Predictors of twin live birth following cryopreserved double embryo transfer on day 3

Daniel J. Kaser · Stacey A. Missmer · Katharine F. Correia ·
S. Temel Ceyhan · Mark D. Hornstein · Catherine Racowsky

Received: 13 May 2013 / Accepted: 20 June 2013 / Published online: 4 July 2013
© Springer Science+Business Media New York 2013

Abstract

Purpose To determine embryo and cycle-specific parameters associated with twin live birth in day 3 cryopreserved double embryo transfer (DET) cycles, and to propose a new prediction model for external validation.

Methods All cycles with autologous or donor cryopreserved DET of day 3 embryos from 2002 to 2011 at a single academic institution with a singleton or twin live birth were included ($n=207$). Patient characteristics, post-thaw embryo morphology and freeze-thaw parameters were compared between patients with a single and twin live birth.

Results Demographic characteristics were similar, except that patients delivering twins were younger at age of cryopreserved embryo transfer (CET), fewer were parous and more were anovulatory. Duration of embryo storage, time in culture post-thaw, endometrial thickness and use of assisted

hatching were comparable. Six predictors of twin delivery were identified: patient age <35 year at CET, intact survival of the lead embryo, resumption of mitosis, 7–8 viable cells in the non-lead embryo, transfer of a lead embryo with ≥ 7 cells and a sum of ≥ 14 viable cells in the two transferred embryos. Regression modeling predicted a step-wise increase in the probability of twins with addition of each predictor; with all six present, the risk of twins was predicted to be 53 % and with none present, the risk decreased to 6 %.

Conclusions The six identified variables associated with twin live birth following day 3 cryopreserved DET have been applied to derive a prediction model for estimating the risk of twin delivery. External validation of the model is required prior to clinical application.

Keywords Cryopreserved · Double embryo transfer · Frozen · Prediction model · Pregnancy · Twins

Capsule A prediction model has been derived to estimate an individualized risk of twin live birth following transfer of two thawed cleavage stage embryos.

D. J. Kaser · S. A. Missmer · K. F. Correia · M. D. Hornstein ·
C. Racowsky (✉)

Department of Obstetrics, Gynecology and Reproductive Biology,
Brigham and Women's Hospital, Division of Reproductive
Medicine, Harvard Medical School, 75 Francis Street, Boston,
MA 02115, USA
e-mail: cracowsky@partners.org

S. A. Missmer
Channing Division of Network Medicine, Department of Medicine,
Brigham and Women's Hospital, Harvard Medical School, Boston,
MA 02115, USA

S. A. Missmer
Department of Epidemiology, Harvard School of Public Health,
Boston, MA 02115, USA

S. T. Ceyhan
Gülhane Military Medical School, Department of Obstetrics and
Gynecology, Center for Assisted Reproduction, Etlik, 06018
Ankara, Turkey

Introduction

Since the introduction of assisted reproduction, the twin live birth rate has increased by 76 % from 18.9 per 1,000 live births in 1980 to 33.2 per 1,000 live births in 2009 [20]. Of the 7,424 live births reported in the Society for Assisted Reproductive Technology (SART) database from cryopreserved non-donor embryos in 2009, 22.3 % were twins, compared to an approximate 1 % incidence of spontaneously conceived twins in the United States; the mean number of embryos transferred in these cycles varied with recipient age and ranged from 2.0 to 2.3 [5].

The adverse maternal and neonatal outcomes associated with twins are well-documented and include gestational hypertension, pre-eclampsia and eclampsia, cesarean section, postpartum hemorrhage and maternal death, along with pre-term delivery, low and very low birth weight, congenital malformations, perinatal and neonatal death, and long-term neurodevelopmental disability [6, 18, 33, 37]. Compared to

singletons, twins are ten more times likely to weigh less than 1500 g at birth and eight times more likely to die within the first month of life [13].

With increased focus on elective single embryo transfer (eSET), defining criteria for appropriate application of eSET in cryopreserved embryo transfers (CETs) as well as fresh embryo transfers is critical to optimize cumulative pregnancy rates and reduce the overall risk of multiple gestations. Several studies have shown a relationship between implantation rates and various parameters associated with thawed cleavage stage embryos including: survival of an intact embryo [4], resumption of mitosis in post-thaw culture [10], fresh embryo morphology [29], percent blastomere survival after thaw [7], the use of laser assisted hatching after thaw [2], lead cell number at transfer [9] and endometrial thickness [8]. Factors such as duration of embryo storage, post-thaw embryo morphology, length of time in post-thaw culture and age and body mass index (BMI) of the patient at embryo transfer have not been thoroughly evaluated. The purpose of this study was to derive a prediction model based on these parameters to estimate an individualized risk of twins following double embryo transfer (DET) of day 3 cryopreserved embryos.

