Morphokinetic parameters using time-lapse technology and day 5 embryo quality: a prospective cohort study

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Abstract

Purpose The aims of this prospective study were to evaluate whether time-lapse parameters can aid in the prediction of day 5 embryo quality and also to assess their discriminatory capacity. **Methods** In this prospective cohort study, we used time-lapse technology to record specific timings of key events for 380 day 5 blastocysts (originating from 108 patients). Generalized estimating equation regression models were used to evaluate the capacity of these markers to identify a top-quality blastocyst. Multivariable regression models were also constructed, aiming to identify the model with the highest capacity to predict a top-quality blastocyst. The discriminatory capacity of single predictors or composite models was assessed with the use of receiver operating characteristic (ROC) analyses.

Results Eight significant predictive parameters of a top-quality blastocyst were identified: s3, t6, t7, t8, tM, tSB, tB and tEB. A ROC analysis of the identified parameters found s3 (area under the curve—AUC 0.585, 95 % CI 0.534–0.635) to have the best individual discriminatory capacity to predict a top-quality blastocyst prior to embryo compaction. The parameter tEB (AUC 0.727, 95 % CI 0.675–0.775) was the best predictor regardless of embryo stage. A model containing s3,

t8 and tEB showed a slightly increased discriminatory capacity for top-quality blastocyst prediction (AUC 0.748, 95 % CI 0.697–0.794).

Conclusions The identified morphokinetic parameters and their cutoffs, albeit of limited clinical value, add to the increasing knowledge concerning the potential predictive markers of a top-quality blastocyst. Additional evidence is necessary before validated time-lapse parameters can be used for embryo selection in IVF laboratories.

Keywords Blastocyst · Morphokinetics · Prediction · Time-lapse · Quality

Introduction

In vitro fertilization (IVF) laboratories have relied on morphologic assessment of embryos at distinct time-points to choose the best one for transfer since the early days of assisted reproductive technology [1, 2].

For this purpose, several grading systems have been developed which, although not perfect, have allowed the non-invasive selection of the embryo with presumably the highest implantation potential [3–5]. These systems require that embryos be graded at different stages of development (cleavage and blastocyst stage) by visual assessment of a static image under light microscopy. A major limitation of these methods is the need to evaluate embryo development multiple times by removing it from the incubator and thus exposing embryos to sub-optimal conditions [6, 7]. Also, since these systems involve judgment by an embryologist, there may be significant inter-observer variability affecting their overall reproducibility [8].

Time-lapse monitoring (TLM) has emerged as a non-invasive method that offers the ability to monitor embryos continuously without disturbing their stable culture conditions

Capsule Morphokinetic parameters using time-lapse technology can predict the quality of day 5 embryos, although their clinical usefulness might be limited.

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[9, 10]. This seems to be important as it is well documented that fluctuations in humidity, temperature, light and pH negatively impact on embryo development and quality [6, 7]. In addition, TLM provides significantly more information to the embryologist regarding embryo development and allows for the identification of key events that might be associated with its ability to form a top-quality blastocyst and eventually with its implantation potential. Some TLM systems, such as the EmbryoScope (Unisense Fertilitech, Aarhus, Denmark), allow for a more standardized method of grading embryos by using computer-assisted annotations [8]. Using image analysis software (EmbryoViewer Unisense Fertilitech, Aarhus, Denmark), all embryo developmental events (e.g. division to a two-cell embryo) can be recorded, along with recording the corresponding timing of these events in hours after intracytoplasmic sperm injection (ICSI). This also allows for retrospective analysis of images for each embryo [10].

The continuous monitoring of embryo development, along with the ability to perform automatic annotations, has made way for the identification of morphokinetic markers based on specific events during embryo growth that have been suggested to predict blastocyst formation [11–14] and/or implantation potential [9, 10, 15, 16]. Some studies have combined multiple time-lapse parameters into a cumulative algorithmic model in an attempt to predict blastocyst development [11, 12, 14]. Although there have been cases where these models were validated in subsequent studies [11, 17], a recent systematic review has concluded that overall the different parameters used are inconsistent between most studies and cannot be directly compared [18]. Furthermore, some algorithms that have been shown to be predictive of blastocyst morphology include day 3 standard morphology grading [19].

