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Article

Live births after embryo selection using morphokinetics versus conventional morphology: a retrospective analysis

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Simon Fishel, Founder and President of the CARE Fertility Group, worked with IVF pioneer and Nobel Laureate Robert Edwards from 1975 to 1985 at Cambridge University and as Deputy Scientific Director of the first IVF clinic, Bourn Hall, from 1980. In 1978 he received the prestigious Beit Memorial Fellowship and was elected a Research Fellow of Churchill College, Cambridge, publishing over 200 papers and four books. He was the first to publish on the adaptation of the mammalian to its environment, and in 1984 on the secretion of hCG by the human embryo. In 1992 he founded the world's first degree course in IVF and in 2009 was honoured by the Liverpool John Moores University with their highest award of 'University Fellow' for "*outstanding contribution to science and to humanity*".

KEY MESSAGE
Incidence of live birth after embryo transfer using morphokinetic algorithms during uninterrupted culture to select embryos was increased by 19% compared with conventional morphology and standard incubation in women younger than 38 years. Incidence of aneuploidy may limit their effectiveness in women older than 37 years.

A B S T R A C T

The increasing corpus of clinical studies using time-lapse imaging for embryo selection demonstrates considerable variation in study protocols and only limited-sized study cohorts. Outcome measures are based on implantation or clinical pregnancy; some predict blastulation from early cleavage-stage data, and few have evaluated live birth. Erroneously, most studies treat the embryos as independent variables and do not include patient or treatment variables in the statistical analyses. In this study, cohort size was 14,793 patients and 23,762 cycles. The incidence of live birth (n = 973 deliveries) after embryo selection by objective morphokinetic algorithms was compared with conventional embryology selection parameters (n = 6948 deliveries). A 19% increase in the incidence of live birth was observed when morphokinetic data were used to select embryos for the patient cohort aged younger than 38 years (OR 1.19 with 95% CI 1.06 to 1.34) using their own eggs, and an increase of 37% for oocyte recipients aged over 37 years (OR 1.370; 95% CI 0.763 to 2.450). This is the largest study of the prospective use of time-lapse imaging algorithms in IVF reporting on live birth outcome, although the nature of purely a closed system versus standard incubation could not be assessed.

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Introduction

The success of IVF during the last 35 years has evolved from aspirational live birth rates of around 15% (Edwards et al., 1984; Fishel et al., 1984, 1985) to rates of between 35 and 60% (HFEA, 2013). This is largely dependent on female age, and is a result of advances in follicular stimulation regimens, multiple embryo transfer and improvements in the culture and selection of the human embryo for transfer. In recent years, with the more responsible adoption of single- or double-embryo transfer after IVF, permitted by further improvements in culture media, the need for embryo-selection tools has been ever more pressing to ensure high live birth rates. Human conception is inefficient, with estimates of up to 70% of human zygotes being non-viable in their progression to a healthy full-term delivery (Zinaman et al., 1996). The first in-vitro evidence for aneuploidy as a potential cause of human embryo failure was published in the early 1980s (Angell et al., 1983, 1986). Irrespective of chromosome copy number, since the origins of IVF, human morphokinetics was enormously important in predicting viable embryos (De Neubourg et al., 2004; Edwards et al., 1984; Fishel et al., 1983). For many clinics, the goal is single embryo transfer while still improving the incidence of healthy live birth outcomes to levels unimaginable only 2 decades ago, thereby minimizing the unacceptable risk of high-order multiple pregnancies. For the highest chance of success per cycle, however, IVF practice involves ovarian stimulation usually resulting in several embryos, from which one, or at most two, are selected for transfer. Confidence in the elective transfer of a single embryo has been achieved, in part, by advances in embryo culture and technology. The development of time-lapse imaging (TLI) in recent years (Lemmen et al., 2008; Meseguer et al., 2011) has enabled better embryology laboratory practice in several domains: workflow, consistent objective selection, and zygote and embryo protection as part of the putative improved culture conditions using continuous, undisturbed culture system.

The evidence for using sophisticated time-lapse systems for morphokinetic algorithms that are predictive of successful outcome, however, is limited (Kaser and Racowsky, 2014). In this retrospective analysis of treatments undertaken from January 2010 to January 2015, live birth outcome of a large cohort using our time lapse imaging protocols as a prospective selection tool for embryo transfer was compared with those patients undergoing conventional embryology. The time-lapse system used closed incubation (EmbryoScope), and generated the algorithms from morphokinetic data for the embryo selection, compared with standard incubation and the use of conventional morphological embryo selection according to The Istanbul Consensus (Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology, 2011).

