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Female age, serum anti-Mullerian Hormone (AMH) and the number of oocytes affect the rate and number of euploid blastocysts in IVF/ICSI cycles

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Running Title: AMH and blastocyst euploidy in IVF/ICSI cycles

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Abstract

Objective: To study the relative role of female age and ovarian reserve, measured through serum anti-Mullerian Hormone (AMH) in determining the rate and number of euploid blastocysts in IVF/ICSI cycles

Des ign: Retrospective analysis of cycles performed in 2014-15

Setting: A tertiary referral IVF center

Patients: 578 infertile couples undergoing IVF/ICSI and PGS analysis

Interventions: All embryos were cultured and biopsied at the blastocyst stage. The method involved whole genome amplification followed by array-CGH. Serum AMH was measured with the modified AMH generation II assay.

Main Outcome Measures: The rate and number of euploid blastocysts and their correlation with ovarian reserve and response to stimulation

Results: The mean (±SD) age of patients was 37.6±4.1 years and the mean number of blastocyst per patient was 3.1±2. The total number of blastocysts available to the analysis was 1814 and 36% of them were euploid after PGS. Age and serum AMH were significantly and independently related to the rate of euploid blastocysts available for patients. As an effect of the cohort size, the number of mature oocytes positively affected the total number of euploid blastocysts per patient.

Conclusions In this study a strong positive age-independent relationship between AMH and the rate of euploid blastocysts has been found**.** This confirms that the measurement of ovarian reserve by means of AMH has high relevance when counselling infertile patients.

Key words: AMH, mature oocytes, female age, IVF/ICSI, euploid blastocyst

Introduction

The age associated decline in female reproductive function due to the reduction of the ovarian reserve and the quality of oocytes has been well established. The age related reduction in ovarian reserve has been well established and very clearly reflected by the continuos decrease in the ovarian primordial follicle pool with ageing through atresia or due to ovulatory recruitment. As a consequence the pool of follicles becomes exhausted, and the reproductive function is considered ultimate (1,2).

Oocyte quality is a more complex characteristic of ovarian reserve to be defined and measured rather than quantity. Nowadays the association between female age and declining fertility is thought to be the result of abnormalities in the oocyte in the first place. The association between advancing maternal age and increased risk of chromosomal abnormality in the embryos is well known; the association is mainly due to chromosomal abnormalities occurring in the egg. The abnormalities include modifications in the mechanism for assembly of the meiotic spindle, leading to errors in chromosome alignment and the microtubule matrix (3), increased rates of chromosome degeneration into unassociated chromatids (4) and increased rates of chromosome non-disjunction (5). Aneuploidy affects more than half of human embryos and it is the main reason for implantation failure and miscarriages in IVF/ICSI cycles (6-8). In a previous study performed in our centre (9), we found that the aneuploidy rate increases by about 10% per year of female age, with 48.1%, 41.3%, 29.7% and 10.3% of euploid blastocysts in patients with mean female age ≤32, 33-36, 37-41 and ≥42 years old, respectively. This was in line with what reported by Franasiak and collaborators (10), who performed a study on 15169 consecutive trophectoderm biopsies from 2701 patients with female age ranging from 22 to 49 years (10). They found that the rate of aneuploidy rose steadily with age, showing the lowest risk for embryonic aneuploidy between ages 26 and 30, while older women had an increased risk for aneuploidy (10).

Both quantitative and qualitative aspects of the ovarian reserve are inversely related to age, hence the relationship existing between quantity and quality may only be indirect and due to their strong relationship with the third variable, namely female age. However there is the possibility that they may also be directly related; hence independently of age, a reduced ovarian reserve may lead to reduced oocyte quality. Infact some evidence in literature indicates a direct link between quantity and quality of ovarian reserve. Mothers of children with trisomy 21 had significantly higher serum levels of FSH (indicating low ovarian reserve) than age-matched controls (11,12). Similarly women with aneuploid spontaneous miscarriage had a reduced ovarian reserve compared to women with euploid miscarriage (13). Thus, we can make the assumption that a reduced ovarian reserve may be *per se* associated to an accelerated onset of oocyte aneuploidy leading to increase in the aneuploidy rate in embryos.