A critical test for any predictive algorithm is that it is both sensitive and specific and also has an acceptable discriminatory capacity when externally validated. The few studies that have been carried out to validate previously designed algorithms in this field have found limited predictive ability in different clinical settings [20, 21]. Hence, although the use of time-lapse for prediction of blastocyst morphology appears to be promising, it is clear that the accumulation of further data evaluating this technology is required.

The aim of this prospective study was to evaluate whether time-lapse parameters can aid in the prediction of top-quality blastocyst morphology and additionally to assess their discriminatory capacity.

Materials and methods

Study design and participants

This was a single-center, prospective cohort study performed at IVF Australia between May 2013 and February 2015.

Written informed consent was obtained from all participants before inclusion in the study. Patients had either a fresh transfer or a freeze-all cycle on day 5 of embryo development, and both donor and autologous cycles were included. Patients had to be undergoing ICSI treatment and were not eligible for this study if they had laser-assisted hatching, polarized light microscopy for spindle assessment [22], surgical sperm collection, pre-implantation genetic diagnosis or screening or if they did not have any of their embryos develop to the blastocyst stage.

Ovarian stimulation, oocyte retrieval and ICSI

Ovarian stimulation was performed with the use of gonadotrophins, the starting dose of which ranged from 100 to 450 IU and was determined by the treating physician based on age, anti-Müllerian hormone (AMH) and previous response to ovarian stimulation. Prevention of premature luteinising hormone (LH) surge was achieved with the use of gonadotrophin-releasing hormone (GnRH) agonists or antagonists. Follicular growth was monitored with the use of regular ultrasounds and serum estradiol assessments. When follicular growth was deemed satisfactory by the treating physician (usually when more than two follicles of at least 17 mm were present at ultrasound), final oocyte maturation was triggered with the use of 250 µg of recombinant human chorionic gonadotrophin (rhCG). Oocyte retrieval occurred 36 h after rhCG injection. Following follicle aspiration, oocytes were collected in G-MOPS PLUS medium (Vitrolife, Sweden) before being cultured in G-1 PLUS medium (Vitrolife, Sweden) at 37 °C, 5 % O₂ and 6 % CO₂ in a bench-top incubator (MINC, COOK, Sydney, Australia). Denudation of cumulus cells was performed in an EmCell (HD Scientific, Australia) by mechanical pipetting in hyaluronidase (30 IU/ml, Hyalase, Sanofi Aventis, Australia) in G-1 PLUS medium at 6 % CO₂ and 37 °C just prior to ICSI. All metaphase II oocytes were injected using standard ICSI procedures at ×400 magnification 39–41 h after rhCG injection. The oocytes were injected in 5 µl G-MOPS PLUS droplets under mineral oil (Ovoil, Vitrolife, Sweden) in a pre-warmed dish, and prepared sperm was placed in a separate droplet of 7 % polyvinyl pyrrolidone (CooperSage, USA).

Embryo culture

At post-injection, each zygote was individually placed in a pre-equilibrated culture slide (EmbryoSlide, Unisense Fertilitech, Aarhus, Denmark). The EmbryoSlide has a central depression containing 12 straight-sided circular wells, each filled with 20 µl of G-1 PLUS medium. The depression containing the wells was then filled with an overlay of 1.4 ml of mineral oil to prevent evaporation. It was ensured that the order of injection at ICSI was preserved from ICSI dish to

culture slide. The culture slides were pre-equilibrated (37 °C and 6 % CO₂) using fresh G-1 PLUS medium and mineral oil. Any air bubbles that formed during pre-equilibration were removed before the zygotes were positioned in the wells. The culture slide was placed in an EmbryoScope at 37 °C, 5 % O₂ and 6 % CO₂ immediately after ICSI. The EmbryoScope is a tri-gas incubator capable of taking photographs every 7 to 20 min at three to seven focal planes (depending on the frequency of photographs taken). The media change was performed on the morning of day 3 of embryo culture in pre-equilibrated dishes containing G-2 PLUS (Vitrolife, Sweden) medium, prepared an hour in advance using the same method as day 0 dish preparation.

Embryo assessment

Successful fertilization was assessed at 16–19 h after insemination based on digital images acquired using the time-lapse incubator. Embryo morphology was also evaluated using digital images on day 2 (44–48 h post-injection) and day 3 (68–72 h post-injection) for number of blastomeres, symmetry and percentage of fragmentation. Blastocysts were graded on day 5 (112–120 h post-injection) according to the Gardner criteria [3] based on the expansion of the blastocoel cavity and the number and integrity of both the inner cell mass (ICM) and trophectoderm cells without removal from the EmbryoScope. The same person was assigned to embryo assessment for all included patients to ensure that there was no inter-observer assessment error. Blastocysts with ICM and trophectoderm grade AA or BA were considered to be top quality, grade AB or BB was considered to be good quality [5], while blastocysts with grade C ICM or trophectoderm were considered to be of poor quality [3].

