The Non-linear Association Between Serum Level of Anti-Müllerian Hormone and *in vitro* Fertilisation Outcome

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Aim of the study was to analyse the relationship between Anti-Müllerian Hormone (AMH) serum level and in vitro fertilisation (IVF) with and without intra-cytoplasmic sperm injection (ICSI) outcome. We performed a retrospective study which included 1073 patients (mean age 34.68±4.28 years, mean body mass index 22.7±15.65 kg/m2) who performed IVF or IVF/ICSI between January 2013 and December 2016.We found that AMH serum level was age-independent positively related with oocytes (beta=0.329, p<0.0001) and zygotes number (beta=0.248, p<0.0001) and negatively correlated with fertilization rate (beta=-0.108, p=0.001). In multivariate regression, after adjustment for confounders, only oocytes number, but not AMH serum level, was associated with zygotes number (beta=0.814, p<0.0001) and fertilisation rate (beta=-0.133, p=0.001). Patients with AMH in the range 1.1-5 ng/mL had significantly higher biochemical (65.3% versus 56.6%, p=0.009) and clinical pregnancy rates (57.7% versus 49.2%, p=0.014) in comparison with patients with AMH below 1.1 ng/mL and higher clinical pregnancy rates in comparison with patients with AMH above 7 ng/mL (57.7% versus 44%, p=0.011). Logistic regression analysis showed that AMH was positively associated with biochemical (OR 1.19, p=0.003) and clinical pregnancy (OR 1.16, p=0.009) independently of age and number of good embryos transferred in patients with AMH below 5 ng/mL. In turn, when only patients with normal ovarian reserve were analysed (AMH above 1.1 ng/mL), we found an age-independent negative association between AMH and clinical pregnancy (OR 0.93, p=0.014). AMH serum level is associated with both quantitative response (oocytes number) and qualitative parameters (pregnancy rate) during IVF/ICSI. We also found a bimodal relationship between AMH and pregnancy rates, which were positively associated in patients with AMH below 5 ng/mL, although higher AMH values seem to have a negative impact on pregnancy chances.

Keywords: AMH, IVF outcome, oocytes competence, fertilisation rate, pregnancy

Infertility is an increasingly more common condition, affecting 15% of the reproductive age couples. The treatments choices include intrauterine insemination, ovulation induction, in vitro fertilisation (IVF) or intracytoplasmic sperm injection (ICSI). Despite significant progress in assisted reproductive technologies in the last decades, the success of IVF remains modest, not exceeding 43% live birth rate per fresh cycle even in the best prognosis patients [1]. Thus, improving IVF success is a constant concern for those involved in the field of reproductive medicine. The use of prediction models which would allow the clinician to administrate the optimal gonadotropins doses in order to obtain the best IVF outcome is a tempting possibility, but ideal prediction markers are not identified yet. Ovarian reserve markers like antral follicle count, follicle stimulating hormone (FSH) and anti-müllerian hormone (AMH) were studied as possible predictors of live birth rate, the final IVF outcome, but their predictive value is controversial [2,3]. However, AMH is widely recognised as a good predictor of the ovarian response to controlled ovarian stimulation [4]. Thus, its poor predictive value for the final IVF outcome suggests that other factors involved in pregnancy occurrence, like oocytes' and embryos' competence and endometrium receptivity, might not be associated with the ovarian reserve and, therefore, with AMH serum level. Indeed, there is an ongoing discussion regarding the relationship between AMH and qualitative parameters of ovarian stimulation, the data reported in the literature being divergent [5-10]. Moreover, it is possible that the relationship between AMH serum level and reproductive parameters to be different according to the ovarian reserve category or AMH serum level category [3,11], although available data are limited. Identifying the relationship between AMH serum level and oocytes number and competence and early pregnancy is an essential aspect in IVF. Therefore, the study aims to analyse whether the AMH serum level is associated with ovarian response to ovarian stimulation, oocytes

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competence and pregnancy rate in a large cohort of infertile women performing IVF and whether this relationship varies according to AMH category.

