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ARTICLE

Morphokinetic parameters in chromosomal translocation carriers undergoing preimplantation genetic testing

BIOGRAPHY

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KEY MESSAGE

Although significant morphokinetic differences exist between balanced and unbalanced embryos in translocation carriers undergoing preimplantation genetic testing for structural rearrangement cycle without aneuploidy screening, no relevant morphokinetic predictor of embryo chromosomal status could be found.

ABSTRACT

Research question: Can embryo morphokinetic parameters help identify unbalanced embryos in translocation carriers?

Design: This retrospective study was conducted in 67 translocation carriers undergoing 105 preimplantation genetic testing cycles for chromosomal structural rearrangements (PGT-SR) without aneuploidy screening (PGT-A). Using time-lapse imaging analysis, morphokinetic parameters of balanced and unbalanced embryos were compared, as well as the frequency of abnormal cellular events. The performance of a previously published prediction model of aneuploidy was also tested in this population.

Results: Significant differences were observed between balanced and unbalanced embryos for some morphokinetic parameters: t5 ($P = 0.0067$), t9+ ($P = 0.0077$), cc2 ($P = 0.0144$), s2 ($P = 0.0003$) and t5–t2 ($P = 0.0028$). Also, multinucleation at the two- or four-cell stages, abnormal division and cell exclusion at the morula stage were significantly (all $P < 0.05$) more frequent in unbalanced than in balanced embryos. None, however, could accurately predict embryo chromosomal status. A previously published morphokinetic prediction model for embryo aneuploidy did not adequately classify balanced and unbalanced embryos.

Conclusions: No significant morphokinetic predictor of chromosomal status could be found. Time-lapse should not be used as a diagnostic tool for chromosomal status in translocation carriers.

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KEYWORDS

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Translocation

INTRODUCTION

Preimplantation genetic testing (PGT) is a procedure developed in the early 1990s for couples with a high risk of transmitting a genetic abnormality or with a high risk of miscarriage because of chromosomal structural rearrangement (*Harton et al., 2011*). Pre-implantation genetic testing for aneuploidy (PGT-A) consists of the evaluation of embryo ploidy, as reflected by the number of copies of each chromosome, and allows the selection of euploid embryos for transfer. Although the exact clinical benefit of PGT-A in subgroups of patients is still discussed and remains to be calculated, this approach is generally considered to result in a shorter time before pregnancy and higher pregnancy rate per transfer than conventional IVF and intracytoplasmic sperm injection (ICSI) (*Dahdouh et al., 2015*). Indeed, aneuploidy is commonly observed in human embryos obtained by IVF procedure, even in embryos developing to the blastocyst stage with good morphology, and accounts for the relatively limited implantation rate observed in human IVF cycles. The presence of chromosomal translocation in one or both partners in a couple is a situation with a particularly high risk of embryo aneuploidy. When authorized by regulation, the most recent techniques allow the simultaneous use of preimplantation genetic testing cycles for chromosomal structural rearrangements (PGT-SR) and PGT-A to optimize clinical efficiency and cost-effectiveness for couples with a genetic abnormality or chromosomal structural rearrangement (*Capalbo et al., 2016a; 2016b*). Both PGT-SR and PGT-A are based on the genetic analysis of embryonic blastomeres biopsied either at the cleavage stage (day 3) or at the blastocyst stage. Therefore, the success of PGT-SR and PGT-A greatly depends on the number and quality of the embryos available for biopsy.

