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ASSISTED REPRODUCTION

Ovarian reserve evaluation: state of the art

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Abstract

Purpose Revise role of hormonal basal and dynamic tests, as well as ultrasonographic measures as ovarian reserve markers, in order to provide better counseling to subfertile couples.

Methods Review of publications on the topic, with an emphasis on recent well designed articles.

Results Currently available ovarian reserve tests do not provide sufficient evidence to be solely considered ideal, even for premature ovarian senescence patients who do not present subfertility complaints. However, these markers occupy important place in initial approach to treatment of subfertile couples, predicting unsatisfactory results that could be improved by differentiated induction schemes and reducing excessive psychological and financial burdens, and adverse effects.

Conclusions In order to remedy the limitations due to the scarcity of strong evidence about this topic, future studies should try to clarify predictive value of markers in groups of specific diseases-related subfertility and pay special attention to propaedeutic multivariate models including anti-Müllerian hormone and antral follicle count.

Keywords Infertility · IVF/ICSI outcome · Ovarian reserve · Reproductive aging · Reproductive potential · Ovarian senescence

Capsule The key to better counseling subfertile couples may reside in joint analysis of distinct ovarian reserve markers, providing information needed to formulate adequate stimulation protocols.

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Introduction

Since the mid-twentieth century, when the progressive loss of follicular population between 30 and 40 years of age was estimated to be of approximately 75% [1], more attention has been devoted to the reproductive potential of women in the presence of ovarian ageing. Considering modern trends of maternity postponement [2] and the increasing demand for assisted reproduction techniques (ART), the evaluation of functional ovarian reserve has arisen in an attempt to better counsel interested couples and guide the elaboration of stimulation protocols, with a reduction of emotional and financial burdens of a hard and stressful process.

Although follicular exhaustion is considered to be a concrete fact starting in the fourth decade of life, the changes mainly occur in the gonadal environment and many women in this age range continue to have apparently normal menstrual cycles, with a consequent great challenge for the identification of those with a lower reproductive potential [3]. Thus, a test for the evaluation of ovarian reserve would be ideal if it permitted to infer the size and quality of the follicle pool remaining in the ovaries after a normal menstrual cycle and therefore to identify candidates

for ART with a lower chance of obtaining a pregnancy, even after repeated attempts.

Within this context, serum and ultrasonographic markers have been enthusiastically tested to infer the gonadal reserve of women, but without a consensus about their relevance for use in the evaluation of ART candidates. Although the low invasiveness of these tests renders them reasonably attractive, they may not always reflect the complex follicular dynamics and none of them has been shown to be strongly correlated with the population of primordial follicles remaining in the gonads. In other words, these tests may not reveal the pool of still inactive follicles responsible for the continuity of ovulatory cycles and therefore for the long-term reproductive potential [4–6].

Broekmans et al. recently published a systematic review of the literature demonstrating that the predictive properties of the tests available for the evaluation of ovarian reserve were modest and that their use was still insufficient for clinical needs [7]. In the present report we shall deal with the tests commonly used for the evaluation of ovarian reserve in patients who are candidates for assisted reproduction cycles, dividing them into static markers—measured during the early follicular phase (estradiol, follicle-stimulating hormone, inhibin-B, and anti-Müllerian hormone), dynamic markers (tests of stimulation with clomiphene citrate, gonadotropins and gonadotropin-releasing hormone analogues) and ultrasonographic markers (antral follicle count and ovarian volume).

Static markers

Basal estradiol

Determination of basal serum estradiol (E2) has been proposed as a predictor of the ovarian response in assisted reproduction (AR) cycles, and therefore as an indirect determinant of functional gonadal reserve. This hypothesis was supported by the study of Evers et al., in which hormonal levels >60 pg/mL were found to be able to predict a higher cancellation rate and a smaller number of aspirated oocytes compared to lower levels, leading to the conclusion about the potential of basal E2 as an important prognostic determinant [8]. Fratarelli et al. supported this hypothesis by demonstrating a higher rate of cycle cancellation among patients with basal levels <20 or ≥80 pg/mL [9].

Many other studies, however, have failed to support the clinical applicability of basal serum E2 for the prediction of ovarian reserve, and even studies favorable to its use for this purpose were unable to demonstrate a significant correlation with follicular development [9] or to predict the occurrence of pregnancy [10–12]. It has also been recently demonstrated that basal E2 levels did not differ significantly between patients who were poor and good responders to AR cycles [13]. This had already been proposed two decades ago by Lee et al., who showed no significant differences between women aged 24 to 52 years in relation to hormone levels [14].