Material and methods

This study was approved by the Partners' Healthcare Institutional Review Board for record review.

Experimental design

All cryopreserved autologous and donor cycles performed at Brigham and Women's Hospital from January 1, 2002 to September 30, 2011 were reviewed ($n=2,502$). Cycles with slow freeze and thaw of day 3 embryos with subsequent transfer of two day 3 embryos were included, provided that the outcome was a singleton or dizygotic twin live birth. Only one cycle per patient was included. Demographic, cryopreservation, embryo and cycle parameters were compared between patients with a single live birth and those with a twin live birth. Exclusion criteria were pronuclear, day 2 or day 5 cryopreservation, day 5 transfer, blastomere or polar body biopsy, cycles involving embryos frozen from multiple stimulations, no long luteal GnRH pre-treatment for pituitary suppression, and no luteal phase support (i.e. natural cycles). Cycles with missing data were likewise excluded. In addition, cycles with luteal support using preparations other than intramuscular progesterone were excluded, as live birth rates in CET cycles supplemented with micronized vaginal progesterone (8 % Crinone) have been found to be significantly lower than those supplemented with intramuscular progesterone [12]. Application of these inclusion and exclusion criteria left 207 cycles for subsequent analysis.

Clinical and laboratory protocols

Clinical protocols for controlled ovarian hyperstimulation (COH), insemination and endometrial preparation were the same as previously described [25]. All thaw cycles were programmed with luteal GnRH. Estradiol acetate (Estrace, Warner Chilcott, Rockaway, NJ, USA) was used to achieve a target endometrial thickness of ≥ 7 mm based on a previous study showing a significant reduction in implantation when the endometrial thickness was < 7 mm [8]. Cycles were supplemented with intramuscular progesterone 25–50 mg daily starting 3 days prior to transfer through 10 weeks' gestational age or negative hCG.

Supernumerary day 3 embryos were cryopreserved if they had at least 6 cells, < 10 % fragmentation and the blastomeres did not exceed moderate asymmetry in size and shape as evaluated using the scoring system of Racowsky et al. [24]. The one-step slow freezing and thaw protocols of Liebo were used [16]. Upon thawing, embryos were serially washed in HTF+HEPES supplemented with 10 % human serum albumin and incubated for 10 min at room temperature prior to being placed in growth media (G1.5, Vitrolife, Göteborg, Sweden or Global, LifeGlobal, Guilford, CT USA) for a minimum of 2 h before embryo transfer. Day 3 embryos were scored for cell number, fragmentation and asymmetry immediately after thaw and again just before transfer. Transfer was performed with or without ultrasound-guidance according to physician preference, using a Wallace catheter (Marlow/Cooper Surgical, Shelton, CT, USA). Two embryos were transferred in all cycles included in this study dataset.

Outcome variables

Patient demographics, such as age at embryo freeze and age at transfer, gravidity, parity, BMI, day 3 FSH, infertility diagnosis, number of prior failed cycles and outcome from the corresponding fresh cycle were recorded. Cycle-level parameters, such as endometrial thickness on the day orders were set to thaw the embryos (mapping), use of assisted hatching with Acid Tyrode's, number of embryos thawed and total number of cells transferred were compared between patients with a single live birth and those with a twin live birth. Likewise, embryo-specific characteristics such as resumption of mitosis during post-thaw culture (defined as division of at least one blastomere), number of blastomeres pre- and post-thaw, degree of post-thaw fragmentation and blastomere asymmetry were compared between the two patient groups. An embryo was termed 'intact' if the number of cells after thaw was equal to the number of cells prior to freeze. A 'good quality' embryo was defined as having ≥ 7 cells on day 3 without regard to fragmentation or asymmetry, while a 'superior quality' embryo was defined as having ≥ 7

cells, a fragmentation score of <10 % and with blastomeres with either no asymmetry or moderate asymmetry (as compared with severe asymmetry). The ‘lead embryo’ was defined as having the best composite morphology score per Racowsky et al. [24], and the ‘non-lead’ embryo was the other one transferred. Live birth was defined as delivery of a viable infant after 24 weeks gestational age.

Model building and statistical analyses

Statistical Analysis Software (SAS®) version 9.2 (SAS Institute, Inc., Cary, NC, USA) was used for all analyses. Relative risks (RR) and 95 % confidence intervals (CI) were calculated using log binomial regression, after adjusting a priori for age at freeze, age at transfer and cell number (<7 cells, 7–8 cells, >8 cells) of each of the two transferred embryos. When CI's excluded 1.0 or P-values were <0.05, variables were regarded as statistically significant and included in the final model. If multiple predictors of the same factor (e.g. cell number and whether the embryo was a slow, appropriate or fast cleaver) were significant in the crude analysis, the number of parameters and the strength of the associations were considered when choosing which predictor to include in the final model in an effort to limit collinearity. To calculate the risk of twins from the log probability, the estimates were exponentiated according to the final model (refer to the section ‘[Prediction of twins in DET cycles](#)’ under Results for the regression equation). Given the small sample size, all cycles were used as a training set for derivation of the final model.

