

IN VITRO STUDY AND CORRELATION OF AGE-SPECIFIC AMH LEVELS AS A PREDICTOR OF EUPLOIDY IN INFERTILE WOMEN

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Abstract

Background: The frequency of abnormal chromosome numbers is estimated to be in the range of 10-30% for fertilized human eggs, the majority of which are monosomic or trisomic. These are closely linked to miscarriage, and account for almost a third of all miscarriages. This is more related to biological age than chronological age. The finite oocyte pool theory proposes that the depletion of the pool of oocytes in the right stage of growth contributes to the problem. Anti-Müllerian Hormone (AMH) is a clinical marker commonly used to determine ovarian reserve, and is associated with egg euploidy and pregnancy success rates.

Methods: A laboratory study was performed to assess euploidy rates of females of different ages through Preimplantation Genetic Diagnosis (PGD). AMH was quantified in serum samples via ELISA. Different statistical approaches such as Principal Component Analysis (PCA) and Partial Least Squares (PLS) were used to assess the relationship between age, AMH levels, and euploidy.

Objective: To assess the association between age, AMH, and euploidy rate in vitro.

Results: This study showed that increasing AMH levels have a positive effect on the rate of euploidy, especially in women between 25 and 35 years of age. Older women showed a reduction in euploidy with lower AMH levels. Age was negatively correlated with euploidy, while AMH was positively correlated. A higher ovarian reserve was associated with more euploid blastocysts. Indeed, statistical testing revealed a negative effect of age on ovarian outcomes, and a positive effect of AMH.

Conclusion: Age and AMH are important indicators of embryo euploidy. Age has a negative effect on ovarian reserve and euploidy, whereas AMH levels improve fertility. This shows the importance of AMH as a valuable tool in fertility testing and treatment.

INTRODUCTION:

Aneuploidy, or the presence of an abnormal number of chromosomes, is a major cause of failed implantation, miscarriage and birth defects in humans. It's estimated that 10-30% of human fertilized embryos are chromosomally abnormal, with most cases of monosomies and trisomies

leading to early pregnancy loss and birth defects(1). Humans have higher frequencies of meiotic errors, especially during oogenesis, than other species, making chromosomal instability a significant problem in human reproduction(2). Chromosomal instability, the cause of aneuploidy, is thought to be derived from errors in meiotic

segregation, such as non-disjunction in meiosis I and II. These lead to embryos with abnormal number of chromosomes, causing an imbalance. Research has indicated that around one-third of spontaneous abortions are due to chromosomal abnormalities, therefore emphasising the importance of studying the cause of aneuploidy(3). Additionally, aneuploidy is a significant genetic cause of mental retardation and birth defects, further emphasising its importance in both fertility and development(4).

A key factor affecting aneuploidy is age of the mother. Ever since the beginning of the 20th century, studies have shown that advancing maternal age is strongly associated with chromosomal abnormalities, specifically trisomy 21(5). This association is thought to be due to the long duration of meiotic arrest of the oocyte (which can last for decades), and subsequent increased risk of errors in chromosome segregation(6). The number and quality of oocytes decrease with age, which increases the risk of meiotic errors and decreases fertility(7).

The "oocyte pool hypothesis" explains age-related aneuploidy. The hypothesis suggests that women are born with a certain number of oocytes, which are lost through atresia and ovulation as they age(8). As women age, the remaining oocytes are more likely to be of lower quality, and thus more susceptible to aneuploidy. Furthermore, age-associated alterations in the processes involved in spindle formation, sister chromatid cohesion and recombination also lead to an increase in aneuploidy (9).

In recent years, efforts have been made to identify markers that may better predict the ovarian reserve and fertility potential. Anti-Müllerian hormone (AMH), a glycoprotein secreted by granulosa cells of pre-antral and small antral follicles, has gained recognition as a good predictor of ovarian reserve(10).AMH is not affected by the menstrual cycle or gonadotropins making it a reliable and stable marker(11).

