

Research Article

Higher likelihood of success in pregnancy in patients with high serum basal anti-müllerian hormone concentrations: An age-stratified analysis of 4516 women undergoing in vitro fertilization

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Abstract

The aim of this study was to evaluate whether anti-Müllerian hormone (AMH) was a predictor of pregnancy rate after ovarian stimulation for in vitro fertilization (IVF). A total of 4516 patients were included in this retrospective analysis: 3535 patients for fresh embryo transfer and 981 for frozen embryo transfer after at least 3 months of whole embryo vitrification. Blood samples of all patients were taken on days 3-5 of their menstrual cycle for testing AMH in serum by using enzyme-linked immunosorbent assays. Four age groups (<35, 35-37, 38-40 and >40 years) were divided into AMH subgroups according to their serum AMH levels (undetectable, 0.06-0.49, 0.5-0.99, 1-1.49, 1.5-1.99, 2-2.99, 3-3.99, 4-4.99, and ≥ 5 ng/mL). We found that serum AMH levels were positively correlated with the number of retrieved oocytes, fertilized oocytes, developed embryos and clinical pregnancy rate among the four age groups. In conclusion, this study demonstrates that women with high AMH levels had a higher probability of pregnancy across all age groups. However, due to our retrospective design, future randomized controlled trials are needed to corroborate these results

Introduction

The reproductive lifespan is related to the number and quality of oocytes. The depletion of the primordial follicle pool is associated with the decline of ovarian reserve. Although the number and quality of oocytes decrease with age, fertility varies among women of the same age. Consequently, a large number of measurements are available to evaluate the functional ovarian reserve, disclosing a prognostic value for ovarian response and helpful to decide the appropriate stimulation protocol of IVF procedures. Although reliable markers of the oocyte quality are not yet developed, Anti-Müllerian hormone (AMH) has been shown to represent a good marker of ovarian reserve.

The AMH, discovered in 1947 by Alfred Jost, is a homodimeric glycoprotein whose gene is located on the chromosome 19p13.3 and belongs to the transforming growth factor-beta superfamily that is produced by the granulosa cells surrounding pre-antral and small antral follicles, of independently of follicle-stimulating hormone (FSH) stimulation [1]. AMH is secreted into the circulation from the time of birth until menopause with only minimal fluctuations during the menstrual cycle. The serum level of AMH has been shown to correlate with the number of small follicles. Previous studies have demonstrated that AMH could reliably predict the quality of oocytes in *in vitro* fertilization (IVF) [2], the extremes of ovarian response to stimulation [3], and outcomes of IVF [4], although the predictive role of AMH may be only indirect and related to quantitative ovarian response that has achieved after the gonadotropin stimulation.

The predictive value of AMH from published evidence, the objectivity and potential standardization of AMH levels, and the convenience of testing at any time throughout the menstrual cycle

lean toward AMH levels becoming the gold standard biomarker to evaluate ovarian reserve and predict ovarian response to stimulation [5]. Nevertheless, the ability of AMH to predict implantation rate, pregnancy rate, ongoing pregnancy rate, and live birth rate is poor [6,7], and the observational studies that have examined the association of AMH and outcomes of IVF have either been small [8-10] or restricted to specific subpopulations of infertility patients [11-12].

The aim of this study was to provide a large-sample observational study to determine the accuracy of AMH's predictive value with respect to pregnancy rate in the population after ovarian stimulation for IVF.

