

Associations among morphological parameters, clinical factors and euploid blastocyst formation

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ABSTRACT

Objective: To evaluate the association among embryonic morphological parameters, clinical factors and euploid blastocyst formation.

Methods: This prospective cohort study included 422 blastocysts from 135 patients who had undergone preimplantation genetic analysis after intracytoplasmic sperm injection (ICSI).

Results: Of 422 blastocysts, 200 (47.4%) were euploid and 222 (52.6%) aneuploid. Women aged older than 38 years were more likely to develop aneuploid embryos (OR: 3.4, CI: 2.2-5.4, $p < 0.001$). Poor ovarian reserve (OR: 3.3, $p < 0.001$), increased male age (39.0 versus 40.7, $p = 0.019$), and decrease in sperm percentage with normal morphology (2.5% vs. 1.9%, $p = 0.047$) were associated with aneuploidy. Type C trophoctoderm (TE) and type C inner cell mass were associated with a high risk of embryo aneuploidy, with OR of 4.1 (CI: 2.2-7.7, $p < 0.001$) and 1.7 (CI: 1.01-3.0, $p = 0.048$), respectively. Logistic regression analysis revealed maternal age and type C TE as the main risk factors for aneuploidy. Among combinations of factors, the best marker for the risk of aneuploidy was maternal age older than 38 years, combined with a type-C embryo with trophoctoderm, which showed a positive predictive value of 88.6% and a specificity of 97.5%.

Conclusions: Trophoctoderm and type-C inner cell mass are the main embryo risk factors for aneuploidy, explaining approximately 71% and 60% of the risk, respectively. Among clinical factors, advanced maternal and paternal age (older than 38 and 36 years, respectively), antral follicles (< 5), and a low percentage of sperm with normal morphology increased the risk of embryonic aneuploidy.

Keywords: aneuploidy, PGT-A, trophoctoderm, blastocyst, inner cell mass

INTRODUCTION

Aneuploidy is the most common type of chromosomal abnormality and the leading cause of implantation failure, miscarriage and congenital abnormalities in humans (Lee *et al.*, 2015). Approximately 30-35% of miscarriages in women over 35 are due to chromosomal abnormalities (Hodes-Wertz *et al.*, 2012). Preimplantation genetic testing for aneuploidies (PGT-A) can be used to select an euploid embryo for transfer, to prevent chromosomal abnormality-induced abortions due to embryonic aneuploidies after in vitro fertilization (IVF) (Brezina *et al.*, 2012).

PGT-A improves embryo transfer implantation rates and reduces spontaneous abortion rates, particularly in patients at an increased risk of producing aneuploid embryos (Lee *et al.*, 2015). PGT-A increases embryo implantation rates to 70-80%; as a result, many couples, especially those at an increased risk of forming aneuploid embryos, due to advanced maternal age for instance, or who fear generating aneuploid embryos, are seeking this technology (Lee *et al.*, 2015). However, despite these advantages, approximately 45%-50% of biopsied embryos are aneuploid or mosaic—i.e. many embryos that are subjected to PGT-A are not viable, causing an increase in costs for couples due to the need to analyze several embryos (Platteau *et al.*, 2006; Friedenthal *et al.*, 2018; Lawrenz *et al.* 2019; Friedenthal *et al.*, 2020).

Although preimplantation genetic analysis can help select embryos for transfer, it does not change the final pregnancy rate and may even decrease this rate due to the aggressive manipulation of embryos during biopsy (Zhang *et al.*, 2016; Guzman *et al.*, 2019; Munné *et al.*, 2019). As such, couples undergoing IVF treatment, in addition to having difficulty achieving pregnancy, now also need to decide whether to undergo PGT-A to avoid the risk of pregnancy with an aneuploid embryo. To do so, these couples need to bear the high costs of this technology, which on the one hand, prevents the transfer of aneuploid embryos, but on the other hand, yields a similar or even lower pregnancy rate than in couples who do not choose an embryonic biopsy (Zhang *et al.*, 2016; Guzman *et al.*, 2019; Munné *et al.*, 2019). It is noteworthy that the costs of PGT-A depend on the number of biopsied embryos, and approximately 50% of them are aneuploid (Gazzo *et al.*, 2020).

Researchers have attempted to correlate embryonic morphology with euploidy, to try to predict which embryos are most suitable for biopsy. Some found a higher risk of aneuploidy in embryos with a higher number of blastomeres at the cleavage stage (Kroener *et al.*, 2015), and others found that blastocysts with higher inner cell mass (ICM) and trophoctoderm (TE) scores are more likely to be euploid (Barash *et al.*, 2017; Wang *et al.*, 2018). Having embryonic culture in time-lapse incubators, in addition to the morphological aspect, and embryonic morphokinetics has also begun to be evaluated as a possible predictor of the formation of euploid blastocysts (Zaninovic *et al.*, 2017). However, due to its high cost, few centers have access to this time-lapse technology; thus, the morphological criteria for embryonic classification and selection are still the most commonly used.

