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NUMBER OF FOLLICLES AT THE TIME OF TRIGGER AND MEAN NUMBER OF OOCYTES RETRIEVED: A CORRELATION OF AMH WITH AGE, MEAN RETROSPECTIVE STUDY

***Dr. Anitha Adaboina**

Laxmi Narsimha IVF Center, 2-2-316, Beside Pushpanjali Function Hall, Nayeem Nagar, Hanamkonda, Warangal, Telangana 506001

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ABSTRACT

Introduction: For assisted reproductive technology [ART] to be successful, patients should be evaluated and correct ART should be selected. The ovary reserve is associated with oocyte quality and yield, it represents the reproductive potential of women. While various method are available to assess ovarian reserve, few methods are currently available to assess oocyte quality. The anti-mullerian hormone [AMH] is a member of transforming growth factor beta family. It is secreted by granulosa cells in the preantral and antral follicles in the ovaries.AMH plays a role in regulating ovarian activity. **Methods:** This prospective study of 75 patients were received antagonist protocol. AMH levels were measured and mean number of follicles at the time of trigger were counted. The patients were divided into four groups i.e very low [AMH <0.5], low [AMH 0.5 TO 1.1], normal [AMH 1.2 TO 3.5], High [AMH >3.5] 1. **Results:** In our observed study very low [AMH<0.5]group showed mean number of follicles at the time of trigger is 2.3 and mean number of oocytes retrieved is 2.3, low [AMH 0.5 to 1.1]group showed 6.5 and 3.8, normal [AMH 1.2 to 3.5]group showed 7.5 and 6.0, high [AMH >3.5]group showed 16.5 and 14.4. The mean number of follicles at the time of trigger and mean number of oocytes retrieved correlates with AMH. **Discussions:** AMH can be used to predict the number of oocytes that can be collected during treatment. Basal AMH is used as an indicator to determine ovarian reserve. High AMH correlates with increased number of follicles and oocytes retrieved. Low AMH correlates with decreased number of follicles and decreased number of oocytes retrieved. **Clinical Relevance:** AMH is used as a marker to determine number of oocytes to be collected with controlled ovarian stimulation

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INTRODUCTION

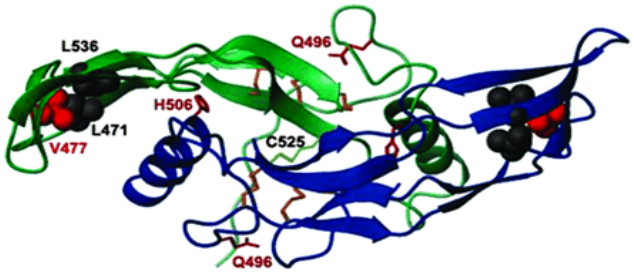
Anti-Mullerian hormone also known as AMH is a protein that, in humans, is encoded by the AMH gene (Cate *et al*., 1986). It inhibits the development of the mullerian duct (paramesonephric ducts) in the male embryo (Behringer, 1994). It has also been called Mullerian inhibiting factor (MIF), Mullerian-inhibiting hormone (MIH), Mullerian-inhibiting substance (MIS), and Anti-paramesonephric hormone (APH) (Minkoff, 2004). It is named after Johannes Peter Muller. AMH was for the first time reported by Prof. Alfred jost in 1940. Although the AMH receptor is expressed in both male and female fetuses, AMH expression has been isolated to male sertoli cells (Rey *et al*., 2003).

Corresponding author: **Dr. Anitha Adaboina,**
Laxmi Narsimha IVF Center, 2-2-316, Beside Pushpanjali Function Hall, Nayeem Nagar, Hanamkonda, Warangal, Telangana 506001.