Ovarian reserve can now be accurately measured in vivo by measuring circulating levels of Anti-Mullerian Hormone (AMH). This hormone is produced and secreted by granulosa cells of primary and mainly secondary follicles up to the stage of 6-8 millimiters (14). Serum AMH concentrations are strongly related to the pool of primordial follicles and decline with advancing female age becoming undetectable some years before menopause. Due to the strong relationship with ovarian reserve, serum AMH measurement may allow to predict the age at menopause and the number of retrieved oocytes (15, 16).

In the present study we aimed to investigate the relative role of female age and ovarian reserve, as measured by serum AMH, in determining the rate of euploid embryos and the probability of having at least one euploid blastocyst in patients undergoing IVF/ICSI cycles.

Materials and Methods

Study subjects

This is a retrospective analysis of ART cycles performed at the European Hospital, Rome, Italy during 2014 and 2015. Only patients with AMH measured before the treatment and with complete patient records on clinical, IVF/ICSI cycle characteristics, Preimplantation Genetic Screening (PGS) analysis could be included in the retrospective analysis. PGS was proposed to the couples for the following reasons: advanced maternal age, recurrent miscarriage, repeated implantation failure, severe male infertility and finally to all good prognosis patients who desire information regarding the health status of their embryos. Data were obtained without applying any particular exclusion criteria regarding baseline characteristics of patients such as age, main cause of infertility, ovarian reserve or body mass index (BMI). In total data from 578 patients were obtained for the analysis. Data are reported in table 1.

IVF/ICSI treatment protocol

Both the standard GnRH agonist and antagonist protocol were used for controlled ovarian stimulation. Briefly, the long GnRH agonist protocol was based on the administration of daily leuprorelin or triptorelin (Enantone die, Takeda, Italy; Fertipeptil, Ferring, Italy) on the mid-luteal phase before the stimulation cycle. The administration of recombinant FSH (rFSH; GonalF Merck Serono, Italy and Puregon, MSD, Italy) or human menopausal gonadotropin (hMG, Meropur, Ferring, Italy) or urinary FSH (Fostimon, IBSA, Switzerland) was started when pituitary desensitization was achieved (~14 days after the initiation of GnRH agonists), as evidenced by the absence of ovarian follicles >10mm and endometrial thickness <4 mm on transvaginal ultrasound examination. In the GnRH antagonist protocol (Cetrotide, Merck Serono, Italy, Orgalutran, MSD, Italy) treatment with FSH or hMG was started on day 2-3 of the stimulation cycle and the GnRH antagonist was added when one or more follicles had reached a diameter ≥ 14mm. The starting dose

of FSH/hMG was based on age, body weight and ovarian reserve thus ranging between 100 to 225 IU per day as per internal protocol of the clinic.

An ovarian ultrasound was performed on stimulation day 5 to 6 and gonadotrophin doses were adjusted according to the ovarian response. When at least one follicle reached ≥ 18 mm, 10000 IU of hCG or 250 mcg of rhCG were administrated and 34-36 hours later follicles were aspirated under patient sedation.

Oocyte insemination, Embryo culture and biopsy

All biological procedures were performed as described elsewhere (9). Briefly, Cumulus Corona Complex (COCCs) after retrieval were incubated in fertilization medium (Quinn's Advantage Protein Plus Fertilization Medium, SAGE) for 2-3 hours at 37°C under the gas phase of 5%O2 and 6%CO2 until denudation. For all oocytes, denudation was performed by exposure to 20 IU/ml of hyaluronidase fraction VIII (Hyaluronidase 80 U/mL in HEPES-HTF, Sage, USA) in hepes-buffered medium (Quinn's Advantage® Medium with Hepes, Sage, USA). Subsequently, oocytes were aspirated in and out of a plastic pipette (Flexipet, 170 and 140 µm i.d., COOK, Australia) to allow the removal of cumulus and corona cells. Only oocytes with first polar body extruded (metaphase II) were treated by ICSI immediately after the denudation procedure. Finally, injected oocytes were moved to single drops of cleavage medium (Quinn's Advantage Protein Plus Cleavage Medium, SAGE) under oil at 37°C, 5%O2 and 6%CO2. On day-3, for all the developing embryos a media change-over (Quinn's Advantage Blastocyst Medium, SAGE) for sequential culture was performed. On day 3, a non-contact 1.48 µ diode laser (17) was used to create a circular 6-9µ diameter opening in the zona pellucida in cleavage stage embryos, in order to allow the trophectoderm cells to herniate. Depending on the embryo's development, the blastocyst stage can be reached on day 5, 6 or 7. On the day of biopsy, 5-10 trophectoderm cells were gently aspirated into the biopsy pipette (COOK, Ireland Ltd, Limerick, Ireland) followed by a laser assisted removal

