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ASSISTED REPRODUCTION TECHNOLOGIES

A comparison of morphokinetic markers predicting blastocyst formation and implantation potential from two large clinical data sets

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Abstract

Purpose To demonstrate whether the standard morphokinetic markers used for embryo selection have a similar relationship to blastocyst formation and implantation in two large clinical data sets.

Methods This is a retrospective cohort analysis striving to answer two distinct questions utilizing data sets from two large IVF clinics. Blastocysts (BL) and implanted blastocysts (I) in both clinics, IVI-Valencia (BL = 11,414, I = 479) and WMC (BL = 15,902; I = 337), were cultured in a time-lapse system (EmbryoScope, Vitrolife, Sweden). The study was designed to assess the relationship between early morphokinetic hallmarks and BL development, with a secondary analysis of implantation rates following single-embryo day 3 and day 5 transfers.

Results We performed a detailed graphical analysis for t3, t5, duration of the second cell cycle (cc2) (t3-t2), and the ratio (t5-t3)/(t5-t2). The t5 timing was not affected between the clinics. However, Weill Cornell Medicine's (WCM) proportions were significantly affected by having BL vs. not. A significant decrease of blastocysts with longer t5 in WCM data, while t5 was more informative in the IVI data set for the implantation rate.

Conclusions Morphokinetic intervals for early cleavages were distributed differently between the clinics. Incorporation of embryo-selection algorithms depends on the individual clinic's selected developmental hallmarks, all of which must be validated before incorporation into clinical practice.

Keywords Time-lapse incubator · Morphokinetics · Embryo selection · Implantation rate

Introduction

Current efforts in IVF have been aimed at reducing the incidence of multiple pregnancies by transferring fewer embryos [1]. Although traditional subjective morphological evaluation is still the primary method used to evaluate and select embryos, it is difficult to accurately and objectively identify embryos with the highest implantation potential using this approach [2]. In recent years, the focus has shifted toward the use of time-lapse imaging [3]. This well-known, noninvasive technique maintains embryo integrity while enabling the continuous recording of morphological changes during embryo development, thus allowing embryologists to analyze morphokinetic changes [4].

As previously shown [5–8], time-lapse imaging helps us to understand better the intricate events that occur during embryo development by capturing changes that we cannot otherwise perceive. It also allows us to determine the exact timing of embryo cleavages in a clinical setting [9].

Numerous reports have attempted to correlate morphokinetic variables with several outcome parameters: pregnancy rate [10, 11], implantation potential [5, 12], blastocyst formation [13–17], and aneuploidies [6, 18–20]. In 2012, Meseguer et al. [11] proposed a multivariable model to classify embryos according to their implantation probability by using a decision tree that combined morphokinetic exclusion and selection criteria. Since then, additional predictive algorithms have been reported [11, 17, 21–23]. In 2014, the first

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prospective randomized control trial was performed to determine the efficacy and utility of morphokinetic parameters in efforts to improve implantation rates [9].

The universal application of the TLM algorithms and their overall routine use remains questionable [24–26]. Morphokinetic selection criteria published to date are based on clinic-specific embryo cohorts and therefore may not be valid universally, as several clinical and/or laboratory factors (oxygen concentration, culture media, patient infertility) have been shown to impact an embryo's morphokinetic profile [26–30]. According to the relevant morphokinetic variables, each center develops algorithms based on particular sets of patients, environmental conditions, stimulation protocols, and media culture [26]. For this reason, published algorithms could lose their predictive value if applied externally and might not be universally applicable. To address this, we compared previously published "standard" morphokinetic markers for blastocyst prediction and implantation potential from two large clinical data sets. We tried to establish key universal developmental hallmarks and demonstrated that predictive morphokinetic markers have similar relationships to blastocyst (BL) development and implantation in both data sets.

Materials and methods

This research project was conducted at Instituto Valenciano de Infertilidad (IVI) in Valencia, Spain, and at Weill Cornell Medicine (WCM) in New York. The procedure and protocol for embryo analysis were approved by the IVI and WCM Institutional Review Boards: IVI: IRB CODE 1407-MAD-053-NB; WCM: 1401014735. The data sample was selected from a total of 28,132 human embryos after intracytoplasmic sperm injection (ICSI) cycles. Both data sets were analyzed using embryos that were cultured in time-lapse incubators and evaluated for exact timing of developmental events.

Cycle selection

In this retrospective cohort study, the following embryo populations were analyzed: (a) 27,316 embryos cultured until the blastocyst stage (11,414 from IVI and 15,902 from WCM) and (b) 816 implanted embryos following single-embryo transfers on day 3 (fetal heartbeat, FH) (479 from IVI and 337 from WCM).

The study included data from patients using homologous and donated oocytes. The selection criteria for IVI donors, as mandated by Spanish law, can be found in a previous study [31]. The mean maternal age was 38.5 ± 3.5 years for IVI and 38.2 ± 4 years for WCM. Patients using preimplantation genetic diagnosis and screening (PGD/S) were excluded from the study.

