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PREDICTIVE VALUE OF ANTI MULLERIAN HORMONE LEVEL IN SERUM AND FOLLICULAR FLUID FOR OVARIAN RESPONSE IN WOMEN UNDERGOING OVARIAN STIMULATION FOR IN VITRO FERTILIZATION

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ABSTRACT

Objective: to evaluate the predictive value of follicular and serum AMH for extremes of ovarian response in women undergoing controlled ovarian hyperstimulation. **Design**: Prospective cohort study. **Setting**: Tertiary referral center for reproductive medicine at the IVF unit of EL-Hussin Hospital and EL-Galaa Maternity Teaching Hospital which are two referral hospitals located in cairo governorate, the Egyptian capital, between October and December 2016. **Patient(s):** 50 women undergone their cycles of controlled ovarian hyperstimulation for in vitro fertilization (IVF). **Materials and Methods:** Basal serum level of AMH and follicular fluid AMH(Anti mullerian hormone) measured in 50 patients. All patients were followed prospectively and their cycle outcomes recorded. **Main Outcome Measure(s):** the predictive value of follicular and serum AMH for extremes of ovarian response of stimulation. **Result(s):** out of 50 cycles in 50 women according to inclusion criteria**,** total 33 pregnancies were acheived (clinical pregnancy rate 66.0% confirmed 4 weeks later from day of retreival by U/S). Further, significantly higher levels of FF AMH in conception (n = 33) cycles compared to nonconception (n=17) cycles (mean 53.14 vs. 16.84 ng/ml; P < 0.001). Also**,** fertilization rate with both serum &FF indcated an extremely significant difference when **(**P<0.001). Since there was an extremely significant difference in serum AMH levels between conception and nonconception cycles (mean 3.05 vs 1.14 ng/ml, P < 0.001).they were subsequently presented data about the relation between high level of AMH in serum and FF and The N. of embryos transferred in all 50 cycles., there is an invers relation between AMH serum and FF and age of female and BMI. Finally**,** a direct propotional between levels of serum and FF AMH shows highly statistically significant deference between AMH serum and AMH FF when p-value was <0.001*. **Conclusion(s):** Serum AMH levels can predict ovarian response during first IVF treatment cycles.AMH in the pooled FF and serum can be used as a potent biochemical indicator as a predictive value for success in conventional IVF cycles, FF AMH levels acts as an index of oocyte maturity and therefore it's quality.

KEYWORDS: Antral follicle count, anti-M€ullerian hormone, controlled ovarian hyperstimulation, exogenous gonadotrophins, follicle stimulating hormone, ovarian response.

INTRODUCTION

In recent times postponement of childbearing has led to increased rates of age-related female subfertility among couples trying to conceive. As result of this trend fertility clinicians have been faced with the challenge of determining the degree of ovarian reserve to better tailor assisted reproductive technology (ART) treatment. The costly drug regimens **(Macklon,et al., 2006)**, the discomfort to patients **(Fauser et al.,2009)** and the significant risk of complications associated with ovarian stimulation, all justify the need for obtaining clinically relevant information before commencement of treatment. In a climate where safety and cost-effectiveness are central to ART, unexpected excessive and poor responses to ovarian stimulation.

Female reproductive performance and probability of live birth after in vitro fertilization (IVF) decline with increasing chronologic age **(Templeton et al.,1996)**. It is therefore appropriate to speculate that age per sex is an important predictor of success in IVF cycles, and should always be the first step in ovarian reserve assessment. Nevertheless, the relationship between age and ovarian reserve is highly variable **(te Velde et al., 2002).**

Over the last 2 decades a number of ovarian reserve tests have been investigated and proposed as accurate predictive markers of the primordial follicle pool, ovarian response, and IVF outcome **(Broekmans FJ et al., 2006)**.