AMH is important in the process of folliculogenesis, as it controls the activation of primordial follicles. The amount of AMH in the bloodstream is directly correlated with the number of follicles left, thus reflecting the quantitative aspect of ovarian reserve(12). Crucially, AMH

concentrations decrease with age, and are undetectable at menopause, reflecting a loss of the ovarian follicular pool (13). In practice, AMH is commonly employed to predict ovarian response to stimulation, evaluate fertility potential and inform decisions regarding assisted reproduction(14).

In addition to its quantitative implications, there is growing evidence that AMH can also provide insight into the qualitative aspect of ovarian reserve. A number of studies have suggested a link between AMH and embryo quality, implantation and pregnancy outcomes(15). This has prompted speculation that higher AMH levels may be associated with an increased probability of euploid embryo formation, but research in this field is still conflicting and needs to be further explored (16).

The development of assisted reproductive technologies, such as Preimplantation Genetic Diagnosis (PGD) and Preimplantation Genetic Screening (PGS), has allowed the testing of embryo chromosomal status before implantation. This enables the selection of euploid embryos, leading to higher implantation rates and lower miscarriage rates(17). Current methods, including single nucleotide polymorphism (SNP) arrays and array comparative genomic hybridization (aCGH), allow for an analysis of all chromosomes, increasing diagnostic sensitivity(18).

While these technologies have improved, predicting embryo euploidy is still complex. Although the woman's age is a good predictor, it fails to explain the variability in reproductive potential among women of the same age. A younger woman may have poor quality embryos, while an older woman may still have good quality, euploid embryos. This suggests that other predictive factors, like AMH, should be used for better clinical management(19).

In addition, several studies have indicated that ovarian aging rather than chronological age is a key factor affecting fertility. Women with low ovarian reserve may have reproductive characteristics of older women despite their young age(20).This further underscores the value of considering age in conjunction with hormonal biomarkers in fertility evaluation.

While maternal age is a well-known predictor of aneuploidy, it is not sufficient to account for the

variability in embryo quality and reproductive outcomes. An indicator of ovarian reserve, anti-Müllerian hormone (AMH), may offer further understanding of the number and quality of oocytes. Yet, the association between AMH and embryo euploidy has not been well understood and is understudied, especially in infertile women receiving assisted reproductive technologies.

Accordingly, this study aims to explore the relationship between maternal age and serum AMH on embryo euploidy using an in vitro model. This understanding could aid in better predicting fertility outcomes, improving embryo selection, and ultimately improving success rates with assisted reproduction.

Materials and Methods

This study was an in vitro observational cohort study that examined the association between age, levels of Anti-Müllerian Hormone (AMH) and euploidy of embryos in women undergoing infertility treatment. The

study involved infertile women of different ages. The following variables were assessed for each woman: age, number of embryos, number of euploid embryos and AMH levels. Chromosomal analysis of embryos was carried out by Preimplantation Genetic Diagnosis (PGD). Oocytes were obtained using conventional intracytoplasmic sperm injection (ICSI) techniques and incubated in laboratory conditions. Before the biopsy, all media and solutions were prepared, including calcium and magnesium free media for the embryos, specific fixation and spreading solutions. Biopsy was performed on the embryos using holding and biopsy needles under a micromanipulator. Embryos were transferred to a biopsy dish filled with suitable media and covered with oil for optimal conditions. The laser-assisted method was adopted to make a hole in the zona pellucida and