Time-lapse monitoring and annotation

In this study, the EmbryoScope recorded images of each embryo every 7 min in five focal planes. Analysis of acquired images was performed with the use of image analysis software (EmbryoViewer Unisense Fertilitech, Aarhus, Denmark). Various stages of embryo development were annotated (Table 1), and their exact timing relative to the time of sperm injection during ICSI was determined. The same person performed the annotations for all embryos.

Outcome measures

The primary outcome of this study was prediction of top-quality blastocyst morphology as assessed on day 5 (112–120 h post-ICSI) according to the Gardner criteria. The secondary outcome measure was prediction of top- or good-quality blastocyst morphology on day 5 according to the Gardner criteria.

Table 1 Parameters evaluated for their capacity to identify a top-quality blastocyst in the current study

Parameter	Description
Pn_t1	Time of pronuclei formation
NEBD	Nuclear envelope break down
Cytokinesis	First cytokinesis
t2	Time of cleavage to a two-cell embryo
t3	Time of cleavage to a three-cell embryo
t4	Time of cleavage to a four-cell embryo
t5	Time of cleavage to a five-cell embryo
t6	Time of cleavage to a six-cell embryo
t7	Time of cleavage to a seven-cell embryo
t8	Time of cleavage to an eight-cell embryo
tM	Time to full compaction
tSB	Time to the first signs of blastulation
tB	Time to full blastocyst
tEB	Time to expanded blastocyst
tHB	Time to hatching blastocyst
s1	Time between NEBD and subsequent division to two cells
s2	Time between division to three cells and subsequent division to four cells
s3	Time between division to five cells and subsequent division to eight cells
t4int	Time between division to four cells and subsequent division to five cells
t5-t2	Time between division to two cells and subsequent division to five cells
cc2	Duration of the second cell cycle
cc3	Duration of the third cell cycle

Statistical analysis

All continuous variables are presented as mean and standard deviation (SD). Categorical variables are presented as proportions. Parameters were also converted to their standardized values (mean=0, SD=1) to facilitate a more direct comparison of the predictive effect of each variable on the odds of the outcome of interest. Due to the clustering nature of data (i.e. some oocytes and/or embryos originate from the same patient), the analysis of data was performed with the use of generalized estimating equations (GEE), which adjust for any auto-correlation between the observations. Logit regression models were constructed for both the unstandardized and standardized values of the independent variables, and clustered robust standard errors were calculated.

Initially, for each one of the time-lapse parameters, we performed a bivariate regression analysis, in which the time-lapse parameter was entered as an independent variable and the outcome of interest (e.g. top-quality blastocyst and top- or good-quality blastocyst) as the dependent variable. Those

identified as significant ($p \leq 0.05$) predictors were subsequently included in multivariable regression models aiming to identify the model with the highest capacity to predict top-quality and top- or good-quality blastocysts. Female age was also added in this model. In a backward stepwise fashion, variables with $p < 0.10$ were removed.

Composite models were constructed including (a) only parameters prior to embryo compaction and (b) all parameters regardless of embryo stage.

Moreover, using the results of the aforementioned GEE regression analyses, receiver operator characteristic (ROC) analysis was performed in order to determine the area under the curve (AUC) and identify cutoffs with the best discriminatory capacity in regards to the outcome examined.

Results

A total of 125 couples signed the consent form prior to initiation of their stimulation cycle, of which 108 fulfilled the inclusion criteria and were analysed in this study. These couples contributed 110 cycles in total. The mean female age was 34.9 (SD 4.4), while the mean number of oocytes retrieved was 14.1 (SD 7.5). The mean number of oocytes injected was 11.4 (SD 6.2). A total of 380 blastocysts were available on day 5 for this analysis. Baseline characteristics are presented in Table 2.