Clinical protocols and laboratory procedures

Clinical stimulation and laboratory protocols for each clinic have been previously described, including controlled ovarian stimulation: gonadotropins, hCG, or GnRH agonists, were used to trigger ovulation; laboratory procedures including ICSI and embryo culture, embryo evaluation, and grading; and embryo transfer (see [32] for WCM protocols and [9, 33] for IVI protocols).

Notably, embryos in both clinics were cultured in a time-lapse system (EmbryoScope®, Vitrolife, Sweden) but with different culture media. Immediately after ICSI, the injected oocytes were placed individually in culture dishes (EmbryoSlide, Vitrolife, Sweden) and were cultured at 37 °C (5.5% CO_2 at IVI and 5.8% CO_2 at WMC). All embryos were cultured under 5% O_2 incubation conditions, and the injected oocytes were covered with 1.2 mL of mineral oil to prevent evaporation until embryo transfer at day 3 or day 5. At IVI, cleavage medium was used from days 0 to 3, and blastocyst medium was used from days 3 to 5 (Cook, Australia). At WCM, C1 and C2 media developed in-house were used from days 1 to 3 and from days 3 to 6, respectively.

The number of embryos transferred and the day of the transfer (day 3 vs. day 5) were determined based on embryo number, quality, and patient clinical history. Embryos were selected for transfer according to a combination of standard morphological grading [34] and/or an internal laboratory system. Supernumerary embryos with good quality were frozen using standard vitrification techniques [35, 36].

The positive β-hCG value on day 13 after embryo transfer was detected and clinical pregnancy was confirmed when a gestational sac with a fetal heartbeat was detected by ultrasound examination 5 weeks after embryo transfer.

Image acquisition during embryo culture and evaluation of morphokinetic parameters

Time-lapse image acquisition was performed automatically in the EmbryoScope every 15 min. For each embryo, we acquired stacks of five images at different focal planes to enable an accurate assessment of embryo morphology and time points. The images were acquired with low-intensity red light with exposure times of 15–30 ms per image. Thus, the total light exposure using this methodology was lower than that used during routine microscopy [12, 37].

Retrospective analysis of the acquired images was performed with an external computer (EmbryoViewer® workstation, Vitrolife, Sweden) using image analysis software that annotates all embryo developmental events with the corresponding timing of those events expressed as "hours after ICSI" which was the average time to inject all oocytes. The precise time of cell divisions was annotated from time to 2

cells (t2) to t9. Two subgroups of embryos were generated from the starting population: those cultured until day 3 and those cultured until day 5.

The parameters for comparison between the two laboratories were selected from the most informative morphokinetic ("standard") markers, which predict blastocyst formation and implantation potential reported in published models [12, 38]. These include t3, t5, second cycle duration (cc2 = t3-t2), and the (t5-t3)/(t5-t2) ratio. All average times in the general population, as well as the dispersion data for each variable, were analyzed and interpreted.

The blastocyst formation was defined as the formation of a "full blastocyst" when the blastocoele cavity filled the embryo, and the inner cell mass and the trophectoderm tissues were distinguishable and well defined. The blastocyst quality was assessed on the morning of day 5 (at 114–122 h after ICSI), according to a combination of standard morphological grading [34].

Statistical analysis

Data analysis was performed using MATLAB and Statistics Toolbox software (release 2017b, The MathWorks). Quartiles were determined from each data set after removing all the empty entries in either the parameter described or the binary response. Confidence intervals for all statistics were determined by bootstrap sampling with replacement (2000 samples). The area under the receiver operating curve of the morphokinetic parameters was determined by quadratic logistic regression of the binary response (outcome) against the particular parameter (AUC values are considered excellent when ranges between 1 and 0.9 excellent, 0.80–0.90 good, 0.70–0.80 fair, 0.60–0.70 and 0.50–0.60 fail). This procedure was incorporated to adequately capture instances in which the proportion of successful outcomes exhibited a nonlinear behavior concerning the parameter, specifically, cases in which the maximum value of the proportion of successful outcomes was in the mid-region of the observed values for the parameter.

Results

Our study included morphokinetic parameters from two groups of embryos: (a) 27,316 embryos cultured until the blastocyst stage and (b) 816 implanted embryos following single-embryo transfers on day 3.

The correlation between morphokinetic markers and blastocyst formation and implantation

We compared general distributions of morphokinetic parameters between two large clinical data sets. Cleavage times for t3, t5, and cc2 (t3-t2) were compared between the clinics, and a detailed graphical analysis was performed to determine whether the timing of early embryo division was associated with the probability of blastulation and implantation. As shown in Table 1, receiver operating characteristic (ROC) curves were employed to test the predictive value of temporal events on implantation. ROC curve analysis provides an area under the curve (AUC) value between 0.5 and 1, which is interpreted as a measurement of the global classification ability. The data show that there were no significant differences in

Table 1 Morphokinetic factors and its prediction abilities (ROC values) for blastulation and implantation prediction

