Time-lapse morphokinetic assessment has low to moderate ability to predict euploidy when patient– and ovarian stimulation–related factors are taken into account with the use of clustered data analysis

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Objective: To study whether time-lapse morphokinetic (TLM) assessment predicts ploidy status when patient– and ovarian stimulation–related factors are taken into account.
Design: Retrospective cohort study.
Setting: Private IVF clinic.
Patient(s): In total, 103 consecutive patients (415 blastocysts) were included. All embryos were individually cultured in a time-lapse incubator from intracytoplasmic sperm injection up to trophectoderm biopsy. Following trophectoderm biopsy on day 5 or 6, blastocysts were vitrified and 23 TLM parameters were analyzed.
Intervention(s): Correlations between patient– and ovarian stimulation–related factors and TLM parameters were tested in a multilevel mixed-effects linear regression model and assessed by means of intraclass correlation coefficient (ICC).
Main Outcome Measure(s): Predictive ability of TLM parameters for euploidy.
Result(s): The majority of TLM parameters had ICCs of 16%–47%. None of the patient– or ovarian stimulation–related factor had any systematic effect on any TLM parameter; however, body mass, total FSH dose, duration of infertility, number of previous cycles, antral follicle count, ovarian stimulation protocol, and E_2 on the trigger day had a significant impact on some TLM parameters. With the use of multilevel mixed-effects logistic regression analysis, of the ten TLM parameters that were initially noted to be significantly different among euploid and aneuploid blastocysts in the univariate analysis, only five remained significant. However, the areas under the receiver operating characteristic curves at regression analysis were low, ranging from 0.55 to 0.63.
Conclusion(s): Five TLM parameters, all related to timing of blastocyst development, have limited ability to predict euploidy when patient– and ovarian stimulation–related factors are taken into account. (Fertil Steril® 2017;107:413–21. ©2016 by American Society for Reproductive Medicine.)
Key Words: Time-lapse, morphokinetic parameters, aneuploidy, preimplantation genetic testing, confounding, cluster analysis

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The contemporary goal of in vitro fertilization (IVF) is to maximize live birth rates with the use of single-embryo transfer. An objective assessment tool to evaluate embryo ploidy status and viability is of critical importance for selection of the best embryo to be transferred. Blastocyst-stage embryo transfer may enhance embryo selection (1), but embryo morphology, even at the blastocyst stage, might be misleading (2).

Aneuploidy is the main contributor to implantation failure (3) and increased risk of miscarriage (4) in IVF. Currently, blastocyst-stage embryo biopsy is the method of choice for assessment of the ploidy status (5). Despite the lack of any detrimental effect of trophectoderm biopsy on implantation rate (5), noninvasive assessment of ploidy status with high validity would be very useful.

Morphokinetic assessment of preimplantation embryo development has been a breakthrough in human embryology in the past decade. Sophisticated time-lapse incubators along with single-step medium permitted not only uninterrupted in vitro culture and embryo development but also provided continuous information about dynamic changes during the preimplantation period. There have been efforts to predict aneuploidy by means of various time-lapse morphokinetic (TLM) parameters, six studies reporting a significant association with some TLM parameters and the ploidy status (6–11), and four refuting any such association (12–15).

The main drawback of the available ten studies is that each embryo is treated as an individual, ignoring the fact that all of the embryos from the same patient may act in a similar fashion affected by patient–and ovarian stimulation–related factors (16). The aim of the present study was to evaluate the association between various TLM parameters and ploidy status at the blastocyst stage with the use of clustered data analysis.

MATERIAL AND METHODS

Study Design and Participants

In this retrospective cohort study, 103 consecutive patients undergoing 103 cycles of intracytoplasmic sperm injection (ICSI) and preimplantation genetic screening (PGS) at the Anatolia IVF and Women's Health Center, Ankara, from April 2015 to April 2016 were enrolled. Only one ICSI cycle per patient was included; for those patients who underwent multiple ICSI cycles during this time period, only the chronologically first cycle was included.

A total of 416 blastocysts were biopsied. No result, owing to amplification failure, was noted in six blastocysts (1.4%); of those six blastocysts, one lost viability at warming and was therefore excluded. Rebiopsy followed by revitrification was undertaken for the remaining five blastocysts. Thus, a total of 415 blastocysts were included in the current analysis.

The main indication for PGS was advanced maternal age (AMA; $\geq$38 years; n = 87). Because in our setting, we do PGS routinely along with preimplantation genetic diagnosis (PGD) for single-gene disorders and balanced translocations, 16 couples undergoing PGD for single-gene disorders (n = 5) and chromosomal translocations (n = 11) were also included. Because clustered data analysis was performed to overcome patient– and ovarian stimulation–related factors as confounding (16), patients with at least two blastocysts to be biopsied were included (17).

Protocols for ovarian stimulation, procedures performed in the IVF laboratory regarding trophectoderm biopsy, vitrification and warming process of blastocysts, and methodology of genetic testing with the use of array comparative genomic hybridization are presented in detail in Supplemental Appendix 1 (Supplemental Appendix 1, Supplemental Fig. 1, and Supplemental Tables 1 and 2 are available online at www.fertstert.org).

Time-lapse Imaging and Assessment

All embryos were individually cultured in a time-lapse incubator (Embryoscope; Vitrolife) from ICSI up to the stage of trophectoderm biopsy.

Images were recorded with the use of the integrated microscope of the Embryoscope every 15 minutes from seven different focal planes. For this purpose, 15-μm intervals, 1,280 Å $\approx$ 1,024 pixels, 3 pixels per mm, monochrome, 8-bit, 0.5 seconds per image, and single 1-W red light-emitting diode were used. A time point was automatically assigned to each image, reported as hours after time zero (t0); t0 was defined as the time of injecting the sperm into the oocyte. Various TLM parameters included in our analysis are defined in Supplemental Table 1.

All annotations were made in a prospective fashion by two experienced senior embryologists. Before the present study, high intra- (kappa score = 0.95) and interobserver (kappa score = 0.91) clinical agreement was noted between these two embryologists (data not presented).

Statistical Analysis

Distribution characteristics of variables were visually assessed with the use of histograms, box plots, and Q-Q plots and analyzed with the use of Kolmogorov-Smirnov and Shapiro-Wilk tests. Continuous variables were expressed as mean $\pm$ SD or median and interquartile range (IQR) as appropriate. Comparisons were made with the use of independent-samples *t* test or Mann-Whitney *U* test according to distribution characteristics.