Association between parameters and achievement of a top-quality and top- or good-quality blastocyst

Multiple bivariate GEE logit regression analyses were used to select the parameters that predict top-quality and top- or good-quality day 5 blastocyst morphology. Table 3 provides an overview of all parameters assessed and the bivariate regression analyses performed for both the unstandardized and the standardized values of the independent variables. Eight significant predictive parameters of a top-quality blastocyst were identified (Table 3): s3, t6, t7, t8, tM, tSB, tB and tEB. A total of 14 parameters predicted top- or good-quality blastocyst morphology (Table 3): Pn_t1, NEBD, Cytokinesis, s1, s3, t2, t4, t6, t7, t8, tM, tSB, tB and tEB. In terms of effect sizes, the regression analyses of the standardized independent variables reveal that prior to embryo compaction, s3 and s1 appear to have the most important effect on the prediction of top-quality and top- or good-quality blastocysts, respectively. After embryo compaction, tEB is the most significant predictor of both top-quality and top- or good-quality blastocysts.

Tables 4 and 5 show the time-ranges for each of the statistically significant variables identified in the logistic regression analyses, as well as the results of the ROC analyses. For top-quality blastocyst prediction using a single parameter, the maximum AUC was 0.727 (95 % CI 0.675–0.775) for tEB, while for top- or good-quality blastocyst prediction the maximum AUC was 0.826 (95 % CI 0.780–0.866) also for tEB. Considering only the parameters prior to embryo compaction,

Table 2 Baseline characteristics

Characteristic	Mean	Standard deviation	Range
Number of patients	108	N/A	N/A
Maternal age	34.9	4.4	26–46
BMI	25.3	4.9	17.2–38.9
Primary infertility— <i>n</i> (%)	61 (56.5)	N/A	N/A
Secondary infertility— <i>n</i> (%)	47 (43.5)	N/A	N/A
Number of cycles	110	N/A	N/A
Starting FSH dose (IU)	182.0	67.1	75–450
Duration of stimulation (days)	10.1	1.8	5–16
Oocytes retrieved	14.1	7.5	3–33
Oocytes injected	11.4	6.2	3–32
Fertilization rate (%)	75.4	19.3	14.3–100.0
Total number of blastocysts available on day 5	380	N/A	N/A
Top quality— <i>n</i> (%)	139 (36.6)	N/A	N/A
Good quality— <i>n</i> (%)	159 (41.8)	N/A	N/A
Poor quality— <i>n</i> (%)	82 (21.8)	N/A	N/A
Number of cycles with embryo transfer	101	N/A	N/A
Number of embryos cryopreserved/cycles	2.2	2.0	0–10
Number of freeze-all cycles	9	N/A	N/A
Number of clinical pregnancies— <i>n</i> (%)	49 (48.5 %)	N/A	N/A

N/A not applicable

Table 3 Bivariate GEE logit regression analyses of time-lapse parameters for development into a top-quality blastocyst and a top- or good-quality blastocyst

