2 lateraled intra-abdominal testes. The deferent ducts originate in the testis, penetrate the uterine wall where they run until they reach a superior vaginal pouch that empties into the posterior urethra.

AMHR2 is only expressed in the gonads, where AMH inhibits both the development and the function of reproductive organs via paracrine and autocrine pathways [7, 8, 20].

When AMH binds to AMHR2, type 1 receptor is recruited and a heterodimer [AMHR1-AMHR2] is formed. Type 2 receptor phosphorylates the serin and threonin residues in the intracellular domain of AMHR1, inducing its kinase activity. This activates an intracellular signaling pathway that is dependent on Smad proteins [42] (fig. 1b). This signaling pathway is connected with other known signaling pathways, in particular with those that involve β-catenin and Nuclear Factor κB (NF-κB) which is involved in the mediation of inflammatory responses. These secondary signaling pathways might play a minor role in the biological action of AMH [7, 8, 19, 47, 49].

**Gene and regulation of gene expression**

AMH is encoded by a gene located on chromosome 19 (19p13). Its characteristics are well known: it weighs 2.8 kilobases and comprises 5 exons. AMH gene mutations can cause the persistent Mullerian duct syndrome previously described. As opposed to the other members of TGF-β-family which are widely expressed and have a broad spectrum of biological activities, the AMH gene is exclusively expressed in the gonads (in the Sertoli cells in males, and in the granulosa cells in females). Its expression is highly regulated. Its promoter contains a number of binding sites for transcription factors such as SF-1 and Sox-9. Some hormones such as FSH or androgens also regulate AMH expression [2, 20, 32, 35]. However, the regulation of AMH synthesis by GC is not well understood. In females, AMH expression increases moderately at puberty. Secretion then decreases progressively with adulthood, becoming undetectable by menopause. In non-menopaused females, AMH synthesis begins with initial follicular recruitment. Synthesis is intense in the granulosa cells of the growing follicles (preantral follicles and small antral follicles). Then synthesis progressively decreases as the follicles grow and differentiate (fig. 1a). GC in atretic follicles produce very little AMH, while primordial follicles and the corpus luteum produce none [7].

**Physiological activity of AMH in the ovary**

Studies in AMH knock-out mice have shown that AMH has a paracrine inhibiting role on the initial recruitment of promordial follicles: mice were born with a normal amount of follicles, but ovarian reserve rapidly decreased as a greater number of follicles were recruited and began to grow. This was confirmed *in vitro* in ovaries that were cultured with no AMH. It seems that AMH prevents early follicular depletion by inhibiting initial recruitment. Other studies have highlighted the modulating role of AMH in cyclic follicular recruitment [10]: AMH knock-out mice demonstrated more growing antral follicles than control mice, despite low levels of FSH. It appears that AMH decreases antral follicular sensitivity to FSH, thus modulating cyclic recruitment [9] (fig. 1a). This was confirmed by *in vitro* studies that showed that AMH inhibits FSH-dependent follicular growth [41]. In addition, AMH regulates ovarian steroidogenesis [41], in particular by decreasing the expression of aromatase in granulosa cells, which in turn lowers estradiol production. Last, AMH
reduces the number of LH receptors on granulosa cells stimulated by FSH [16, 48] (fig. 1b).

RELEVANCE OF AMH IN REPRODUCTIVE BIOLOGY

Literature review

Over the past 4 years, many teams have investigated the potential contribution of AMH to reproductive medicine for the information it provides on ovarian reserve and for its predictive value of ovarian response and pregnancy onset in an IVF cycle. Most of these studies now concur in showing that serum AMH levels are a very reliable marker of ovarian reserve [6, 13, 40, 44-46]. But the reports published after 2002 differ in population, objectives, recorded data and immunoassays. Table I compiles most of the literature published to date pertaining to the subject. Ovarian reserve provides an indication on ovarian status, and its evolution has a prognostic value for ovarian stimulation in IVF. Ovarian reserve indicates the pool of primordial follicles and the quality of the oocytes. It decreases with age. Because it is impossible to count primordial follicles, preantral follicles are counted as their number is correlated with that of primordial follicles. Therefore a marker of the number of preantral follicles having made the transition from the primordial pool to that of growing follicles would be a good though indirect indicator of ovarian reserve, as is the sonographic antral follicles count (AFC) performed on day 3 of a spontaneous cycle. AMH, which is involved in recruitment, is mainly secreted by small antral follicles before FSH-dependent growth begins, and might prove useful to indirectly assess ovarian reserve [1, 7, 21, 22, 27, 43]. The relevance of serum AMH measurement is very close to that of sonographic count of antral follicles, to which it is strongly correlated [6, 13, 16, 45], even though antral follicle count remains highly operator- and machine-dependent. In addition, AMH is the best hormonal predictive marker of ovarian response to stimulation: it is better correlated with ovarian reserve estimated by sonographic count than are any of the other hormonal markers, i.e., FSH, LH, estradiol or inhibin B. Thus is provides an early and precise estimation of a possible alteration in ovarian reserve [14, 17, 28-30, 39].