Results

Patient demographics and cycle parameters

Patients who delivered a singleton were similar to those who delivered twins with respect to the following characteristics: age at freeze, day 3 FSH, BMI, number of prior failed fresh and frozen cycles, duration of embryo storage, use of assisted hatching at CET and endometrial thickness at mapping (Table 1). Women delivering twins were younger at the time of CET (mean±standard deviation (SD): 33.1±4.9 vs. 36.0±6.2 year), slightly fewer were parous (40.8 % vs. 46.8 %) and more had anovulatory infertility (22.4 % vs. 9.6 %). In contrast, those delivering a singleton were more than twice as likely to have diminished ovarian reserve (26.1 % vs. 10.2 %). The incidence of prior failed cycles, use of assisted hatching in the CET cycle and the thickness of the endometrium on the day of mapping were similar between the two patient groups. Among the 207 CETs, 61 were from oocyte donation cycles (twins: 18.4 % vs. singleton: 32.9 %).

Day 3 embryo survival, morphology and post-thaw culture

The number of embryos thawed was similar between the singleton and twin groups (3.7±1.9 vs. 3.1±1.4). The combined number of cells at freezing for all embryos thawed was slightly greater in the singleton group (29.6±15.1 vs. 25.8±11.9), although the combined number of viable cells after thawing was equivalent (17.0±6.7 vs. 17.8±6.1), as was the combined number of viable cells transferred (14.0±4.2 vs. 14.9±3.2) (Table 1). The post-thaw mean fragmentation and

Table 1 Patient demographic and cycle parameters for 207 cryopreserved double embryo transfers by live birth outcome^a

	Singleton (n=158)	Twins (n=49)
Age at embryo freeze	30.3±4.5	30.5±3.8
Age at embryo transfer	36.0±6.2	33.1±4.9
Time in freezer (y)	1.3±1.3	1.0±1.0
Donor oocyte	52 (32.9)	9 (18.4)
Gravid	108 (68.4)	34 (69.4)
Parous	74 (46.8)	20 (40.8)
Day 3 FSH from corresponding fresh cycle	6.3±2.0	6.3±2.3
BMI of embryo recipient ^b (kg/m ²)	24.0±4.9	24.6±3.9
Infertility diagnosis		
Unexplained	26 (16.6)	9 (18.4)
Male factor	50 (31.8)	14 (28.6)
Tubal factor	8 (5.1)	6 (12.2)
Anovulation	15 (9.6)	11 (22.4)
Endometriosis	9 (5.7)	1 (2.0)
Diminished ovarian reserve	41 (26.1)	5 (10.2)
Uterine factor	2 (1.3)	0 (0)
Gestational carrier	6 (3.8)	2 (4.1)
Other	0 (0)	1 (2.0)
Number of prior failed cycles (fresh and CET)		
0	26 (16.5)	9 (18.4)
≥1	132 (83.5)	40 (81.6)
AH ^c	28 (17.7)	7 (14.3)
Endometrial thickness at mapping (mm) ^d	9.8±2.7	10.2±2.9
No. embryos thawed	3.7±1.9	3.1±1.4
No. cells pre-thaw	29.6±15.1	25.8±11.9
No. viable cells post-thaw	17.0±6.7	17.8±6.1
No. viable cells transferred ^e	14.0±4.2	14.9±3.2
No. ≥7-cell embryos transferred		
0	38 (24.1)	3 (6.1)
1	59 (37.3)	22 (44.9)
2	61 (38.6)	24 (49.0)

^a Values represent mean ± standard deviation or n (%)

^b BMI = body mass index

^c AH = assisted hatching

^d Mapping indicates the day on which orders were set for thaw

^e Combined number of viable cells in the two transferred embryos

asymmetry scores were likewise similar (fragmentation: 0.90 ± 0.49 vs. 0.83 ± 0.50 ; asymmetry: 1.72 ± 0.42 vs. 1.62 ± 0.43). Duration of culture of thawed embryos before the CET was not associated with number of liveborn: the incidence of twins in the quartile of embryos with the longest duration of culture (>5 h) was 24.5 % vs. 23.7 % in the remaining quartiles (adjusted RR 1.02, CI=0.59–1.78).