Multilevel mixed-effects models account for the correlation among observations in the same cluster and give an estimate of this correlation. Because embryos generated from a patient do not provide independent information, multilevel models were used. Intraclass correlation coefficients (ICCs) were calculated from the specified models to delineate to what extent the variation in each TLM is explained by patient–and ovarian stimulation–related factors. In a multilevel random-effects model (level one: embryo; level two: patient), ICC corresponds to the correlation of measurements within the same individual as well as to the proportion of variance explained by the individual random effect.

Multilevel mixed-effects linear regression analysis was performed for all 23 TLM parameters to determine which had any significant effect on the ploidy status adjusted by

confounders (18, 19). The patient– and ovarian stimulation–related confounders included in the model were female age, body mass index (BMI), number of previous cycles, duration of infertility, antral follicle count, ovarian stimulation protocol, total FSH dose/100, E_2 level on the day of triggering final oocyte maturation/100, and number of retrieved oocytes.

We used the following methodology for selection of patient– and ovarian stimulation–related factors as independent variables in the multilevel mixed-effects logistic regression model. First, those previously known or defined confounders, including female age, number of previous cycles, BMI, and total FSH dose (16) were included. In addition, we enrolled ovarian stimulation protocol and E_2 level on the day of triggering, because they might have a potential impact on TLM parameter(s). Finally, any patient– or ovarian stimulation–related factor that had significant effect on timing of any of the TLM parameter (duration of infertility, antral follicle count, number of retrieved oocytes) was also included.

Receiver operating characteristic (ROC) regression and Youden index were used to discriminate the predictive value of TLM parameters and optimum cutoff points for the ploidy status taking the patient– and ovarian stimulation–related factors into account.

All statistical analyses were performed with the use of the statistical package Stata for Windows, academic trial version 14.0 (Stata Corp.). R 3.0.0 software was used to draw Supplemental Figure 1.

The Institutional Review Board of Hacettepe University approved the study protocol (GO-16/422-43).

RESULTS

The baseline demographic features of the 103 patients are presented in Supplemental Table 2. The mean female age was 38.0 ± 4.7 years, and the mean number of oocytes retrieved was 10.3 ± 4.7. A total of 415 blastocysts that were biopsied on day 5/6 and whose ploidy statuses were known were included in the current analysis.

Univariate Analysis of Euploid and Aneuploid Embryos

The univariate analysis of euploid and aneuploid blastocysts is presented in Table 1. Of the studied 23 parameters, 15 were time points and the remaining eight were calculated from the former. There was statistically significant delay in tPNa, t2, t7, t8, t9, tM, tSB, tB, EB, and t9 − t2 in aneuploid compared with euploid blastocysts. However, because the range for all of the studied time events was wide for both euploid and aneuploid blastocysts, there was an overlap of euploid and aneuploid blastocysts regarding nearly all time events, as depicted in Supplemental Figure 1.

TABLE 1

Comparison of time-lapse morphokinetic (TLM) parameters of euploid and aneuploid blastocysts.

	Euploid (n = 173)					Aneuploid (n = 242)					
TLM parameter (n)	Mean ± SD	Median	Min.	Max.	IQR	Mean ± SD	Median	Min	Max	IQR	*P* value
tPB2 (415)	3.8 ± 2.3	3.5	1.6	23.5	1.1	4.1 ± 1.9	3.6	0.7	23.5	1.2	.386
tPNa (415)	8.6 ± 2.0	8.5	5.2	24.1	2.1	9.4 ± 3.0	8.8	5.5	28.6	2.4	.018
tPNf (415)	24.5 ± 2.8	24.2	19.2	37.4	3.6	24.8 ± 3.1	24.5	18.1	36.5	3.7	.182
t2 (411)	26.7 ± 2.9	26.4	21.0	39.7	4.1	27.3 ± 3.3	26.9	20.6	44.1	4.1	.033
t3 (343)	36.8 ± 4.7	36.9	23.6	55.6	5.0	37.6 ± 4.5	37.6	22.9	52.7	5.6	.095
t4 (392)	38.8 ± 4.5	38.6	27.3	57.1	5.4	39.3 ± 4.7	38.7	27.8	64.4	5.0	.233
t5 (353)	48.6 ± 7.1	49.7	31.4	70.8	9.0	49.7 ± 7.2	50.2	26.5	69.6	9.3	.172
t6 (360)	51.4 ± 6.6	51.6	32.6	71.8	8.1	52.4 ± 7.2	52.3	30.8	74.8	8.5	.181
t7 (350)	53.5 ± 7.5	54.0	31.6	80.6	9.1	55.3 ± 7.4	54.2	33.0	94.1	8.0	.027
t8 (391)	56.9 ± 8.1	55.9	31.9	76.7	10.8	58.8 ± 9.3	56.7	43.0	109.7	10.0	.028
t9 (398)	68.4 ± 10.3	69.0	34.4	100.6	12.6	71.6 ± 11.4	71.7	42.2	118.4	13.7	.005
tM (415)	91.4 ± 8.9	91.0	60.4	111.4	11.6	94.3 ± 9.2	93.0	72.2	130.9	11.3	.001
tSB (415)	99.4 ± 8.7	98.2	76.6	127.1	11.1	102.4 ± 9.3	101.1	81.8	143.7	12.0	.001
tB (415)	108.1 ± 9.1	107.5	87.1	139.5	12.2	112.5 ± 10.4	111.9	89.1	148.9	12.0	< .001
tEB (317)	113.8 ± 9.6	111.7	95.4	140.1	10.7	118.7 ± 11.2	116.4	97.1	160.5	15.1	< .001
CC2 (339)	10.3 ± 3.0	11.3	0.5	16.9	1.9	10.4 ± 3.2	11.3	0.2	17.5	2.0	.655
CC3 (297)	12.4 ± 4.1	12.8	0.5	22.3	3.0	12.4 ± 4.7	13.4	0.2	28.5	3.5	.900
S2 (325)[a]	2.1 ± 3.1	1.0	0.2	14.3	1.6	1.8 ± 3.1	0.7	0.2	25.3	1.0	.371
S3 (336)[a]	8.7 ± 7.2	6.3	0.5	31.5	11.3	9.7 ± 8.1	5.9	0.5	42.5	11.7	.245
t9 − t2 (395)	42.1 ± 9.1	43.1	16.3	69.5	9.8	44.2 ± 10.0	44.9	13.8	80.0	13.0	.026
t5 − t2 (349)	22.2 ± 6.0	23.8	4.7	34.0	6.0	22.5 ± 6.3	24.0	4.0	41.8	6.7	.612
CC3/CC2 (293)[a]	2.0 ± 3.4	1.2	0.1	25.0	0.3	2.2 ± 5.8	1.2	0.1	56.5	0.3	.983
Blastulation (415)	9.5 ± 4.1	9.2	2.0	23.0	5.5	10.4 ± 5.6	9.5	2.5	45.2	5.4	.052