Laboratory	Factor	Population	Outcome	*N*	ROC-AUC	ROC-ACU 95% CI	S
WCM	t5	D3T FHR 1T	Implantation	455	0.57	0.51–0.63	
IVI	t5	D3T FHR 1T	Implantation	320	0.57	0.50–0.64	
WCM	t3-t2	D3T FHR 1T	Implantation	479	0.65	0.59–0.70	*
IVI	t3-t2	D3T FHR 1T	Implantation	337	0.54	0.48–0.60	
WCM	t3	D3T FHR 1T	Implantation	479	0.57	0.51–0.63	
IVI	t3	D3T FHR 1T	Implantation	337	0.61	0.54–0.67	
WCM	t3	t2 > 6 h	120 h blastocyst	26,778	0.69	0.68–0.69	*
IVI	t3	t2 > 6 h	120 h blastocyst	16,594	0.54	0.53–0.55	
WCM	t3-t2	t2 > 6 h	120 h blastocyst	26,778	0.72	0.72–0.73	*
IVI	t3-t2	t2 > 6 h	120 h blastocyst	16,594	0.61	0.60–0.62	
WCM	t5	t2 > 6 h	120 h blastocyst	24,747	0.68	0.67–0.69	*
IVI	t5	t2 > 6 h	120 h blastocyst	14,662	0.55	0.54–0.56	
WCM	t3-tpnf	t2 > 6 h	120 h blast	19,988	0.73	0.727–0.741	*
WCM	t5-tpnf	t2 > 6 h	120 h blast	18,582	0.73	0.724–0.738	*
WCM	t3	Day 5 SET	Implantation	462	0.53	0.482–0.587	
IVI	t3	Day 5 SET	Implantation	264	0.53	0.456–0.598	
WCM	t5	Day 5 SET	Implantation	462	0.54	0.491–0.594	
IVI	t5	Day 5 SET	Implantation	262	0.53	0.458–0.597	
WCM	t3-t2	Day 5 SET	Implantation	462	0.50	0.449–0.554	
IVI	t3-t3	Day 5 SET	Implantation	264	0.50	0.426–0.567	

the location of quartile means or limits between the clinics.

– t5 general distribution and blastocyst formation rate

We studied t5 in further detail, as it is the most significant morphokinetic parameter based on the published literature. Figure 1 shows the frequency of t5 between the clinics based on the quartile distribution (Q1, Q2, Q3, and Q4). The distribution of the timing was compared in the embryos that did (Y-Blast, blue line) or did not (N-Blast, red line) reach the blastocyst stage both at IVI ($n = 11{,}414$ embryos) and WCM ($n = 15{,}902$).

In WCM embryos, t5 was tightly distributed as opposed to the IVI embryos, where its distribution was more heterogeneous. In the IVI data, embryos whose cleavages were completed in the two central quartiles displayed the highest blastocyst rate. In contrast, first-quartile (fast-growing) and fourth-quartile (slow-growing) embryos were found to reach the blastocyst stage at a low rate. Compared to IVI data, a prominent tail of lagging embryos was found in WCM embryos that did not reach the blastocyst stage. A significant decrease in the proportion of blastocysts with longer t5 times was observed, indicating that slower embryos are unlikely to reach the blastocyst stage.

– t5 distribution and implantation rate

Figure 2 shows the predictive capability of t5 on implantation rates, showing a similar distribution as in blastocyst prediction. A higher implantation rate in the IVI data is likely due to maternal age (higher number of donor cycles). Nevertheless, no significant differences in the location of quartile means or limits were found, data was presented for D3 (Fig. 2) and day 5 (Fig. 8). The AUC value shows that the predictive capability of t5 is equally informative in both data sets (0.572) (Table 1).

– t3, cc2 (t3-t2) distribution, and blastocyst and implantation rate

T3 timing distribution, day 3 (Figs. 3 and 4) and day 5 (Fig. 8) and second cell cycle duration (Figs. 5 and 6) were analyzed between the two clinics. Our analysis of the parameter t3 indicated that the WCM embryo population developed more slowly than that of IVI, specifically in Q2–Q4, with approximately 3 h of slower development in the Q4 population. In the IVI population, the proportion of implanted day-3 embryos in Q2 was the highest, while there were no significant changes in the proportion of implanted day-3 embryos in the WCM population. As the AUC value shows, this parameter was more informative for predicting the outcome in the IVI than in the WCM data (0.608 vs. 0.568, respectively).

On the contrary, cc2 (t3-t2) was a more informative parameter for the WCM data than for that of IVI regardless of D3 or D5 (Table 1). Also, the second cell cycle duration had a lower proportion of positive outcomes in Q1 and Q4 distribution for WMC.

– t5-t3/t5-t2 [38]

The ratio between the duration of the second and third cleavage adequately captured the positive outcome in both data sets (data not shown). This parameter was slightly more informative for WMC than for IVI (ROC-AUC 0.571 vs. 0.542) (data not shown).