Expression of AMH is activated by SOX9 in the male sertoli cells and causes the irreversible regression of the Mullerian ducts (Taguchi *et al*., 1984). Because AMH expression is critical to sex differentiation at a specific time during fetal development, it appears to be tightly regulated by SF1, GATA factors, DAX1 and FSH (Shen *et al*., 1994; Nachtigal *et al*., 1998; Viger *et al*., 1998). Mutations in both the AMH gene and the type II AMH receptor have been shown to cause the persistence of Mullerian derivatives in males that are otherwise normally virilized (Belville *et al*., 1999). AMH expression also occurs in ovarian granulosa cells of females postpartum, and serves as a molecular biomarker for relative size of the ovarian reserve (Weenen *et al*., 2004). In humans, the number of cells in the follicular reserve can be used to predict timing of menopause (van Disseldorp *et al*., 2008). In bovine, AMH can be used for selection of females in multi-ovulatory embryo transfer programs by predicting the number of antral follicles developed to ovulation (Rico *et al*., 2011).

Source: AMH is secreted by sertoli cells of the testis during embryogenesis of the fetal male. In females, it is secreted by the granulosa cells of ovarian follicles.

Structure: AMH is a protein hormone structurally related to inhibin and activin.It belongs to a member of transforming growth factor β. It's a dimeric glycoprotein with molecular weight of 140 KD.



Fi. 1. Structure of Anti mullerian hormone

Gene: Gene for human AMH is located on short arm of chromosome 19 and is composed of 275 nucleotide bases., while the gene for AMHR2 codes for its receptor on chromosome 12 (Imbeaud *et al*., 1995).

Embryology: In males AMH is produced by sertoli cells of the testis from 5th week of embryonic development. It is also formed in females in ovaries from 36th week of gestation. In mammals, AMH prevents the development of the mullerian ducts into the uterus and other mullerian structures. The effect is ipsilateral i.e each testes supresses mullerain development on its own side. In humans this action takes place during first 8 weeks of gestation. If no hormone is produced from gonads, mullerian ducts automatically develops, while wolffian ducts automatically die. At the bipotential stage, both Mullerian and Wolffian ducts are present.

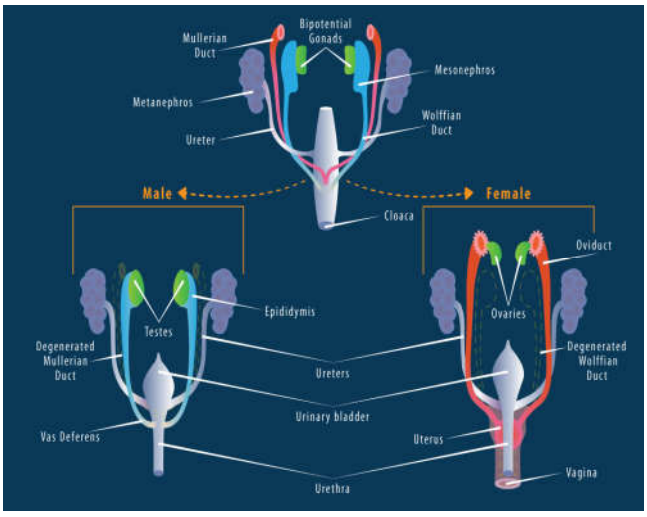


Fig. 2. Sex differentiation

Bottom left: In the male embryo, the Mullerian duct degenerates under the influence of AMH, secreted by the testicular Sertoli cell, and each testis connects to the Wolffian duct through a series of tubules. During further development, the Wolffian duct gives rise to the efferent ductules, epididymis, ductus deferens, ejaculatory duct, and the seminal vesicle under the control of androgens produced by Leydig cells.

Bottom right: In the female embryo, the Wolffian duct degenerates, while the Mullerian duct contributes to the formation of the female reproductive organs. The distal ends of the paired Mullerian ducts fuse to form the vagina and uterus. The proximal un-fused portions become the oviduct.

