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Predictive Value of Serum versus Follicular Fluid Anti-Mullerian Hormone in Intra Cytoplasmic Sperm Injection Outcome

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Abstract: Background: Although multiple studies have established the association between serum Anti-Mullerian Hormone (AMH) levels and evaluation of ovarian reserve and successful outcomes in Intra Cytoplasmic Sperm Injection (ICSI) procedure. However, there is still debate about the exact role of follicular fluid (FF) AMH in ICSI outcomes. **Aim of the work:** To compare serum and FF AMH levels as predictors of intra cytoplasmic sperm injection (ICSI) outcomes. **Patients and methods:** This prospective cross-sectional study included 85 ladies with different indications for ICSI. After ovarian stimulation, both serum and FF AMH were assessed. Both of the previous parameters were correlated with oocyte maturation, fertilization capacity, embryos quality and pregnancy outcomes. **Results:** The mean value of serum AMH was 1.91 ng/ml (range, 1 – 3.3), while follicular fluid AMH had mean values of 1 ng/ml (range, 0.2 – 2.8). No significant correlation was reported between serum and FF AMH levels (p = 0.965 – r = 0.005). Serum AMH had significantly higher levels in pregnant cases (2.09 vs. 1.66 ng/ml in non-pregnant cases – p = 0.001). However, follicular fluid AMH did not significantly differ between the two groups. Using a cut-off value of 1.55 ng/ml, serum AMH had sensitivity and specificity of 72 and 60% respectively to predict pregnancy. Serum AMH had significant correlation with the number of retrieved oocytes, meiotic status, oocyte score, fertilization capacity, and embryo quality. **Conclusion:** Serum AMH appear to be more useful than its FF levels. Its levels showed a significant difference between pregnant and non-pregnant subjects.
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Key words: Anti-Mullerian hormone; Follicular fluid; ICSI outcomes.

1. Introduction

Recently, assisted reproductive technology (ART) including in vitro fertilization (IVF) and Intra Cytoplasmic Sperm Injection (ICSI) procedures have been used by many couples complaining of infertility with hopeful outcomes **[1, 2]**. However, the success of ICSI procedures depends on many factors including maternal age, ovarian reserve, and previous reproductive capacity **[3]**.

Ovarian reserve could be expressed as the size and number of the remaining ovarian oocytes. It can be accessed via multiple sonographic and biochemical methods **[4]**. Serum anti-Mullerian hormone (AMH) has been supposed as a good method to assess the ovarian reserve **[5, 6]**. AMH is a glycoprotein hormone secreted by the preantral and antral follicles. Of note, both serum and intrafollicular AMH are reported to correspond to follicular maturation rate **[7]**.

Although multiple studies have established the association between serum AMH levels and successful outcomes after ART. However, there is still debate about the exact role of follicular fluid (FF) AMH in oocyte maturation, quality and ART outcomes **[3, 7]**. FF AMH could have as direct autocrine and paracrine effects on granulosa cells, oocyte or embryo quality **[8, 9]**.

The current study was conducted to compare between serum and FF AMH levels as predictors of intra cytoplasmic sperm injection (ICSI) outcomes.

2. Patients and methods

After obtaining approval from the local ethical committee of Al-Azhar University, the current prospective cross-sectional study was conducted at a special fertility center in Damietta, Egypt, in collaboration with Al Azhar university hospitals, New Dameitta, Egypt. The study cases were collected and

procedures were done and monitored during the period between July 2018 till January 2020.

The sample size was calculated using the Epi-Info software statistical package. Using a 95% confidence limit, 80% power, and an expected favorable outcome reaching up to 85% compared to 65% as unfavorable, the sample size was calculated to be more than 73 subjects. However, the number was increased up to 85 to improve the validity of our results.

We included cases having an indication for ICSI, aged between 20 and 35 years, with BMI between 18 and 30 kg/m2, and having normal levels of serum AMH (between 1 and 4 ng/ml). Contrarily, cases with polycystic ovary syndrome (PCOS), uterine anomalies, ovarian endometrioma, previous history of unilateral oophorectomy, and poor responders were excluded.

