Human Reproduction, Vol.31, No.10 pp. 2245–2254, 2016
Advanced Access publication on September 2, 2016 doi:10.1093/humrep/dew183

human reproduction **ORIGINAL ARTICLE** ***Embryology***

Correlation between aneuploidy, standard morphology evaluation and morphokinetic development in 1730 biopsied blastocysts: a consecutive case series study

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Submitted on March 10, 2016; resubmitted on May 31, 2016; accepted on June 24, 2016

STUDY QUESTION: Are there correlations among human blastocyst ploidy status, standard morphology evaluation and time-lapse kinetics?

SUMMARY ANSWER: Correlations were observed, in that euploid human blastocysts showed a higher percentage with top quality inner cell mass (ICM) and trophectoderm (TE), higher expansion grades and shorter time to start of blastulation, expansion and hatching, compared to aneuploid ones.

WHAT IS KNOWN ALREADY: Embryo quality has always been considered an important predictor of successful implantation and pregnancy. Nevertheless, knowledge of the relative impact of each morphological parameter at the blastocyst stage needs to be increased. Recently, with the introduction of time-lapse technology, morphokinetic parameters can also be evaluated. However, a large number of studies has reported conflicting outcomes.

STUDY DESIGN, SIZE, DURATION: This was a consecutive case series study. The morphology of 1730 blastocysts obtained in 530 PGS cycles performed from September 2012 to April 2014 that underwent TE biopsy and array comparative genomic hybridization was analyzed retrospectively. A total of 928 blastocysts were cultured in a time-lapse incubator allowing morphokinetic parameters to be analyzed.

PARTCIPANTS/MATERIALS, SETTING, METHOD: Mean female age was 36.8 ± 4.24 years. Four hunderd fifty-four couples were enrolled in the study: 384, 64 and 6 of them performed single, double or triple PGS cycles, respectively. In standard morphology evaluation, the expansion grade, and quality of the ICM and TE were analyzed. The morphokinetic parameters observed were second polar body extrusion, appearance of two pronuclei, pronuclear fading, onset of two- to eight-cell divisions, time between the two- and three-cell (cc2) and three- and four-cell (s2) stages, morulae formation time, starting blastulation, full blastocyst stage, expansion and hatching timing.

MAIN RESULTS AND THE ROLE OF CHANCE: Of the 1730 biopsied blastocysts, 603 were euploid and 1127 aneuploid. We observed that 47.2% of euploid and 32.8% of aneuploid blastocysts showed top quality ICM ($P < 0.001$), and 17.1% of euploid and 28.5% of aneuploid blastocysts showed poor quality ICM ($P < 0.001$). Top quality TE was present in 46.5% of euploid and 31.1% of aneuploid blastocysts ($P < 0.001$), while 26.6% of euploid and 38.1% of aneuploid blastocysts showed poor quality TE ($P < 0.001$). Regarding expansion grade,

81.1% of euploid and 72.4% of aneuploid blastocysts were fully expanded (Grade 5–6; $P < 0.001$). The timing of cleavage from the three- to four-cell stage, of reaching four-cell stage, of starting blastulation, reaching full blastocyst stage, blastocyst expansion and hatching were 2.6 (95% confidence interval (CI): 1.7–3.5), 40.0 (95% CI: 39.3–40.6), 103.4 (95% CI: 102.2–104.6), 110.2 (95% CI: 108.8–111.5), 118.7 (95% CI: 117.0–120.5) and 133.2 (95% CI: 131.2–135.2) hours in euploid blastocysts, and 4.2 (95% CI: 3.6–4.8), 41.1 (95% CI: 40.6–41.6), 105.0 (95% CI: 104.0–106.0), 112.8 (95% CI: 111.7–113.9), 122.1 (95% CI: 120.7–123.4) and 137.4 (95% CI: 135.7–139.1) hours in aneuploid blastocysts ($P < 0.05$ for early and $P < 0.0001$ for later stages of development), respectively. No statistically significant differences were found between euploid and aneuploid blastocysts for the remaining morphokinetic parameters.

A total of 407 embryo transfers were performed (155 fresh, 252 frozen–thawed blastocysts). Higher clinical pregnancy, implantation and live birth rates were obtained in frozen–thawed compared to fresh embryo transfers ($P = 0.0104$, 0.0091 and 0.0148, respectively). The miscarriage rate was 16.1% and 19.6% in cryopreserved and fresh embryo transfer, respectively. The mean female age was lower in the euploid compared to aneuploid groups (35.0 ± 3.78 versus 36.7 ± 4.13 years, respectively), We found an increasing probability for aneuploidy with female age of 10% per year (odds ratio (OR) = 1.1, 95% CI: 1.1–1.2, $P < 0.001$).

LIMITATIONS, REASONS FOR CAUTION: The main limitation of morphology assessment is that it is a static system and can be operator-dependent. In this study, eight embryologists performed morphology assessments. The main limitation of the time-lapse technology is that it is impossible to rotate the embryos making it very difficult to observe them in case of blastomere overlapping or increased cytoplasmic fragmentation.

WIDER IMPLICATIONS OF THE FINDINGS: Although there seems to be a relationship between the ploidy status and blastocyst morphology/development dynamics, the evaluation of morphological and morphokinetic parameters cannot currently be improved upon, and therefore replace, PGS. Our results on ongoing pregnancy and miscarriage rates suggest that embryo evaluation by PGS or time-lapse imaging may not improve IVF outcome. However, time-lapse monitoring could be used in conjunction with PGS to choose, within a cohort, the blastocysts to analyze or, when more than one euploid blastocyst is available, to select which one should be transferred.

