Correlations between follicular fluid AMH and IVF/ICSI outcomes among polycystic ovary syndrome women using different controlled hyperstimulation protocols

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Research Article

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Abstract

Background

Anti-Müllerian hormone (AMH) is a glycoprotein that plays an important role in the regulation of ovarian folliculogenesis. However, the data link between its follicular fluid levels (FF AMH) and the IVF/ICSI outcomes in polycystic ovary syndrome (PCOS) women are limited, contradicted, and mainly obtained from Long-GnRH agonist cycles. Thus, we conducted this study to compare the correlations between the FF AMH levels and the IVF/ICSI outcomes in PCOS women during different controlled hyperstimulation protocols.

Methods

The current study is a re-analysis of our previous work. The data were adopted from a prospective trial that was conducted on women who were referred to the Assisted Reproductive Unit of Orient Hospital, Damascus, Syrian Arab Republic, from December 2019 to August 2021. A total of 75 PCOS women (Rotterdam criteria) (GnRH agonist group, PCOS-A, n = 53; GnRH antagonist group, PCOS-Anta, n = 22) were included. Follicular fluid samples were collected on the retrieval day, and the FF AMH levels were measured using ELISA Kits. In addition, the embryological and clinical IVF/ICSI outcomes were detected. Spearman rank correlation coefficients were computed to assess the correlations among the studied parameters. The area under the receiver operating characteristic (ROC) curve (AUC) was used to evaluate the accuracy of FF AMH levels in predicting pregnancy rates.

Results

The patients' baseline characteristics were comparable between the PCOSA and the PCOSAnta groups. FF AMH levels were negatively correlated with the total FSH dose, the number of retrieved, MII, MI, germinal vesicle, immature, and fertilized oocytes, and with the number of obtained embryos in the PCOSA group, but not in the PCOSAnta one. Nevertheless, there were not any correlations between FF AMH levels and the rates of oocyte maturation or fertilization, or with the highquality embryo rate, embryos cleavage rate or implantation rate in any of the studied groups. In addition, no significant differences were noted in FF AMH levels between pregnant and non-pregnant women in any of the studied groups, which also was confirmed by the Receiver Operating Characteristic (ROC) Curve analysis.

Discussion

Since the FF AMH levels do not correlate with the maturation rate, fertilization rate, or embryos cleavage rate, the negative correlations in the PCOSA group arise from the negative impacts of the FF AMH on the number of retrieved oocytes. During the long agonist protocol, since PCOS follicles have good follicular angiogenesis, the number of retrieved oocytes would mostly depend on the negative effects of the FF AMH on folliculogenesis. However, during the GnRH antagonist protocol, the limited angiogenesis acts together with the high levels of FF AMH to reduce the number of retrieved oocytes.

Conclusions

High FF AMH negatively affects ovarian folliculogenesis during both; the long GnRH agonist protocol and the flexible GnRH antagonist one. However, the different patterns of follicular angiogenesis during the two protocols would affect the dependency of the oocytes' yield on the follicular fluid levels of AMH.

Study registration:

The data were adopted from a prospective clinical trial that was registered on the clinicaltrials.gov site by registration numbers NCT04727671.

Introduction

Polycystic ovary syndrome (PCOS) is the most common endocrine disorder among females of reproductive age [1] and represents the principal cause of anovulation cases referring to infertility clinics [2]. The principal manifestations of this syndrome are ovulatory dysfunction, hyperandrogenism, and polycystic ovarian morphology [1]. In-vitro Fertilization/ Intra-Cytoplasmic Sperm Injection (IVF/ICSI) technologies are considered the third-line treatment option for PCOS women after the failure of other approaches of ovulation induction [3]. One of the fundamental steps of controlled ovarian hyper-stimulation (COH) of IVF/ICSI cycles is to prevent premature luteinizing hormone (LH) surge while the follicles are still immature, which has usually accomplished by using one of the Gonadotropin-releasing hormone (GnRH) analogues [4].