In addition to embryonic morphology, clinical factors have been associated with the risk of aneuploidy,

especially advanced maternal and paternal ages, and low ovarian reserves (García-Ferreira *et al.*, 2015; Shahine *et al.*, 2016). Other clinical factors associated with infertility, such as endometriosis (Juneau *et al.*, 2017), recurrent miscarriage (Hodes-Wertz *et al.*, 2012), polycystic ovary syndrome (PCOS) (Wang *et al.*, 2016) and male factors (Mazzilli *et al.*, 2017), have also been investigated to assess the risk of aneuploid embryo formation. Many of these clinical factors have shown conflicting results and need to be further evaluated regarding their association with embryonic aneuploidy.

Therefore, due to the increased use of PGT-A, the need to find markers associated with embryonic euploidy to better select embryos, and especially the need to better help couples regarding embryonic euploidy prognoses in different situations, the aim of this study was to evaluate the association among morphological parameters, clinical factors and euploid blastocyst formation determined by PGT-A.

MATERIALS AND METHODS

Patients

We ran a prospective cohort study with 430 blastocyst embryos that underwent PGT-A, by next-generation sequencing (NGS) from 135 patients who underwent intracytoplasmic sperm injection (ICSI) during the study period, from June 2018 to June 2019, at the Pronatus Reproductive Medicine Center, Belém, Pará State - Brazil. The National Ethics in Research Committee approved the study and by the Ethics Committee of the Instituto de Ciências da Saúde da Universidade Federal do Pará under CAAE number 12779919.0000.0018.

All couples were evaluated for the probable cause of infertility according to the following criteria: tubal factor (defined by change on hysterosalpingography or videolaparoscopy); male factor (defined as sperm count below 15 million/ml - according to the criteria of the World Health Organization) (Cooper *et al.*, 2010); a low ovarian reserve (defined as <5 antral follicles evaluated by transvaginal ultrasound performed on the 2nd or 3rd day of the menstrual cycle); repeat abortion (two or more consecutive abortions); polycystic ovary syndrome (PCOS); endometriosis (presence of endometriosis foci on videolaparoscopy or endometrioma on imaging studies); and undetermined cause.

Ovarian stimulation

The ovarian stimulation protocol began on the second day of the menstrual cycle, after transvaginal ultrasound was performed to determine the antral follicle count; recombinant follicle-stimulating hormone (FSH, Elonva 150[®], Organon) was injected subcutaneously. After day 6 of the application, transvaginal ultrasound was performed every two days to monitor follicle development. When one of the largest follicles reached 14 mm, the gonadotrophin-releasing hormone (GnRH) antagonist (Orgalutran[®], Organon) was administered daily and subcutaneously until the use of recombinant human chorionic gonadotropin (rhCG). After day 9 of the induction, we used recombinant FSH (Puregon[®], Organon) at a dose of 150 IU per day, until rhCG was used. When three or more follicles reached 17 mm in diameter, we stimulated oocyte maturation with rhCG (Ovidrel 250 mcg; Serono). Transvaginal ultrasound-guided oocyte recovery was performed 35h after hCG application. After denudation, we classified the collected oocytes into metaphase II (MII), metaphase I (MI), germinal vesicle (GV), ruptured or atretic.

Assessment of fertilization, embryo quality and embryo biopsy

We subjected all MII oocytes to ICSI, and we performed embryonic culture in a Thermo Scientific CO₂ Incubator. We used the CSCM-C IRVINE[®] culture medium for the Single Step system-type with the pH adjusted to 7.3 to obtain a CO₂ pressure of 7.4%, and the embryonic development was carried out according to the Istanbul consensus (Alpha Scientists in Reproductive and Embryology, 2011).

The same embryologist performed all embryonic evaluations and biopsies. Additionally, in all evaluations, we photographed the embryos and stored the images in a database so that, if necessary, we could run a retrospective evaluation of the embryonic classification.

On day 1 (D1, evaluation 17 hours post insemination (HPI)), the ideal fertilized oocyte was spherical and there were two polar corpuscles and two centrally located, juxtaposed pronuclei of uniform size, with distinct membranes. The nuclear precursor corpuscles classification was divided into three categories (1: symmetrical, 2: asymmetrical, 3: abnormal).