Experimental part

Study subjects

The database of infertile couples who performed IVF/ICSI between January 2013 and December 2016 in the Reproductive Medicine Department of Medlife Hospital was reviewed retrospectively. Only patients with the following inclusion criteria were selected: 1. The presence of the following data in the database: age, body mass index (kg/m2), AMH serum level, gonadotropins doses used for controlled ovarian stimulation, the number of oocytes retrieved at egg collection, zygotes number, fertilisation rate, number and quality of embryos transferred, serum beta-HCG measurement 12 days after embryo transfer, transvaginal ultrasound in order to identify foetal heartbeat 4 weeks after embryo transfer; 2. Serum AMH measured in the laboratory of Medlife Hospital; 3. Only cycles with fresh embryos transferred. Patients treated with antagonist protocol were excluded from the study group for the uniformity of the study population. The causes of infertility were categorised as male factor, endometriosis, anovulation, tubal factor and idiopathic. Patients with polycystic ovary syndrome (PCOS) were excluded from the study because of the reduced number which performed fresh embryo transfer, most of these patients being treated with a freeze-all strategy.

Total doses of gonadotropins were calculated based on the content of the products administrated. Thus, one vial of highly purified human menopausal gonadotropin (HP-HMG) of 75 IU was considered to correspond to 75 IU of human FSH and 75 IU of human luteinizing hormone (LH). Therefore, the total FSH dose was calculated as the FSH dose from HP-HMG added to the total dose of recombinant FSH (recFSH). LH dose was calculated only based on the LH activity from HP-HMG.

Fertilisation rate, zygotes number and biochemical and clinical pregnancy were considered in this study as indirect indicators of oocytes and embryos competence.

Patients were divided into four categories according to AMH serum level in order to analyse the relationship between reproductive parameters and ovarian reserve category: group 1 patients with AMH below 1.1 ng/mL, group 2 patients with AMH $\geq$ 1.1 and < 5 ng/mL, group 3 patients with AMH $\geq$ 5 and < 7 ng/mL and group 4 patients with AMH $\geq$ 7 ng/mL. The value of 1.1 ng/mL was used to delineate patients with normal and low ovarian reserve [12]. The cut-off value of 5 ng/mL was used to identify patients with possible polycystic ovary as previously suggested [13], and the cut-off value of 7 was arbitrarily chosen to delineate patients with extreme AMH values.

Treatment protocol

Only one clinician established the treatment protocol according to the patient profile. All the patients were treated with a long agonist protocol, and COS was performed with variate proportions of HP-HMG and recFSH (mixt protocol). Dose adjustments were performed according to serum estradiol determinations and ovarian follicles number and dimensions evaluated at the transvaginal ultrasound. In all the patients the treatment started with a combined oral contraceptive pill administration for 10-21 days, followed by administration of triptorelin 0.1 mg/day starting in the last two days of the combined oral contraceptive pill administration till the day before triggering. The ovarian stimulation was carried out with recFSH (follitropinum alpha or follitropinum beta) in association with HP-HMG starting after seven days of pill-free interval. Oocytes maturation was triggered with a single dose of 10 000 UI urinary HCG when at least two follicles reached 17 mm. Oocytes retrieval was performed 36-38 hours after HCG administration. The day of the embryo transfer and the number of embryos transferred were decided according to ESHRE and ASRM guidelines.

During the study period, the same culture system was used, and the same embryology team reported the oocytes and zygotes number and quality.

Beta HCG was measured 12 days after embryo transfer, and a value above 12 mUI/L was considered indicative of biochemical pregnancy. Foetal heart activity noticed at transvaginal ultrasound four weeks after embryo transfer was necessary to diagnose clinical pregnancy.

The fertilisation rate was calculated as the number of two pronuclei divided by the total number of oocytes used for insemination in IVF patients or as the number of zygotes divided by the total number of metaphase II oocytes injected at ICSI. The embryologist evaluated the quality of the embryos according to The Istanbul consensus workshop on embryo assessment criteria [14]. Blastocysts were graded according to Gardner classification [15].

Assays

AMH was measured in the serum of the patients in any day of the menstrual cycle no more than three months before IVF. AMH levels were measured by a commercial ELISA kit (AMH Gen II ELISA kit from Beckman-Coulter) with the AMH limit of detection of 0.08 ng/mL.

Statistical analysis

Statistical analysis was performed with SPSS for IBM statistics version 22. Data are expressed as mean and standard deviations (continuous variables) or percentages (categorical variable) as appropriate. Comparisons of the continuous variables between groups were performed with students t-test (two groups) or ANOVA test (more than two groups). Chi-square test was used for categorical variables. Correlations were tested using Pearson analysis. Few models of logistic regression and multivariate linear regression were constructed in order to adjust for covariates. All the multivariate models were adjusted for infertility cause. A p-value below 0.05 was chosen as indicative of statistical significance.