Oocyte developmental competence is one of the determining factors that affect the ability of a female gamete to maturate, be fertilized, and promote embryo development during pre-implantation and full-term phases [5,6]. A healthy intra-follicular environment is critical for oocyte competency [7], and disruptions in the levels of follicular fluid components may influence the final outcomes of IVF/ICSI cycles. Controlled ovarian stimulation perturbs the follicular milieu [8–11], but it is still unclear whether these alterations may differ among different COH protocols. Several studies have reported differences in follicular fluid levels of Anti-Müllerian hormone (FF AMH) levels between unstimulated and stimulated cycles [10–13], AMH, also known as Müllerian inhibiting substance (MIS), is a dimeric glycoprotein that belongs to the transforming growth factor (TGF)-β family and plays an important role in the regulation of ovarian folliculogenesis [13,14]. AMH levels increase both in serum and follicular fluid samples of PCOS women [15–19]. Although the cause of this elevation is still unknown, current evidence suggests that it may be a result of the increased number of pre-antral and small antral follicles seen in PCOS ovaries besides the overproduction of AMH per granulosa cell (GC) [20,21]. In addition, some studies reported an abnormality in AMH/AMHR regulation system in PCOS subjects [22–25]. Serum AMH has widely been used in infertility clinics as a marker for ovarian reserve [26]. Yet, it is considered a poor predictor for natural pregnancy [27], and its role in predicting pregnancy after IVF/ICSI cycles in PCOS women is still controversial [17,28–30]. Similarly, data regarding the predictive value of FF AMH in predicting IVF/ICSI outcomes in PCOS subjects are limited, contradicted, and mainly obtained from Long-GnRH agonist cycles [17,31–35]. Thus, further research is needed to clarify whether the FF AMH effects on IVF/ICSI outcome would depend on the COH protocol used.

Objectives

This study aimed to evaluate the effects of FF AMH on the IVF/ICSI outcomes in PCOS women and determine the usefulness of their levels in predicting pregnancy achievement in this population. In addition, to clarify whether the results would still be consistent among the most commonly used GnRH analogues protocols; the long GnRH agonist protocol and the flexible GnRH antagonist protocol.

Materials And Methods

Study Design

The current study is a re-analysis of our previous work. The data were adopted from a prospective clinical trial [36] that was registered on the clinicaltrials.gov site on registration numbers NCT04727671. The trial was conducted on women who were referred to the Assisted Reproductive Unit of Orient Hospital, Damascus, Syrian Arab Republic, from December 2019 to August 2021. The Ethical Committee of Damascus University approved the study protocol, and a written informed consent was obtained from all participants.

Participants

In this study, we included a total of 75 PCOS women (GnRH agonist group, PCOS-A, n=53; GnRH antagonist group, PCOS-Anta, n=22). Both the patients and the doctors were aware of the allocated arm.
PCOS diagnosed was according to the Rotterdam criteria [37]; the presence of at least two of the following three criteria: (1) oligo or anovulation, (2) clinical and/or biochemical signs of hyperandrogenism, (3) polycystic ovarian morphology on ultrasound examination (defined as the presence of 12 or more follicles in each ovary measuring 2–9 mm in diameter and/or an ovarian volume >10 ml) with the exclusion of other possible etiologies. Patients who aged ≥ 40 years; or were diagnosed with androgen-secreting tumors, Cushing's syndrome, congenital adrenal hyperplasia, hyperprolactinemia, thyroid disorders, epilepsy, diabetes mellitus, cardiovascular diseases, liver diseases, kidney diseases, cancer; or had any conditions that might affect IVF outcomes like endometriosis, uterine fibroids, hydrosalpinx, adenomyosis, or autoimmune diseases were excluded. Women with three or more previous IVF failures, poor responders (Bologna criteria [38]), and those who were previously undergone unilateral oophorectomy were also excluded.

Controlled ovarian stimulation protocols:

Agonist Group (Long protocol):

The pituitary down-regulation in this group was carried out using 0.05-0.1 mg of Triptorelin acetate subcutaneously (SC) once daily from the mid-luteal phase (day 21) of the menstrual cycle until the ovulation triggering day. When the suppressive effect was obtained (E2 < 50 pg/ml, no cysts or follicles > 1 cm maximum diameter detected by ultrasound, endometrial thickness < 5 mm), ovarian stimulation was

commenced with recombinant Follicle-Stimulating Hormone (r-FSH) and/or human Menopausal Gonadotropin (hMG), and the dose was adjusted according to the ovarian response, which was monitored by transvaginal ultrasound (Voluson ^{TM} E10, GE Healthcare Ultrasound, USA).

Antagonist Group (Conventional Flexible protocol):

The ovarian stimulation in this group was started with recombinant Follicle-Stimulating Hormone (r-FSH) and/or human Menopausal Gonadotropin (hMG) on the third day of the menstrual cycle, and the dose was adjusted according to the ovarian response, which was monitored by transvaginal ultrasound (Voluson ^{TM} E10, GE Healthcare Ultrasound, USA). The initiation of 0.25 mg of GnRH antagonist, Cetrorelix, took place after detecting a leading follicle diameter ≥ 14 mm and continued till the day of ovulation triggering.