In view of the low predictive accuracy and the lack of standardization of a cut-off with high sensitivity and specificity, the use of basal E2 should be avoided as a determinant of the inclusion of patients in AR programs [7]. Thus, the role of this marker is limited to that of a modest prognosticator of response to exogenous gonadotropin stimulation and it should be used strictly for purposes of initial counseling of subfertile couples who are candidates for ART, specially when follicular growth is observed before stimulation start.

Follicle-stimulating hormone

Basal follicle-stimulating hormone (FSH) appears to be the test most frequently used to determine ovarian reserve, being contemplated in the broadest collection of well-designed studies published in the literature on this topic [7].

Watt et al. evaluated basal FSH as a predictor of success in cycles of in vitro fertilization (IVF) for women older than 40 years and determined a basal cut-off of 11.1 mIU/mL for the non-occurrence of pregnancy. However, they did not evaluate the follicular response properly, with possible interference of male factor as determinants of treatment success [15]. In our service, after assessing the response of women older than 30 years to AR cycles, we observed that basal serum FSH (with a cut-off of 10 IU/mL) had sensitivity, specificity, and positive and negative predictive values of 87%, 100%, 100% and 94.7%, respectively, regarding the growth of at least four follicles, what means indication of a good response [16].

In a study on 212 patients submitted to IVF cycles, Ashrafi et al. observed that women with FSH levels ≥15 IU/mL had fewer aspirated oocytes and a larger number of canceled cycles than women with lower levels, with no significant difference in gonadotropin doses administered [17]. In partial agreement, Klinkert et al. associated even lower pregnancy rates with high basal FSH levels, although they did not detect the same cut-off value [18].

Although day 3 FSH is widely used as an ovarian reserve test, its accuracy in predicting a poor response is adequate only when very high thresholds are used. Clinically, we observe a low frequency of such thresholds, as the value of FSH usually maintains within normal ranges and tend to rise only when ovarian function is deeply compromised, so its level cannot be used as a criterion of couple exclusion from AR cycles [7]. In a previous study, van Montfrans et al. had already suggested that determina-

tion of basal FSH should not be added to the initial management of subfertile women with clinically normal cycles, with pregnancy occurring in about half the women with elevated serum levels [19].

The few studies published over recent years were unable to validate basal FSH as a marker of ovarian reserve, but opened some new perspectives. The study by Letterie et al., for example, pointed out the different molecular isoforms of FSH secreted as the result of variants in the process of glycosylation influenced by hormonal variations depending on cyclicity and age of the patient. After chromatographic separation of the FSH isoforms, the authors detected a significant difference in the pH range from 5.1 to 5.0 between poor and good responders to previous gonadotropin stimulation. On this basis, they raised the hypothesis that concentrations of certain FSH isoforms may interfere differently with follicular dynamics and reduce oocyte quality, suggesting new possibilities about the follicular response to an exogenous stimulus [20].

van der Steeg et al. studied predictive value of basal FSH for spontaneous pregnancy occurrence in ovulatory subfertile women younger than 40 years and observed reduced chances when the levels exceeded 8 IU/L, whereas no association could be determined for lower levels [21]. The controversy continued with the study by Luna et al., in which pregnancy rates for women younger than 35 years and with high FSH levels were better than those for older women with normal levels of this gonadotropin. The authors recommended a more careful counseling for patients aged 35 to 40 years, especially regarding the larger number of canceled cycles and the lower ovarian response to the stimulus, but did not consider a high FSH level to be an obstacle in AR programs [22].

Thus, even though basal FSH still is the most frequently used marker of ovarian reserve in AR services all over the world [23], a search for more representative results has shifted the emphasis to other markers such as inhibin-B, anti-Müllerian hormone (AMH), initial antral follicle count (AFC) [5, 7, 24], and ovarian volume [3].

Inhibin-B

The inhibins are glycoproteic hormones of the superfamily of transforming growth factors β (TGF-β) [25] secreted by granulosa and theca cells [26], which are selectively responsible for pituitary inhibition of FSH secretion [27]. Inhibin-B is particularly outstanding in the execution of this function and in its paracrine action on developing follicles, stimulated by the association of FSH itself with insulin-like growth factor I (IGF-I) [28, 29].