– t5-tPNF general distribution and blastocyst formation rate

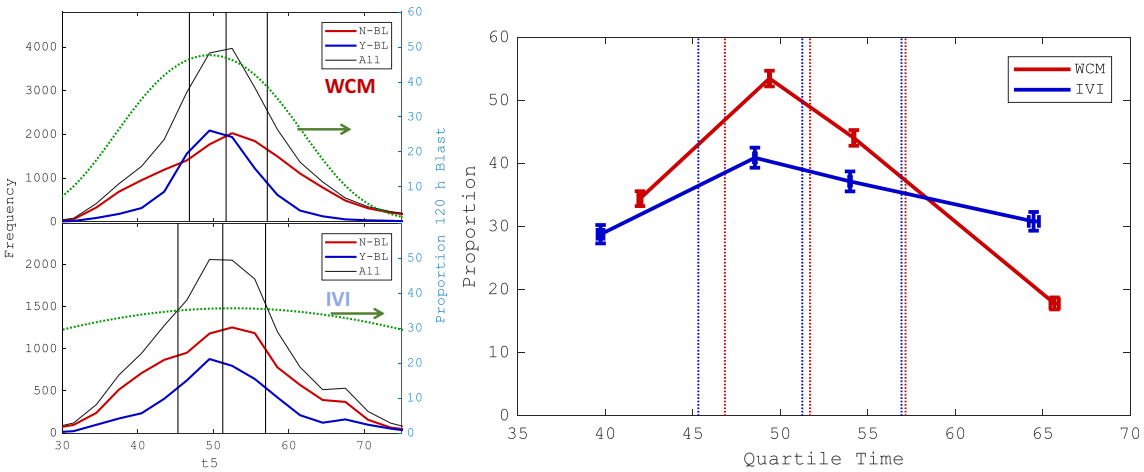

Fig. 1 Histograms (left) for the t5 in WCM (top) and IVI (bottom). Quartile plot of the proportion of 120-h blastocysts in WCM and IVI. The dashed lines indicate the quartile limits; the proportions were plotted at the mean value of the quartile with the corresponding 95% CI (for the proportion and the mean). Quartile distributions were characterized by the shape and location of the overall distribution and the associated proportion of the outcomes in each location

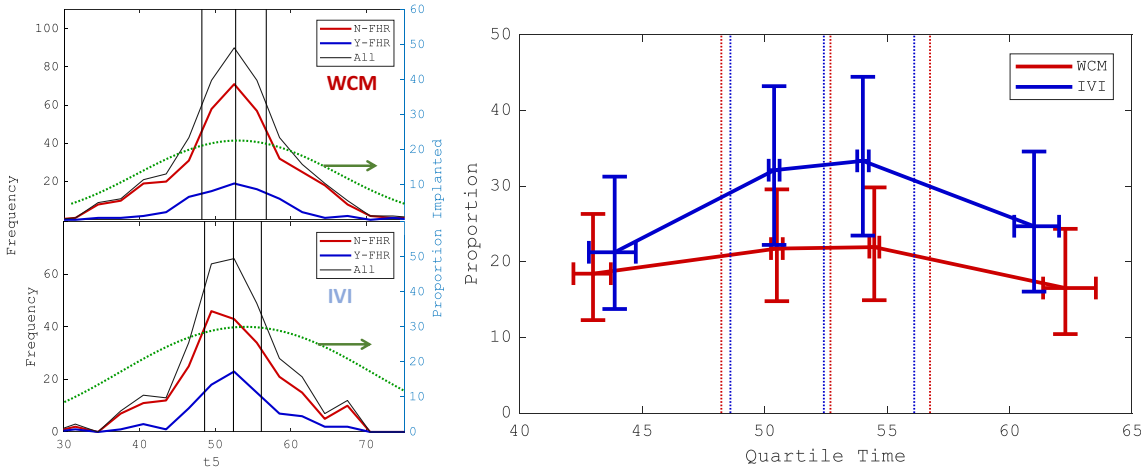

Fig. 2 Histograms (left) for the t5 in WCM (top) and IVI (bottom). Quartile plot of the proportion of implanted embryos (fetal heart, FH) in WCM and IVI. The dashed lines indicate the quartile limits; the proportions were plotted at the mean value of the quartile with the corresponding 95% CI (for the proportion and the mean)

To avoid the inaccurate zero ICSI time, we applied analysis using tPNf as a starting point. For the lab that recorded the data of tPNf (WCM), we have added the analysis for the t5-tPNf which shows a marginally better AUC compared to the absolute parameters (Fig. 9 and Table 1).

Comparison and distribution analysis of the t3-t2 vs. t5 morphokinetic marker between two data sets for the application of the universal models

Finally, we performed a density distribution study to describe the second cell cycle (t3-t2) vs. t5 in both populations of embryos. With this analysis, we demonstrated which morphokinetic parameters at both clinics were the most significant regarding blastocyst formation. Although both clinics presented similar distributions, the WCM embryos presented more concentrated values than those from IVI, which illustrated greater scattering. Interestingly, the values associated with embryos reaching the blastocyst stage were very similar between the two clinics (Fig. 7).

