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ARTICLE

No evidence of association between blastocyst aneuploidy and morphokinetic assessment in a selected population of poor-prognosis patients: A longitudinal cohort study

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Laura Rienzi, Senior Clinical Embryologist, has 20 years experience in the field of assisted reproductive technology. She has academic degrees in biology and reproductive medicine, and has written almost 100 articles, reviews and book chapters. Laura is President of the Italian Society of Reproductive Embryology and Research, and Laboratory Director of four IVF centres in Italy. Her current areas of interest include human embryo culture, studies of gamete, zygote and embryo, as well as cryopreservation. Laura has played a key role in the clinical application of oocyte vitrification in Italy.

Abstract Recent studies involving a limited number of patients have indicated a correlation between aneuploidy and various morphokinetic parameters during preimplantation development. The results among different groups, however, have been inconsistent in identifying the parameters that are able to predict chromosomal abnormalities. The aim of this study was to investigate whether aneuploidy of human blastocysts was detectable by specific morphokinetic parameters in patients at increased risk of aneuploidy because of advanced maternal age, history of unsuccessful IVF treatments, or both. A longitudinal cohort study was conducted using 455 blastocysts from 138 patients. Morphokinetic features of preimplantation development were detected in a timelapse incubator. Blastocysts were subjected to trophectodermal biopsy and comprehensive chromosomal screening. Analyses were conducted by means of logistic mixed-effects models, with a subject-specific intercept. No statistical correlation between 16 commonly detected morphokinetic characteristics of in-vitro embryo development and aneuploidy was found. Results suggest that morphokinetic characteristics cannot be used to select euploid blastocysts in poor-prognosis patients regarded as candidates for pre-implantation genetic screening.

KEYWORDS: biopsy, blastocyst, PGS, qPCR, time-lapse

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Introduction

Improvement of culture conditions, extended culture and elective single blastocyst transfer were major achievements in human assisted reproduction during the past decade, resulting in a considerable reduction of twin pregnancies while increasing implantation and maintaining pregnancy rates. Results, however, are still far from optimal. It has been revealed that the aneupolidy rate of in-vitro produced embryos can exceed 60% (Fragouli et al., 2011), resulting in implantation failures or spontaneous abortions. Advanced maternal age, a common problem in human assisted reproduction, further increases the risk of aneuploidy. Consequently, a safe method of selecting euploid blastocysts with high in-vivo developmental competence would be crucial to improve overall efficiency further.

The most commonly used approach for blastocyst selection is the application of semiquantitative grading system based on the static inverted microscopic view before transfer (Gardner and Sakkas, 2003; Gardner and Schoolcraft, 1999). Although routinely applied worldwide, the limitations of this method to detect aneuploidy have been revealed and new approaches sought. Definitive diagnosis with high accuracy can be obtained with blastocyst biopsy (Capalbo et al., 2013; de Boer et al., 2004; Jansen et al., 2008; McArthur et al., 2005) and comprehensive chromosomal screening (CCS) for aneuploidies. Although blastocyst biopsy was found to be harmless, and the intervention increased implantation and delivery rates considerably (Scott et al., 2013a), the invasiveness of the procedure and the complexity of the task still discourage many specialists, and search for alternative, non-invasive solutions has been intensified (Cohen et al., 2013).

Morphokinetic analysis of embryo development *in vitro* has become one of the most attractive advances of our decade in human embryology. Sophisticated timelapse equipment and new culture conditions allow uninterrupted embryo development, continuous control over the cohort and detailed analysis of various events (e.g. syngamy, cleavages, compaction and blastulation). Studies have increasingly focused on the possible value of these parameters to detect aneuploidies and to indicate implantation potential. Various correlations have been found (Campbell et al., 2013a, 2013b; Chamayou et al., 2013; Chavez et al., 2012; Meseguer et al., 2011; Wong et al., 2010), but conclusions are controversial (Kaser and Racowsky, 2014).

The aim of this study was to investigate the following: the correlation between individual morphokinetic time-lapse parameters of in-vitro embryo development; molecular karyotype investigated by trophectoderm biopsy and 24 chromosomes screening based on quantitative polymerase chain reaction; and implantation rate after single blastocyst transfer in a selected group of patients prone to aneuploidies. Moreover, the reproducibility of previous published algorithms based on different morphokinetic parameters to predict embryo aneuploidy was also analysed.

