

The use of morphokinetics as a predictor of embryo implantation†

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BACKGROUND: Time-lapse observation presents an opportunity for optimizing embryo selection based on morphological grading as well as providing novel kinetic parameters, which may further improve accurate selection of viable embryos. The objective of this retrospective study was to identify the morphokinetic parameters specific to embryos that were capable of implanting. In order to compare a large number of embryos, with minimal variation in culture conditions, we have used an automatic embryo monitoring system.

METHODS: Using a tri-gas IVF incubator with a built-in camera designed to automatically acquire images at defined time points, we have simultaneously monitored up to 72 individual embryos without removing the embryos from the controlled environment. Images were acquired every 15 min in five different focal planes for at least 64 h for each embryo. We have monitored the development of transferred embryos from 285 couples undergoing their first ICSI cycle. The total number of transferred embryos was 522, of which 247 either failed to implant or fully implanted, with full implantation meaning that all transferred embryos in a treatment implanted.

RESULTS: A detailed retrospective analysis of cleavage times, blastomere size and multinucleation was made for the 247 transferred embryos with either failed or full implantation. We found that several parameters were significantly correlated with subsequent implantation (e.g. time of first and subsequent cleavages as well as the time between cleavages). The most predictive parameters were: (i) time of division to 5 cells, t5 (48.8–56.6 h after ICSI); (ii) time between division to 3 cells and subsequent division to 4 cells, s2 (≤ 0.76 h) and (iii) duration of cell cycle two, i.e. time between division to 2 cells and division to 3 cells, cc2 (≤ 11.9 h). We also observed aberrant behavior such as multinucleation at the 4 cell stage, uneven blastomere size at the 2 cell stage and abrupt cell division to three or more cells, which appeared to largely preclude implantation.

CONCLUSIONS: The image acquisition and time-lapse analysis system makes it possible to determine exact timing of embryo cleavages in a clinical setting. We propose a multivariable model based on our findings to classify embryos according to their probability of implantation. The efficacy of this classification will be evaluated in a prospective randomized study that ultimately will determine if implantation rates can be improved by time-lapse analysis.

Key words: embryo / cell division / pregnancy / exact timing / time-lapse

Introduction

Evaluation of embryos *in vitro* has improved greatly over the past 20 years. Classical embryo assessment has been supplemented by the evaluation of several additional morphological characteristics that allow prediction of the developmental potential of an embryo and the probability of achieving pregnancy for an infertile couple e.g. review in Baczkowski *et al.* (2004). Several publications have proposed additional morphological evaluations to assess the timing of embryonic cell divisions that appear to be related to embryo viability (Shoukir

et al., 1997; Sakkas *et al.*, 1998; Lundin *et al.*, 2001; Ciray *et al.*, 2006; Lemmen *et al.*, 2008; Mio and Maeda, 2008). Many of these studies have investigated the relationship between the timing of the first embryonic division and the embryo quality summarized in Table 5 in Hesters *et al.* (2008). The underlying reason for variation in the time of the first cell division is not clear; it could be related to culture conditions as well as intrinsic factors of the oocyte and sperm, maturity, genetic competence and metabolism (Lundin *et al.*, 2001). Early cleavage in first embryonic division, operationally defined as an early cell division resulting in a 2-cell embryo at a time

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of inspection 25–27 h post ICSI, and its impact on pregnancy rate in humans was first published by the Edwards group (Edwards et al., 1984). Subsequently, many studies have used this concept as the basis for their publications (Shoukir et al., 1997; Sakkas et al., 1998, 2001; Bos-Mikich et al., 2001; Lundin et al., 2001; Salumets et al., 2001; Fenwick et al., 2002; Tsai et al., 2002; Ciray et al., 2006) and all have found that the transfer of early cleavage embryos results in higher implantation and pregnancy rates compared with embryo transfers with delayed division. However, many transfers in these studies involved more than one embryo and many included a mix of early and late cleaving embryos; thus, it is difficult to obtain conclusive evidence that the implantation can be attributed to the early cleavage (Shoukir et al., 1997; Sakkas et al., 1998, 2001; Bos-Mikich et al., 2001; Salumets et al., 2001; Fenwick et al., 2002; Tsai et al., 2002; Ciray et al., 2006). In one fascinating study, Van Montfoort et al. (2004) investigated this, by comparing embryo transfers that composed entirely of early cleaving embryos with transfers that composed entirely of late cleaving embryos in 253 double transfers and 165 single transfers. They found that there were significantly higher pregnancy rates in the early cleavage group in both single and double transfers. The blastocyst formation rate for early cleaving embryos also increased, and the miscarriage rate decreased compared with the late cleaving group (Van Montfoort et al., 2004).

It is important to highlight that it is unclear if early cleavage is an independent predictor of pregnancy or if it is correlated with other variables such as embryo morphology and cell number. Several studies have shown that early cleavage embryos have significantly higher numbers of cells and better viability compared with late-cleaving embryos (Shoukir et al., 1997; Sakkas et al., 1998, 2001; Lundin et al., 2001; Salumets et al., 2001; Fenwick et al., 2002).

A key consideration in many studies of timing of first cleavage is the limited number of observations, which restricts temporal assessment of a given phenomenon to a determination if an event occurred before or after a particular time point. Knowledge of the exact time a given event occurred cannot be obtained with a limited number of discrete observations. Indeed, one of the fundamental problems of embryo quality assessment is the static evaluation of a dynamic developing entity. Current classification scores analyze the morphology at a few predefined time points during embryo development preimplantation, with the consequent lack of information about what happened between the analyzed time points. Thus, continual monitoring might provide one strategy to collect a complete picture of embryo developmental kinetics.

Using a time-lapse photography system, Lemmen et al. (2008) found that embryos that implant have an earlier disappearance of pronuclei and first division and an increased cell number on Day 2 of embryonic development. They also found a correlation between a higher pregnancy rate and synchronicity in re-appearance of nuclei in the two blastomeres formed after the first division (Lemmen et al., 2008). In a more recent study, Wong et al. (2010) found that development of human embryos to the blastocyst stage was correlated with: (i) the duration of the first cytoplasmic cleavage from 1 cell to 2 cells; (ii) time between division to 2 cells and subsequent division to 3 cells and (iii) time between division to 3 cells and subsequent division to 4 cells. However, none of the embryos in that study were transferred. It is thus unclear if embryos with the suggested morphokinetic cleavage pattern would have implanted (Wong et al., 2010).

Here, we present a clinical study with time-lapse imaging of embryo development for 247 transferred embryos. The study presents—to our knowledge—the largest set of transferred embryos after time-lapse analysis and thus a novel opportunity to correlate morphokinetic parameters to implantation and ongoing pregnancy. The purpose of this study is to generate and evaluate a tool for the selection of viable embryos based on the exact timing of embryo development events together with morphological patterns by using an automatic time-lapse system to monitor embryo development.

Materials and Methods

This research project was conducted at the Instituto Valenciano de Infertilidad—IVI, Valencia. The procedure and protocol were approved by an Institutional Review Board, (IRB), which regulates and approves database analysis and clinical IVF procedures for research at IVI. The project complies with the Spanish Law governing Assisted Reproductive Technologies (14/2006). The present study sample was drawn from a total of 2903 oocytes from which 2120 embryos were generated in 285 IVF treatment cycles between September 2009 and September 2010. All embryos were obtained after fertilization by ICSI and were part of our standard ($n = 188$) and ovum donation program ($n = 97$). Embryos were investigated by detailed time-lapse analysis measuring the exact timing of the developmental events in hours post insemination by ICSI.

Implantation of transferred embryos was confirmed at an ultrasound scanning for gestational sacs with fetal heart beat after 7 weeks of pregnancy. A single gestational sac after dual embryo transfer was frequently observed. Treatments with partial implantation were excluded from further analysis because it was not possible to ascertain which of the two transferred embryos implanted. Only 247 embryos from treatments where the number of gestational sacs matched the number of transferred embryos (full implantation) and embryos from treatments where no biochemical pregnancy was achieved (no implantation) were included in the analysis.

The exclusion criteria for standard patients and recipients with respect to this study were: low response (less than five metaphase II oocytes), endometriosis, polycystic ovary syndrome (PCOS), hydrosalpinx, BMI $> 30 \text{ kg/m}^2$, uterine pathology (myomas, adenomyosis, endocrinopathies, thrombophilia, chronic pathologies, acquired or congenital uterine abnormalities), recurrent pregnancy loss, maternal age over 39 years old for standard patients and 45 for oocyte donation recipients (aging uterus), or severe masculine factor (presenting less than 5 million motile sperm cells in total in the ejaculate).