from the rest of the blastocyst. The obtained trophectoderm cells were washed in sterile phosphate-buffered saline solution (PBS) and then placed in microcentrifuge tubes containing 2 µL of PBS, spinned down for few seconds and sent to GENOMA laboratory for analysis (18).

Preimplantation genetic analysis

For whole genome amplification (WGA), trophectoderm cells and negative controls were first lysed and genomic DNA was randomly fragmented and amplified using the SurePlex DNA Amplification System (BlueGnome, Cambridge, UK), according to the manufacturer's instructions. WGA products were processed as reported elsewhere (18,19) according to the BlueGnome 24sure V3 protocol (available at www.cytochip.com). Briefly, WGA products were fluorescently labelled and competitively hybridized to 24sure V3 arrays (BlueGnome, Cambridge, UK) with a matched control in an array-CGH experiment format. A laser scanner InnoScanw 710 AL (INNOPSYS, Carbonne, France) was used to excite the hybridized fluorophores and read and store the resulting images of the hybridization. Scanned images were then analyzed and quantified by algorithm fixed settings in BlueFuse Multi Software (BlueGnome, Cambridge, UK), a software package that performed the steps of grid placement, quantification, normalization and post-processing automatically. The software combines in an automatic way the data from the single channel sample experiments with both male and female references from the hybridized reference subarrays, to produce a single fused result compared with a sex matched and a mismatched reference. Once a specific amplification was observed (i.e. low autosomal noise), autosomal profiles were analyzed for gain or loss whole chromosomal ratios using a 3 x SD assessment, greater than ±0.3 log2 ratio call, or both. To pass hybridization quality control, female samples hybridized with a male reference DNA (sex mismatch) had to show a consistent gain on chromosome X and a consistent loss of chromosome Y (Gutierrez-Mateo et al., 2011).

Assays

The modified Beckman Coulter AMH Gen II assay was used for all AMH analyses in this study. The AMH Gen II assay is a two-step, sandwich-type enzymatic, microplated assay. Problems with the robustness of the Gen II assay were solved with a modified version of the AMH Gen II assay kit (reference A79765), including an additional assay step before calibrators were added (premixing). This additional step eliminates the complement and thereby overcomes the not optimal assay reproducibility of the original AMH Gen II assay. The standards cover a range of 0–22 ng/mL. The sensitivity is reported to be 0.1–0.21 ng/mL. Reported intra- and interassay CVs were <2% and <12%, respectively.

Outcomes and statistical analysis

Data are presented as mean ± SD when they had a Gaussian distribution or median (25th–75th range) when they were not normally distributed. Parametric and non-parametric tests were used to compare baseline characteristics and outcomes as appropriate. To identify characteristics that may be associated with the rate and number of euploid blastocysts, multivariate logistic regression analysis was performed. Univariate regression analyses were performed to identify factors that predict the rate and number of euploid blastocysts. Variables found to have tendency of association with the primary outcome ($P < 0.25$) in the univariate analysis were included in the multivariate analysis. All independent variables were simultaneously entered into the logistic regression model. The Hosmer–Lemeshow goodness-of-fit test, assessed the goodness-of-fit of the normal regression models. All statistical tests used a two-tailed α of 0.05. Statistical analysis was done using Stata 12.

193 *Ethics approval*

194 Full ethics committee approval was not required because of the retrospective design of the study
195 and the anonymized handling of the data. The synopsis and objective of the study were
196 communicated to local Institution review board. All women were treated at the European Hospital,
197 Rome and routinely they provided informed consent for their clinical data as anonymous records to
198 be used for research purposes.