Follicular development in females

20 weeks of gestation	→	6-7 million oocytes
↓		↓
Birth	→	1-2 million oocytes
↓		↓
Menarche	→	3-4 lakhs
↓		↓
Reproductive age	→	300-400 oocytes ovulate
↓		↓
Menopause	→	No oocytes left

During fetal life, germ cells populate the ovary and become surrounded by somatic cells forming so called primordial follicle. Around 6 million follicles are formed at 20 weeks of gestation. About 1-2 million oocytes are present at birth which gradually decreases and only 3 lakhs primordial follicles are left at menarche (faddy *et al* 1992). Throughout life, follicles leave primordial follicle pool, majority of which are lost due to atresia until they are rescued by FSH. Now FSH rescue starts only after hypothalamic pituitary ovarian axis is activated that is at puberty. Among cohort of rescued follicles, only one follicle is selected to become dominant follicle which will ovulate under influence of LH (Mc Gee and Hsueh 2000). This continue throughout life till primordial follicle pool is exhausted and there are no available growing follicles and then menopause ensue.

AMH: AMH is expressed by granulosa cells of the ovary during the reproductive years. AMH is secreted by early antral follicles (≤ 4-6mm). It reflects both number of small growing follicles and the primordial pool at gonadotropin independent folliculogenesis (Trbovich *et al*., 2004). AMH is not detectable at birth, increases after puberty and declines towards menopause.

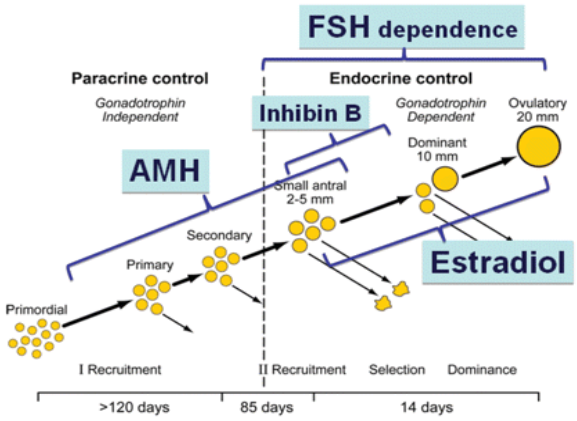


Fig. 3.

AMH secreted by early antral follicles: AMH is useful in assessing excessive, poor and normal ovarian reponse such as polycystic ovarian syndrome and premature ovarian failure (Broer *et al*., 2011; Visser *et al*., 2006). but it does not appear to add any predictive information about success rates of an

already established pregnancy after IVF[20]. Additionally AMH levels are used to determine a womens remaining egg supply.[21]

Possible actions of AMH: Inhibition of initial follicular recruitment Inhibition of FSH dependent growth and selection of antral follicles

AMH Serum levels: AMH is accurate to assess ovarian reserve. serum AMH Peak at age 25 and decreases with aging, it's an early marker of decreased ovarian reserve (Wang *et al*., 2009). AMH can be measured any time on any day of menstrual cycle. It is cycle independent. It has low inter- cycle and intra- cycle fluctuations.

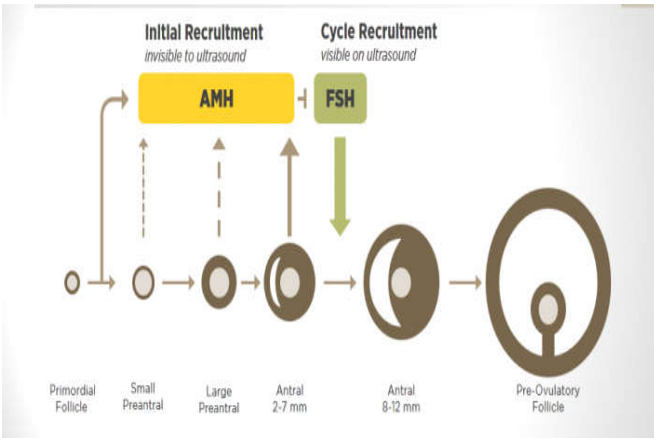


Fig. 4.