Many of these tests, which relate mainly to the quantitative aspects of ovarian reserve, have become part of the routine diagnostics for response prediction in subfertile women undergoing ART. However, their added value to chronologic age as determinants to predict ovarian performance and pregnancy prospects is still unclear. Clinically there is a need to identify women of relatively young age with reduced ovarian reserve as well as women whose fertility is naturally impaired by age who may still have satisfactory ovarian potential Should this become possible, then management can be individualized and optimized using modified stimulation approaches, and the risks of extremes of response and drop-out rates be significantly reduced while maximizing the chance of pregnancy **(Fauser et al., 2009).**

Anti-M€ullerian hormone (AMH), also known as M€ullerian Inhibiting Substance, is a member of the transforming growth factor-beta superfamily, which is synthesized in the granulosa cells of preantral and small antral follicles **(Modi et al., 2006)**. In the ovary, AMH inhibits initial primordial follicles recruitment and decreases the sensitivity of preantral and small antral follicles to FSH **(Durlinger et al., 2002)**, hence suggesting its role in intrafollicular and interfollicular coordination of follicle development **(Salmon et al., 2004)**. It has been reported that human antral follicles measuring <6mm express the greatest amount of AMH, and that levels decline as antral follicles increase in size **(Weenen et al., 2004)**. Furthermore, AMH has been found to be elevated in women with polycysitc ovary syndrome (PCOS) compared with controls, leading to the hypothesis that the exaggerated AMH tone could be involved in the follicular arrest seen in polycystic ovaries **(Pigny et al., 2006)**. Anti Meullerian hormone is strongly correlated to antral follicle count (AFC), and both predictors have a linear relationship with age **(deVos et al., 2012)** confirming the decline of ovarian reserve with age. Several investigators have shown that basal AMH levels accurately reflect the total developing follicular cohort and ovarian response to gonadotrophins in ART cycles **(Nelson et al., 2013).** As yet, AMH testing, like other predictive markers do not appear to predict accurately the probability of pregnancy after IVF treatment **(Broer et al., 2013).**

The aim of this prospective cohort study is to evaluate the clinical value of basal anti-Mullerian hormone (AMH) measurements in the prediction of ovarian response to gonadotrophin stimulation for In Vitro Fertilization procedures.

MATERIALS AND METHODS

Subjects and Assays

50 women undergone their cycles of controlled ovarian hyperst-imulation. All patients were followed prospectively and their cycles outcomes recorded to evaluate the predictive value of follicular and serum AMH for extremes of ovarian response of stimulation. **The inclusion criteria:** (1) Women younger than 40 years. (2) First cycle for ART programes (IVF) and ovarian hyperstimulation (3). No history of previous ovarian surgeries (4) Both ovaries are present on transvaginal ultrasound (5) Body mass index $\geq 19 \leq 30 kg/M^2$.

Exclusion criteria: (1) Women older than 40 years of age. (2) Women with polycystic ovary syndrome (PCOS as defined according to the Rotterdam consensus). (3) Women with endometriosis as diagnosed by laparoscopy (4) Cycles where no oocytes were retrieved on the day of aspiration (5) History of previos trial for IVF.

All the selected patients for the study were chosen according to the inclusion criteria and all of them were revised according to : regular menstrual cycles, day2 or 3 FSH, LH, E2, Prolactin was assessed, testosterone and TSH also were recorded**,** HSG for all patients**,** history of diagnostic laparoscopies and hystrescopies if it were done, pre-antral follicles were measured to all patients during menses, complete semen analysis for all partners, basal serum AMH was measured as a standard for the study from the start and follicular fluid AMH obtained during oocyte retrieval.

Fertilization rate and pregnancy rate were recorded after embryo transfer by chemical pregnancy tests and U/S for detection of cardiac activity after 4 weeks of embryo transfer.

Implantion rates also calculated according to:

$$\frac{Total\ number of\ gestational\ sacs}{Total\ number of\ embryos\ transferred} x\,100$$

Main outcome measures: Functional viability of oocyte comprising of the following: (a)Morphological assessment of oocyte quality (b)Fertilization rate (c) Clinical pregnancy: Gestational sac with positive cardiac activity observed at ultrasound at around 4th week of embryo transfer was defined as confirmation of clinical pregnancy.