STUDY FUNDING/COMPETING INTEREST(S): No specific funding was obtained for this study. None of the authors have any competing interests to declare.

Key words: time-lapse / embryo morphology / blastocyst biopsy / PGS / morphokinetic / aneuploidy / fresh embryo transfer / cryopreserved embryo transfer

Introduction

The primary goal in ART is to identify the embryo with a high implantation potential, in order to achieve a single healthy live birth. Embryo quality has always been considered an important predictor of successful implantation and pregnancy. Historically, standard morphological evaluation has been the most widespread approach for embryo selection, and it is still the main strategy applied (Gardner and Schoolcraft, 1999; Gardner and Sakkas, 2003). However, knowledge of the relative impact of each morphological parameter needs to be increased, and this evaluation method has some limitations. First, despite the recent collaborative study between the ESHRE and Alpha Scientists in Reproductive Medicine societies with the aim to establish common criteria and terminology for grading oocytes, zygotes and embryos (ALPHA and ESHRE, 2011a,b), it remains a very subjective assessment strategy. Second, static embryo evaluations are performed at only a few time points (perhaps established more on convenience than on a rationale), taking no notice of what happens when the embryos are not checked (Meseguer *et al.*, 2011; Cruz *et al.*, 2012). Third, embryo morphology can vary widely just in a few hours, introducing high variability in embryo scoring (Gardner *et al.*, 2015).

Recently, time-lapse monitoring has been introduced as a noninvasive strategy for embryo selection. This technology, integrating frequent image capture with undisturbed culture conditions, permits a more accurate embryo morphological evaluation combined with developmental kinetic analysis. Despite the high number of recent studies, conflicting data are reported, and there is still considerable disagreement regarding which parameters are useful to predict blastocyst formation, implantation potential and ploidy status of the embryos (Kaser and Racowsky, 2014; Gardner *et al.*, 2015). In addition, it has been reported that morphokinetics can be influenced by other factors, such as culture media (Ciray *et al.*, 2012), stimulation protocols (Muñoz *et al.*, 2013), insemination method (Lemmen *et al.*, 2008; Dal Canto *et al.*, 2012), obesity (Bellver *et al.*, 2013), smoking (Fréour *et al.*, 2013) and oxygen tension (Kirkegaard *et al.*, 2013a). Therefore, it is likely that models developed employing time-lapse technology may not be directly applied across different infertility centers, but local adjustments should be performed according to their own protocols and procedures (Campbell *et al.*, 2013a; Kirkegaard *et al.*, 2014; Fréour *et al.*, 2015). The first attempt to develop a time-lapse hierarchical model in order to classify the embryos on the basis of their implantation potential was introduced by Meseguer and collaborators (2011). Their model was based on a combination of morphological screening, absence of exclusion criteria, the timing of division to five cells, synchrony of division from two to four cells and duration of the second cell cycle. This classification produced 10 categories of embryos with increasing implantation potential. In a subsequent retrospective study (Fréour *et al.*, 2015), this model failed when applied in an unselected population of patients, being unable to replicate the sensitivity reported in the original publication. More recently, another morphokinetic noninvasive prediction model has been proposed, based on the time of starting blastulation and the time when the full

blastocyst stage was reached, in order to select blastocysts with high, medium or low risk of being aneuploid (Campbell *et al.*, 2013a,b). Subsequently, two groups tried to apply the same criteria to their morphokinetic data, but no significant difference in the proportion of euploid or aneuploid embryos was found in any of the three classes of risk (Kramer *et al.*, 2014; Rienzi *et al.*, 2015).

PGD is an invasive method of embryo selection. Initially it was used in fertile patients with a risk of transmitting genetically inheritable sex-linked monogenic diseases to their offspring (Handyside *et al.*, 1990). Later, after the introduction of different techniques allowing comprehensive chromosomal screening for analyzing all 24 chromosomes, PGD was applied to investigate the genetic status of the embryos (PGS) obtained from infertile patients (Greco *et al.*, 2013). It was hypothesized that this approach could lead to improved clinical outcomes especially in some categories of patients, such as women with advanced maternal age, recurrent implantation failure or recurrent miscarriage (Kahraman *et al.*, 2000; Rubio *et al.*, 2003; Greco *et al.*, 2014). Moreover, due to the advancement of highly sophisticated culture and cryopreservation techniques, it has been observed that performing the biopsy at the blastocyst stage was more convenient that at cleavage stage, producing higher ongoing and clinical pregnancy rates (Hodes-Wertz *et al.*, 2012; Harton *et al.*, 2013; Scott *et al.*, 2013a; Adler *et al.*, 2014; Minasi and Greco, 2014).

The aim of our study was to evaluate possible relationships among blastocyst ploidy status, standard morphology and time-lapse morphokinetics in PGS with array comparative genomic hybridization (aCGH) cycles.