To predict excessive response: Cut –off point of 3.5ng/ml (nardo *et al*. 2011) – high sensitivity (88%), specificity (70%) and accuracy (0.81). To predict decreased ovarian response: cut –off point of 1.4ng/ml (kwee *et al*)[25] – high sensitivity (76%) and specificity (86%)

Amh measurement methods: Several ELISA assays are available for accurate measurement (Broer *et al*., 2014):

DSL (diagnostic system laboratories) (pmol/l)
Immunotech beckmen-coulter assay (IBL) (ng/ml)
Beckman – coulter generation II (Hybrid of DSL and Immunotech)

Fully automated ELISA assay

AMH levels are about 5 fold lower with DSL kit than with Beckman coulter kit

Conversion ng/ml to pmol/l =value in ng/ml × 7.14

Reproductive Process

Normal reproductive mechanism requires mainly 3 things:

- Normal ovulatory function
- Patent tubes
- Normal semen

Blocked tubes and abnormal semen parameters can be overcome by using IVF/ICSI. But we need to know about ovarian reserve and ovarian responsiveness. There are many tests to show how ovaries will respond to gonadotropin stimulation and how many oocytes will get (AMH being one of them). But they have their own advantages and disadvantages. Some of the tests we use frequently are –

- FSH and serum Estradiol levels
- Clomiphene citrate challenge test
- Inhibin B levels
- Antral follicular count and ovarian volume
- AMH
- Ovarian reserve tests –
- Clomiphene citrate challenge test (CCCT)
- Exogenous FSH ovarian reserve testing (EFFORT)
- Gonadotropin agonist stimulation test (GAST)

Review of literature: Serum AMH appears to be solely of ovarian origin since AMH was undetectable in women 3–5 days after bilateral oopherectomy (Marca *et al*., 2005; Marca *et al*., 2005). Moreover, since serum AMH reflects ovarian origin, reduction in the number of pre-antral and antral follicles will result in serum AMH reduction. In the last few years, many authors have been able to confirm the strong association between serum AMH and the ovarian pool (Disseldorp *et al*., 2009; Marca *et al*., 2010; Marca, 2006; La Marca, 2005; La Marca, 2005; La Marca *et al*., 2006; La Marca *et al*., 2006; La Marca *et al*., 2007; La Marca *et al*., 2009; Sowers *et al*., 2008). Van rooij *et al* studied the relationship between AMH levels and ovarian response during ovarian stimulation for IVF. Since then number of ovarian follicles declines with increasing age, AMH levels might be used as a marker for ovarian aging (Van Rooij *et al*., 2005). La Marca showed that the variations of AMH during the menstrual cycle are not significant, and this was confirmed by Tsepelidis and Streuli (Streuli *et al*., 2009; Tsepelidis *et al*., 2007). In addition, GnRH agonist treatment does not affect AMH levels[55]. Also, serum AMH is not affected by pregnancy and oral contraceptive pills use (La Marca *et al*., 2005; La Marca, 2007). This makes serum AMH an ideal marker for ovarian reserve. Many authors have shown that circulating AMH levels are higher in PCOS patients, and that AMH levels correlate with the severity of the syndrome (La Marca *et al*., 2010; La Marca *et al*., 2009).

Pigny reported that AMH levels have higher specificity and sensitivity (92% and 67%, respectively) as a diagnostic marker for PCOS (Plante *et al*., 2010). Gracia has shown that non-PCOS obese women have reduced AMH levels, and concluded that obesity may be associated with impaired ovarian reserve[57]. The effect of obesity on ovarian reserve is still controversial since there are several studies revealing that the low serum AMH levels in obese women is physiological and is not indicative of impaired ovarian reserve. Several factors may be related to reduced serum AMH levels such as smoking, alcohol use, and race (Nardo *et al*., 2007; Mohamed *et al*., 2006; Seifer *et al*., 2008). Plante measured serum AMH levels on day 2, 3 and 4 of the menstrual cycles in women aged 38 to 50 years. He concluded that active smoking is associated with decreased serum AMH in late reproductive age and peri-menopausal women confirming the effect of smoking on the depletion of antral follicles (Plante, 2010). Several studies investigating the role of AMH as a marker of ovarian response to controlled ovarian stimulation have shown a decline in AMH levels during gonadotropin administration (Fanchin *et al*., 2007; La Marca *et al*., 2004; Aflatoonian *et al*., 2009). This decline is caused by a negative effect of FSH on AMH production by the small growing follicles.

