Type of chromosome abnormality affects embryo morphology dynamics

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Objective: To study the differences in the cleavage time between types of embryo chromosomal abnormalities and elaborate algorithm to exclude aneuploid embryos according to the likelihood to be euploid.
Design: Retrospective cohort study.
Setting: University affiliated private center.
Patient(s): Preimplantational genetic screening patients (n = 112) including cases of advanced maternal age, repeated implantation failure, and recurrent miscarriage. A total of 485 embryos were analyzed.
Intervention(s): None.
Main Outcome Measure(s): All biopsied embryos were cultured in an incubator with time-lapse technology, cleavage timing from insemination to day 3 and all kinetic parameters that have been described in previous studies by our group.
Result(s): Logistic regression analysis were used to identify morphokinetic parameters and some were strongly associated with complex aneuploid embryos; t3 (odds ratio = 0.590, 95% confidence interval 0.359–0.971) and t5–t2 (odds ratio = 0.151, 95% confidence interval 0.082–0.278).
Conclusion(s): Embryo morphokinetics are affected by chromosome aneuploidy and further analysis of the chromosome content reveals higher differences when the complexity in the chromosome disorders is increased. The use of time-lapse monitoring, although not able to detect an abnormal embryo, may be potentially useful to discard those embryos with high risk of complex chromosomal abnormalities. (Fertil Steril® 2017;107:229–35. ©2016 by American Society for Reproductive Medicine.)
Key Words: Embryo kinetics, complex abnormalities, array CGH, time lapse

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Selecting an embryo according to its morphology has the disadvantage of the moment we observed it and the subjectivity of the observer (1–4). Another concern that has been historically used in assisted reproductive technique (ART) is multiple pregnancies. We must avoid those at-risk pregnancies, and to achieve that, the best solution is transferring only one embryo (5–7).

One of the great inconveniences about selecting an embryo using morphological criteria is that we are not considering the chromosomal content of the embryo (8). The correlation between embryo morphology and implantation potential is relatively weak (9, 10). Even embryos considered to be morphologically good do not always succeed in implanting in the uterus. Before implantation, chromosomal abnormality is extremely common, affecting more than half of whole cohort of embryos produced by women >35 years of age (11, 12). We cannot obviate that parameter. In addition, in patients with repeated implantation failure or recurrent miscarriage, the only way to determine an euploid embryo is by using preimplantation genetic screening (PGS). In many of these patients, the use of PGS implies a huge effort (economically and psychologically). In addition, this genetic diagnosis cannot always be done, for many reasons, like clinical infrastructure, moral reasons, legal reasons, or for any other reason.

Time lapse is a noninvasive method that allows us to observe embryos for 24 hours. Therefore, we can increase the amount and quality of information about the embryo without affecting negatively the culture conditions (13–16). This type of image analysis is not a new technology; it has been commercialized and has become accessible to many clinics around the world (17–20).

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Observing the embryo kinetics has helped us determine the behavior of those embryos and know that we can propose another tool for those patients in whom PGS cannot be performed. Recent publications show that chromosomally normal and abnormal embryos have different kinetic behaviors. Davis (21), Chavez (22), and Basile (23) and their colleagues reported differences between euploid and aneuploid embryos during the early stages of development. Yet Campbell et al. (24) observed no difference between euploid and aneuploid embryos during those early stages of development. However, in the periblastulation phase, aneuploid embryos had a significant delay in development compared with euploid embryos.

Nevertheless, all chromosomal abnormalities are not equal; depending on each specific abnormality, the development of the embryo will be different (25). Our goal was to separate the embryos according to each chromosomal abnormality and to find a correlation between the type of aneuploidy and embryo morphokinetic. The objective of the present study was to compare embryo kinetics depending on the type of chromosome abnormality (monosomy, trisomy, and complex) in euploid embryos and try to build a model based on the kinetic variables that can predict the chromosomal status of an embryo.