Women who delivered twins were more likely to have had two ≥ 7 -cell embryos transferred (49.0 % vs. 38.6 %) and fewer had no ≥ 7 -cell embryos transferred (6.1 % vs. 24.1 %) (Table 1). Furthermore, the proportion of embryos having 7–8 cells was greater for the twin group compared with the singletons (lead embryos: 67.3 % vs. 52.5 %; non-lead embryos: 38.8 % vs. 20.3 %).

Association of embryo and clinical variables and the risk of twin live birth

The associations between the risk of twin live birth and post-thaw morphology of the lead and non-lead embryos are shown in Table 2. The remaining patient, embryo and cycle-specific variables are summarized in the forest plot in Fig. 1. When adjusted for patient age at embryo freeze and age at transfer, four variables significantly increased the risk of twins: 1) resumption of mitosis in either of the transferred embryos during post-thaw culture; 2) intact survival of the lead embryo (i.e. the same number of cells pre- and post-thaw); 3) the lead embryo having ≥ 7 cells, regardless of fragmentation or

asymmetry scores; and 4) the lead embryo having ≥ 7 cells, <10 % fragmentation, and without severe asymmetry (i.e. having the pre-defined criteria for a superior embryo). Inclusion of these fragmentation and asymmetry scores when the lead embryo had ≥ 7 cells, interestingly, did not further increase the risk of a twin live birth; in fact, the association was not as strong as that for cell number alone: 27.3 % (adjusted RR 2.37, CI=1.01–5.59) vs. 27.9 % (adjusted RR 3.65, CI=1.20–11.10). When the non-lead embryo survived intact or met the criteria of a superior embryo, the risk of twins was also increased, but the CI's crossed 1.0 (adjusted RR 1.54, CI=0.96–2.46 and adjusted RR 1.42, CI=0.84–2.40, respectively).

Three variables were associated with a decreased risk of twins: 1) age ≥ 35 years at the time of CET; 2) number of cells in the non-lead embryo; and 3) the sum of the viable cells in the two transferred embryos. When age at freeze and age at transfer were considered together, the incidence of twins was 32.4 % when both were <35 year (referent), 14.9 % for age at freeze <35 year and age at transfer ≥ 35 year (RR 0.46, CI=0.24–0.87), and 12.5 % for age at freeze ≥ 35 year regardless of age at transfer (RR 0.39, CI=0.15–1.00; p-trend=0.006). In the crude analysis, the risk of twins was greatest when the non-lead embryo had 7–8 cells (37.3 % twins) compared to when the non-lead embryo had either <7 cells (20.5 % twins; RR 0.55, CI=0.33–0.91) or >8 cells (14.7 % twins, RR 0.39, CI=0.16–0.96). The effect of cell number in the non-lead embryo was attenuated and not

Fig. 1 Forest plot of associations between clinical variables, post-thaw embryo morphology and the risk of twin live birth in cryopreserved double embryo transfers. ^a Adjusted for patient age at embryo freeze and transfer; relative risks (RR) are from log binomial models. ^b Obesity = body mass index ≥ 30 kg/m². ^c Represents >3 rd quartile for duration of embryo storage. ^d Represents >3 rd quartile for duration of post-thaw culture. ^e Either lead or non-lead embryo resumed mitosis in post-thaw culture (defined as division of at least one blastomere). ^f Assisted hatching

with Acid Tyrode's. ^g Pregnancy outcome in corresponding fresh cycle was live birth. ^h Intact signifies that the number of cells after thaw was equal to the number of cells prior to cryopreservation (i.e., 100 % thaw survival). ⁱ Referent group is non-lead embryo with <7 cells. ^j Referent group is non-lead embryo with 7 or 8 cells. ^k Superior morphology indicates that an embryo has ≥ 7 cells, a fragmentation score of <10 % and with blastomeres not exhibiting severe asymmetry. ^l Sum of cells in the lead and non-lead embryo; referent group is 14–18 cells transferred

significant after adjusting for age at freeze, age at transfer and cell number in the lead embryo. Finally, when there were less than 14 viable cells in the two transferred embryos combined, the risk of twins was significantly lower than when embryos with 14–18 cells were transferred (17.1 % vs. 31.6 %, respectively; adjusted RR 0.57, CI=0.33–0.98).

Notable variables that did not significantly affect the twin rate were duration of embryo storage (range 0.1–6.1 year; median 0.7 year) and time in post-thaw culture (range 0.3–8.5 h; median 3.5 h), use of assisted hatching, endometrial thickness ≥ 7 mm on day of mapping, pregnancy outcome in corresponding fresh cycle and BMI of the woman having the CET (Figure 1).