Discussion

Time-lapse analysis has provided us with a new and improved view of embryo development. In the last few years, data obtained from time-lapse imaging have allowed us to identify several morphokinetic variables as markers of embryo viability and implantation potential [6, 12, 15, 17, 39]. However, morphokinetic selection criteria published to date are based on specific embryo cohorts and therefore may not be valid for all clinics, as several factors have been shown to impact

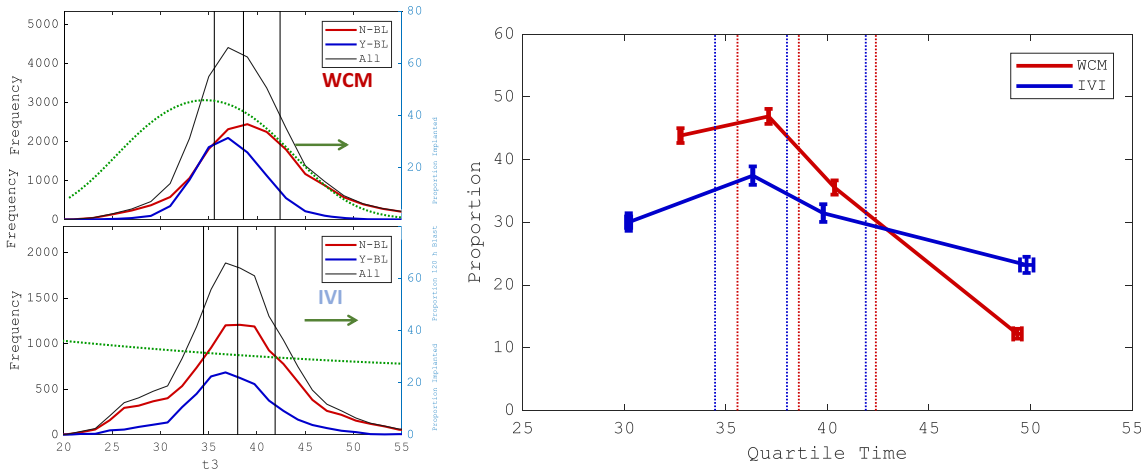

Fig. 3 Histograms (left) for the t3 in WCM (top) and IVI (bottom). Quartile plot of the proportion of 120-h blastocysts in WCM and IVI. The dashed lines indicate the quartile limits; the proportions were plotted at the mean value of the quartile with the corresponding 95% CI (for the proportion and the mean)

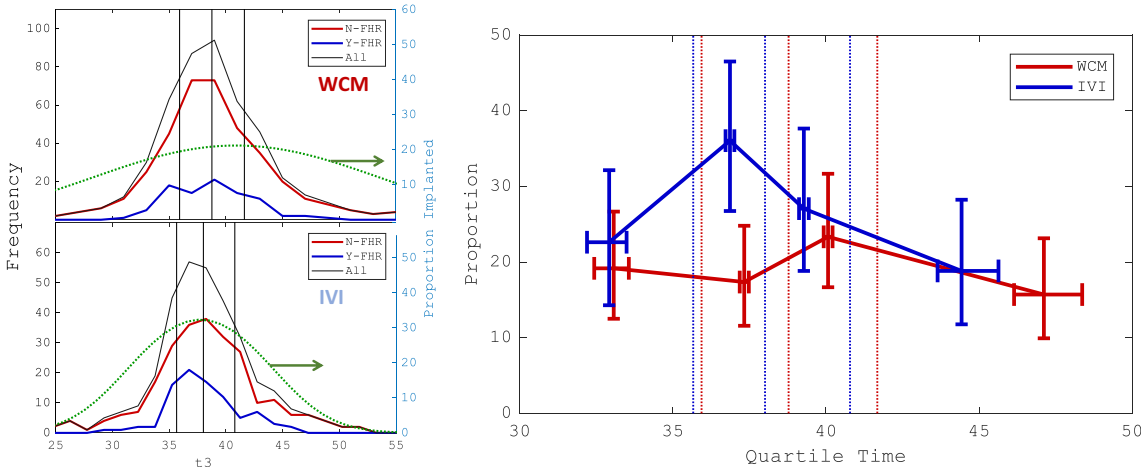

Fig. 4 Histograms (left) for the t3 in WCM (top) and IVI (bottom). Quartile plot of the proportion of implanted embryos (fetal heart, FH) in WCM and IVI. The dashed lines indicate the quartile limits; the proportions were plotted at the mean value of the quartile with the corresponding 95% CI (for the proportion and the mean)

morphokinetics [16, 28–30]. To shed light on this issue, our main objective in this study was to determine if the morphokinetic patterns developed on a data set from a specific clinic are likely to differ for other culturing embryo conditions, or if embryo-selection markers already described in the literature could be applied universally.

This retrospective work compares, for the first time, the developmental timing of more than 20,000 embryos from two large data sets. We attempted to analyze the predictive potential of different published morphokinetic markers. Ideally, it would be very useful if a morphokinetic algorithm could be applied universally to support embryologists' decisions in selecting embryos for transfer. Although validated through a large data set from two experienced time-lapse clinics, we are aware that the retrospective nature of this analysis is not ideal.

Determining when embryos reach the blastocyst stage, as an outcome, can vary due to human biases and differences in definitions. We also analyzed unbiased FH implantation data following day-3 embryo transfers. From our analysis of the developmental timing of both embryo populations (IVI and WCM), we can conclude that the most optimal embryos (ones that reach the blastocyst stage or implant) are divided in a narrow and intermediate time range. However, the timing shows different embryo distributions between quartiles depending on the population origin. For example, we observed slower embryo development, probably affected by increased maternal age, in the WCM population. A previous publication [3] reported distributions of timings of embryos from IVI clinics, by using several endpoints (all embryos, all without direct cleavage, achieved 8 cells on day 3, reached blastocyst stage, and finally implanted). We did not have the chance to compare with another clinical setting as WCM and we did not perform any predictive test nor any quartile distribution analysis (Figs. 8 and 9).