Materials and methods

This multicentre study included 14,793 patients from six CARE Fertility centres from January 2010 to January 2015: CARE Nottingham, CARE Northampton, CARE Manchester, CARE Sheffield, Beacon CARE Fertility Dublin and CARE London; representing a total of 21,235 treatment cycles in the standard treatment group and 2527 in the EmbryoScope treatment group, with 6948 (32.7%) and 973 (38.5%) live births observed, respectively . 'Patients' or 'non-recipients' (n = 21,466 cycles) comprised those who used all their own oocytes (n = 20,664) and patients who were also oocyte-share donors (n = 802); 'recipients' comprised those women undergoing oocyte donation (n = 2296 cycles). All protocols for patient treatments complied with UK regulation (Human Fertilisation and Embryology Act, 1990, 2008) and all UK facilities are regularly inspected by the Human Fertilisation and Embryology Authority (HFEA). The retrospective analysis, or the use of time-lapse imaging algorithms for embryo selection, did not require ethical or institutional review board approval, as it was carried out according to previously validated procedures, and practised under license from the HFEA. All patients were counselled and gave signed consent. Time lapse imaging (TLI) was undertaken using the EmbryoScope (Vitrolife, Sweden) with strict adherence to annotation protocols, and all embryos were selected for transfer based on their in-house derived TLI algorithm score for each stage of transfer; patient choice dictated whether they used EmbryoScope associated with morphokinetic algorithm for embryo selection or standard incubation. The primary end-point of this study was live birth events; that is, the number of patients achieving a delivery of a live born infant. Only 'fresh' embryo transfer cases were included, and all pre-genetic implantation screening cases were excluded.

The following clinical variables were categorized for inclusion in the analysis: patient age, day of embryo transfer, number of embryos transferred, donor age (where applicable), body mass index (BMI), anti-Müllerian hormone (AMH), antral follicle count (AFC), gonadotrophin type, gonadotrophin dosing days, and gonadotrophin total dose. The groupings applied are presented for each variable in **Table 1**. Patient age was considered as a binary variable in the modelling (younger than 38 years and 38 years and over), corresponding to the common grouping used by HFEA, which is familiar to patients. The groupings for categorizing BMI, AMH, and AFC were chosen to correspond to clinically meaningful categories, i.e., to reflect what might be considered to be above, below or within a normal or healthy range. The donor ages (less than 29 years, 29–32 years and 33 years and over), gonadotrophin number of days of dosing and the total dose variables were categorized on the basis of quantiles of the observed distribution to ensure that sufficient information was present in each of the categories for a robust analysis.

The following definitions were used for the BMI, AMH and AFC categories, relating to the data presented: BMI less than 18.5 (underweight), 18.5 and less than 25 (healthy weight), 25 and less than 30 (overweight), 30 and less than 40 (obese), and 40 upwards (extremely obese); AMH (pmol/L): less than 6 (low), 6 and less than 24 (normal), 24 and less than 70 (high), 70 upwards (very high); AFC (number of follicles seen on ultrasound scan): less than 4 (extremely low), 4 and less than 10 (low), 10 and less than 14 (somewhat low), 14 and less than 22 (normal), 22 and less tha 35 (high), 35 upwards (very high).

Ovarian stimulation protocols

Pituitary suppression for ovarian stimulation was carried out either with gonadotrophin-releasing hormone agonist (Suprecur; 0.5 ml subcutaneously daily; Sanofi Aventis, UK) or antagonist (Cetrotide; 0.25 mg daily; Merck Serono, UK). Ovarian stimulation was achieved using human menopausal gonadotrophin (Menopur, Ferring, UK), recombinant FSH (Gonal-F; Merck Serono), or both (as previously described (Campbell et al., 2013b; Fishel et al., 2016).

Table 1 – Clinical data available for the cycles in each treatment arm (EmbryoScope/standard).

