

# Analysis of Anti-Mullerian Hormone along Different Clinical Parameters in Infertile Women Undergoing Intracytoplasmic Sperm Injection

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**Abstract:** Anti-Mullerian hormone (AMH) is a homodimeric disulfide-linked glycoprotein that controls not only different reproductive functions in both sexes but also outcomes of ovarian hyperstimulation and syndrome, live birth rate following *in vitro* fertilization, and cancer prognosis. This study was therefore conducted to analyze changes in AMH serum concentration in patients, with different clinical parameters, undergoing intracytoplasmic sperm injection. In this retrospective study, 51 patients were hyperstimulated with follitropin alfa and cetrorelix before undergoing oocyte recovery, ICIS, and embryo transfer. Levels of AMH were analyzed on cycle day two before the ovarian hyperstimulation, and clinical pregnancy outcomes were evaluated five weeks following embryo transfer. Our analysis showed that AMH levels were significantly lower in infertile women with BMI >30 ( $P < 0.001$ ) or older than 35 years ( $P < 0.0001$ ) in comparison to women with BMI <30 or younger than 35 years. In addition, correlation analyses confirmed that AMH levels are negatively correlated with BMI ( $P = 0.02$ ) and age ( $P = 0.04$ ). In addition, infertile women with polycystic ovary syndrome showed elevated AMH levels when compared to infertile women without polycystic ovary ( $P < 0.01$ ). Finally, a positive correlation was noted between increased AMH levels and numbers of recovered oocytes ( $r = 0.79$ ,  $P = 0.006$ ); however, AMH levels were similar between pregnant and non-pregnant women ( $P = 0.8$ ). It is concluded that both age and BMI negatively affect AMH levels, and that high levels of AMH results in high oocyte collection rates following ovarian hyperstimulation. However, AMH cannot be considered a reliable tool to predict pregnancy outcomes following intracytoplasmic sperm injection.

**Keywords:** Anti-Mullerian hormone; *in vitro* fertilization; infertility; intracytoplasmic sperm injection

## 1. Introduction

Early in mammalian fetal development, both Mullerian and Wolffian ducts are present in the urogenital ridge. In early fetal embryology of females, the Mullerian duct develops to the upper third of vagina, cervix, uterus, Fallopian tubes, and the surface epithelium of ovaries, whereas in early fetal embryology of males, the Wolffian duct develops into the epididymis, vas deferens, and seminal vesicles (1-2). In this context, the anti-Mullerian hormone (AMH), from Sertoli cells of fetal testis, causes the regression of the Mullerian duct while testosterone stimulates the differentiation of the Wolffian duct during male embryogenesis. Conversely, in female embryogenesis, the Mullerian duct differentiates spontaneously in the absence of AMH, while the Wolffian duct regresses in the absence of testosterone. Interestingly, AMH continues to be produced by Sertoli cells even after the regression of the Mullerian duct and after birth but then decreases markedly to low levels at puberty (3-4). However, ovarian production of AMH starts postnatally and is highest in the pubertal and adult ovary (4). The secretion of AMH by the ovary and by the testis after Mullerian duct regression suggests that AMH has extra-Mullerian biological functions. During the last two decades, AMH was found to control germ cell maturation and gonadal differentiation (5), induce testicular descent (6), suppress lung maturation (7), and inhibit cell transformation and tumor growth (8).

In female reproduction, AMH was found to be produced in high concentrations by granulosa cells of small antral follicles, whereas low or undetectable concentrations of AMH were observed in follicles  $\geq 10$  mm (9). The cessation of production of AMH from large follicles suggests that

AMH may play an important role in selection of dominant follicle(s) and ovulation. Neither AMH staining nor AMH mRNA expression were observed in oocytes, corpus luteum, atretic follicles or theca cells in mice, rats or human ovaries, confirming that granulosa cells are the only source of AMH in the ovary (9-10). Moreover, it has been recently found that oocytes from early preantral, late preantral, and preovulatory follicles up-regulate AMH mRNA levels in granulosa cells in a fashion that is dependent upon the developmental stage of oocytes (9). These findings suggest that oocyte regulation of granulosa cell gene expression occurs during late periods of follicle development, and that oocyte regulation of AMH expression in granulosa cells may play a role in follicle development.