Materials and methods

Study population

The present study included data from 4516 women undergoing an IVF protocol with ovarian stimulation at IVF center of Maternal and Child Health Care Hospital of Chongqing between January 2012 and

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Received: August 16, 2016; **Accepted:** September 19, 2016; **Published:** September 22, 2016

February 2014. The patients were recruited according to the following inclusion criteria: (1) serum basal AMH assay within 3 months before ovarian stimulation, (2) first attempted cycle for ovarian stimulation and IVF/intracytoplasmic sperm injection (ICSI), (3) first attempted embryo transfer regardless fresh or frozen embryos transfer, and (4) embryos transfer on day 3. The exclusion criteria were: (1) received hormonal therapy in the previous 3 months, (2) any genetic diseases (3) polycystic ovarian syndrome (PCOS) or endometriosis, (4) azoospermia with male partner and (5) a history of endocrine disorders. All patients signed an informed consent form approved by the Ethics Review Board of our Hospital, allowing retrieval of clinical data from patients' records in accordance with Good Clinical Practice guidelines.

AMH assays

The serum AMH concentration was measured using an enzyme-linked immunosorbent assay (ELISA) kit, according to the manufacturer's instructions (AMH ELISA, AnshLabs, UK). The minimal detectable concentration for AMH was 0.06 ng/mL. The serum AMH level <0.06 ng/mL was defined undetectable value. Before treatment, blood samples of all patients were taken for testing AMH in serum. Blood was drawn on days 3-5 of the menstrual cycles. Specimens were frozen within 24 h and stored at -20°C until analysis was performed within 1 week.

Ovarian stimulation protocols

Different ovarian stimulation protocols were used, including long GnRH agonist, short GnRH agonist and mild stimulation protocols. Stimulation protocol was decided according to individual patient characteristics. Gonadotropins were prescribed at the discretion of the physicians according to the ovarian reserve tests (AMH, antral follicular count AFC, bFSH) and age. The dosage was then adjusted according to follicular growth until the day of hCG administration.

For the long protocol, as described in our previous publication [13]. Briefly, after down-regulation with a GnRH agonist (Triptorelin Acetate, Ipsen Pharma, France), the ovaries were stimulated with gonadotropins. There were 4231 patients included in the long protocol.

For the short protocol, daily GnRH agonist (Triptorelin Acetate, Ipsen Pharma, France) and gonadotropins stimulation were started on day 2 of the menstrual cycle. There were 214 patients included in the short protocol.

For the mild stimulation protocol, Clomiphene Citrate (CC) 50 or 100 mg/d was started on day 3 of the menstrual cycle until the follicular maturation. Gonadotropins were started when dominant follicles size less than 14 mm after CC was given 5 days. There were 71 patients included in the mild stimulation protocol.

Human chorionic gonadotropin (HCG) (Ovidrel, Merck Serono, Switzerland) was administered when at least three follicles measured diameter >18 mm. Transvaginal oocyte retrieval was performed 36 h after HCG injection and embryo transfer was performed on day 3 after oocyte retrieval.

Embryo culture

Cumulus-enclosed oocytes were collected for insemination in 2.5 mL of IVF medium (G-IVF, Vitrolife Sweden AB, Sweden) and incubated in 5% O₂ and 6% CO₂ in compressed air at 37°C in a humidified incubator. Insemination (ICSI or IVF) and embryo culture were performed as previously described [14,15]. Cleavage-stage embryo assessment was conducted according to the criteria of Dale

[16] and embryos graded 1-3 were deemed developed embryos; these were transferred or vitrified on day 3 post-insemination following doctor's advice or patient's request. Embryo culture was carried out in 50 µL micro-drops (1 normal fertilized embryo per drop) at 37°C in an incubator with humidified atmosphere of 5% O₂ and 6% CO₂ in compressed air.

Vitrification and warming

Embryos from patients who cancelled their transfers because of potential ovarian hyperstimulation syndrome (OHSS), improper endometrial preparation, or personal reasons, as well as surplus embryos after transfer, were vitrified for cryopreservation. All of embryos were frozen at day 2 or 3. First, we equilibrated embryos in VS1 solution for 5 min before exposure to the vitrification solution (VS 2). The vitrification method followed the instructions from commercial kits (Kita, Toyota, Japan). After placing embryos on a Cryotop sheet, excess vitrification solution was removed by aspiration with a pipette before the carrier was plunged into LN₂.