one or more blastomeres were removed from each embryo. After biopsy, the blastomeres were removed from the biopsy dish, placed onto labeled slides, and spread and fixed. The nuclei were identified, fixed in methanol-acetic acid fixative, and dehydrated in a series of alcohol washes (70%, 90% and 100%). Chromosomes were evaluated using fluorescent methods, such as CEP XY probes to identify the chromosomes and then hybridised using a thermobrite system. The samples were washed, counter-stained with DAPI and examined under a fluorescent microscope for euploidy or aneuploidy of embryos. Serum AMH concentrations were quantified using an electrochemiluminescence immunoassay (ECLIA) method on the Cobas Elecsys AMH analyzer. The assay used sandwich immunoassay with monoclonal antibodies against AMH. Serum samples were obtained and handled following laboratory guidelines, with appropriate storage conditions. In the assay, AMH in the sample bound to biotinylated and ruthenium-labeled antibodies, which then bound to streptavidin-coated microparticles. The chemiluminescent response was detected and AMH levels determined using a standard curve. The assays were conducted according to the manufacturer's instructions with appropriate quality control procedures. We performed statistical analysis to assess the association between age, AMH and euploidy. Scatter plots and bar graphs were employed to detect patterns and associations. Principal Component Analysis (PCA) was used to simplify the data and evaluate the effects of variables. Moreover, Partial Least Squares (PLS) regression analysis was applied to build a model for prediction and to assess the magnitude and direction of the relationships between the variables. These statistical methods allowed us to assess the effects of both individual and combined effects of maternal age and AMH on embryo euploidy

Results:

Table 1: Females of different age groups were analysed for the levels of serum AMH and Euploid embryos in the cohort study.

Age	E.B	No.of Euploid Embryos	AMH
36	4	1	1
34	6	2	3.37
31	8	5	3.77
37	16	11	12.75
32	5	2	7.55
30	4	1	3.3
44	1	1	1
35	7	4	2.99
19	6	1	5.58
34	2	1	0.68
29	6	3	2.91
36	15	8	5.65
29	5	1	2.76
35	10	2	1.28
30	8	2	3.06
34	7	2	3
37	5	0	1.52
30	8	4	5.4
35	6	3	2.99
41	2	0	0.845
36	6	4	1.2
28	4	1	1.71
36	1	0	0.35
26	6	3	2.18
33	9	4	2.79
44	3	0	0.9
30	9	5	3.02
35	7	2	4.54
35	3	0	1.6
37	1	0	0.38
37	4	0	3.59
43	1	0	0.55
32	4	3	4.94
38	6	0	2.14
40	7	1	3.66
26	5	2	3.16
32	5	2	2.26
27	9	4	6.95
38	1	0	0.897
26	2	0	2.48
30	4	2	2.1
27	0	0	9
30	1	3	10.3

28	13	6	4.36
30	19	8	7.29
28	44	1	2.41
38	1	1	0.5
35	9	5	2.65
45	4	1	0.89
42	2	0	0.2
31	7	4	4.32
34	3	1	0.47