Materials and methods

Study design, target population and outcome measures

In this longitudinal cohort study, 455 blastocyts obtained from 138 consecutive patients undergoing an intracytoplasmic sperm injection (ICSI) cycle with preimplantation genetic screening (PGS) at the GENERA Centre, Rome, recruited between December 2012 and December 2013 were included. Infertile patients of advanced maternal age (>36 years) ($n = 102$), with a history of unsuccessful IVF treatments (more than two failed IVF cycles) ($n = 16$), previous spontaneous abortion (more than two spontaneous abortions) ($n = 20$), or all three, were offered PGS.

All embryos were individually cultured in a timelapse incubator (EmbryoScope, Unisense, Denmark) from the insemination procedure up to blastocyst development. All biopsies and subsequent cryopreservations were carried out at blastocyt stage. Full morphokinetic information (from pronuclear formation up to blastocyst expansion) and chromosomal status (as assessed by comprehensive chromosomal screening) were obtained for each included blastocyst. At warming, single euploid blastocyst transfers were carried out. Implantation rate was defined as number of fetuses with heart activity beyond 12 weeks of gestation per transferred embryo.

Blastocysts were classified as euploid (normal chromosomal complement) or aneuploid (presence of monosomy or trisomy). Aneuploid embryos were further separated as single or complex aneuploid ones (with two or more chromosomal errors in the trophectoderm cell samples for the latter). The relationship between morphokinetic data and euploidy was then assessed. Moreover, euploid implanted embryos were compared with euploid not implanted embryos for all morphokinetic parameters.

The study and the informed consent were approved by the Institutional Review Board of the Clinic on 14 August 2014.

Sample size

In this equivalence study, the power of the statistical tests were asessed on the basis of the planned sample size. A minimal clinically significant effect for each predictor, according to Cohen (1988) convention, was given by an odds ratio of 1.49 for the increase of one standard deviation. At a significance level of 5%, our samples gave a power of 95% to detect an odds ratio of 1.41 or more for each standard deviation. The resulting minimal odds ratio per unit increase of each predictor was often very close to 1 (**Appendix: Supplementary Table S1**). Consequently, a lack of significance at the 5% level could be interpreted as equivalence (i.e. a true odds ratio above the minimally clinically significant level only with 5% or lower probability). Even at the Bonferroni corrected level of $5/16 = 0.31\%$, our sample gave a satisfactory power of 80%, and similarly minimally clinically significant odds ratios only slightly larger.

Ovarian stimulation, oocyte collection, denudation, insemination and embryo evaluation

Two protocols were used for ovarian stimulation: gonadotrophin-releasing hormone (GnRH) agonist long protocol and GnRH-antagonist protocol, as described previously (Ubaldi et al., 2010).

Details of laboratory procedures have been described previously (Rienzi et al., 2010). Briefly, oocytes were collected

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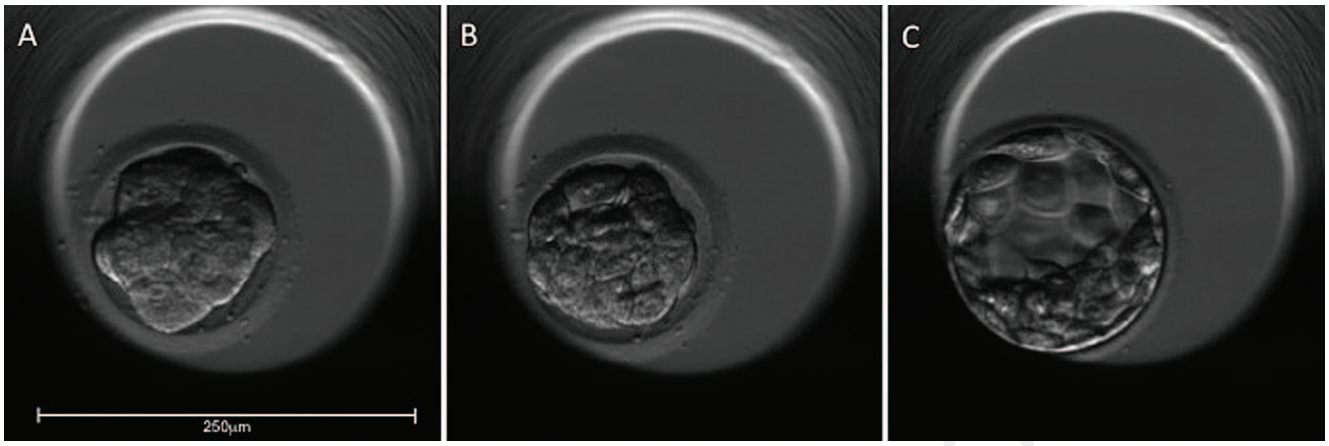