Variable	Group/summary Statistic	Standard (*n* = 21235)	Embryoscope (*n* = 2527)	Total (*n* = 23762)
Patient age, years	<38	13447 (63.3)	1414 (56.0)	14861 (62.5)
	38 +	7788 (36.7)	1113 (44.0)	8901 (37.5)
Day of embryo transfer	2	4150 (19.5)	234 (9.3)	4384 (18.4)
	3	8790 (41.4)	924 (36.6)	9714 (40.9)
	4	419 (2.0)	146 (5.8)	565 (2.4)
	5 or 6	7876 (37.1)	1223 (48.4)	9099 (38.3)
Number of embryos transferred	1	10118 (47.6)	1057 (41.8)	11175 (47.0)
	2	10463 (49.3)	1470 (58.2)	11933 (50.2)
	3	654 (3.1)	0 (0.0)	654 (2.8)
Patient type	Oocyte share donor	774 (3.6)	28 (1.1)	802 (3.4)
	Donor recipient	1810 (8.5)	486 (19.2)	2296 (9.7)
	Standard	18651 (87.8)	2013 (79.7)	20664 (87.0)
Donor age, years	<29	585 (2.8)	182 (7.2)	767 (3.2)
	29–32	649 (3.1)	165 (6.5)	814 (3.4)
	33 +	576 (2.7)	139 (5.5)	715 (3.0)
	Not applicable	19425 (91.5)	2041 (80.8)	21466 (90.3)
ICSI	No	10515 (49.5)	763 (30.2)	11278 (47.5)
	Yes	10720 (50.5)	1764 (69.8)	12484 (52.5)
Intralipid	No	19364 (91.2)	2214 (87.6)	21578 (90.8)
	Yes	1871 (8.8)	313 (12.4)	2184 (9.2)
BMI[a], kg m^2	Underweight	167 (0.8)	24 (0.9)	191 (0.8)
	Healthy	6933 (32.6)	799 (31.6)	7732 (32.5)
	Overweight	3952 (18.6)	355 (14.0)	4307 (18.1)
	Obese	1322 (6.2)	152 (6.0)	1474 (6.2)
	Extremely obese	30 (0.1)	2 (0.1)	32 (0.1)
	Unavailable	8831 (41.6)	1195 (47.3)	10026 (42.2)
AMH, pmol/L[a]	Low	1071 (5.0)	139 (5.5)	1210 (5.1)
	Normal	1900 (8.9)	264 (10.4)	2164 (9.1)
	High	790 (3.7)	109 (4.3)	899 (3.8)
	Very high	57 (0.3)	6 (0.2)	63 (0.3)
	Unavailable	17417 (82.0)	2009 (79.5)	19426 (81.8)
AFC number of follicles seen on ultrasound scan[a]	Extremely low	484 (2.3)	59 (2.3)	543 (2.3)
	Low	1797 (8.5)	238 (9.4)	2035 (8.6)
	Somewhat low	1667 (7.9)	185 (7.3)	1852 (7.8)
	Normal	2539 (12.0)	213 (8.4)	2752 (11.6)
	High	1978 (9.3)	174 (6.9)	2152 (9.1)
	Very high	1111 (5.2)	67 (2.7)	1178 (5.0)
	Unavailable	11659 (54.9)	1591 (63.0)	13250 (55.8)
Gonadotrophin type	Other	353 (1.7)	63 (2.5)	416 (1.8)
	Gonal-F	2994 (14.1)	482 (19.1)	3476 (14.6)
	Menopur	9929 (46.8)	1452 (57.5)	11381 (47.9)
	Unavailable	7959 (37.5)	530 (21.0)	8489 (35.7)
Gonadotrophin dosing days	<10	2178 (10.3)	412 (16.3)	2590 (10.9)
	10–11	3156 (14.9)	569 (22.5)	3725 (15.7)
	12 +	8227 (38.7)	1035 (41.0)	9262 (39.0)
	Unavailable	7674 (36.1)	511 (20.2)	8185 (34.4)
Gonadotrophin total dose, iuiu iu	<1800	1973 (9.3)	420 (16.6)	2393 (10.1)
	1800–2699	3929 (18.5)	576 (22.8)	4505 (19.0)
	2700–3599	3822 (18.0)	464 (18.4)	4286 (18.0)
	3600 +	3442 (16.2)	532 (21.1)	3974 (16.7)
	Unavailable	8069 (38.0)	535 (21.2)	8604 (36.2)
Total previous cycles	Mean (SD)	2.4 (1.82)	2.5 (1.78)	2.4 (1.81)
	Median (range)	2.0 (0–25)	2.0 (0–18)	2.0 (0–25)
Total numbr of previous live births	Mean (SD)	0.3 (0.63)	0.3 (0.58)	0.3 (0.62)
	Median (range)	0.0 (0–9)	0.0 (0–6)	0.0 (0–9)
Total number miscarriages	Mean (SD)	0.4 (0.88)	0.5 (0.89)	0.4 (0.88)
	Median (range)	0.0 (0–15)	0.0 (0–7)	0.0 (0–15)
Duration of infertility	Mean (SD)	3.6 (2.85)	3.4 (2.63)	3.6 (2.83)
	Median (range)	3.0 (0–31)	3.0 (0–25)	3.0 (0–31)
Total number of ectopics	Mean (SD)	0.1 (0.35)	0.1 (0.28)	0.1 (0.34)
	Median (range)	0.0 (0–5)	0.0 (0–3)	0.0 (0–5)
Number of eggs collected	Mean (SD)	9.6 (4.98)	10.1 (5.20)	9.7 (5.02)
	Median (range)	9.0 (1–38)	9.0 (1–41)	9.0 (1–41)

(*continued on next page*)

Table 1 – (*continued*)

Variable	Group/summary Statistic	Standard (*n* = 21235)	Embryoscope (*n* = 2527)	Total (*n* = 23762)
Number of eggs inseminated	Mean (SD)	7.9 (4.34)	8.6 (4.71)	8.0 (4.40)
	Median (range)	7.0 (1–35)	8.0 (1–41)	7.0 (1–41)
Ratio of mature (metaphase 2) to total eggs collecte	Mean (SD)	0.3 (0.54)	0.2 (0.42)	0.3 (0.53)
	Median (range)	0.1 (0–12)	0.1 (0–7)	0.1 (0–12)
Number of eggs fertilized	Mean (SD)	5.2 (3.27)	5.9 (3.60)	5.3 (3.33)
	Median (range)	5.0 (0–26)	5.0 (1–30)	5.0 (0–30)
Patient age	Mean (SD)	35.5 (5.17)	36.7 (4.80)	35.6 (5.15)
	Median (range)	36.0 (19–54)	37.0 (22–50)	36.0 (19–54)
Recipient patient age	Mean (SD)	40.7 (4.92)	41.4 (4.40)	40.8 (4.83)
	Median (range)	41.0 (21–50)	42.0 (24–50)	42.0 (21–50)
Donor age (where applicable)	Mean (SD)	30.1 (3.91)	29.5 (4.05)	29.9 (3.94)
	Median (range)	31.0 (18–45)	30.0 (19–42)	30.0 (18–45)