Since the release of the first time-lapse system in 2009, several laboratories around the world have implemented this technology to improve embryo culture conditions and evaluate embryo quality according to various morphokinetic parameters and related algorithms (*Barrie et al., 2017*). Considering that embryo ploidy is a critical factor for implantation, but that PGT is an invasive and expensive technology, a number of authors have raised the hypothesis that the morphokinetic pattern of embryos can indirectly reflect embryo ploidy and thus be used as a surrogate for PGT-A and PGT-SR. If true, this approach could be particularly relevant in countries in which PGT-SR, PGT-A, or both, are prohibited. These studies carried out in patients referred for PGT-A because of advanced maternal age, recurrent implantation failure or recurrent pregnancy loss yielded predictive models with either no or moderate sensitivity and specificity for the identification of aneuploid embryos up to now (reviewed in *Reignier et al., 2018*). As far as we know, such studies have not been specifically conducted in translocation carriers referred for PGT-SR until now. In France, PGT is allowed for specific inherited genetic abnormalities, such as translocations, whereas PGT-A is prohibited by regulation. As translocation carriers present great risks of having unbalanced embryos (*Tobler et al., 2014*), we wondered whether these embryos would display a specific morphokinetic pattern. Therefore, our first study aim was to compare the morphokinetic parameters of balanced and unbalanced embryos in couples referred for PGT-SR. We then performed an external validation study of a published prediction model of embryo ploidy based on PGT-A results (*Basile et al., 2014*) to evaluate its performance in our local PGT-SR population referred for chromosomal rearrangement.

MATERIALS AND METHODS

Patients

This monocentric retrospective cohort study was conducted in couples referred for PGT-SR because of a chromosomal rearrangement in one of the partners. We analysed the clinical and biological data of all consecutive patients who had undergone an ICSI–PGT–SR cycle with autologous oocyte and embryo culture performed using the Embryoscope® between May 2013 and April 2016 in our University Fertility Centre. All patients gave consent for the anonymous use of their data registered in this database. This protocol was approved by the local ethics committee on 12 July 2017.

Ovarian stimulation

Before stimulation, all women had complete ovarian reserve exploration, including FSH, LH, oestradiol, anti-Müllerian hormone and antral follicle count (AFC). All patients underwent ovarian stimulation with the antagonist protocol. A gonadotrophin starting dose was chosen according to female age, ovarian reserve and previous IVF cycles, if they had been undertaken. Cycle monitoring consisted of hormonal assays and ultrasonography, and ovulation was triggered with recombinant HCG when at least three follicles reached 18 mm in diameter.

Oocyte retrieval and embryo culture for PGT

Oocyte retrieval was carried out 34–36 h later. After denudation with hyaluronidase (SynVitro® hyadase, Origio, Måløv, Denmark), all mature oocytes were microinjected and immediately placed in individual microwells within a specific culture dish (Embryoslide®, Vitrolife®, Stockholm, Sweden) before being loaded into the Embryoscope® (Vitrolife®). Embryo culture was carried out at 37°C under a controlled atmosphere with low oxygen pressure (5% O_2, 6% CO_2). Sequential media was used for embryo culture (G1plus® and G2plus®)

Time-lapse analysis

Each embryo was investigated by detailed time-lapse analysis measuring the exact timing of the developmental events in hours after ICSI procedure, as described by *Ciray et al. (2014)*. The terms t2, t3, t4, t5, t6, t7 and t8 were used for the exact timings of appearance of embryos with 2, 3, 4, 5, 6, 7 and 8 well-defined blastomeres, respectively. The mean ± SD duration of cell cycle between each cleavage was also considered. The term s2 is used to illustrate the synchrony of the second cell cycle, i.e. the transition from a two-cell to four-cell embryo. Also, cc2 is defined as the time of the second cell cycle (t3-t2) and cc3 as the time of third cell cycle (t5-t3). Abnormal division referred to chaotic cleavage, reverse cleavage or direct cleavage, all of which have been shown to lead to extremely low implantation rates (*Rubio et al., 2012; Zhan et al., 2016*).

The hierarchical model developed by Basile *et al*. (2016) was applied to all of the embryos biopsied on day 3. This model was based on the morphokinetic differences observed between euploid and aneuploid embryos and included the most relevant parameters identified in their database, i.e. t5-t2 and cc3, to classify the embryos into four categories with a decreasing probability of euploid status (A to D).