Note: IQR = interquartile range; tPB2 = appearance of second polar body; tPNa = appearance of pronuclei (2PN); tPNf = both pronuclei faded/syngamy; t2, t3, t4, t5, t6, t7, t8, and t9 = time between intracytoplasmic injection and 2-, 3-, 4-, 5-, 6-, 7-, 8-, and ≥9-cell stages, respectively; tM = time from insemination to formation of a morula; tSB = time from insemination to start of blastulation; tB = time from insemination to formation of a full blastocyst; tEB = time from insemination to expanded blastocyst; CC2 = length of second cell cycle (t3 − t2); CC3 = length of third cell cycle (t5 − t3); S2 = synchrony in division from 3 to 4 cells (t4 − t3); S3 = synchrony in division from 5 to 8 cells (t8 − t5); blastulation = start of blastulation to formation of a full blastocyst (tB − tSB).
[a] Not normally distributed.

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TABLE 2

Results of multilevel mixed-effects linear regression model analyzing confounding effects of patient– and ovarian stimulation–related factors.

TLM parameter (n)	Age (y)	BMI (kg/m^2)	Duration of infertility (y)	No. of previous cycles
tPB2 (415)	−0.01 (−0.11 to 0.09)	−0.07 (−0.19 to 0.02)	0.01 (0.00–0.02)[a]	0.07 (−0.13 to 0.27)
tPNa (415)	−0.03 (−0.12 to 0.07)	−0.14 (−0.26 to −0.02)[a]	0.01 (−0.01 to 0.01)	0.13 (−0.08 to 0.34)
tPNf (415)	−0.01 (−0.15 to 0.12)	−0.13 (−0.24 to −0.03)[a]	−0.00 (−0.01 to 0.01)	0.10 (−0.11 to 0.33)
t2 (411)	−0.02 (−0.13 to 0.10)	−0.16 (−0.27 to −0.04)[a]	−0.00 (−0.01 to 0.01)	0.11 (−0.12 to 0.35)
t3 (343)	−0.03 (−0.19 to 0.12)	−0.15 (−0.31 to 0.01)[b]	−0.00 (−0.02 to 0.01)	0.21 (−0.11 to 0.54)
t4 (392)	0.03 (−0.12 to 0.17)	−0.20 (−0.36 to −0.05)[a]	−0.00 (−0.01 to 0.01)	0.21 (−0.09 to 0.51)
t5 (353)	−0.08 (−0.31 to 0.13)	−0.26 (−0.51 to −0.02)[a]	0.00 (−0.01 to 0.02)	0.26 (−0.19 to 0.72)
t6 (360)	0.02 (−0.21 to 0.26)	−0.31 (−0.56 to −0.06)[a]	0.00 (−0.02 to 0.02)	0.17 (−0.31 to 0.66)
t7 (350)	−0.03 (−0.26 to 0.20)	−0.32 (−0.57 to −0.07)[a]	0.01 (−0.01 to 0.03)	0.12 (−0.36 to 0.60)
t8 (391)	−0.11 (−0.38 to 0.16)	−0.30 (−0.58 to −0.02)[a]	−0.00 (−0.02 to 0.02)	0.02 (−0.53 to 0.58)
t9 (398)	0.05 (−0.31 to 0.42)	−0.37 (−0.74 to 0.01)[b]	0.02 (−0.01 to 0.05)	0.18 (−0.57 to 0.92)
tM (415)	−0.15 (−0.41 to 0.12)	−0.10 (−0.38 to 0.15)	−0.01 (−0.02 to 0.01)	0.31 (−0.20 to 0.89)
tSB (415)	0.06 (−0.05 to 0.01)	−0.29 (−0.58 to 0.00)[b]	−0.00 (−0.02 to 0.02)	0.03 (−0.57 to 0.63)
tB (415)	0.12 (−0.21 to 0.46)	−0.29 (−0.62 to 0.02)[b]	0.01 (−0.02 to 0.03)	−0.12 (−0.75 to 0.49)
tEB (317)	0.06 (−0.29 to 0.41)	−0.28 (−0.66 to 0.09)	0.01 (−0.03 to 0.03)	0.23 (−0.49 to 0.94)
CC2 (339)	−0.02 (−0.12 to 0.08)	0.01 (−0.09 to 0.12)	−0.00 (−0.01 to 0.01)	0.15 (−0.06 to 0.36)
CC3 (297)	−0.07 (−0.19 to 0.05)	−0.09 (−0.22 to 0.05)	0.00 (−0.01 to 0.01)	0.08 (−0.16 to 0.32)
S2 (325)	0.02 (−0.06 to 0.11)	−0.10 (−0.19 to −0.01)[a]	−0.01 (−0.01 to 0.01)	−0.03 (−0.20 to 0.15)
S3 (336)	−0.05 (−0.27 to 0.16)	−0.07 (−0.31 to 0.17)	−0.01 (−0.02 to 0.02)	−0.22 (−0.66 to 0.22)
t9–t2 (395)	0.05 (−0.24 to 0.34)	−0.18 (−0.48 to 0.13)	0.02 (−0.01 to 0.04)	0.07 (−0.51 to 0.66)
t5 − t2 (349)	−0.10 (−0.27 to 0.07)	−0.07 (−0.26 to 0.11)	0.00 (−0.01 to 0.01)	0.16 (−0.17 to 0.51)
CC3/CC2 (293)	0.08 (−0.05 to 0.22)	−0.10 (−0.25 to 0.04)	0.00 (−0.01 to 0.01)	−0.02 (−0.29 to 0.25)
Blastulation (415)	0.06 (−0.08 to 0.19)	0.02 (−0.13 to 0.17)	0.01 (−0.01 to 0.02)	−0.19 (−0.47 to 0.08)

Note: Estimates are reported as predictive difference in hours per unit variable. In the column of ovarian stimulation protocol, GnRH antagonist protocol is compared with long GnRH agonist protocol. CI = confidence interval; ICC = intraclass correlation coefficient; other abbreviations as in Table 1.

[a] $P<.05$.

[b] $P=.05–.1$.

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Association between TLM Parameters and Patient– and Ovarian Stimulation–related Factors

In general, moderate ICCs were noted for the majority of TLM parameters, indicating that embryos from one patient elicit clustering at a patient level (Table 2).