FSH causes an enlargement of the follicles which subsequently lose their AMH expression; in addition, FSH stimulates the production of estradiol which down regulates AMH production in the ovary. Therefore, AMH measurement should be performed prior to the start of gonadotropin administration. In 2009, Nardo performed a prospective cohort study of 165 women undergoing their first IVF cycle (Nardo *et al*., 2007). AMH, FSH and AFC were measured. Compared with FSH and AFC, AMH has the ability to predict both excessive response and poor response to gonadotropins (La Marca *et al*., 2010; Nardo *et al*., 2007; La Marca *et al*., 2009). There is a strong correlation between basal AMH level and the number of retrieved oocytes (La Marca *et al*., 2010; Nardo *et al*., 2007; La Marca *et al*., 2006; La Marca *et al*., 2006; La Marca *et al*., 2007; La Marca *et al*., 2009; Sowers *et al*., 2008; Pigny *et al*., 2006; Broekmans *et al*., 2008). Seifer was the first to report an association between serum AMH and ovarian response to controlled ovarian stimulation (Seifer *et al*., 2002). Themmen published a review article and compared AMH to other hormonal markers including FSH, estradiol, and inhibin B (Themmen, 2005). Again, AMH was found to be a better marker to predict the response to gonadotropin stimulation than age, day 3 FSH, estradiol, and inhibin B. Recently, Broer performed a meta-analysis and reviewed a total of 30 studies to compare the role of AMH and AFC in predicting ovarian response (Broer *et al*., 2008). He concluded that AMH and AFC have the same accuracy level in predicting ovarian response (Broer *et al*., 2008). Also, this was confirmed by a recent prospective study that showed that small AFC (2–6 mm) and AMH are equally accurate predictors of ovarian response (Aflatoonian Abbas *et al*., 2009).