On D2 and D3 (D2: assessment 44 HPI, D3: assessment 68 HPI), embryonic cleavage along with the blastomere count, degree of fragmentation, cell size and presence of multinucleation were observed and classified as grade 1 (<10% fragmentation, stage-specific cell size, no multinucleation), grade 2 (10-25% fragmentation, stage-specific cell size for most cells, no evidence of multinucleation) and grade 3 (severe fragmentation > 25%, cell size not stage-specific, evidence of multinucleation).

On the morning of day 5 (D5), approximately 116+2 HPI, blastocyst classification was performed by assessing the developmental stage (1: initial, 2: blastocyst, 3: expanded, 4: hatched/hatching), ICM (A: prominent, B: easily discernible, C: difficult to distinguish), and TE (A: many cells forming a cohesive epithelium, B: few cells forming a loose epithelium, C: few cells). Expanded blastocysts were deemed usable and considered for biopsy if they displayed a single adequately cellular and compact ICM. Expanded blastocysts were biopsied as soon as they met these criteria. Notably, if a blastocyst was collapsed at the time of assessment, it was re-evaluated 1-2h later. All embryos not meeting these criteria on the morning of day 5 remained in culture until the morning of day 6, at which point they were again assessed.

Blastocyst biopsy

Only blastocysts that presented at least 3 degrees of expansion (complete cavity), a detectable internal cell mass and detectable trophoctoderm cells were biopsied. Approximately 5-8 trophoctoderm cells were excised using a laser (OCTAX Laser Shot[™] System - Infrared Diode Laser - 1.48 μm Wave Length) and 1- to 2,800-mm pulses to break apart cell junctions in the trophoctoderm layer for tissue removal. All embryos were vitrified on average 30 minutes after the biopsy. The removed cells were then sent for aneuploidy analysis (PGT-A).

For the PGT-A analysis, 5-8 cells of the trophoctoderm from each blastocyst were analyzed. The test detects numerical chromosomal abnormalities and aneuploidies in the 24 chromosomal types by next-generation sequencing (NGS). This analysis checks for gains and losses of small chromosomal fragments. We used Ion ReproSeq PGS and Ion Chef System kits (Thermo Fisher Scientific, USA) for the examination, and we used the Ion Reporter software for the analysis, in which the readings were aligned

using the latest compilation of human genome data (hg19) (Thermo Fisher Scientific, USA).

After the results, we ran statistical analyses to assess the association of the risk of aneuploidy with the characteristics of embryonic development and the couple's clinical infertility factors.

Statistical analysis

We assessed continuous variables with a normal distribution and equal variances using the Student's t-test for independent samples. We used the Chi-squared test for categorical variables. We ran a logistic regression model to determine the association between embryonic developmental characteristics, couple clinical factors and aneuploidy. The threshold for statistical significance was 5%. We performed the statistical tests using the Statistical Package for the Social Sciences 20 (SPSS, Inc., Chicago, IL, USA). We ran a receiver operating characteristic (ROC) curve analysis according to the area under the ROC curve (AUC) and compared the according to the 95% confidence interval by the Hanley and McNeil method using MedCalc. The best cutoff to maximize sensitivity and specificity was selected according to the ROC curve. The sensitivity, specificity, positive predictive value, negative predictive value, positive likelihood ratio and negative likelihood ratio were determined after choosing the best cutoff point for aneuploidy risk. Our results showed sufficient power, Fisher's test and mid-P test results between 99.98% and 100%, to analyze the fertilization rate and blastocyst formation rate. Therefore, our findings are robust and valuable, considering an adequate power greater than 80%.

RESULTS

Of the 430 embryos subjected to PGT-A from 135 couples who underwent ICSI, 8 embryos were excluded from the analysis due to failed DNA amplification; in all, 200 (47.4%) were euploid (euploid group), and 222 (52.6%) were aneuploid (aneuploid group). The most frequent aneuploidies found were trisomy of 16 (1.9%), 9 (1.9%), 21 (1.4%) and 6 (0.9%); and monosomies of 15 (1.6%), 16 (1.6%), 21 (1.4%) and 7 (1.4%). Most aneuploid embryos showed two or more chromosomal changes. Four blastocysts had mosaicism (included in the aneuploid group), and two embryos with amplification failure were re-biopsied, both of which were aneuploid.

Table 1 depicts the effects of maternal age, ovarian reserve, paternal age and semen quality on the risk of embryonic aneuploidy. Advanced maternal age was associated with the risk of aneuploidy (33.9 years in the euploid group *versus* 36.4 years in the aneuploid group, $p < 0.001$). We found that women over 38 years of age had an odds ratio (OR) of 3.4 (CI: 2.2-5.4, $p < 0.001$) for forming aneuploid embryos. We also found that infertility time was associated with risk of aneuploidy, but this risk is probably due to the increase in the age of couples secondary to this time. Consequently, the infertility time was not analyzed in the logistic regression. In addition, a low ovarian reserve with an antral follicle count (AFC) of less than 5 had an OR of 3.3 (CI: 1.5-7.0, $p < 0.001$) for forming aneuploid embryos. An advanced paternal age was also associated with a higher risk of aneuploid embryo formation (39.0 years in the euploid group *versus* 40.7 years in the aneuploid group, $p = 0.019$).