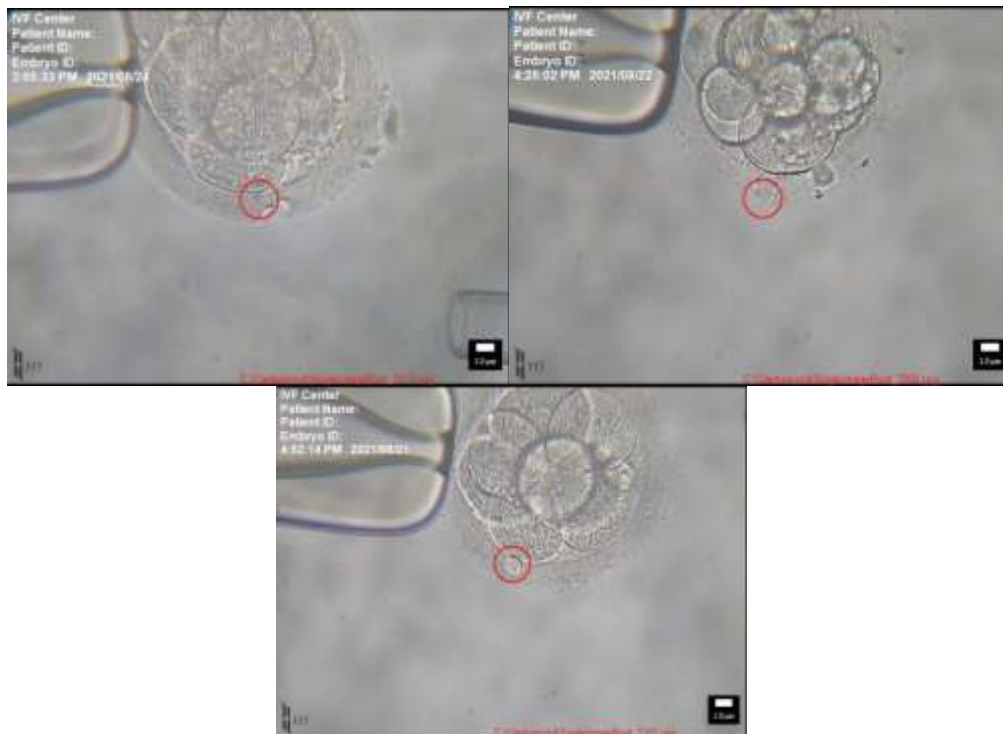


Figure1. Microscopic Images

Scatter Plots

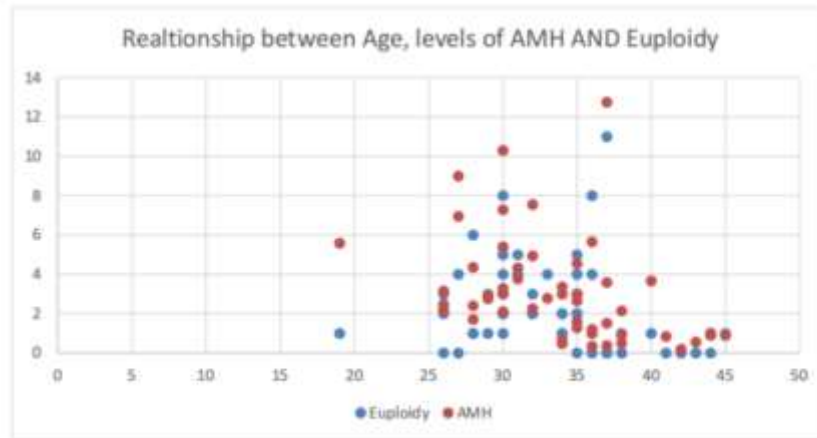


Figure2: It is presented that increase in the levels of AMH (red) which is indicative of ovarian reserves is directly related to increased rate of Euploidy (blue) between the age span of 25-35 and after this certain age the euploidy rates tends to decline followed by decrease in AMH levels.

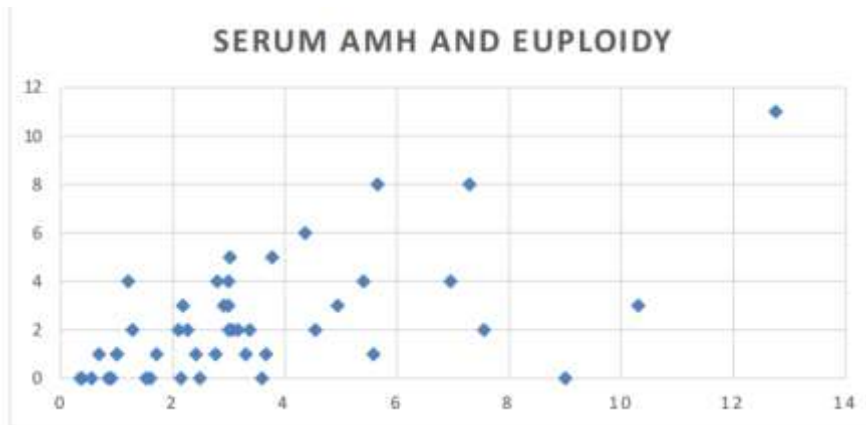


Figure3: The independent relationship between serum AMH levels and Euploidy rates generally, indicates that with increase of AMH the rate of euploidy increases respectively.