Most recently, Broer performed a review of the role of AMH in assisted reproductive technology (ART) outcome (Broer Simone *et al*., 2010). He reported that ovarian reserve is considered normal when 6–14 oocytes are retrieved after ART, and this resulted in optimal live birth rate. He concluded that AMH is an excellent predictor of ovarian response to controlled ovarian stimulation, but cannot predict pregnancy after ART (Simone *et al*., 2010). Gnoth reviewed 132 oocyte retrievals and reported that an AMH cut off level $\leqslant$1.26 ng/ml detected poor responders ($\leqslant$4 oocytes) with a sensitivity of 97%, and a 98 % prediction of normal response if levels were above 1.26 ng/ml (Gnoth, 2008), while levels <0.5 ng/ml predicted 88% of very poor responders ($\leqslant$2 oocytes). However, AMH levels $\geqslant$0.5 ng/ml are not significantly correlated with clinical pregnancy rates (Gnoth, 2008). Studying AMH in the donor oocyte population is very useful due to their homogenous nature. Nakhuda measured AMH in 104 oocyte donors between the ages of 21–32 years[31]. In this study, AMH was correlated with the number of oocytes retrieved, peak estradiol, and suggested that AMH appears most useful in the prediction of gonadotropin sensitivity, allowing individualization of dosing protocols (Nakhuda *et al*., 2009). In 2010, Gleicher *et al*. compared the concordance and discordance between FSH and AMH. He concluded that women with normal FSH and abnormal AMH will have reduced oocyte yield (women with normal FSH and normal AMH have the best oocyte yield), showing again that AMH is a better marker than FSH (Gnoth *et al*., 2008). Also, the same authors compared the predictive values of AMH and baseline FSH with respect to IVF outcomes and oocyte yield in 76 women. They reported that an AMH $\leqslant$0.5 ng/ml has a sensitivity of 87 % and specificity of 84% in predicting poor response. In contrast, FSH has sensitivity and specificity of 64.5% and 82.2 %, respectively (Gleicher *et al*., 2010; Barad *et al*., 2009). Predicting poor response and cycle cancellation to avoid treatment side effects, expense, and psychological stress is very crucial in the field of infertility, and identifying poor responders before undergoing expensive and time consuming treatment is paramount. A total of 16 prospective and two retrospective studies investigated the role of AMH in predicting poor response (Broer *et al*., 2008; La Marca *et al*., 2010; La Marca, 2009; Pigny, 2006; Aflatoonian, 2009). The reported sensitivity and specificity ranged between 44–97% and 41–100%, respectively. The cut off values for AMH vary between studies; however, the use of low cut off values implies that many poor responders will pass unrecognized. La Marca reported that AMH level of 0.7–0.75 ng/ml has a good sensitivity and specificity of identifying 75% of poor responders[41]. On the opposite end of the spectrum, prediction of hyper response and avoiding OHSS remain difficult tasks. Four prospective studies reported on the importance of basal serum AMH in the prediction of hyper response and OHSS. The cut off value was about 3.5 ng/ml, above which hyper response and/or OHSS may occur (Nelson, 2007; Kwee *et al*., 2007; Lee, 2008; Nardo, 2008). Many studies indicate that measuring AMH follicular level is useful in the prediction of oocyte and embryo quality, as well as clinical pregnancy, with mixed results (Fanchin *et al*., 2007; Pigny *et al*., 2006). Nelson in 2007 concluded that basal AMH has a very good correlation with the number oocytes retrieved but, like basal FSH, does not seem to predict clinical pregnancy (Nelson *et al*., 2007). Ficicioglu *et al* found a relationship between antral follicle counts and the number of oocytes collected in their study. They reported that AMH levels reflected the extent of the antral follicle pool and concluded that the AMH level can be used as a marker to determine the number of oocytes to be collected with controlled ovarian stimulation (Ficicigolu *et al*., 2006). Seifer *et al* found a relationship between early follicular phase serum AMH levels and the number of oocytes obtained after the induction of ovulation. Specifically, they collected a higher number of oocytes and more mature oocytes in patients with higher serum AMH levels (Seifer *et al*., 2002). In study performed by Hazout *et al* AMH demonstrated a better correlation with the number of oocytes collected and antral follicle counts compared to inhibin B and FSH (Hazout, 2004). In their study, Van Rooij *et al* did not demonstrate a relationship between FSH, E_2, and inhibin B levels and pregnancy conception (Van Rooij, 2002). The ovary reserve is related to both the quantity and quality of the ovary follicle pool. Although direct measurement of the primordial follicle pool is not possible, the antral follicle count is known to be proportional to the size of the primordial follicle pool. For this reason, the antral follicle count is believed to reflect the quantitative changes that may occur in ovaries with age. Unfortunately, there is no marker currently available to directly assess oocyte quality. Ebner *et al* performed an early study focusing on this area in which they assessed oocyte quality according to AMH levels. AMH is released by granulosa cells during the early follicular phase. Decreased AMH levels may be associated with impaired secretions from granulosa cells and may result in irreversible damage to the germ cells.

Aim and objectives of thesis: The aim of this thesis is the correlation of AMH with age , mean number of follicles at the time of trigger and mean number of oocytes retrieved in patients undergoing ICSI Cycle.

This includes

- Relationship of AMH and age
- Relationship of age with mean number of follicles at the time of trigger and mean number of oocytes retrieved
- Relationship of AMH with mean number of follicles at the time of trigger and mean number of oocytes retrieved

MATERIALS AND METHODS

This retrospective study was conducted at Laxmi narasimha ivf center, hanamkonda, warangal from January 2017 to December 2017.
A total of 150 patients receiving antagonist protocol were included in this study.