Ovarian stimulation in standard patients and oocyte donors

All donors were from our egg donation program. Only patients who did not meet the exclusion criteria were included in the study. The selection criteria for donors can be found in Garrido et al. (2002) as stated by Spanish law. The mean age of the male patients of our study population was 37.9 years (SD = 5.2). The mean age of our female population (for oocyte donation treatments, we only considered the age of the acceptors) was 36.9 years (SD = 4.9). All donors had normal menstrual cycles of 26–34 days duration, normal weight (BMI of 18–28 kg/m^2), no endocrine treatment (including gonadotrophins and oral contraception) in 3 months preceding the study, normal uterus and ovaries at transvaginal ultrasound (no signs of PCOS), and antral follicle count >20 on the first day of gonadotrophin administration, after down-regulation with GnRH agonist (Meseguer et al., 2011).

Prior to controlled ovarian stimulation (COS), we used cycles with GnRH agonist protocols (Melo *et al.*, 2010).

For COS, we proceeded as previously described (Melo *et al.*, 2010). HCG (Ovitrelle, Serono Laboratories, Madrid, Spain) was administered subcutaneously when at least eight leading follicles reached a mean diameter of ≥ 18 mm. Transvaginal oocyte retrieval was scheduled 36 h later.

Protocol for endometrial preparation of recipients can be found in Meseguer *et al.* (2008) and Meseguer *et al.* (2011).

After embryo transfer for luteal phase support, standard patients received a daily dose of 200 mg and oocyte recipients received a daily dose of 400 mg vaginal micronized progesterone (ProgeffikEffik, Madrid Spain) every 12 h.

Ovum pick-up and ICSI

Follicles were aspirated and the oocytes were washed in Quinn's Advantage medium (QAM) (SAGE, Rome, Italy). After washing, oocytes were cultured in Quinn's Advantage Fertilization medium (QAFM; SAGE) at 5.2% CO₂ and 37°C for 4 h before oocyte denudation. Oocyte denudation was carried out by mechanical pipetting in 40 IU/ml of hyaluronidase in the same medium (QAFM). Subsequently, ICSI was performed in a medium containing HEPES (QAM) (Garcia-Herrero *et al.*, 2011). ICSI was performed at $\times 400$ magnification using an Olympus IX7 microscope. Finally, the oocytes were placed in pre-equilibrated slides (EmbryoSlide[®], Unisense FertiliTech, Aarhus, Denmark).

Incubation

The EmbryoSlide[®] is constructed with a central depression containing 12 straight-sided cylindrical wells, each containing a culture media droplet of 20 μ l Quinn's Advantage Cleavage medium. The depression containing the 12 wells was filled with an overlay of 1.4 ml mineral oil to prevent evaporation. The slides were prepared at least 4 h in advance and left in an incubator to pre-equilibrate at 37°C in the 5.0% CO₂ atmosphere. After pre-equilibration, all air bubbles are meticulously removed before the oocytes are placed individually in EmbryoSlide[®] microwells and incubated in the time-lapse monitoring system at 37°C in 5.0% CO₂ concentration and $\sim 20\%$ O₂ concentration until embryo transfer ~ 72 h later.

The time-lapse instrument EmbryoScope[™] (ES) (Unisense FertiliTech, Aarhus, Denmark) is a tri-gas incubator with a built in microscope to automatically acquire images of up to 72 individual embryos during development.

Imaging system

The imaging system in the ES uses low intensity red light (635 nm) from a single light-emitting diode with short illumination times of 30 ms per image to minimize embryo exposure to light and to avoid damaging short wavelength light (Oh *et al.*, 2007; Ottosen *et al.*, 2007; Takenaka *et al.*, 2007). The optics comprise a modified Hoffmann contrast with a 20 \times specialty objective, NA 0.4, long working distance (LWD; Leica, Wetzlar, Germany) to provide optimal light sensitivity and resolution for the red wavelength. The digital images are acquired by a highly sensitive CCD camera (1280 \times 1024 pixels per image). The CCD chip is a 1/2" sensor with a sensitive cell size for each pixel of 4.65 μ m \times 4.65 μ m. Combining the objective magnification with $\times 0.8$ tube optics gives an on chip resolution of 3.5 pixels per μ m. The ultimate magnification of the resulting digital image displayed on a computer screen will depend on the size of the displayed image, which vary between representations. A highly magnified view of the observed area of 286 \times 286 μ m is measuring 27 \times 27 cm on the computer screen when shown with the normal annotation software. The magnification in this representation is close to $\times 1000$, yet the effective resolution is limited by the pixel count in the camera and the optical limitations of the 20 \times LWD objective used. Image stacks

were acquired at five equidistant focal planes every 15 min during embryo development inside the ES (i.e. from about 1 h after insemination to transfer on Day 3 about 72 h after insemination). Embryo exposure to light during incubation was measured with a scalar irradiance microsensors with a tip diameter of 100 μ m placed within the ES at the position of the embryo in the EmbryoSlide[®]. Similar measurements were made on standard microscopes used in fertility clinics. The total exposure time in the time-lapse system during 3 day culture and acquisition of 1420 images was 57 s, which compares favourably with the 167 s microscope light exposure time reported for a standard IVF treatment (Ottosen *et al.*, 2007). As the light intensity measured within the ES with the scalar irradiance microsensors was much lower than the light intensity in microscopes used in IVF clinics, we found the total light dose during 3 day incubation in the time-lapse system to be 20 J/m² (i.e. 0.24 μ J/embryo) as opposed to an exposure of 394 J/m² during microscopy in normal IVF treatments (i.e. 4.8 μ J/embryo) based on average illumination times (Ottosen *et al.*, 2007) and measured average intensities with the scalar irradiance microsensors. Furthermore, the spectral composition of the light in the ES was confined to a narrow range centered around 635 nm, and thus devoid of low wavelength light < 550 nm, which has been shown to be inhibitory to embryo development (Oh *et al.*, 2007; Takenaka *et al.*, 2007) and comprises $\sim 15\%$ of the light encountered in a normal IVF microscope.

Embryo score and culture conditions

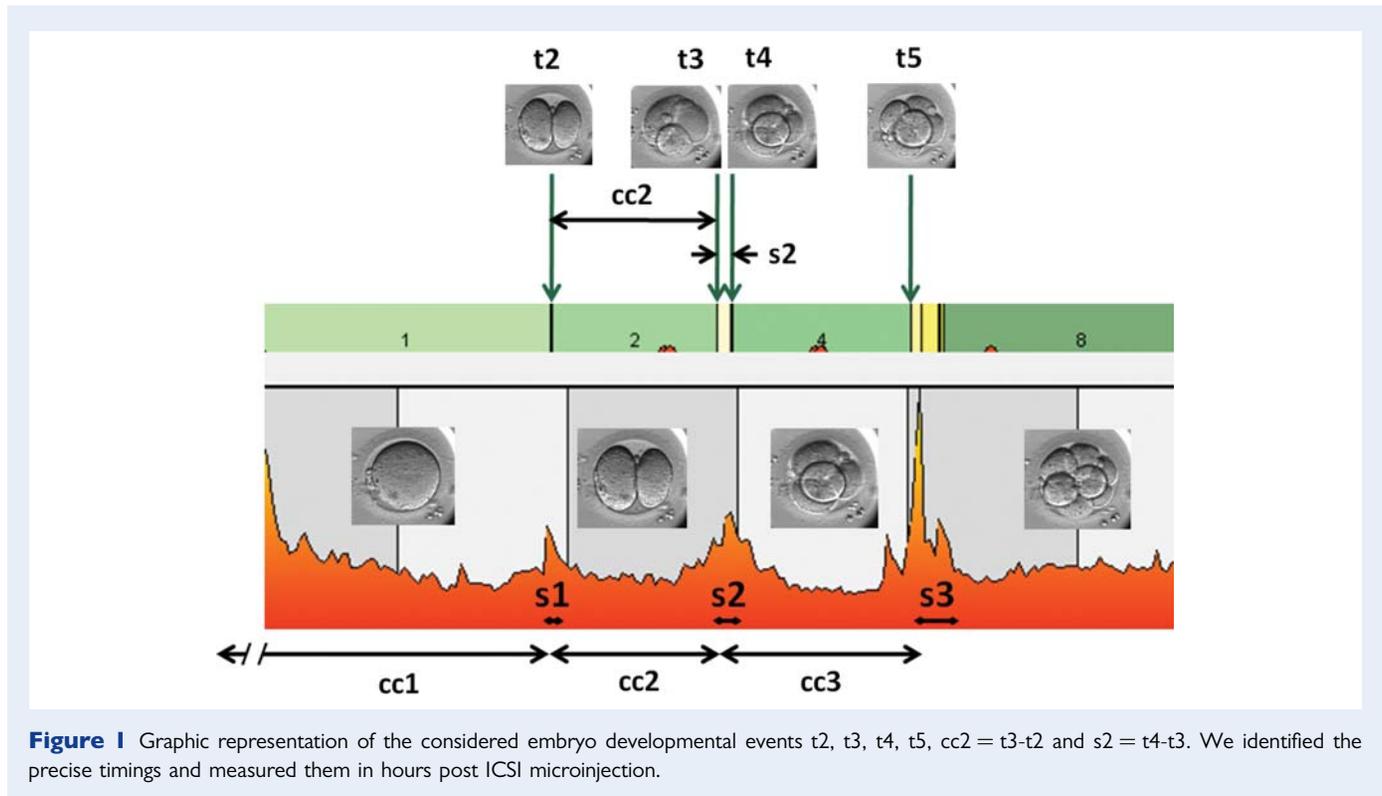
Successful fertilization was assessed at 16–19 h post ICSI based on digital images acquired with the time-lapse monitoring system. Embryo morphology was evaluated on Days 2 (44–48 h post ICSI) and 3 (64–72 h post ICSI) based on the acquired digital images, taking into account the number, symmetry and granularity of the blastomeres, type and percentage of fragmentation, presence of multinucleated blastomeres and degree of compaction as previously described (Alikani *et al.*, 2000). Embryo selection was performed exclusively by morphology based on: (i) the absence of multinucleated cells; (ii) between 2 and 5 cells on Day 2; (iii) between 6 and 10 cells on Day 3; (iv) total fragment volume $< 15\%$ of the embryo and (v) the embryo must appear symmetric with only slightly asymmetric blastomeres (Meseguer *et al.*, 2006, 2008; Muriel *et al.*, 2006). A total of 522 embryos were transferred to 285 patients.