In normal ovulatory cycles, the serum concentration of inhibin-B is inversely correlated with FSH concentration and increases insidiously up to the mid-point of the follicular phase, when it reaches a maximum peak together with the mass of granulosa cells. A progressive decrease occurs thereafter to low concentrations that persist in the luteal phase, except for a brief new elevation after the LH surge [30]. This behavior along the cycle permits us to assume that inhibin-B plays a role in follicular development, reflecting ovarian function and follicular reserve, thus acting as a marker of the functional reserve of the gonad.

Studies by Seifer et al. and Hofmann et al. generated good perspectives regarding the use of inhibin-B for the prediction of ovarian reserve. The first authors demonstrated greater estrogen responses and number of oocytes obtained after stimulation among women with serum inhibin-B levels ≥45 pg/mL, whereas cancellations were three times more frequent among patients with lower levels [31]. The second group comprised women with a supposedly adequate ovarian reserve based on the test of stimulation with clomiphene citrate and demonstrated that inhibin-B levels were more elevated among women with a normal response [32]. Tinkanen et al. investigated infertile women aged 24 to 40 years and detected a significant negative correlation between serum inhibin-B levels and FSH, as well as a significant positive correlation between inhibin-B and initial antral follicles counted by ultrasound [33].

Other studies, however, have not reproduced the results favorable to the use of inhibin-B as a marker of ovarian reserve [34–36], which remains a subject to be studied further. No studies have confirmed what was postulated more than one decade ago by Franchimont et al. and Fowler et al., who proposed a possible association of inhibin-B concentrations with oocyte quality in AR cycles [37, 38].

Regarding supposed potential in prediction of ovarian response, it has been categorically stated that the high rate of false-positive results in the determination of basal inhibin-B would lead to the unnecessary exclusion of women from IVF programs. It has been also pointed out that, even using very low levels, the accuracy in the prediction of poor response would be only modest, with inhibin-B being inferior to other markers currently used, even though it can be used as a tool for counseling [7].

Anti-Müllerian hormone

Anti-Müllerian hormone (AMH) is also a glycoprotein hormone of the TGF-β superfamily, known to be produced by testicular Sertoli cells and to be responsible for the regression of the paramesonephric ducts during the sexual differentiation of male human embryos [39]. Absent during female differentiation, AMH is expressed in granulosa cells as soon as the first primordial follicles are recruited, at about the 36th week of intrauterine life [40], and at higher concentrations starting at puberty [24]. After activation of the hypothalamus–pituitary–ovarian axis, its expression is

maintained until the follicles reach about 6 mm in diameter, when the differentiation into antral follicles itself is enough for dominance [41] and follicular growth follows controlled by FSH action [42].

The biological role of AMH in women is still unclear but recent data suggest that it may act as a modulator of follicle recruitment and a regulator of ovarian steroidogenesis [24, 43]. AMH is known to have an inhibitory effect on the population of primordial follicles, acting on pre-granulosa cells in order to limit the number of recruitable follicular units [42]. The greater sensitivity of follicular cells to the action of FSH in the absence of AMH, demonstrated both in vitro and in vivo, supports the hypothesis that this hormone acts as a decisive factor in permitting the FSH-dependent growth of ovarian follicles [43, 44]. di Clemente et al. supported this hypothesis by demonstrating reduced expression of aromatase and LH receptors in granulosa cells cultured in the presence of exogenous AMH [45].

The inhibitory function of AMH was also demonstrated in animal studies comparing wild to AMH-knock-out mice, demonstrating that the latter species presents three times greater quantities of growing ovarian follicles as well as an early depletion of the follicular population [43, 46]. Thus, it has been postulated that inhibition of sensitivity to FSH by AMH may be an important factor in follicular selection and that progression to antral follicle may occur due to the variable expression of AMH receptors among recruited units, with the persistence of those having a greater sensitivity to FSH (lower AMH expression), until reaching single dominance [44].

AMH determination has been proposed in clinical practice for the prediction of ovarian reserve because it signals pool of inactive and initially growing follicles, it means, the stock of primordial follicles [4, 47]. In other words, AMH is considered to be a marker that can estimate the quantity and activity of retrievable follicle units in early stages of maturation, thus being more reliable for the prediction of ovarian reserve [24, 36, 42, 48–51].

