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ARTICLE

Association between blastocyst morphology and outcome of single-blastocyst transfer

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Abstract The aim of this study was to assess the ability of three individual blastocyst morphology parameters — expansion and hatching (EH) stage, inner cell mass (ICM) grade and trophectoderm grade — to predict outcome of a cycle with single-blastocyst transfer. The study was a secondary analysis of data prospectively collected in a large multicentre trial. A total of 618 intracytoplasmic sperm injection patients undergoing ovarian stimulation in a gonadotrophin-releasing hormone antagonist cycle with compulsory single-blastocyst transfer on day 5 were included. In the simple logistic regression analysis, all three blastocyst morphology parameters were statistically significantly ($P < 0.005$ for each) associated with positive human chorionic gonadotrophin, clinical and ongoing pregnancy rates and live birth rates, while only the ICM grade was significantly ($P = 0.033$) associated with early pregnancy loss rate. Blastocyst EH stage was the only significant predictor of live birth ($P = 0.002$) in the multiple logistic regression. In conclusion, although all three blastocyst morphology parameters were related to treatment outcome of fresh single-blastocyst cycles, selection of high-quality blastocysts for transfer should consider first the EH stage. Transfer of a blastocyst with ICM grade A may reduce the risk of early pregnancy loss.

KEYWORDS: blastocyst morphology, early pregnancy loss, live birth, prediction, single-embryo transfer

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Introduction

Weighing the benefits and risks of assisted reproduction technology, the birth of a single healthy child is the ultimate objective in patients undergoing ovarian stimulation for IVF/intracytoplasmic sperm injection (ICSI) (Land and Evers, 2003). Single-embryo transfer would appear to be the most effective approach to ensure this objective. The advantages of reducing multiple pregnancies in terms of obstetric risk and child health outcome are obvious, but the impact on the overall efficacy of single- versus multiple-embryo transfer has been debated (Bergh, 2005; Gelbaya et al., 2010; Martikainen et al., 2001; McLernon et al., 2010; Pandian et al., 2009; Thurin et al., 2004). Systematic reviews have concluded that while single-embryo transfer is associated with lower live birth rate than double-embryo transfer in fresh cycles, the cumulative live birth rates from fresh and frozen single-embryo transfer cycles are similar to those in patients undergoing fresh double-embryo transfer (Gelbaya et al., 2010; McLernon et al., 2010; Pandian et al., 2009).

Embryo quality is considered a major predictor of implantation and pregnancy (Ahlström et al., 2011; della Ragione et al., 2007; Richter et al., 2001; Terriou et al., 2001; Thurin et al., 2005; Van Royen et al., 1999; Ziebe et al., 1997). Selecting the embryo(s) with the best implantation potential is essential for securing each couple the highest chance of achieving pregnancy after assisted reproduction. The ability to make the optimal choice has become even more important with the growing implementation of single-embryo transfers. In the early 1990s, knowledge of metabolic requirements of the developing embryo increased and sequential embryo culture media were introduced (Gardner and Lane, 1999). This rapidly increased the proportion of embryos developing to the blastocyst stage. Extended embryo culture to the blastocyst stage has allowed for assessment of embryo morphology beyond genomic activation and thus contributed to improved embryo selection. There is increasing evidence that an embryo's ability to reach the blastocyst stage *in vitro* improves prediction of clinical pregnancy (Blake et al., 2007; Rehman et al., 2007) and that transfer of blastocysts results in higher live birth rates than those achieved with the same number of cleavage-stage embryos (Papanikolaou et al., 2006, 2008).