Prediction of twins in DET cycles

The final model contained the following variables which, from the crude analysis, were all found to be significantly associated with the risk of twins: age at transfer, intact survival of the lead embryo after thawing, resumption of mitosis, cell number of the non-lead embryo, transfer of a superior quality lead embryo and number of viable cells transferred for the two embryos combined. When these parameters were included in the final model, none remained a significant predictor of twin live birth. Nonetheless, when considered together in various clinical scenarios, the expected probability of twins varied from 6 % to 53 % (Tables 3). The individualized risk of twins following cryopreserved DET may be estimated according to the following exponential equation:

$$\text{Probability of twin birth} = e^{[A]},$$

where $A = -2.2497 - 0.6840(\text{age at freeze} \geq 35) - 0.5306(\text{age at freeze} < 35, \text{ age at transfer} \geq 35) + 0.4940(\text{lead embryo has superior quality}) + 0.1163(\text{non-lead embryo has 7–8 cells}) - 0.5477(\text{non-lead embryo is a fast cleaver}) + 0.2972(\text{resumption of mitosis in either embryo}) + 0.2195(\text{total number of viable cells transferred is 14–18}) + 0.1410(\text{total number of viable cells transferred is } < 14) + 0.4863(\text{lead embryo is intact})$. For a given variable in the model, the value is set to equal 1 where the statement is true and 0 where false.

Discussion

To our knowledge, this is the first study designed specifically to identify variables in a day 3 freeze-thaw cycle that are predictive of a twin live birth following transfer of two day 3 embryos. Our results show that the risk of twins following day 3 cryopreserved DET varied according to specific patient characteristics and post-thaw embryo morphology, viability and cell number. Parameters that were predictive of twins unique to our analysis were age at CET, post-thaw embryo morphology and total number of viable cells transferred. The strongest predictor of twins was transfer of a lead embryo having ≥ 7 cells without regard to fragmentation or symmetry (a 3.65-fold increase in relative risk). Factors such as live birth in the corresponding fresh cycle, duration of cryopreservation, length of time in post-thaw culture, use of assisted hatching, endometrial thickness at mapping and recipient BMI did not significantly affect the risk of twins.

While we identified a total of six distinct variables that significantly affected the risk of twins, all but one was related

Table 2 Associations between the risk of twin live birth and morphology of the lead and non-lead embryos post-thaw

	Lead embryo ^a		Non-lead embryo ^b	
	Twin birth n (%)	Adjusted RR (95 % CI)	Twin birth n (%)	Adjusted RR (95 % CI)
Cell Number				
<7 cells	3 (7.1)	0.29 (0.09–0.92)	25 (20.5)	0.77 (0.46–1.28)
7–8 cells	33 (28.5)	1.00 (Referent)	19 (37.3)	1.00 (Referent)
>8 cells	13 (26.5)	1.14 (0.66–1.98)	5 (14.7)	0.44 (0.18–1.08)
Fragmentation score ^c				
Not fragmented (0)	14 (26.4)	1.00 (Referent)	12 (28.6)	1.00 (Referent)
Fragmented (1–4)	35 (22.9)	0.95 (0.57–1.60)	36 (22.6)	0.77 (0.42–1.22)
Symmetry score ^d				
Perfect (1)	25 (32.9)	1.00 (Referent)	15 (23.1)	1.00 (Referent)
Not perfect (2 or 3)	24 (18.5)	0.62 (0.39–0.99)	33 (24.3)	1.13 (0.68–1.88)

^a Lead embryo is defined as the best embryo transferred based on cell number, fragmentation score and symmetry score; relative risks are from log binomial models adjusted for age at freeze, age at transfer and developmental stage of non-lead embryo (<7 cells, 7–8 cells, >8 cells)

^b Non-lead embryo is defined as the second best embryo transferred, based on standard morphologic scoring; relative risks are from log binomial models adjusted for age at freeze, age at transfer and developmental stage of lead embryo (<7 cells, 7–8 cells, >8 cells)

^c Fragmentation scores of 0–4 were assigned to each embryo (0 %, 1–9 %, 10–25 %, 26–50 % or >50 %, respectively)

^d Symmetry scores of 1–3 were assigned to each embryo (perfect symmetry, moderate asymmetry and severe asymmetry, respect

Table 3 Examples of expected probabilities of twin live birth following cryopreserved double embryo transfer according to final adjusted model

Example patient	Patient age (yr)	Lead embryo is superior quality ^a	No. of viable cells in non-lead embryo	Mitosis resumed ^b	Total no. viable cells transferred ^c	Lead embryo is 100 % intact ^d	Probability of twin birth
1	Age at freeze <35, age at transfer <35	Yes	7–8 cells	Yes	14–18	Yes	53 %
2	Age at freeze <35, age at transfer <35	Yes	< 7 cells	No	14–18	Yes	35 %
3	Age at freeze <35, age at transfer ≥35	Yes	< 7 cells	No	14–18	Yes	21 %
4	Age at freeze ≥35	Yes	< 7 cells	No	14–18	Yes	18 %
5	Age at freeze ≥35	No	< 7 cells	No	<14	No	6 %