[a] Cross-tabulations, showing the number (and percentage) of observations in each group, are provided for the binary or categorical variables considered in the analysis versus treatment. Summary statistics, mean (and SD) and median (and range), are provided for each count variable by treatment. Continuous summaries of patient age, donor age (where applicable) and patient age by donor status are also provided for completeness. [antr]For definition so fhte categories see Materials and Methods.

AFC, antral follicle count; AMH, anti-Müllerian hormone; BMI, body mass index; ICSI, intracytoplasmic sperm injection.

Oocyte retrieval, denudation and intracytoplasmic sperm injection

Female sedation was achieved with a combination of propofol (Braun, Germany), fentanyl (Auden McKenzie, UK) and midazolam (Hamelyn, UK), and transvaginal ultrasound-guided oocyte retrieval took place about 36 h after HCG injection (10,000 IU; Pregnyl; Organon, UK; or Ovitrelle; Merck Serono) or agonist trigger (Buserelin 0.5 ml sc; Suprefact, Sanofi S.A, France), using an aspiration needle (Vitrolife, Sweden) connected to a vacuum pump (Rocket Medical, UK). Oocyte–cumulus complexes were recovered from follicular aspirates using a stereomicroscope in a class II hood with a heated stage, washed and cultured in Ferticult IVF medium (Fertipro, Belgium) at 5% CO_2 in air, 37.0 ^{0}C, maximum humidity, Oocytes allocated for ICSI were cultured for 2–4 h before cumulus cell denudation with 15–20 IU/ml cumulase (Origio, Denmark) in the same medium and complete removal of the coronae radiatae with a 140 µm pipette (EZ Squeeze; Research Instruments, UK). Oocytes at the metaphase-II stage underwent insemination by intracytoplasmic sperm injection (ICSI) within 2 h of denudation. Oocytes allocated for IVF were inseminated after preparation using SupraSperm density gradient (Origio, Denmark) and washing in Ferticult IVF medium (Fertipro, Belgium) at a concentration of 0.2 M/ml, between 3 and 6 h after oocyte recovery and cultures in standard incubators for 18 + /– 1 h before fertilization was assessed.

Embryo culture and incubation

For time-lapse imaging, after ICSI or IVF, oocytes or zygotes, respectively, were placed individually in microwells of EmbryoSlides™ (Vitrolife, Sweden)) in 25 µl Global IVF medium (LifeGlobal) supplemented with 10% dextran serum supplement (Irvine Scientific) and were overlaid with 1.4 ml mineral oil (Fertipro, Belgium) and placed in the EmbryoScope. EmbryoSlides were prepared with medium and oil that had equilibrated overnight. Once loaded with the ICSI-inseminated oocytes, or zygotes after IVF, EmbryoSlides were placed into the EmbryoScope time-lapse incubator at 37.0^{0}C in 5.5–6.0% CO_2, 5% O_2 and 89.5% N_2 for up to 6 days. The built-in microscope was used to acquire images of each fertilized oocyte every 10–20 min through seven focal planes.

For standard incubation, fertilization and embryo culture was carried out in small volume box, or flatbed incubators (Galaxy 48R, New Brunswick, UK; Miri, ESCO, Japan), using the same media, mineral oil as described for the EmbryoScope (Vitrolife (Sweden) CE marked plasticware and 25 µl culture drops). As this was a multicentre study, up to 2012, less than 25% of standard incubation was atmospheric O_2 with an increasing switch to 5% O_2, resulting in the last 18 months of the study being 100% low O_2. Analysis of the data showed no significant effect of atmospheric O_2 on the analyses (data not shown). During the early phase of this work, the culture medium was refreshed on day 3 but after CARE pilot studies, this step was no longer used. All embryos were cultured singly in culture drops, irrespective of incubation method. Selection of the embryos from standard incubation was based primarily on morphological criteria (Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology, 2011).