Recent clinical studies have shown that AMH controls not only different reproductive functions in both sexes but also can predict the outcomes of ovarian hyperstimulation and live birth rate following *in vitro* fertilization (11). To the best of our knowledge, in our region, only Mawlood (2015) studied the impact of different demographic and clinical parameters on AMH levels, and subsequent effect of AMH on live birth rate in infertile patients undergoing Intracytoplasmic Sperm Injection (ICSI [12]). This study was therefore conducted to further investigate the changes in AMH concentrations in infertile women with different age, body mass index (BMI), and causes of infertility. The effect of AMH on number of retrieved oocytes, and subsequent pregnancy following ICSI, will also be evaluated.

## 2. Materials and Methods

### 2.1 Setting

This study was conducted at the YadIVF Center in Erbil, Kurdistan Region, Iraq. This center was established in April 2014 and is one of the main referral centers for infertility treatment in the State of Erbil. For this retrospective clinical comparative study, data collection started from January 1<sup>st</sup>, 2016 to November 1<sup>st</sup>, 2018.

### 2.2 Patients and ethical approval

Among 51 infertile women that were qualified for this study, 15 women were confirmed to have polycystic ovary syndrome (PCOS) based on Rotterdam criteria, 10 women with tubal factor, 8 with male factor, 10 with endometriosis, and 8 with unexplained infertility. Infertile women, because of male factors, were considered control patients. Table one shows the demographic data, including women and men age, women BMI, infertility duration, and attempt numbers of ICSI cycle treatment. Inclusion criteria included women with primary or secondary infertility, 20-44 year old, and who were referred to our facility, for assisted reproduction, to have their first, second, or third ICSI treatment cycle. Exclusion criteria included infertile women with endocrine abnormalities related to primary hyperprolactinemia, thyroid dysfunction, Cushing's syndrome, and/or congenital adrenal hyperplasia. This study was approved by the Research Ethics Committee of the College of Medicine/Hawler Medical University at March 27<sup>th</sup>, 2018. Informed consents were obtained from all participants.

### 2.3 Ovarian hyperstimulation and ICSI

The ovarian stimulation was started 2-3 cycles after the patient's first visit to the center. Infertile women were started on a daily intradermal dose of 75-150 IU of follitropin alfa (Gonal F®, Serono, Switzerland) from day two or three; based on age, body mass index (BMI), basal FSH, and patient response. Ovarian response was monitored with serum estradiol measurement and transvaginal ultrasonography starting on day six of the cycle. When one or two leading follicles reached >12mm size, gonadotropin releasing hormone antagonist compound named cetrorelix (Cetroid®, Serono, Switzerland) of 0.25mg was started daily till the day intramuscular administration of an ovulatory stimulus of 10,000 IU of human chorionic gonadotropin (hCG; Choriomon®, IBSA-Institut Biochimique S.A.CH-6903 Lugarno-Switzerland). It is noteworthy to mention that hCG was administered when two or three follicles reached 18-22mm in diameter.

Transvaginal oocyte retrieval was conducted 34-36hr after the hCG administration. Oocytes were first washed in G-Mops plus medium (Vitrolife, Sweden) and incubated in G-IVF Plus medium supplemented with human serum albumin (Vitrolife, Sweden) at 37°C and 6% CO<sub>2</sub> in humidified air for 2hr. Oocytes were then denuded and evaluated for meiotic maturation and morphology. Briefly, oocytes that showed a nuclear membrane and intact nucleolus were classified as meiotically immature oocytes at the germinal

vesicle stage (GV). Oocytes that had undergone GV breakdown, but did not have a polar body, were classified as metaphase I (MI) oocytes, whereas those with first polar bodies were classified as metaphase II (MII) oocytes [10]. Oocytes at the GV or MI stage were *in vitro* matured before ICSI. In all patients, a transvaginal ultrasonographic screening with 6.5 MHz vaginal transducer (GE LOGIQ P3 Expert/Logic P3, probe destination E8CS/E8C, USA) was used during the follicular phase and later for oocyte recovery.