MATERIALS AND METHODS

This research project was conducted at the Instituto Valencianode Infertilidad (IVI Valencia and Madrid) and was performed from March 2013 until August 2014. The procedure and protocol for analysis of embryos were approved by an Institutional Review Board (1407-MAD-053-NB), which regulates and approves database analysis and clinical IVF procedures for research at IVI. In addition, the project complies with the Spanish law governing ART (14/2006). The present retrospective cohort study was drawn from a total of 112 cycles from patients undergoing PGS due to advanced maternal age (>39 years old), recurrent pregnancy loss, or repeated implantation failure. Recurrent miscarriage was defined as two or more miscarriages before 20 weeks of pregnancy and repeated implantation failure was defined as the absence of a gestational sac on ultrasound at ≥ 5 after ET and after transferring three high quality embryos (26). Embryo development was retrospectively analyzed using a time-lapse imaging system (Embryoscope, UnisenseFertilitech). A total of 485 embryos were successfully biopsied on day 3 of development and analyzed with array comparative genome hybridization (CGH) for all chromosomes. The IVF centre (IVI Madrid and Valencia) and the PGD laboratory (IGENOMIX) complies with all quality controls, external and internal. They are accreditation ISO 15189 and report dates about cycles of PGD to the European Society for Human Reproduction and Embryology (ESRHE) and the Spanish health authorities. Embryo biopsies and genetic analyzes were performed by a highly qualified staff.

Ovarian Stimulation and Oocyte Retrieval

For ovarian stimulation and oocyte retrieval, the patients were treated as described previously (27). Briefly, the women received a starting dose of recombinant FSH (Puregon, Organon; Gonal F, Serono) ranging from 150–225 IU (maximum) and 0.25 mg of the GnRH antagonist ganirelix (Orgalutran, Organon) daily starting on day 5 or 6 after FSH administration. The patient's cycle was monitored according to the individual policy of the clinic. Recombinant hCG (Ovitrelle, Serono) was administered as soon as two leading follicles reached a mean diameter of ≥ 17 mm, and oocyte retrieval was performed 36 hours later.

Embryo Culture Evaluation and Embryo Biopsy

Fertilization was confirmed 16–20 hours after insemination by the presence of two pronuclei (2PN) and extrusion of the second polar body. Normal fertilized oocytes were cultured in a microdroplet of culture media (Vitrolife, Scandinavian IVF) until the day of blastomere biopsy. Embryos were evaluated on days 2 and 3. Cell number, fragmentation pattern (defined as the embryonic volume occupied by the enucleated cytoplasm and expressed as a percentage), symmetry, and multinucleation were recorded.

Embryo biopsy was performed on day 3 in embryos with more than five cells and <25% fragmentation (28). The zona pellucida (ZP) was perforated using laser technology (OCTAX). Biopsied embryos were cultured up to day 5. Not biopsied embryos were discarded on day 3. Embryo transfer was performed on day 5 when a chromosomal normal embryo was available. The maximum number of embryos transferred was two.

CGH Analysis

Array CGH was performed as described elsewhere (28). Briefly, a single cell from embryos was amplified using the Sureplex DNA amplification system (BlueGnome). Amplification quality was ensured by gel electrophoresis (Lonza). Cy3 and Cy5 fluorophores were used to label the sample and control DNA, respectively. Labeling mixes were combined and hybridized on a 24sure array (BlueGnome) for 6–12 hours. Final results were obtained on day 5 using a laser scanner (710 Innoscan, Innopsys; and Powerscanner, TECAN). BlueFuse software was used to analyze the date (BlueGnome). The entire protocol for array CGH analysis was completed in 24 hours.

For internal quality control, measures were as specified by the array manufacturer for labeling, hybridization, and scanning. The quality and accuracy of the profile and results from reference male and female DNA samples also were checked as an internal quality control. All parts of the work-up and diagnostic procedure from the initial referral to the delivery of the final report were monitored with suitable controls and calibrators. Regular audit of these parameters enables the laboratory to assess the performance of their service and objectively measure improvements. Two main groups of embryos were studied: chromosomally normal embryos and chromosomally abnormal embryos. In the latter group we performed a further separation according to the chromosomal abnormality, as follows: embryos with monosomies (those embryos that lose a chromosome); trisomies

(those embryos that have an extra copy of a chromosome); chaotic embryos (have all of chromosomes altered); complex (embryos that have more than one chromosome altered); and other abnormalities (embryos that have partial abnormalities). Embryo transfer was performed on day 5 when an euploid embryo was available.