Figure 1 Time-lapse imaging of a forming blastocyst. (A) begining of the compaction process; (B) begining of the blastulation process; and (C) formation of the full blastocyst and begining of the expansion process (<10% diameter increase). Grading performed according to Campbell et al. (2013).

at 35 h after HCG administration. After 24-h incubation, cumulus-oocyte complexes were exposed to 40 IU/ml hyaluronidase solution in fertilization medium (Sage In-Vitro Fertilization, Cooper-Surgical Inc., Trumbull, CT, USA), and the corona radiata was removed mechanically with plastic pipettes of defined diameters (denuding pipettes; COOK Ireland ltd, Limerick, Ireland) in a controlled CO2 and temperature environment (Unica, IVF Tech, Denmark). Insemination of oocytes by ICSI was carried out immediately after denudation. Each inseminated oocyte was then placed in 25 µl of culture media covered by pre-equilibrated mineral oil (Quinn's Advantage®, Cleavage Medium, Cooper Surgical) in a microwell of the EmbryoSlide, and loaded into the EmbryoScope (Unisense, Denmark). The integrated microscope of the EmbryoScope was programmed to acquire images of each oocyte/embryo every 15 min through seven different focal planes. Embryo culture was carried out until blastocyst development in 6% carbon dioxide and 5% oxygen tension with blastocyst medium change over carried out on day 3 after fertilization (Quinn's Advantage®, Blastocyst Medium, Cooper Surgical).

Time-lapse images were used for the assessment of fertilization, embryo morphology and timing of developmental events up to the point of biopsy. Various morphokinetic parameters were assessed at cleavage stage: time between the end of the ICSI procedure and syngamy, completion of cleavage to two, three, four, five and eight cells (T2, T3, T4, T5, T8, respectively); length of the first, second and third cell cycle (CC) (CC1, CC2, and CC3 respectively); and synchrony in the division from three to four and five to eight cells (S2 and S3, respectively). Standard blastocyst morphological assessment was carried out according to the criteria reported by Gardner and Schoolcraft (1999).

The blastocysts were evaluated according to the degree of expansion, quality of the inner cell mass and of the trophectoderm cells. The inner cell mass was evaluated according to the number of cells, and the degree of compaction, whereas the trophectoderm cells were evaluated according to the number, dimension of the cells and the appearance of the epithelium (cohesive or loose).

Morphokinetic parameters related to blastocyst formation were also recorded and, in particular, initiation of compaction, initiation of blastulation and completion of blastulation, respectively (Figure 1).

Data were recorded by three different certified senior embryologists with no information about the embryos chromosomal status. The consistency of embryo scoring between the different embryologists was validated before starting the study. In accordance with a recent study (Sundvall et al., 2013), time-lapse parameters showed high intra- and inter-observer correlation. Each annotation used for this study was carried out by two of the abovementioned three embryologists, and mean values were used for the data analysis (Appendix: **Supplementary** Table S2).

Blastocyst biopsy and pre-implantation genetic screening

At 120–160 h from insemination, all expanded blastocysts, independently from standard morphology quality, underwent trophectoderm biopsy. In particular, all blastocysts with a visible blastocoele where an inner cell mass could be identified and with at least a few cells forming the trophectoderm epithelium, were included.

All biopsy procedures were conducted on a heated stage in a dish prepared with three droplets of 10 µl of HEPES-buffered medium (Quinn's Advantage®, Cooper Surgical) overlaid with pre-equilibrated mineral oil. A diode laser (Research Instruments, Cornwall TR11 4TA, UK) was used to assist the opening of a 10–20 µm hole in the zona pellucida. Five to 10 trophectoderm cells were then aspirated into the trophectoderm biopsy pipette (Research Instruments) followed by laser-assisted removal of the target cells from the body of the embryo.

Aneuploidy screening of trophectoderm biopsies

Trophectoderm biopsies were sent to a reference genetic laboratory for the analysis (GENETYX srl, Marostica, Italy). All samples were processed for CCS by placing them in an alkaline lysis buffer and performing real-time polymerase chain

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reaction as previously described by Treff et al. (2012). In brief, multiplex amplification of 96 loci (four for each chromosome) was carried out, and a method of relative quantitation (Schmittgen and Livak, 2008) was applied to predict the copy number status of each chromosome. This methodology was designed to specifically identify whole chromosome, not segmental, aneuploidy, and was validated in preclinical (Treff et al., 2012) and clinical studies (Scott et al., 2013b). A karyotype prediction was made for each embryo by a certified cytogeneticist.