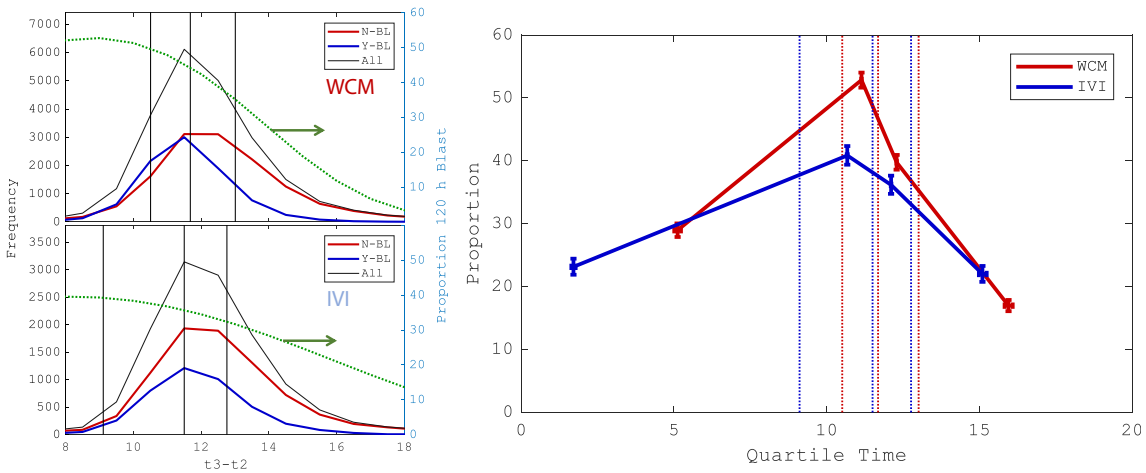

Fig. 5 Histograms (left) for the t3-t2 in WCM (top) and IVI (bottom). Quartile plot of the proportion of 120-h blastocysts in WCM and IVI. The dashed lines indicate the quartile limits; the proportions were plotted at the mean value of the quartile with the corresponding 95% CI (for the proportion and the mean)

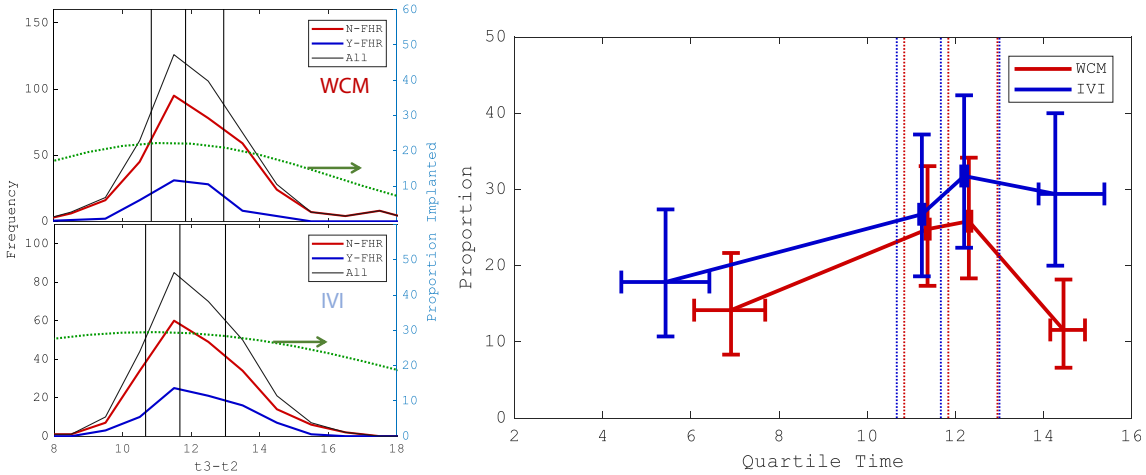

Fig. 6 Histograms (left) for the t3 in WCM (top) and IVI (bottom). Quartile plot of the proportion of implanted embryos (fetal heartbeat, FHB) in WCM and IVI. The dashed lines indicate the quartile limits; the proportions were plotted at the mean value of the quartile with the corresponding 95% CI (for the proportion and the mean)

The information obtained from the ROC curves shows that the predictive power of the parameters analyzed varies depending on the origin of the data (IVI or WCM). However, none of the individual parameters analyzed were able to predict implantation with a high degree.

It has been shown that embryo morphokinetic profiles are greatly altered in different culture media (sequential vs. single-step media) [27] or in different $C0_2$ and 0_2 gas concentrations [40, 41]. Inevitably, culture conditions vary between centers, and fundamental differences must be considered before we apply universal external algorithms or predictive markers. Early reviews and meta-analyses, performed on the limited number of studies, indicated no benefit of using TLM [24, 25]. The lack of a universally accepted TLM nomenclature and morphologic features can be contributed to an inability to validate universal TLM implementation and indicate the need to develop internal selection algorithms [26]. However, the most recent meta-analysis indicates the beneficial use of the TLM showing a higher clinical pregnancy and live birth rate [42].