The interest of AMH also lies in its small variation during a cycle. Circulating concentrations of AMH in a female with normal ovulation measured at the onset of the follicular phase showed no significant difference with those measured at ovulation or during the luteal phase [5]. Therefore the slight increase in serum AMH level at the time of ovulation proves to be moderate and theoretically, serum AMH levels can be measured at any
time of the cycle. Concerning whether or not AMH production correlates with embryo development and initiation of a pregnancy, literature is less abundant and somewhat contradictory on account of the heterogeneity of populations. Silberstein et al. (2006) recently showed that serum AMH measured on the day of ovulation correlated with the quality of the embryo. This same team, along with that of Hazout et al. (2004), found that AMH was a good predictive marker of IVF outcome, while two other studies could not conclude on this same question [14, 31].

The polycystic ovary syndrome (PCOS) is a particular situation in which AMH might be of interest. PCOS is the first cause of ovulation disorders and hyperandrogenism among young women. The mechanisms that lead to anovulation are poorly understood. It seems that AMH might contribute to the follicular arrest in relation with the excess of intrafollicular steroids. One of the characteristics of PCOS is the increased number of preantral follicles, which are the main source of AMH. It is not surprising then that many studies have noted higher levels of serum AMH in women with PCOS than in women with normal ovulation [3, 4, 24, 34]. Some authors have even suggested that serum AMH should be included in the definition criteria of PCOS [33]. Another concurring finding is that PCOS patients with biological androgenism have even higher levels of serum AMH [11], a fact that could not be confirmed by another team [22].

Beyond reproductive medicine, AMH is also a tumoral marker that is both sensitive and specific of granulosa tumors. Its levels are monitored to assess treatment

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<td>van Rooij et al 2002 [45]</td>
<td>n = 130</td>
<td>— (Day 3) AMH, FSH, Inhibin B</td>
<td>— CFA, number of eggs collected</td>
<td>AMH is strongly correlated with antral follicle count (AFC) and ovarian response.</td>
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<td>Seifer et al 2002 [39]</td>
<td>n = 107</td>
<td>— (D3) AMH, E2, FSH</td>
<td>— number of oocytes retrieved and maturity</td>
<td>AMH is better correlated with ovarian response than E2 or FSH.</td>
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<td>de Vet et al 2002 [6]</td>
<td>n = 41 normo-ovulatory women</td>
<td>— (D3) AMH, FSH, CFA, Inhibin B (3 years evolution)</td>
<td>— correlation with age and AFC</td>
<td>Serum AMH level decreases with advancing age before changes occur in other aging-related variables.</td>
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<td>Muttukrishna et al 2004 [29]</td>
<td>n = 69</td>
<td>— (D5) AMH, Inhibin B, FSH</td>
<td>— ovarian response</td>
<td>Delta Inhibin B and AMH are good markers in predicting ovarian response.</td>
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<td>Penarrubia et al 2005 [31]</td>
<td>n = 80</td>
<td>— (D and Stimulation day 5) AMH, FSH, E2</td>
<td>— — AMH concentration is lower in the cancelled group.</td>
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<td>Silberstein et al 2006 [40]</td>
<td>n = 257</td>
<td>— (day of HCG) AMH and E2, (D3) FSH</td>
<td>— morphological assessment of embryos, pregnancy</td>
<td>AMH is interesting in predicting the likelihood of cancellation, but not the reproductive outcome (pregnancy).</td>
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<td>Ficicioglu et al 2006 [14]</td>
<td>n = 50</td>
<td>— (D3) AMH, FSH, E2 max, AFC, age</td>
<td>— number of eggs collected, pregnancy</td>
<td>Correlation of FSH, E2 and AMH with ovarian response.</td>
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Our results
In 2005 we led a prospective study of 69 patients undergoing ovarian stimulation for in vitro fertilization. The data we gathered on patients and ovarian stimulation included number of mature oocytes collected and IVF outcome (pregnancy or not). Our objective was to assess the prospective value of serum AMH assays in reproductive medicine for both ovarian response and outcome of the cycle and to determine, if possible, cut-off values for serum AMH.

Material and Methods
Patients (mean age 30.2 years) were following a lengthy treatment (daily agonist) and underwent extensive hormonal testing prior to being included. FSH, LH and estradiol levels were assayed on day 3, and only patients with normal levels were included (mean FSH ± SD=6.1 ±1.3 UI/L). Serum AMH was measured in each patient on day 0 of gonadotrophic stimulation, when pituitary desensitization was confirmed both by ultrasonography and hormonal levels. AMH was assayed using the Immunotech® ELISA kit 2nd generation.