Bar Graph

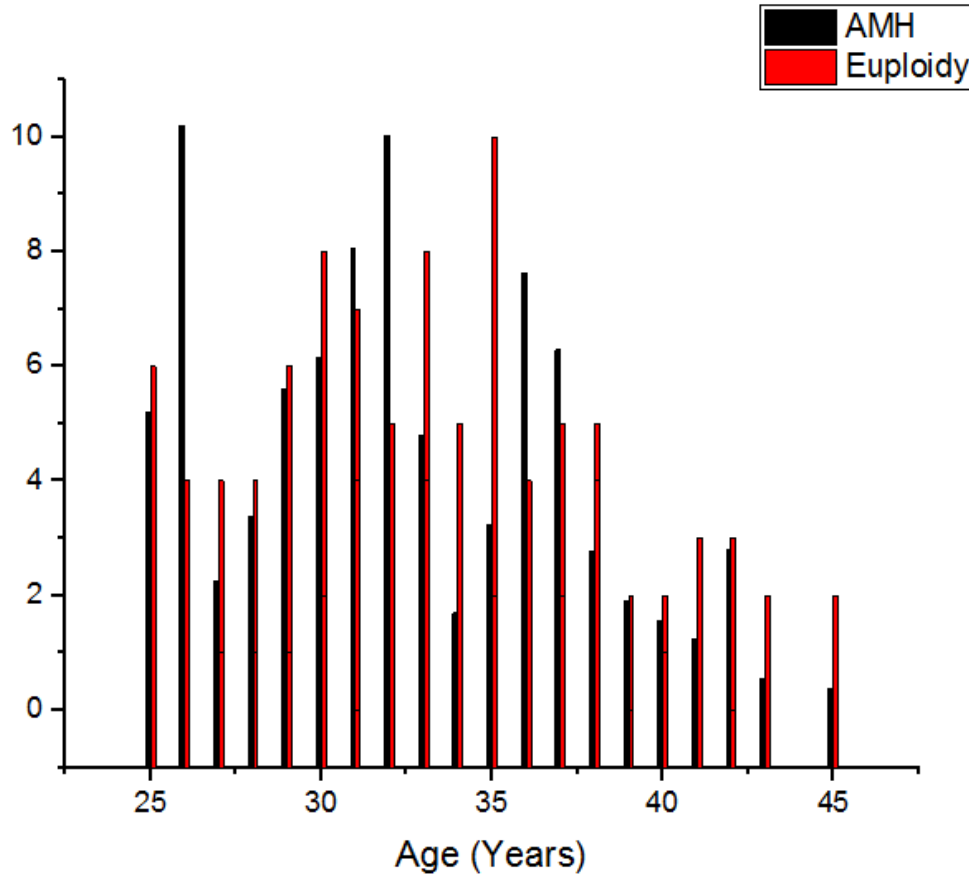


Figure 4: The bar graph represents the relationship between three variable given here i.e. Age, AMH concentrations and Euploidy, it can be seen that during a particular period of younger age euploidy tends to increase with the increase in AMH.

Principal Component Analysis.

Complexities in the high dimensional data is simplified by the Technique of Principal

Component Analysis. Which also retains trends and patterns. The data is first transformed into fewer dimensions which is used as features summaries.