Embryo biopsy, genetic analysis and embryo transfer

Embryo biopsy was carried out on day 3 for all embryos with at least six blastomeres, less than 25% fragmentation and fair evenness. Embryos were first briefly placed in Ca/Mg-free medium (G-PGD, Vitrolife®) for a few minutes, before laser-assisted zona pellucida hatching (ZilosTK, Hamilton Thorn®, Beverly, MA, USA). One or two cells were then gently aspirated for subsequent genetic analysis depending on the number of blastomeres (one cell in six- to seven-cell embryos, two cells in embryos with eight or more cells). On average, 1.81 cells were removed per embryo. Each biopsied blastomere was lysed and the nucleus spread on a separate poly-l-lysine-coated slide. Interphase fluorescence in-situ hybridization (FISH) analyses were carried out according to the following procedures. The bacterial artificial chromosome contig probes covering 1 Mb of the subtelomeric region of the chromosomes involved in translocations were used to make FISH probes. All probes were directly labelled by nick translation with SpectrumOrange, SpectrumGreen and Diethylaminocoumarin fluorophores. First, slides were pretreated with a 0.05% pepsin solution at 37°C for 3 min to remove any remaining cytoplasmic proteins, followed by washing with PBS and serial ethanol dehydration. A mix containing 60–80 pg of probes was applied to each slide and sealed with rubber cement. Denaturation was carried out at 73°C for 3 min and hybridization at 37°C overnight. After hybridization, slides were washed in 2 x SSC/1%NP40 at 72°C for 2 min. The slides were then air-dried and mounted in Vectarshield (Vector Laboratories, USA) anti-fade medium containing 1 ng/ml 40,6-diaminidino-2-phenyolindole to counterstain the nuclei. Slides were analysed under the fluorescence microscope. The FISH signals were counted following the criteria described by *Wilton et al. (2009)*.

Balanced embryos were selected for transfer on day 4 according to post-biopsy development for practical and organizational reasons. Indeed, many patients live far away from our PGT centre and remain close to the centre after ovum retrieval up to the time of embryo transfer. Therefore, embryo transfer is generally carried out as soon as possible (day 4) to allow patients to return home quickly. Moreover, day-4 embryo transfers have been shown to perform as well as day-5 transfers (*Feil et al., 2008*). Single or double embryo transfer was chosen by a joint decision between medical staff and the couple. A pregnancy test was carried out 11 or 12 days after embryo transfer, and, if positive, clinical pregnancy was confirmed ultrasonographically 4–5 weeks later by the detection of a gestational sac and fetal heart activity.

Statistics

Student's or Wilcoxon's tests were used for continuous variables and chi-squared or Fisher's tests for categorical variables. The non-parametric Mann–Whitney test was used for non-normally distributed variables. GraphPad Prism® software was used for statistical analysis. $P < 0.05$ were considered to denote significant differences.

RESULTS

Study group

A total of 67 couples undergoing 105 PGT-SR cycles for chromosomal translocation were included in the analysis. Among them, 42 couples (62.7%) undergoing 71 cycles were referred for paternal chromosomal translocation, whereas 25 (37.3%) undergoing 34 cycles were referred for maternal translocation. The mean ± SD female and male ages were 32.0 ± 3.6 and 34.26 ± 4.87 years, respectively. The mean ± SD female BMI was 24.3 ± 4.2 kg/m^2. The average ± SD total FSH dose was 2511 ± 967 units. The average ± SD number of mature oocytes collected was 11.7 ± 5.0. A total of 1176 oocytes were microinjected and cultured in the Embryoscope®, with 749 being normally fertilized (63.7%).