Compared to FSH, inhibin-B and E2, AMH has the advantage of reduced variability of its serum concentrations along the menstrual cycle [3, 6, 52], with consequent credibility, uniformity of evaluation and malleability regarding the time of determination. Using an enzyme immunoassay (ELISA), Elgindy et al. detected mean values of 1.4±1.1 ng/mL, 1.43±1.08 ng/mL and 1.35±1.02 ng/mL in follicular, ovulatory and midluteal phases, respectively [3], corroborating results of previous studies and confirming the absence of an effect of FSH or LH on AMH production [52, 53]. Similarly, Tsepelidis et al. obtained a mean of 2.4±1.1 ng/mL along the menstrual cycle in normo-ovulatory [54].

The reproducibility of AMH between cycles was demonstrated by Fanchin et al., who studied its behavior in subfertile women aged 20 to 40 years and detected lower variations in serum levels between consecutive cycles than those detected for FSH, inhibin-B and estradiol, in addition to antral follicle count (AFC). In that study, there was a positive correlation of the response to ovulation induction with initial AFC, but this marker was found to be more susceptible to variations between cycles on a short-term basis [6].

In addition to the studies cited above, other investigations have provided evidence indicating that AMH really is a good serum marker of gonadal reserve. de Vet et al. demonstrated a decline in AMH levels after menopause in women with previously regular menstrual cycles and also detected a significant correlation between serum AMH levels and ultrasound AFC [55]. In AR cycles, van Rooij et al. detected reduced pre-induction AMH levels and number of antral follicles in poor responders (women with canceled cycles or from whom fewer than four oocytes were aspirated) compared to women with a good response to the exogenous stimulus. There was also a strong correlation between the two markers, who were found to be important predictors of the response [48].

Muttukrishna et al. prospectively evaluated women older than 38 years with a basal FSH >10 UI/L, who were previously poor responders (fewer than four follicles with a diameter >15 mm). When comparing women with complete and incomplete AR cycles, they detected significantly elevated FSH levels and reduced AMH and inhibin-B levels in the second group. AMH was considered to be the best single marker of the ovarian response to the exogenous stimulus [56]. One year later, the same group restated that AMH is the biochemical marker that best predicts the response in high complexity AR cycles and the cancellation of ovulation induction, with 87% sensitivity and 64% specificity, for a cut-off of 0.2 ng/mL [51].

Tremellen et al. constructed a curve for AMH and demonstrated that the hormone decreases after 30 years of age, a fact not observed at the same intensity for FSH, which increases in an insidious manner. They also detected a significant difference in mean AMH levels between poor and good responders (≤4 and ≥8 aspirated oocytes, respectively). Evaluating poor response prediction potential, they detected 80% sensitivity and 85% specificity, with positive and negative predictive values of 67% and 92%, respectively, for the cut-off point of 8.1 pmol/L, and concluded that AMH is more sensitive than habitual markers [5].

Despite these data, a systematic review by Broekmans et al. identified only two well-designed studies involving AMH and the prediction of the ovarian response, a fact that did not permit to consider AMH to be superior to other tests [7]. Since then, many other studies have been conducted on the role of the hormone in the evaluation of ovarian reserve.

In a study by Fiçicioglu et al., serum AMH levels were significantly higher in women with good ovarian response to induction (0.67±0.41 pg/mL) compared to poor responders (0.15±0.11 pg/mL) [13], supporting the results of previous studies [3, 48]. In the prediction of low number of oocytes aspirated, basal AMH presented the largest area under the curve (AUC=0.92) among all variables tested, followed by AFC (AUC=0.78) and age (AUC=0.63). Considering the cut-off value of 0.25 pg/mL, the sensitivity of AMH was 90.9% and its specificity 90.9%, with positive and negative predictive values of 96.8% and 76.9%, respectively [13]. La Marca et al. ratified the use of AMH for the prediction of a poor response by demonstrating 80% sensitivity and 93% specificity in AR cycles when a threshold of 0.75 ng/mL was considered [57]. The same conclusion was reached with higher thresholds [58], with the lack of a cut-off combining satisfactory sensitivity and specificity for routine use continuing to represent a considerable drawback.

Attempts have also been made to correlate serum AMH levels with the occurrence of pregnancy in AR cycles. Values higher than 2.7 ng/mL were associated with higher rates of implantation and pregnancy (although not significantly so) and the levels measured on the day of human chorionic gonadotropin (hCG) administration were superior (AUC=0.647) to those of basal FSH regarding the prediction of embryo quality [23]. Recent studies carried out to determine the value of AMH as a marker of the response to the stimulus were unable to establish its value as a predictor of pregnancy [58].