Time-lapse evaluation of morphokinetic parameters

Retrospective analysis of the acquired images of each embryo was made with an external computer, EmbryoViewer[®] workstation (EV) (Unisense FertiliTech, Aarhus, Denmark), using an image analysis software in which all the considered embryo developmental events were annotated together with the corresponding timing of the events in hours after ICSI microinjection. Subsequently, the EV was used to identify the precise timing of the first cell division. This division was the division to 2 cells and a shorthand notation of t2 is used in the following. We likewise annotated the second (i.e. to 3 cells, t3), third (4 cells, t4) and fourth (5 cells, t5) cell division (Fig. 1). For the purpose of this study, we define time of cleavage as the first observed time point when the newly formed blastomeres are completely separated by confluent cell membranes. The time of all events is expressed as hours post ICSI microinjection.

We defined the duration of the second cell cycle (cc2), as the time from division to a two blastomere embryo until division to a three blastomere embryo (cc2 = t3–t2), i.e. the second cell cycle is the duration of the period as two blastomere embryo.

We defined the second synchrony s2, as the duration of the transition from a two blastomere embryo to a four blastomere embryo (s2 = t4–t3),



which corresponds to the duration of the period as three blastomere embryo.

The detailed analysis was performed on transferred embryos with full implantation (i.e. where the number of gestational sacs matched the number of transferred embryos) ($n = 61$) and on embryos with no implantation (where no biochemical pregnancy was achieved) ($n = 186$).

Embryo transfer

The number of embryos transferred was normally two, but in some cases, one or three embryos were transferred because of embryo quality or patient wishes. Supernumerary embryos were frozen for potential future transfers using IVF standard vitrification technique (Cobo et al., 2010). The β -hCG value was determined 13 days after embryo transfer and the clinical pregnancy was confirmed when a gestational sac with fetal heartbeat was visible by ultrasound examination after 7 weeks of pregnancy.

Morphology categories for comparison with time-lapse categories

To make a comparison with the time-lapse classification categories, we retrospectively evaluated the morphology of the transferred embryos using the following categories:

Category 1: The two pronuclei (2PN) embryo consists of 2 cells at 27 h post insemination, 4 cells at Day 2 and 8 cells at Day 3. Even blastomere size at the 2, 4 and 8 cell stage, no multinucleation is observed at any time and the fragmentation is $<10\%$.

Category 2: The 2PN embryo consists of 1–2 cells at 27 h, 3–4 cells at Day 2 and 6–8 cells at Day 3. Only one mismatch is allowed, i.e. either 1 cell at 27 h, 3 cells at Day 2 or 6–7 cells at Day 3. Blastomeres are even sized at the 2, 4 and 8 cell stage; no multinucleation is observed at any time and the fragmentation is $<20\%$.

Category 3: The 2PN embryo consists of 1–2 cells at 27 h, 2–4 cells at Day 2 and 6–8 cells (or morula) at Day 3. The embryo can have asymmetric blastomeres and multinucleation can be observed in maximally one blastomere at each stage. The degree of fragmentation is $<20\%$.

Category 4: The 1PN or 2PN embryo consists of 1–2 cells at 27 h, 2–6 cells at Day 2 and 4 to more than 8 cells or morula at Day 3. The embryo can have asymmetric blastomeres and be multinucleated. The degree of fragmentation is $<50\%$.

Category 5: The embryo consists of any number of cells at 27 h, Day 2 and Day 3. Asymmetric blastomere size, multinucleation and any degree of fragmentation is allowed. Atretic embryos and embryos with arrested development belong to this category.

Statistical analysis

The times, in hours after ICSI microinjection, of embryo events in implanted embryos largely followed normal distributions, but that was typically not the case for the not implanted embryos (Shapiro–Wilk test). The distributions of the not implanted embryos typically had long tails extending to later timing values. To investigate whether the variances in the exact timings of embryo events were different between the implanted and not implanted embryos, the Brown–Forsythe’s test for homogeneity of variances was used, since it does not demand normality of the tested distributions. The Mann–Whitney U -test was used to test whether the median values in the exact timings of embryo events were significantly different between the implanted and not implanted embryos.

To describe the distribution of the probabilities of implantation, timings were converted from continuous variables into categorical variables by dividing them into groups based on their quartiles. By this procedure, we avoided bias due to differences in the total number of embryos in each category. We then calculated the percentage of embryos that implanted for each timing quartile to assess the distribution of implantation in the different categories.

Continuous data were analyzed using Student's *t*-test when comparing two groups, and analysis of variance followed by Bonferroni's and Scheffe's *post hoc* analysis when multiple groups were considered. Chi-square tests were used to compare between categorical data. For each timing variable, an optimal range was defined as the combined range spanned by the two quartiles with the highest implantation rates. Additionally, a binary variable was defined with the value inside (outside) if the value of the timing variable was inside (outside) the optimal range.

The odds ratio (OR) of the effect of all binary variables generated on implantation was expressed in terms of 95% confidence interval (95% CI) and significance. By conducting the logistic regression analysis, the effect of optimal ranges and other binary variables on implantation were quantified. Significance was calculated using the omnibus test (likelihood ratio), and the uncertainties uncovered by the model were evaluated by Nagelkerke R^2 —a coefficient that is analogous to the R^2 index of the linear regression analysis. Receiver operating characteristic (ROC) curves were employed to test the predictive value of all the variables included in the model with respect to implantation. ROC curve analysis provides AUC values (area under the curve) that are comprised between 0.5 and 1 and can be interpreted as a measurement of the global classification ability of the model.

The degree of sorting implantation rate (IR_{sort}) of the embryos after implantation probability in both a time-lapse and a morphology classification system was evaluated. The evaluation was done by calculating the mean absolute difference from the overall average IR experienced by the $n = 247$ transferred embryos in each of the categorization systems. In other terms, IR_{sort} was calculated as $IR_{\text{sort}} = \sum_i n_i \text{Abs}(IR_i - IR) / n$ where IR_i is the implantation rate and n_i is the number of embryos in each category i . IR is the average implantation rate for all n embryos.

Statistical analysis was performed using the Statistical Package for the Social Sciences 17 (SPSS Inc., Chicago, IL) and MedCalc Software (Ghent, Belgium).

Results

The primary etiology of female infertility was: poor oocyte quality: 34.7% ($n = 99$); advanced maternal age (>45): 24.6% ($n = 70$); premature ovary failure: 6.0% ($n = 17$); Unknown: 23.8% ($n = 68$), tubal obstruction: 2.5% ($n = 7$); low ovary response: 8.4% ($n = 24$). Average estradiol levels prior to hCG injection were 1701 (SD = 991) pg/ml. A total of 201 embryos were implanted successfully (gestational sac with fetal heartbeat) out of the total 522 transferred, giving rise to a 38.5% implantation rate. The biochemical pregnancy rate per transfer was 55.1% ($n = 157$) and ongoing pregnancy rate per transfer was 49.8% ($n = 142$).

All treatment cycles with either full or no implantation were selected for further retrospective analysis. This analysis considers 247 embryos; 61 from cycles with full implantation (number of gestational sacs matched the number of transferred embryos) and 186 from cycles with no implantation (no biochemical pregnancy was achieved).