Parameter	Top-quality blastocysts OR (95 % CI)		Top- or good-quality blastocysts OR (95 % CI)	
	Unstandardized ^a	Standardized ^b	Unstandardized ^a	Standardized ^b
Pn_t1	0.95 (0.82–1.11)	0.93 (0.74–1.17)	<i>0.84 (0.72–0.98)</i>	<i>0.76 (0.60–0.97)</i>
NEBD	0.94 (0.85–1.05)	0.86 (0.65–1.14)	<i>0.90 (0.81–0.99)</i>	<i>0.76 (0.59–0.99)</i>
Cytokinesis	0.94 (0.84–1.05)	0.85 (0.64–1.13)	<i>0.89 (0.81–0.98)</i>	<i>0.73 (0.57–0.95)</i>
s1	0.70 (0.39–1.27)	0.69 (0.37–1.28)	<i>0.43 (0.20–0.90)</i>	<i>0.41 (0.19–0.89)</i>
cc2	1.02 (0.93–1.12)	1.05 (0.86–1.30)	1.02 (0.87–1.19)	1.05 (0.73–1.49)
s2	0.82 (0.61–1.10)	0.70 (0.42–1.17)	0.89 (0.77–1.02)	0.82 (0.64–1.04)
s3	<i>0.94 (0.89–0.98)</i>	<i>0.67 (0.50–0.89)</i>	<i>0.93 (0.89–0.97)</i>	<i>0.65 (0.50–0.83)</i>
cc3	1.01 (0.94–1.09)	1.04 (0.87–1.26)	1.06 (0.94–1.20)	1.19 (0.84–1.68)
t2	0.93 (0.84–1.03)	0.82 (0.62–1.08)	<i>0.87 (0.79–0.95)</i>	<i>0.67 (0.52–0.86)</i>
t3	0.99 (0.92–1.06)	0.96 (0.75–1.22)	0.94 (0.86–1.03)	0.80 (0.58–1.12)
t4	0.95 (0.88–1.02)	0.83 (0.64–1.09)	<i>0.91 (0.84–0.98)</i>	<i>0.71 (0.53–0.94)</i>
t4int	1.05 (0.98–1.13)	1.19 (0.95–1.48)	1.08 (0.99–1.19)	1.30 (0.96–1.78)
t5	1.00 (0.96–1.04)	0.99 (0.79–1.23)	0.99 (0.92–1.06)	0.95 (0.67–1.37)
t5-t2	1.03 (0.97–1.08)	1.12 (0.91–1.37)	1.05 (0.96–1.16)	1.23 (0.86–1.76)
t6	<i>0.95 (0.90–0.99)</i>	<i>0.76 (0.59–0.99)</i>	<i>0.93 (0.88–0.98)</i>	<i>0.70 (0.53–0.92)</i>
t7	<i>0.95 (0.91–0.98)</i>	<i>0.70 (0.54–0.91)</i>	<i>0.94 (0.90–0.98)</i>	<i>0.67 (0.52–0.87)</i>
t8	<i>0.96 (0.93–0.99)</i>	<i>0.72 (0.57–0.92)</i>	<i>0.95 (0.92–0.98)</i>	<i>0.67 (0.53–0.85)</i>
tM	<i>0.94 (0.91–0.97)</i>	<i>0.63 (0.50–0.80)</i>	<i>0.92 (0.88–0.95)</i>	<i>0.53 (0.41–0.69)</i>
tSB	<i>0.92 (0.88–0.96)</i>	<i>0.52 (0.37–0.72)</i>	<i>0.88 (0.85–0.92)</i>	<i>0.40 (0.30–0.52)</i>
tB	<i>0.90 (0.86–0.94)</i>	<i>0.42 (0.29–0.61)</i>	<i>0.86 (0.82–0.89)</i>	<i>0.27 (0.19–0.39)</i>
tEB	<i>0.89 (0.85–0.94)</i>	<i>0.40 (0.26–0.61)</i>	<i>0.83 (0.79–0.88)</i>	<i>0.22 (0.14–0.35)</i>

OR values in italics indicate significant outcomes

OR odds ratio, CI confidence interval

^a The odds ratio represent the expected effect on the dependent variable with a one-unit increase of the independent variable^b The odds ratio represent the expected effect on the dependent variable with a one-standard-deviation increase of the independent variable

s3 had the highest capacity to discriminate between embryos that will form top-quality blastocysts (AUC 0.585, 95 % CI 0.534–0.635) and top- or good-quality blastocysts (AUC 0.624, 95 % CI 0.573–0.673).

A backward stepwise regression analysis including female age and significant parameters prior to embryo compaction for the prediction of top-quality blastocyst identified a model with s3, t6 and t8, which was associated with an AUC of 0.596 (95 % CI 0.545–0.646). This was not significantly different than the AUC of s3 (diff. +0.011, 95 % CI −0.031 to +0.054) (Fig. 1a). When female age and every significant time-lapse predictor (regardless of embryo stage) were entered in a backward stepwise regression analysis, a model with s3, t8 and tEB was constructed. This model was characterized by a greater AUC for prediction of top-quality blastocysts (AUC 0.748, 95 % CI 0.697–0.794), which, however, was not significantly different than the AUC of tEB (diff. +0.021, 95 % CI −0.012 to +0.053) (Fig. 1b).

Regarding the prediction of top- or good-quality blastocysts, a stepwise regression analysis of female age and time-

lapse parameters prior to embryo compaction resulted in the construction of a model combining t2 and s3 with an AUC of 0.621 (95 % CI 0.570–0.670). This AUC was not significantly different than the AUC of s3 (diff. −0.003, 95 % CI −0.060 to +0.054) (Fig. 2a). When female age and all the significant time-lapse parameters (regardless of embryo stage) were included in the stepwise regression analysis, a model combining t2, s3, t8, tSB and tEB was constructed with an AUC of 0.849 (95 % CI 0.806–0.886), which, however, was not significantly different than the AUC of tEB (diff. +0.023, 95 % CI −0.008 to +0.054) (Fig. 2b).