Table2: Extracted Eigenvectors

	Coefficients of PC1	Coefficients of PC2
Age	-0.64969	0.29762
AMH	0.65828	-0.23999
Euploidy	0.38023	0.92403

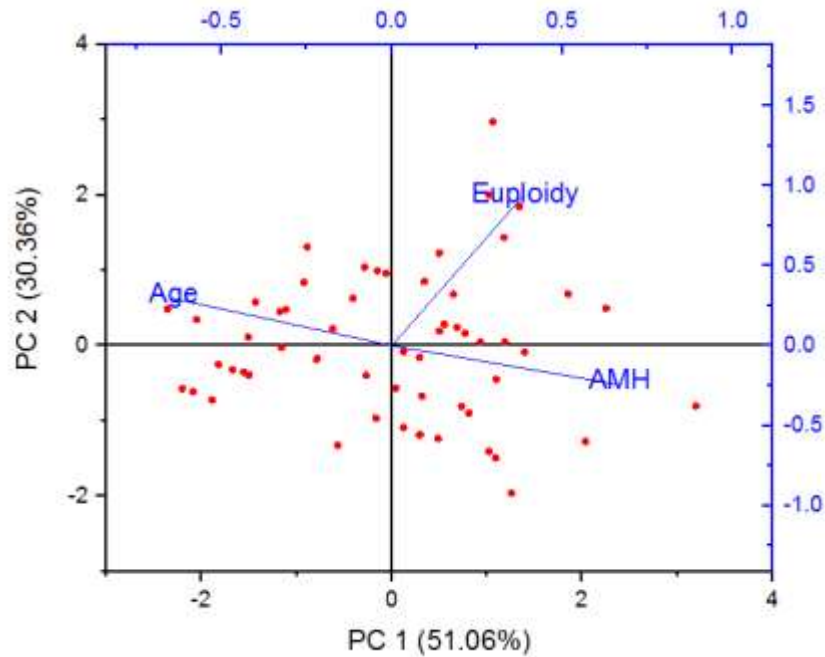


Figure 5: Principal component analysis of the given components have been plotted to find out the role of each variable, the results indicated that the age factors plays a negative role in the euploidy conditions while the levels of AMH are found to exert a positive effect in the development of eiploidy rates.

Partial Least squares analysis

The method of Partial Least Square Regression is used for the prediction to a smaller set of predictors by reducing the number of variables. A regression is performed by utilizing these

predictors. There is programing which differentiate PLS 1 and PLS 2, whereas PLS 1 corresponding to only dependent variable.

Table 3: Percent of Variance

Number of Factors	Variance Explained for X Effects(%)	Cumulative X Variance(%)	Variance Explained for Y Responses(%)	Cumulative Y Variance(%)
1	72.01431	72.01431	3.33965	3.33965
2	27.98569	100	0.01628	3.35593