Time-lapse Analysis

Zygotes were monitored in an incubator with a built-in camera designed to automatically acquire images at specific time points. Images were taken every 15 minutes and in up to 5 focal planes. The exact times for embryo division was recorded in hours after microinjection and was retrospectively analyzed using an Embryo Viewer software workstation (UnisenseFertiliTech). Development markers included the appearance of 2PN; PN fading when both PN were no longer visible; the first cell division leading to two cells (t2); and subsequently the second (3 cells, t3), third (4 cells, t4), and fourth (5 cells, t5) cell divisions. The intervals between two consecutive cleavages were studied and defined as cc2 = t3 - t2 and cc3 = t5 - t3. The second synchrony we defined (s2 = t4 - t3) as the time from the division to a three-blastomere embryo until the time to the division to a four-blastomere embryo and the interval between 2 and 5 cells as the variable t5-t2, which combines the concepts of cell cycle and synchrony.

Regarding annotations and the quality of the information provided, we perform it internally on a yearly basis, which is based in the annotation of 25 embryos by all the embryologist in the clinic. We use for reference intraclass correlation coefficients >0.8 as observed cleavage divisions until t8.

Statistical Analysis

The times, in hours after intracytoplasmic sperm injection (ICSI) microinjection, of embryo events did not always follow normal distributions, but that was typically not the case. Nevertheless we take into consideration the central limit theorem that overcome the differences between parametric (analysis of variance [ANOVA]) and nonparametric test (Mann-Whitney *U* test) when samples sizes are >30 cases per arm, in which case both tests were performed and checked for significance ($P<.05$). Analysis of variance and Mann-Whitney *U* were used to test whether the mean times for embryonic events were significantly different in euploid and aneuploid (complex, monosomy, trisomy) embryos. To describe the distribution of the probability of complex aneuploidy, times were converted from continuous variables into categorical variables by dividing them into groups based on their quartiles. Using this procedure, we avoided bias caused by differences in the total number of embryos in each category. Next, we calculated the percentage of complex abnormalities for each time quartile to assess the distribution of those abnormalities in the different categories. Chi-square tests were used to compare categorical data. For each time variable, the optimal range was defined as the combined range spanned by the two quartiles or as the range spanned by the one quartile with the highest proportion of a complex abnormalities. A binary variable was defined by the value inside the optimal range if the value of the time variable was inside that optimal range, and vice versa for outside. The odds ratio (OR) of the effect of all binary variables generated for complex abnormalities was expressed in terms of the 95% confidence interval (95% CI) and significance. The effects of optimal ranges and other binary variables on complex abnormalities were quantified and weighted by logistic regression analysis. Logistic regression analysis depicted the binary variables relevant for complex aneuploidy. The relationship between chromosomal abnormalities and the proportions of embryos that reached blastocyst stage was analyzed by χ^2 test, including 95% CI calculations.

Due to the retrospective nature of the analysis we performed a power determination of the main variables that were demonstrated significant differences between euploid and complex abnormality embryos. For perform this calculation we used MACRO SPSS! N2IM V2006.01.27 (c) JM. Domenech, A. Bonillo & R. Granero for SAMPLE SIZE (MEAN) & POWER DETERMINATION: Two independent samples.

In any case, the significant differences observed are properly powered. Data were analyzed with the Statistical Package for the Social Sciences software program (SPSS Inc.).