Ovulation triggering and oocytes retrieval:

Ovulation was triggered by the administration of 10,000 IU of Human Chorionic Gonadotropin (hCG) when at least three follicles become more than 16-17 mm. After 35±2 hours of ovulation triggering, the oocytes were retrieved by transvaginal ultrasound-guided follicle aspiration.

IVF procedure and embryological outcomes assessment:

An Intra-Cytoplasmic Sperm Injection (ICSI) technique was used for insemination. The embryological outcomes were assessed by independent highly-trained embryologists. Each studied outcome was assessed by a single assessor for all groups to limit inter-assessor variations. The same media and culturing methodology were used for the two groups. The Thermo Scientific HERACELL 150i incubator (Thermo Fisher Scientific, USA) was used for COCs and oocytes cultures (humidified atmosphere at 37ºC, CO2 level at approximately 6%, and culture medium pH between 7.28-7.35), and the K-Systems G210 InviCell (K-Systems Kivex Biotec Ltd. Denmark) was used for Embryos cultures.

Oocytes denudation and maturation assessment:

Retrieved oocytes were first rinsed in G-MOPS ^{TM} Plus media (G-MOPS ^{TM} Plus, Vitrolife, Sweden) then maintained in G-IVF ^{TM} Plus culture (G-IVF ^{TM} Plus, VitroLife, Sweden) covered with paraffin oil (OVOIL, VitroLife, Sweden) before cumulus cell removal. The surrounding cumulus cells were removed within 2 hours after retrieval by the exposure to hyaluronidase (HYASE-10× in G-Mops ^{TM} Plus media, Vitrolife, Sweden) for several seconds before being transferred to G-MOPS ^{TM} Plus media where they were mechanically dissociated from the oocyte.

The denuded oocytes were classified according to their level of maturation using a Nikon SMZ1500 stereoscope. The number of Metaphase II Oocytes (MII; identified as oocytes with the extrusion of the first polar body), Metaphase I Oocytes (MI; identified as oocytes lack the presence of both the germinal vesicle

and the polar body), Germinal Vesicle Oocytes (GV; identified as oocytes with Germinal Vesicle), and Atretic Oocytes (oocytes with signs of degeneration) were documented. The Maturation Rate was calculated by dividing the number of mature (MII) oocytes by the number of retrieved oocytes. In addition, the ovarian sensitivity index (OSI) was calculated by dividing the number of retrieved oocytes by the total dose of FSH used and multiplying the results by 1000 [39].

Insemination and fertilization assessment:

Microinjections were performed at X400 magnification on a 37ºC heated stage inverted Nikon Eclipse Ti2 (Nikon, Tokyo, Japan). A Petri dish containing a microdroplet of ICSI ™ media in the center (ICSI ™, VitroLife, Sweden) under paraffin oil (OVOIL, VitroLife, Sweden) was used for sperms selection and immobilization. On the same dish, a microdroplet of G-Gamete ™ culture medium (G-Gamete ™, Vitrolife, Sweden) was used for placing the oocytes for microinjection. A single sperm was mechanically immobilized using the tip of the microinjection needle (Origio, USA) and then was aspirated inside the needle. The oocyte was held in place using a 35-degree angle holding micropipette (Origio, USA) with the polar body in the 6 or 12 o'clock position. Injection of a single spermatozoon within the oocyte cytoplasm was performed by using a micromanipulator (TransferMan® 4r, eppendorf, Germany). After ICSI, injected oocytes were cultured in G1-Plus ™ medium (G1-Plus ™, VitroLife, Sweden). Fertilization was confirmed by the presence of two pronuclei and the extrusion of the second polar body approximately 16-18 h after ICSI. The Fertilization Rate was calculated by dividing the number of obtained zygotes (2PN) by the number of injected oocytes.

Embryos Grading, Cleavage rate, and high-quality embryos rate:

Embryos were morphologically evaluated using Nikon SMZ1500 stereoscope microscope (Nikon, Tokyo, Japan) and were graded based on ESHRE criteria (2011) [40]. According to these criteria, high-quality cleavage-stage embryos are defined as those with all of the following characteristics: 2-4 cells on day 2 or 6-8 cells on day 3, <10% fragmentation, symmetric blastomeres, and absence of multinucleation. Cleavage rate was calculated by dividing the number of cleavaged embryos by the number of obtained zygotes (2PN), while High-Quality Embryos Rate was calculated by dividing the number of high-quality embryos (Grade I) obtained by the total number of cleavaged embryos obtained.