Inclusion criteria

- Bilateral tubal block
- Endometriosis
- Unexplained infertility
- Polycystic ovarian syndrome
- Diminished ovarian reserve
- Male factor infertility

Exclusion criteria

Bilateral oophorectomy
Premature ovarian failure

AMH measurement was done on any random day in the cycle by fully automated system using enzyme linked immunosorbent assay.Patients were categorized in 2 ways-

According to AMH

High AMH : > 3.5ng/ml
Normal AMH: 2-3.5 ng/ml
Low AMH: < 2 ng/ml

According to Age

< 30 years
30- 35 years
>35 years

All enrolled women are started with antagonist protocol. Following process was used for all patients –

*Baseline scan on day 2/3 to assess AFC

*Gonadotropins (either FSH or HMG) started on day 2 (dose is decided by AMH and AFC)

*Follicular monitoring started from day 6 of stimulation with ultrasound and serum E_2 estimation

*Antagonist is started once the follicle reaches 14 mm size. when maximum number of follicles are between 18-19 mm size Inj. HCG was given as trigger for normal and low response.

*For hyperresponse Gnrh agonist is used as trigger.

*Oocytes are aspirated by transvaginal guidance under anaesthesia.

*Ovarian response is defined by number of oocytes obtained during the ooyte aspiration procedure.

RESULTS

Relationship between AMH and AGE: illustrates AMH to be inversely related to age of the patient. When patients seen according to age, mean AMH found in age < 30 yrs is 4.55ng/ml, age 30- 35 yrs is 3.9 ng/ml and age >35 yrs is 3.2ng/ml

Table 1.1

Age group	Age < 30 yrs	Age 30- 35 yrs	Age >35 yrs
Mean AMH	4.55ng	3.95 ng	3.28 ng

Table 1.2

Group	Age < 30 years	Age 30 - 35 years	Age >35 years
mean number of follicles at the time of trigger	11. 61 (35.08%)	10.55 (48.45%)	8.09 (16.46%)
mean number of oocytes aspirated	10.08 (36.43%)	9.22 (46.23%)	7.12 (17.33%)

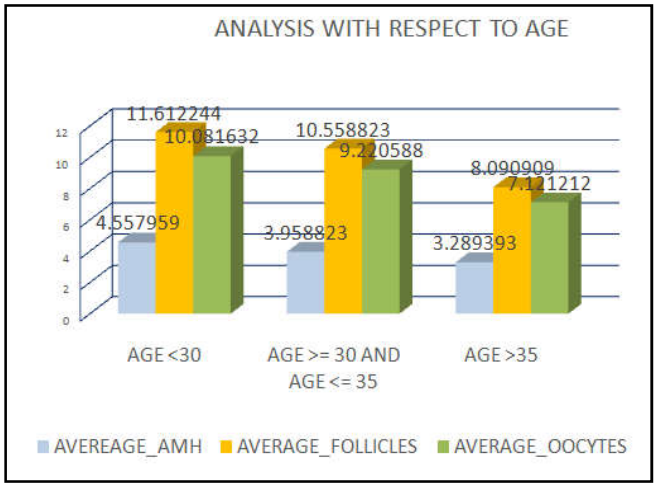
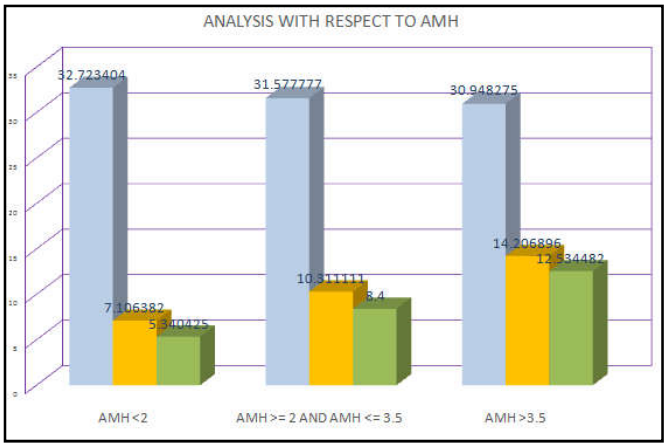


Table 1.3.