In view of the scarcity of studies and of the lack of standardization of a cut-off with high sensitivity and specificity, it is still too early to determine the real importance of AMH in the prediction of a response and of ovarian reserve. However, based on the favorable results reported and on the proven significant correlation with important variables such as AFC and the quantity of matured or aspirated oocytes [13, 57], we believe in a promising future for this marker.

Dynamic markers

Clomiphene citrate challenge test

The clomiphene citrate challenge test (CCCT) was first described by Navot et al. more than two decades ago [59] and, together with the determination of basal FSH, is one of the tests most extensively used to predict the ovarian response [60]. The physiological explanation of the test is based on the property of clomiphene to antagonize estrogen in its pituitary receptors, simulating temporary estrogen deprivation, with a compensatory increase in FSH and in follicle retrieval. In patients with a good ovarian reserve, this recruitment would be successful, producing E2 and again reducing FSH levels, whereas in patients with a compromised reserve, follicle recruitment would fail despite the elevation in FSH, with a lower E2 production and a slow reduction of FSH levels. In addition, despite regular cycles, in women with a reduced ovarian reserve the production of inhibin-B by granulosa cells would be lower, a fact that, due to the absence of a negative pituitary feedback, would lead to excessive elevation of circulating FSH levels after administration of the drug [32, 59].

In the original study by Navot et al., clomiphene citrate was administered to women aged 35 years or older at the oral dose of 100 mg between days 5 and 9 of the menstrual cycle. Basal FSH, LH and E2 levels were determined on days 2 and 3 of the menstrual cycle and the response was determined on days 9 to 11 of the cycle. The prediction of a poor ovarian response was considered to occur in women whose sum of basal and post stimulation with clomiphene FSH levels corresponded was higher than 26 mIU/mL, as confirmed by the significantly lower pregnancy rates in relation to patients considered to have an adequate gonadal reserve [59]. A few years later, Loumaye et al. supported previous results, but suggested that a poor response would occur when the sum of basal and post-stimulus levels was higher than 22.5 IU/mL [61].

The CCCT has been accepted as a prognostic indicator of reproductive performance in assisted cycles, with a higher negative than positive predictive value. However, as is the case for other markers of ovarian reserve and follicular response to the exogenous stimulus, at present the opinions about its use are controversial.

Kwee et al. evaluated CCCT in the prediction of a poor ovarian response to stimulation (<6 aspirated oocytes) and detected an AUC=0.88 in a population aged 18 to 39 years, with no influence of age on the response to the test [62]. However, a study carried out in our service by Franco et al. had already demonstrated that sensitivity of basal FSH alone was higher than that of CCCT for prediction of follicular development regarding the values suggested by both Navot et al. and Loumaye et al. [16].

Corson et al. detected some prognostic value of the test in women older than 35 and in women with a history of poor response, but correlation with the other hormonal markers was low both for basal levels and levels on the tenth day of the menstrual cycle [35]. Thus, many recent reports have demonstrated that CCCT has no advantage over basal measurement of FSH [60, 63, 64], especially for younger women.

Regarding the prediction of pregnancy, Watt et al. observed that the CCCT is not superior to the evaluation of basal FSH, even in women older than 40 [15]. On this basis, its value is reduced when questions such as costs and

potential adverse effects of ovarian stimulation are considered, with its indication being reserved for specific cases.

Gonadotropin analogue stimulation test

The gonadotropin analogue stimulation test (GAST) is based on the induction of the FSH, LH and E2 peaks (flare up response) that occur within 24 h of its administration, followed by prolonged pituitary inhibition. The levels of E2 and inhibin-B appear to reflect ovarian follicle integrity, as is also the case for FSH levels, as interpreted after the administration of clomiphene [65].

Although this was not the main objective of their study, Scheffer et al. demonstrated that 24 h after the administration of the GnRH agonist, the production of estradiol and inhibin-B was significantly elevated [66], supporting data obtained in other studies over the last decade [65, 67] and the impression that they were superior to basal levels when the objective is to determine the ovarian follicle reserve. Ravhon et al. detected a significant positive correlation between the ovarian response and variables such as the sum of basal and post-stimulus inhibin-B levels, and the increase in E2 after the stimulus [67].

However, the value of GAST as a test of ovarian reserve is not consensual. Hendriks et al. evaluated 57 women candidates for IVF and, although they recognized some value of the test for this purpose or as a predictor of pregnancy, they reported that the it did not show a better clinical performance than the determination of basal inhibin-B or AFC, even when subsequent tests were performed [63]. Similarly, McIlven et al. also detected a predictive value of elevated E2 levels after GAST for the cancellation of AR cycles in women older than 39, with a previous poor response or a basal FSH known to be elevated [68]. This, however, cannot be extrapolated to the general population of subfertile women.