Embryos transfer and luteal phase support:

The Selected embryos were treated with EmbryoGlue® media (EmbryoGlue®, VitroLife, Sweden) before being transferred using a Sure-Pro Ultra catheter (Wallace, USA) under transvaginal ultrasound guidance on day 2-3 after insemination (cleavage stage embryos). Luteal phase support was achieved using vaginal micronized progesterone gel (Crinone ® 8%, Merck Serono). It was started from the day of oocyte retrieval and continued for 14 days when a pregnancy was carried out. If pregnancy was confirmed, progesterone administration was continued until the 12th week of pregnancy.

Embryo transfer was cancelled, and elective embryo cryopreservation was performed in cases that were highly suspected of developing life-threatening (critical) OHSS [41,42] or fulfill the criteria for OHSS hospitalization [43]. Cycle Cancellation Rate (CCR) was calculated by dividing the number of cycle cancellation cases by the total number of participants.

Follicular fluid collection and analysis:

Follicular fluid was aspirated from all follicles (>15) mm, and then it was centrifuged at 3000 g for 10 min at room temperature, and the supernatant was stored at −80 °C until assayed. Follicular fluid concentrations of AMH were assayed using an ELISA kit from Biorex diagnostics (United Kingdom). The intra-assay and inter-assay coefficients of variation were less than 5% and less than 10%, respectively.

Pregnancy assessment and follow up:

A serum pregnancy test (serum hCG) was performed 14 days after embryo transfer. All women with a positive test received a transvaginal ultrasound scan after one-two weeks. Clinical pregnancy was defined as the presence of at least one gestational sac on ultrasound after 3-4 weeks of embryo transfer. In addition to intra-uterine pregnancy, it included a clinically documented ectopic pregnancy [44]. The Implantation rate was calculated by dividing the number of gestational sacs observed by the number of embryos transferred. Then the pregnancy was followed up till week 12 of gestation (ongoing pregnancy).

Statistical analysis

All statistical analyses were performed using a Statistical Package for the Social Sciences (SPSS) software version 24.0 (IBM Corp., Armonk, NY, USA). Continuous variables were expressed as mean ± standard deviation and categorical variables as counts with percentages. Between-group comparisons were performed using the independent t-test for normally distributed variables, the Mann–Whitney U test for non-normally distributed variables, and chi-square or Fisher's exact test as appropriate for categorical variables. Spearman rank correlation coefficients were computed to assess the correlation among the studied parameters. The area under the receiver operating characteristic (ROC) curve (AUC) was used to evaluate the accuracy of the FF AMH levels in predicting pregnancy rates. For testing all hypotheses, tests were two-tailed, and values less than 0.05 were considered statistically significant.

Results

The patients' baseline characteristics and the cycles' characteristics were comparable between the PCOS-A and the PCOS-Anta groups, as shown in Table 1 and Table 2, except for the stimulation duration, which was significantly shorter in the PCOS-Anta group. As shown in Table 3, FF AMH levels were negatively correlated with the total FSH dose (r = -0.346, P = 0.011), the number of retrieved oocytes (r = -0.418, P = 0.002), MII oocytes (r = -0.354, P = 0.009), MI oocytes (r = -0.429, P = 0.001), GV oocytes (r = -0.286, P = 0.038), immature oocytes (r = -0.413, P = 0.002), fertilized oocytes (r = -0.309, P = 0.024), and the number of

obtained embryos (r = -0.299, P = 0.030) in the PCOS-A group, but not in the PCOS-Anta one. Similar correlations were noted between the FF AMH levels and the studied IVF/ICSI outcomes after including the total number of participants, except for the correlations with the number of fertilized oocytes and the number of obtained embryos, which became insignificant. Nevertheless, there were not any correlations between FF AMH and the rates of oocyte maturation or fertilization, or with the high-quality embryo rate, embryos cleavage rate, or implantation rate in any of the studied groups. In addition, no significant differences were noted in FF AMH levels between pregnant and non-pregnant women in any of the studied groups, as shown in Table 4, which also was confirmed by the Receiver Operating Characteristic (ROC) Curve analysis (Table 5).