Results and discussions

One thousand seventy-three patients were included in the study with a mean age of 34.68±4.28 years and mean BMI of 22.7±15.65 kg/m^2 (Table 1). Most of the patients in the study group had an AMH serum level below 5 ng/mL (n=917 patients, 85.5%). When patients were divided into groups according to AMH serum level we found that 321 patients (29.9 %) were in group 1, 596 patients (55.5%) were in group 2, 72 patients (6.7%) were in group 3 and 84 patients (7.8%) were in group 4.

Parameter	
Age (years)	34.68 ± 4.28
BMI (kg/m²)	22.7 ± 15.65
AMH (ng/mL)	2.85 ± 3.03
FSH dose (IU)	2514.4±618.7
LH dose (IU)	1757±387
Fertilization rate	0.7 ± 0.25
Oocytes (no)	7.55 ± 4.78
Embryos (no)	4.9 ± 3.34
Transferred embryos (no)	2.15 ± 0.8
Good quality embryos transferred (no)	1.54 ± 1

Table 1
THE CLINICAL AND REPRODUCTIVE CHARACTERISTICS OF THE STUDY GROUP

The relationship between AMH and oocytes number, embryos number and fertilization rate

We found that AMH serum level was positively correlated with the number of the oocytes retrieved at ovum pickup ($r = 0.499$, $p < 0.0001$) (Figure 1) and the zygotes number ($r = 0.397$, $p < 0.0001$) (Figure 2) and negatively correlated with the fertilization rate ($r = - 0.103$, $p = 0.001$) (Figure 3).

AMH serum level was an independent predictor of oocytes (beta = 0.329, $p < 0.0001$) and zygotes number (beta = 0.248, $p < 0.0001$) and fertilization rate (beta = - 0.108, $p = 0.001$) after adjustment for age, BMI and gonadotropins doses. In turn, multivariate linear regression showed that oocytes number, but not serum AMH, was positively related with zygotes number (beta=0.814, $p<0.0001$) and negatively related with fertilization rate (beta - 0.133, $p = 0.001$).

When the patients were divided into four categories according to AMH serum level we noticed that age gradually decreased (36.27±3.62 years in group 1, 34.11±4.1 years in group 2, 33.58±3.97 in group 3 and 32.21±3.66 years in group 4, $p<0.0001$ between groups) (Table 2), while the oocytes (4.14±2.75 in group 1 versus 8.08±4.18 in group 2, $p<0.05$) and zygotes number (3.01±2.1 in group 1 versus 5.23±3.12 in group 2, $p<0.05$) gradually increased, but reached a plateau in patients in groups 3 (10.87±4.17 oocytes, 6.76±3.29 zygotes) and 4 (11.70±5.53 embryos, 6.89±3.89 zygotes, p=NS group 3 versus group 4) (Table 2). In turn, fertilisation rate was lower in patients in groups 2 (0.70±0.23, $p<0.05$ versus group 1), 3 (0.68±0.21, $p<0.05$ versus group 1) and 4 (0.63±0.2, $p<0.05$ versus group 1) in comparison with group 1 (0.75±0.27), but only in group 4 was lower than in group 2 ($p<0.05$) (Table 2).

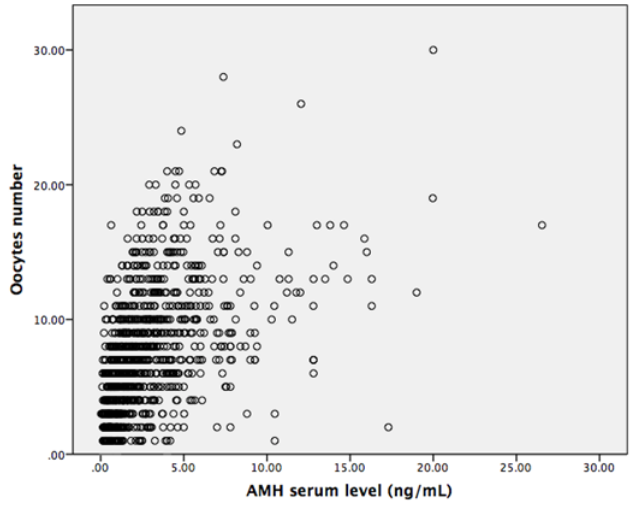

Fig.1. The correlation between AMH serum level and the number of oocytes retrieved at ovum pick-up