GAST accuracy in predicting follicular response is similar to that obtained with AFC, opening perspectives for further research [7]. However, its use is questioned in view of the high financial cost and the risks of exogenous stimulation involved, without a direct relation to a real attempt to obtain a pregnancy.

Exogenous FSH ovarian reserve test

Like a-GnRH, exogenous FSH has also been administered in order to assess ovarian reserve. The exogenous FSH ovarian reserve test (EFFORT) is applied after the subcutaneous administration of 300 IU of recombinant FSH (rFSH) on cycle day 3. After the evaluation of E2 and inhibin-B in a previously collected blood sample, the increase in these hormones is evaluated 24 h after the administration of rFSH in order to determine the functional condition of the ovarian apparatus [62].

In a first study, Kwee et al. stated that increased levels of E2 and inhibin-B after EFORT had the best predictive value for the number of follicles obtained after stimulation, whereas CCCT was not superior to basal hormone levels [69]. Furthermore, the same group later evaluated EFORT for prediction of a poor ovarian response, when sensitivity was found to be good, with an AUC=0.86 for the increase in basal inhibin-B levels in the age range of 18 to 39 years. When basal FSH was associated with this increase, the AUC=0.87 [62].

It is probable that the test of stimulation with exogenous FSH will improve the prediction of ovarian response compared to basal markers [7]. However, its routine use involves elevated costs and the potential adverse effects of exogenous stimulation without a direct relation with a real attempt to obtain a pregnancy. Thus this test should be reserved for exceptional cases in which the estimated risks of hyperstimulation are carefully considered.

Ultrasonographic markers

Antral follicle count

Ultrasound antral follicle count (AFC) before the exogenous gonadotropin stimulus has been considered to be a good predictor of the response in AR cycles, thus reflecting ovarian follicular patrimony. A prospective study evaluating 120 women candidates for the first in vitro fertilization (IVF) cycle concluded that AFC is the most reliable basal marker of ovarian reserve in the prediction of a poor response [70].

Scheffer et al. experimentally evaluated predictors of ovarian aging and detected superiority of AFC (diameter 2 to 10 mm) both compared to biochemical markers such as E2, inhibin-B and FSH, and to ovarian volume, although a strong correlation was established between all of them [66]. According to Muttukrishna et al., AFC can identify 89% of patients who are poor responders before induction of ovulation with exogenous gonadotropins and, despite a reduced specificity of 39%, these investigators detected a significant association with the number of oocytes obtained and the probability of chemical pregnancy [51].

Although in their systematic review Broekmans et al. did not determine the value of AFC for the prediction of poor ovarian response, they admitted its importance as a screening test for couples who are candidates for AR [7], encouraging researchers to test it and permitting its gradual incorporation into protocols of pre-induction evaluation. Although its value is not yet universally recognized, recent studies have shown significant correlations with classically used serum markers [64] and with AMH [24, 42], as well as significant differences between women with a normal

response and with a poor response to gonadotropic stimulus [7]. In a recent study, Elgindy et al. evaluated AFC up to 10 mm in diameter and detected significant different amounts of 10.1±3.0 and 5.7±1.0 follicles for the two groups, respectively [7].

Special attention has been paid to small antral follicles which, like total AFC, have been shown to decrease significantly in number with age, whereas the pool of larger follicles may remain practically unchanged until about 45 years of age [64]. Klinkert et al. demonstrated that frequency of a normal response to stimulation was significantly higher in patients with AFC≥5 U with a diameter of 5 mm or less, and was accompanied by higher pregnancy rates [18]. In agreement, Haadsma et al. pointed out a significant correlation of follicles up to 6 mm in diameter with all the endocrine tests of ovarian reserve, in contrast to what is observed with larger follicles, which correlate only with ovarian volume and inhibin-B [64].

In summary, literature data seem to agree about the value of AFC as a predictor of ovarian response. On this basis and considering that AFC can be performed during ultrasound exams normally requested for routine gynecological evaluation, we believe that its inclusion as a marker of gonadal reserve in the management of all candidates to AR cycle is promising, with the count of at least five follicles measuring up to 6 mm predicting an adequate response to the stimulus.