Blastocyst vitrification and warming procedures

Vitrification and warming procedures used for our study have been described by Cobo et al. (2008). Biopsied blastocysts were vitrified by using the Cryotop device and solutions (Kitazato BioPharma Co., Japan). The first equilibration was carried out in 7.5% ethylene glycol and 7.5% dimethylsulphoxide at room temperature for 12–15 min. Subsequently, blastocysts were transferred into 15% ethylene glycol, 15% dimethylsulphoxide and 0.5 M sucrose for 1 min, then placed on the film strip of the Cryotop in a single small drop. The excess solution was removed to leave just a thin layer around each embryo, and the Cryotop was submerged into liquid nitrogen, the strip was covered with the cap and the sample was stored submerged in liquid nitrogen.

At warming, the cap was removed under liquid nitrogen and the film strip of Cryotop was quickly submerged into 1 ml of 37°C warming solution containing 1.0 M sucrose for 1 min, then blastocysts were transferred to a room temperature solution containing 0.5 M sucrose, and incubated for 3 min. After two subsequent washing in basic medium at room temperature for 6 min each, blastocysts were placed into 1 ml culture medium (Cleavage medium, Sage). Surviving blastocysts were then transferred singularly in the course of a natural cycle. The luteal phase was supported by vaginal micronized progesterone, 400 mg/day (Progeffik 200 mg, Effik, Cinisello Balsamo, Milan, Italy) starting on the day of oocyte retrieval and warming.

Statistical analysis

Continuous data were expressed as median +/− inter quartile range, and categorical data as percentages. The relationship between each of the 16 predictors and the categorical outcomes was assessed by means of bivariate generalized mixed models. Outcome probabilities were parameterized by means of local logit transformations, building a linear logistic model. To take account of dependence arising from different blastocysts collected from the same woman, a Gaussian subject-specific intercept was used. All effects were adjusted for age of the individual.

A hierarchical classification of euploid embryos was built on the basis of criteria recently described by Basile et al. (2014): interval t5-t2 and the duration of the third cell cycle (CC3). The algorithm classified embryos into four categories based on the expected percentage of chromosomally normal embryos. A parametric bootstrap method was used to compute significance. A parametric bootstrap was used to plan the sample size. All analyses were carried out using the statistical software R version 2.14.2 (Free Software Foundation, Inc., Boston, MA).

Results

Comprehensive chromosome screening of the 455 blastocysts found euploidy in 186 (40.9%), single aneuploidy in 167 (36.7%) and complex aneuploidies in 102 (22.4%). Morphokinetic parameters of preimplantation embryo development described in **Table 1** were similar between euploid

Table 1 Comparison of morphokinetic parameters between euploid and aneuploid embryos.

	Euploid (n = 186)				*Aneuploid (n = 269)*			
	Median	*Interquartile range*	*Minimum*	*Maximum*	*Median*	*Interquartile range*	*Minimum*	*Maximum*
imumSyngamy	24.06	3.97	13.64	35.80	24.11	4.00	17.60	37.23
T2	26.61	4.53	20.32	38.80	26.63	4.36	20.13	50.40
T3	37.48	5.64	23.5	57.40	37.74	5.20	23.91	62.41
T4	38.62	6.10	27.49	58.15	39.17	5.91	27.92	63.16
T5	51.15	7.27	33.52	89.03	51.83	8.48	34.48	93.49
T8	59.83	13.47	43.69	110.12	58.46	14.41	41.50	111.99
CC1	2.50	0.75	0.5	9.14	2.50	0.51	0.76	13.17
CC2	11.5	1.96	0.26	19.10	11.64	1.82	0.25	16.26
S2	0.75	1.01	0.24	14.48	0.75	1.21	0.25	17.28
S3	5.91	13.26	0.5	43.53	6.01	13.26	0.66	39.52
CC3	13.95	3.30	0.5	31.63	13.66	3.50	0.25	43.00
CC3/CC2	1.20	0.23	0.05	40.38	1.20	0.28	0.02	51.66
T5 to T2	25.19	4.62	3.25	50.73	25.27	4.93	2.26	55.75

Syngamy,T2, T3, T4, T5, T8 is the time (h) between intracytoplasmic injection and syngamy, two, three, four, five and eight-cell stage, respectively; CC1, CC2, CC3 is the length (h) of the first, second and third cell cycle, respectively; S2, S3 is the synchrony (h) in division from three to four and five to eight cells, respectively. No statistically significant differences were found between the two groups.

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Table 2 Comparison of timings of compaction and blastocyst development between euploid and aneuploid embryos.