^a Superior quality refers to an embryo with ≥7 cells, < 10 % fragmentation and without severe asymmetry

^b Either embryo resuming mitosis in post-thaw culture (defined as division of at least 1 blastomere)

^c The sum of viable cells in the two transferred embryos

^d An intact embryo has the same number of blastomeres pre- and post-thaw

to characteristics of the transferred embryos. The one clinical variable identified as a predictor was, surprisingly, the patient age at CET rather than the patient age at embryo freeze. Currently, no consensus exists regarding the effect of uterine senescence on pregnancy rates [22]. Several studies have evaluated whether or not patient age at transfer affects pregnancy rates, and the results are conflicting: Legro et al. [15] and Paulson et al. [23] reported that advanced uterine age is not associated with decreased implantation potential, while Rosenwaks et al. [28], Borini et al. [3], Moomjy et al. [22] and Toner et al. [35] demonstrated that embryo recipients over a particular age threshold have decreased implantation rates, independent of donor age and embryo morphology. Specifically, Moomjy et al. [22] reported decreased implantation rates in women ≥ 35 years (29 % vs. 37 %, $P=0.002$), Borini et al. [3] specified a higher age threshold (age ≥ 40 years: 24.5 % vs. 47.3 %, $P<0.05$) and Toner et al. [35] found the effect was limited to women ≥ 50 years (15.7 % vs. 22.4 %, $P=0.008$). Interestingly, in the largest study, Toner et al. analyzed over 17,000 donor cycles in the SART-database and found that this effect of recipient age was noted in fresh embryo transfer cycles only, and not in CET cycles. Furthermore, twin live birth was more common in younger recipients (44.2 % of patients age 25–29 years delivered twins, compared to 35.9 % of patients age 35–39 years; $P=0.01$) [35]. The reason for this age-related effect of the uterus remains unclear, but may be attributed to decreased uterine blood flow, impaired sensitivity to progesterone and altered endometrial receptivity with advancing age [21, 34].

Embryo cryopreservation has the potential to reduce multiple gestation rates through an eSET paradigm in which embryos from the same stimulation are transferred, one at a time, in subsequent cycles. Repeat SET was shown to be equivalent to or outperform repeat DET in the towardSET Collaboration predictive model in terms of cumulative live birth rate and the risk of twins, provided that loss of viability

due to freeze-thaw was moderate or less [26]. Dizygotic twinning was also eliminated in the repeat SET model, whereas the repeat DET model predicted a 20.9 % twin rate with a lower overall live birth rate (39.0 % vs. 41.4 %; [26]). Moreover, there is accumulating evidence that both pregnancy rates [1, 27, 30–32] and obstetric and neonatal outcomes [11, 19] may be improved in cryopreserved cycles, specifically due to uncoupling the processes of COH and embryo transfer. Thus, as routine cryopreservation may emerge as a method to optimize the safety and efficacy of assisted reproduction, the development of prediction models specific to eSET in cryopreserved cycles will become increasingly important, as recently published for fresh embryo transfers [14, 36].

There are several limitations to the current study. Given the small sample size resulting from our strict inclusion criteria, there were insufficient cycles to allow us to split the data into training and validation sets. Accordingly, we cannot comment on the model's discrimination or calibration functions. As adequate performance with both internal and external validation is a requisite for use in clinical practice, our model for predicting twin pregnancy requires validation prior to implementation [17]. Also, because of the limited size of our dataset, it was not possible to model the distinction between those women who had DET due to clinical history and overall embryo quality versus those who had DET because only two embryos were suitable for transfer. Without this distinction, the data are more heterogeneous, as the a priori risk of twins may be different between these groups. Finally, this study applies to a specific subset of patients only; namely, those with slow-freeze, thaw and transfer of cleavage stage embryos. While this may not represent contemporary practice in all clinics, supernumerary day three embryos are likely stored in most cryopreservation programs, and as such, it still is relevant to define optimal transfer criteria. Other strengths of this study include the use of log binomial regression analyses, which allowed

the relative contribution of each covariate to be determined, and the comprehensive evaluation of both embryo and cycle-specific parameters. Another important strength that enhances the internal validity of our dataset is the consistency of our laboratory and clinical protocols during the ten-year study period (e.g. the embryo cryopreservation and thaw protocols, and the protocols for morphological grading, transfer techniques and luteal phase support).