For all the included cases, an informed written consent was obtained after complete explanation of the steps, benefits and drawbacks of each intervention. Data access was also accepted from the included participants after ensuring keeping their privacy. All cases were subjected to detailed history taking, gynecological examination, and routine laboratory investigations (including FSH, LH, estradiol and prolactin). Additionally, transvaginal ultrasonography was ordered for all cases.

Serum AMH was measured for all ladies by collecting 5ml of venous blood from each patient on day 2-3 of stimulation cycle, serum was separated and analyzed by using the Elecsys ® AMH assay (Roche, La Roche Ltd, Germany) on a Cobas e 411 analyzer at the central laboratory of clinical pathology department, Al Azhar university hospitals, New Dameitta.

The "long agonist" protocol was commenced for all ladies, starting on day 21 (midluteal phase) of the pre-stimulation cycle when subcutaneous injection of triptoreline acetat (Decapeptyl® 0.1mg, Ferring Kiel, Germany) was done.

After confirming pituitary suppression, controlled ovarian hyperstimulation was initiated in the second day of the stimulated cycle by intramuscular administration of human menopausal gonadotropin (Merional® IBSA, Switherland) (active ingredient contains: Human Follicle Stimulating Hormone (FSH) 75 or 150 I.U & Human Luteinizing Hormone (LH) 75 or 150 I.U) and Human follicle stimulating hormone (Fostimon ®, IBSA, Switherland) (active ingredient contains urofollitropin, FSH, 75 or 150 I.U).

The starting dose of gonadotrophins was prescribed according to age, body mass index and antral follicles count. Then the dose was adjusted (increased or decreased) according to the ovarian response detected by folliculometry.

Folliculometry with serial ultrasound was ensured using GE Voluson P6 ultrasound machine to monitor follicle size and count. When at least two follicles reached 18 mm in diameter, final oocyte maturation was achieved by human chorionic gonadotropin (hCG 10000 IU IM). (Choriomon ® active ingredient per vial contains Chorionic Gonadotropin 5000 I.U, IBSA, Switherland). Ovum pick up was planned to be 34 – 36 hours following that under ultrasonographic guidance using ovum aspiration needle.

During oocyte retrieval, aspiration of the FF was done, then it was kept in a sterile Polystyrene Falcon tube 5 ml, centrifuged for 10 minutes at 1500 rpm to isolate the fluid from its cellular components. It was stored at -40°C till assay of AMH. FF AMH measurements were carried out using the same steps as serum AMH was measured.

Following proper handling of the oocytes, it was carefully examined regarding its meiotic status and morphology. Meiotic status was graded as GV (immature germinal vesicle), metaphase 1 (MI), Mature oocyte (MII), and atretic or aged oocyte (PM). Besides, oocyte morphology was classified according to the "modified Xia's morphological criteria" **Table (1) [10]**.

Table (1): Modified Xia's morphological criteria

	0	**1**	**2**
First polar body	–	Fragmented	Intact
Perivitelline space	Large	Normal	Normal
Cytoplasmic granulation	Present	Absent	Absent

Morphology scores were measured for all oocytes retrieved with a minimum score of 0 and a maximum score of 6. The mean morphology scores for each patient was done

Fertilization by a single sperm using micro-insemination was done for each oocyte. then fertilization rate (percentage of transformation of micro injected oocytes into two pronuclei) for each patient was measured by dividing the number of fertilized oocytes to the number of injected oocytes.

Following microinjection and fertilization, the resulting embryo was graded according to Society for Assisted Reproductive Technology grading system (SART) **[11]**.

Embryo transfer was done 3 – 5 days after oocyte retrieval, and luteal support was mainuined by IM progesterone administration (Prontogest 100 mg® ampoule, IBSA, Switherland). We defined clinical pregnancy as the detection of gestational sac using transvaginal ultrasonography 21 days after embryo transfer **[7]**.

Statistical analysis

Data were statistically analyzed using SPSS software version 26 for Mac. Quantitative data were expressed as mean ± standard deviation and range. Pearson's correlation coefficient and spearman's correlation coefficient were used to test the association between two quantitative parametric and non-parametric variables respectively. The diagnostic ability of a quantitative variable to test the occurrence of categorical outcomes was assessed using receiver operator characteristic curve and was expressed in the following terms; sensitivity, specificity, positive predictive value, negative predictive value and accuracy. Probability (p value) ≤ 0.05 was considered to be statistically significant.