In addition to the differences in morphokinetic annotations among embryologists, each center has unique sets of patients, culture environments, and stimulation protocols that may influence predictive algorithms [26]. Based on our data, any algorithm or predictive marker loses its value for external

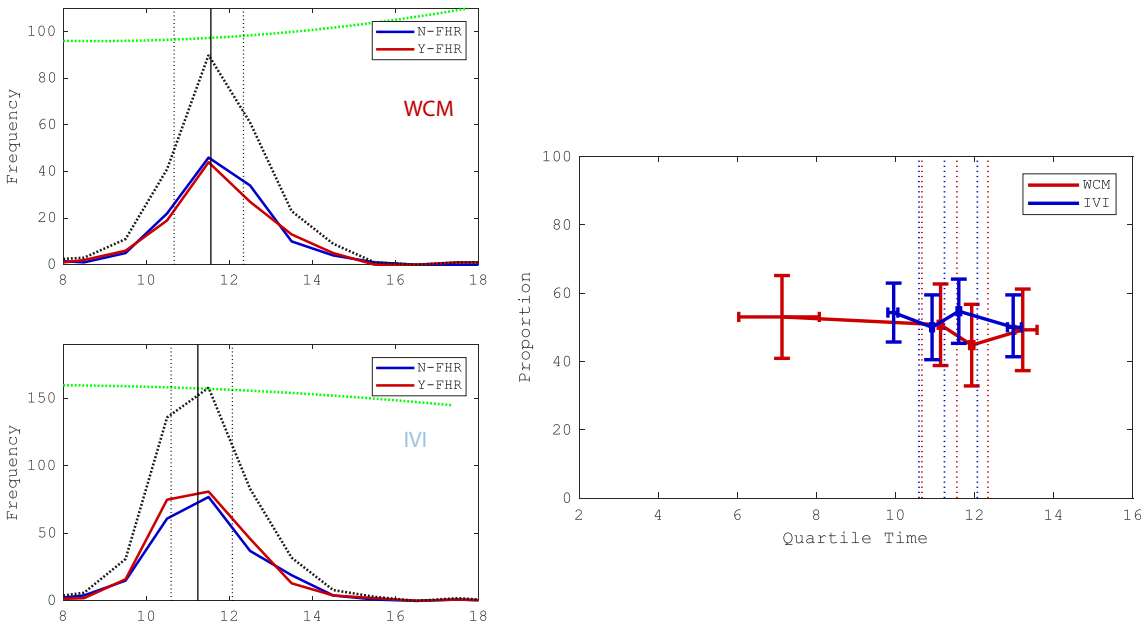

Fig. 7 Density plot distributions of embryo values for t5 (*X*-axis) and t3-t2 (*Y*-axis) by WCM and IVI. Left graphics represent embryos that achieved blastocyst at 120 h (day 5), and center plots represent no blastocyst populations. The right panel shows embryos that reached the blastocyst stage and their frequencies related to t3-t2 and t5

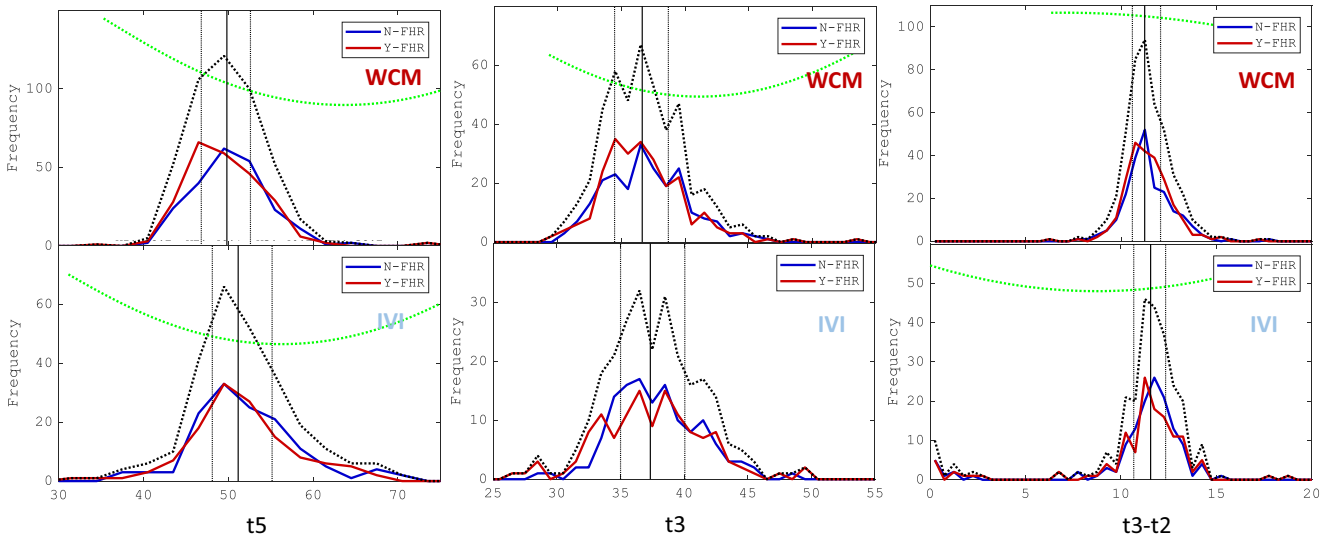

Fig. 8 Histograms for the t5, t3, and t3-t2 in WCM (top) and IVI (bottom) comparing between implanted and not implanted blastocyst (day 5 of transfer)

application without a careful assessment of clinic-specific data distribution and parameters.