RESULTS

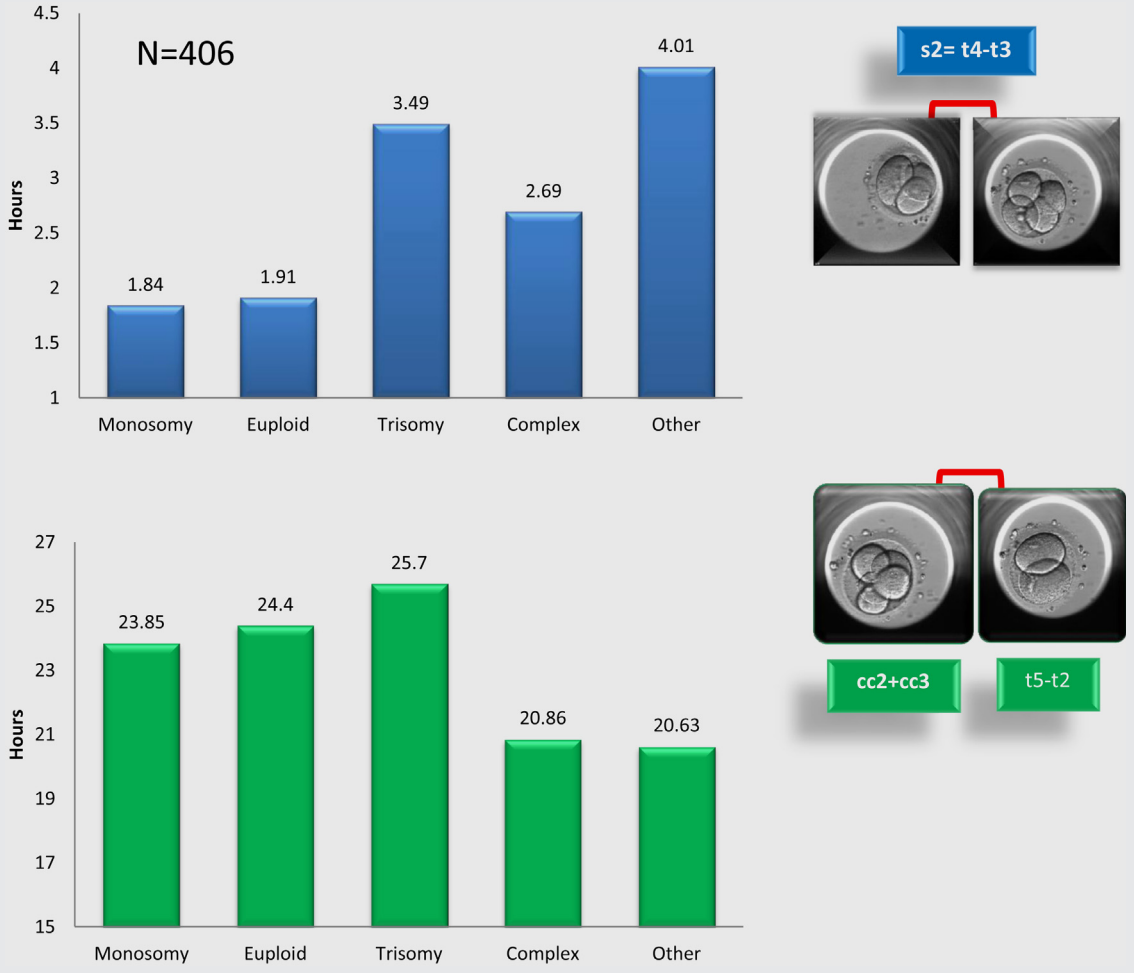
The study included 112 cycles. The mean age of our female population was 35.5 years. From these 112 cycles, 47 patients (42%) had recurrent miscarriage, 34 (30.3%) had repeated implantation failure, and 31 patients (27.7%) had advanced maternal age. A total of 485 embryos were biopsied and screened for aneuploidy using array CGH.

Of the 485 analyzed embryos, 185 had normal chromosome content and 300 had abnormal chromosome content. Among abnormal embryos, 44 (14.6 %) had trisomies, 68 (23.0%) had monosomies, and 29 were chaotic embryos (9.9%) All of the chromosomes were altered; 149 (49.6%) had complex abnormalities (more than one chromosome altered) and 10 (3.3%) had other abnormalities (partial abnormalities). Supplemental Table 1 (available online) shows the mean times for each division and for the intervals between consecutive divisions.

Embryos with trisomies showed very similar kinetics to normal embryos for all the variables studied; there was no statistically significant difference. The embryos with monosomies showed an intermediate kinetics between complex and normal. Complexes embryos showed faster divisions than normal embryos. The difference was statistically significant for t3, t5, cc2, cc3, s2 and t5-t2, as shown in Figure 1. Power calculations for the differences reported by cc2, cc3, and t5-t2 were 100%.

This makes it possible to determine the optimal range for t3 >34.7 hours, t5 >45.9 hours, cc2 >11 hours, cc3 >11.6 hours, and t5-t2 >21.01 hours. Table 1 describes the distribution in more detail: embryos outside of these ranges for t3, t5, cc3, and t5-t2 exhibited a significantly greater proportion of normal embryos than those outside of these ranges (64%, 64%, 61.2%, 64.3%, and 66.8% vs. 36%, 36%, 38.8%, 35.7%, and 33.2%). Using logistic regression, we can

FIGURE 1



Kinetic behavior according to the type chromosome abnormality: complex, monosomy, and trisomy.