3. Results

Starting with demographic data, the mean age of the included cases was 28.56 years (range, 20 – 35). In addition, BMI had mean value of 27.38 kg/m^2 (range, 24 – 30). When it comes to hormone levels, the mean value of serum AMH was 1.91 ng/ml (range, 1 – 3.3), while follicular fluid AMH had mean values of 1 ng/ml (range, 0.2 – 2.8). The previous data are illustrated at table (2).

Table (2): Mean and standard deviation of studied cases in relation to age, body mass index, serum and FF AMH.

Characteristics	Range	Mean ± SD
Age in years	20-35	28.56 ± 4.32
Body mass index	24-30	27.38 ± 1.98
S AMH (ng/ml)	1.0-3.3	1.91±0.67
FF AMH (ng/ml)	0.2-2.8	1.00±0.66

As shown in table (3), there was no significant correlation between serum and follicular fluid AMH levels (p = 0.965 – r = 0.005).

Table (3): Correlation between serum and FF AMH.

Hormones	Follicular fluid AMH	
	r	p
Serum AMH	0.005	0.965

A significant positive correlation was noted between serum AMH and retrieved oocytes number (r = 0.618 – p = 0.001). On the other hand, no significant correlation was detected between FF AMH and oocyte number (p = 0.400). While evaluating the meiotic status, no significant correlation was noted between different meiotic stages with either serum or FF AMH levels (p > 0.05), apart from MI and MII that had a significant positive correlation with serum AMH (p = 0.034 and 0.001 respectively). There was a significant positive correlation between serum AMH and oocyte score (r = 0.510 – p = 0.001). However, FF AMH did not have a significant correlation with the same parameters (p = 0.320). Table (4) summarizes these data.

Table (4): Correlations between serum, and FF AMH levels, number of retrieved oocytes (quantitative), oocyte meiotic status evaluation and oocyte score (according to modified Xia score).

Hormones	Number of retrieved oocytes		oocyte meiotic status								Oocyte score (modified Xia score)	
			GV		M I		M II		PM			
	r	p	r	p	rho	p	r	p	rho	p	r/rho	p
S AMH	0.618	0.001*	-0.159	0.015	0.230	0.034*	0.611	0.001*	-0.191	0.080	0.510	0.001*
FF AMH	-0.093	0.400	0.015	0.893	0.023	0.838	-0.129	0.240	-0.016	0.884	-0.109	0.320

Serum AMH and injected oocyte number had a significant positive correlation (p = 0.001), the number of fertilized oocytes (p = 0.001). Fertilization rate showed the same relation (p = 0.006). Nevertheless, no significant correlation was noted between the FF AMH levels and either of the previous parameters (p > 0.01). Table (5) shows these data.

Table (5): Correlations between serum, and FF AMH levels and fertilization capacity.

Hormones	Fertilization capacity					
	Injected oocytes		Fertilized oocytes		Fertilization rate	
	r	p	r	p	r	p
S AMH	0.598	0.001*	0.626	0.001*	0.294	0.006*
FF AMH	-0.071	0.519	-0.154	0.159	-0.162	0.139

A significant positive correlation was detected between serum AMH and embryos number (r = 0.567 – p = 0.001), whereas FF AMH had no significant correlation with that parameter (p = 0.059). When it comes to the embryo grading, serum AMH had a significant positive correlation with either of good and fair quality embryos (p = 0.003 and 0.001 respectively), while there was no significant correlation with poor quality embryos. FF AMH had a significant negative correlation with fair quality embryos (p = 0.028), with no reported significant correlation with other embryo grades. Table (6) summarizes these data.

Table (6): Correlations between serum, and FF AMH levels, embryos number and embryo grading according to SART group 2010.

Hormones	Embryos number		Embryo grading according to SART group 2010					
			Good quality		Fair quality		Poor quality	
	rho	p	r	p	r	p	rho	p
S AMH	0.567	0.001*	0.318	0.003*	0.390	0.001*	-0.060	0.588
FF AMH	-0.206	0.059	-0.066	0.551	-0.238	0.028*	0.131	0.233

As shown in table (7), serum AMH had significantly higher levels in pregnant cases (2.09 vs. 1.66 ng/ml in non-pregnant cases – p = 0.001). However, FF AMH did not show a significant difference (p = 0.485).