The results of multilevel mixed-effects linear regression model analyzing the confounding effects of patient– and ovarian stimulation–related factors are also presented in Table 2. In general, no single factor was noted to elicit a systematic influence on any TLM parameter. However, BMI appeared to have a significant impact on cleavage-stage parameters including tPNa, tPNf, t2, t4, t5, t6, t7, t8, and S2. Notably, higher BMI was associated with significantly earlier occurrence of these time events. The total FSH dose was also significantly related to tB, tEB, and blastulation: the higher the FSH dosing, the later those time events (Table 2). In addition, subtle nonsystematic but significant effects between some patient– and ovarian stimulation–related factors and TLM parameters were noted, including duration of infertility–tPB2, antral follicle count–tM, and number of retrieved oocytes–blastulation.

Multilevel Mixed-effects Logistic Regression Model in Clustered Data to Assess the Ploidy Status

With this model, when patient– and ovarian stimulation–related factors are taken into consideration as confounding, of the ten TLM parameters that were initially noted to be significantly different among euploid and aneuploid blastocysts in the univariate analysis (Table 1), only five (t9, tM, tSB, tB, and tEB) remained significant (Table 3).

ROC regression analysis was formed for those five significant TLM parameters to delineate the areas under the ROC curves (AUCs) and optimal cutoff points for euploidy prediction. The AUCs (95% confidence interval [CI]) for t9, tM, tSB, tB, and tEB were, respectively, 0.55 (0.47–0.62), 0.56 (0.48–0.64), 0.57 (0.50–0.65), 0.61 (0.55–0.68), and 0.63 (0.55–0.72). The respective optimal cutoff points to yield best sensitivity and specificity, respectively, for these TLM parameters in our laboratory set-up were 71.1 hours (39.4% and 72.2%), 89.8 hours (51.4% and 60.7%), 99.7 hours (36.8% and 80.2%), 107.5 hours (40.8% and 79.2%), and 112.2 hours (50.0% and 75.9%).

Validation of Previously Defined Cutoff Points in Our Clustered Dataset

We tested previously defined cutoff points for various TLM parameters (6, 8–10) in our clustered data analysis. Only tSB with 96.6 hours as the cutoff (10) had significant predictive ability (odds ratio 1.74, 95% CI 1.10–2.73) in our database.

DISCUSSION

In the present study, moderate ICCs (16%–47%) were noted for the majority of TLM parameters, indicating that embryos from one patient elicited clustering at a patient level. When

TABLE 2

Continued.

Antral follicle count	Ovarian stimulation protocol	Total FSH dose/100 (IU)	E_2 level on the day of triggering/100 (pg/mL)	No. of retrieved oocytes	ICC (95% CI)
0.01 (−0.09 to 0.11)	−0.37 (−1.84 to 0.51)	0.03 (−0.02 to 0.09)	−0.01 (−0.05 to 0.02)	0.01 (−0.13 to 0.15)	0.76 (0.71–0.83)
−0.02 (−0.12 to 0.08)	−0.48 (−1.39 to 0.43)	0.02 (−0.02 to 0.08)	−0.02 (−0.05 to 0.01)	0.01 (−0.11 to 0.18)	0.64 (0.55–0.72)
−0.02 (−0.88 to 1.01)	0.06 (−0.88 to 1.09)	0.04 (−0.02 to 0.09)	0.03 (−0.02 to 0.07)	−0.02 (−0.16 to 0.14)	0.47 (0.36–0.57)
−0.02 (−0.14 to 0.10)	−0.06 (−1.07 to 0.96)	0.02 (−0.03 to 0.08)	−0.00 (−0.04 to 0.03)	−0.01 (−0.16 to 0.15)	0.45 (0.35–0.56)
−0.02 (−0.18 to 0.14)	−0.47 (−1.88 to 0.94)	0.04 (−0.03 to 0.10)	−0.01 (−0.04 to 0.03)	−0.06 (−0.29 to 0.16)	0.32 (0.22–0.46)
−0.03 (−0.18 to 0.11)	−0.50 (−1.79 to 0.79)	0.02 (−0.04 to 0.09)	0.00 (−0.04 to 0.04)	−0.01 (−0.21 to 0.19)	0.25 (0.16–0.38)
−0.06 (−0.29 to 0.16)	−0.81 (−2.81 to 1.18)	0.06 (−0.04 to 0.16)	0.01 (−0.06 to 0.08)	−0.11 (−0.42 to 0.21)	0.21 (0.11–0.35)
0.02 (−0.21 to 0.26)	−0.65 (−2.74 to 1.45)	0.04 (−0.05 to 0.15)	−0.01 (−0.08 to 0.06)	−0.06 (−0.39 to 0.26)	0.29 (0.18–0.42)
−0.04 (−0.28 to 0.20)	−0.67 (−2.76 to 1.42)	0.07 (−0.04 to 0.18)	−0.01 (−0.08 to 0.08)	0.07 (−0.26 to 0.40)	0.21 (0.12–0.35)
−0.14 (−0.42 to 0.14)	0.72 (−1.68 to 3.13)	0.08 (−0.05 to 0.20)	0.00 (−0.08 to 0.08)	0.10 (−0.28 to 0.47)	0.20 (0.11–0.33)
−0.24 (−0.65 to 0.16)	1.57 (−1.65 to 4.79)	0.06 (−0.10 to 0.23)	0.00 (−0.11 to 0.11)	−0.11 (−0.40 to 0.62)	0.31 (0.21–0.43)
−0.41 (−0.69 to −0.14)[a]	−0.77 (−3.08 to 1.54)	0.08 (−0.24 to 0.15)	0.06 (−0.01 to 0.16)	−0.05 (−0.43 to 0.32)	0.20 (0.12–0.31)
−0.12 (−0.43 to 0.19)	−1.12 (−3.70 to 1.44)	0.07 (−0.06 to 0.21)	0.05 (−0.04 to 0.14)	−0.15 (−0.55 to 0.61)	0.28 (0.19–0.39)
−0.03 (−0.33 to 0.26)	−1.89 (−4.46 to 0.76)	0.17 (0.02–0.33)[a]	0.02 (−0.07 to 0.11)	0.07 (−0.34 to 0.49)	0.20 (0.12–0.32)
0.03 (−0.32 to 0.38)	−0.02 (−3.16 to 3.13)	0.19 (0.03–0.36)[a]	0.01 (−0.10 to 0.12)	0.08 (−0.41 to 0.57)	0.21 (0.11–0.35)
0.02 (−0.08 to 0.12)	−0.42 (−1.33 to 0.49)	0.02 (−0.03 to 0.06)	0.01 (−0.02 to 0.04)	−0.07 (−0.21 to 0.08)	0.16 (0.07–0.31)
−0.05 (−0.17 to 0.06)	−0.45 (−1.51 to 0.61)	0.02 (−0.03 to 0.08)	−0.01 (−0.03 to 0.03)	−0.00 (−0.17 to 0.17)	0.00 (0.00–0.00)
−0.05 (−0.13 to 0.04)	−0.08 (−0.85 to 0.70)	−0.02 (−0.06 to 0.02)	0.01 (−0.02 to 0.03)	0.05 (−0.08 to 0.17)	0.06 (0.01–0.26)
−0.05 (−0.27 to 0.17)	1.07 (−0.86 to 3.00)	0.03 (−0.07 to 0.13)	0.01 (−0.05 to 0.08)	0.11 (−0.18 to 0.42)	0.07 (0.02–0.25)
−0.26 (−0.58 to 0.07)	1.47 (−1.10 to 4.83)	0.03 (−0.10 to 0.17)	0.01 (−0.08 to 0.09)	0.08 (−0.33 to 0.48)	0.18 (0.09–0.31)
−0.06 (−0.23 to 0.10)	−0.75 (−2.25 to 0.76)	0.04 (−0.04 to 0.12)	0.01 (−0.04 to 0.06)	−0.13 (−0.37 to 0.11)	0.07 (0.02–0.28)
−0.01 (−0.13 to 0.12)	−0.58 (−1.76 to 0.60)	−0.04 (−0.10 to 0.02)	−0.02 (−0.06 to 0.02)	0.12 (−0.06 to 0.32)	0.00 (0.00–0.00)
0.08 (−0.04 to 0.22)	−0.52 (−1.70 to 0.61)	0.11 (0.04 to 0.17)[a]	−0.03 (−0.07 to 0.01)[b]	0.25 (0.07–0.42)[a]	0.09 (0.03–0.22)