Table 1. Influence of maternal age, ovarian reserve, paternal age and seminal quality on the risk of embryonic aneuploidy.

	Euploid Group N=200	Aneuploid Group N=222	Odds Ratio (CI)	P
Infertility time (years), mean (SD)	3.0±2.5	3.6±3.2		0.041*
Maternal age (years), mean (SD)	33.9±3.8	36.4±4.5		<0.001*
<38 years, %	56.4%	48.4%	3.4 (2.2-5.4)	<0.001 [†]
>38 years, %	27.2%	72.8%		
Maternal weight (kg), mean (SD)	63.3 (7.9)	62.6 (9.0)		0.442*
Maternal height (m), mean (SD)	1.62 (0.06)	1.61 (0.05)		0.549*
Maternal body mass index, mean (SD)	24.1 (2.8)	23.9 (3.1)		0.557*
Antral follicle count, %				
Low ovarian reserve (≤5)	25.6%	74.4%	3.3 (1.5-7.0) [‡]	<0.001 [†]
Intermediate ovarian reserve (6-10)	37.3%	62.7%		
Normal ovarian reserve (> 10)	53.4%	46.6%		
Paternal age (years), mean (SD)	38.5±7.0	40.0±7.5		0.000*
Seminal collection type, % (N)				
Masturbation	46.9% (189)	53.1% (214)		0.605 [†]
Percutaneous epididymal sperm aspiration	60.0% (9)	40.0% (6)		
Testicular sperm extraction	50.0% (2)	50.0% (2)		
Semen concentration (10⁶ /ml), mean (SD)	57.9±44.5	56.8±49.0		0.817*
Semen motility (%), mean (SD)	51.6±22.2	49.8±49.0		0.435*
Sperm preparation 10⁶/ml (swim-up), mean (SD)	29.3±31.5	21.3±30.0		0.073*

SD: standard deviation

CI: confidence interval

* T-test

[†] Chi-squared test

[‡] Risk of aneuploidy: (low reserve) x (normal reserve).

[§] Risk of aneuploidy: (low reserve + intermediate reserve) x (normal reserve).

^{||} Only data for semen collected by masturbation were included in this analysis.

To assess whether or not the increased paternal age could cause a false association, we performed multivariate analysis to assess the influence of each of these factors (paternal age and maternal age) with the risk of embryo aneuploidy. We found that these factors were independently associated with the risk of aneuploidy (dependent variable: aneuploidy; independent variables: maternal age ($B=0.106$; $\text{Sig}<0.001$; $\text{Exp}(B)=1.112$), paternal age ($B=0.026$; $\text{Sig}=0.025$; $\text{Exp}(B)=1.026$), and constant ($B=-4.767$; $\text{Sig}<0.001$; $\text{Exp}(B)=0.009$).

Of the parameters analyzed regarding the seminal quality of samples collected by masturbation, only a decrease in the number of sperm with normal morphology was associated with the risk of aneuploidy (2.5% of normal forms in the euploid group *versus* 1.9% in the aneuploid group, $p=0.047$). Other factors (type of collection, concentration, motility, and sperm preparation) were not associated with embryonic aneuploidy.

Table 2 depicts the influence of infertility factors on the risk of embryonic aneuploidy. None of the factors analyzed (tubal factor, male factor, repeat abortion, endometriosis, PCOS, cryopreserved oocyte) were associated with the risk of aneuploidy.

Table 3 shows the influence of embryonic morphological quality on D1 (assessment 17 h after ICSI), D2 and D3 on the risk of embryonic aneuploidy. None of the morphological parameters on D1 (pro-nucleus, type of nuclear precursor corpuscles, polar corpuscles), D2 and D3 (number of blastomeres, embryonic classification) showed an association with the risk of embryonic aneuploidy.

The influence of the morphological quality of blastocysts on the risk of embryonic aneuploidy is shown in Table 4. Type-C TE was associated with a high risk of embryonic aneuploidy; the risk of aneuploidy was 36.5%, 51.5% and 69.7% for types A, B, and C TE, respectively ($p<0.001$). In comparing types (A + B) TE *versus* type C TE, we found an OR of 2.7 (CI: 1.6-4.3, $p<0.001$) for the risk of aneuploidy, and in comparing type A TE *versus* type C TE, we found an OR of 4.1 (CI: 2.2-7.7, $p<0.001$) for the risk of aneuploidy. In the evaluation of the ICM, only a type-C

ICM compared with a type-A ICM was associated with a higher risk of aneuploidy, with an OR of 1.7 (CI: 1.01-3.0, $p=0.048$). The degree of embryonic expansion and the day of embryonic biopsy were not associated with the risk of embryonic aneuploidy.