Among the 480 embryos undergoing blastomere biopsy on day 3, 190 (39.6%) had nine cells or more, 196 (40.8%) had eight cells, 66 (13.8%) had seven cells and 28 (5.8%) were at the six-cell stage. A total of 427 embryos (89%) could be analysed by FISH, with 177 displaying a balanced chromosomal status (41.5%) and 250 (58.5%) being unbalanced with various chromosomal patterns. No results could be obtained in 53 embryos (11%), because of the absence of nuclei, dubious results or technical problems. Significant differences were observed between balanced and unbalanced embryos for t5 ($P = 0.0067$), t9+ ($P = 0.0077$), cc2 ($P = 0.0144$), s2 ($P = 0.0003$) and t5–t2 ($P = 0.0028$) (TABLE 1), with t5 and t9 + occurring significantly earlier in unbalanced than in balanced embryos.

According to cell cycles and synchrony, cc2 and t5–t2 were significantly longer and s2 shorter in balanced than in unbalanced embryos. The classification of embryos inside or outside of the optimal range for each of these parameters (i.e. t5, t5-t2, cc2) based on quartiles (second and third quartiles represent the optimal range) did not allow us to identify relevant thresholds with acceptable sensitivity and specificity for the identification of balanced versus unbalanced embryos (Supplementary TABLE 1). Additionally, the frequency of multinucleation at the two- or four-cell stages (31.6% [$n = 79$]) versus 19.8% [$n = 35$]; $P < 0.05$), abnormal division (32.8% [$n = 82$] versus 11.3% [$n = 20$]; $P < 0.05$) and cell exclusion at the morula stage (36.4% [$n = 52$] versus 32.8% [$n = 40$]; $P < 0.05$) was significantly higher in unbalanced embryos than in balanced embryos. The repartition of balanced and unbalanced embryos according to conventional morphological criteria is presented in Supplementary TABLE 2.

Hierarchical classification of embryos according to *Basile et al. (2014)*

In the second phase, we tested the performance of the morphokinetic predictive model for embryo aneuploidy developed in a PGT-A population and published by *Basile et al. (2014)* in our PGT-SR population of couples with chromosomal rearrangements. The proportion of balanced embryos for the chromosomes involved in the translocation was not significantly different between the four groups (46.95%, 43.90%, 26.92% and 25.93%, respectively) (FIGURE 1**A**). As we observed a similar proportion of balanced embryos in groups A and B on the one hand and in groups C and D on the other, we tested the performance of a simplified model only, including the most significant morphokinetic variable in the original publication, i.e. the t5–t2 interval (FIGURE 1**B**). We found a significantly higher proportion of balanced embryos in the group [A + B] than in the group [C + D] (46.56% versus 26.17%, respectively) (FIGURE 1**B**). The performance of this simplified model, however, was low (sensitivity 47%,

TABLE 1 MORPHOKINETIC PARAMETERS ACCORDING TO THE ABSENCE OR PRESENCE OF CHROMOSOMAL STRUCTURAL REARRANGEMENTS

	Balanced embryos (*n* = 177)		Unbalanced embryos (*n* = 250)		Significant *P*-values
	n	Mean ± SEM	*n*	Mean ± SEM	
tPB2	177	3.690 ± 0.1053	250	3.958 ± 0.1393	
tPNa	177	7.820 ± 0.1671	250	7.987 ± 0.1582	
tPNf	177	25.25 ± 0.2495	250	25.44 ± 0.2438	
T2	177	27.76 ± 0.2519	250	28.07 ± 0.2554	
T3	177	38.92 ± 0.3258	250	37.90 ± 0.3560	
T4	177	40.12 ± 0.3322	250	40.24 ± 0.3076	
T5	177	51.01 ± 0.4792	250	49.45 ± 0.4687	0.0067
T6	177	53.44 ± 0.4099	250	53.02 ± 0.4312	
T7	175	55.24 ± 0.4453	232	56.15 ± 0.5668	
T8	165	59.17 ± 0.8216	192	58.29 ± 0.7001	
T9+	144	73.99 ± 1.013	179	70.61 ± 1.011	0.0077
tPGT	177	69.87 ± 0.1207	250	70.01 ± 0.2372	
tSC	153	89.01 ± 0.7485	176	89.70 ± 0.7083	
tM	122	94.93 ± 0.8118	143	95.88 ± 0.8043	
tSB	93	103.6 ± 1.191	86	102.4 ± 1.099	
tB	56	111.0 ± 1.425	47	110.6 ± 1.447	
tEB	32	109.6 ± 1.916	25	112.0 ± 2.107	
T5–t2	177	23.25 ± 0.3989	250	21.38 ± 0.4203	0.0028
Cc2 (t3–t2)	177	11.16 ± 0.2155	250	9.829 ± 0.2768	0.0144
S2 (t4–t3)	177	1.195 ± 0.1934	250	2.343 ± 0.2488	0.0003