Material and Methods

In this consecutive case series study, the morphology of 1730 blastocysts, obtained in 530 PGS cycles performed in 454 infertile couples (384, 64 and 6 of them performed single, double or triple PGS cycles, respectively) from September 2012 to April 2014, with trophectoderm (TE) biopsy and aCGH, were retrospectively analyzed. Of these, 802 were cultured in standard incubators (Multigas incubator; Sanyo, Panasonic Biomedical, the Netherlands), ensuring that a single incubator was used for each patient couple for the duration of the cycle, and 928 blastocysts were cultured in time-lapse incubators (EmbryoScope; UnisenseFertiliTech, Denmark), allowing also the morphokinetic parameters to be analyzed. All the blastocysts enrolled in this study were individually cultured under oil, in sequential media (Quinn's Advantage Medium; SAGE, USA), at 37°C, 5% O_2, 6% CO_2. On Day 3, a media changeover was performed (Minasi *et al.*, 2015). Blastocysts having an exact number of chromosomes (46XX or 46XY) were considered euploid. Blastocysts having monosomy and/or trisomy were considered aneuploid. Mosaic embryos were excluded from the study.

Ovarian stimulation, oocyte denudation and insemination

Controlled ovarian stimulation was performed using recombinant FSH (Gonal F; Merck Serono, Geneva, Switzerland) and a GnRH agonist suppression protocol (short or long) or GnRH antagonist flexible protocol according to ovarian reserve and anti-Mullerian hormone values, as described elsewhere (Greco *et al.*, 2007, 2014). Triggering was performed by injection of hCG (Gonasi, 10.000 IU; IBSA, Lodi, Italy) intramuscularly. Denudation was performed by brief exposure to 20 IU/ml hyaluronidase (Hyaluronidase 80 U/ml in HEPES-HTF; SAGE) in Hepes-buffered medium (Quinn's Advantage Medium with Hepes; SAGE) under oil. Subsequently, oocytes were gently aspirated in and out of a plastic pipette (Flexipet, 170 and 140 µm i.d.; COOK, Australia) to allow the complete removal of cumulus and corona cells. All metaphase II embryos underwent ICSI immediately after denudation.

Morphological and morphokinetic evaluation

Embryo morphology was checked daily using the scoring system reported by Rienzi and collegues (1998). Briefly, the embryos were classified on the basis of blastomere number, symmetry and fragmentation. Type A embryos showed ≥7 cells on Day 3, ≤20% fragmentation and equal-sized blastomeres; Type B and C embryos showed 20–50% and >50% of fragmentation, respectively. Blastocysts were classified according to Gardner and Schoolcraft (1999), giving a number based on the degree of expansion and hatching status (from 1 to 6). Due to the subjectivity to discern among six levels of expansion, the blastocysts enrolled in this study were divided in three groups: 1–2, 3–4 and 5–6 of expansion degree. For fully developed blastocysts (Grades 3–6), a second scoring step was performed to assess the inner cell mass (ICM) and TE quality. For the ICM, Grades A, B or C corresponded to many cells tightly packed, several cells loosely grouped and very few cells, respectively. For the TE, Grades A, B or C, corresponded to many cells forming a cohesive epithelium, few cells forming a loose epithelium and very few large cells, respectively (Fig. 1). A retrospective analysis of the acquired images of each blastocyst was made with an external computer workstation (EmbryoViewer; UnisenseFertiliTech) annotating the timing of the events in hours, from the time of insemination throughout the whole culture period. The morphokinetic parameters analyzed were second polar body extrusion (tPB2), pronuclei (PN) appearance (tPNa), pronuclear fading (tPNf), onset of 2- to 8-cell divisions (t2, t3, t4, t5, t6, t7, t8, t9+), time between 2- and 3-cell stage (cc2) and between 3- and 4-cell stage (s2) and timing of morulae formation (tM), starting

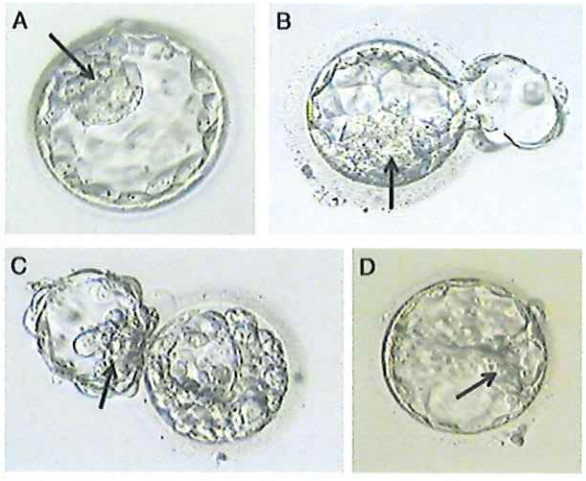

Figure 1 Examples of human blastocyst morphology evaluation. (**A**) High-quality blastocyst: Type A ICM, Type A TE and degree of blastocoel expansion 4; (**B**) High-quality blastocyst: Type B ICM, Type A TE and degree of blastocoel expansion 5; (**C**) Low-quality blastocyst: Type C ICM, Type B TE and degree of blastocoel expansion 5; (**D**) Low-quality blastocyst: Type C ICM, Type C TE and degree of blastocoel expansion 4. The arrows indicate the ICM. Pictures are at magnification ×400. ICM, inner cell mass; TE, trophectoderm.

blastulation (tSB), full blastocyst stage (tB), blastocyst expansion (tEB) and blastocyst hatching (tHB) (Meseguer et al., 2011).