Semen was collected by masturbation after an abstinence period of three-four days. Thirty to forty minutes were allowed for semen to liquefy at 37°C before seminal analysis according to World Health Organization criteria. On day of insemination, sperm preparation was carried out using the swim-up method. In cases of severe poor semen parameters, spermatozoa were prepared by mixing equal volumes of GMOPS+ medium and semen, centrifuging twice, and re-suspending the pellet in small volume of media before injection. Inseminated oocytes were washed and incubated in groups of three oocytes in 40µl G1+ medium droplet (Vitrolife, 101128, Sweden) covered with mineral oil. The oocytes were then cultured for 18-20h before evaluation for fertilization under a high power inverted microscope. Fertilization was considered normal when two clearly distinct pronuclei were observed plus the 2nd polar body. If a single pronucleus was observed, a second evaluation was carried out after an additional 4hr. Embryos were cultured at 37°C and 6% CO<sub>2</sub> in humidified air before transferred on day three after fertilization. On average 3-4 embryos of high grades were selected and transferred using G2+ media (vitrolife, Sweden) and embryo transfer catheters (Cook, K-JETS-7019-SIVF, Australia).

### 2.4 Luteal phase support

Women were treated orally with 10mg tablet of synthetic progesterone (dydrogesterone, duphaston®, Abbott, USA), tid, and 400mg pessaries of natural progesterone (cyclogest®, Actavis, UK) once a day. Both luteal support therapies were started one day after the oocyte retrieval and continued for two weeks. If the β-hCG assay showed a positive result after two weeks from the transfer, both medications were continued for an additional 12-16 weeks.

### 2.5 Clinical pregnancy and ICSI outcomes

Patients were considered to have positive chemical pregnancy when β-hCG was >20 IU/l. The test was repeated two days later to confirm the results. Patients were considered to have clinical pregnancies when a gestation sac and a viable fetal heart beat were observed five weeks from the transfer. Early pregnancy loss or miscarriage was defined as the loss of a pregnancy before 20 weeks.

### 2.6 Statistics

The independent-sample t-test was used to evaluate the statistical significance that existed between mean numbers of two independent continuous groups. The one-way ANOVA was used to determine the statistical differences among mean numbers of more than two continuous

independent groups. Significant ANOVA analysis was followed by the Bonferroni post hoc test to determine significant differences in all possible pairwise comparisons. Pearson correlation coefficient was used to determine the strength and direction of linear relationships that existed between two numerical variables. The Pearson's chi-square test of independence was used to determine statistical differences between two categorical variables. In the latter test, when more than 20% of all expected cell frequencies were less than five, the Fisher's exact test was considered. Data are considered statistically different when the alpha level of P value was less than 0.05. Data are presented as mean $\pm$ SEM when parametric analyses were used or as percent from the total number of cases where non-parametric analyses were used. All statistical analyses were carried out using the Statistical Package for Social Sciences (SPSS Inc., version 19, Chicago, IL).

### 3. Results

#### 3.1 Levels of AMH in patients with different BMI

In our first analysis, we investigated AMH levels in infertile women with normal weight (BMI 18.0-24.9), overweight (BMI 25.0-29.9), obese (BMI 30.0-35.0), or very obese (BMI >35.0). Our analysis shows that AMH levels were significantly lower in obese or very obese infertile women than in infertile women with normal or over-weight ( $P<0.001$ , Figure 1). However, similar levels of AMH were noticed in women with normal weight and overweight ( $P>0.05$ ). In addition, a correlation analysis confirmed this observation and showed that AMH levels are negatively correlated with BMI. In other words, infertile women with small or normal body weights tend to have high AMH levels, whereas infertile women with high body weights tend to have low AMH levels ( $r = -0.56$ ,  $P=0.02$ , Figure 2).