AMH and ovarian response
Ovarian response was estimated by the number of mature oocytes collected before being inseminated or microinjected. We found that serum AMH and the number of mature oocytes collected were positively correlated (fig. 2a). Note that serum FSH levels were significantly less correlated with ovarian response (fig. 2b). This concords with literature [13]. We then divided patients into 3 categories according to oocyte collection criteria that are commonly used in reproductive medicine: (1) “adequate” ovarian response with over 10 oocytes collected (2) “intermediate” ovarian response with 6 to 9 oocytes collected and (3) “inadequate” ovarian response with less than 6 oocytes collected. Figure 3 depicts serum AMH concentrations in all 3 categories. Serum AMH was significantly higher in group (1) with adequate ovarian response (Kruskall Wallis test followed by Dunn’s test, p<0.05). If the “adequate” and “intermediate” ovarian response groups are pooled (total of 60 patients), a serum AMH norm can be defined for patients with acceptable ovarian response. According to our results, this norm was 1.5 to 9.9 μg/L (10,7 à 70,7 pmol/L). It was interesting to note that the 4 patients with AMH levels under 1,30 μg/L (9,3 pmol/L) had inadequate ovarian response, in fact stimulation was interrupted for 2 of them.

AMH and pregnancy
The final criterion that defines the success of a stimulation cycle is the onset of pregnancy. In our cohort, 23 patients became pregnant (33%). Our results did not pinpoint a significant difference in serum AMH between both groups of patients, with levels that reached 4.15 vs. 4.17 μg/L in the non-pregnant and pregnant groups, respectively. However we did find that pregnant patients had a serum AMH over 1.40μg/L (10 pmol/L) at the beginning of stimulation, while the 46 patients that did not become pregnant had AMH levels under 1.40μg/L (fig. 4). This leads us to suggest that serum AMH concentrations lower than 1.40μg/L with the
DISCUSSION AND CONCLUSION

One of the areas that needs to be improved in the management of IVF couples is our capacity to assess ovarian response and to predict ovarian response to stimulation in order to optimize treatment and avoid useless cycle interruptions when there is no chance of success. AMH appears to be a promising marker that might contribute to better assess the ovarian situation before beginning treatment cycles.

Our results are in agreement with the corpus of data found in literature [17, 29, 39, 45] and clearly signify that AMH is a good indicator of ovarian reserve and has good predictive value for ovarian response. We suggest a normal range for serum AMH levels: 1.5 – 9.9µg/L or 10.7 à 70.7 pmol/L in patients with adequate ovarian response, though it will need to be refined with larger cohorts. The superiority of AMH over other hormonal markers (FSH, estradiol or inhibin B) and over chronological age in terms of pertinence and precocity now seems to be widely accepted [13]. The sonographic AFC appears to be the only other test that is as informative as serum AMH concentration on ovarian reserve and prediction of ovarian response. The decisional process that will determine when to use of each one of these two tests still needs to be established, even though they might be more complementary than redundant as they both provide specific information. But though sonographic count is of undeniable interest, its results must be tempered by the fact that they are highly operator-dependent.

Very few studies have investigated the correlation between serum AMH levels and cycle outcome (pregnancy or not), and their results diverge [14, 17, 31, 40]. It appears however that serum AMH concentrations are not quantitatively correlated with outcome, but that there is a cut-off value that might predict the chances of becoming pregnant (and not only ovarian response), which would be a very useful clinical tool. We showed that there was a threshold at 1.4 µg/L (10 pmol/L) under which the probability of becoming pregnant is very low. The size of our cohort was small, but another recent study found a similar cut-off value of 1.10 µg/L (7.8 pmol/L) with the Immunotech® kit [17]. The Eldar-Geda et al. team found that a serum AMH concentration of 18 pmol/L (2.5 µg/L) was associated with a chance of becoming pregnant that reached 67%. Chances of becoming pregnant for a patient with serum AMH levels below 18 pmol/L were lower (39%) [12].

But we can only advocate caution as to the use of this cut-off predictive value because of the few studies that have been published on the subject, their heterogeneity, and overall because of the small total number of patients included. At this point there is no consensus as to the day AMH should be measured, even though it seems preferable to include it in the hormone tests performed

Figure 3: Distribution of AMH values in the 3 groups according to the ovarian response. The horizontal bar indicates the average.

Figure 3 : Répartition et moyenne de l’AMH sérique dans les 3 groupes en fonction de la qualité de la réponse ovarienne.

Immunotech® kit on day 0 of stimulation is of bad prognosis for the onset of pregnancy.
on day 3 of a spontaneous cycle. Also, there are several kits for AMH assaying and they all use different standards [15]; there is no international standard yet. Therefore results must be interpreted in light of the kit that was used to measure them, and cut-off values suggested in literature can obviously not be transposed to other kits. That is why the French Society of Clinical Biology is currently establishing a work group on gonadal peptides that will strive to establish normal values. Once the kits are standardized, studies of larger cohorts will need to be set up to determine cut-off values that are so crucial in daily decision processes. This should help physicians integrate serum AMH assay into the routine hormone testing and sonograms that are run in reproductive medicine. But the interpretation of results will clearly not be identical for every patient: age, history, sonogram, and other hormonal results will necessarily modulate clinical interpretation. Therefore studies of well-characterized homogeneous sub-populations are needed to enhance the pertinence of the information provided by such a promising marker in reproductive biology.

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