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Results

In total, data from 578 patients were available for analysis. Characteristics of the patients are reported in table 1. Briefly, the mean (±SD) age was 37.6±4.1 years (range 27-43) and the mean number of blastocysts per patient was 3.1±2 (range 1-11) . The total number of blastocysts available for the analysis was 1814 and 654 (36%) of them were euploid after PGS. The mean number of euploid blastocyst per patient was 1.1±1.2 (Table 1)

The mean (±SD) rate of euploid blastocysts per patient was 38% (±36.2%), and as expected was negatively associated to female age (Fig.1). The rate of euploid blastocysts was also positively and significantly related to serum AMH and to the number of retrieved mature oocytes in univariate analysis (Fig.1). Multiple regression analysis showed that female age and serum AMH, but not MII oocytes number were independent predictors of the rate of euploid embryos in IVF cycles (Table 2). For the two variables (age and AMH), ANOVA analysis showed a statistically significant effect in determining the rate of euploid blastocysts per patient (female age: F ratio=30, $p<0.001$; serum AMH: F ratio=65, $p<0.001$) (supplemental figure 1). No significant association was found between the rate of euploidy and other patient's or cycle characteristics such as type and duration of infertility, body mass index, the protocol, type and dose of drugs for ovarian stimulation, the indication to the PGS and the day of the blastocyst biopsy (data not shown).

While the total number of mature oocytes did not affect the rate of euploid blastocysts, it strongly and expectedly influenced the total number of euploid blastocysts available for each single patient (supplemental figure 2). This was due to a positive size-cohort effect of the number of oocytes. When using the regression analysis the total number of euploid blastocyst per patient was related to age, AMH and to the number of mature oocytes. The relationship between the different variables with the total number of euploid blastocysts was then examined using univariate and multivariate regression analysis (Table 2).

Finally, in order to measure the effect size of this association, the probability of having at least one euploid blastocyst available for transfer was chosen as outcome. The odds ratio for female age (1 year) was 0.84 (CI 95% 0.79-0.89), while for AMH (1ng/ml) and MII oocytes (1 oocyte) it was 1.27 (CI 95% 1.14-1.41) and 1.09 (95%CI 1.04-1.14), respectively ($y=5.3+0.24AMH-0.16Age+0.08MII$; R2: 0.24; model fit: chi-square 170.9, df 3, $p<0.0001$). This indicates that for two women of similar age, higher serum AMH and more oocytes lead to increased probability if having at least one euploid blastocyst for the embryo transfer. In figure 3 the effect of female age, serum AMH and number of retrieved oocytes on the rate of euploidy, the number of euploid blastocysts per patient and probability of having at least one euploid blastocyst is clearly reported.

Discussion

To our knowledge, the present study is the first one to clearly investigate the independent relationship between female age, ovarian reserve (as assessed by circulating AMH) and the rate of embryo euploidy in ART. We found that independently of age, the increase in ovarian reserve is associated to increased rate of euploid blastocysts .

The results of our study at least in part support the hypothesis that quality and quantity of the follicular pool are directly related. Qualitative and quantitative aspects of the ovarian reserve are inversely related to women's age. However the decrease in oocyte quantity and quality could progress regardless of female ageing (20). Independently of age, selection of oocytes may become impaired when few oocytes are available or physiological modifications secondary to follicle loss may affect oocyte competence (20,21, 22, 23). The fact that serum AMH is related to the euploidy of blastocysts in IVF cycles independently of female age, could explain our previous observation that AMH was a weak but statistically significant age-independent predictor of live birth in IVF (24,25). This once again highlights the possible role of serum AMH as a qualitative other than quantitative marker of ovarian reserve.

According to our findings, in women with the same ovarian reserve, young age may increase the rate of euploidy in blastocysts. From this point of view young female age at least in part may be a protective factor in women with low ovarian reserve that generally have a low prognosis in IVF. Indeed it is has generally been assumed that younger women with poor response still have a good prognosis for pregnancy while older women with poor response have an extremely poor outcome with IVF. In the present study we had a low incidence of poor response and could not address this point, hence a specific investigation should be designed to address this relevant question.