A Dewar flask of LN₂ containing the carriers was placed close to the microscope. Forceps were used to grasp the straw in the LN₂ and place it into a dish containing 3 mL of 1.0 mol/L sucrose at 37°C for 1 min. Embryos were transferred sequentially to 0.5 and 0.25 mol/L sucrose solutions at room temperature for 3 min each. The embryos were then washed several times in Quinn's 1024 (Cooper Surgical, CT, USA) solution and placed in G1 medium (Vitrolife, Kungsbacka, Sweden) for further culture. Post-thaw survival of cryopreserved embryos was defined as survival with more than half of the original cells intact.

Endometrial preparation of artificial cycle for frozen embryo transfer (FET)

On day 3 of the menstrual cycle, patients commenced oral Estradiol Valerate (Progynova, Bayer, Germany) at a dosage of 4-8 mg/d. After 14 d of estradiol preparation, an ultrasound was performed. If no dominant follicle was present, and the endometrial thickness was ≥ 8 mm, progesterone 100 mg/d intramuscular injection (Li Zhu, China) was added to the regime, and cleavage embryos were thawed on day 3 after progesterone administered and transferred on day 4 after progesterone administered.

Embryo transfer

Laser-assisted hatching [16,17] by zona drilling was performed on transferred embryos in all cases immediately before the embryo transfer. The embryo transfers were all performed with ultrasonographic guidance. Patients received luteal support (combination of estrogen and progesterone) until the blood HCG test day (14 d after embryo transfer). A clinical pregnancy was established when the gestational sac was detected by transvaginal ultrasound 28 d after embryo transfer.

Outcome measurements and age-group stratification defined

In determining clinical pregnancy, the denominator was the number of patients who underwent embryo transfer. In determining the developed embryo rate, the denominator was the number of cleaved 2PN zygotes. In determining fertilization rate, the denominator was the number of oocytes used for insemination. In determining metaphase II (MII) oocyte rate, the denominator was the number of oocytes obtained. The MII oocytes were decided at cumulus cell removal after 2-4 h of egg insemination in conventional IVF or at ICSI. The insemination with early cumulus cells removal made the MII oocyte rate measurement more objective than overnight insemination. The age stratification was

classified according to the 2013 Assisted Reproductive Technology National Summary Report published by the U.S. Centers for Disease Control and Prevention (CDC, 2013) [18].

Statistical analyses

Continuous data are presented as mean ± sd and 25th, 50th, and 75th percentiles. One-way ANOVA with Tukey's test for multiple comparisons and Chi-square test were performed in different age groups for patient characteristics and treatment outcomes of each AMH subgroup. Proportions of pregnancy rates were compared using trend Chi-square tests. A two-tailed value of P < 0.05 was considered statistically significant.

Results

A total of 4516 patients were included in this retrospective analysis (Figure 1); 3535 underwent their first fresh embryo transfer, and 981 underwent their first frozen embryo transfer after at least 3 months of whole embryo vitrification. The mean age of the 4516 patients who underwent oocyte retrieval was 31.2 ± 4.63 years. Their mean serum AMH level was 3.88 ± 3.18 ng/ml, BMI was 22.07 ± 2.69 kg/m² and oocyte number was 11.43 ± 6.07 (Table 1).

With respect to analysis stratified by age (<35, 35-37, 38-40 and >40 years) in the subgroups of various baseline serum AMH