Cycle monitoring

Downregulation protocol: Pituitary desensitization involving treatment with Gonadotropin Releasing Hormone (GnRH) agonists (500µg/day of leuprolide acetate) was started in the midluteal phase of the menstrual cycle 7 days prior to the earliest expected date of menstruation. A comprehensive down regulation was confirmed by measurement of serum Follicular Stimulating Hormone (FSH) and estradiol (E2) levels below 1.0mIU/mL and >20 pg/mL respectively, either on the day of onset of menstruation or 1/2 days at the most, after onset.

Ovarian stimulation protocol

After confirmation of compr-ehensive down regulation with GnRH agonist, a standard long protocol was used for controlled ovarian hyperstimulation. Ovarian stimulation was effected with daily administration of recombinant FSH using a starting dose from 150 IU/day

in age <35 years to a dose 225 IU/day in age >35 years and also may be increased with increasing BMI. Transvaginal ultrasound scan was done on days 8 to 10 of ovarian stimulation and every 1 or 2 days thereafter, as required. Final oocyte maturation and trigger for ovulation was induced by administering human Chorionic Gonadotropin (hCG) 10000 IU, when there was at least one leading follicle reaching a mean diameter of 18 mm and at least 2 to 4 other follicles reaching mean diameter of 16mm, endometrial thickness >8mm with trilamenal halo appear,E2 level 100-150 pg/ml.

Oocyte pickup

Transvaginal ultrasound-guided oocyte retrieval under patient sedation was done between 34 and 36h after hCG administ-ration. Insemination was done after 4h of oocyte incubation.

Hormonal estimation

FF obtained on the day of oocyte retrieval was estimated for AMH levels by enzyme-linked immunosorbent assay technique using diagnostic kits of serum AMH. Protocol was followed as per manufacturer's instructions. Theoretical sensitivity or lowest detection limit was 0.006ηg/mL. FF levels obtained in ηg/mL were expressed as a ratio of their corresponding total protein content to remove any bias due to volume variability. Protein estimation was performed by folin phenol reagent method.

Fertilization assessment

Fertilization rate, which was assessed 16-18h after insem-ination, was characterized by the presence of two pronuclei and two polar bodies. The position of pronuclei (whether centric or eccentric), number, and alignment of nucleoli/nucleolar precursor bodies at junction of two pronuclei, granulation of cytoplasm, and presence of halo (indicative of nuclear membrane breakdown) were also noted. Embryo development was monitored daily and cleavage stage embryos were assessed before transfer. **(Ebner et al. 2003).** Embryo transfer, only sedation or light anathesia may be used or no need as technique is minimal. The duration of the procedure is brief with a range between 31-47 seconds, the lithotomy position as used for transcervical transfer. The vagina is just cleaned by saline only. Trans-cervical transfer of day three or five cleavage stage embryos was performed using a soft-tipped embryo transfer catheter eg; labotaect by using abdominal U/S or TV U/S in case using THE Twako Transmy-ometrial Embryo Transfer Set **(Sharif and Kato et al.,1998)**. Immediately after ET, catheter was flushed and the media was examined microscopically to confirm absence of embryos. Micronized progesterone 200mg twice daily was administered to support luteal phase starting from evening of day ET until day 14 of ET. On day 14 of ET, serum β-hCG concentration <50mIU/mL was considered as positive indicator of pregnancy. Clinical pregnancy was confirmed by presence of gestational sac with positive cardiac activity after four weeks of Embryo transfer. Finally, 50 cycles were considered for oocyte quality assessment, fertilization, and clinical pregnancy. Ethical consent from the scientific comitte of Al-Azhar university, Informed consents were taken from all patients for participation in the study.

Statistical Analysis

Statistical presentation and analysis of the present study was conducted, using the mean, standard Deviation, unpaired student t-test, analysis of variance [ANOVA] test and linear correlation coefficient tests by **SPSS v. 20.** Unpaired Student T-test was used to compare between tow groups in quantitative data. Analysis of variance [ANOVA] tests**,** According to the computer program SPSS for Windows. ANOVA test was used for comparison among different times in the same group in quantitative data.