Validation of previously identified time-lapse markers

Only one other study could be identified that used proposed cutoffs to identify top-quality blastocysts [12]. This study suggested t3, t5, cc2 and s2 to be able to predict a good-morphology blastocyst. Table 6 presents the outcomes of the logistic regression analysis performed to validate these predictive parameters. Regarding the prediction of a top-quality

Table 4 ROC analysis of predictive time-lapse parameters for development into a top-quality blastocyst

Parameter	Min–max (h)	AUC (95 % CI)	Optimal cutoff (h)	Sensitivity %	Specificity%	LR+	LR–
s3	0.58–26.17	0.585 (0.534–0.635)	<3.50	69.10	50.20	1.39	0.62
t6	41.01–62.38	0.550 (0.498–0.601)	<51.98	66.20	46.50	1.25	0.71
t7	41.71–68.06	0.558 (0.506–0.609)	<54.25	71.22	41.49	1.22	0.69
t8	42.41–77.47	0.516 (0.508–0.610)	<52.46	48.64	65.56	1.44	0.77
tM	71.70–104.22	0.601 (0.550–0.650)	<97.33	89.93	26.14	1.22	0.39
tSB	82.88–116.30	0.665 (0.615–0.712)	<102.13	83.45	45.23	1.53	0.37
tB	89.53–127.51	0.713 (0.665–0.758)	<107.69	71.22	68.05	2.22	0.42
tEB	94.55–130.79	0.727 (0.675–0.775)	<112.86	78.68	62.69	2.11	0.34
Model with s3, t6 and t8	N/A	0.596 (0.545–0.646)	>0.33 ^a	84.17	33.20	1.23	0.54
Model with s3, t8 and tEB	N/A	0.748 (0.697–0.794)	>0.48 ^a	64.12	79.27	3.09	0.45

AUC area under the curve, LR+ positive likelihood ratio, LR– negative likelihood ratio

^a This is the cutoff in the score produced by the regression function

blastocyst, none of the proposed parameters had statistically significant predictive ability in our study population. Only the parameter cc2 was able to significantly predict top- or good-quality blastocyst outcome (OR 1.88, 95 % CI 1.11–3.19).

Discussion

This study identified eight individual morphokinetic parameters (s3, t6, t7, t8, tM, tSB, tB and tEB) that could be used to predict top-quality blastocyst morphology. Prior to embryo compaction, s3, t6 and t8 could be used as independent predictors of top-quality blastocyst morphology, whereas when

all parameters were considered (regardless of embryo stage), s3, t8 and tEB were found to have an independent capacity to predict a top-quality blastocyst and were included in a composite model.

Regarding the prediction of a top- or good-quality blastocyst, the parameters that were identified as predictors in this study were Pn_t1, NEBD, Cytokinesis, s1, s3, t2, t4, t6, t7, t8, tM, tSB, tB and tEB. When all of these parameters were evaluated, t2, s3, t8, tSB and tEB were found to be independent predictors of a top- or good-quality blastocyst and were included in a composite model. When only parameters prior to embryo compaction were considered, t2 and s3 were found to be independent

Table 5 ROC analysis of predictive time-lapse parameters for development into a top- or good-quality blastocyst

Parameter	Min–max (h)	AUC (95 % CI)	Optimal cutoff (h)	Sensitivity %	Specificity %	LR+	LR–
Pn_t1	5.00–15.33	0.552 (0.501–0.603)	<6.88	22.48	90.24	2.30	0.86
NEBD	17.77–30.04	0.557 (0.506–0.608)	<26.04	82.21	31.71	1.20	0.56
Cytokinesis	20.23–32.83	0.563 (0.511–0.613)	<28.10	78.19	35.37	1.21	0.62
s1	1.75–3.74	0.567 (0.515–0.617)	<2.66	67.79	48.78	1.32	0.66
s3	0.47–27.47	0.624 (0.573–0.673)	<2.92	52.68	67.07	1.60	0.71
t2	20.34–32.94	0.572 (0.521–0.623)	<28.40	79.87	35.37	1.24	0.57
t4	30.42–46.51	0.563 (0.511–0.613)	<39.12	64.77	48.78	1.26	0.72
t6	41.01–66.01	0.556 (0.505–0.607)	<56.38	88.26	24.39	1.17	0.48
t7	41.58–74.50	0.571 (0.520–0.622)	<56.78	82.55	32.93	1.23	0.53
t8	42.41–78.13	0.574 (0.522–0.624)	<60.63	84.90	30.49	1.22	0.50
tM	71.70–109.18	0.647 (0.597–0.695)	<97.33	84.23	36.59	1.33	0.43
tSB	82.88–116.30	0.723 (0.675–0.767)	<102.71	77.18	58.54	1.86	0.39
tB	89.53–134.95	0.787 (0.743–0.827)	<115.40	90.60	54.88	2.01	0.17
tEB	94.55–136.12	0.826 (0.780–0.866)	<114.81	76.72	79.03	3.66	0.29
Model with t2 and s3	N/A	0.621 (0.570–0.670)	>0.78 ^a	68.79	53.66	1.48	0.58
Model with t2, s3, t8, tSB and tEB	N/A	0.849 (0.806–0.886)	>0.71 ^a	90.84	66.13	2.68	0.14