In the clinical practice, the availability of euploid blastocysts for patients is extremely important considering that the rate of cumulative success of IVF itself is strictly dependent on this figure. Very recent studies clearly confirmed that the number of cryopreserved embryos following the fresh embryo transfer is directly related to the cumulative live birth rate that results form the success of the fresh and successive frozen embryo transfers (26). In the present study we showed that the total number of euploid blastocysts is dependent on female age, serum AMH and number of retrieved mature oocytes. Of course this may be expected and is secondary to the fact that for any given rate of blastocyst euploidy, the higher the number of oocytes, the higher the number of euploid embryos that will be obtained. This confirm previous findings by Ata and coll (27), that found that the number of euploid embryos in IVF/ICSI cycles was significantly decreased by increasing female age and was significantly increased by every additional embryo available for analysis. In the present study we calculated that the probability of having at least one euploid blastocyst available at transfer in order to measure the effect size of the association of the predictors and found it was reduced by 16% per every year of female age, raised by 27% for every increase in AMH by 1 ng/ml and increased by 9% for each additional mature oocyte.

The strength of our study is that observations are made on a very large number of blastocysts (n=1814) and that AMH was centrally measured. Another strong point is related to the fact that the preimplantation aneuploidy testing was based on the array comparative genomic hybridization (array-CGH) technology that has been sufficiently validated using cells of a known genotype (27) and is now used worldwide. At the same time we recognize the retrospective design as the main limitation of the study.

In conclusion in the present study we could confirm the very well know negative effect of female age on euploidy of embryos in IVF/ICSI cycles and also reported the original finding of the independent and positive role of ovarian reserve. Serum AMH, independently of age, significantly affects a very relevant outcome in ART, namely the rate of euploid blastocysts per patient. The

probability of having at least one euploid blastocyst is finally dependent on female age, serum AMH and the number of mature oocytes.

- Authors' contributions

ALM, EG, MGM.: study design and data analysis; MGM, PG, FF: cohort collection and characterization; ALM, MGM, GS, PG, CA, VG, FF, EG: preparation and approval of the final version of the manuscript.

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Legends of Figures

Fig. 1 The relationship between the rate of euploid blastocysts, female age (r:-0.326 [95%CI -028, -0.43], p<0.0001), MII (r:0.11 [95%CI 0.03, 0.19] p: 0.006), and serum AMH (r:0.21[95%CI 0.13, 0.29], p<0.0001).

Fig2. Mean number of euploid blastocysts (n, diamonds), mean rate of euploidy (%, circles) and the probability of having at least one euploid blastocisyst (%, squares) in women according to female age, serum AMH and the number of mature oocytes . Percentiles are for age: 20^{th} 34 years, 40^{th} 37 years, 60^{th} 39 years and 80^{th} 41 years. Percentiles are for AMH: 20^{th} 1.2 ng/ml, 40^{th} 2.2ng/ml, 60^{th} 3.6ng/ml and 80^{th} 5.5ng/ml. Percentiles are for mature oocytes: 20^{th} 4 oocytes, 40^{th} 6 oocytes, 60^{th} 9 oocytes and 80^{th} 12 oocytes.

Supplemental Figure 1 A. Box and whisker plots of the rate of euploid blastocysts by quintiles of serum AMH levels. Values are median (lines), 25^{th} to 75^{th} percentiles (boxes) and 10^{th} to 90^{th} percentiles (whiskers). Percentiles are for AMH: 20^{th} 1.2 ng/ml, 40^{th} 2.2ng/ml, 60^{th} 3.6ng/ml and 80^{th} 5.5ng/ml. B. Box and whisker plots of the rate of euploid blastocysts by quintiles of female age. Values are median (lines), 25^{th} to 75^{th} percentiles (boxes) and 10^{th} to 90^{th} percentiles (whiskers). Percentiles are for age: 20^{th} 34 years, 40^{th} 37 years, 60^{th} 39 years and 80^{th} 41 years.

Supplemental Figure 2 The relationship between female age, serum AMH, mature oocytes and the total number of euploid blastocysts