Linear Correlation Coefficient [r], Linear Correlation coefficient was used for detection of correlation between two quantitative variables in one group. **S**ignificant level**,** Non Significant >0.05 Significant <0.05* High Significant <0.001*.

RESULTS

In our study, out of 50 cycles in 50 women according to inclusion criteria**,** total 33 pregnancies were acheived (clinical pregnancy rate 66.0% confirmed 4 weeks later from day of retreival by U/S) **(**table 1&fig 1). Further, our results indicated significantly higher levels of FF AMH in conception (n=33) cycles compared to nonconception (n=17) cycles (mean 53.14 vs. 16.84ng/ml; P<0.001). Also**,** fertilization rate with both serum &FF indcated an extremely significant difference when **(**P<0.001). (Table 2&fig. 2). Since there was an extremely significant difference in serum AMH levels between conception and nonconception cycles (mean 3.05vs 1.14 ng/ml, P<0.001) **(**table 2& fig 2). They were subsequently presented data about the relation between high level of AMH in serum and FF and The N. of embryos transferred in all 50 cycles, for example 5 embryos transferred show a**(**mean 66.08ng/ml for AMH FF, and mean 3.10ng/ml in AMH serum ; P < 0.001) and for 1 embryo transferred (mean 17.37 ng/ml for AMH FF**,** and mean 1.16 ng/ml in AMH serum; P < 0.001) **(**Table 3).Our study also shows, there is an invers relation between AMH serum and FF and age of female and BMI. Also, there's an inverse relation with FSH and that was showed in (Table 4).Finally**,** a direct propotional between levels of serum and FF AMH Table (5) shows highly statistically significant deference between AMH serum and AMH FF when p-value was <0.001*. Implantation Rate =45 %.

Table(1): Positive pregnancy test in the study group

Pregnancy test	N	%
Negative	17	34.0
Positive	33	66.0
Total	50	100.0

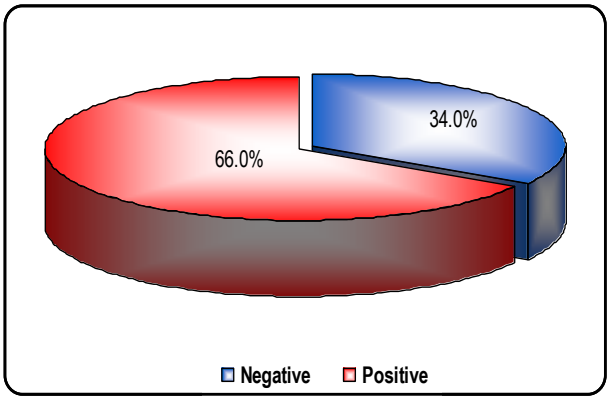

Fig. (1): Positive pregnancy tests in the study group

Table (2): Relation between AMH in serum and FF and pregnancy tests, this table show highly statistically significant deference when p-value was <0.001.

	Pregnancy test					
	Negative		Positive		T-test	
	Mean	SD	Mean	SD	t	P-value
AMH serum	1.14	0.27	3.05	0.84	9.057	<0.001*
AMH FF	16.84	5.01	53.14	12.58	11.397	<0.001*

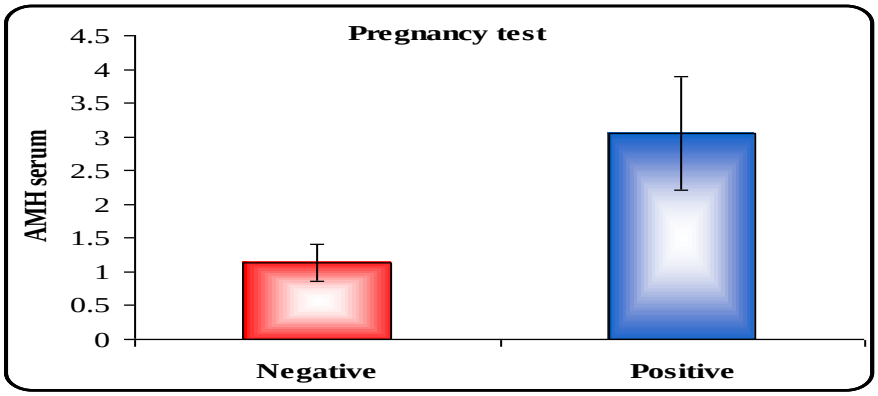

Fig. (2): Relation between AMH in serum and FF and pregnancy tests.