Morphokinetic and morphological events and implantation rate

Of the total 247 (19.4%) embryos, 48 exhibited one or more of the following morphological events: (i) direct cleavage from zygote to three blastomere embryo, defined as: $cc2 = t3 - t2 < 5$ h ($n = 8$). (ii) uneven blastomere size at the 2 cell stage during the interphase where the nuclei are visible ($n = 26$). Blastomeres were considered uneven sized if the average diameter of the large blastomere was

$>25\%$ larger than the average diameter of the small blastomere. (iii) multinucleation at the 4 cell stage during the interphase where the nuclei are visible ($n = 23$). The embryo was considered multinucleated if more than one distinct nucleus was observed in one (or more) blastomeres. From those 48 embryos, only four implanted (8%) (two with uneven blastomere size and two that were multinucleated) and we suggest using the listed observations as exclusion criteria for embryo selection. We found 29 transferred embryos that exhibited $>20\%$ of fragmentation, out of which four implanted (14% IR); consequently, we did not consider ' $>20\%$ fragmentation' to be an exclusion criterion.

Timing of embryo development events and implantation

Cleavage times for the first four divisions are shown in Fig. 2 as percentages of embryos that have completed their cell division at different time points after insemination by ICSI. The four blue curves represent the successive divisions of the 61 embryos that implanted, and the four red curves the 186 embryos that did not.

It is apparent that there is a tighter distribution of cleavage times for implanting embryos as opposed to non-implanting embryos. A prominent tail of lagging embryos was found for the non-implanting embryos (red curves). At least for the late cleavages ($t3$ – $t5$) there appeared a leading tail of too early cleaving embryos that were found to not implant.

More detailed evaluation of the distribution of all divisional timings was made. An example, the timing for cell division to 5 cells, $t5$, is shown in Fig. 3. The distribution of cleavage times for 61 implanting embryos (positive) is indicated by blue dots and for 186 non-implanting embryos (negative) by red dots. The left panel shows the overall distributions of $t5$ for the respective embryo types. The right panel shows a normal quartile plot of observed $t5$ cleavage times for the two embryo types. A straight line on this type of plot indicates a normal distribution. Both types of embryos approximate to a normal distribution. The mean value of $t5$ is similar for both groups as the lines intersect at 0.5, but the slopes of the lines differ, indicating that the standard deviation for the two types of embryos are not the same. The slope of the full-implantation group is more horizontal and the variance thus significantly lower for $t5$ from implanting embryos than for non-implanting embryos.

The average timing of $t2$, $t3$, $t4$ and $t5$, together with $cc2$ and $s2$ for the those implanted and not implanted embryos, is presented in Table 1. Exact timings of embryo events follow normal distributions for the implanted embryos for all parameters (except $s2$). The exact timings of embryo events for the not implanted embryos do not follow normal distributions (except for $t5$, see also Fig. 3).

As expected from the distributions of cleavage times shown in Fig. 2, all the distributions of parameters from implanted embryos are characterized by significantly smaller variances than the distributions of parameters from the non-implanting embryos.

The median values were not significantly different between the implanting and non-implanting embryos for any of the parameters except for $s2$, with a median value of 0.50 h for implanted and 1.00 h for non-implanted embryos ($P = 0.0040$). The four quartiles for the timing of each of the investigated parameters are presented in Table 2, together with percentages of implanting embryos in each quartile. The categories defined by these quartiles were used to

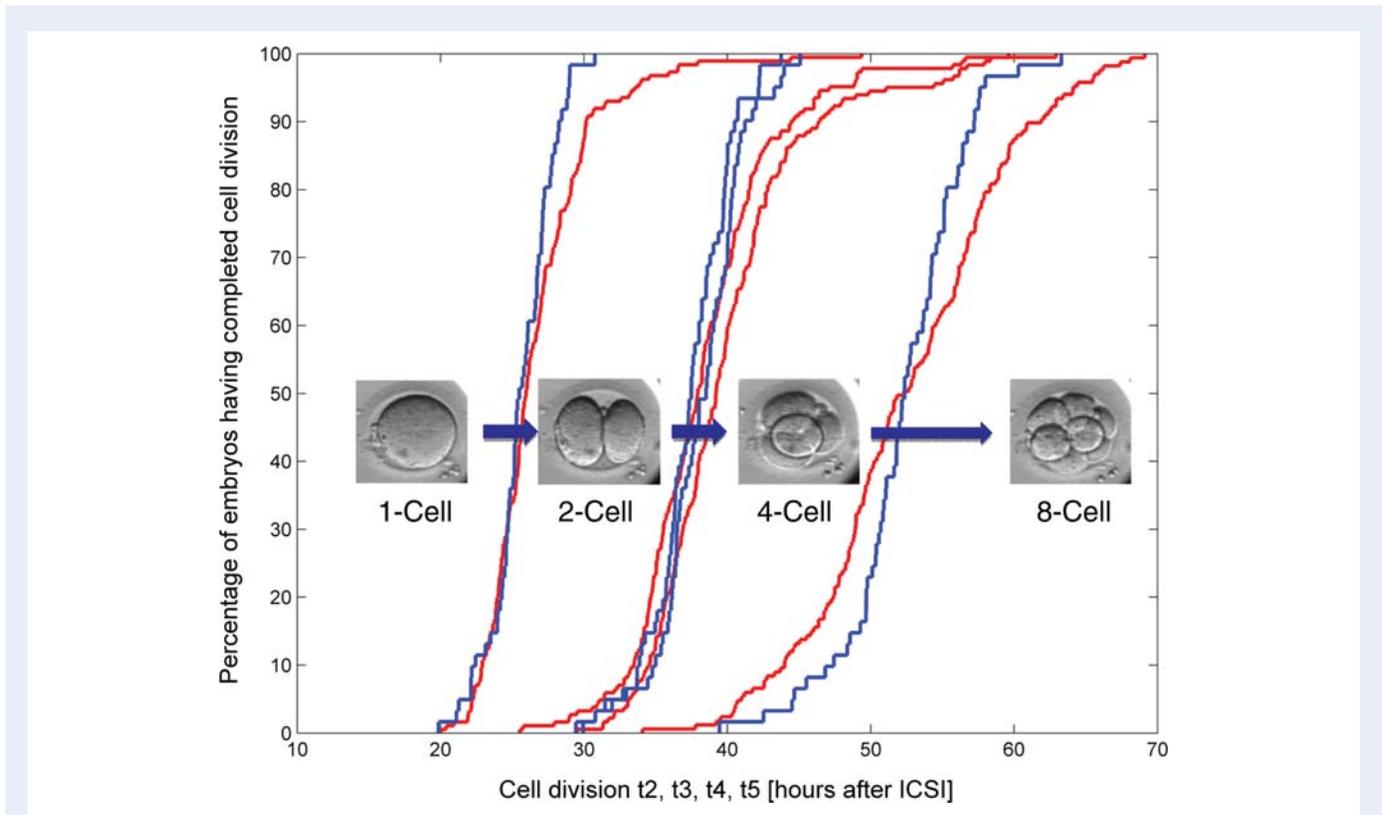


Figure 2 Percentage of embryos having completed a cell division by a given time after insemination. Blue curves represent implanting embryos; red curves represent embryos that did not implant. Four curves of each color represent completion of the four consecutive cell divisions from 1 to 5 cells i.e. t_2 , t_3 , t_4 and t_5 .