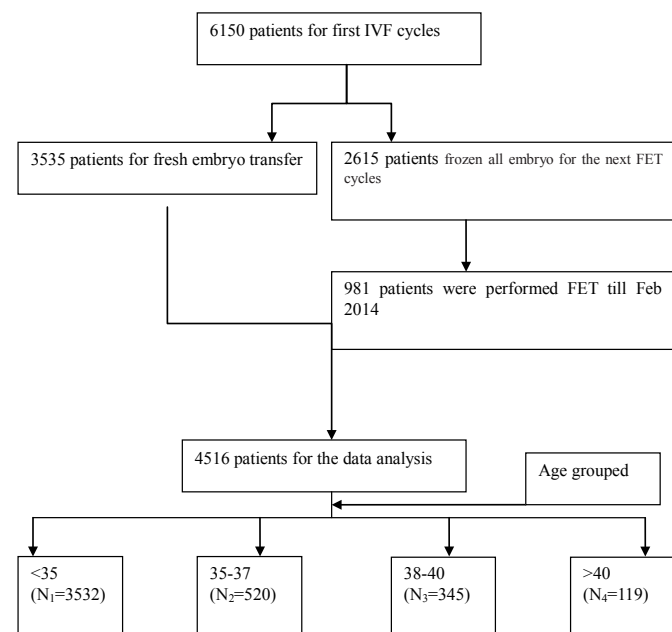


Figure 1. The flow chart of data recruitment.

Table 1. Patient characteristics according to AMH percentile in oocyte retrievals (n=4516).

	Mean ± sd	Minimum	25 th percentile	50 th percentile	75 th percentile	Maximum
Age (years)	31.2 ± 4.64	20	28	31	34	48
AMH (ng/mL)	3.876 ± 3.179	undetectable	1.63	3.03	5.17	28.12
BMI (kg/m ²)	22.07 ± 2.69	14.98	19.88	21.48	23.44	37.33
No. of oocytes	11.43 ± 6.071	1	7	11	15	37
No. of MII oocyte	11.43 ± 5.99	1	7	11	15	37
MI rate (%)	0.89 ± 0.12	0.38	0.83	0.92	1	1
No. of fertilized oocytes	9.12 ± 5.36	1	5	9	13	35
Fertilization rate (%)	0.81 ± 0.21	0.02	0.71	0.86	1	1
No. of developed embryos	4.56 ± 3.32	1	2	4	6	27
Developed embryo rate(%)	0.64 ± 0.26	0.01	0.45	0.64	0.83	1

(undetectable,0.06-0.49, 0.5-0.99, 1-1.49, 1.5-1.99, 2-2.99, 3-3.99, 4-4.99, and ≥5 ng/mL), it demonstrated the number of retrieved oocytes, fertilized oocytes and developed embryos positively correlated with baseline serum AMH in all age groups (<35, 35-37, 38-40 and >40 years) (p<0.05), and the number of matured oocytes positively correlated with baseline serum AMH in the age <35 group (p<0.01). However, the rate of mature oocytes and fertilization in all age groups did not differ according to baseline serum AMH (p>0.05). The study also demonstrated the rate of developed embryos in all age groups except age >40 group which negatively correlated with baseline serum AMH (p<0.05). For age >40 group, the rate of developed embryos did not differ according to baseline serum AMH (p=0.63) (Table 2-5).

The trend Chi-square tests showed that clinical pregnancy rates among age <35, 35-37 and 38-40 groups were positively correlated with baseline serum AMH (p<0.05) (Table 6).

Discussion

AMH has demonstrated its effectiveness in many areas. It is the best hormonal marker of ovarian reserve, and is a useful tool to predict low response to controlled ovarian stimulation and conversely the risk of excessive response [19]. Our study showed that higher AMH was related to more retrieved oocytes independent of age, which are consistent with this previous report on ovarian reserve. High AMH expression was detected in primary, secondary, pre-antral, and early antral follicles <4 mm in diameter, but this sharply decreased in follicles 8 mm in diameter [20,21]. Due to peripheral circulating concentrations of AMH being proportional to the primordial follicle pool, AMH may indirectly represent the ovarian reserve and ovarian response to gonadotropins stimulation.