Evaluation of time-lapse images

Time-lapse images were collected for the duration of the culture period, to the point of embryo transfer, and were used for the assessment of fertilization after ICSI and during all in-vitro embryo development. The time of insemination by ICSI was programmed into the EmbryoScope when the slide was loaded, as the time point midway through the ICSI procedure. For IVF, the time of insemination was recorded as the time sperm was added to the oocytes. The EmbryoViewer® image analysis software (Vitrolife) was used to log and display the precise timing of developmental events as they were annotated by the embryologists studying the time-lapse images. The definitions of morphokinetic variables has been described in detail previously (Campbell et al., 2013a, 2013b). For the present study, the early cleavage model avoided the selection of embryos where irregular or rapid division occurred cc2 < 2h and cc2>/=2 h or cc3<5 h and cc3>/=5 h and where t2>/=27.1 h and t2 <27.1 h. All times were after ICSI insemination, and a 1.95 h adjustment was made for IVF (based on our in-house data, unpublished data). The duration of cc2 (t3–t2) relative to cc2+cc3 (t5–t3) {relcc2} was an effective splitting criterion if the aforementioned irregular or rapid divisions were not present. The optimum value for relCC2 was more than 0.44 and less than 0.47.

Embryos with multinucleation at the four-cell stage or smooth endoplasmic reticulum clusters were also avoided. For blastocysts, these were preferentially selected with a tSB (time to the start of blastulation in hours after insemination) ≤ 93.1. If this criterion was not met, preference was given to embryos where dB (tB–tSB) ≤ 12.5 h.

All times were recorded in hours after insemination. All annotations were made before any decision on embryo transfer, with the annotation criteria outlined above being the arbiter of which embryo to transfer.

Embryology annotation protocols and quality control

After training in annotation and competency assessment, CARE embryologists participate in regular quality assurance exercises and use a centralized annotation quality-assurance protocol whereby example embryos are annotated by each practitioner and their values compared with those of their colleagues. Intra-correlation coefficients are calculated for each morphokinetic value. Annotation quality is considered assured where the intra-correlation coefficients are greater than 0.9, demonstrating close correlation between practitioners, and competent annotation.

Embryo selection

After TLI, embryos were objectively selected using user defined time-lapse algorithms programmed into the 'Compare and Select' software (EmbryoViewer®, Vitrolife, Sweden – Class 1 Medical Device). Model scores were used to rank embryos according to their implantation or live birth potential. An evolving series of models were used during this study period as they were fine-tuned based on increasing outcome data. Models differed according to insemination method and day of transfer. IVF models were developed using an adjustment of 1.95 h for the time taken for the sperm to fertilize the oocytes. Early cleavage stage models used morphokinetic variables t2, duration of the second cell cycle (t3–t2) and a calculated variable (t2/t5–t2). Blastocyst models were similar to the published aneuploidy risk classification model using tSB and duration of blastulation to rank embryos using the calculated variables cc2 (t3-t2), cc3 (t5-t3), dB (tB [time to blastulation from insemination]-tSB). A Wallace (UK) embryo transfer catheter under ultrasound guidance was used for embryo transfer.

Statistics

Descriptive statistics were calculated to summarize the clinical data available for the cycles in each treatment arm (EmbryoScope/standard). For the binary and categorical variables, cross-tabulations of the frequencies compared with treatment were produced. For the count variables, summary statistics (including measures of central tendency and dispersion) split by treatment were calculated. Continuous summaries of patient age, donor age (where applicable) and patient age by donor status were also calculated for completeness. A mixed-effects logistic regression model (Agresti, 2013; Jiang, 2007) was fitted to the live birth event outcome, modelling the probability of the binary outcome as a function of the explanatory variables. To account for the fact that multiple treatment cycles were observed for individual patients included in the study, a patient random effect was included in the model. Generalized linear models assume that observations are independent, whereas results from the same patient will be related. To account for the fact that multiple treatment cycles were observed for individual patients included in the study, a patient random effect was included in the model. This appropriately accounts for non-independence by modelling the correlation among patients' multiple outcomes, preventing an artificial increase in the sample size. To explicitly control for differences in the patient populations between the treatment arms, potential confounding variables from the available clinical data were also considered as other explanatory variables in the model (as fixed effects). To choose which of the available variables to include as fixed effects in the model, a stepwise variable selection procedure was undertaken. Starting with an initial model that included just the patient effect, new explanatory variables were added one at a time to look for the model that has the best value of the Akaike information criterion. This stepwise selection procedure carries on adding variables until no further improvement in the Akaike information criterion (a measure of model fit that has a penalty term for the number of parameters in the model) can be achieved (**Table 2**).

A number of interactions that allow the level of one or more variables to change the effect of another variable were made available to the variable selection procedure. Any combination of variables may

Table 2 – Variables offered to the model during the stepwise selection procedure. Those marked Y were included in the final model.