Group	AMH < 2ng/ml	AMH 2-3.5 ng/ml	AMH >3.5 ng/ml
mean number of follicles at the time of trigger	7.10 (20.59%)	10.31 (28.60%)	14.20 (50.80%)
mean number of oocytes aspirated	5.34 (18.51%)	8.4 (27.87%)	12.53 (53.61%)

Relationship of age with mean number of follicles an mean number of oocytes aspirated: This table 1.2 illustrates the mean number of follicles and mean number of oocytes aspirated decreases as age increases i.e at >35yrs. mean number of follicles and oocytes aspirated are more at age <30 years.

Relationship of AMH with mean number of follicles at the time of trigger and mean number of oocytes aspirated: This table 1.3 illustrates low AMH is associated with less number of follicles and less number of oocytes aspirated.



High AMH is associated with increase in the number of follicles and increase in the number of oocytes aspirated.

Statistical Methods: Pearson correlation coefficient test was used for comparison of the AMH groups and the variables. Since all the p-values with follicles at the time of trigger and oocyte retrieved with respect to AGE and AMH are less than alpha 0.05, the data is considered to be statistically significant**.**

DISCUSSION

Relationship between amh and age: Table 1.1 illustrates mean AMH decreases as age increases. Mean AMH found in age < 30 yrs is 4.55ng/ml, age 30- 35 yrs is 3.9 ng/ml and age >35 yrs is 3.2ng/ml. This inverse relationship is also found in the study by Van Rooij *et al* who reported that AMH levels decline with age in normal females with proven fertility.

Relationship between age with mean number of follicles at the time of trigger and mean number of oocytes retrieved: Table 1.2 illustrates mean number of follicles and oocytes retrieved decreased with increasing age. At age < 30 mean number of follicles are 11.61 and mean number of oocytes retrieved are 10.08 . At age >35 mean number of follicles are 8.09 and mean number of oocytes retrieved are 7.12. Similar results were reported by La Marca *et al*. (2007) and Te Velde *et al*. (2002).

Relationship of amh with mean number of follicles at the time of trigger and mean number of oocytes retrieved: Table 1.3 illustrates Mean number of follicles (14.20) and oocytes retrieved (12.53) with high AMH group (>3.5) and were seen low mean number of follicles (7.10) and low number of oocytes retrieved (5.34) in low AMH group (< 2). Similar results were seen in the study done by Seifer *et al* (2002).

Conclusion

The present study is done in 150 patients. The aim of the study is correlation of AMH with age, mean number of follicles at the time of trigger and mean number of oocytes retrieved. Conclusions that can be drawn from this study are:

Mean AMH decreases as age increases. Also mean number of follicles at the time of trigger and mean number of oocytes retrieved decreases with increasing age. At high AMH (> 3.5), more mean number of follicles and mean number of oocytes retrieved.

At low AMH (<2), less mean number of follicles and mean number of oocytes retrieved. AMH in combination with AFC (Antral follicular count) are the best markers of ovarian reserve with both markers having similar accuracy. In conclusion basal AMH levels can be used as a guide to predict ovarian response during IVF treatment cycle.AMH is useful in predicting the number of oocytes, the number of mature oocytes, and the number of oocytes that can be fertilized during treatment. This study confirmed previously published data related to AMH measurement. Age has the predictive ability to modify AMH therefore affecting number of oocytes. At young age there is no difference in outcome based on AMH. With advancing age, low AMH is associated with low number of oocytes. Based on our finding AMH and AFC are the predictive markers of ovarian reserve.

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