Table (3): Relation between AMH in serum and FF and No. of Embryos transferred, This table show highly statistically significant deference when p-value was <0.001*.

	No. of Embryos transferred											
	1		2		3		4		5		ANOVA	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	f	P-value
AMH serum	1.16	0.51	2.49	1.22	2.75	0.91	3.28	0.55	3.10	0.84	7.076	<0.001*
AMH FF	17.37	8.45	39.26	18.51	48.91	13.51	56.63	10.40	66.08	12.55	13.029	<0.001*

Table (4): Correlation between AMH serum and AMH FF with others patients variables.

	AMH serum		AMH FF	
	r	P-value	r	P-value
Age of females	-0.570	<0.001*	-0.627	<0.001*
BMI	-0.494	<0.001*	-0.492	<0.001*
AFC	0.721	<0.001*	0.645	<0.001*
FSH	-0.577	<0.001*	-0.601	<0.001*
LH	-0.315	0.026*	-0.319	0.024*
Prolactin	-0.152	0.293	-0.124	0.392
E2	0.700	<0.001*	0.709	<0.001*
Number of follicles	0.703	<0.001*	0.892	<0.001*
Size of follicles	0.544	<0.001*	0.507	<0.001*
Fertilization rate	0.618	<0.001*	0.771	<0.001*

This table show highly statistically significant deference between AMH serum and AMH FF and(Age of females, BMI, AFC, FSH, E2, Number of follicles, Size of follicles and fertilization rate) when p-value was <0.001*.

Table (5): This table show highly statistically significant deference between AMH serum and AMH FF when p-value was <0.001*

	AMH serum	
	r	P-value
AMH FF	0.861	<0.001*

DISCUSSION

The present study has taken a holistic approach towards AMH measurement by estimating its levels in pooled fluid obtained from the follicular fluid during day of oocyte has been retrieved in each cycle. This seems logical not only because it is practically more feasible, but also because it is a more comprehensive reflection of the dynamic milieu that the FF microenvironment represents and for serum also. Moreover, the practice of carrying studies in individual lead follicles may be too time-consuming, and feasible. Our results, associating high FF AMH levels on day of retreival with conception cycles and indicating a direct correlation of FF AMH with clinical pregnancy, also serum AMH was measured as a part of routine investigation were taken from our patients. completely confirm that they are in conjunction with earlier reports of a progressive increase in AMH levels mainly obtained from follicular fluid in the day of oocytes retreival after ovarian stimulation, A recent study carried out in monofollicular fluid (FF obtained from each individual follicle) of stimulated cycles by **(Takahashi et al.,2008)** reported correlation of higher FF AMH levels with higher rates of fertilization. However, they could not associate it with pregnancy outcome. Moreover, their study involved comparison of FF AMH between two broad groups namely fertilization success versus fertilization failure. In 2008, **Wunder et al.** correlated higher FF AMH with reproductive outcome in IVF-ICSI cycles. Another study carried out by **(Fanchin et al.,2007)** in monodominant follicles (single lead follicle) of unstimulated cycles, reported correlation of FF AMH with implantation rates and pregnancy outcome but not with fertilization rates. Other recent study of **(Aflatoonian et al.,2010)** who correlated FF AMH with fertilization and embryo quality.