Table (7): Correlations of serum and follicular fluid anti-Mullerian hormone in relation to occurrence of pregnancy.

Variables	Pregnancy negative		Pregnancy positive		t	p
	Range	Mean+SD	Range	Mean+SD		
S AMH	1.0-3.2	1.66±0.59	1.1-3.3	2.09±0.67	7.569	0.001*
FF AMH	0.3-2.8	1.09±0.67	0.2-2.8	0.93±0.65	0.701	0.485

Using a cut-off value of 1.55 ng/ml, serum AMH had sensitivity and specificity of 72 and 60% respectively to predict pregnancy. Table (8) and figure (1) show these data.

Table (8): Recipient- Observer- Characteristic curve (ROC-Curve) for prediction of pregnancy by serum AMH.

ROC results	
Area under the curve	0.700
P	0.002*
Cut off value	1.55
Sensitivity	72.0%
Specificity	60.0%

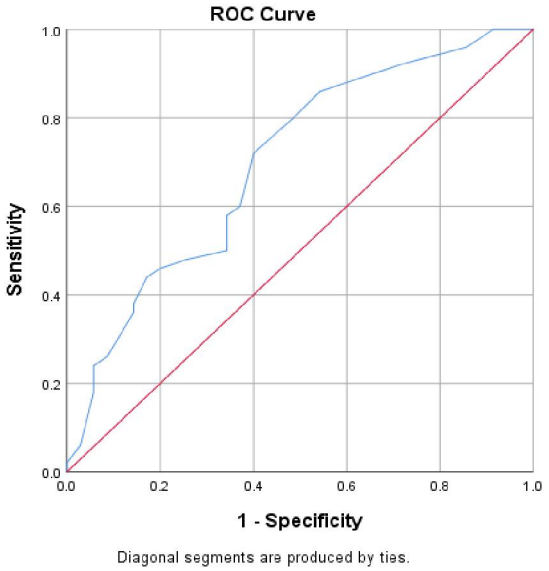

Figure (1): ROC curve showing the ability of serum AMH to predict pregnancy

4. Discussion

Previous studies reported that serum AMH levels along with antral follicle count are the most important tools used for the evaluation of ovarian reserve before IVF **[12, 13]**. As a significant positive correlation was reported between the previous two parameters, serum AMH has been used as an indicator of antral and pre-antral follicle count **[14, 15]**.

FF, secreted from both granulosa and theca cells, helps in the formation of optimum microenvironment for oocyte development as it contains certain biochemical substances that are crucial for oocyte quality, fertilization capacity and embryo development **[16]**. After extensive literature details, great debate exists about the role of FF AMH as a predictor of ICSI outcomes. Hence, we conducted the present study to compare serum and FF AMH levels as predictors of ICSI outcomes.

In our study, significant negative correlation was detected between serum AMH and both age (r = - 0.529 – p = 0.001) and BMI (r = - 0.461 – p = 0.001). On the contrary, no significant correlation was detected between follicular fluid AMH and either of these two parameters (p = 0.429 and 0.114) respectively. A previous study has reported that serum AMH had significant negative correlation with patient age (r = - 0.311 – p = 0.043), and that agrees with our findings. Conversely, no significant correlation was detected between follicular fluid AMH and the same parameter (p = 0.264) **[17]**.

Of note, our results also showed significant negative correlation with serum AMH and BMI (r = - 0.461 – p = 0.001), whereas FF AMH had no significant correlation with either age nor BMI. To the best of our knowledge, there a paucity of studies in the existing literature that handle the previously mentioned correlations.

In the current study, no significant correlation was detected between serum and FF AMH levels (p = 0.965 – r = 0.005). Nevertheless, in a previous study handling the same perspective, significant positive correlation was also detected between serum and FF AMH levels in both pregnant (r = 0.78 – p < 0.001) and non-pregnant cases (r = 0.86 – p < 0.001) **[3]**. Another study also confirmed the previous findings (r = 0.47 - P < 0.0001) **[18]**. The heterogeneity between different studies could be due to different sample size, or statistical tests, and that should emphasize the need for further studies to clearly elucidate this relationship.