These results are in line with current studies that have attempted to apply previously published models [26]. The data presented herein suggest that morphokinetic parameters should not yet be used universally and that models should be created contingent on specific data sets.

Although validated throughout a large data set of two experienced time-lapse user clinics, the retrospective nature of the analysis is limited and less than ideal.

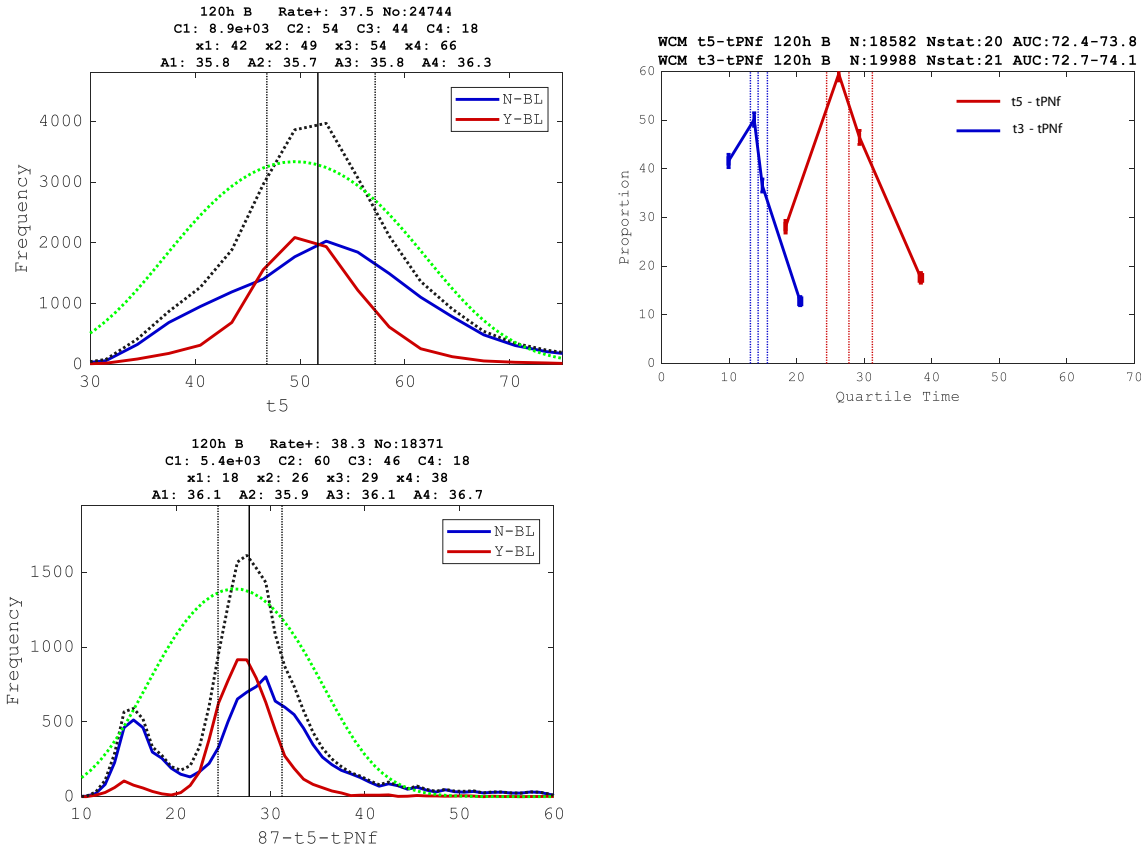

Fig. 9 Left: Histograms for t5 (top) and t5-tPNf (bottom) in WCM. Right: Quartile plot of the proportion of 120-h blastocysts in WCM t3-tPNf (blue) and t5-tPNf (red). The dashed lines indicate the quartile limits; the proportions were plotted at the mean value of the quartile with the corresponding 95% CI (for the proportion and the mean)

In summary, our data indicate differences between two centers related to the distribution of the parameters and outcomes. Embryos were selected for transfer according to a combination of morphological grading and morphokinetics. In particular, embryos with optimal or good morphology may potentially affect the relationship between implantation potential and cleavage timings (differences between WCM and IVI may be even larger), however, only in those transferred and not in embryos that reached or did not reached blastocyst stage. The type and accuracy of annotations, maternal age, culture media, and culture conditions may explain the varied results between the laboratories as the parameters are sensitive to specific attributes of the data and should not be universally applied. Therefore, models that attempt to estimate any outcome need to be carefully reviewed contingent on data sets of interest. Finally, based on the data presented here, it is unlikely that a universal model can be developed without considering laboratory and clinical variabilities. Additionally, it would be beneficial to create universal annotation criteria that will help standardize morphokinetic parameters.

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