	Euploid (n = 186)				*Aneuploid (n = 269)*			
	Median	*Interquartile range*	*Minimum*	*Maximum*	*Median*	*Interquartile range*	*Minimum*	*Maximum*
Initiation of compaction	90.35	9.88	61.29	123.1	91.07	12.12	63.59	118.74
Initiation of bastulation	103.77	10.84	86.29	138.77	102.52	11.54	68.11	143.92
Completion of bastulation	117.05	15.64	98.31	162.82	117.32	14.64	94.33	163.51

Initiation of compaction and initiation and completion of bastulation is time (h) between intracytoplasmic sperm injection and initiation of compaction, initiation of blastulation, and completed blastulation, respectively. No statistically significant differences were found between the two groups.

and aneuploid embryos. The time-period from ICSI to start of compaction, start of blastulation and completed blastulation did not differ between the two groups (**Table 2**). Blastocyst aneuploidy rate was significantly correlated with advancing female age ($P < 0.01$). Logistic regression analysis adjusted for female age did not find correlation between aneuploidy and the investigated morphokinetic parameters (**Table 3**). Euploid and aneuploid blastocysts had a similar pattern of distribution when the time of onset of blastulation was shown in relation to the time of completed blastulation as suggested by Campbell et al. (2013a, 2013b) (**Figure 2**). Moreover, when embryos were divided into four categories according to the hierarchical classification proposed by Basile *et al.* (2014), based on the interval between t5 and t2, and the duration of the third cell cycle (CC3), no significant difference in the percentage of chromosomally normal embryos was found (**Figure 3**). In particular, the embryo was categorized as A or B if the value of t5 to t2 was over 20.5 h; on the other hand, if the value was outside this range, the embryo was categorized as C or D. Moreover, if the value of CC3 fell inside the range 11.7–18.2 h, the embryo was categorized as A or C depending on t5 to t2; if the value of CC3 fell outside the range, the embryo was categorized as B or D depending on t5 to t2.

Table 3 Logistic regression analysis adjusted for female age: relationship between morphokinetic parameters and comprehensive chromosomal screening data of blastocysts categorized as euploid or aneuploid.

Variables	*Odds ratio*	*Confidence interval low 95%*	*Confidence interval up 95%*
Syngamy	0.016	−0.046	0.077
T2	0.01	−0.048	0.068
T3	0.02	−0.023	0.063
T4	0.01	−0.028	0.055
T5	0.01	−0.017	0.036
T8	0.00	−0.018	0.019
CC1	−0.055	−0.283	0.174
CC2	0.037	−0.028	0.102
S2	−0.009	−0.075	0.056
S3	−0.003	−0.023	0.018
CC3	0.011	−0.034	0.055
CC3/CC2	0.025	−0.067	0.017
T5-T2	0.011	−0.021	0.044
Initiation of compaction	0.004	−0.018	0.026
Initiation of bastulation	−0.004	−0.025	0.018
Completion of bastulation	0.012	−0.006	0.030

Syngamy,T2, T3, T4, T5, T8 = time (h) between ICSI and syngamy, two, three, four, five and eight-cell stage, respectively; CC1, CC2, CC3 = length (h) of the first, second and third cell cycle, respectively; S2, S3 = synchrony (h) in the division from three to four and five to eight cells, respectively; initiation of compaction, initiation of bastulation and completion of bastulation is time (h) between ICSI and initiation of compaction, initiation of blastulation, and completed blastulation, respectively. No statistically significant correlations were identified.

Direct cleavage (second cell division in less than 4 h) was found in 13 embryos: five out of 186 (2.7%) were euploid, four out of 167 (2.4%) were with single aneuploidy, and four out of 102 (3.9%) were with complex aneuploidies.

Finally, irregular cell divisions (transition from 1- to 3- and 2- to 5-cell stage) were found in two out of 455 (0.4%) blastocysts, one of them was euploid and one aneuploid.

A total of 101 euploid blastocysts were transferred individually after warming, and 55 transfers resulted in a positive HCG outcome (54.5%). Among them, four resulted in biochemical pregnancies. Two spontaneous abortions were recorded before 12 weeks of gestation. A total of 49 blastocysts resulted in an ongoing implantation (>20 gestational weeks) and live birth (48.5%).

Discussion

The chance of pregnancy decreases with female age after natural conception and IVF (Fauser, 2008a). Data from PGS suggest that most pre- and post-implantation losses are associated with chromosomal abnormalities, predominantly aneuploidies in gametes and embryos (Campbell et al., 2013b; Fragouli and Wells, 2012; Fragouli et al., 2011; Macklon et al., 2002). With advanced maternal age, aneuploidy rates increase dramatically, in parallel with decreased implantation rates (Munne et al., 2007; Scott et al., 2012). A reliable method for selecting single euploid blastocysts is, therefore, crucial for successful single embryo transfer to increase implantation and decrease multiple pregnancy rates.