Table 1. Patients Baseline Characteristics:

	ALL N=75	GnRH Agonist N=53	GnRH Antagonist N=22	P value
Female age (years)	27.64 ± 4.72	27.87 ± 4.57	27.09 ± 5.15	0.411
Male age (years)	35.25 ± 6.54	35.51 ± 6.41	34.64 ± 6.96	0.838
Infertility % (n):				0.272
Primary	64.0% (48/75)	67.9% (36/53)	54.5% (12/22)	
Secondary	36.0% (27/75)	32.1% (17/53)	45.5% (10/22)	
Infertility cause % (n):				0.195
PCOS only	21.4% (16/75)	18.9% (10/53)	27.3% (6/22)	
PCOS + Male factor	77.3% (58/75)	81.1% (43/53)	68.2% (15/22)	
PCOS + Tubal factor	1.3% (1/75)	0.0% (0/53)	4.5% (1/22)	
PCOS + Male factor + Tubal factor	0.0% (0/75)	0.0% (0/53)	0.0% (0/22)	
Infertility duration (years)	5.90 ± 3.74	5.75 ± 3.35	6.25 ± 4.62	0.995
Smoker Female % (n)	24.0% (18/75)	18.9% (10/53)	36.4% (8/22)	0.106
Smoker Male % (n)	54.7% (41/75)	50.9% (27/53)	63.6% (14/22)	0.315
Female alcohol-consuming % (n)	0.0% (0/75)	0.0% (0/53)	0.0% (0/22)	-
Male alcohol-consuming % (n)	1.3% (1/75)	1.9% (1/53)	0.0% (0/22)	1.000
Male classification % (n):				0.284
Normozoospermia	20.0% (15/75)	13.2% (7/53)	36.4% (8/22)	
Mild-Moderate Male factor	32.0% (24/75)	32.1% (17/53)	31.8% (7/22)	
Oligoasthenoteratozoospermia	28.0% (21/75)	32.1% (17/53)	18.2% (4/22)	
Azoospermia	13.3% (10/75)	15% (8/53)	9.1% (2/22)	
Necrozoospermia	2.7% (2/75)	3.8% (2/53)	0.0% (0/22)	
Cryptozoospermia	4.0% (3/75)	3.8% (2/53)	4.5% (1/22)	

PCOS: Polycystic Ovary Syndrome.

Table 2. Cycles Characteristics:

	ALL N=75	GnRH Agonist N=53	GnRH Antagonist N=22	P value
Types of stimulator % (n):				0.579
r-FSH	86.6% (65/75)	88.7% (47/53)	81.9% (18/22)	
hMG	2.7% (2/75)	1.9% (1/53)	4.5% (1/22)	
r-FSH + hMG	10.7% (8/75)	9.4% (5/53)	13.6% (3/22)	
FSH starting dose (units)	227.00 ± 74.97	227.83 ± 72.00	225.00 ± 83.45	0.690
Total FSH dose (units)	1842.33 ± 675.05	1868.40 ± 668.29	1779.55 ± 702.87	0.432
Stimulation duration (days)	7.92 ± 0.96	8.04 ± 0.81	7.64 ± 1.22	**0.011**
Sperms Source % (n):				0.093
Ejection	76.0% (57/75)	75.5% (40/53)	77.3% (17/22)	
Tesa	18.7% (14/75)	22.6% (12/53)	9.1% (2/22)	
Pesa	0.0% (0/75)	0.0% (0/53)	0.0% (0/22)	
Frozen	1.3% (1/75)	0.0% (0/53)	4.5% (1/22)	
Ejection + Tesa	4.0% (3/75)	1.9% (1/53)	9.1% (2/22)	
Day of transfer				0.683
Day 2	61.8% (42/68)	63.3% (31/49)	57.9% (11/19)	
Day 3	38.2% (26/68)	36.7% (18/49)	42.1% (8/19)	
Cycle cancellation Rate % (n)	9.3% (7/75)	7.5% (4/53)	13.6% (3/22)	0.412
Cycle cancellation Rate due to risk of OHSS % (n)	5.3% (4/75)	5.7% (3/53)	4.5% (1/22)	1.000

hMG: human Menopausal Gonadotropin, OHSS: Ovarian Hyperstimulation Syndrome, Pesa: Percutaneous Epididymal Sperm Aspiration, r-FSH: recombinant-Follicle Stimulating Hormone, Tesa: Testicular Sperm Aspiration.