On the journal of human reproductive science April **2013, Bindu et al.,** a study similar to our study but the FF AMH was obtained from the largest follicle during the day of retreival, this study, although, it's making a different from other previous studies but it seems to be more expensively costed and increasing the study group. Other study by **(Broekmans et al., 2003)**, included relation between serum AMH and FSH as tests for ovarian reserve. Others, **(Renato et al.,2005)**, show Predictive value of FSH, AMH, and AFC for extremes of ovarian response to stimulation. Thus, all these studies seem to have contrasting results as regards association of FF AMH and serum with fertilization rates and pregnancy outcome. Interestingly, all these studies correlated higher FF AMH levels with either higher rates of implantation/clinical pregnancy or fertilization or number of embryos transferred or even embryos quality, respectively. These findings seem paradoxical and incongruous with earlier statements by the same authors that corpus lutea, large antral follicles size and number, oocytes and thecal, and interstitial cells express little or no AMH. Rather, its production by granulosa cells of large follicles has been reported to interfere with follicular maturation and luteinization **(Fanchin et al., 2005)**. Results could partly be explained by the fact that the study was carried out in unstimulated monodominant follicle cycles where GnRH antagonist and human menopausal gonadotropin was administered only to prevent a premature leuteinizing hormone (LH) surge and to control recruitment of additional secondary follicles. Experiments in rats have indicated that FSH administration reduces AMH levels. Extrapolating from murine data by **(La Marca et al., 2006),** reported that in women, the reduction in AMH levels may be due to the supraphysiological increase in E2 levels observed when exogenous FSH is administered. Therefore, it may be argued that in the absence of FSH stimulation for multiple follicular growth, AMH levels were found to be higher in study. However, **(Fanchin et al.,2007)** in the same study refer to the presumable FSH independent production of AMH. **(Fanchin et al.,2007)** have also made reference to an observation by **(Salmon et al.,2004)** that in the mice preovulatory follicles, AMH gene expression is directly activated by the oocyte, which may facilitate fertilization. However, no such claim has yet been made in the human preovulatory follicle to substantiate the presence of higher levels of AMH in FF.Application of AMH and FSH by **(Bancsi et al., 2000)** as a predictor of ongoing pregnancy appears to be limited in view of the fact that they only represent the quantitative aspect of ovarian reserve,, but, in this study we found direct correlation between AFC and AMH in both serum and FF which was investigated in our study. In our study, the association of high FF AMH and serum with higher fertilization, embryo implantation, and clinical pregnancy rates is also supported and substantiated by the observation of significantly higher levels of FF and mean of serum AMH. This finding corroborates and is in confirmity with the fact that as the follicle matures to the preovulatory stage, the granulosa cells undergo developmental changes which transform the follicle from being steroidogenically quiescent to one that is capable of producing large quantities of oestrogen. From the midfollicular phase, with it's increasing capacity to aromatize and rostenedione, the follicle destined to ovulate begins to synthesize estradiol. AMH

is reported to have an inhibitory effect on aromatase activity and estrogen production. In cultured fetal rodent ovaries, AMH has been shown to suppress the expression of cytochrome P450 aromatase, the enzyme in granulosa cells that converts androgens to E2. The intrafollicular androgen to estrogen ratio acts on oocyte function; and AMH appears to play a major role in the regulation of this ratio. Animal studies have revealed that not only does AMH decrease aromatase activity of FSH-stimulated granulosa cells, but it also decreases the number of LH receptors, and regulates testosterone production in theca cells. Moreover, many workers have associated higher levels of FF and high level of serum AMH with better chances of achieving pregnancy. It is, therefore, logical that FF levels of AMH and serum AMH share a direct relationship as demonstrated by our study.We observed a high correlation of AMH with ovarian response, as expressed by the number of oocytes retrieved. Also increasing number of antral follicles showed by (MEAN ~13.22) may produce more AMH as a reflection of their high granulosa cell number, the smaller size follicles may also contribute significantly to serum AMH on the basis of their larger number. This will reflect on the overall viability of the oocyte, thus influencing its quality, fertilization rate as well as clinical pregnancy and embryo implantation rates. Also, an inverse relation with age, BMI and FSH obtained through the analysis which need much more other reviews. This study had successfully explored AMH in the pooled FF and serum as a potent biochemical indicator as a predictive value for success in conventional IVF cycles.

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