Ovarian volume

As was the case for AFC, an attempt was made to associate ovarian volume (OV) determined by ultrasound with the functional patrimony of the gonad and with successful AR cycles. In a study conducted on 261 women aged 23 to 46 years, Syrop et al. demonstrated a clear reduction of E2 peaks, number of oocytes obtained and pregnancy rates with decreasing volume of the smaller ovary of patients submitted to AR cycles [71]. However, in a recent review of ten studies of ovarian volume as a marker of ovarian reserve, Broekmans et al. concluded that this marker has little clinical applicability for the prediction of a poor pregnancy response [7].

Other studies followed that systematic review, but controversy persists. While Bowen et al. demonstrated a significant correlation between reduction of ovarian measurements, increased age and elevated circulating FSH levels [72], Elgindy et al. did not detect significant differences in mean OV between women up to 37 years old with a normal or poor response to the stimulus (mean values of 4.1±0.66 and 3.36±0.71 cm^3, respectively) [3]. McIlven et al. reached similar conclusion when evaluating women at high risk for the cancellation of AR procedures [68].

Thus, no predictive value should be attributed to the measurement of OV, although we believe that, because of its easy execution, this measurement could be included in the preparatory protocols, adding information to the patient's medical records and providing data for the continuity of research.

Combined markers

Since no study succeeded in selecting a single marker of ovarian reserve of satisfactory sensitivity and specificity, the combination of markers has been proposed for a better estimate of functional gonadal capacity. However, a recent meta-analysis of 11 studies reporting various models of evaluation concluded that these combinations were similar to AFC alone to predict a poor response to IVF stimulation [73].

As a matter of fact, multivariate models evaluated were not sufficiently varied for the quantity of individual exams available, with few studies including AMH and with no study considering the combination of AMH and AFC, which are currently being extensively investigated in the evaluation of ovarian reserve. More studies evaluating this combination and others will be necessary to rule out the use of multivariate propaedeutic models which still seem to be the best strategies for the management of subfertile couples.

Role of ovarian reserve markers in the diagnosis of premature ovarian senescence

Premature ovarian ageing (POA) and premature ovarian failure (POF) compound a spectrum of ovarian dysfunction causing not just damage on fertility potential, but all clinical consequences of hypoestrogenism. POF is clinically defined as the complete absence of menstrual cycles before the age of 40 [74], whereas POA represent a milder degree of gonadal dysfunction in patients who still demonstrate some chance of pregnancy with autologous gametes up to menopausal FSH levels [75].

In spite of being associated to a vast spectrum of conditions, from gonadal dysgenesis to gonadotropin resistance [76], the etiology of premature ovarian senescence processes remains unknown in the majority of cases, mainly those with normal karyotypes [77]. Recent researches on POF diagnose has focused on mutations of the FSH receptor (FSHR) gene [78, 79], other genetic diseases which involves intermediate stages on mutation expansion of X-linked genes [80], autoimmune function [81] and abnormal AMH expression in granulosa cells, leading to impaired follicular development [77]. Yet, special attention is being modernly given to cancer survivors, who normally present with ovarian reserve damage associated to chemotherapy and/or radiotherapy repeated cycles.

In regard of an accurate clinical investigation in such cases, several ovarian reserve markers have been tested, but no confident conclusions have been achieved until now. Although currently used in secondary amenorrhea investigation, diagnostic criteria for the establishment of ovarian senescence stages have not been definitively delineated. According to Conway, POF is considered in women with at least 4 months of amenorrhea associated with elevated FSH serum levels in two occasions [82], but it has been demonstrated that plasma levels of FSH have been considered of limited value in POF patients ovarian reserve prediction, which means it is not solely accurate to make a diagnosis of irreversible ovarian failure, even for amenorrheic women or those presenting menopausal symptoms [83–86].

Even E2 and inhibin-B, which should directly reflect granulosa cells function, do not supply clinical needs for the estimation of the gonadal functional patrimony [86]. Actually, the mentioned markers seem to be altered only in late stages of the ovarian aging process. A recent study of Tsigkou et al. suggested inhibin-B should sign an autoimmune etiology for ovarian insufficiency as a result of precocious thecal destruction with preservation of granulosa cells [87].

Low consistent results in literature lead to researches on new markers, like AMH. Massin et al. considered its serum levels, histological analysis of ovarian follicles and AMH immunoexpression in POF women granulosa cells; the authors found out low or under detection threshold serum levels of the hormone in patients with diminished follicular population and observed that antral follicle AMH immunostaining in POF gonads was dissimilar from the diffuse labeling observed in healthy granulosa cells. However, in spite of these promising results, positive and negative predictive values of 66,7% and 60% were not sufficient for sustaining this marker as a sole predictor of early ovarian decline in POF patients [77]. Sonographic evaluation was either performed in the study of Massin et al., but the authors assumed that it is not of predictive value in determining the presence of follicles within the gonad, once histological analysis could not display follicles in almost half of patients whose ultrasound scan had suggested their existence [77].