The logistic regression analysis results regarding factors of aneuploidy risk (dependent variable: aneuploidy; independent variables: maternal age, ovarian reserve, paternal age, sperm with normal morphology, TE quality, and ICM quality) are shown in Table 5. Logistic regression analysis showed that the main factors for the risk of aneuploidy are maternal age (OR 1.1 per year of age), type B TE (risk: 3.5), and type C TE (risk: 4.8).

To better assess the role of maternal and paternal age in aneuploidy risk, ROC curve analysis was performed to determine the best maternal and paternal age cutoffs for determining the frequency of aneuploidy. The ROC curve showed that the best cutoff point for maternal age was 38 years (AUC:0.709, $p<0.001$, sensitivity: 46.5, specificity: 82.4, +LR: 3.68, -LR: 0.57), as shown in Figure 1; the best cutoff age point for male age was 36 years, as shown in Figure 2 (AUC: 0.591, $p<0.001$, sensitivity: 64.7, specificity: 49.5, +LR: 1.28, -LR: 0.71).

To assess the associations of the identified markers with embryonic aneuploidy, we analyzed the predictive value, likelihood ratio, sensitivity, and specificity for diagnosis of embryonic aneuploidy of the risk factors separately and in association (Table 6). Of the independent factors, maternal age and antral follicle count less than five had the best positive predictive value (72.7% and 72.5%, respectively), with high specificities (81.5% and 94.0%, respectively). Among the combinations of factors, the best marker for the risk of aneuploidy was maternal age older than 38 years, associated with an embryo with trophoctoderm type C, with a positive predictive value of 88.6% and a specificity of 97.5%.

DISCUSSION

We found that approximately 53% of the embryos analyzed were aneuploid, similar to the findings of other studies

Table 2. Influence of infertility factors on the risk of embryonic aneuploidy.				
	Euploid Group N=200	Aneuploid Group N=222	Odds Ratio (CI)	p*
Tubal factor †				
No	45.2% (95)	54.8% (115)	0.8 (0.5-1.2)	0.409
Yes	49.4% (88)	50.6% (90)		
Male factor ‡				
No	48.6% (138)	51.4% (146)	1.1 (0.7-1.7)	0.599
Yes	45.5% (46)	54.5% (55)		
Recurrent miscarriage				
No	47.1% (169)	52.9% (190)	0.9 (0.4-2.0)	0.901
Yes	48.3% (14)	51.7% (15)		
Endometriosis				
No	46.2% (168)	53.8% (196)	0.4 (0.1-1.1)	0.059
Yes	66.7% (16)	33.7% (8)		
Polycystic ovary syndrome				
No	44.0% (85)	56.0% (108)	0.7 (0.5-1.1)	0.219
Yes	50.3% (99)	49.7% (98)		
Cryopreserved oocyte				
No	48.7% (170)	51.3% (179)	1.3 (0.8-2.2)	0.236
Yes	41.1% (30)	58.9% (43)		

*Chi-squared test

† Tubal factor identified on hysterosalpingography and/or videolaparoscopy

‡ Male factor defined by a sperm count of less than 15 million/ml

Table 3. Influence of embryonic morphological quality on D1 (17 HPI), D2 (44 HPI) and D3 (HPI) on the risk of embryonic aneuploidy			
	Euploid Group N=200	Aneuploid Group N=222	p
Number of embryonic pro-nuclei			
0	30.8% (4)	69.2% (9)	0.318*
1	0	100% (1)	
2	47.8% (188)	51.8% (202)	
Nuclear precursor corpuscles			
Type 1	47.8% (142)	52.2% (159)	0.916*
Type 2	48.1% (37)	51.9% (40)	
Type 3	53.3% (8)	46.7% (7)	
Number of polar corpuscles			
1	50.0% (3)	50.0% (3)	0.903*
2	47.5% (189)	52.5% (209)	
Number of blastomeres on D2, mean	4.0 (0.9)	3.9 (0.9)	0.184†
Embryonic classification on D2, % (N)			
Type 1	47.0% (183)	53.0% (206)	0.950*
Type 2	46.2% (6)	53.8% (7)	
Type 3	0% (0)	0% (0)	
Number of blastomeres on D3, mean	7.6 (1.5)	7.6 (1.5)	0.576†
Embryonic classification on D3, % (N)			
Type 1	46.4% (159)	53.6% (184)	0.322*
Type 2	56.3% (18)	43.8% (14)	
Type 3	100% (1)	0% (0)	