Blastocyst biopsy, whole-genome amplification and aCGH protocol

All biopsies were performed at blastocyst stage. On Day 3, a non-contact 1.48 µ diode laser (Fragouli et al., 2008) was used to create a circular 6- to 9-µ-diameter opening in the zona pellucida in cleavage stage embryos, in order to allow the TE to herniate at the time of blastocyst expansion. On the day of biopsy, 5–10 TE cells were gently aspirated with a biopsy pipette (inner and outer diameters 35 and 49 µ, respectively; COOK Ireland Ltd, Limerick, Ireland) followed by a laser-assisted cut. The TE cells were washed in sterile phosphate-buffered saline solution (PBS) and then placed into microcentrifuge tubes containing 2 µl PBS and sent to GENOMA laboratory for the genetic analysis (Greco et al., 2014). For whole-genome amplification (WGA), TE cells were first lysed and genomic DNA was randomly fragmented and amplified using the SurePlex DNA Amplification System (BlueGnome, Cambridge, UK). For each sample, a negative control underwent the same treatment. WGA products were processed as previously reported (Fiorentino et al., 2014; Greco et al., 2014) according to the BlueGnome 24sure V3 protocol (available at www.cytochip.com). Briefly, WGA products were fluorescently labeled and competitively hybridized to 24sure V3 arrays (BlueGnome) with a matched control in an aCGH 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) (Gutiérrez-Mateo et al., 2011).

Blastocyst vitrification, warming and embryo transfer

Vitrification was carried out using the Kuwayama protocol with Cryotop (Kuwayama, 2007). The media employed were vitrification and warming kits (Kitazato Vitrification Kit; BioPharma, Shizouka, Japan). Briefly, blastocysts were incubated in equilibration and vitrification solutions for 15 minutes and 30–60 seconds at room temperature, respectively. Blastocysts were individually loaded onto Cryotops in a volume of <0.1 µl and quickly plunged into liquid nitrogen. Warming was performed by placing the Cryotop in the thawing solution for 60 seconds at 37°C. Then the blastocyst was moved to the dilution solution for 3 minutes at room temperature, followed by two steps in washing solution for 5 minutes each. Blastocysts were transferred 1–2 hours post-warming. Single or double embryo transfer was performed, both fresh or after cryopreservation. Fresh embryo transfers were carried out in the morning of Day 6, after receiving the genetic result. Frozen–thawed embryo transfers were performed both after preparation by combining GnRH agonist and estrogen pills (Progynova; Bayer, New Zealand Limited, Auckland, New Zealand) or in a spontaneous cycle. All transfer procedures were carried out with the use of a catheter (Wallace; Smiths Medical, Dublin, Ireland) under direct ultrasound guidance as previously described (Brown et al., 2010). Only euploid blastocysts were selected for transfer. No blastocyst transfer was performed with an endometrium thickness of <7 mm.

Statistical analysis

Continuous data are presented as mean and standard deviation, and categorical variables are presented as absolute and percentage frequency. In order to account for multiple data from the same patient, several mixed logistic models (with patient identification code as random factor) were used to evaluate associations between presence of aneuploid blastocysts and morphological variables. Odds ratio (OR) and their 95% confidence interval (CI) were reported. Adjusted OR were also reported after adjusting for female age. Analyses were performed on all samples, and separately by age classes. Differences between euploidy and aneuploidy in morphokinetic variables were calculated by mixed linear regression (with patient identification code as random factor and each kinetic parameter as the dependent variable), and marginal means and their 95% CI were reported for all samples. Stata 13.1 (Stata; Data Analysis and Statistical Software, Texas, USA) was used for all analyses. A P-value < 0.05 was considered statistically significant.

The Institutional Ethics Committee of the European Hospital approved this study proposal in accordance with the Helsinki Declaration. An informed consent was signed from all the patients enrolled in this study.

Results

From September 2012 to April 2014, 530 PGS cycles were performed. The general outcomes of the cycles are reported in Table I. The TE biopsy with aCGH was performed in 1748 blastocysts. In 1730 of them, it was possible to obtain the genetic result and 603 (34.9%) of these were euploid; the other 18 (1.0%) produced no results, probably due to amplification failure. At time of writing, a total of 407 embryo transfers were performed, 155 of them with fresh and 252 with frozen–thawed blastocysts; 274 blastocysts were thawed and 271 (98.9%) of them survived. Overall, a positive hCG test was obtained in 228 (56.0%) patients, 45 of them had a biochemical pregnancy, 3 of them an ectopic pregnancy and the other 180 were clinical pregnancies. Significantly higher clinical pregnancy, implantation and live birth rates were obtained in frozen–thawed compared to fresh embryo transfers (49.2%, 47.2% and 41.3% in frozen–thawed and 36.1%, 34.1% and 29.0% in fresh embryo transfers, $P = 0.0104$, 0.0091 and 0.0148, respectively). In addition, a trend towards a reduced biochemical pregnancy rate was found with frozen embryo transfer (16.2% versus 26.3% in cryopreserved and fresh embryo transfer, respectively, $P = 0.0819$), but this was not statistically significant. The miscarriage rate was 16.1% and 19.6% in cryopreserved and fresh embryo transfer, respectively (NS, Table I).