Table 3. Correlations between FF AMH and IVF/ICSI outcomes:

	ALL N=75		GnRH Agonist N=53		GnRH Antagonist N=22	
	Correlation Coefficient	P value	Correlation Coefficient	P value	Correlation Coefficient	P value
Female age (years)	0.099	0.400	0.247	0.074	-0.164	0.467
Infertility duration (years)	0.141	0.226	0.203	0.144	-0.020	0.930
Total FSH dose (units)	-0.304	**0.008**	-0.346	**0.011**	-0.200	0.373
Stimulation duration (days)	-0.066	0.574	-0.057	0.687	-0.194	0.387
Endometrial thickness on hCG day (mm)	0.100	0.396	0.178	0.202	0.009	0.969
Number of Retrieved Oocytes	-0.404	**<0.001**	-0.418	**0.002**	-0.330	0.134
Ovarian Sensitivity Index	-0.220	0.058	-0.198	0.156	-0.277	0.212
Number of Metaphase II Oocytes	-0.306	**0.008**	-0.354	**0.009**	-0.081	0.721
Number of Metaphase I Oocytes	-0.388	**0.001**	-0.429	**0.001**	-0.262	0.239
Number of GV Stage Oocytes	-0269	**0.020**	-0.286	**0.038**	-0.154	0.494
Number of Immature Oocytes (GV + MI)	-0.393	**<0.001**	-0.413	**0.002**	-0.218	0.329
Number of Atretic Oocytes	-0.086	0.462	-0.074	0.600	-0.109	0.630
Number of Fertilized Oocytes	-0.201	0.084	-0.309	**0.024**	0.035	0.879
Number of Embryos Obtained	-0.185	0.111	-0.299	**0.030**	0.054	0.812
Maturation Rate (%)	0.090	0.442	0.076	0.590	0.116	0.608
Fertilization Rate (%)	0.068	0.564	-0.022	0.876	0.156	0.487
High-quality Embryos Rate (%)	0.067	0.565	0.053	0.706	0.056	0.806
Cleavage Rate (%)	0.127	0.277	0.074	0.600	0.221	0.324
Implantation Rate %	-0.064	0.583	-0.003	0.982	-0.209	0.351

FSH: Follicle-Stimulating Hormone, GV: Germinal Vesicle, hCG: human Chorionic Gonadotropin.

Table 4. FF AMH levels between pregnant and non-pregnant women:

Group	Clinical pregnancy			Ongoing Pregnancy		
	Pregnant	Non-Pregnant	P Value	Pregnant	Non-Pregnant	P Value
All	15.43 ± 19.02	14.06 ± 14.12	0.640	11.14 ± 9.44	16.31 ± 18.38	0.185
GnRH Agonist	16.34 ± 21.23	11.83 ± 9.54	0.971	10.25 ± 8.08	15.21 ± 17.54	0.331
GnRH Antagonist	13.02 ± 12.26	19.16 ± 20.79	0.365	13.02 ± 12.26	19.16 ± 20.79	0.365

Table 5. Receiver Operating Characteristic (ROC) Curve to evaluate the accuracy of FF AMH levels in predicting pregnancy rates:

Group	Clinical Pregnancy					Ongoing Pregnancy				
	AUC	Std. Error	Sig.	95% CI		AUC	Std. Error	Sig.	95% CI	
				Lower Bound	Upper Bound				Lower Bound	Upper Bound
All	0.468	0.071	0.640	0.329	0.606	0.406	0.071	0.185	0.266	0.545
GnRH Agonist	0.503	0.084	0.971	0.338	0.668	0.417	0.086	0.331	0.249	0.584
GnRH Antagonist	0.375	0.131	0.339	0.118	0.632	0.375	0.131	0.339	0.118	0.632

AUC: area under curve, 95% CI: 95% confidence interval.