*Chi-squared or Fisher's test

† T-test

HPI: hours post insemination

Table 4. Influence of the morphological quality of the blastocyst on the risk of embryonic aneuploidy						
	Type	Euploid Group % (N)	Aneuploid Group % (N)	p*	OR	p
Degree of expansion	2	45.4% (124)	54.6% (149)	0.215		
	3	49.3% (69)	50.7% (71)			
	4	77.7% (7)	22.2% (2)			
Trophectoderm	A	63.5% (54)	36.5% (31)	<0.001	2.5 (1.5-4.1) † 4.0 (2.1-7.4) †	<0.001 † <0.001 †
	B	48.9% (116)	51.1% (121)			
	C	30.3% (30)	69.7% (69)			
Inner cell mass	A	54.1% (72)	45.9% (61)	0.117	1.7 (1.1-3.0) †	0.048†
	B	45.9% (95)	54.1% (112)			
	C	40.2% (33)	59.8% (49)			
Biopsy day	D5	51.7% (76)	48.3% (71)	0.247		
	D6	45.1% (123)	54.9% (150)			

*Chi-squared test

† Risk of aneuploidy: (TYPE A + TYPE B) x (TYPE C)

* Risk of aneuploidy: (TYPE A) x (TYPE C)

(Platteau *et al.*, 2006; Friedenthal *et al.* 2018; Lawrenz *et al.*, 2019). We sought to evaluate whether alterations in embryonic and clinical morphology factors of the couple were predictive of forming aneuploid blastocysts. Of the embryonic morphology factors, poor blastocyst TE and ICM quality were associated with a higher risk of aneuploidy, whereas morphology factors during cleavage, blastocyst expansion and blastocyst formation were not associated with the risk of aneuploidy. Of the clinical factors, maternal age and antral follicle count less than five had the best positive predictive value, with high specificities.

The transfer of embryos in the blastocyst phase, especially those with better morphology, increases the rate of pregnancy per transfer cycle due to a better embryo selection enabled by prolonged cultivation (Alfarawati *et al.*, 2011). PGT-A, which enables better embryo selection, has raised costs without effectively increasing final pregnancy rates (Munné *et al.*, 2019), and even causing this rate to decrease (Zhang *et al.*, 2016; Guzman *et al.*, 2019; Munne *et al.*, 2019). Next-generation sequencing for PGT-A has led to an increase in reports of chromosomal mosaicism in trophectoderm biopsies (Sachdev *et al.*, 2017;

	B	Sig.	Exp (B)	95% CI for Exp (B)
Maternal age	0.126	<0.001	1.134	1.071 - 1.202
Antral follicle count >11		0.019		
Antral follicle count 6-10	-1.079	0.069	0.340	0.106 - 1.085
Antral follicle count < 5	1.408	0.059	4.089	0.948 - 17.635
Paternal age	0.015	0.637	1.016	0.952 - 1.083
Sperm with normal morphology	-0.203	0.077	0.816	0.652 - 1.023
Type A trophoctoderm		0.008		
Type B trophoctoderm	1.275	0.004	3.577	1.519 - 8.422
Type C trophoctoderm	1.573	0.017	4.820	1.322 - 17.571
Type A inner cell mass		0.042		
Type B inner cell mass	0.174	0.664	1.190	0.543 - 2.610

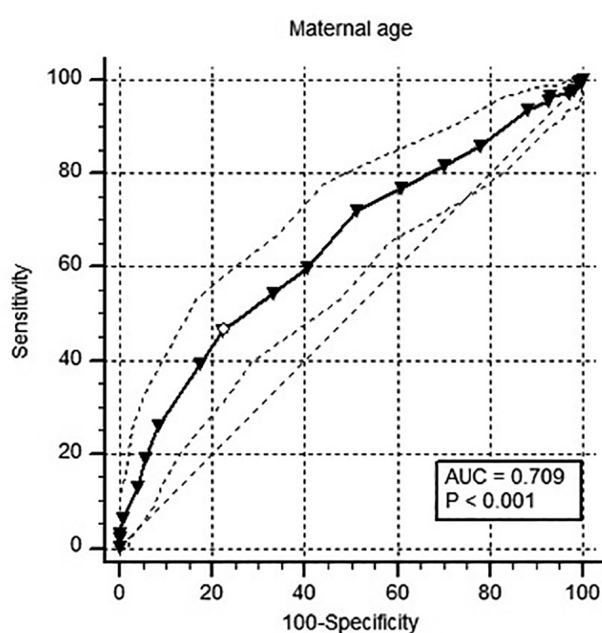


Figure 1. Evaluation of the best maternal age cut off (>38 years) to predict the risk of aneuploidy.