In conclusion, our model indicates that the individualized risk of twin live birth following cryopreserved DET varies considerably (from 6 % to 53 %) according to post-thaw embryo morphology, viability and developmental stage, resumption of mitosis in post-thaw culture, total number of cells transferred and patient age at CET. External validation of this model is now required in order to prove that the individualized risk of twin birth can be estimated from our derived exponential equation. If validity is proven, our prediction model will be useful for physicians when counseling patients regarding the risk of twins following transfer of two thawed day 3 embryos.

References

1. Afatoonian A, Oskouian H, Ahmadi S, Oskouian L. Can fresh embryo transfers be replaced by cryopreserved-thawed embryo transfers in assisted reproductive cycles? A randomized controlled trial. *J Assist Reprod Genet.* 2010;27:357–63.
2. Balaban B, Urman B, Yakin K, Isiklar A. Laser-assisted hatching increases pregnancy and implantation rates in cryopreserved embryos that were allowed to cleave in vitro after thawing: a prospective randomized study. *Hum Reprod.* 2006;21:2136–40.
3. Borini A, Bianchi L, Violini F, Maccolini A, Cattoli M, Flamigni C. Oocyte donation program: pregnancy and implantation rates in women of different ages sharing oocytes from single donor. *Fertil Steril.* 1996;65:94–7.
4. Burns WN, Gaudet TW, Martin MB, Leal YR, Schoen H, Eddy CA, et al. Survival of cryopreservation and thawing with all blastomeres intact identifies multicell embryos with superior frozen embryo transfer outcome. *Fertil Steril.* 1999;72:527–32.
5. Centers for Disease Control and Prevention, American Society for Reproductive Medicine, Society for Assisted Reproductive Technology. 2009 Assisted Reproductive Technology Success Rates: National Summary and Fertility Clinic Reports. Atlanta: U.S. Department of Health and Human Services; 2011.
6. Conde-Agudelo A, Belizán JM, Lindmark G. Maternal morbidity and mortality associated with multiple gestations. *Obstet Gynecol.* 2000;95:899–904.
7. El-Toukhy T, Khalaf Y, Al-Darazi K, Andritsos V, Taylor A, Braude P. Effect of blastomere loss on the outcome of frozen embryo replacement cycles. *Fertil Steril.* 2003;79:1106–11.
8. El-Toukhy T, Coomarasamy A, Khairy M, Sunkara K, Seed P, Khalaf Y, et al. The relationship between endometrial thickness and outcome of medicated frozen embryo replacement cycles. *Fertil Steril.* 2008;89:832–9.
9. Gabrielsen A, Fedder J, Agerholm I. Parameters predicting the implantation rate of thawed IVF/ICSI embryos: a retrospective study. *Reprod Biomed Online.* 2006;12:70–6.
10. Guerif F, Bidault R, Cadoret V, Couet ML, Lansac J, Royere D. Parameters guiding selection of best embryos for transfer after cryopreservation: a reappraisal. *Hum Reprod.* 2002;17:1321–6.
11. Kalra SK, Ratcliffe SJ, Coutifaris C, Molinaro T, Barnhart KT. Ovarian stimulation and low birth weight in newborns conceived through in vitro fertilization. *Obstet Gynecol.* 2011;118:863–71.
12. Kaser DJ, Ginsburg ES, Missmer SA, Correia K, Racowsky C. Intramuscular progesterone versus 8% Crinone vaginal gel for luteal phase support for day 3 cryopreserved embryo transfer. *Fertil Steril.* 2012;98:1464–9.
13. Kochanek KD, Kirmeyer SE, Martin JA, Strobino DM, Guyer B. Annual summary of vital statistics: 2009. *Pediatrics.* 2012;129:338–48.
14. Lannon BM, Choi B, Hacker MR, Dodge LE, Malizia BA, Barrett B, et al. Predicting personalized multiple birth risks after in vitro fertilization-double embryo transfer. *Fertil Steril.* 2012;98:69–76.
15. Legro RS, Wong IL, Paulson RJ, Lobo RA, Sauer MV. Recipient's age does not adversely affect pregnancy outcome after oocyte donation. *Am J Obstet Gynecol.* 1995;172:96–100.
16. Leibo SP. A one step method for direct nonsurgical transfer of frozenthawed bovine embryos. *Theriogenology.* 1984;21:767–90.
17. Leuhuis E, van der Steeg J, Steures P, Bossuyt PMM, Eijkemans MJC, van der Veen F, et al. Prediction models in reproductive medicine: a critical appraisal. *Hum Reprod Update.* 2009;15:537–52.
18. Luke B, Keith LG. The contribution of singletons, twins and triplets to low birth weight, infant mortality and handicap in the United States. *J Reprod Med.* 1992;37:661–6.
19. Maheshwari A, Pandey S, Shetty A, Hamilton M, Bhattacharya S. Obstetric and perinatal outcomes in singleton pregnancies resulting from the transfer of frozen thawed versus fresh embryos generated through in vitro fertilization treatment: a systematic review and meta-analysis. *Fertil Steril.* 2012;98:368–77.
20. Martin JA, Hamilton BE, Ventura SJ, Osterman MJ, Kirmeyer S, Mathews TJ, et al. Births: final data for 2009. *Natl Vital Stat Rep.* 2011;60:1–104.
21. Meldrum DR. Female reproductive aging—ovarian and uterine factors. *Fertil Steril.* 1993;59:1–5.
22. Moomjy M, Cholt I, Mangieri R, Rosenwaks Z. Oocyte donation: insights into implantation. *Fertil Steril.* 1999;71:15–21.
23. Paulson RJ, Hatch IE, Lobo RA, Sauer MV. Cumulative conception and live birth rates after oocyte donation: implications regarding endometrial receptivity. *Hum Reprod.* 1997;12:835–9.
24. Racowsky C, Combelles CMH, Nureddin A, Pan Y, Finn A, Miles L, et al. Day 3 and day 5 morphological predictors of embryo viability. *Reprod Biomed Online.* 2003;6:323–31.
25. Reichman DE, Jackson KV, Racowsky C. Incidence and development of zygotes exhibiting abnormal pronuclear disposition after identification of two pronuclei at the fertilization check. *Fertil Steril.* 2010;94:965–70.
26. Roberts SA, McGowan L, Mark Hirst W, Vail A, Rutherford A, Lieberman BA, et al. Reducing the incidence of twins from IVF treatments: predictive modeling from a retrospective cohort. *Hum Reprod.* 2011;26:569–75.
27. Roque M, Lattes K, Serra S, Solà I, Geber S, Carreras R, et al. Fresh embryo transfer versus frozen embryo transfer in in vitro fertilization cycles: a systematic review and meta-analysis. *Fertil Steril.* 2013;99:156–62.
28. Rozenwaks Z, Davis OK, Damario MA. The role of maternal age in assisted reproduction. *Hum Reprod.* 1995;10:165–73.
29. Salumets A, Tuuri T, Makinen S, Vilska S, Husu L, Tainio R, et al. Effect of developmental stage of embryo at freezing on pregnancy outcome of frozen-thawed embryo transfer. *Hum Reprod.* 2003;18:1890–5.
30. Shapiro BS, Daneshmand ST, Garner FC, Aguirre M, Hudson C, Thomas S. Evidence of impaired endometrial receptivity after