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determine which of these variables are best to distinguish between normal embryos and complex aneuploid embryos.

The result showed that the variable t3 (OR = 0.59, 95% CI 0.359–0.971) and the variable t5-t2 (OR = 0.151, 95% CI 0.082–0.278) were the best to predict whether an embryo was normal or abnormal complex. As a result, we developed a hierarchical model using the corresponding decision tree procedure, which subdivided embryos into four categories (A–D), as shown in Figure 2. The classification system based on the binary variables t3 and t5-t2 was between normal embryos and abnormal complex embryos and was as follows. If the value of t5-t2 was inside the optimal range, the embryo was categorized as A or B; if the value of t5-t2 was outside the optimal range, the embryo was categorized as C or D. If the value of t5-t2 was inside the optimal range, the embryo was categorized as A or B depending on t3; similarly, if the value of t5-t2 was outside the optimal range, the embryo was categorized as C or D depending on t3. Figure 3 describes a significant decrease in the percentage of normal embryos between the categories (A = 70.1%; B = 62.6%; C = 35.6%; and D = 14.6%). Regarding blastocyst formation rate as stratified by chromosomal disorder, results are presented on Supplemental Table 2, available online.

DISCUSSION

This study has combined time-lapse technology and PGS to demonstrate the kinetic behavior between chromosomally normal and abnormal embryos, but only those with complex abnormalities (more than one chromosome affected). Embryos with single trisomy or monosomy are overlapped with euploid embryos and may not be detected by time-lapse analysis. These differences have allowed us to obtain an algorithm that is a noninvasive tool to decrease the probability of selecting complex abnormal embryos.

We have chosen the best embryo from a static morphological point of view or left the embryo in sequential culture. This selection is imprecise and may lead to chromosomally

TABLE 1

Predefined embryo time ranges for t3, t5, cc2, cc3 (t5-t3), and t5-t2 linked with complex chromosomal abnormalities.

Embryo time range	N	Abnormal complex, % (95% CI), n/N	Normal, % (95% CI), n/N
t3			
Out	168	52.4 (44.8–59.9), 88/168	47.6 (40.0–55.1), 80/168
34.7–40.5 h	164	36.0 (28.7–43.4), 59/164	64.0 (56.7–71.4), 105/164
P value			<.001
t5			
<45.9 h	77	68.8 (58.4–79.2), 53/77	31.2 (20.9–41.6), 24/77
>45.9 h	225	36.0 (26.7–42.3), 81/225	64 (57.7–70.3), 144/225
P value			<.001
cc2 (t3-t2)			
<11 h	69	65.2 (54.0–76.4), 45/69	34.8 (23.6–46.1), 24/69
>11 h	263	38.8 (32.9–44.6), 102/263	61.2 (55.3–67.9), 161/263
P value			<.01
cc3 (t5-t3)			
<11.6 h	81	67.9 (57.7–78.1), 55/81	32.1 (21.9–42.3), 26/81
>11.6 h	221	35.7 (29.4–42.0), 79/221	64.3 (25.9–38.3), 142/221
P value			<.001
t5-t2			
<21.0 h	76	77.6 (68.2–87.0), 59/76	22.4 (13.0–31.7), 17/76
>21.0 h	226	33.2 (27.1–39.3), 75/226	66.8 (66.6–72.9), 151/226
P value			<.001

Note: Data presented as percent (95% CI), n/N. CI = confidence interval.

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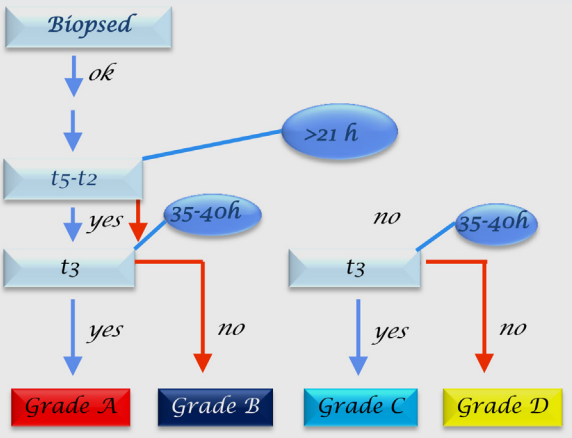
abnormal embryos (10, 29, 30). Several studies have demonstrated that chromosomal abnormalities are one of the most common causes of poor clinical outcomes in IVF (30–36). Therefore we need a more accurate method to choose embryos for transfer. Time-lapse technology allows the precise determination of the onset, duration, and interval between cell divisions and dynamic morphologic characteristics of a developing human embryo.