Variable	Included?
Embryoscope (y/n)	Y
Patient age (<38/38+), years	Y
Day of embryo transfer	Y
Numbr of embryos transferred	Y
Patient type (standard/oocyte share donor/ donor recipient)	Y
Donor age (where applicable)	Y
Total previous cycles	Y
Total number of previous live births	Y
ICSI (y/n)	N
Total number of miscarriages	Y
Intralipid (y/n)	Y
Duration of infertility	Y
Total number of ectopics	N
BMI (underweight/healthy/overweight/obese/ extremely obese/unavailable)	N
AMH (low/normal/high/very high/unavailable)	N
AFC (extremely low/low/somewhatlow/normal/high/ very high/unavailable)	Y
Gonadotrophin type (other/Gonal-F/Menopur/unavailable)	Y
Gonadotrophin dosing days (<10/10 to 11/12 + /unavailable)	Y
Gonadotrophin total dose (<1800 iu/1800 to 2699 iu/ 2700 to 3599 iu/3600 iu +/ unavailable)	Y
Number of eggs collected	N
Numbr of eggs inseminated	N
Ratio of metaphase 2 (mature) to total eggs	N
Number of eggs fertilized	Y
Patient age/donor recipient interaction	Y
Day of embryo transfer/number of embryos transferred interaction	Y
Embryoscope/patient age interaction	Y
Embryoscope/donor recipient interaction	Y
Embryoscope/patient age/donor recipient interaction	Y
Embryoscope/donor age interaction	N
Embryoscope/day of embryo transfer interaction	N
Embryoscope/number of embryos transferred interaction	N
Embryoscope/day of embryo transfer/number of embryos transferred interaction	N

AFC, antral follicle count; AMH, anti-Müllerian hormone; BMI, body mass index; ICSI, intracytoplasmic sperm injection.

interact with each other; therefore, as the number of potential interactions is enormous, we limited the available interactions to the following: patient age with donor recipient status, to allow for the effect of the patient's age to vary with whether they are an oocyte recipient; the day of embryo transfer with the number of embryos transferred, allowing the effect of the number of embryos to vary by transfer stage; the EmbryoScope variable, i.e. treatment effect, with each patient's age, oocyte recipient indicator, donor age (where applicable), day of embryo transfer and the number of embryos transferred; three-way interactions between EmbryoScope, recipient and patient age, and between EmbryoScope, day of embryo transfer and the number of embryos transferred. These were included as the corresponding two-way interactions without EmbryoScope were included. Importantly, these interactions were included to allow for the possibility that the size and direction of the EmbryoScope effect varied for different groups of patients.

The model-estimated effect sizes (presented as odds ratios that describe the relative difference in the odds of a live birth between different treatment cycles) are accompanied by confidence intervals that quantify the uncertainty in the odds ratio estimates arising from the sample data. The confidence intervals are constructed using case bootstrapping to achieve appropriately conservative estimates for the statistical significance of the effects of interest and sensible estimates for their uncertainty, where no analytically tractable formulas are available for the standard errors (Booth, 1995; Carpenter and Bithell, 2000; Thai et al., 2013). The observed data are resampled (with replacement) to obtain a large number of pseudo datasets (n = 1000). For each pseudo-dataset the final model is refitted and the effect size of interest calculated. The result is a large sample of estimates of the effect size that mimics the variability that we would expect to observe if we were to repeat the study many times on new sets of similar patients. The confidence interval is derived from the percentiles of the sample of estimates.

Finally, likelihood ratio tests were carried out for each of the explanatory variables that were considered but not retained in the model, after the stepwise variable selection procedure. These tests evaluate the null hypothesis that including the variable in question does not improve the model fit as measured by the likelihood.

The statistical software package R version 3.3.1 (R Core Team, 2016) was used for analyses. The *lme4* package was used to implement the generalized linear mixed effects modelling (Bates et al., 2015).

Results

The summary statistics for each clinical variable considered in the analysis versus treatment (standard/embryoscope) are shown in **Table 1**; the variables selected for inclusion in the final model are presented in **Table 2**. The interactions between the EmbryoScope and each of donor age, day of embryo transfer, number of embryos transferred, and the three-way interaction between EmbryoScope, day of embryo transfer and number of embryos transferred were omitted after carrying out the likelihood ratio tests for each, which returned a *P*-value larger than 0.1 for each of these cases, indicating no evidence to reject the null hypothesis (**Table 3**); hence, no evidence was found that the EmbryoScope morphokinetic algorithm effect varies with donor age, day of embryo transfer, the number of oocytes recovered in total, as a ratio of mature (metaphase 2) to total or the number of embryos transferred. In each case, however, the corresponding main effects were included in the model, as these variables did affect the incidence of live birth events.

Table 3 – *P*-values returned by likelihood ratio tests for including the listed variables in the model.

Variable	LRT *P*-value[a]
ICSI (y/n)	0.616
Total number of ectopics	0.595
BMI (underweight/healthy/overweight/obese/extremely obese/unavailable)	0.465
AMH (low/normal/high/very high/unavailable)	0.153
Embryoscope/donor age interaction	0.476
Embryoscope/day of embryo transfer interaction	0.553
Embryoscope/number of embryos transferred interaction	0.179
Embryoscope/day of embryo transfer/number of embryos transferred interaction	0.125
Number of eggs collected	0.245
Number of eggs inseminated	0.458
Ratio of metphase II (mature) to total number of eggs	0.257

[a] $P > 0.1$ indicate that there is no evidence to reject the null hypothesis.
AMH, anti-Müllerian hormone; BMI, body mass index; ICSI, intracytoplasmic sperm injection; LRT, likelihood ratio test.