clustering is not taken into account, as has been the case in previous studies, ten TLM parameters were initially noted to be significantly different between euploid and aneuploid blastocysts in univariate analysis. However, when patient– and ovarian stimulation–related factors, as potential sources of confounding, were taken into account by multilevel mixed-effects logistic regression analysis, we noted that only five TLM parameters (t9, tM, tSB, tB, and tEB), all related to timing of blastocyst development, remaining significant predictors of ploidy status. Of note, aneuploid embryos appeared to have significantly delayed time to blastocyst development. However, the AUCs in the ROC regression analysis for these five significant parameters were still in the range of 0.55–0.63, implying only low to moderate predictive ability. To our knowledge, this study is the first to assess the prediction of ploidy status with the use of TLM parameters in clustered data analysis.

Currently, there are ten studies that aimed to predict the ploidy status of preimplantation embryos with the use of TLM parameters, six reporting significant associations between some TLM parameters and ploidy status (6–11), and the remaining four refuting any such significant association (12–15) (Table 4). Models for ploidy prediction were suggested by two of the studies (8, 9), which were not validated by different datasets (13, 14).

The main drawback of the previously available studies in this context is that none had taken the clustering effect into consideration. In all of them, even though multiple embryos are generated from one couple, each embryo was treated as

TABLE 3

Multilevel mixed-effects logistic regression model analysis for time-lapse morphokinetic (TLM) parameters to predict the ploidy status of embryos.

TLM parameter (n)	β coefficient (95% CI)[a]	*P* value
tPB2 (415)	−0.002 (−0.118 to 0.113)	.952
tPNa (415)	0.815 (−0.020 to 0.184)	.128
tPNf (415)	0.035 (−0.039 to 0.109)	.363
t2 (411)	0.052 (−0.019 to 0.122)	.150
t3 (343)	0.028 (−0.023 to 0.080)	.279
t4 (392)	0.006 (−0.042 to 0.054)	.815
t5 (353)	0.013 (−0.019 to 0.045)	.442
t6 (360)	0.013 (−0.021 to 0.046)	.457
t7 (350)	0.029 (−0.004 to 0.061)	.084
t8 (391)	0.026 (−0.000 to 0.052)	.051
t9 (398)	0.021 (0.000–0.041)	.047
tM (415)	0.029 (0.004–0.056)	.025
tSB (415)	0.031 (0.007–0.057)	.013
tB (415)	0.042 (0.018–0.065)	.001
tEB (317)	0.045 (0.019–0.072)	.001
CC2 (339)	0.004 (−0.064 to 0.072)	.912
CC3 (297)	−0.002 (−0.058 to 0.053)	.927
S2 (325)	−0.040 (−0.116 to 0.034)	.290
S3 (336)	0.021 (−0.009 to 0.052)	.173
t9–t2 (395)	0.016 (−0.006 to 0.039)	.156
t5–t2 (349)	0.002 (−0.035 to 0.039)	.914
CC3/CC2 (293)	0.014 (−0.037 to 0.066)	.585
Blastulation (415)	0.048 (−0.003 to 0.098)	.067

Note: Abbreviations as in Tables 1 and 2.

[a] β coefficient of each TLM parameter for prediction of euploidy, taking patient– and ovarian stimulation–related factors (body mass index, female age, number of previous cycles, duration of infertility, antral follicle count, number of retrieved oocytes, peak E_2 level, total FSH dose, and ovarian stimulation protocol) into account.

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TABLE 4

Published studies investigating the relationship between time-lapse morphokinetic (TLM) parameters and ploidy status.