Currently, PGS is the only available diagnostic procedure to detect aneuploidies. Although the basic technology has been available since 1990 (Handyside et al., 1990), until recently no convincing evidence was available on it efficiency (i.e. increase pregnancy and birth rates) (Audibert et al., 2009; Fauser

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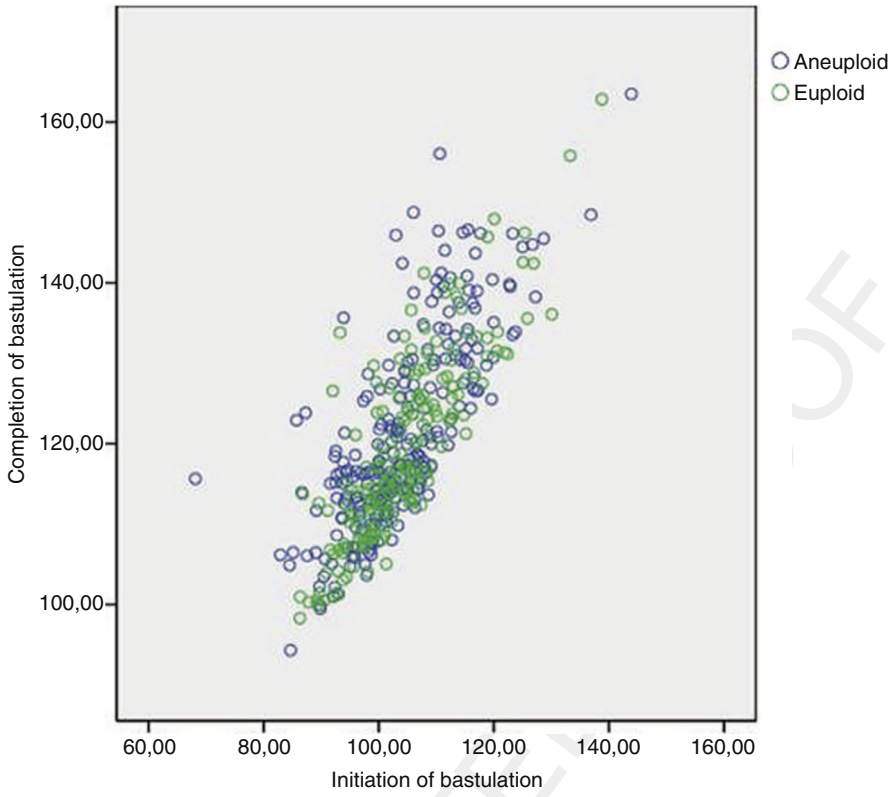

Figure 2 Distribution of euploid (green dots) and aneuploid (blue dots) embryos according to time (h) between intracytoplasmic sperm injection and completed versus initiated blastulation as suggested by Campbell et al. (2013).

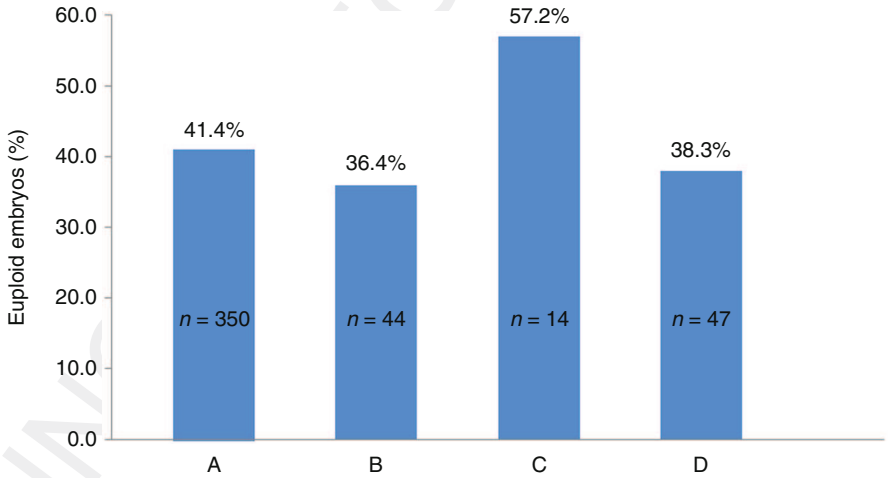

Figure 3 Percentage of euploid embryos according to hierarchical classification as suggested by Basile et al. (2014). The embryo is categorized as A or B if the value of completion of cleavage from two to five cells is more than 20.5 h; on the other hand, if the value is outside this range, the embryo is categorized as C or D. Moreover, if the value of the third cell cycle fell inside the range 11.7–18.2 h, the embryo is categorized as A or C depending on completion of cleavage from two to five cells; if the value of the third cell cycle fell outside the range, the embryo was categorized as B or D depending on completion of cleavage from two to five cells.