The use of time lapse with PGS can provide additional information on the differences in the kinetics between normal and abnormal embryos. In addition it delivers evidence related to the type of chromosome abnormality. Previous studies on this topic only focused on two categories. They considered any chromosomal abnormality, whether single or multiple.

Davis et al. (21) reported delayed first and second cleavage divisions and prolonged transition between the two- and four-cell stages as significantly correlated with chromosome aneuploidy, particularly multiple aneuploidies. However, when aneuploidies are detected at cleavage stages they may include those of paternal and maternal meiotic origin, as well as postzygotic errors caused by malsegregation in cleavage. Therefore further work will be required to determine whether the association is related to the origin of the aneuploidy. Chavez et al. (22) observed that chromosomally normal embryos display strict and tightly clustered cell cycle parameters up to the four-cell stage, but only 30% of aneuploid embryos exhibited values within normal time ranges. Basile et al. (23) observed that chromosomally normal and abnormal embryos have different kinetic behaviors. Campbell et al. (24) reported no difference between euploid and aneuploid embryos during the early stages of development. However, in the periblastulation phase, aneuploid embryos had a significant delay in development compared with euploid embryos.

This is a detailed analysis that depends on the type of chromosome abnormality. Embryos have different kinetics depending on the type of chromosome abnormality and we observed that the higher chromosomal abnormality rate, the faster the development until arrest. Embryos with trisomies behave very similarly to euploid embryos, which was already assumed, considering that they implant and present optimal morphology in many cases. Embryos with complex anomalies present with the shortest division times,

FIGURE 2



Hierarchical classification of embryos based on the embryos available for biopsy, division three cells (t3) and interval t5-t2. The algorithm classified embryos into four categories based on the expected percentage of chromosomally normal embryos.

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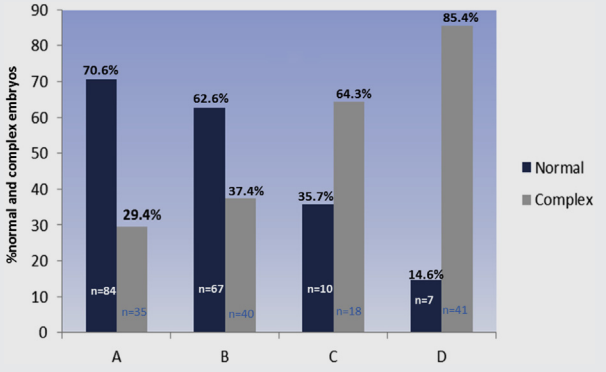
FIGURE 3



Percentage of chromosomally normal and complex embryos according to hierarchical classification. n represents the total number of embryos in each category. $P<.01$.

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whereas embryos with monosomies fall between complex and trisomy embryos. Selection of embryos with time-lapse technology should not be considered as a replacement for PGS, as there are embryos with trisomies that behave like euploid. Nonetheless, it could be useful to increase the chances of implantation. In patients who are not indicated for PGS or those that for any legal, social, or economic reasons who do not wish or cannot have PGS performed, have a potential benefit with morphokinetic screening and selection using our algorithm.

We performed embryo biopsy of a single blastomere on day 3 with the existing probability of mosaicism. However, in our program, confirmation rates on embryos biopsied on day 3 diagnosed as abnormal and rebiopsied on day 5, are similar to those embryos biopsied directly on day 5. The results have close to 97% concordance (28).

As a limitation within the present study, we may underline the mosaicism, as the embryos that were not transferred (aneuploid) were not tested for confirmatory diagnosis, especially given their development to day 5. Those embryos diagnosed as abnormal were discarded as considered not viable embryos. We believe, by our experience, that mosaicism has no clinical relevance, as the concordance between an abnormal embryo on day 3 is confirmed on day 5 in close to 97% of the cases. But there still is a 3% uncertainty, which represents an important weakness. As observed in Supplemental Table 2, it may be possible that those embryos, exhibiting complex aneuploidies, were slightly less likely to blastulate. Additional larger studies are needed to confirm the value of the time-lapse assessments.