In our study, significant positive correlation was reported between serum AMH and retrieved oocyte number (r = 0.618 – p = 0.001). On the other hand, no significant correlation was detected between FF AMH and oocyte number (p = 0.400). Since it is produced by small follicles, AMH has been proposed as a predictor for ovarian response. However, its association with the growing follicle is complex in nature despite the numerous studies handling that perspective **[5, 6, 19]**.

Çapkın et al. reported that serum AMH had significant positive correlation with oocyte numbers (r = 0.343 - p = 0.024) **[17]**. Additionally, Wunder et al. and Fong et al. confirmed the same findings **[20, 21]**. On the contrary, Zargar et al. denied any significant correlation between serum AMH with oocyte number (p = 0.96) **[3]**. Regarding FF AMH, another two studies also negated any significant correlation between follicular fluid AMH and the number of oocytes **[3, 22]**. The latter two studies confirmed our findings regarding follicular fluid AMH and its relation with oocyte number.

On oocyte meiotic status evaluation in the current study, no significant correlation was noted between different meiotic stages with either serum or follicular fluid AMH levels (p > 0.05), apart from MI and MII that had significant positive correlation with serum AMH (p = 0.034 and 0.001 respectively). Çapkın et al. reported significant positive correlation between serum AMH and MII oocyte number (r = 0.389, p= 0.01) which confirmed our results. However, no significant correlation was detected between the same variable and follicular fluid AMH level (p = 0.361) **[17]**. The correlation with serum AMH was confirmed in another study **[23]**.

In our study, there was a significant positive correlation between serum AMH and oocyte score, (r = 0.510 – p = 0.001). However, FF AMH did not have a significant correlation with the same parameters (p = 0.320). In 2011, Irez et al. showed that serum AMH may act as a surrogate marker for oocyte quality **[24]**. Wang et al. reported that higher serum AMH levels were associated with better pregnancy outcomes due to better oocyte quality (p < 0.01) **[25]**, which is in accordance with our findings. On the other hand, another study by Guerif et al. denied any correlation between serum AMH and oocyte quality or development **[26]**.

Another study reported that serum AMH had significant positive correlation with oocyte morphology scores (p < 0.001) which is in accordance with our findings. However, the same authors noted a significant association between FF AMH and oocyte score which was not reported by us **[27]**. However, another study reported that FF AMH levels expressed positive correlation (r = 0.331; P = 0.015) with the matched embryo score three days after fertilization **[28]**.

A previous Chinese meta-analysis confirmed the positive relationship between serum AMH level and oocyte quality but FF AMH yielded ambiguous results **[29]**. Another study reported that FF AMH level cannot be used as a predictor for oocyte quality **[27]**.

The previous two reports are in line with our findings regarding FF AMH. On the contrary Yilmaz et al. and Mehta et al. demonstrated an inverse correlation between FF AMH and either of oocyte growth, maturation, and quality **[30, 31]**.

In the current study, there was a significant positive correlation between serum AMH and the injected oocyte number (p = 0.001), fertilized oocyte number (p = 0.001), and the fertilization rate (p = 0.006). Nevertheless, no significant correlation was noted between FF AMH levels and either of the previous parameters (p > 0.01). A previous study also confirmed the nonsignificant correlation between FF AMH and fertilization rate (p = 0.344). However, the same authors also reported a nonsignificant correlation between fertilization rate with serum AMH (p = 0.854) which is in contrast to our findings **[17]**. Another study also negated any significant impact of follicular fluid AMH on fertilization rates (p = 0.0102) whereas the opposite was detected on serum AMH analysis (p = 0.007) **[22]**, which is in accordance with our findings.

In our study, significant positive correlation was detected between serum AMH and the number of embryos (r = 0.567 – p = 0.001), whereas follicular fluid AMH had no significant correlation with that parameter (p = 0.059). Another study confirmed the previous findings that serum AMH on Day 3 was significantly correlated to the number of good quality embryos **[32]**.