2008b; Harper et al., 2010; Practice Committee of Society for Assisted Reproductive Technology and Practice Committee of American Society for Reproductive Medicine, 2008). Retrospectively, most of the problems could be attributed to the inappropriate biopsy technology, low coverage screening methods and inadequate embryo culture conditions (Fragouli and Wells, 2012; Fragouli et al., 2011; Kirkegaard et al., 2012b; Ly et al., 2011; Scott et al., 2013c; Xu and Montag, 2012). Recently, it has been revealed that blastocyst biopsy does not compromise the developmental competence of embryos (Scott

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et al., 2013b), and, in combination with comprehensive chromosome screening, is highly predictive of developmental potential of blastocysts (Scott et al., 2012). This upgraded form of PGS significantly improves implantation both with fresh transfer or after vitrification (Schoolcraft and Katz-Jaffe, 2013; Scott et al., 2013b). Accordingly, despite the latest suggestions from practice committees, increasing number of laboratories offer PGS either for a narrow or wide segment of infertile patients (Meldrum, 2013).

The costs of PGS are considerable, as it is an invasive procedure and requires a well-equipped laboratory to exploit fully its potential (Ly et al., 2011; Meldrum, 2013). As the commonly used static morphological assessment is inappropriate for this purpose (Alfarawati et al., 2011; Forman et al., 2012), extensive efforts were made to find a non-invasive and reliable approach to predict the probability of euploidy and high developmental competence with high accuracy. Among the various methods used for this purpose reviewed recently by Montag et al. (2013), continuous observation of morphokinetic parameters by a purpose-built time-lapse equipment has become the most popular approach spreading rapidly among human IVF laboratories worldwide. It has been revealed that time-lapse monitoring does not compromise embryo development (Cruz et al., 2011; Kirkegaard et al., 2012a; Nakahara et al., 2010), and evaluation of morphokinetic data can be carried out with high consistency between observers (Sundvall et al., 2013).

By far the most important potential application of time-lapse investigations is the selection of the most competent embryo(s) for fresh transfer, cryopreservation, or both. Even before the application of time-lapse machines, various parameters of pre-compaction stage development were suggested to predict in-vitro developmental compentence (Scott et al., 2007), implantation potential (Lawler et al., 2007) or euploidy (Finn et al., 2010; Magli et al., 2007). Since the first reported baby born after embryo selection based on time-lapse parameters (Pribenszky et al., 2010), extensive research has been conducted to establish the most relevant morphokinetic parameters to predict embryo viability. Parameters investigated include duration of time of visible pronuclei, syngamy, T2, T3, T4, T5, T8, CC1, CC2, CC3, s2, s3, initiation of compaction, initiation of bastulation and completion of bastulation. All these parameters are reported to have an optimum range, indicating good developmental potential (Campbell et al., 2013a, 2013b; Chamayou et al., 2013; Chavez et al., 2012; Conaghan et al., 2013; Cruz et al., 2012; Dal Canto et al., 2012a; Hashimoto et al., 2012; Hlinka et al., 2012; Kirkegaard et al., 2013; Lemmen et al., 2008; Meseguer et al., 2011; Rubio et al., 2012; Wong et al., 2010). Negative signs included multinucleation, micronuclei, fragmentation, blastomere asymetry, and direct cleavage from 1 to three cells (Chavez et al., 2012; Kirkegaard et al., 2013; Meseguer et al., 2011; Montgomery et al., 2013). Some of these parameters were investigated individually, others in groups or in a hierarchic classification procedure.

Most studies have focused on the pre-compaction period, and the intention was to predict further development *in vitro*. Observed parameters and identified correlations show a wide variation between investigators. Contradictory results on the relation of morphokinesis and in-vivo developmental competence have also been published. Meseguer et al. (2011) have observed strong positive and negative correlations between various parameters of early embryo development and implantation. Dal Canto et al. (2012b) found a correlation between short early cell cycles and high implantation potential. Other publications found only one or two parameters indicative for in-vivo developmental potential, the duration of the first cytokinesis (Kirkegaard et al., 2013); length of the third cell cycle (Chamayou et al., 2013); and time from insemination to onset and completion of blastulation (Campbell et al., 2013a). According to Hong et al. (2013) early times of cavitation were associated with reduced prevalence of aneuploidy, although the magnitudes were modest. In a recent study, Herrero et al. (2013) found a correlation between various pre- and post-implantation outcomes and preimplantation morphokinetic parameters.