Discussion

AMH is produced mainly by the granulosa cells (GCs) of follicles in ovaries, but the production level depends on the follicle development stage [45–48]. Based on the data adapted from in vitro studies, AMH may regulate ovarian folliculogenesis during both FSH-independent and FSH-dependent stages by inhibiting primordial follicles recruitment [49–51], attenuating the follicular responsiveness to FSH [52,53], and inhibiting granulosa cell aromatase [53,54]. Nevertheless, the impact of AMH on oocyte competence is still ambiguous. Based on the results of the in vitro maturation (IVM) studies on animals, AMH may improve oocyte competence via up-regulating the expression of the Bone Morphogenetic Protein-15 (BMP-15) and the Growth Differentiation Factor-9 (GDF-9) in mice [55], but not in cows [56]. A recent study on human germinal vesicle oocytes (GV) showed that adding recombinant AMH to the IVM medium improved the maturation rate. However, poor oocyte maturation was noted in IVM medium supplemented with AMH, FSH, and hCG, which had been explained by the authors by the antagonistic action of these hormones [57]. We have to take into account that AMH function and regulation may be species-specific and differ between mono-ovulatory

and poly-ovulatory species [58,59], e.g., AMH inhibits primordial follicles recruitment rate in mice [49,50] and rats [51] but not in sheep [60]. Data on human follicles were inconsistent [61,62], but these discrepancies may also arise from differences in the experiment's conditions. Thus, more studies are needed to clarify the exact role of AMH in regulating folliculogenesis, oocyte maturation, fertilization, and embryo development in humans.

Based on our results, the FF AMH levels were negatively correlated with the total dosage of gonadotropins in the PCOS-A group, but the correlation was insignificant in the PCOS-Anta one. Previously, several reports showed lower FF AMH levels in stimulated cycles compared to natural cycles [10–13]. According to the available data, the gonadotropins' effects on AMH expression depend on the follicle's developmental stage. Inhibition gonadotropin reduces AMH expression from granulosa cells of early-preantral follicles of the marmoset ovary [63] while treating human granulosa cells obtained from the follicular fluid samples of IVF/ICSI cycles with FSH represses AMH expression [64], which explain the negative correlation between the FF AMH and the total FSH dose that was observed in the current study. However, the lower gonadotropin requirements [65] during the GnRH antagonist protocol might hinder the correlation from reaching significance levels in the PCOS-Anta group. Similarly, we noted significantly negative correlations between the FF AMH levels and the number of retrieved oocytes, MII oocytes, MI oocytes, GV oocytes, immature oocytes, fertilized oocytes, and the number of obtained embryos in the PCOS-A group only. Nevertheless, there were not any correlations between the FF AMH levels and the oocyte maturation or fertilization rates, or with the embryos cleavage rate neither in the PCOS-A group nor in the PCOS-Anta one, which suggested that the negative correlations in the PCOS-A group all arose from the negative impacts of FF AMH on the oocytes yield. In addition, FF AMH were comparable between pregnant and non-pregnant women, independently of the protocol used, which also had been confirmed by the ROC curve analysis. According to our recent work, lower levels of placental growth factor (PlGF) can be noted in the follicular fluid of PCOS women during the flexible GnRH antagonist protocol compared to the long agonist one [36]. In addition, in the PCOS population, FF PlGF levels positively correlate with the number of MII oocytes and the OSI index during the GnRH antagonist protocol, but not the long agonist protocol (). PlGF is an angiogenic growth factor that plays a pivotal role in regulating ovarian angiogenesis, follicular development, and ovulation [66–68]. Since FF AMH levels in PCOS subjects are not only related to the antral follicle counts (AFCs) but also to the overproduction of AMH per GC [20,21], the results of the current study and of our previous work ([36]) suggest that the negative effect of FF AMH on folliculogenesis and in subsequence on the number of retrieved oocytes would be noted during both; the long GnRH agonist protocol and the flexible GnRH antagonist one as well. However, other factors like follicular angiogenesis and the FF PlGF levels can also influence the oocytes' yield. During the long agonist protocol, since PCOS follicles possess enough levels of PlGF to promote good follicular angiogenesis and development, the number of retrieved oocytes would mostly depend on the FF AMH levels. However, during the GnRH antagonist protocol, the high levels of FF AMH together with the lower levels of PlGF negatively affect the follicles' growth and reduce the number of retrieved oocytes. Therefore, the negative correlation between FF AMH levels and the number of retrieved oocytes is more potent in the GnRH agonist protocol than in the GnRH antagonist one.