tB, timing of full blastocyst formation; tEB, timing of blastocyst expansion; tM, timing of fully compacted morula; tPB2, timing of extrusion of the second polar body; tPGT, timing of embryo biopsy for preimplantation genetic testing; tPNa, timing of appearance of pronuclei; tPNf, timing of pronuclei fading; tSB, timing of onset of blastocyst cavitation; tSC, timing of onset of compaction; t2 to t9+, timings of appearance of embryos with 2, 3, 4, 5, 6, 7, 8 and 9 or more well-defined blastomeres.

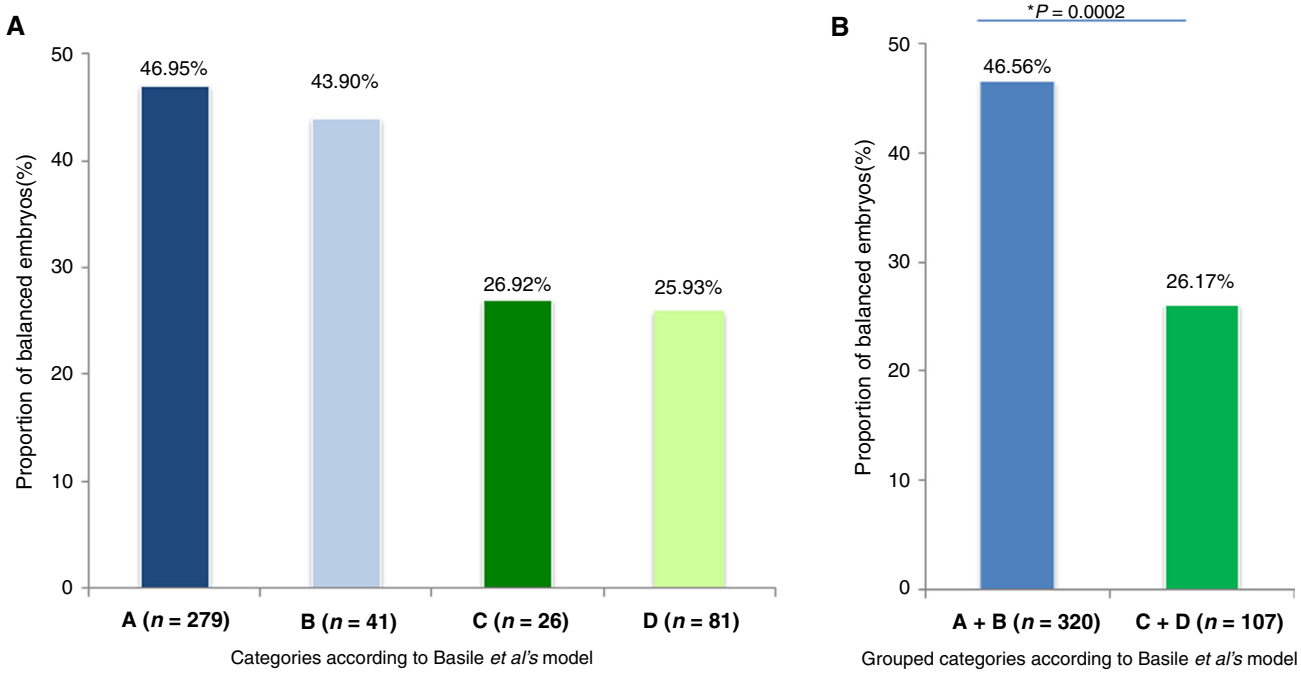

FIGURE 1 (A) Proportion of balanced embryos according to the four categories described in *Basile et al.'s model (2014)* and (B) after simplification into only two categories based on the most significant morphokinetic variable in the original publication, i.e. the t5–t2 interval.

specificity 73%, positive predictive value 84% and negative predictive value 32%).

DISCUSSION

In this study, we showed that significant morphokinetic differences exist between balanced and unbalanced embryos in translocation carriers. No significant predictor of embryo chromosomal status, however, could be identified.

Although we did not screen embryo aneuploidy but only chromosomes involved in translocation for regulatory reasons, these findings are consistent with other studies (*Campbell et al., 2013a; Mumusoglu et al., 2017*), where the association between morphokinetic parameters and embryo ploidy status using PGT-A was evaluated. Indeed, these studies demonstrated that some morphokinetic parameters were significantly different between euploid and aneuploid embryos, either in the early stages of embryo development or during the later stages (*Campbell et al., 2013a; Mumusoglu et al., 2017*). The relevance and clinical value of this strategy, however, was questioned (*Rienzi et al., 2015; Minasi et al., 2016; Reignier et al., 2018*).

As PGT-A is not allowed in France, we tested this approach in patients undergoing PGT-SR cycles for chromosomal translocation to evaluate its predictive value for chromosomal balance. We found that two cell cleavages (t5 and t9+) occurred significantly earlier in unbalanced than in balanced embryos, and that some cellular intervals (cc2, s2, t5-t2) were significantly different between unbalanced and balanced embryos. A