Study, year	Biopsy day; PGS/PGD indications	Patients (n); cycles (n); embryos (n)	Age (y, mean ± SD); aneuploidy rate (%)	Amplification failure (%); genetic testing method	Evaluated TLM parameters	Statistical methods	Significantly differing TLM parameters	Defined cutoffs for ploidy status
Chavez, 2012 (7)[a]	Day 2; surplus embryos from successful IVF cycles	45; NA; 53	33.5 ± NA; 75.5	15.0; aCGH	tPNa, t2, t3, t4 (monitoring by custom-built miniature microscope system)	Mean comparison	Long duration of 1st cytokinesis and 2nd to 3rd mitosis	None
Campbell, 2013 (8)	Day 5–6; AMA, RIF, RM, severe male factor, previous aneuploidy	25; NA; 98	38.6 ± 3.6–61.2	NA; SNP, aCGH	tPNf, MN2, MN4, t2, t3, t5, t8, tSC, tM, tSB, tB, tEB, tHB, CC2, CC3, S2, S3, t3−t1 <5 h, t5−t2 <5 h, blastulation	Mean comparison, ROC curve, decisions tree model	tSC, tSB, tB	tSB <96.2 h and tB <122.9 h more likely to be euploid, -AUC = 0.72
Yang, 2014 (12)	Day 5; RIF, RM	138; NA; 263	36.6 ± 2.4–51.3	2.7; aCGH	t2, t3, t5, t8, tM, tSB, tB, tEB, CC2, S2	Mean comparison	None	None
Kramer, 2014 (14)[a]	Day 5; AMA, RM, translocation, family balancing	25; NA; 149	37.3 ± 3.9–57.0	2.7; aCGH	tPB2, tPNa, tPNf, t3, t4, t5, t8, tSC, tM, tSB, tB, CC2, S2, tM−tSC	Mean comparison of variance, ROC curve, 2-way analysis of variance	None	None
Basile, 2014 (9)	Day 3; RIF, RM	87; 125; 504	36.1 ± 0.8; 71.7	NA; aCGH	tPNa, tPNf, t2, t3, t4, t5, CC2, CC3, S2, t5−t2	Mean comparison, logistic regression, ROC curve	t5, t5−t2, CC2, CC3	t5−t2 >20.5 h and CC3 = 11.7–18.2 h more likely to be euploid; AUC = 0.63
Campbell, 2014 (10)	Day 5–6; AMA, RIF, RM, severe male factor, previous aneuploidy	NA; NA; 195	NA; NA	NA; SNP, aCGH	tSB, tB	Logistic regression, ROC curve	tSB, tB	tSB <96.6 h and tB <118.1 h more likely to be euploid; AUC = 0.67
Rienzi, 2015 (13)	Day 5; AMA (>36 y), RIF, RM	138; 138; 455	NA; 59.1	NA; aCGH	tPNf, t2, t3, t4, t5, t8, tSC, tSB, tEB, CC1, CC2, CC3, CC3/CC2, S2, S3, t5−t2	Mean comparison, logistic regression	None	None
Chawla, 2015 (6)	Day 3; sex selection	132; 132; 496	32.9 ± NA; 57.1	7.2; aCGH	tPB2, tPNa, tPNf, t2, t3, t4, t5, CC2, CC3, S2, t5−t2	Mean comparison, logistic regression, ROC curve	tPNf, t2, t5, t5−t2, CC2, CC3	CC3 >10.0 h more likely to be euploid (AUC = 0.63); t5−t2 >20.0 h more likely to be euploid (AUC = 0.63)

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TABLE 4

Continued.

Study, year	Biopsy day; PGS/PGD indications	Patients (n); cycles (n); embryos (n)	Age (y, mean ± SD); aneuploidy rate (%)	Amplification failure (%); genetic testing method	Evaluated TLM parameters	Statistical methods	Significantly differing TLM parameters	Defined cutoffs for ploidy status
Patel, 2016 (15)	Day 3; AMA (≥35y), RIF, RM	26; 29; 167	32.94 ± 3.19; 75.4	NA; aCGH	tPB2, tPNa, tPNf, t2, t3, t4, t5, t6, t7, t8, t9, tM, tSB, tB, tEB, tHB, CC2, CC3	Mean comparison, logistic regression	None	None
Minasi, 2016 (11)	Day 5–6; NA	NA; NA; 928	36.8 ± 4.24; 68.3	1.0; aCGH	tPB2, tPNa, tPNf, t2, t3, t4, t5, t6, t7, t8, t9, tM, tSB, tB, tEB, tHB CC2, S2	Mean comparison	t4, tM, tSB, tB, tEB, tHB, s2	None
This study, 2016	Day 5–6; AMA (≥38 y), translocation, single-gene disorders	103; 103; 415	38.0 ± 4.7; 58.3	1.4; aCGH	tPB2, tPNa, tPNf, t2, t3, t4, t5, t6, t7, t8, t9, tM, tSB, tB, tEB, CC2, CC3, S2, S3, CC3/CC2, t5−t2, t9–t2, blastulation	Mean comparison, multilevel mixed-effects logistic regression, ROC regression curve	t9, tM, tSB, tB, tEB	tEB <112.2 h more likely to be euploid; AUC = 0.63

Note: aCGH = array comparative genomic hybridization; AMA = advanced maternal age; AUC = area under receiver operating characteristic curve; MN2 = multinuclearity at the 2-cell stage; MN4 = multinuclearity at the 4-cell stage; NA = not available; PGD = preimplantation genetic diagnosis; PGS = preimplantation genetic screening; RIF = recurrent implantation failure; RM = recurrent miscarriage; ROC = receiver operating characteristic; SNP = single-nucleotide polymorphism; tHB = time from insemination to hatching of blastocyst; tSC = earliest sign of compaction. other abbreviations as in Table 1.

[a] Morphokinetic time parameters assessed from syngamy but not from insemination.

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an individual, which actually is not the case. Indeed, embryos from the same patient exhibit clustering (16). The multilevel mixed-effects linear regression model analysis is a perfect tool to assess correlations among observations in the same cluster (a patient in our study) and give an estimate on how much of the overall variation of a parameter (TLM parameter in our study) is explained by the clustering effect, as expressed with the use of ICC (19). In other terms, ICC gives an estimate of how much of the variation in any TLM parameter can be explained by patient–and ovarian stimulation–related factors. In our study, the majority of ICCs for various TLM parameters were moderate, in the range of 16%–47% (Table 2), meaning that 16%–47% of the observed variation of those TLM parameters was due to patient–or ovarian stimulation–related factors. Our results clearly demonstrate that embryos from the same patient exhibited similar developmental timing events compared with those embryos from other patients. Therefore, it would be erroneous to consider each embryo from a patient as an individual. Apart from introducing a confounding effect, clustering might also reduce the power of a study, because a cohort of embryos from the same individual would not provide independent unique information (18). Moreover when clustering is not taken into account, statistical tests for comparison of means, such as Mann-Whitney *U* test, Student *t* test, and analysis of variance, assuming each embryo as an independent observation may end up with overestimation of the reported influence (16, 19). Similarly, ordinary logistic regression analysis with inclusion of tightly correlated TLM parameters, as was performed in five studies (6, 9, 10, 13, 15), may be erroneous.

The impact of clustering and confounding would be more pronounced if the study population is more heterogeneous (18). For nine of the reported studies, the inclusion criteria were AMA (8, 13–15), recurrent implantation failure (8, 9, 12, 13, 15), recurrent miscarriage (8, 9, 12–15), severe male factor (8), sex selection (6), and balanced translocation in either partner (14), and in one study inclusion criteria were not available (11). In the present study, 103 consecutive patients (103 cycles), during a specific time frame, requiring PGS (with or without PGD) were included; the inclusion criteria were AMA, balanced translocation, or single-gene disorder in either partner. Significant impact of clustering on the results of prediction of euploidy with the use of TLM parameters would not be unexpected, with such heterogeneous patient populations in the available ten studies plus ours, if clustering had not been taken into account.