The ability of AMH to predict pregnancy is contradictory, as a meta-analysis of individual patient data clearly demonstrated that AMH does not predict ongoing pregnancy in IVF [22]. However, another meta-analysis of diagnostic accuracy showed that AMH adds some value in predicting live birth, and that this is independent of age or immune assayable AMH [7]. To the best of our knowledge, this is the largest retrospective analysis investigating the relationship between serum AMH and the rate of clinical pregnancy after first IVF cycles. Our retrospective stratified analysis found that clinical pregnancy rate among age <35, 35-37 and 38-40 groups were positively correlated with baseline serum AMH independent of age. Although several investigators have evaluated an AMH cut off value for predicting clinical pregnancy rate and live birth rate in certain women undergoing IVF [2,4,7,10,23,24], only one study indicated that follicle fluid AMH was significantly correlated with clinical pregnancy rate and live birth rate [25]. Most of the previous reports regarding the use of AMH in prediction of outcomes in IVF were diagnostic trials, or where data were demonstrated to be in extracted form, raw data were omitted,

Table 2. Treatment outcomes according to baseline serum AMH in age<35 group (n=3532).

	Undetectable	0.06-0.49	0.5-0.99	1-1.49	1.5-1.99	2-2.99	3-3.99	4-4.99	≥5	P
No. patients	2	63	200	335	385	701	568	375	903	
No. oocytes	11	4.43 ± 2.72	6.77 ± 3.43	8.32 ± 3.98	9.71 ± 3.95	10.82 ± 4.68	12.49 ± 4.86	13.35 ± 4.94	14.65 ± 5.87	<0.01
No. MII oocytes	10 ± 1.41	3.5 ± 2.06	5.26 ± 2.18	7.42 ± 3.99	8.66 ± 3.91	9.48 ± 4.55	11.38 ± 4.99	11.99 ± 5.02	12.11 ± 5.77	<0.01
MIH oocyte rate	0.9 ± 0.1	0.875 ± 0.14	0.89 ± 0.11	0.91 ± 0.12	0.85 ± 0.16	0.89 ± 0.12	0.89 ± 0.12	0.86 ± 0.13	0.91 ± 0.11	0.073
No. fertilized oocytes	7.5 ± 0.7	3.11 ± 2.44	4.86 ± 3.08	6.81 ± 3.73	7.85 ± 3.63	8.84 ± 4.29	10.25 ± 4.41	10.56 ± 4.54	11.68 ± 5.24	<0.01
Fertilization rate	0.9 ± 0.22	0.86 ± 0.22	0.83 ± 0.28	0.84 ± 0.28	0.82 ± 0.24	0.84 ± 0.25	0.85 ± 0.24	0.83 ± 0.18	0.81 ± 0.18	0.11
No. developed embryos	2	2.11 ± 2.44	3.99 ± 3.08	4.81 ± 3.13	5.48 ± 2.61	5.84 ± 4.29	5.05 ± 3.07	5.06 ± 3.14	5.8 ± 3.36	<0.01
Developed embryo rate	0.25	0.76 ± 0.27	0.69 ± 0.29	0.67 ± 0.27	0.62 ± 0.24	0.6 ± 0.25	0.6 ± 0.24	0.59 ± 0.25	0.6 ± 0.24	<0.01

Table 3. Treatment outcomes according to baseline serum AMH in age35-37 group (n=520).