considerable overlap, however, existed between the distribution of these variables in unbalanced and balanced embryos. Nevertheless, we used the same approach as other investigators (*Basile et al., 2014*) based on quartiles to try to build a prediction model. The classification of embryos according to their morphokinetic optimal range did not allow us to generate a relevant classification tree. We defined the optimal range as the interval between the 25th and 75th percentile, i.e. quartiles 2 and 3.

We also tested the performance of a previously published aneuploidy prediction model based on two morphokinetic parameters in our dataset of PGT-SR cycles (*Basile et al., 2014*). We found that the original version of this model had a low clinical value for the classification of balanced versus unbalanced embryos. A simplified version of the model, however, performed slightly better and allowed embryos to be grouped into two categories with significantly different chances of being balanced. The main explanation of these slightly different results probably lies within the different populations being considered. Indeed, we specifically included couples undergoing PGT because of chromosomal rearrangements in one of the partners, whereas *Basile et al. (2014)* included patients undergoing PGT-A for recurrent miscarriage and repeated implantation. Moreover, we could only look at the chromosomes involved in the translocation, not others, for regulatory reasons. Although it has been largely reported that a significant proportion of embryos obtained from translocation carriers have chromosome imbalances unrelated to the rearrangement carried in the couple (*Alfarawati et al., 2011; Fiorentino et al., 2011*), we were prevented from extensively evaluating embryo chromosomal status, whereas *Basile et al. (2014)* carried out PGT-A analysis on all 46 chromosomes. Interestingly, in our previously published validation study aimed at evaluating the performance of a pregnancy prediction model based on morphokinetic parameters, we found that a simplified version of the model performed significantly better than the original one (*Fréour et al., 2015*). In both cases, the difference in atmosphere, i.e. low versus atmospheric oxygen pressure, could partly explain these slight discrepancies.