To our knowledge, the impact of clustering effect on TLM parameters was first reported by Kirkegaard et al. (16). In that study, 243 patients aged <38 years with at least eight harvested oocytes were included. A total of 1,507 embryos were monitored for 6 days in a time-lapse incubator. The authors concluded that 16%–31% of the observed variation in timing of embryo development was due to patient- and treatment-related factors. In general, no single patient–or ovarian stimulation–related factor was reported to elicit a systematic influence on the overall timing from the cleavage to the blastocyst stage. However, female age, number of previous cycles, and cumulative FSH dose had an influence on timing of blastocyst development (16). Similarly in our study, we did not note a systematic influence of patient–and ovarian stimulation–related factors on TLM parameters. However, we noted that BMI had a significant impact on several cleavage-stage parameters, including tPNa, tPNf, t2, t4, t5, t6, t7, and t8. In addition, the total FSH dose, duration of infertility, number of previous cycles, antral follicle count, and number of retrieved oocytes had a significant impact on some TLM parameters.

We also tested the previously reported cutoff points for various TLM parameters but only noted significant predictive ability of tSB with 96.6 hours as the cutoff point (10). However, one should bear in mind that differences in laboratory and in vitro culture conditions might contribute to differences in time events.

The main drawback of our study is the lack of automated annotation; however, the high kappa scores for intra- and interobserver agreement were reassuring. Limited sample size is a limitation of the current study. The inclusion of AMA cases as the majority in the current series limits the generalizability of our results to different patient populations. Finally, the association of various morphologic parameters (e.g., multinucleation, direct uneven cleavage, and embryo fragmentation) with ploidy status was not studied.

We conclude that aneuploid embryos appear to be delayed in timing at the post-cleavage stages, as manifested by some TLM parameters; however, the predictive ability of these significant TLM parameters is low to moderate when patient– and ovarian stimulation–related factors as potential sources of confounding are taken into account. Therefore, caution should be exercised in predicting ploidy status by means of TLM assessment.

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SUPPLEMENTAL APPENDIX

Protocols for ovarian stimulation, laboratory procedures for trophectoderm biopsy, vitrification, warming, and genetic testing

OVARIAN STIMULATION AND PROCEDURES PERFORMED IN THE IVF LABORATORY

Long GnRH agonist (Lucrin; Abott) protocol (n = 57 cycles) and GnRH antagonist protocol (Cetrotide [Merck Serono] or Orgalutran [MSD]) (n = 46 cycles) were used for ovarian stimulation. Depending on ovarian reserve, daily recombinant FSH (Gonal-F; Merck) and/or highly purified hMG (Menopur; Ferring) with initial doses of 150–450 IU/d were used for ovarian stimulation. After 5 days of stimulation, ovarian response was monitored with the use of transvaginal ultrasonography and serum E_2 measurements to adjust daily gonadotropin dosing. For GnRH antagonist use, fixed protocol was used starting the GnRH antagonist on the 5th or 6th day of ovarian stimulation. Triggering of final oocyte maturation was performed with the use of recombinant hCG (Ovitrelle) or GnRH agonist triptorelin (Decapeptyl; Ferring), as soon as there were three follicles >17 mm in diameter. Oocyte retrieval was carried out under general anesthesia with the use of transvaginal ultrasound–guided puncture of follicles 34–36 hours after triggering final oocyte maturation.

After 2–4 hours of incubation, cumulus-oocyte complexes were denuded by exposure to 80 IU/mL hyaluronidase solution diluted tenfold with G-MOPS Plus medium (Vitrolife), and also mechanically by plastic pipettes of defined diameters (denuding pipettes; Origio). Insemination of oocytes by means of intracytoplasmic sperm injection (ICSI) was carried out immediately after denudation. Each inseminated oocyte was then placed in 25 μL culture medium, covered by pre-equilibrated mineral oil (Ovoil; Vitrolife) in a microwell of the Embryoslide, and loaded into the Embryoscope (Unisense Fertilitech). Embryo culture was carried out in 6.8% CO_2 and 5.0% O_2. Single step medium (G-TL; Vitrolife) was used. Following ICSI, no refreshment was made until day 5; refreshment on day 5 was made if in vitro culture was extended to day 6 or 7. Time-lapse images were used for the assessment of fertilization, embryo morphology, and timing of developmental events up to the point of biopsy.

BLASTOCYST BIOPSY AND PREIMPLANTATION GENETIC SCREENING

Trophectoderm biopsy was performed to expanding, expanded, and hatched blastocysts after 120–160 hours from insemination (day 5 or 6). In particular, all blastocysts with a defined inner cell mass and 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 6 μL G-MOPS-Plus buffered medium (Vitrolife) overlaid with pre-equilibrated mineral oil. A diode laser (Research Instruments) was used to assist an opening of 10–15 μm in the zona pellucida. Five to ten 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.

GENETIC TESTING

Trophectoderm biopsies were sent to a reference genetic laboratory for the analysis (Genlab). All samples were processed for whole-genome amplification (WGA) and array comparative genomic hybridization (aCGH). The WGA of the biopsy samples was performed with the use of the Sureplex DNA Amplification System (Bluegnome). One nanogram of genomic DNA and one reagent-negative control sample were subjected to WGA. The WGA products and reference DNA were labeled with Cy3 and Cy5 fluorophores for 2–4 hours. Labeled DNA was then resuspended in the hybridization buffer and hybridized onto the 24sure slides under cover slides for 4–6 hours. After washing and drying, the slides were scanned at 10 μm with the use of a laser scanner (Innoscan 710). The scanned data were then analyzed and quantified by algorithm-fixed settings in Bluefuse Multi (Bluegnome), a software package that performed the steps of grid placement, quantification, normalization, and post-processing automatically. Once a specific amplification was observed (i.e., low autosomal noise), autosomal profiles were analyzed for gain or loss of whole chromosomal ratios with the use of a 3 × SD assessment, greater than ±0.3 log2 ratio call, or both, according to the manufacturer's instructions.

BLASTOCYST VITRIFICATION AND WARMING PROCEDURES

The vitrification and warming procedures that we used in our laboratory setting were previously described by Cobo et al. (1). After trophectoderm biopsy, blastocyst vitrification was performed with the use of the Cryotop device and solutions (Kitazato Biopharma). The first equilibration was carried out in 7.5% ethylene glycol and 7.5% dimethylsulphoxide at room temperature for 12 minutes. Subsequently, blastocysts were transferred into 15% ethylene glycol, 15% dimethylsulphoxide, and 0.5 mol/L sucrose for 1 minute, and then placed on the filmstrip 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 0.5 mL 37°C warming solution containing 1.0 mol/L sucrose for 1 minute, then blastocysts were transferred to a room temperature solution containing 0.5 mol/L sucrose and incubated for 3 minutes. After two subsequent washings in basic medium at room temperature for 6 minutes each, blastocysts were placed into 25 μL culture medium (G-TL Plus, Vitrolife) and covered with oil.