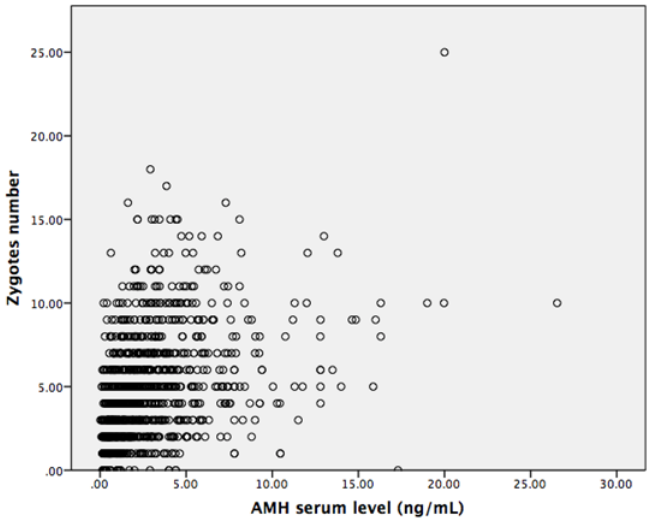

Fig. 2. The correlation between AMH serum level and zygotes number

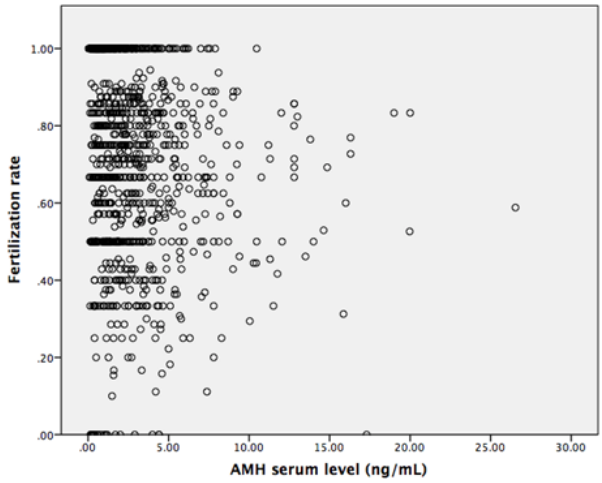

Fig. 3. The correlation between AMH serum level and fertilization rate

The association between AMH and pregnancy

Biochemical pregnancy was significantly higher only in patients in group 2 versus patients in group 1 (65.3% vs 56.6%, p=0.009) (Table 2). On the other hand, clinical pregnancy was significantly higher in group 2 in comparison with patients in group 1 (57.7% vs 49.2%, p=0.014) and group 4 (57.7% vs 44%, p=0.011), but similar in groups 1, 3 and 4 (Table 2).

When only patients in the groups 1 and 2 were analysed, in logistic regression analysis, after adjustment for age, the AMH serum level was positively associated with both biochemical (OR=1.18, p=0.003) and clinical pregnancy (OR=1.15, p=0.009). When the number of good quality embryos transferred was introduced as an independent variable in the logistic regression model, AMH continued to be an independent predictor of biochemical (OR=1.19, p=0.003) and clinical pregnancy rates (OR=1.16, p=0.009).

When patients in group 1 were excluded from the analysis, we found that AMH serum level was negatively associated with clinical pregnancy (OR=0.93, p=0.014), but not with biochemical pregnancy after adjustment for age.

Our study shows that AMH serum level is related to quantitative responses to COS (oocytes number), and this relationship is independent of age. Our results are concordant with the data available in the literature, which strongly support the predictive value of serum AMH for quantitative parameters [4,3]. The positive linear relationship between AMH and the number of retrieved oocytes observed in our study mirrors the value of AMH as a marker of ovarian reserve. Thus AMH is secreted by the granulosa cells of the growing follicles, the primary source of circulating AMH being pre-antral and small antral follicles, which are considered a proxy for the primordial follicles pool. In the same time, antral follicles represent the pool of follicles responsive to FSH stimulation and can be selected during COS, giving rise to oocytes yield at egg collection. This is the most probable explanation for the close correlation between AMH serum level and the oocytes yield.