Standard morphology evaluation

Out of the 1730 blastocysts from which the genetic result was obtained after the biopsy, 114 were not fully expanded (Grade 2) making it impossible to do a complete morphological evaluation of the ICM and TE; in the other 1616 blastocycts, the ICM and TE quality were evaluated. No correlation was found between the ploidy status of the embryos, and either the standard morphology evaluation performed on day 3 cleavage stage or the day when the blastocysts formed and were biopsied (Table II). A higher proportion of euploid, compared to aneuploid blastocysts, was found in correlation with top quality ICM, TE and with the degree of the blastocyst expansion. Particularly, when the ICM was of Grade B or C, the blastocysts showed, respectively, 60% or 150% higher probability of being aneuploid (OR = 1.6, 95% CI: 1.3–2.1, $P = 0.001$ and OR = 2.5, 95% CI: 1.8–3.3, $P < 0.001$, respectively), compared to Grade A ICM. Similarly, when the TE was of Grade B or C, the blastocysts showed, respectively, 80% or 120% higher probability of being aneuploid (OR = 1.8, 95% CI: 1.3–2.3, $P < 0.001$ and OR = 2.2, 95% CI: 1.7–2.9, $P < 0.001$, respectively), compared to Grade A TE. Finally,

Table I Biological and clinical outcomes of 530 PGS with aCGH cycles performed from September 2012 to April 2014.

PGS + aCGH	September 2012 to April 2014
Number of cycles	530
Mean female age (years, ±SD)	36.8 ± 4.24
Mean male age (years, ±SD)	39.4 ± 5.37
Number of Retrieved oocytes	5811
Mature oocytes (%)	4463 (76.8%)
Injected oocytes	4463
Fertilized oocytes (%)	3495 (78.3%)
Day 3 embryos obtained (%)	3463 (99.1%)
Day 3 good/excellent quality embryos (%)	3011 (86.9%)
Blastocysts obtained (%)	1812 (52.3%)
Blastocyst analyzed for PGS (%)	1748 (96.5%)
Blastocysts with genetic result (%)	1730 (98.5%)
Blastocysts no result (%)	18 (1.0%)
Aneuploid blastocysts (%)	1127 (65.1%)
Euploid blastocysts (%)	603 (34.9%)
Transferred blastocysts	
Total	435
In fresh ET	164
In frozen–thawed ET	271
ETs	
Total	407
Fresh	155
Frozen–thawed	252
hCG-positive test (%)	
Total	228 (56.0%)
In fresh ET	80 (51.6%)
In frozen–thawed ET	148 (58.7%)
Biochemical pregnancies (%)	
Total	45 (19.7%)
In fresh ET	21 (26.3%)
In frozen–thawed ET	24 (16.2%)
Clinical pregnancies (CPR%)	
Total	180 (44.2%)
In fresh ET	56 (36.1%)
In frozen–thawed ET	124 (49.2%)
Fetal hearts (IR%)	
Total	184 (42.3%)
In fresh ET	56 (34.1%)
In frozen–thawed ET	128 (47.2%)
Ectopic pregnancies	
Total	3
In fresh ET	3
In frozen–thawed ET	0
Miscarriages (%)	
Total	31 (17.2%)
In fresh ET	11 (19.6%)
In frozen–thawed ET	20 (16.1%)
Deliveries (LBR%)	
Total	149 (36.6%)
In fresh ET	45 (29.0%)
In frozen–thawed ET	104 (41.3%)

aCGH, array comparative genomic hybridization; ET, embryo transfer; CPR, clinical pregnancy rate; IR, implantation rate; LBR, live birth rate.

blastocysts displaying expansion degree 5–6 had 40% less probability of being aneuploid (OR = 0.6, 95% CI: 0.4–0.9, $P = 0.026$), compared to expansion degree 1–2 (Table II).

The mean female age was lower in the euploid compared to aneuploid groups (35.0 ± 3.78 versus 36.7 ± 4.13 years, respectively), with an increasing probability to be aneuploid of 10% per year (OR = 1.1, 95% CI: 1.1–1.2, $P < 0.001$) (Table II; Figs. 2–4). In order to eliminate the effect of mean female age, the same evaluations were performed dividing the patients into four age classes: ≤32, 33–36, 37–41, ≥42 years (Supplementary Table SI).

Morphokinetic evaluation

Out of the 1730 biopsied blastocysts, 928 were cultured in time-lapse incubators, allowing the morphokinetic parameters to be evaluated (Table III). The four-cell stage was reached significantly earlier in euploid (40.0 hours, 95% CI: 39.3–40.6) compared to aneuploid blastocysts (41.1 hours, 95% CI: 40.6–41.6, $P = 0.002$). In addition, the cleavage from three- to four-cell stages (s2) was faster in euploid embryos compared to aneuploid ones: 2.6 hours (95% CI: 1.7–3.5) and 4.2 hours (95% CI: 3.6–4.8), respectively ($P = 0.004$). Finally, the euploid blastocysts started to form, reached the full stage, expanded and hatched significantly faster, compared to aneudploid ones: tSB 103.4 (95% CI: 102.2–104.6) versus 105.0 (95% CI: 104.0–106.0, $P = 0.007$), tB 110.2 (95% CI: 108.8–111.5) versus 112.8 (95% CI: 111.7–113.9, $P < 0.001$), tEB 118.7 (95% CI: 117.0–120.5) versus 122.1 (95% CI: 120.7–123.4, $P < 0.001$) and tHB 133.2 (95% CI: 131.2–135.2) versus 137.4 (95% CI: 135.7–139.1, $P < 0.001$) hours, respectively. Comparisons between euploid and aneuploid blastocysts for the other morphokinetic parameters did not reach statistical significance (Table III). The same evaluations were performed dividing the patients into four age classes: ≤32, 33–36, 37–41, ≥42 years (Supplementary Table SII).