#### 3.2 Levels of AMH in patients with different age

The second analysis in this experiment included evaluating AMH levels in patients with different age. For this purpose, patients were divided into five age group; 20-25, 25-30, 30-35, 35-40, and 40-45 years old. We observed that infertile women in age groups of 20-25, 25-30, and 30-35 years old have significantly higher levels of AMH than infertile women in the age group of 35-40 and 40-45 years old ( $P<0.0001$ , Fig. 3). Furthermore, women ranked in the age group of 35-40 had higher levels of AMH than women ranked in the age group 40-45 years old ( $P<0.05$ ). To further validate these results, we also conducted a correlation test that revealed a reversible relationship between age and AMH levels. In other words, young women tend to have elevated levels of AMH and as they grow old, the AMH levels decrease ( $r=-0.036$ ,  $P=0.04$ , Fig. 4).

#### 3.3 Analyses of levels of AMH in infertile women with different causes of infertility

Infertile women were diagnosed with causes of infertility. We were able to determine five causes of infertility named tubal, endometriosis, PCOS, unexplained and male infertility factor. We have considered couples with male infertility factors as our control group, since the reason of infertility

was the male partner. In this regard, AMH levels were similar in control women and infertile women with tubal, unexplained, or endometriosis infertility factor. However, infertile women with PCOS showed elevated AMH levels when compared to control women or infertile women because of tubal, unexplained, or endometriosis ( $P=0.01$ , Fig. 5).

#### 3.4 Effect of different levels of AMH on oocyte recovery in hyperstimulated women

The effect of different AMH levels on numbers of oocyte recovered following hyperstimulation with cetorelix was evaluated. A positive correlation was noted between increased AMH levels and numbers of recovered oocytes. Low AMH concentrations were associated with few numbers of oocytes recovered 36h following the hCG injection, whereas high levels of AMH was correlated with increased numbers of recovered oocytes ( $r=0.79$ ,  $P=0.006$ , Fig. 7).

#### 3.5 Levels of AMH in pregnant or non-pregnant women

The hormone was assayed on cycle day two before the initiation of the hyperstimulation program. Those patients underwent then oocyte recovery, *in vitro* fertilization (ICSI), and embryo transfer. Patients with or without clinical pregnancy were then compared to their initial AMH levels to define the effect of AMH on pregnancy prognosis in infertile women. It was observed that AMH levels were similar between pregnant or non-pregnant women ( $P=0.8$ , Fig. 8).

### 4. Discussion

In this research, we have investigated the levels of AMH in infertile women with different demographic and clinical profiles. We found that AMH levels increase as the BMI decreases, and significantly low levels of AMH were observed in obese and very obese women with BMI>30. However, similar levels of AMH were noticed in women with normal weight and overweight, indicating a negative correlation between AMH and BMI. Numerous studies have shown that AMH levels inversely correlates with weight in overweight, obese, and very obese patients (9, 13-14). Interestingly, our study reveals a similar statistical significance, confirming a negative correlation between AMH and weigh. Therefore, our results are in agreements with previous studies that also found low AMH levels in obese and overweight women. This means that it is the weight factor, besides the ovarian state, that impacts the AMH levels in infertile patient. Future studies designed to investigate the molecular mechanism of weight effect on AMH levels are merited.

Our results also showed that AMH levels are negatively related to patient age. Production of AMH from granulosa cells of small antral follicles has become an established fact. It is also an established fact that the level of serum AMH reflects the numbers of growing follicles at the primordial stage till the antral stage. This also means that AMH levels reflect the state of ovarian reserve. Indeed, follicles  $\geq 10$  mm produce low or undetectable concentrations of AMH (9, 15). In addition, this ovarian reserve of follicles is under



continuous recruitment of oocytes, and it depletes as women grow older. This means that both ovarian reserve and AMH are decreasing with age (16-17). Similarly, our results of negative correlation between AMH and age are reasonable and in agreement with previous reports, which also confirmed this relationship, especially in women older than 25 years old (9, 15-17).