Efforts were also made to relate morphokinetic patterns to the chromosomal status of embryo. By analysing frozen-thawed human zygotes for 2 days with a dark-field timelapse microscopy, Chavez et al. (2012) observed diversity in cleavage parameters, blastomere asymmetry and fragmentation in aneuploid embryos. Data on a small cohort of embryos, presented by Davies et al. (2012) in an abstract, described delayed cleavage division and prolonged transition between 2- and 4-cell stages in aneuploid embryos (Davies et al., 2012). Basile et al. (2014) reported, analysing a similar sample size as the one used in the present study, an increased probability of selecting chromosomally normal embryos using an algorithm based on the interval between t5 and t2, and the duration of CC3 (Basile et al., 2014). The algorithm developed in the present study, however, was not prospectively tested in a validation cohort of embryos proving limited evidence of clinical translation so far. In-vitro studies of Hong et al. (2013) and Melzer et al. (2013) found an association between reduced prevalence of aneuploidy and early times of cavitation or short duration of compaction, respectively. Campbell et al. (2013a) detected delayed initiation of compaction and blastulation in aneuploid embryos. In a subsequent study, based on similar criteria (time from insemination to the onset and completion of blastulation; initiation and completion of bastulation, respectively) correlations between these parameters and fetal heart beat were found, and live birth rates were found to be attributed to aneuploidy detectable by timelapse investigation (Campbell et al., 2013b). As emphasized in a letter to the Editor, Ottolini et al. (2014), answered by Campbell et al. (2014), raised serious concerns about the usefulness of the hypothesized triple correlation (delayed blastulation, low implantation potential, aneuploidy) for aneuploidy prediction in a given cohort. With standard morphological investigation and trophoectoderm CCS analysis, no correlation was found between faster compared with slower growing embryos and aneuploidy in the same age group (Capalbo et al., 2014). Female age may have a considerable effect on developmental competence (Cohen et al., 2012), implantation potential (Scott et al., 2012), and blastocyst formation (Porter et al., 2002). Accordingly, a correlation was found between aneuploidy and implantation potential in a non-age controlled study, population may not be a proof that timelapse predicts euploidy among embryos within the same IVF cycle. Furthermore, a recent prospective study with sibling embryos and combined time-lapse microscopy and array comparative genomic hybridization analysis found no significant differences between aneuploid and euploid embryos in the observed 10 morphokinetic parameters (Yang et al., 2014).

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According to a recent systematic review (Kaser and Racowsky, 2014), in spite of the considerable potential of time-lapse monitoring, no high-quality data support its use for selection of human preimplantation embryos.

In the present cohort study conducted on 455 blastocysts in a selected population with increased maternal age, history of unsuccessful IVF treatments, or both, no correlation was found between aneuploidy and the observed 16 morphokinetic parameters from ICSI to completed blastulation. Furthermore, in contrast to previous publications, a similar distribution of euploid and aneuploidy blastocyst in relation to time from insemination to the onset and completion of blastulation (**Figure 2**), and to the classification model proposed by Basile et al., (2014) (**Figure 3**) was found. Moreover, the proportion of euploid embryos was higher in our dataset compared with that reported by Basile et al., 2014. This difference is probably a result of different biopsy technique and CCS method used. Day 3 biopsy (in contrast to blastocyst biopsy) and single blastomere analysis used by Basile et al. (2014) is known to be subject to more mosaicism and particularly to a higher false positive error rate owing to single cell analysis (Van Echten-Arends et al., 2011). In the present study, the aneuploidy screening used did not allow a distinction to be made between meiotic- and mitotic-derived aneuploidies. This could be a key point for future studies to specifically investigate the role of precompaction morphokinetic parameters in predicting mosaic aneuploidies. It cannot be excluded from our data that mosaic embryos may display a distinct precompaction morphokinetic profile as a consequence of the occurrence of a chromosomal error during cleavage stage mitotic divisions. In this case, morphokinetic evaluation could be of great value in assisting data analysis and interpretation of blastocyst stage PGS programmes.

In conclusion, the correlation between morphokinetic parameters up to the blastocyst stage and aneuploidy diagnosed by trophectoderm biopsy and comprehensive chromosomal screening could not be confirmed. The difference between our findings and that of other groups may be related to variations between laboratories and embryo culture systems as emphasized by Montag (2013). On the basis of these contradicting outcomes, we suggest caution when timelapse results are connected to chromosomal status of the embryo, and urge further, large scale multicentre studies to clarify the possible relation.

Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.rbmo.2014.09.012.

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