We also found a positive correlation between AMH and zygotes number, which lost its significance after adjustment for oocytes number in a multivariate regression model. In this model, only oocytes number was significantly associated with zygotes number. Thus, more oocytes available for fertilisation will result in more zygotes irrespective of age, gonadotropins doses, BMI and AMH serum level.

Table 2
REPRODUCTIVE PARAMETERS OF THE STUDY PATIENTS ACCORDING TO AMH SERUM LEVEL CATEGORY

	Group 1 AMH<1.1 ng/mL (n=321)	Group 2 AMH ≥1.1 and < 5 ng/mL (n=596)	Group 3 AMH ≥ 5 and < 7 ng/ml (n=72)	Group 4 AMH ≥7 ng/mL (n=84)	P value
Age (years)	36.27±3.62	34.11±4.1[a]	33.58±3.97[a]	32.21±3.66[a,b,c]	<0.0001
Oocytes number	4.14±2.75	8.08±4.18[a]	10.87±4.17[a,b]	11.70±5.53[a,b]	<0.0001
Zygotes number	3.01±2.1	5.23±3.12[a]	6.76±3.29[a,b]	6.89±3.89[a,b]	<0.0001
Fertilization rate	0.75±0.27	0.70±0.23[a]	0.68±0.21[a]	0.63±0.2[a,b]	<0.0001
Biochemical pregnancy (%)	56.6	65.3[a]	59.7	58.3	0.06
Clinical pregnancy (%)	49.2	57.7[a]	50.7	44[b]	0.02

[a] p<0.05 versus group 1; [b] P<0.05 versus group 2; [c] p<0.05 versus group 3

Our study showed a negative relationship between AMH serum level and the fertilisation rate. However, after adjustment in a multiple regression model, only the retrieved oocytes' number, but not AMH, was an independent negative predictor of the fertilisation rate. These data suggest that the negative relationship between AMH and fertilisation rate is probably explained by the close relation between AMH and oocytes number. This negative association between oocytes number and fertilisation rate was previously reported [5], although the data regarding this aspect are limited in human studies. The study of Kok et al. [5] showed that decreased fertilisation rate associated with high oocytes yield could be the consequence of the increasing proportions of immature oocytes [5].

Similarly, an earlier study found that patients with more than 11 oocytes obtained after COS had significantly higher cytogenetic abnormalities in the unfertilized oocytes, suggesting a high cytoplasmic immaturity in this group [16]. An important finding of our study is the positive association between oocytes and zygotes number despite decreased fertilisation rate, demonstrating that the negative impact of a high oocytes yield on the fertilisation rate is mild and does not negatively affect the zygotes number. Both oocytes and zygotes number were showed to be significant predictors of live birth in large cohorts [17]. Thus their association with AMH indicate that AMH might be a valuable tool in the evaluation of pregnancy outcome and counselling patients undergoing IVF.

Our data showed a gradual increase in oocytes and zygotes number which reached a plateau in patients with AMH above 5 ng/mL, in association with similar fertilisation rate in patients with AMH between 1.1 ng/mL and 7 ng/mL, which decreased when AMH serum level exceeded 7 ng/mL. Despite a linear relationship between AMH and oocytes number, zygotes number and fertilisation rate, patients in group 4 seem to have no further benefit in terms of reproductive parameters in comparison with patients in group 3. Thus, our data suggest that AMH levels exceeding a cut off value can be associated with limited reproductive benefits, although this finding should be interpreted with caution due to a limited number of patients in higher AMH groups. It is possible that some of these patients to have mild phenotype PCOS since this disease is known to be associated with higher AMH values [13]. In PCOS the relationship between AMH and the ovarian reserve is different from patients without this disease [18], explaining the limited value of AMH as a predictor of reproductive outcome in IVF reported by other authors [3]. Although we excluded patients with PCOS from our study group for the reasons already mentioned, it is possible to miss the diagnosis in patients with mild phenotypes. For example, in a patient with regular menstrual cycles, without hyperandrogenism, but with polycystic ovaries on ultrasound, the presence of oligo-ovulation is challenging to be identified if we evaluate only one cycle.