Patients with PCOS showed high levels of AMH in comparison to women with other causes of infertility such as endometriosis, tubal blockade, unexplained infertility, or male factor. It has been agreed that women with ovarian reserve of antral follicle count greater than 12 follicles (measuring 2–9 mm in diameter) are considered to have PCOS. Since AMH is secreted from the granulosa cells of small antral follicles, and such follicles were observed more frequent in PCOS patients than in other patients without PCOS, one would expect to see higher AMH levels in PCOS than in patients with other causes of infertility. In addition, data from other laboratories have shown that AMH production from granulosa cells was 75 times higher in infertile women with PCOS than in infertile women without PCOS (18). Furthermore, concentrations of AMH were found to be five times higher in follicular fluids of women with PCOS than in women without PCOS (18). More interestingly, follicles from women with PCOS were found to produce significant higher AMH levels than their sized-matched counterpart follicles from women without PCOS (9). Put all together, PCOS patients produce higher levels of AMH than women without PCOS, because of the increased antral follicle counts in PCOS patients and that AMH production from ovarian follicles in PCOS patients are higher than those in patient without PCOS.

Numbers of recovered oocytes were also found to be positively correlated with AMH. This increase in mean number of recovered oocytes in infertile patients with elevated AMH levels is expected, as those patients would ovulate more oocytes due to their high bulk of antral follicle counts and rich ovarian reserve responsible on production of AMH, as stated earlier. In fact, it has been reported that AMH levels can accurately predict recovery rate of both mature oocytes and immature oocytes at the metaphase I stage (12). It is now believed that AMH levels may become a more sensitive marker for predicting ovarian response than serum follicular stimulating hormone (FSH), inhibin B, and estradiol assayed at day two or three. For instance, high FSH levels do not always predict pregnancy outcomes (19). Moreover, the hormone is age dependent. It increases at early menstrual cycles with advancing reproductive age. Another shortcoming with FSH is that assays of FSH have significant inter- and intra-cycle variability that limits their reliability (20). As for the prediction capacity of estradiol for the ovarian response, vast majority of studies have found that basal estradiol levels do not differ between women with and without diminished ovarian response to ovarian stimulation or women with positive or negative pregnancy outcomes (21-22). Similar to basal FSH, basal estradiol levels have poor inter- and intra-cycle reliability (23). Inhibin B was also evaluated as a predictor for outcomes of ovarian hyperstimulation. The large majority of studies have demonstrated that the routine use of inhibin B as a measure

of ovarian reserve or stimulation is not recommended (22, 24).

Levels of AMH, assayed on cycle day two, were similar between pregnant or non-pregnant women following ICSI. We found that AMH lacks the ability to predict the pregnancy outcomes in infertile women. Data regarding the influence of circulating AMH on pregnancy outcomes are conflicting. For instance, Xi and co-workers reported that AMH levels on day three of stimulation cycle positively predict ovarian response, but high AMH levels significantly decreased fertilization and pregnancy rates in PCOS women (25). Similarly, La Marca et al. (2010) and Lee et al. (2008) concluded that AMH measurement is useful in the prediction of poor ovarian response, cycle cancellation, and ovarian hyperstimulation syndrome (17, 26). Some other studies have concluded that AMH is not associated with pregnancy in agreement with our study (27-28). On the other hand, other investigators reported positive correlation between circulating AMH and pregnancy rates (29-30). Unfortunately, studies examining the association of AMH and live birth have either been small (31-32) or restricted to specific subpopulations of infertile patients (33-34). It is concluded from these reports that association of AMH with pregnancy outcomes after assisted conception is still inconclusive.

In summary, AMH levels in infertile women undergoing ovarian hyperstimulation and ICSI were analyzed along patient different clinical profiles. We conclude that AMH levels inversely correlate with age and BMI. High levels of AMH were also observed in patients with PCOS in comparison to other infertility patients. We report that AMH serum levels positively correlate with oocyte recovery rates but fail to correlate or predict live birth rates.

## 5. Conflict Of Interest

The authors declare no conflict of interest.

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Tables

Table 1: Demographic data of all patients recruited in this study.