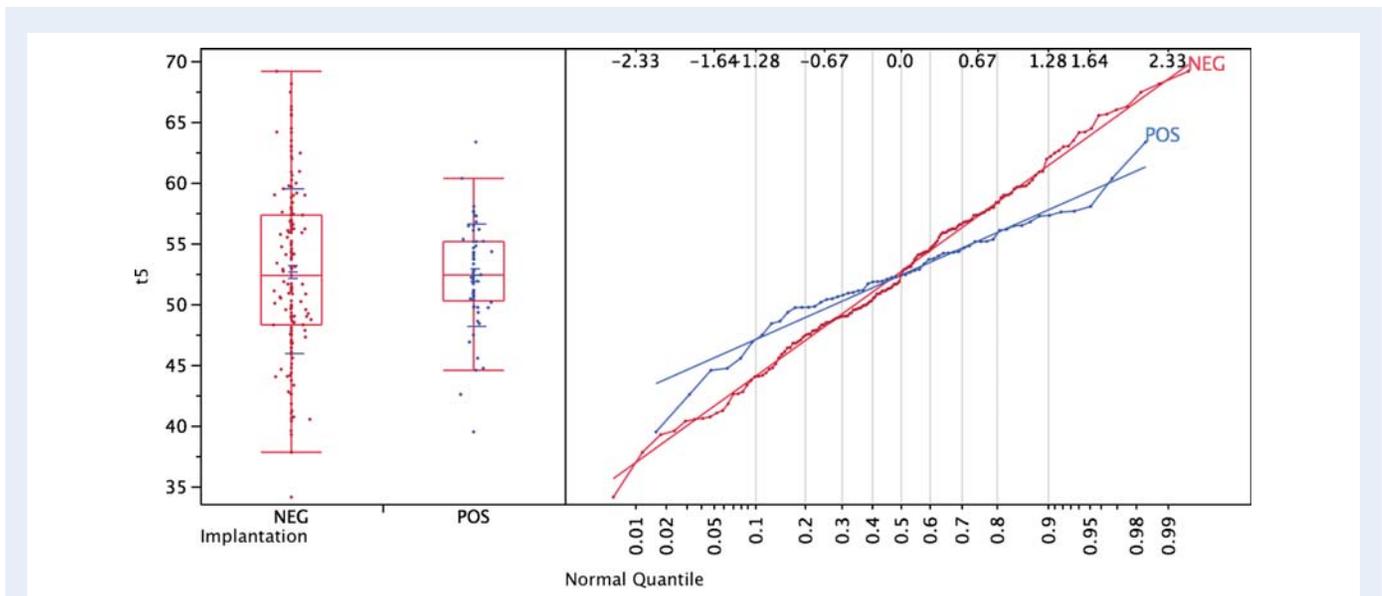


Figure 3 Distribution of the timing for cell division to 5 cells, t_5 , for 61 implanting embryos (positive, blue dots) and for 186 non-implanting embryos (negative, red dots). The left panel shows the overall distributions of cleavage times. Short blue lines demarcate standard deviations, means and 95% confidence limits for the mean. Red boxes denote the quartiles for each class of embryos. The right panel shows the distribution of observed t_5 cleavage times for the two types of embryos (red = non-implanted; blue = implanted) plotted as normal quantiles on a plot where a normal distribution is represented by a straight line. The two fitted lines represent normal distributions corresponding to the two types of embryos.

Table I Exact timing of embryo events analysed from transferred implanted and not implanted embryos.

Parameter	Implanted embryos				Not implanted embryos				Homogeneity of variances
	Mean (h)	SD (h)	n	Normal dist.	Mean (h)	SD (h)	n	Normal dist.	P-value
t2	25.6	2.2	61	Yes	26.7	3.8	186	No	0.022
t3	37.4	2.8	61	Yes	38.4	5.2	185	No	0.002
t4	38.2	3.0	61	Yes	40.0	5.4	182	No	0.004
t5	52.3	4.2	61	Yes	52.6	6.8	167	Yes	<0.001
cc2	11.8	1.2	61	Yes	11.8	3.3	185	No	0.006
s2	0.78	0.73	61	No	1.77	2.83	182	No	0.016

Table II Exact timing of the first cleavages grouped in quartiles (Q1, Q2, Q3 and Q4) from 247 transferred embryos.

Parameter	Q1		Q2		Q3		Q4	
	Limit (h)	Implantation (%)	Limit (h)	Implantation (%)	Limit (h)	Implantation (%)	Limit (h)	Implantation (%)
t2	<24.3	23	24.3–25.8	32	25.8–27.9	30	>27.9	15
t3	<35.4	18	35.4–37.8	39	37.8–40.3	32	>40.3	11
t4	<36.4	23	36.4–38.9	36	38.9–41.6	31	>41.6	10
t5	<48.8	16	48.8–52.3	37	52.3–56.6	40	>56.6	14
cc2	<11.0	23	11.0–11.9	39	11.9–12.9	18	>12.9	19
s2	<0.30	36	0.30–0.76	28	0.76–1.50	20	>1.50	16

Additionally, the percentage of implanting embryos in each quartile is shown. Numbers in bold indicate the two quartiles with the highest implantation percentages.

establish optimal ranges based on the two consecutive quartiles with highest implantation probabilities (entries in bold typeface in Table II). Observed parameters with significantly higher implantation rate for parameters inside the optimal range when compared with those outside the range are presented in Figs 4 and 5.

For all cleavage times assessed (t2, t3, t4 and t5), embryos whose cleavage were completed in the two central quartiles displayed the highest implantation rates, and were consequently combined in an optimal range for each parameter (Fig. 4). For all cleavage times, there is a significant difference in implantation rate between embryos within the optimal range as opposed to those outside the range (Fig. 4). However, it should be noted that the discrimination between implantation rates within the two best quartiles and the implantation rate outside these quartiles increases with successive cell divisions. For t2, the difference in implantation rate is 12%, for t3 we found 21% difference and for t5 it amounts to 24%. The implantation rate of embryos with t5 cleavage within the range is 2.6 times the implantation rate for embryos outside this range. Selection based on the timing of cleavage to the 5 cell stage, thus provides the best single criteria to select embryos with improved implantation potential.

For both the duration of the second cell cycle, cc2, and the synchrony of cell cleavages in the transition from 2 cell stage to 4 cell stage, s2 (i.e. the duration of the 3 cell stage), we found that embryos cleaving in the two first quartiles have significantly higher implantation rate that those cleaving in the last two quartiles (Fig. 5). If we had eliminated the embryos, where we observed abrupt cell division from 1 cell to 3 or 4 cells from this analysis (i.e.

8 of 247), the implantation rate in the first quartile for cc2 would be higher (26% instead of 23%) as none of these embryos implanted.

Evaluation of potential selection parameters based on a logistic regression analysis

A logistic regression analysis was used to select and organize which observed timing events (expressed as binary variables inside or outside the optimal range as defined earlier) should be used together with the morphological exclusion criteria. The model identified the time of division to 5 cells, t5 OR = 3.31 (95% CI 1.65–6.66) followed by synchrony of divisions after the 2 cell stage, s2 OR = 2.04 (95% CI 1.07–4.07) and the duration of the 2 cell cycle, cc2 OR = 1.84 (95% CI 0.95–3.58) as the most promising variables characterizing implanting embryos.

By using exclusion variables plus t5, s2 and cc2, we defined a logistic regression model. An ROC curve analysis to determine the predictive properties of this model with respect to probability of implantation gave an AUC value of 0.720 (95% CI 0.645–0.795).

These data were used to generate the hierarchical selection model described later.

Embryo scoring based on a classification tree to select embryos with higher implantation probabilities

The observed correlations between morphokinetic parameters and embryo implantation form the basis for a proposed hierarchical classification procedure to select viable embryos for transfer with a high

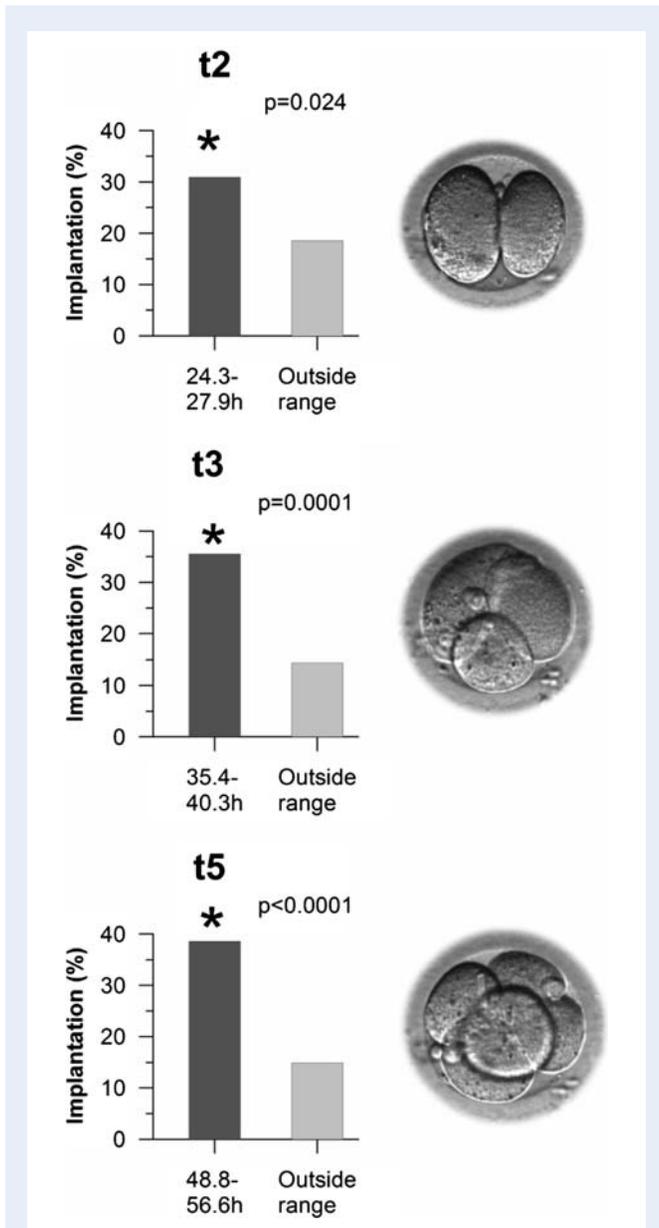


Figure 4 Percentage of implanting embryos with cell division times inside or outside ranges defined by quartile limits for the total data set. The three panels show ranges and implantation for: (i) division to 2 cells, t2; (ii) division to 3 cells, t3 (iii) and division to 5-cells, t5. As the limits for the ranges were defined as quartiles, each column represents the same number of transferred embryos with known implantation outcome, but the frequency of implantation was significantly higher for embryos within the ranges as opposed to those outside the ranges. Data for division to 4 cells, t4, are very similar to division to 3 cells and are thus not shown. Values for t4 are included in Tables I and II.

implantation potential. The classification tree depicted in Fig. 6 represents a sequential application of the identified selection criteria in combination with traditional morphological evaluation.