Method: Wold's Iteration
Standardize: Yes

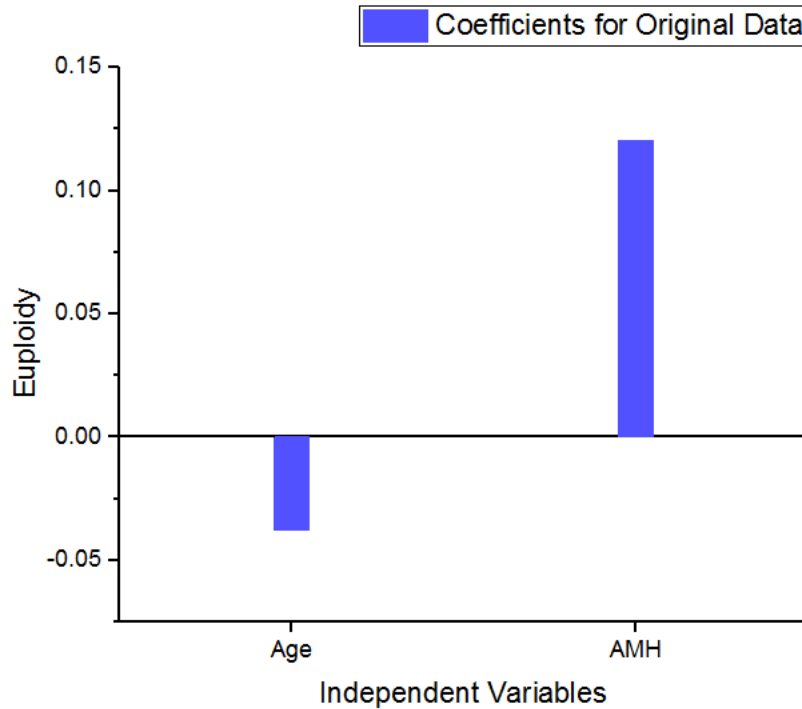


Figure 6 : Partial Least Square diagram have been created in order to obtain a predictive model based upon the data from second cohort of embryos which have provided us very clear insights that the factor of increasing age have a negative effect on the euploidy states whiles the number of ovarian reserves as indicated by the levels of AMH has been found to have positive effect on the number of euploid embryos i.e. cause an increase in the rate of euploidy.

DISCUSSION

The current study examined the association between maternal age, maternal serum Anti-Müllerian Hormone (AMH) and embryo euploidy and shows that these factors are significantly correlated. It found that maternal age is inversely associated with euploidy, while AMH levels are positively related to euploidy. This is consistent with previous studies indicating that age-related decline in oocyte quality leads to increased chromosomal abnormalities due to meiotic errors, particularly non-disjunction events during oogenesis(1, 6). The prolonged meiotic arrest of oocytes and age-related deterioration in spindle integrity and chromosomal cohesion are considered major contributors to this phenomenon(9).

In addition to age, ovarian reserve, as reflected by serum AMH levels, showed a significant influence on embryo quality. Women with higher AMH levels demonstrated better euploidy rates,

particularly within the age group of 25–35 years. This supports the concept that AMH is not only a marker of ovarian quantity but may also reflect oocyte competence. Similar findings have been reported in earlier studies, where AMH levels were positively associated with improved implantation and live birth rates in assisted reproductive techniques(15). Furthermore, AMH production by granulosa cells of growing follicles directly reflects the size of the follicular pool, which is known to decline with advancing age(12, 13).

This study's findings also confirm the "limited oocyte pool hypothesis" that as the number of high-quality oocytes diminishes with age, aneuploidy increases [8]. As ovarian reserve decreases, so does the chance of ovulation of poor quality oocytes, leading to an increased risk of aneuploidy. This may explain why women with low ovarian reserve, despite being younger in age, may have lower fertility potential than other women of similar age. Previous studies have also

stressed the importance of ovarian age, as well as chronological age, in determining fertility(20).

Similarly, in this study, using advanced technology such as Preimplantation Genetic Diagnosis (PGD) allowed for the identification of euploid embryos, which might suggest that genetic and hormonal profiling in practice may be used together to enhance fertility. Maternal age is the strongest predictor of aneuploidy, but does not account for individual differences. So, AMH analysis might be more informative. However, although the results from the current study are important, the variability in AMH levels and the variability in predictions made, means that we need to conduct more large-scale studies to verify these findings and improve the clinical value of this prediction.

CONCLUSION

Age and Anti-Müllerian Hormone (AMH) are significant predictors of embryo euploidy, according to the current study. Age was inversely correlated with euploidy, as age went up, AMH and euploidy rate came down. In contrast, increased AMH levels, particularly between the ages of 25-35 years, was associated with greater ovarian reserve and a greater likelihood of a euploid embryo. This finding indicates ovarian reserve is a significant predictor of embryo quality, aside from age.

Additionally, the research shows that AMH can be a useful marker not only to predict ovarian reserve but also embryo quality. Indeed, the statistical analysis (PCA, PLS models) demonstrated that age has a negative effect on euploidy, whereas AMH has a positive effect. Thus, age and AMH together offer a better picture of the reproductive potential. This study suggests the need for a combined approach of hormone assessment and genetic screening technologies to select healthy embryos and for better outcomes of fertility treatments.

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