Total number of women	51
Female Age (year)	39.4±5.9
Male Age (year)	35.8±6.6
Female BMI (Kg/m <sup>2</sup> )	31.3±4.8
Fertility Duration (year)	4.8±2.7
1 <sup>st</sup> Attempt	38
2 <sup>nd</sup> Attempt	9
More than 2 attempts	2
[AMH] on day 2 (ng/ML)	3.4±3.1

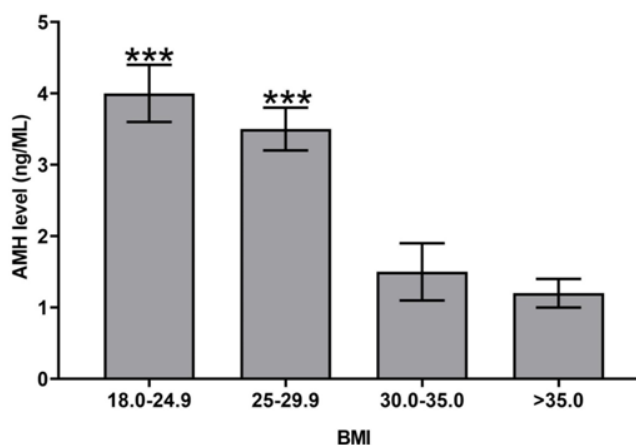


Figure 1: AMH levels in infertile women with normal weight (BMI 18.0-24.9), overweight (BMI 25.0-29.9), obese (BMI 30.0-35.0), or very obese (BMI >35.0). Values with different superscripts are significantly different (\*\*P < 0.001).

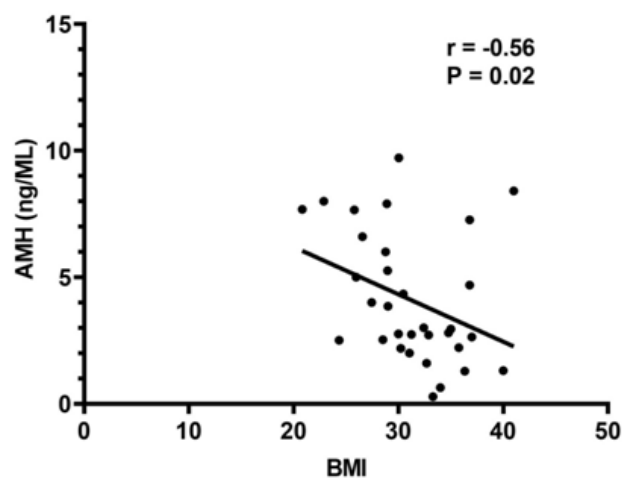


Figure 2: Correlation analysis between patient AMH levels and BMI.

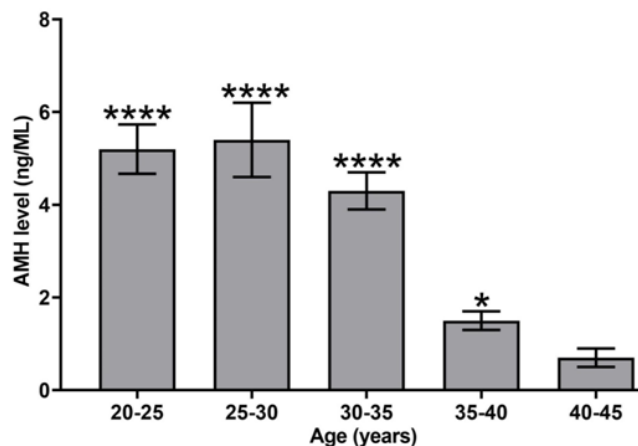


Figure 3: Evaluation of AMH levels in patients with different age. Values with different superscripts are significantly different (\*\*\*\*P < 0.001).