Using the data presented here, we made a hierarchical model representing a classification tree, which subdivided embryos into six

categories from A to F. Four of these categories (A–D) were further subdivided into two sub-categories (+) or (–) as shown in Fig. 6, giving a total of 10 categories. The hierarchical classification procedure starts with a morphological screening of all embryos in a cohort to eliminate those embryos that are clearly NOT viable (i.e. highly abnormal, atretic or clearly arrested embryos). Those embryos that are clearly not viable are discarded and not considered for transfer (category F). Next step in the model is to exclude embryos that fulfill any of the three exclusion criteria: (i) uneven blastomere size at the 2 cell stage; (ii) abrupt division from one to three or more cells or (iii) multi-nucleation at the 4 cell stage (category E). The subsequent levels in the model follow a strict hierarchy based on the binary timing variables t5, s2 and cc2. First, if the value of t5 falls inside the optimal range (48.8–56.6 h), the embryo is categorized as A or B. If the value of t5 falls outside the optimal range (or if t5 has not yet been observed at 64 h), the embryo is categorized as C or D. If the value of s2 falls inside the optimal range (≤ 0.76 h) the embryo is categorized as A or C depending on t5; similarly, if the value of s2 falls outside the optimal range, the embryo is categorized as B or D depending on t5. Finally, the embryo is categorized with the extra plus (+) if the value for cc2 is inside the optimal range (≤ 11.9 h) (A+/B+/C+/D+) and is categorized with a minus (–) as (A–/B–/C–/D–) if the value for cc2 is outside the optimal range.

The hierarchical classification procedure divides all the 247 evaluated embryos in 10 different categories, containing approximately the same number of transferred embryos but with largely decreasing implantation potential (i.e. from 66% for A+ to 8% for E). In Table III, the implantation potential is listed for all categories A+ to E, as well as for the combined categories: A (52%), B (27%), C (19%), D (14%) and E (8%). No implantation potential is listed for the non-viable category F because none of the 247 transferred embryos were classified in this category. It should be noted that each of the 10 sub-categories contains only about 25 embryos, and therefore, one implanting embryo more or less would change the implantation rate with $\sim 4\%$ (1/25).

Comparison between morphology and time-lapse categories

The 247 transferred embryos were split into the morphology category 1 ($n = 35$, IR = 43%), 2 ($n = 56$, IR = 32%), 3 ($n = 92$, IR = 21%), 4 ($n = 54$, IR = 13%) and 5 ($n = 10$, IR = 20%). A comparison between the time-lapse categories A–E and the morphology categories 1–5 was made, dividing the 247 embryos into the sub-categories between the two categorization systems. For each subcategory in Fig. 7, the number of embryos is proportional to the area of the pie chart, and the fraction of implanting embryos is proportional to the blue parts of the pie charts. The highest implantation rate (67%) is found in the subcategory of the best time-lapse category ‘A’ and best morphology category ‘1’. The time-lapse category with highest implantation rate (‘A’, $n = 54$, IR = 52%) contains more embryos and has a higher implantation rate than the best morphology category (‘1’, $n = 35$, IR = 43%). Note that when inspecting the implanting embryos in time-lapse category ‘A’, they are highly represented in all the morphology categories; consequently, the time-lapse categorization seems to better find the embryos with good probability of implantation than the morphology categories, although the number

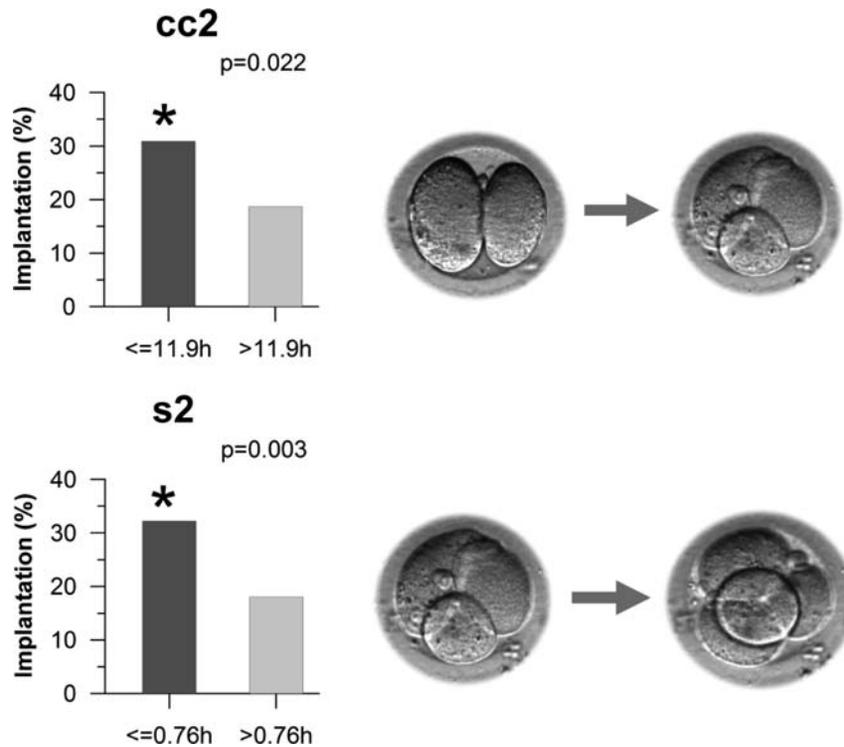


Figure 5 Percentage of implanting embryos with cell division parameters below or above the median values. The two panels show classification for: (i) duration of second cell cycle, cc2; (ii) synchrony of divisions from 2-cell to 4-cell stage, s2. As the limits are defined as median values for all 247 investigated embryos with known implantation outcome, each column represents the same number of transferred embryos and the frequency of implantation was significantly higher for embryos with parameter values below the median.

of embryos in each subcategory is very small. In the left side of Fig. 7, a column of pie charts illustrates the overall distribution of embryos in each of the morphology categories (the number of embryos in each category is again proportional to the area of the chart). The row of pie charts at the bottom of Fig. 7 illustrates the size of each of the time-lapse categories. It is seen that the time-lapse categories have almost equal sizes, whereas the sizes of the morphology categories are unequal, with the middle category '3' being much larger than the other categories.

Logistic regression on the morphology categories 1–5 gives an AUC of 0.64, whereas a logistic regression on the simplified time-lapse categories A–E gives an AUC of 0.72. The higher AUC for the time-lapse categories supports the possibility of improved embryo selection using time-lapse. The degree of sorting (IR_{sort}) was calculated for each of the categorization systems and was 8.5% for the morphology and 12.6% for the time-lapse categorization, supporting that the time-lapse categorization indeed sorts the embryos better than the morphology system.

Discussion

In the present study, we aimed at discovering specific temporal developmental markers that predict implantation. Six discriminative morphokinetic parameters were identified (t_2 , t_3 , t_4 , t_5 , cc2, s2) for a set of 247 transferred embryos with either full or no implantation. For each of the identified parameters, we selected the two quartiles with the highest frequency of implanting embryos and combined this

classification with morphologic exclusion criteria (first cleavage asymmetry, abrupt first division to more than 2 cells and multinucleation in the 4-cell stage embryos). This classification was chosen in order to describe correlations between time taken to reach each developmental milestone and the implantation potential of that specific embryo. We identified an optimal range for each parameter, which was correlated with a significantly higher probability of implantation. There are many plausible explanations for the observed association that could be directly or indirectly related to cellular processes implicated in cell division. Observed variations in the timing of embryonic development may be related to culture conditions that can affect embryo metabolism coupled with intrinsic factors within the oocyte such as ooplasm maturity (Escrich *et al.*, 2010) and/or sperm, paternal effect which may affect the duration of synthesis phase (S-phase). Furthermore, chromosomal abnormalities may delay DNA replication (Lechniak *et al.*, 2008), thus altering the length of cell cycles and divisions.

The search for prognostic factors that predict embryo development and the outcome of IVF treatments has attracted considerable research attention as it is anticipated that the knowledge of such factors may improve future IVF treatments (Mastenbroek *et al.*, 2007; Scott *et al.*, 2008; Seli *et al.*, 2011).