Discussion

Oocyte and embryo aneuploidies, affecting more than half of embryos produced and increasing with advancing maternal age, is the main

Table II Correlation between the human Day 3 embryo morphological evaluation, day of the TE biopsy, ploidy status of the blastocysts, ICM morphology, TE morphology, degree of expansion (EXP) of the blastocysts and their ploidy status.

	Euploid blastocysts ($N = 603$)	Aneuploid blastocysts ($N = 1127$)	OR	95% CI	P^c	OR adj[b]	95% CI	P^d
Day 3 Grade A embryos	474 (78.6%)	863 (76.6%)	1[a]					
Day 3 Grade B embryos	114 (18.9%)	244 (21.7%)	1.2	0.9–1.5	0.207	1.2	0.9–1.5	0.224
Day 3 Grade C embryos	15 (2.5%)	20 (1.8%)	0.8	0.4–1.6	0.456	0.7	0.3–1.4	0.339
Day 4 biopsy	9 (1.5%)	10 (0.9%)	0.4	0.1–1.3	0.124	0.4	0.1–1.3	0.130
Day 5 biopsy	402 (66.7%)	731 (64.9%)	0.6	0.3–1.1	0.092	0.6	0.3–1.2	0.165
Day 6 biopsy	178 (29.5%)	343 (30.4%)	0.6	0.3–1.2	0.144	0.7	0.3–1.3	0.229
Day 7 biopsy	14 (2.3%)	43 (3.8%)	1[a]					
Grade A ICM	270 (47.2%)	342 (32.8%)	1[a]					
Grade B ICM	204 (35.7%)	404 (38.7%)	1.6	1.3–2.1	<0.001	1.5	1.2–2.0	0.001
Grade C ICM	98 (17.1%)	298 (28.5%)	2.5	1.8–3.3	<0.001	2.3	1.7–3.2	<0.001
Not evaluable ICM quality	31	83						
Grade A TE	266 (46.5%)	325 (31.1%)	1[a]					
Grade B TE	154 (26.9%)	321 (30.7%)	1.8	1.3–2.3	<0.001	1.7	1.3–2.2	<0.001
Grade C TE	152 (26.6%)	398 (38.1%)	2.2	1.7–2.9	<0.001	2.1	1.6–2.7	<0.001
Not evaluable TE quality	31	83						
EXP 1–2	31 (5.1%)	83 (7.4%)	1[a]					
EXP 3–4	83 (13.8%)	228 (20.2%)	1.0	0.6–1.7	0.894	1.0	0.6–1.6	0.879
EXP 5–6	489 (81.1%)	816 (72.4%)	0.6	0.4–0.9	0.026	0.6	0.4–0.9	0.021
Female age			1.1	1.1–1.2	<0.001			

[a]Reference class.
[b]OR adj: odds ratio adjusted by female age.
[c]P-value was calculated by univariable mixed logistic model.
[d]P-value was calculated by mixed logistic model adjusting by female age.

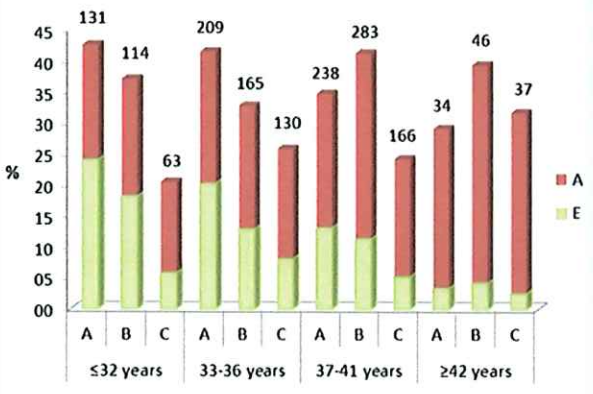

Figure 2 Distribution of euploid (E) and aneuploid (A) human blastocysts according to the ICM morphology in four classes of female ages (≤32, 33–36, 37–41, ≥42 years). The *N* values are at top of bars.

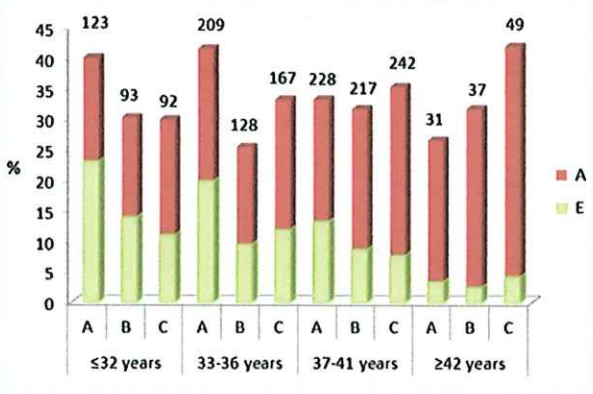

Figure 3 Distribution of euploid (E) and aneuploid (A) human blastocysts according to the TE morphology in four classes of female ages (≤32, 33–36, 37–41, ≥42 years). The *N* values are at top of bars.

reason for implantation failure and abortion in IVF cycles (Macklon *et al.*, 2002; Fragouli and Wells, 2012; Fragouli *et al.*, 2013). Our data confirm this finding: the mean female age in the euploid group was younger compared to the aneuploid one. Furthermore, this study underlines that the aneuploidy rate increases by ~10% per year of female age. Therefore, the establishment of a feasible procedure able to identify euploid embryos without impairing their implantation potential is of crucial relevance.