The relationship between AMH and pregnancy rate in IVF/ICSI is the subject of continuous debate. Although some studies [19,10,20] reported that AMH is a predictor of pregnancy, two meta-analyses showed a poor predictive value of AMH for this outcome [2,3]. The possible explanation for these conflicting results is the heterogeneity of the study populations since it was showed that AMH has a different relationship with the IVF outcome in patients with polycystic ovary syndrome or decreased ovarian reserve [3]. Moreover, the live birth outcome might be influenced by multiple factors. This aspect was evaluated in the study of Kebbon Vaegter et al. [21] which analysed 100 possible predictors of live birth and found that factors that are not related to ovarian response are associated with chances of live birth in assisted reproduction, like infertility cause, treatment history, endometrial thickness, and female height [21]. In our patients we found that when only patients with AMH below 5 ng/mL were analysed, patients with AMH serum level between 1.1 and 5 ng/mL

had increased chances of pregnancy in comparison with patients with AMH below 1.1 ng/mL and AMH serum level was positively associated with biochemical and clinical pregnancy rates after adjustment for age and number of good quality embryos transferred.

On the other hand, when only patients with normal ovarian reserve (AMH ≥1.1 ng/mL) were analysed, we found an age-independent negative association between AMH and clinical pregnancy rates. This negative association might be the consequence of decreased endometrial receptivity in patients with a high response due to increased estradiol levels [22] and premature induction of progesterone receptors leading to advanced endometrium [23]. The poorest reproductive outcome was found in patients with AMH above 7 ng/mL which had the lowest pregnancy rates, while patients with AMH between 1.1 ng/mL and 5 ng/mL and those with AMH between 5 and 7 ng/mL had similar pregnancy rates. Therefore, our results suggest that the relationship between AMH and pregnancy is not linear and can vary according to the ovarian reserve category and probably the underlying cause of infertility. Thus, studies with a heterogeneous population, including patients with variate ovarian reserve categories and a wide range of AMH serum values might not be able to identify the correct relation between AMH and pregnancy. This might be another explanation for the divergent results in the literature regarding the predictive value of AMH for IVF outcome. This non-linear relationship between AMH and pregnancy in IVF contradict other studies [11], possibly due to higher AMH values in our patients.

The relationship between AMH and qualitative parameters of IVF is most controversial. In our study, we found an association between AMH and indirect indicators of oocytes and embryos competence like zygotes number and biochemical pregnancy rate. Moreover, AMH seems to be associated with pregnancy independent of age and the number of good quality embryos transferred, suggesting that AMH can capture other information predictive for pregnancy occurrence. One possible explanation is the fact that AMH can be associated with embryo euploidy as well. This hypothesis is supported by the findings of La Marca et al. [24], which showed a strong positive age-independent relationship between AMH level and the rate of euploid blastocysts.

The strength of our study is the uniformity of the infertility approach in the study group since all the patients were managed by the same clinician, minimising the effect of COS regimen on reproductive parameters as a confounding factor in the relationship between AMH and IVF outcome. Moreover, the AMH was measured with the same method and in the same laboratory in all the patients, therefore avoiding the differences in AMH serum levels due to laboratory conditions. To our regret, our database does not contain data regarding the number and the quality of frozen embryos. Therefore, it was impossible to analyse the relationship between AMH and embryos quality in the entire cohort of embryos obtained. Another limitation of our study is the small number of patients with AMH above 5 ng/mL. Therefore, the findings involving these patients need confirmation in further studies with adequate sample size.

There are few clinical implications of our findings. First, in patients with AMH below 5 ng/mL, higher AMH levels are associated with higher chances of pregnancy. In turn, in patients with AMH values exceeding 5 ng/mL, higher AMH levels might be associated with decreased chances of pregnancy, at least when fresh embryos are transferred. Secondly, in patients with AMH above 7 ng/mL, the clinician might expect a decreased fertilisation rate and lower pregnancy rate in a fresh cycle. However, our results should be used with caution in patients with AMH above 5 ng/mL since these patients were underrepresented in our study.

Conclusions

In conclusion, our study shows that AMH has an age-independent relationship with oocytes number retrieved at egg collection which, in turn, were associated with higher zygotes number despite a negative impact on fertilisation rate in patients performing IVF/ICSI. We also found that AMH has a bimodal relationship with clinical pregnancy, being positively associated with biochemical and clinical pregnancy rate in patients with AMH below 5 ng/mL. In turn, in patients with normal ovarian reserve, high AMH levels seem to be negatively related to clinical pregnancy rates. These aspects should be taken into account when the relationship between AMH and pregnancy in assisted reproduction is analysed in a clinical study.

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