AUC area under the curve, LR+ positive likelihood ratio, LR– negative likelihood ratio

^a This is the cutoff in the score produced by the regression function

Fig. 1 Comparison of ROC curves of the top-quality blastocyst composite models with the best independent predictor: **a** using the parameters prior to compaction, **b** regardless of embryo stage

predictors of a top- or good-quality blastocyst and were included in a composite model.

This study aimed to identify cutoffs using ROC analysis for the prediction of a top-quality blastocyst from parameters derived using time-lapse technology. Previously published studies have identified variables that might be significantly associated with the development of a blastocyst and/or its quality [11, 13, 14, 16]. However, these studies either did not propose

any cutoffs, [14] thus potentially limiting their clinical applicability, or suggested that the mean $\pm$ standard deviation of the parameter should be used to define the optimal range without evaluating its discriminatory capacity using ROC analysis [11, 13, 16].

In the study by Cruz et al., ranges of values for certain parameters that had already been developed by Meseguer et al. [10] in order to predict implantation were assessed for

Fig. 2 Comparison of ROC curves of the top- or good-quality blastocyst composite models with the best independent predictor: **a** using the parameters prior to compaction, **b** regardless of embryo stage

Table 6 Validation of the proposed time-lapse parameter cutoffs proposed by Cruz et al.

Parameter	Cutoff (h)	Top-quality blastocyst OR (95 % CI)	Top- or good-quality blastocyst OR (95 % CI)
t3	35.4–40.3	1.15 (0.73–1.82)	1.59 (0.96–2.61)
t5	48.8–56.6	1.17 (0.73–1.87)	1.37 (0.78–2.39)
cc2	≤11.9	1.29 (0.73–2.29)	<i>1.88 (1.11–3.19)</i>
s2	≤0.2	1.57 (0.94–2.62)	0.91 (0.48–1.74)

OR odds ratio, CI confidence interval

OR values in italics indicate significant outcomes

their ability to identify good-quality blastocysts. The parameters evaluated in that study were t2, t3, t5, cc2 and s2, of which t3, t5, cc2 and s2 showed a significant capacity to predict a good-morphology blastocyst [12]. Evaluating the proposed ranges for these parameters in our study sample showed that only cc2 (≤11.9 h) predicted top- or good-quality blastocyst morphology (Table 6) but could not predict top-quality blastocyst morphology. Other studies have shown cc2 to predict blastocyst development [11, 19]. Wong et al. [11] was able to demonstrate that cc2 (along with s2 and the duration of the first cytokinesis) predicted blastocyst formation and correlated with normal gene expression profiles if the time range fell between 7.8 and 14.3 h. This time-range differs from the cut-off (≤11.9 h) proposed by Cruz et al. Using Eeva time-lapse technology, Conaghan et al. [19] found that cc2 improved the ability of embryologists to choose an embryo on day 3 with the highest probability of forming a usable blastocyst, if assessed in conjunction with day 3 morphology.

Kirkegaard et al. used a logistic regression analysis to identify s1 (OR 0.35, 95 % CI 0.16–0.83) and s2 (OR 0.88, 95 % CI 0.80–0.97) as significant predictive parameters of a high-quality blastocyst on day 6 [14]. In the current study, s1 was found to be predictive only of top- or good-quality blastocysts. The parameter s2 was not shown to be a significant predictor of either top-quality or top- or good-quality blastocysts (Table 3). These differences might be explained by the fact that Kirkegaard et al. [14] evaluated day 6 blastocysts, whereas in the present study only day 5 blastocysts were analysed. However, the effect sizes produced in the current study also suggest that the probability of a type II error is not unlikely.