Similar to our results on the long agonist group, Wiweko et al. [34] reported a significant negative correlation between the FF AMH levels and the number of oocytes among PCOS women. However, they did not mention

any information about the protocol that was used for COH. On the other hand, Pabuccu et al. [32] showed positive correlations between FF AMH and the rates of fertilization, implantation, and clinical pregnancy during the long agonist protocol in PCOS women whose partners had normal semen parameters. Interestingly, Wafaa et al.[31], did not notice any correlation between FF AMH and the number of retrieved oocytes, mature oocytes, fertilized oocytes, fertilization rate, or implantation rate in a similar population (PCOS undergone the long agonist protocol with normal male partners). Nevertheless, they observed a negative correlation between FF AMH and the number of obtained embryos [31]. In addition, our results partially agreed with Abu-Fakher et al.'s study [17], in which FF AMH levels could not predict pregnancy achievement after IVF cycles in the PCOS population during the long agonist protocol. On the other hand, no correlations were observed between the FF AMH levels and the number of retrieved oocytes, mature oocytes, obtained embryos, or the fertilization rate in that study as well [17]. Similarly, Arabzadeh et al. [33] also could not detect any correlations between the FF AMH levels and the number of retrieved oocytes, maturation rate, fertilization rate, high-quality embryos rate, or implantation rate in the PCOS women during the long agonist protocol. On the other side, Chen et al.'s study [35] revealed a positive correlation between the FF AMH levels and the antral follicle counts, but not with the number of retrieved oocytes, fertilized oocytes, or good quality embryos in PCOS subjects that undergone a combination of GnRH analogues protocols. The differences in study population and study design may explain the heterogeneity of these results. The Arabzadeh et al.'s study [33] included endometriotic women, Abu-Fakher et al.[17] and Wafaa et al.[31] studies did not declare excluding such cases, while only Wiweko et al. [34] and Chen et al. [35] studies excluded them. Pabuccu et al. 's study [32] mostly excluded endometriotic women as they mentioned that "no patients suffering any other aetiology of infertility other than PCOS were enrolled". Indeed, including endometriotic women might have influenced the results due to the differences in the pathophysiology between the two conditions [69]. In addition, FF AMH levels are significantly lower in women with endometriosis compared to PCOS women [70,71] and even compared to controls [72]. On the other hand, using a combination of GnRH analogues protocols (59 PCOS women: GnRH agonist, n=30; GnRH antagonist, n=29) with a comparable number of participants in each group in the Chen et al. study [35] could explain lack of association between the FF AMH levels and the number of retrieved oocytes in that study. Moreover, unlike Pabuccu et al. [32], we included male factor infertility, so even though we used the ICSI technique for insemination, fertilization and pregnancy success in our study might have been influenced by the male factor. Above that, variations between measurement methods may also be involved. Currently, twenty-one different commercial immunoassays are available to detect AMH levels. Yet, there is no international reference preparation of AMH to standardize calibration between the various immunoassays and harmonize the results [73,74]. Even though a purified human AMH preparation (code 16/190) has been investigated by the World Health Organization as a potential international reference preparation, its commutability was unsatisfactory [73,74]. One of the reasons for the poor commutability is that the standardized preparation did not include all AMH isoforms [74]. This obstacle may be more serious when detecting AMH levels in follicular fluid samples; as a recent study noted some novel AMH isoforms, both in follicular fluids and granulosa cell extracts, that did not match known consensus forms, which suggests that intra-follicular AMH proteolytic processing is more complex and involves even unknown steps [58].

Strengths, limitations, and future research:

To the best of our knowledge, this is the first study that investigated the correlations between the FF AMH levels and the IVF/ICSI outcomes in PCOS women and the dependency of these correlations on the COH protocol used. In addition, it provided a better understanding of the regulation of ovarian folliculogenesis in PCOS subjects during different GnRH analogues protocols. However, most of the participants in our study had a partner with male factor infertility, which might bias our results on fertilization and pregnancy success. Thus, further research is needed to clarify the effect of FF AMH on fertilization and pregnancy on PCOS with a normal male partner during the different COH protocols.

Conclusions

High FF AMH negatively affects ovarian folliculogenesis during both; the long GnRH agonist protocol and the flexible GnRH antagonist one. However, the different patterns of follicular angiogenesis during the two protocols would affect the dependency of the oocytes' yield on the follicular fluid levels of AMH.

Declarations

Data Availability:

The data that supports the findings are available upon request from the corresponding author.

Ethics declarations:

Competing interests: The authors declare that there are no conflicts of interest regarding the publication of this paper.

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Author contributions:

All authors contributed to conceptualizing and designing the study. M.A. and S.K performed the clinical experiment and were responsible for the work in the field, including patients' recruitment, sample acquisition, and data collection. S.K. performed the statistical analysis and data interpretation. S.K. drafted the manuscript, while A.N. and M.A. revised it critically for important intellectual content. All authors approved the final manuscript.

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