One promising predictive factor is the precise timing of key events in early embryo development (Payne *et al.*, 1997; Lemmen *et al.*, 2008; Mio and Maeda, 2008; Nakahara *et al.*, 2010; Wong *et al.*, 2010). Dynamics of early embryonic patterns can be measured using non-

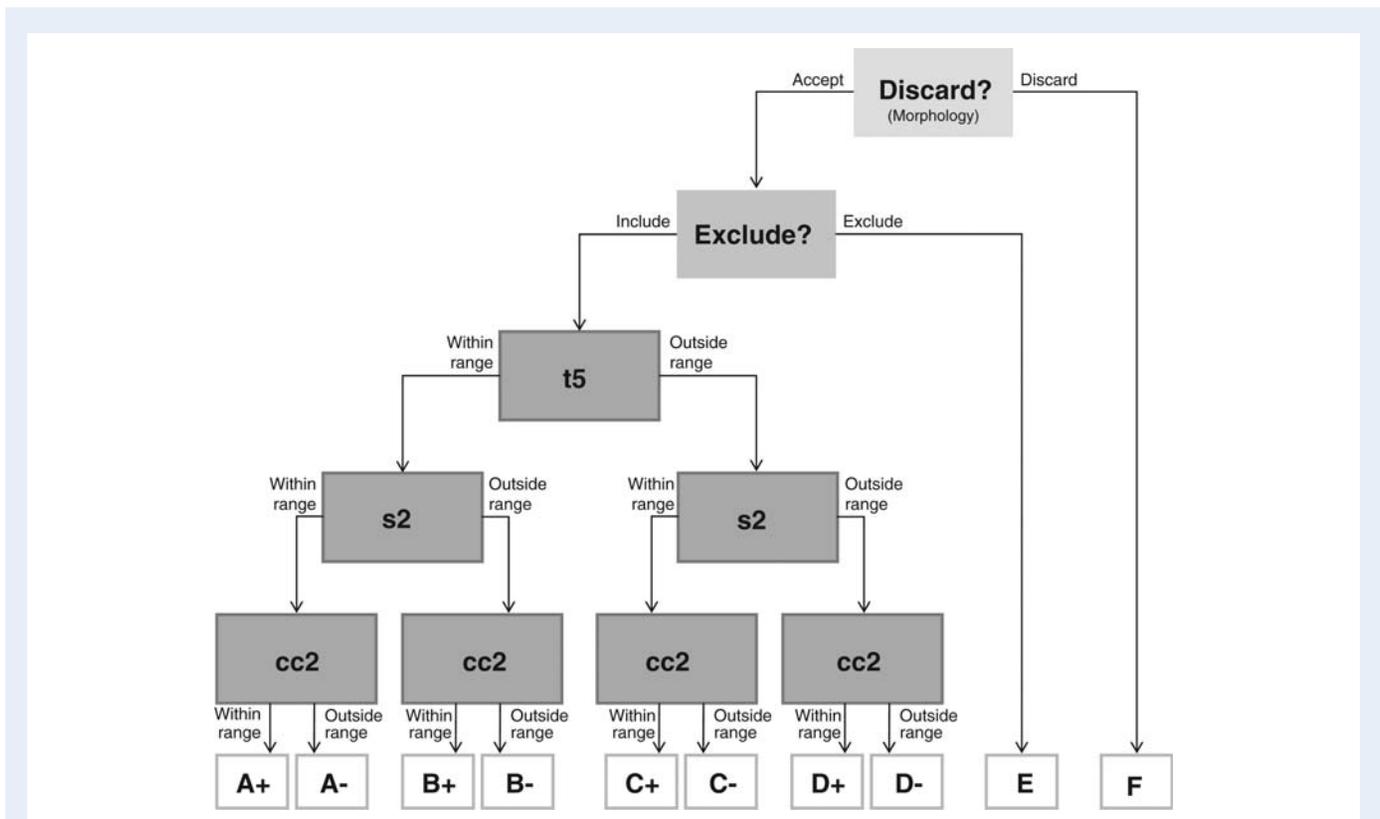


Figure 6 Hierarchical classification of embryos based on: (i) morphological screening; (ii) absence of exclusion criteria; (iii) timing of cell division to 5 cells (t5); (iv) synchrony of divisions from 2 cell to 4 cell stage, s2, i.e. duration of 3 cell stage; (v) duration of second cell cycle, cc2, i.e. the time from division to a two blastomere until division to a three blastomere embryo. The classification generates 10 categories of embryos with increasing expected implantation potential (right to left) and almost equal number of embryos in each.

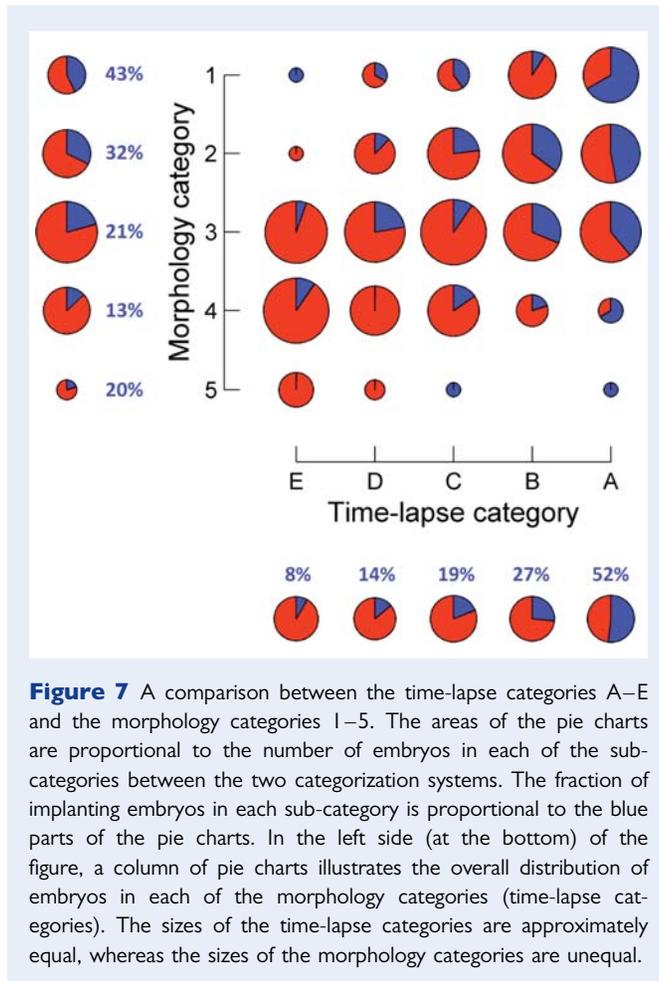
Table III Implantation in the embryo categories of the hierarchical classification tree model.

Embryo category	n total	n implanted	Implantation (%)	Embryo category	Implantation (%)
A+	29	19	66	A	52
A-	25	9	36		
B+	24	7	29	B	27
B-	25	6	24		
C+	32	8	25	C	19
C-	21	2	10		
D+	10	1	10	D	14
D-	33	5	15		
E	48	4	8	E	8

invasive and preferably automated approaches of image recognition, which can be coupled to advanced statistical analysis software and yield objective and consistent data, thereby minimizing reliance on conventional subjective classification of embryo morphology (e.g. Scott, 2003).

Quantitative morphokinetic parameters could be important prognostic factors, but most human embryo research on morphokinetic development have been based on a small number of samples

generated under diverse experimental conditions (Payne et al., 1997; Lemmen et al., 2008; Mio and Maeda, 2008; Nakahara et al., 2010; Wong et al., 2010). Studies that involve imaging have been limited to measurements of early development, such as pronuclear formation and fusion, and time to first cleavage (Nagy et al., 1994; Lundin et al., 2001; Fenwick et al., 2002; Lemmen et al., 2008). Recently Wong et al. sought to overcome these limitations and defined critical pathways and events in human embryo development by correlating imaging profiles



and molecular data throughout preimplantation development from the zygote to the blastocyst stage. They studied supernumerary IVF embryos that had been cryopreserved at the zygote stage; these embryos were frozen with a procedure that significantly affects the outcome after thawing and could not be directly comparable with fresh embryos (Wong *et al.*, 2010). In the present study, we observed important differences in the temporal patterns of development between the embryos that implanted and those that did not. Our data revealed developmental dynamics for transferred embryos related to clinical implantation potential, suggesting that there is an optimal time range for parameters characterizing the early embryonic cell divisions. Embryos that cleave at intermediate time points have significantly improved chance of implantation when compared with embryos that either developed faster or slower. Our observations support the hypothesis that the viability of embryos is associated with a tightly regulated sequence of cellular events that begin at the time of fertilization. In this large clinical study on exclusively transferred embryos, we have corroborated that an embryo's capability to implant is correlated with numerous different cellular events, e.g. timing of cell divisions and time between divisions, as well as uneven blastomere size and multinucleation.