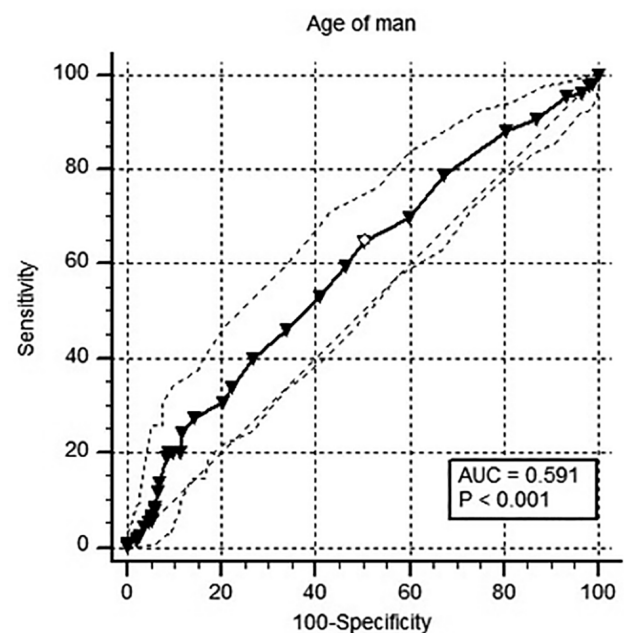


Figure 2. Evaluation of the best paternal age cut off (>36 years) to predict the risk of aneuploidy.

Vera-Rodriguez & Rubio, 2017). Although these embryos may lead to healthy live births, they are linked to poorer clinical outcomes when compared with euploid blastocysts (Munné *et al.*, 2017); thus, we included mosaic embryos in the aneuploid group.

Assessing predictive factors of the risk of aneuploid embryo formation can help us select the embryos to be biopsied and, most importantly, provide patients with more precise information about the real possibility of having an euploid embryo according to their case for each embryo. We found that a type C TE blastocyst had a high risk of being aneuploid (69.7%), with an OR of 4.0, whereas a type A TE blastocyst had a risk of only 36.5%. In addition, compared to ICM type A blastocysts, ICM type C blastocysts had an OR of 1.7 for aneuploidy. Other studies have

also shown that blastocyst-stage embryo morphology is associated with euploidy, especially concerning ICM and TE parameters (Wang *et al.*, 2016; Barash *et al.*, 2017). The reduced number of TE cells can be attributed to cell division failure due to organelle and chromosomal abnormalities, especially aneuploidy (Iwasawa *et al.*, 2019).

When we assessed the risk of aneuploidy in terms of embryonic quality in the cleavage phase, we found no association with aneuploidy. Although some studies have found an association between aneuploidy and the cleavage stage (Kroener *et al.*, 2015), more recent studies have not found this association (Barash *et al.*, 2017). The embryonic morphology in the cleavage phase is associated with blastocyst formation (Lawrenz *et al.*, 2019), but not with blastocyst euploidy. The explanation is that the embryos that became blastocysts are those with the best morphology at

Table 6. Predictive value, likelihood ratio, sensitivity, and specificity for diagnosis of embryo aneuploidy

	Pre-test prevalence	PPV ^a	NPV	LR+	LR-	Sensitivity (%)	Specificity (%)
One factor							
Maternal age > 38 years	52.6	72.7	56.5	2.4	0.7	44.0	81.5
Antral follicle count ≤ 5	52.6	72.5	49.4	2.4	0.9	14.2	94.0
Trophectoderm type C	52.6	70.6	52.8	2.1	0.8	32.1	85.0
Inner cell mass type C	52.6	60.2	49.0	1.4	0.9	22.2	83.5
Two factors							
Maternal age > 38 years + Inner cell mass type C	52.6	81.8	49.6	4.0	0.9	12.0	97.0
Maternal age > 38 years + Trophectoderm type C	52.6	88.6	51.4	7.0	0.8	17.4	97.5
Trophectoderm type C + Inner cell mass type C	52.6	72.2	47.3	2.1	0.9	11.3	94.8
Three factors							
Maternal age > 38 years + Trophectoderm type C + Inner cell mass type C	52.6	85.0	48.8	5.1	0.9	7.6	98.6

PPV: Positive predictive value; NPV: Negative predictive value; LR: Likelihood ratio

the cleavage stage, resulting in a very similar quality at the time of the blastocyst biopsy.