Among the several studies dealing with time-lapse in IVF, some advocated the value of this technique as a deselection tool used to discard embryos with very poor implantation potential rather than a selection tool for the embryos with a high probability of implantation (*Rubio et al., 2012; Liu et al., 2015; Zhan et al., 2016*). For instance, direct cleavage has been reported to be a strong predictor of implantation failure (*Rubio et al., 2012; Zhan et al., 2016*), depending on the cellular stage in which it occurred. Although this remains debated, multinucleation was also reported to be detrimental for implantation (*Aguilar et al., 2016; Desch et al., 2017*). In this study, we found that the frequency of multinucleation at the two- or four-cell stage and abnormal division was significantly higher in unbalanced than in balanced embryos. Whether these abnormal events should be included in a hierarchical classification tree should be tested in further studies. We also observed that cell exclusion at the morula stage was slightly more frequent in unbalanced than in balanced embryos. A recent study of interest evaluated the chromosomal status of these excluded cells and demonstrated that they were more frequently aneuploid than the corresponding blastocysts, suggesting a possible cellular repair mechanism aiming at lowering the aneuploidy rate (*Lagalla et al., 2017*). These preliminary results, however, remain to be confirmed in further studies to determine whether the cell exclusion phenomenon and its pattern should be considered as an indicator of embryo ploidy.

Among the studies evaluating the value of time-lapse as a predictor of embryo ploidy, some were based on the day-3 embryo biopsy (*Basile et al., 2014; Chawla et al., 2015; Del Carmen Nogales et al., 2017*), whereas others used trophectoderm biopsy (*Campbell et al., 2013a; 2013b; Rienzi et al., 2015; Minasi et al., 2016; Mumusoglu et al., 2017*). The advantages of trophectoderm biopsy, such as the higher number of cells and amount of DNA required for analysis and increased euploidy rate in fewer embryos (*Scott et al., 2013*), account for the observed trend towards its increasing use in PGT centres, even though the advantages and pitfalls of embryo biopsy still remain to be deciphered (*Zacchini et al., 2017*). In parallel, some studies have advocated the value of late morphokinetic parameters at the blastocyst stage rather than early ones at the cleavage stage as predictors of embryo ploidy (*Campbell et al., 2013a; 2013b*). Although we recently implemented blastocyst biopsy for PGT-SR, the number of cycles at the time of this study was insufficient to compare with the day-3 biopsy strategy. Therefore, our study should be repeated in a large number of translocation carriers undergoing PGT-SR with trophectoderm biopsy to determine whether late morphokinetic parameters can help to identify balanced embryos and test the relevance of previously published models in this population.

The main limitation of our study is that we could only compare balanced

and unbalanced embryos for specific chromosome rearrangements because of French law. Any attempt to generalize the results to aneuploidy screening should be made with great care. Another limitation lies within the use of FISH for the assessment of chromosomal status. We acknowledge that this technique suffers from some limitations (*Fiorentino et al., 2011; Dahdouh et al., 2015*), exposing a risk of classification error and uninterpretable results (11% of biopsied embryos in this cohort had uninterpretable results). The most critical factors for FISH accuracy are quality of cell fixation, probe hybridization, signal overlap and subjective signal scoring. Our operators, however, were experienced, thus limiting this potential bias. Finally, the retrospective design of this study exposes a risk of bias and prevents the appropriate number of patients for proper statistical power to be calculated.

In conclusion, we found some significant morphokinetic differences between balanced and unbalanced embryos in couples undergoing PGT-SR for chromosomal translocation. The considerable overlap, however, between the variables did not allow the identification of relevant predictors of embryo chromosomal status, as reported previously. We have also shown that a previously published time-lapse model developed for embryo aneuploidy prediction was interesting but had a relatively low performance in our PGT-SR population. The exact clinical value of time-lapse in improving the selection of embryos with low probability of chromosomal abnormality remains to be confirmed.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.rbmo.2018.11.006.

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