The timing of the first cleavage has been investigated extensively and it is generally accepted that early cleavage is a good indicator of developmental competence, with early cleavage always preferred compared

with late cleavage (Lundin *et al.*, 2001; Giorgetti *et al.*, 2007; Terriou *et al.*, 2007). While this certainly holds true for the majority of embryos encountered in IVF treatments, our results indicate that when studying only transferred embryos the cell divisions could occur 'too early'. The percentage of implanting embryos in the first quartile was lower than in the two central quartiles for all cleavages (Table II), although the difference was only significant for t3 ($P = 0.013$) and t5 ($P = 0.002$) (χ^2 test), thus supporting the existence of an optimal time range, or time window, for all cell divisions.

The study by Wong *et al.* based on the retrospective analysis of 100 embryos, suggested that time-lapse-based analysis of the first 48 h of development could predict (with high specificity and sensitivity) which embryos would subsequently develop to the blastocyst stage. Wong *et al.* (2010) included poor quality embryos, which subsequently arrested, in the data analysis, and none of the embryos included in the study were transferred. The retrospective analysis presented in this study is based on a larger set of 247 exclusively transferred embryos, all of which were evaluated based on their ability to implant and form a gestational sac, which largely confirms the findings of the previous studies (Lemmen *et al.*, 2008; Wong *et al.*, 2010). We find the duration of the second cell cycle (cc2) and the synchrony of the second and third cell divisions (s2) to be important indicators that are significantly correlated with embryo implantation. However, by using implantation as our end-point, we assess not only embryo competence for blastocyst formation, but also subsequent highly essential processes such as hatching and successful implantation in the uterus. Thus, our data allows the detection of late developmental predictors of implantation potential. The results indicate that timing of later events such as the cleavage to the 5 cell stage are consistently good indicators of implantation potential, and that the discrimination between implanting and non-implanting embryos is improved when using the later cell division events, e.g. t5 as opposed to the earlier events (t2, t3 and t4). It is likely that timing of events after division to 5 cell stage could be even more indicative of embryo viability; however, exact timing of later divisional events become increasingly difficult to ascertain both manually and through image analysis. Firstly, it is easier to count, for example, 2 cells in an image than to count 8 cells, because it is necessary to keep track on the cells that have already been counted. Secondly, the more cells in the embryo, the more they tend to cover each other and it is therefore necessary to keep track of the cells in several focal planes. Thirdly, distinguishing fragmentation from cells becomes harder the smaller the cells are. In other words, the more objects (cells and fragmentation) there are in the image, the harder it is to count the cells and to determine if a division occurred. Consequently, divisions to more than 6 cells become increasingly difficult to detect. The presented data indicates that incubating the embryos to Day 3, which enables evaluation of timing for cell divisions from 5 to 8 cells, after completion of the third cell cycle, can give additional important information that will improve the ability to select a viable embryo with a high implantation potential.

From Table I, it is seen that the number of transferred embryos, that did not implant is decreasing because some of the embryos were arrested in their development. Specifically, 1 embryo never divided to 3-cells, 4 embryos never divided to 4-cells and 19 embryos never divided to 5-cells within the 64 h of observation time. Consequently, values for t3, t4, t5 and the derived parameters s2 and cc2 do not exist for some of the embryos. Even though 19

of the non-implanting embryos never divide to 5-cells, it is still possible to discriminate the group of implanting and non-implanting based on the SD of t5, emphasizing the power of the later divisional events. One of the main objectives for ART, which is a presently used policy in several European IVF clinics, is to reduce the number of multiple gestations by single embryo transfer. Current morphological and growth-related criteria that are commonly used to assess embryo viability on Day 3 may both underestimate or overestimate embryo potential (Racowsky, 2002). Given the uncertainties associated with evaluation at Day 3, some clinics have turned to extended culture regimens to improve the assessment of embryo implantation potential (Milki et al., 2000; Gardner et al., 2004). Blastocyst culture brings a number of potential advantages over traditional cleavage-stage embryo transfer, since prolonged embryo culture in which some presumably non-viable embryos arrest their development may facilitate the ultimate selection of the blastocyst for transfer. Extended culture has been advocated as a way to increase implantation rate and improve reproductive outcomes (Mercader et al., 2003; Gardner et al., 2004).

However, prolonged *in vitro* culture increases the laboratory workload. Moreover, with prolonged *in vitro* culture—even in greatly improved modern media—we increase the exposure of the developing embryo to artificial culturing conditions. Legitimate concerns have been raised that this extended exposure could affect natural imprinting and lead to altered gene expression patterns or even higher risk of preterm birth or congenital malformations (Niemitz and Feinberg, 2004; Horsthemke and Ludwig, 2005; Källén et al., 2010) and thus result in an increased frequency of epigenetic disorders (Manipalviratn et al., 2009). Thus, optimal duration of embryo cultivation may be somewhere intermediate, optimizing opportunities for selection without affecting imprinting processes. In addition, in-depth understanding of the effects of treatment parameters such as stimulation protocols, media, incubation conditions and handling on embryo morphokinetics are still predominantly unknown (Sifer et al., 2009; Vale and Gardner, 2010).

A possible cause for embryos to deviate from the optimal cleavage pattern is aneuploidies and genetic disorders. While advanced morphokinetic selection is not a guarantee of chromosomal normality, it has been reported that most embryos that fail to follow normal timings for cell divisions may show multiple aneuploidies (Jones et al., 1998). Combining our results with Jones et al. (1998) suggests that morphokinetic parameters could improve selection of genetically normal and viable embryos and thus alleviate the need for invasive procedures but more research is needed to investigate the possible relationship.

In summary, our results demonstrate that routine time-lapse monitoring of embryo development in a clinical setting, (i.e. automatic image acquisition in an undisturbed controlled incubation environment) provides novel information about developmental parameters that differ between implanting and non-implanting embryos, in the sense that the variances for the parameters are larger for the non-implanting than for the implanting embryos. Retrospective analysis of embryo morphokinetics indicated the correlations between developmental events and subsequent implantation after transfer. However, the median values of the morphokinetic parameters (except s2) for transferred embryos do not differ between the implanting and non-implanting embryos—only the variance. It is well known that other

factors besides embryo viability play a vital role in embryo implantation. An embryo can belong to both the best morphology and the best time-lapse category and still not implant, simply because the endometrium is not ready. It is therefore not unexpected that we find non-implanting embryos with the exact same morphokinetic development as implanting embryos. On the other hand, the morphokinetic parameters make rejection of embryos with lower chance of implantation possible, because the variances in the morphokinetic parameters are indeed larger for the non-implanting than for the implanting embryos. The use of morphokinetic parameters could hence be helpful in improving embryo selection in conjunction with currently used morphological parameters.

In clinical practice, our results may be used to improve embryo selection by measuring morphokinetic markers and selecting embryos for transfer that best follow the divisional timings and patterns identified for implanting embryos. We cannot exclude that the morphokinetic concepts presented here are affected by culture media. Consequently, until the influence of culture media on the morphokinetics has been investigated, it is still unclear whether the proposed selection criteria are universally applicable or culture-specific.

The time-lapse categorization system presented with the hierarchical tree is a first attempt of making a model for embryo selection that incorporates time-lapse information. When more data are available, it is desirable to develop a more sophisticated unified model better incorporating and exploiting both the morphology and time-lapse information.

The comparison of the time-lapse hierarchical tree and the morphology categorizations showed that the degree of sorting of the embryos was better in the time-lapse categorization than in the morphology categorization. It suggests that it should be possible to improve pregnancy rates by using the time-lapse information for embryo selection.

Nonetheless, the observed correlations and proposed selection procedures must be tested in randomized prospective trials to evaluate the efficacy of the novel hierarchical selection procedure. The ensuing knowledge building through extensive documentation of embryo development may enable us to discover, test and improve morphokinetic selection criteria for future IVF treatments.

Authors' roles

M.M. played a role in conception and design, data analysis and interpretation, drafting manuscript and final approval. J.H. was involved in drafting the manuscript, acquisition of data and final approval. A.T. revised the article critically, acquired data and took part in final approval. K.M.H. was involved in data analysis and interpretation, drafting the manuscript and final approval. N.B.R. took part in conception and design, data analysis and interpretation, critical revision of the article and final approval. J.R. played a role in critical revising of article, interpretation of data and final approval.

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Conflict of interest

N.B.R. and K.M.H. are employees and shareholders in the company Unisense FertilTech A/S, which develops, manufactures and sells time-lapse incubators (e.g. the EmbryoScope™). Unisense FertilTech has produced the equipment used in the study.

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