Although morphological evaluation has been the main strategy applied in order to choose the embryos to transfer, it was shown that

even aneuploid embryos are able to reach high morphological scores (Hardarson *et al.*, 2003; Munné, 2006; Alfarawati *et al.*, 2011; Fragouli *et al.*, 2014). In accordance with Fragouli and coauthors (2014), the outcomes of this study demonstrate that at cleavage stage the ploidy of the embryo has no effect on its morphology. On the contrary, at blastocyst level, in accordance with previous studies (Alfarawati *et al.*, 2011; Fragouli *et al.*, 2014), we found a greater likelihood of euploidy among blastocysts with good morphology scores and among embryos showing a faster progression to blastocyst stage. A possible explanation is that embryo genome activation starts at the four- to eight-cell stage (Braude *et al.* 1988). Consequently, only after the third day of culture will the embryo gradually express its own genes and the potential genetic abnormalities will start having an effect on embryo development (Alfarawati *et al.*, 2011; Fragouli *et al.*, 2014). It is important to underline that the attempt to select an euploid blastocyst only on the basis of its morphological aspect can be extremely hazardous, because many aneuploid blastocysts are able to reach top quality scores.

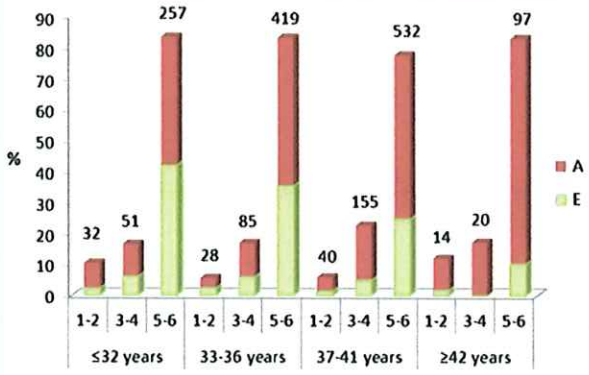

Figure 4 Distribution of euploid (E) and aneuploid (A) human blastocysts according to the degree of expansion in four classes of female ages (≤32, 33–36, 37–41, ≥42 years). The *N* values are at top of bars.

Recently, the time-lapse technology was applied to human embryos and a lot of data analyzing the possible prognostic effect of morphokinetic were reported, with very conflicting results (Kaser and Racowsky, 2014). Azzarello *et al.* (2012), contrary to our finding and to another two previous studies (Chamayou *et al.*, 2013; Kirkegaard *et al.*, 2013b), found that PN breakdown occurred significantly later in embryos leading to live birth, compared to the no live birth group. Proceeding along cellular divisions, we found a higher percentage of euploidy when embryos reached the four-cell stage earlier and cleaved from three- to four-cells stage faster, compared to aneuploid ones. Other studies were partially in accordance with our findings: a highly significant association between implantation (Meseguer *et al.*, 2011; Dal Canto *et al.*, 2012) or blastocyst formation (Hashimoto *et al.*, 2012; Dal Canto *et al.*, 2012) potentials and shorter times to reach the five-cell (Meseguer *et al.*, 2011), seven-cell (Dal Canto *et al.*, 2012) and eight-cell stages (Hashimoto *et al.*, 2012; Dal Canto *et al.*, 2012) as well as a faster time to cleavage from two to three cells (cc2;

Table III Morphokinetic parameters analyzed in euploid and aneuploid human blastocysts.

Kinetic parameter	Euploid blastocysts (*N* = 294)			Aneuploid blastocysts (*N* = 634)			*P*[a]
	N	Mean	95% CI	*N*	Mean	95% CI	
tPB2	294	3.9	3.7–4.0	634	4.0	3.8–4.1	0.390
tPNa	294	10.2	9.8–10.6	634	10.1	9.8–10.4	0.468
tPNf	294	24.4	24.0–24.8	634	24.8	24.4–25.1	0.095
t2	276	28.0	27.5–28.5	602	28.4	28.0–28.9	0.110
t3	99	37.4	36.3–38.4	250	37.2	36.5–37.9	0.798
t4	270	40.0	39.3–40.6	570	41.1	40.6–41.6	0.002
t5	134	50.4	49.1–51.8	282	50.6	49.6–51.6	0.823
t6	170	53.9	52.7–55.2	323	55.2	54.2–56.3	0.069
t7	141	57.8	56.3–59.3	243	58.1	56.8–59.3	0.765
t8	257	61.9	61.5–63.2	556	62.0	61.0–63.0	0.853
9+	232	76.1	74.6–77.6	519	76.8	75.6–78.0	0.369
cc2	90	10.5	9.8–11.2	224	10.4	9.9–10.9	0.787
s2	86	2.6	1.7–3.5	214	4.2	3.6–4.8	0.004
tM	273	94.4	93.0–95.7	607	95.3	94.1–96.4	0.177
tSB	266	103.4	102.2–104.6	598	105.0	104.0–106.0	0.007
tB	263	110.2	108.8–111.5	593	112.8	111.7–113.9	<0.001
tEB	222	118.7	117.0–120.5	493	122.1	120.7–123.4	<0.001
tHB	174	133.2	131.2–135.2	328	137.4	135.7–139.1	<0.001

tPB2, second polar body extrusion; tPNa, appearance of two pronuclei; tPNf, pronuclear fading; t2, t3, t4, t5, t6, t7, t8, t9+, time to 2, 3, 4, 5, 6, 7, 8, 9+ or more cells divisions, respectively; cc2, time between 2- and 3-cell stages; s2, time between 3- and 4-cell stages; tM, morulae formation; tSB, starting blastulation; tB, full blastocyst stage; tEB, blastocyst expansion; tHB, blastocysts hatching. The marginal means reported are expressed in hours.
[a]*P*-value was calculated by linear mixed model.