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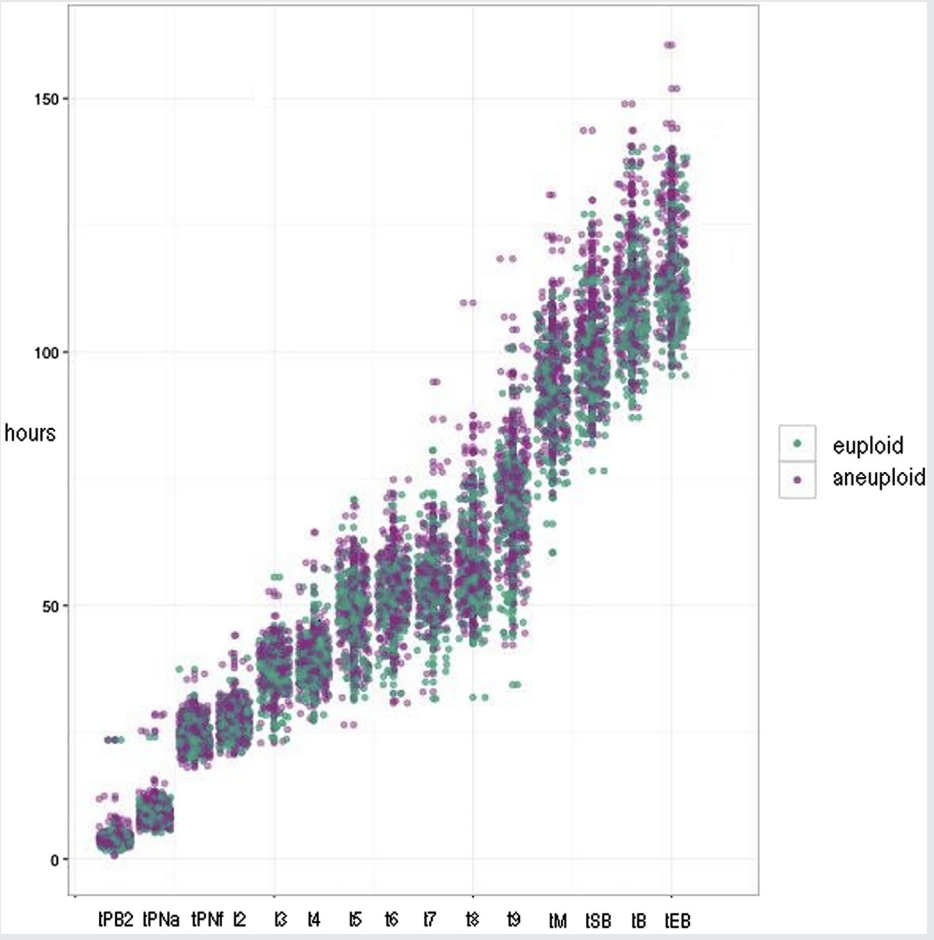
SUPPLEMENTAL FIGURE 1



Distribution of time-lapse morphokinetic parameters of euploid and aneuploid blastocysts. tPB2 = appearance of second polar body; tPNa = appearance of pronuclei (2PN); tPNf = both pronuclei faded/syngamy; t2, t3, t4, t5, t6, t7, t8, and t9 = time between intracytoplasmic injection and 2-, 3-, 4-, 5-, 6-, 7-, 8-, and ≥9-cell stages, respectively; tM = time from insemination to formation of a morula; tSB = time from insemination to start of blastulation; tB = time from insemination to formation of a full blastocyst; tEB = time from insemination to expanded blastocyst.

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SUPPLEMENTAL TABLE 1

Definition of time-lapse morphokinetic (TLM) parameters.

TLM parameter	Definition
tPB2	Appearance of second polar body
tPNa	Appearance of pronuclei (2PN)
tPNf	Both pronuclei faded/syngamy
t2	Time from insemination to division to 2 cells (CC1)
t3	Time from insemination to division to 3 cells
t4	Time from insemination to division to 4 cells
t5	Time from insemination to division to 5 cells
t6	Time from insemination to division to 6 cells
t7	Time from insemination to division to 7 cells
t8	Time from insemination to division to 8 cells
t9	Time from insemination to division to $\geq$9 cells
tM	Time from insemination to formation of a morula, where all of the cells had undergone the compaction process and cell boundaries was unclear
tSB	Time from insemination to start of blastulation, when the first signs of a cavity were visible
tB	Time from insemination to formation of a full blastocyst, when the blastocele filled the embryo with <10% increase in its diameter
tEB	Time from insemination to expanded blastocyst, when the blastocyst had increased in diameter by >30% and the zona pellucida started to thin
CC2	Duration of second cell cycle from 2 to 3 cells (t3 − t2)
CC3	Duration of the third cell cycle from 3 to 5 cells (t5 − t3)
S2	Time of synchrony of the second cell cycle (t4 − t3), from 3 to 4 cells
S3	Time of synchrony of the third cell cycle (t8 − t5), from 5 to 8 cells
t5 − t2	Difference between t5 and t2
t9 − t2	Difference between t9 and t2
Blastulation	Time of blastulation, from start of blastulation to formation of a full blastocyst (tB − tSB)
CC3/CC2	CC3/CC2 ratio

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SUPPLEMENTAL TABLE 2

Baseline demographic features and ovarian stimulation characteristics of the included patients.

No. of patients	103
No. of cycles	103
No. of previous cycles	2.0 (0–9)
Female age, y	38.0 ± 4.7
Female body mass index, kg/m^2	25.2 ± 4.3
Controlled ovarian stimulation protocol	
Long GnRH agonist protocol	57 (55.3)
GnRH antagonist protocol	46 (44.7)
Duration of stimulation, d	9 (6–14)
Total FSH dose, IU	2,250 (630–5,850)
E_2 level on the day of triggering, pg/mL	2,571 (124–9,567)
No. of retrieved oocytes	10.3 ± 4.7
No. of embryos with 2 pronuclei	7.0 ± 3.5
No. of biopsied blastocysts	415
No. of euploid blastocysts	173 (41.7)

Note: Values are presented as n, median (range), mean ± SD, or n (%).

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