Maternal age is known to increase the risk of embryonic aneuploidy and miscarriage, and decrease embryonic implantation rate due to an increased risk of chromosome disruption failure during oogenesis (Ziebe *et al.*, 2001). Our results confirm this association, as we found that women over 38 years of age had a 3.4-fold greater risk of having aneuploid embryos, which agrees with the results of previous studies (Shahine *et al.*, 2016). To better assess the role of maternal age in aneuploidy risk, we performed a ROC curve analysis and found that age 38 was the best cutoff point for predicting aneuploidy risk, suggesting that this could serve as an indication for further tests, such as PGT-A. In addition to maternal age, low ovarian reserve has been associated with the risk of aneuploidy, albeit with conflicting results (Morin *et al.*, 2018). We found that patients with a low ovarian reserve (AFC less than five) had an approximately 73% risk of aneuploid embryos; according to logistic regression, patients with AFC greater than eleven had a lower risk of aneuploidy.

The relationship between paternal age and risk of aneuploidy is very controversial in the literature; some studies have found an association (García-Ferreira *et al.*, 2015; Capelouto *et al.*, 2018), while others have not (Carrasquillo *et al.*, 2019). Our study found an association between increased paternal age and the risk of aneuploidy, and when we evaluated the ROC curve, 36 years was the best cutoff point to predict the risk of aneuploidy. The role of the male factor of infertility often ends up being overlooked as a potential aneuploidy factor. Of all the sperm data evaluated, the only factor we found that was associated with the risk of aneuploidy was a reduction in the percentage of sperm with normal morphology, which corroborates the findings of previous studies (Coban *et al.*, 2018). Our result reinforces the suggestion that men with a reduction in the percentage of sperm with normal morphology should take steps to decrease oxidative stress, which may influence sperm quality and morphology (Smits *et al.*, 2019).

In addition to maternal age, paternal age, ovarian reserve, and seminal quality, other factors in the couple's clinical history could increase the risk of aneuploidy. Some studies have attempted to evaluate PCOS as a possible risk factor for aneuploidy but found no association (Wang *et al.*, 2016), which is in accordance with our findings. Another disease widely studied as a potential risk factor for embryonic aneuploidy is endometriosis; however, we found no association of endometriosis with aneuploidy, similar to other studies (Juneau *et al.*, 2017). In addition, in patients with recurrent miscarriage, the cause could be a high rate of aneuploid embryo formation (Hodes-Wertz *et al.*, 2012; Shahine *et al.*, 2016); however, we did not find this association. Our results indicate that implantation failures and miscarriages that may occur in patients with PCOS, endometriosis and recurrent miscarriage are possibly due to factors other than embryonic aneuploidy, such as implantation, immunologic and thrombophilic factors.

In the logistic regression analysis to identify the factor with the greatest influence on the risk of aneuploidy, we found that the main factors associated were maternal age, with a risk of 1.1 per year, and TE quality, as patients with type C TE had a 4.8-fold greater risk of having aneuploid blastocysts.

Although ours is a cohort study, the number of embryos evaluated as well as the clinical data are representative.

Our findings have shown that evaluating embryonic morphology and couple's clinical factors is important and could enable us to better understand embryonic dynamics and the risk of aneuploidy. On the other hand, the influence of paternal age, seminal alterations and some diseases, such as endometriosis and PCOS, needs to be better evaluated in studies with more suitable designs for this purpose. When we evaluated the best markers to predict aneuploidy, we found that of the independent factors, maternal age and antral follicle count less than five had the best positive predictive value (72.7% and 72.5%, respectively), with high specificities (81.5% and 94.0%, respectively). When we examined the associations of markers to assess the risk of aneuploidy, the best marker for the risk of aneuploidy was maternal age above 38 years combined with an embryo with trophoctoderm type C, with a positive predictive value of 88.6% and specificity of 97.5%. These results can be useful in informing patients and even the embryology laboratory team.

CONCLUSION

Our study shows that trophoctoderm and inner cell mass type C are the major embryo risk factors for aneuploidy, with aneuploidy risks of approximately 71% and 60%, respectively. In addition, the analysis of clinical factors showed that advanced maternal and paternal ages, antral follicle counts less than five, and reduction in the percentage of sperm with normal morphology increase the risk of embryo aneuploidy. Maternal age greater than 38 years is the best cut off for predicting the risk of aneuploidy (72.7%), and if this marker is associated with an embryo with trophoctoderm type C, the positive predictive value is 88.6% with a specificity of 97.5%. These findings are of great importance for the clinical practices of assisted reproduction centers, as they may assist in the selection of embryos to be submitted to PGT-A and in educating patients regarding the real possibility of having an euploid embryo according to the embryonic and clinical characteristics of the couple.

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CONFLICT OF INTEREST

Authors reported no conflict of interest associated with this study.

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