We must acknowledge that time-lapse technology currently involves expensive equipment and, in the short term, the cost differences between PGS and time lapse may be minimal. In the near future, we expect a reduced cost for PGS owing the frequent use of quantitative polymerase chain reaction (PCR), as well as less expensive time-lapse equipment, owing to the existing competition between different brands. A logistic regression model based on the kinetic variables can be proposed to discard embryos with a high probability of having complex abnormalities.

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SUPPLEMENTAL TABLE 1

Mean average for the different variables according to chromosomal normality.

				CI 95%				
Variable	**Chromosomal status**	**N**	**Average**	**Lower limit**	**Upper limit**	**Minimum**	**Maximum**	***P* value**
t2 (h)	Normal	185	26.2	25.6	26.6	20.4	38.5	.038
	Trisomies	44	26.3	25.9	27.0	21.9	32.0	
	Monosomies	68	25.9	25.3	26.6	19.8	31.3	
	Complex	149	27.1	26.4	27.7	19.3	41.6	
t3 (h)	Normal	185	37.8	37.2	38.4	26.8	47.6	.072
	Trisomies	44	36.5	34.9	38.1	24.3	49.0	
	Monosomies	68	37.3	36.2	38.3	23.0	46.0	
	Complex	147	36.5	35.6	37.4	24.0	49.5	
t4 (h)	Normal	184	39.7	39.0	40.3	27.0	56.0	.636
	Trisomies	44	40.0	38.5	41.5	29.6	59.0	
	Monosomies	68	39.1	37.9	40.4	23.0	60.2	
	Complex	145	39.2	38.3	40.0	24.6	51.2	
t5 (h)	Normal	168	51.5	50.6	52.5	27.0	70.5	.000
	Trisomies	42	52.0	49.7	54.3	34.1	70.5	
	Monosomies	62	49.8	48.1	51.5	32.1	72.6	
	Complex	134	48.0	46.6	49.4	26.0	64.9	
cc2 (h)	Normal	185	11.5	11.0	11.9	0	17.0	.000
	Trisomies	44	10.1	8.7	11.5	1.0	17	
	Monosomies	68	11.3	10.5	12.1	0	17	
	Complex	147	9.4	8.5	10.2	0	17	
cc3 (h)	Normal	134	13.9	13.2	14.6	0	35.9	.000
	Trisomies	42	15.6	13.6	17.6	3.0	33.8	
	Monosomies	62	12.6	11.3	13.8	0	28.1	
	Complex	134	11.6	10.4	12.7	0	29.5	
t5-t2 (h)	Normal	168	25.4	24.5	26.2	1.0	47.9	.000
	Trisomies	42	25.6	23.6	27.6	9.5	45.4	
	Monosomies	62	23.8	22.3	25.3	12	42.5	
	Complex	134	20.8	19.5	22.1	0	37.2	
s2(t4-t3)	Normal	184	1.9	1.4	2.3	0	15	.03
	Trisomies	44	3.4	1.7	5.2	0	29	
	Monosomies	68	1.8	1.0	2.6	0	17	
	Complex	145	2.6	2.0	3.3	0	17	

Note: t2 = time to 2 cells; t3 = time to 3 cells; t4 = time to 4 cells; t5 = time to 5 cells; cc2 = t3-t2; cc3 = t5-t3; t5-t2; s2 = t4-t3; CI = confidence interval.

Del Carmen Nogales. Chromosome abnormality and kinetics. Fertil Steril 2016.

SUPPLEMENTAL TABLE 2

Proportion of diagnosed embryos that achieved blastocyst depending on chromosomal status.

			CI 95%		
Chromosomal status	**N**	**Blastocyst rate (%)**	**Lower limit**	**Upper limit**	***P* value**
Monosomies	68	51.4	39.5	63.3	NS
Trisomies	44	47.8	33.0	62.5	
Complex	149	42.3	34.4	50.2	
Other	10	30.0	1.6	58.4	

Note: CI = confidence interval; NS = not significant.

Del Carmen Nogales. Chromosome abnormality and kinetics. Fertil Steril 2016.