- ovarian stimulation for in vitro fertilization: a prospective randomized trial comparing fresh and frozen-thawed embryo transfer in normal responders. *Fertil Steril*. 2011;96:344–8.
31. Shapiro BS, Daneshmand ST, Garner FC, Aguirre M, Hudson C, Thomas S. Evidence of impaired endometrial receptivity after ovarian stimulation for in vitro fertilization: a prospective randomized trial comparing fresh and frozen-thawed embryo transfers in high responders. *Fertil Steril*. 2011;96:516–8.
 32. Shapiro BS, Daneshmand ST, De Leon L, Garner FC, Aguirre M, Hudson C. Frozen-thawed embryo transfer is associated with a significantly reduced incidence of ectopic pregnancy. *Fertil Steril*. 2012;98:1490–4.
 33. Sibai BM, Hauth J, Caritis S, Lindheimer MD, MacPherson C, Klebanoff M, et al. Hypertensive disorders in twin versus singleton gestations. National Institute of Child Health and Human Development network of maternal-Fetal medicine units. *Am J Obstet Gynecol*. 2000;182:938–42.
 34. Steer CV, Tan SL, Mason BA, Campbell S. Midluteal-phase vaginal color Doppler assessment of uterine artery impedance in a subfertile population. *Fertil Steril*. 1994;61:53–8.
 35. Toner JP, Grainger DA, Frazier LM. Clinical outcomes among recipients of donated eggs: an analysis of the U.S. national experience, 1996–1998. *Fertil Steril*. 2002;78:1038–45.
 36. Williams Z, Banks E, Bkassiny M, Jayaweera SK, Elias R, Veeck L, et al. Reducing multiples: a mathematical formula that accurately predicts rates of singletons, twins, and higher-order multiples in women undergoing in vitro fertilization. *Fertil Steril*. 2012;98:1474–80.
 37. Young BC, Wylie BJ. Effects of twin gestation on maternal morbidity. *Semin Perinatol*. 2012;36:162–8.