Considering current knowledge, it is not possible to determine an efficient combination of tests with sufficient accuracy to premature ovarian decline diagnosis and/or prognosis establishment. But, looking for accuracy in these patients' evaluation, AMH levels could be associated with that of FSH, especially in differential diagnosis for women presenting controversial clinical signs, which occur, for example, in cases of hypothalamic dysfunction causing secondary amenorrhea. In fact, recent studies have suggested that AMH does not offer a relevant advantage over commonly used markers [77], but it has been shown it is almost undetectable in women with POF [88]. Also it presents significant variability between ages [89] and, as shown previously in this text, is strongly correlated to ovarian function in regard to assisted reproduction outcomes, corroborating theoretical hypothesis on hormone's physiology.

Final considerations and conclusions

The search for biochemical or ultrasonographic markers that can predict response to AR cycles can reduce the costs and potential side effects of excessive administration of exogenous gonadotropins. For young women, such markers should represent the opportunity of individualized planning by predicting the doses and results based on the estimated cohort of follicles available for retrieval. For older candidates, it would permit to determine the chances of success in the treatment of infertility, as suggested in the literature.

There is general agreement about the idea that the best marker of ovarian reserve should be able to identify women whose chances of pregnancy in AR cycles would be so close to zero that it would not be justified to submit them to the potential adverse effects of exogenous stimulation. Unfortunately, currently available tests do not provide sufficient evidence to be considered ideal and, until new studies will provide more consistent results, directly submitting women to a highly complex technique has been questioned to be an appropriate strategy in order to determine the follicular status.

However, we have no doubt that the markers of ovarian reserve occupy an important place in the initial approach to the treatment of subfertile couples, predicting the possibility of unsatisfactory results that could be improved by differentiated induction schemes, reducing excessive psychological and financial burdens and the occurrence of heterogeneous events. It should be remembered that the ability of a test to predict the gonadal response (follicular development and number of oocytes) usually exceeds the ability to predict pregnancy since the this last one depends on variables such as the characteristics and conservation of the gametes, the fertilization technique used, seminal quality, embryo conservation and evolution, the characteristics of the endometrial cavity and of the pelvic–peritoneal microenvironment. On this basis, and since it is not possible to guarantee the accuracy of a single test for the prediction of ovarian reserve, the key to better counseling may reside in the joint analysis of distinct markers of ovarian reserve that will provide the necessary information for the formulation of appropriate stimulation protocols for each couple.

Although basal FSH, E2 and inhibin-B are being classically used to infer gonadal function, the search for a marker that better reflects the quantity and quality of primordial follicles, and therefore the functional ovarian

reserve, has led to scientific emphasis on AMH. In addition to being present in stable serum levels along the menstrual cycle, its main advantage over other markers, AMH may possibly be the only marker that can potentially assess the follicular population as a whole, including still inactive follicles, since it reflects the transition of follicles from primordial to early antral follicles.

When compared to dynamic markers, basal markers (endocrine or ultrasonographic) have definite advantages regarding practicality, lower costs and harmlessness, so that the use of dynamic tests as markers of ovarian reserve for women candidates to AR is much more an exception than a rule. We believe, however, that the CCCT is still of some value in the prediction of a poor response, involving fewer risks than the GAST and EFORT.

A lower probability of adverse effects confers a role to ultrasound markers in the prediction of ovarian reserve, but their routine use is limited by the subjectivity of examiner-dependent measures and by the possible variability between cycles, which may lead to excessively discrepant results.

Finally, it is important to point out that in the present report we did not consider specific groups of the population of subfertile women, such as women with endometriosis, hyperandrogenic anovulation or other endocrine-metabolic disorders. Thus, the behavior of ovarian reserve markers may differ in these populations and become of higher value in the prediction of success in AR cycles.

In order to remedy the limitations due to the scarcity of strong evidence about the topic, future studies should try to clarify the predictive value of ovarian reserve/response markers, with special attention to specific subfertility-related diseases and propaedeutic multivariate models combining more than one marker, including AMH and AFC in the list, with should be extended to premature ovarian senescence cases, independently from subfertility complaints.

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