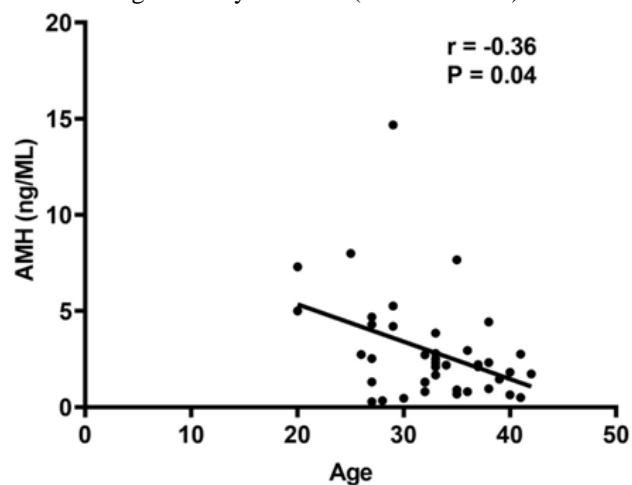


Figure 4: Correlation analysis between patient AMH levels and age.

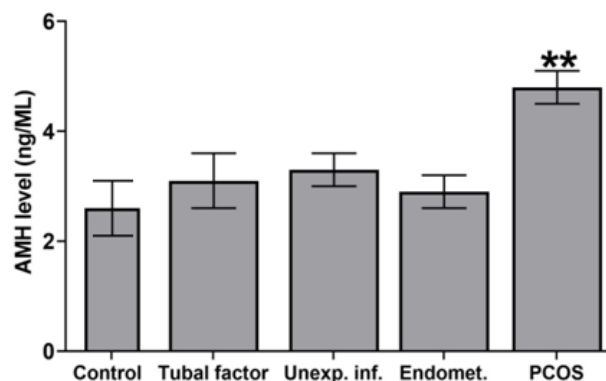


Figure 5: AMH levels in women with different causes of infertility. Values with different superscripts are significantly different (\*\*P < 0.01).

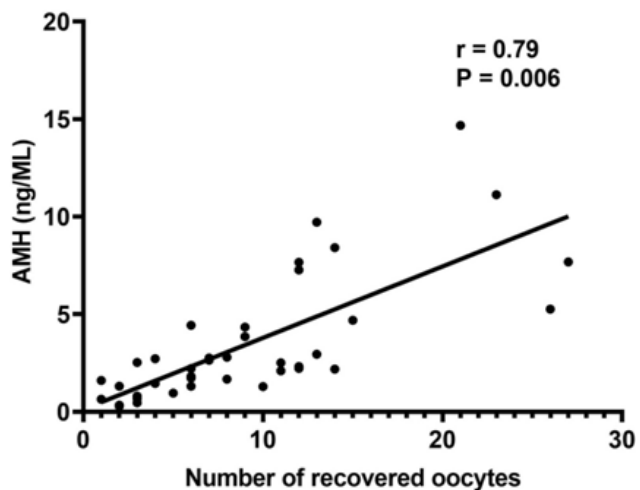


Figure 6: Effect of different levels of AMH on oocyte recovery in hyperstimulated women.

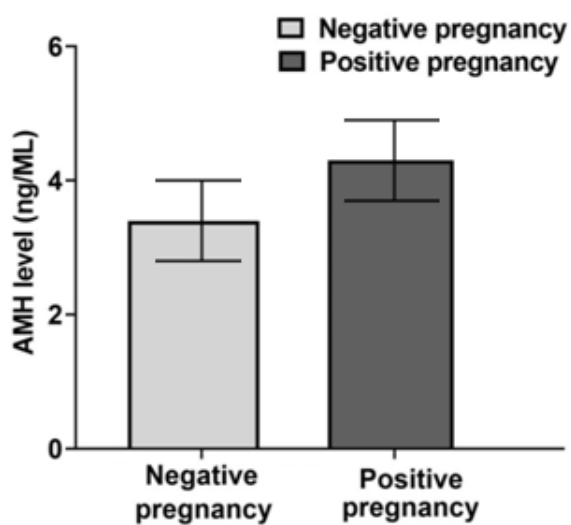


Figure 7: Levels of AMH in pregnant or non-pregnant women ( $P > 0.05$ ).