	Undetectable	0.06-0.49	0.5-0.99	1-1.49	1.5-1.99	2-2.99	3-3.99	4-4.99	≥5	P
No. patients	0	31	66	64	81	107	73	37	61	
No. oocytes	-	4.12 ± 2.43	6.03 ± 3.52	7.41 ± 4.01	9.28 ± 4.11	10.11 ± 3.86	11.42 ± 4.21	13.08 ± 3.83	14.08 ± 5.52	<0.01
No. MII oocytes	-	3.81 ± 2.07	5.85 ± 1.95	5.83 ± 2.45	8.41 ± 3.98	9.24 ± 4.86	11 ± 5.66	11 ± 5.89	11.57 ± 6.33	0.33
MIH oocyte rate	-	0.91 ± 0.12	0.88 ± 0.11	0.92 ± 0.11	0.94 ± 0.08	0.92 ± 0.11	0.93 ± 0.11	0.89 ± 0.11	0.91 ± 0.11	0.58
No. fertilized oocytes	-	3.44 ± 2.11	4.83 ± 2.64	5.77 ± 3.08	7.44 ± 3.76	8.05 ± 3.49	8.85 ± 4.04	9.29 ± 4.51	11.37 ± 4.36	<0.01
Fertilization rate	-	0.86 ± 0.18	0.83 ± 0.18	0.81 ± 0.18	0.81 ± 0.2	0.80 ± 0.19	0.78 ± 0.2	0.79 ± 0.18	0.82 ± 0.19	0.72
No. developed embryos	-	2.12 ± 1.21	2.81 ± 1.72	3.16 ± 1.94	3.68 ± 2.67	4.44 ± 2.29	3.93 ± 2.39	4.73 ± 2.44	5.49 ± 2.51	<0.01
Developed embryo rate	-	0.875 ± 0.22	0.76 ± 0.25	0.71 ± 0.24	0.67 ± 0.25	0.69 ± 0.21	0.58 ± 0.23	0.58 ± 0.21	0.62 ± 0.22	<0.01

Table 4. Treatment outcomes according to baseline serum AMH in age 38-40 group (n=345).

	Undetectable	0.06-0.49	0.5-0.99	1-1.49	1.5-1.99	2-2.99	3-3.99	4-4.99	≥5	P
No. patients	1	28	60	68	53	62	43	13	17	
No. oocytes	13	4.03 ± 2.67	4.67 ± 2.73	6.39 ± 3.21	7.91 ± 4.63	9.74 ± 4.62	11.98 ± 4.99	12.15 ± 2.88	12.53 ± 6.7	0.04
No. MII oocytes	10	3.11 ± 2.51	4.17 ± 2.86	5 ± 1.63	6.33 ± 4.46	8.88 ± 4.07	11.8 ± 3.49	9.12 ± 4.02	10.33 ± 6.66	0.88
MIH oocyte rate	0.76	0.81 ± 0.14	0.94 ± 0.09	0.86 ± 0.12	0.78 ± 0.23	0.92 ± 0.11	0.92 ± 0.15	0.81 ± 0.15	0.83 ± 0.12	0.66
No. fertilized oocytes	7	3.01 ± 2.51	3.73 ± 2.28	4.55 ± 2.88	5.41 ± 3.46	7.93 ± 4.6	9.67 ± 4.3	8.38 ± 3.2	7.35 ± 5.3	0.03
Fertilization rate	0.54	0.91 ± 0.15	0.84 ± 0.21	0.83 ± 0.22	0.81 ± 0.21	0.83 ± 0.17	0.81 ± 0.19	0.85 ± 0.15	0.71 ± 0.3	0.09
No. developed embryos	2	2.21 ± 1.23	2.37 ± 1.43	2.69 ± 1.37	3.45 ± 2.34	3.77 ± 2.18	4.86 ± 3.26	5.23 ± 2.59	4.12 ± 2.57	0.01
Developed embryo rate	1	0.84 ± 0.2	0.8 ± 0.26	0.74 ± 0.26	0.67 ± 0.24	0.63 ± 0.25	0.63 ± 0.25	0.62 ± 0.24	0.63 ± 0.18	0.03

Table 5. Treatment outcomes according to baseline serum AMH in age >40 group (n=119).