Meseguer et al., 2011) and from three to four cells (s2; Meseguer et al., 2011; Hashimoto et al., 2012), was described. Extending the search for predictive time-lapse markers at blastocyst level, we found that the times when the blastocyst starts to form (tSB), is completely formed (tB), expands (tEB) and hatches (tHB) were significantly shorter in the euploid compared to the aneuploid group. These results comply with previous studies reported by Campbell and coauthors (2013a,b) where a delay in starting compaction, starting blastulation and in reaching full blastocyst stage was observed in aneuploid embryos compared to euploid ones. On the contrary, other studies reported a similar development in implanted/not implanted (Chamayou et al., 2013; Kirkegaard et al., 2013b) and euploid/aneuploid (Rienzi et al., 2015) embryos for all the morphokinetic parameters analyzed, at both embryo and blastocyst levels. However, although to date there is the lack of a universally applicable algorithm (Kirkegaard et al., 2013b; Kramer et al., 2014; Basile et al., 2015; Fréour et al., 2015), time-lapse technology has the potential to simplify workflow in the laboratory (Kaser and Racowsky, 2014), being particularly useful in the deselection of embryos showing greatly abnormal development (Liu et al., 2015, 2016). Anyway, evidence of improved clinical outcomes applying this costly technology is still to be obtained (Armstrong et al., 2015), and although there seems to be a relationship between the ploidy status and blastocyst morphology/development dynamics, at this moment morphological and morphokinetic evaluations cannot replace the invasive method of embryo biopsy.

Recently, a variety of technologies for comprehensive chromosome analysis have been developed and are currently available for clinical use (Fragouli et al., 2011; Scott et al., 2012; Fiorentino et al., 2014; Bono et al., 2015). It has been reported that TE biopsy does not impair human embryonic implantation potential and is less invasive compared to blastomere biopsy (Scott et al., 2013b; Minasi and Greco, 2014). In addition, TE karyotype has been demonstrated to be an excellent predictor of ICM karyotype (Johnson et al., 2010). PGS performed at the blastocyst stage with whole-genome screening seems to be a unique procedure, able to provide an accurate assessment of embryo ploidy while maintaining high implantation potential and reducing the time to pregnancy (Scott et al., 2013a,b; Fragouli et al., 2014; Minasi and Greco, 2014). This technology allows performing a single embryo transfer, therefore reducing the multiple pregnancy rates while maintaining the cumulative success rate (Ubaldi et al., 2015). Moreover, a freeze-all strategy, in which all embryos are cryopreserved after the biopsy waiting for the genetic result to be produced, allows a better synchronization with the endometrium, hence reducing the risks of miscarriage and biochemical pregnancy. This strategy prevents the risk of ovarian hyperstimulation syndrome since the subsequent transfer can be performed in a natural cycle, a low-cost and patient-friendly procedure (Pelinck et al., 2002).

The miscarriage and ongoing pregnancy rates obtained in this study are comparable to those that have been routinely achieved without the use of the high-technology methods of PGS or morphokinetic evaluation of embryos. Our results, therefore, suggest that embryo evaluation by PGS or time-lapse imaging, does not enhance *per se* embryo or oocyte quality and may not improve IVF outcome. Indeed similar clinical results, in terms of ongoing pregnancy and miscarriage rates, are reported also in double untested embryo transfers (Ubaldi et al., 2015; Kang et al., 2016). Consequently, the PGS or time-lapse imaging methodologies do not improve general outcomes in the overall patient population. Moreover, considerable controversy still remains about the clinical and economic effectiveness of the PGS approach, and well-designed prospective studies, taking into account different patient characteristics and clinical practice, are still necessary (Lee et al., 2015).

Supplementary data

Supplementary data are available at http://humrep.oxfordjournals.org/.

Authors' roles

M.G.M. is the director of the laboratory, coordinated all the embryological laboratory procedures, was involved in the study conception and design, data analysis and drafting manuscript. A.C. played a central role in laboratory procedures, data analysis and final approval. T.R. and A.R. played a central role in laboratory procedures and final approval. V.C. played a central role in laboratory procedures, in the critical revision of the article and final approval. F.S. (Filomena Scarselli) played a central role in laboratory procedures and final approval, and F.S. (Francesca Spinella) was involved in the genetic analysis and final approval. F.F. was involved in the genetic analysis and final approval. M.T.V. was involved in stimulation protocols, in surgical procedures and final approval. E.G. was involved in stimulation protocols and final approval.

Funding

This work has not received any external financial support from any commercial company.

Conflict of interest

No conflict of interest has to be declared by any of the authors regarding the material discussed in the manuscript.

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