When it comes to the embryo grading in the current study, serum AMH had a significant positive correlation with either of good and fair quality embryos (p = 0.003 and 0.001 respectively), while there was no significant correlation with poor quality embryos. FF AMH had a significant negative correlation with fair quality embryos (p = 0.028), with no reported significant correlation with other embryo grades. Another study showed that baseline serum AMH and FF AMH had significant positive correlations with good quality embryo number **[33]**.

When the included cases were divided according to the pregnancy outcome, cases were divided into two groups; pregnancy positive cases (50 cases – 58.8%) and pregnancy negative cases (41.2%). Serum AMH had significantly higher levels in pregnant cases (2.09 vs. 1.66 ng/ml in non-pregnant cases – p = 0.001). However, follicular fluid AMH did not show a significant difference between the two groups (p = 0.485). Çapkın et al. confirmed these findings as serum AMH had mean values of 1.87 in pregnant ladies versus 0.35 ng/ml in non-pregnant ones (p = 0.017). A significant positive correlation was detected between serum AMH levels and implantation rates **[17]**. Moreover, another study reported that pregnant ladies had mean serum AMH of 5.39 ng/ml versus 3.86 ng/ml in non-pregnant cases, with a significant difference between the two groups (p = 0.012) **[34]**. Conversely, another study denied any significant relationship between serum AMH level and the pregnancy rate **[35]**. Furthermore, Zargar and his coworkers negated any significant difference between pregnant and non-pregnant cases regarding serum AMH levels, which had mean values of 3.14 and 1.82 ng/ml in both groups respectively (p = 0.71) **[3]**.

Our results showed that follicular fluid AMH did not show a significant difference between the two groups (0.93 and 1.09 ng/ml in pregnant and non-pregnant cases - p = 0.485). In another study, there was no significant difference between pregnant and non-pregnant cases as regard follicular fluid AMH levels (p = 0.56), that had mean values of 6.48 and 3.85 ng/ml in the two groups respectively **[3]**. Furthermore, Bolat et al. reported that follicular fluid AMH levels did not significantly differ between pregnant and non-pregnant cases (2.1 vs. 1.66 ng/ml respectively – p = 0.057) **[22]**. On the other hand, limited studies reported the positive impact of FF AMH on implantation rate, as it acts as a mirror for granulosa cell function **[18]**. An additional Turkish study reported a significant elevation of follicular fluid AMH levels in cases who got pregnant compared to who did not (4.1 vs. 1.87 ng/ml respectively – p = 0.028) **[17]**. Hattori et al. also confirmed these findings as follicular fluid AMH had mean values of 16.03 and 9.18 ng/ml in pregnant and non-pregnant cases respectively (p = 0.014) **[34]**.

In our study, using a cut-off value of 1.55 ng/ml, serum AMH had sensitivity and specificity of 72 and 60% respectively to predict pregnancy. In another study, a 1.64 ng/ml cut off value had sensitivity and specificity of 71.4 and 69% respectively **[17]**. In the study conducted by Tolikas et al., for baseline AMH, a cutoff value of 2.74 yielded 69% sensitivity and 70.5% specificity, while day-five AMH cutoff value of 2.7 gave 69% sensitivity and 60.7% specificity for prediction of pregnancy response **[36]**.

Our study has some strength points**,** women with polycystic ovary syndrome were not included in our study as inclusion of PCOS cases may produce heterogeneity in the results due to abnormal secretion of AMH in PCOS patients. Also, The present study had taken a holistic approach towards AMH measurement by estimating its levels in pooled fluid obtained from a cohort of follicles from which an oocyte had been retrieved in each cycle. And 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 the practice of carrying studies in individual lead follicles may be too

time-consuming, cumbersome, and unfeasible for most laboratories.

Our study has some limitations, being conducted in a single center along with the relatively small sample size are the most apparent drawbacks. This necessitates the need for further studies handling the same point of view.

Conclusion

Based on our findings, serum AMH appear to be more useful than its FF levels. Its levels showed a significant difference between pregnant and non-pregnant subjects.

Conflict of interest

The authors report no conflict of interest.

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The laboratory requirements for AMH analysis was self-provided by the corresponding author.

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