The estimated odds ratio for patients younger than 38 years using their own eggs is 1.19, with 95% CI of (1.06 to 1.34), indicating strong evidence that the use of the EmbryoScope with the morphokinetic algorithm in this group is more likely to result in a live birth than standard treatments. The mean (SD) and median (range) for patient age for standard incubation were 35.5 (5.17) and 36.0 (19–54); and for Embryoscope 36.7 (4.80) and 37.0 (22–50), respectively. The total data were 35.6 (5.15) and 36.0 (19–54), respectively.

EmbryoScope treatment in which a single blastocyst is transferred was compared with standard treatment in which two blastocysts were transferred, which needed to take account of the effect of the number of embryos transferred. For the age group younger than 38 years using their own eggs, there is some evidence that transfer of a single blastocyst after EmbryoScope is associated with fewer live births than transfer of two blastocysts after standard treatment (OR 0.854; 95% CI 0.735 to 1.000).

For non-recipients in the group aged 38 years and over, there is strong evidence that transfer of a single blastocyst after EmbryoScope is associated with fewer live births than transfer of two blastocysts after standard treatment (OR 0.603; 95% CI 0.478 to 0.748). For recipients in the group aged 38 years and over, no evidence was found of a difference between transfer of a single blastocyst after EmbryoScope and transfer of two blastocysts after standard treatment (OR 0.981, 95% CI 0.557 to 1.750). These data are important, especially in the context of multiple pregnancies in which the data demonstrated a highly elevated risk of multiple pregnancy in all groups when two embryos where transferred (**Table 4**).

Discussion

In summary, the results demonstrate an 19% increase in live birth rate with EmbryoScope and morphokinetic algorithm embryo selection for patients younger than 38 years, and a 37% increase in live birth rate for recipients aged over 37 years. Recipients aged over 37 years did equally well in live birth outcome, with only a single blastocyst

Table 4 – Multiple birth in relation to age, stage of transfer and age of female.

	Standard (♀ <38)	Standard (♀ >37)	Time-lapse (♀ <38)	Time-lapse (♀ >37)
Single embryo transfer (%)	0.8	1.5	0.7	0.0
Double embryo transfer (%)	31.1	24.2	30.7	26.7
Single blastocyst (%)	0.8	1.5	0.9	0.0
Double blastocyst (%)	38.3	34.7	40.9	35.0

from the EmbryoScope compared with two after standard treatment. The gain of increased incidence of live birth by transferring two embryos needs to be balanced against the increased risk of a multiple pregnancy by about 30– 40%. It is recognised, however, that one of the limitations of such comparative studies is the distinction of the use of algorithms generated by TLI compared with any benefits accruing from the sole use of closed incubation systems; and conversely the use of algorithms of morphokinetic development derived in open culture systems. No adequate study has yet been conducted to evaluate and distinguish these possibilities fully.

The introduction of time-lapse imaging is a recent innovation in IVF (Hlinka et al., 2012a, 2012b; Lemmen et al., 2008; Meseguer et al., 2011), especially since the development of wholly enclosed systems. The key theoretical benefits of using time lapse in human IVF treatment are twofold: providing images, not specifically related to kinetics, that permit assessment of human embryos in unparalleled detail within small (10–20 min) time frames, without disturbing the controlled culture environment (closed system) (Campbell and Fishel, 2015); and to provide detailed morphological and its related kinetic information. The value of both elements of time-lapse in clinical outcome are currently being assessed by many groups. Cruz et al. (2012), working with donor oocytes, used the EmbryoScope time lapse incubator (Vitrolife, Sweden) to compare clinical outcome between standard incubators and the closed incubation provided by the EmbryoScope, without consideration of the additional 'morphokinetic' information acquired and selection of embryos based solely on conventional morphological criteria. Embryos were randomly distributed between the incubators, and the authors reported no differences in their development, blastocyst viability or ongoing pregnancy rate. More recently, a randomized controlled trial (Park et al., 2015) confirmed no significant difference in the mean number of good-quality embryos or ongoing pregnancy rates when comparing closed and conventional incubation, although this study was limited by the short incubation time and day 2 transfer.