	Undetectable	0.06-0.49	0.5-0.99	1-1.49	1.5-1.99	2-2.99	3-3.99	4-4.99	≥5	P
No. patients	6	22	36	23	11	16	1	2	2	
No. oocytes	1.5 ± 0.55	2.3 ± 1.03	3.13 ± 1.89	5.09 ± 3.19	5.22 ± 2.28	7.21 ± 4.39	9	9.5 ± 6.36	17 ± 2.82	0.04
No. MII oocytes	1.4 ± 0.6	2.21 ± 2.12	2.33 ± 2.25	4.25 ± 2.1	4.5 ± 3.53	6.5 ± 0.707	9	9	16	0.91
MIH oocyte rate	0.9 ± 0.2	0.88 ± 0.19	0.86 ± 0.22	0.88 ± 0.21	0.87 ± 0.05	0.85 ± 0.01	1	0.9 ± 0.14	0.9 ± 0.14	0.82
No. fertilized oocytes	1.3 ± 0.55	1.76 ± 0.84	2.07 ± 1.43	4.04 ± 2.91	3.74 ± 2.84	4.19 ± 3.17	8	8.5 ± 7.07	13 ± 1.41	0.04
Fertilization rate	0.9 ± 0.15	0.81 ± 0.26	0.86 ± 0.19	0.91 ± 0.14	0.75 ± 0.21	0.79 ± 0.24	0.89	0.9 ± 0.14	0.77 ± 0.04	0.44
No. developed embryos	1.2 ± 0.5	1.43 ± 0.55	1.86 ± 0.75	2.96 ± 1.02	2.82 ± 1.99	2.87 ± 1.4	5	5 ± 5.66	2	0.05
Developed embryo rate	0.9 ± 0.22	0.91 ± 0.19	0.79 ± 0.29	0.82 ± 0.21	0.78 ± 0.19	0.73 ± 0.23	0.71	0.9 ± 0.13	0.42 ± 0.35	0.63

Table 6. Clinical pregnancy rate according to baseline serum AMH in patients stratified by age.

Age	Undetectable	0.06-0.49	0.5-0.99	1-1.49	1.5-1.99	2-2.99	3-3.99	4-4.99	≥5	Trend P
<35	100% (2/2)	49.2% (31/63)	58.03% (112/193)	58.21% (195/335)	56.36% (217/385)	60.63% (425/701)	60.74% (345/568)	66.40% (249/375)	63.96% (582/910)	0.002
35-37	-	53.13% (17/32)	42.42% (28/66)	53.13% (34/64)	45.58% (37/81)	49.53% (53/107)	39.73% (29/73)	56.76% (21/37)	65% (39/60)	0.000
38-40	- 0/1	35.71% (10/28)	30% (18/60)	39.13% (27/69)	30.19% (16/53)	45.16% (28/62)	41.86% (18/43)	23.07% (3/13)	58.88% (10/17)	0.000
>40	- (0/6)	- (0/21)	25% (9/36)	26.08% (6/23)	14.29% (1/7)	25% (4/16)	- (0/1)	33.33% (1/3)	- (0/6)	-

The AMH undetectable subgroup and the age >40 groups were not calculated for the trend analysis, because the numerators were zero.

or data could only provide limited cues toward further randomized controlled studies [2,4,7,10,23,24]. Although inherent drawbacks exist in the present study due to the retrospective design, our large sample size and specialized infertility distribution made this analysis less

biased. In addition, a narrowing of groups where AMH was less than 5 ng/mL made the results more transparent than those from previous diagnostic trials.

With respect to zygotes and embryos, our study showed that High AMH levels were correlated with high absolute numbers of zygotes, and developed embryos, but augmented rates were not observed; to the contrary, the rate of developed embryo in all age groups except age >40 group which negatively correlated with baseline serum AMH. This might be due to subjective judgments by the embryologist, especially with patients who delivered few oocytes compared with patients where an abundant number of oocytes were retrieved, and normal fertilization (2PN)-derived embryos appear especially important. Although embryonic selection criteria were objective, the embryologist may show bias in judgment, and a cumulative effect due to large sample size may alter the trend in developed embryo rate; this result differs from previous reports [4,24,25].

In conclusion, we demonstrate in the present study that women with high AMH levels have a higher probability of pregnancy across all age groups. However, due to the retrospective experimental design, future RCT studies are needed to corroborate these results.

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