One of the strengths of this study is that the specific injection time for each oocyte during ICSI was calculated, whereas in previous studies only an average ICSI time was documented for each cycle. Similarly, we determined accurate OPU, embryo transfer and freezing time-points. This approach was undertaken to allow for a more precise assessment of key points in embryo development. We also assessed embryos exclusively on day 5 (112–120 h post-ICSI) in an attempt to increase the internal validity of the prediction models. Last but not least, the analysis undertaken has controlled for the clustered nature of data and has adjusted its estimates and standard errors for the auto-correlation present between embryos

originating from the same patients. Such an adjustment unfortunately has only rarely been performed in previous studies, thus the chances of type I error might be increased in these studies.

This study has limitations that need to be discussed. Firstly, the sample size ($n=380$ embryos) is smaller than those of previously published studies [14, 19], and this might translate to reduced statistical power to detect potentially important effect sizes. Another limitation is the fact that this study has attempted to predict blastocyst morphology and not pregnancy rates. It should also be noted that since this study does not include arrested embryos, it does not evaluate blastocyst formation, but the quality of blastocysts formed. Although pregnancy represents the preferred outcome measure when testing the effectiveness of an intervention in IVF, this study has merit since it increases the body of knowledge around the early development of human embryos in culture and enhances our insight into potential predictive markers of blastocyst quality. At the same time, it has been shown that top-quality blastocysts could be used as a surrogate marker for pregnancy [3].

It should be emphasized that the clinical usefulness of the parameters identified in the current study in reliably predicting top-quality blastocyst morphology seems limited since the AUC produced for these variables suggested a poor-to-moderate discriminatory capacity. The combinations of sensitivity and specificity were not optimal, and the likelihood ratios reveal that it is unlikely that these variables can have a significant prognostic impact in everyday practice. Only the composite model predicting top- or good-quality blastocyst morphology seemed to present a slightly improved discriminatory capacity.

Another important aspect that should be taken into account when interpreting the results of this study is the fact that the variables that seemed to perform better were those closer to the actual event of blastocyst formation (i.e. tSB, tB, tEB) which translates to limited clinical value. Ideally, one would aim for a reliable prediction of top-quality blastocysts during the early stages of embryo growth. However, the early variables (prior to embryo compaction) identified were all characterized by poor discriminatory capacity. The best AUC obtained was when a composite model for the prediction of top- or good-quality blastocysts was constructed with the use of s3

and t2 as independent predictors. However, this model also produced a poor to moderate AUC of 0.621.

While a number of studies have demonstrated higher pregnancy and live birth rates after blastocyst transfer than after cleavage stage transfer [23], it has been previously shown that pregnancies resulting from blastocyst-stage embryos have an increased risk of preterm birth [24, 25] and congenital malformations [24]. The identification of early markers of top-quality blastocysts might allow for an earlier selection of cleavage-stage embryos that have the highest probability of forming top-quality blastocysts. This strategy might reduce the occurrence of negative outcomes associated with blastocyst transfer without compromising pregnancy rates. Furthermore, reduced culture time will result in other potential benefits such as cost saving for IVF laboratories. However, based on the findings of the current study, even with the use of TLM, reliably selecting an embryo at cleavage stage with a high chance of forming a top-quality blastocyst might be a challenging task.

The present study is contributing to the overall body of knowledge regarding the applicability of time-lapse morphokinetic parameters. At the same time, it highlights the potential problems in the clinical usefulness of proposed predictive algorithms and the challenges that are present when these algorithms are evaluated by different researchers. Although the technology of time-lapse seems to be promising, significantly more research is required for the objective development of an algorithm that will have significant clinical usefulness and at the same time will be externally valid.

Conclusion

This prospective study has developed specific cutoffs for the identification of blastocysts with the best day 5 morphology using time-lapse technology. It has also shown that the discriminatory capacity of these variables is likely to be limited. This study adds to our knowledge concerning morphokinetic parameters and embryo quality and underlines that further evidence is required in order for externally validated time-lapse morphokinetic parameters to be used for embryo selection in IVF laboratories.

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Compliance with ethical standards The authors declare that they have no conflict of interest. Consent was obtained from all individual participants included in the study.

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