Such technology has captured the imagination of practitioners as both a research tool (Costa-Borges et al., 2016; Hardarson et al., 2015; Mölder et al., 2015), comparing embryos resulting from various clinical conditions (Balakier et al., 2016; Gurbuz et al., 2016; Hashimoto et al., 2016; Kaihola et al., 2016; Lindgren et al., 2016), and as a potential prognosticator in clinical practice (Adamson et al., 2016; Chen et al., 2016; Kong et al., 2016; Liu et al., 2016; Milewski et al., 2015; Mizobe et al., 2016; Rubio et al., 2014; VerMilyea et al., 2014; Wu et al., 2016a, 2016b; Yang et al., 2014), although some studies negate this (Freour et al., 2015; Wu et al., 2016a, 2016b). Bronet et al. (2015) even found distinctive morphokinetic differences between male and female embryos. A few studies have purported to relate discriminating morphokinetics of euploid and aneuploid embryos (Campbell et al., 2013a, 2013b; Minasi et al., 2016; Vera-Rodriguez et al., 2015), although this has been disputed (Rienzi et al., 2015). Much complexity arises in the analysis of embryo morphokinetics and attempting to compare such with clinical outcome. It is important to have consistent ambient conditions for embryo cleavage, but most studies ignore patient and treatment variables that have an effect and are so vital to meaningful analyses (Kirkegaard et al., 2016); some studies also only consider morphokinetics as a prognosticator for blastulation (Conaghan et al., 2013). Kirkegaard et al. (2014) showed that about 50% of embryos classified as non-usable were in fact viable; and further studies have shown such an approach to be no better than morphology assessment (Kieslinger et al., 2016). Other studies are inadequate in relation to number of cases, comparative controls, including the assessment of quality control for annotation, and end-points (Wu et al., 2016a, 2016b). Much of this has been discussed in an excellent review by Kaser and Racowsky (2014).

In clinical practice, it is incumbent on practitioners to continue to improve protocol according to new data. During this period of analysis, it was necessary to use an evolving series of selection models, by which the transferred population is continuously narrowed, in morphokinetic terms, such that the ratio between positive and negative known implantation data may reach a plateau, as well as the area under the receiver operator characteristic curve, commonly used to measure the prognosticative power of a model. When each model was derived, no assumptions were made about the morphokinetic variables to be used. Information was used to derive the models and 'holdback data', not used in model derivation, was used to test before introducing prospectively.

As far as is known, the data presented here are the largest to date investigating the use of TLI algorithms for embryo selection and its effect on the incidence of live birth, with comprehensive morphokinetic variables assessed during embryo cleavage and in relation to other patient and treatment-related factors. It is highly probable that the increasing incidence of aneuploid embryos with age (Lee et al., 2015) has a negating effect of discriminatory morphokinetic factors in woman aged over 37 years.

As the ploidy status of human embryos is critical to successful outcome, early clinical studies were undertaken with TLI to assess any correlation between embryo aneuploidy and morphokinetic parameters. The studies by Campbell et al., (2013a, 2013b) and Campbell (2014) indicated the possibility of ranking embryos in relation to ploidy, but such hierarchical data are only risk-based. Only by evaluating chromosome copy number directly using invasive biopsy procedures would the most accurate knowledge of embryo ploidy be provided. Such studies comparing embryo ploidy and morphokinetics, however, are still fraught with complexity, not least the type of aneuploidy be it one compatible with full-term delivery such as trisomy 21; to those not compatible with implantation, such as many monosomies; and complex versus simple aneuploidy, mosaicism and segmental aneuploidies may well have additional effects on morphokinetics. Additionally, simply having closed systems throughout cleavage *in vitro* as distinct from any morphokinetic differential may have a beneficial effect.

Kong et al. (2016) using time-lapse imaging reported a relationship between early cell division behaviour and developmental potential with elongation or shortening of the cell cycle affecting cell number, respectively. This study concluded that, by excluding such embryos,

the incidence of implantation and live birth after day 3 transfer of embryos increased when the cell number of the embryo was maximal.

In assessing the limitations of this study, the EmbryoScope is a closed system for embryo culture and in itself may procure benefit; this was not assessed. Given that patient factors and some treatments *per se* affect embryo morphokinetics, not all possibilities have been fully explored. In the pragmatic world of patient treatment, we were unable to use morphokinetic data as a blinded randomized control study. Patients were not randomly allocated to EmbryoScope or standard incubation and selection. It is not possible to account for any differences not contained within the available data, and therefore elimination of all confounding effects in a non-randomized study can never be guaranteed. Larger studies using donor oocyte recipients are needed to further evaluate the findings here. The evaluation is based on live birth per embryo transfer to focus on the value of morphokinetic data. Embryo implantation and successful progression to live birth also requires a receptive endometrium and functional placenta.

In summary, many factors feed into embryo morphokinetics that need to be controlled for, and data need to be presented on live birth outcome with sufficiently powered studies that take into account patient and treatment factors. Ideally, prospective randomized controlled studies are needed, but pragmatic difficulties exist in undertaking such studies, not least to obtain sufficient patient recruitment, and for prospective annotation data to be ignored. The present study used morphokinetic data for selection of embryos for transfer; a 19% increase in live birth was observed for women younger than 38 years using their own oocytes. The advantage of the EmbryoScope morphokinetic data was evident too for recipients aged over 37 years, demonstrating that the transfer of a single blastocyst had the same chance of a live birth as two transferred after standard treatment without the risk of multiple pregnancy. Finally, it must always be recognized that such analyses cannot account for all factors affecting successful pregnancy outcome, particularly in the IVF population cohort; normal placental function (Fishel et al., 2016) and endometrial receptivity (Miravet-Valenciano et al., 2015; Nejat et al., 2014) are also essential.

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