A number of classification and grading systems are available for assessing embryo quality at the cleavage stage (Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology, 2011; Cummins et al., 1986; Fisch et al., 2001; Giorgetti et al., 1995; Holte et al., 2007; Prados et al., 2012; Puissant et al., 1987; Terriou et al., 2001; Van Royen et al., 1999), but only a few grading systems have been proposed for evaluating quality at the blastocyst stage. In 1999, Gardner and Schoolcraft introduced a blastocyst grading system in which selection of high-quality blastocysts is based on morphology parameters of the expansion and hatching (EH) stage, the inner cell mass (ICM) grade and the trophectoderm (TE) grade (Gardner and Schoolcraft, 1999). Composite scores of the different morphology parameters (Balaban et al., 2006; della Ragione et al., 2007; Gardner et al., 2000; Goto et al., 2011) have been linked to pregnancy and/or pregnancy loss. Other quantitative measures of blastocyst morphology and their relation to implantation and pregnancy have been evaluated (Richter et al., 2001; Shapiro et al., 2008), but the blastocyst grading system by Gardner and Schoolcraft (1999) remains largely unchallenged. There is, however, a need for increased knowledge of the relative impact of each morphology parameter at the blastocyst stage, as well as their correlations, in predicting the probability of successful implantation and pregnancy. Such data would be most reliable when based on single-blastocyst transfers; however, the majority of the available data on the association between blastocyst quality and outcome parameters have been derived from studies using multiple-blastocyst transfers and, so far, only a few studies reporting data from fresh single-blastocyst transfers exist (Ahlström et al., 2011; della Ragione et al., 2007; Hill et al., 2013; Kresowik et al., 2012).

The main aim of this study was to investigate the relationship between individual morphology parameters and pregnancy, pregnancy loss and live birth, using data obtained from a large multicentre trial with compulsory single-blastocyst transfer (Devroey et al., 2012). A secondary aim was to develop a multiple logistic regression model for prediction of the probability of live birth after single-blastocyst transfer based on blastocyst morphology scoring.

Materials and methods

This study is a secondary analysis of data prospectively collected from all patients ($n = 618$) who had compulsory single-blastocyst transfer on day 5 after oocyte retrieval while participating in a multicentre randomized controlled trial comparing ongoing pregnancy rates after ovarian stimulation with highly purified human menopausal gonadotrophin (HP-HMG; Menopur; Ferring Pharmaceuticals) or recombinant FSH (follitropin β; Puregon; MSD) in ICSI patients following a gonadotrophin-releasing hormone (GnRH) antagonist cycle. The trial was carried out in accordance with the declaration of Helsinki, International Conference on Harmonization Guidelines for Good Clinical Practice and local regulatory requirements. The trial was registered at ClinicalTrials.gov (number NCT00884221) and the protocol (FE 999906 CS08) was approved by the local regulatory authorities and the independent ethics committees covering all participating centres. Written informed consent was provided by all patients before any trial-related examinations were initiated. The trial design, population, methods, conduct and results have been reported previously (Devroey et al., 2012).

Trial population

The main inclusion criteria were women with primary diagnosis of infertility being unexplained infertility or partners with mild male factor, age 21–34 years, body mass index 18–25 kg/m^2, FSH 1–12 IU/l, antral follicle count ≥10 and regular menstrual cycles of 24–35 days. Women with polycystic ovaries, endometriosis stage I–IV or poor response in a previous stimulation cycle were excluded.

Treatment regimen

The starting gonadotrophin dose was fixed at 150 IU daily for the first 5 days and adjusted according to ovarian response from day 6 when the GnRH antagonist (ganirelix acetate; Orgalutran; MSD) was initiated at a daily dose of 0.25 mg and continued throughout the gonadotrophin-treatment period. A single injection of 250 μg HCG (Ovitrelle; Merck Serono) was administered as soon as three follicles of ≥17 mm were observed. Oocyte retrieval took place 36 ± 2 h after the HCG administration.

Blastocyst assessments

All oocytes retrieved were fertilized by ICSI. Fertilization was assessed 19 ± 1 h post insemination and embryos with two pronuclei were cultured individually (in separate droplets) and assessed daily by the local embryologists. Only commercially available culture media were used from retrieval to transfer. On day 5 (120 ± 2 h) post insemination, the blastocyst quality was assessed based on morphological criteria and a single blastocyst of the best quality, according to the local embryologist, was transferred. Remaining blastocysts were cryopreserved individually by vitrification.

The morphological evaluation of blastocysts was performed according to the Gardner and Schoolcraft grading system (Gardner and Schoolcraft, 1999) and included three different parameters: EH stage, ICM grade and TE grade. The EH stage was assessed as one of the following: (1) an early blastocyst, blastocoele being less than half volume of that of the embryo; (2) a blastocyst with a blastocoele whose volume is half of, or greater than half of that of the embryo; (3) a full blastocyst with a blastocoele completely filling the embryo; (4) an expanded blastocyst with a blastocoele volume larger than that of the full blastocyst, with a thinning zona; (5) a hatching blastocyst with the TE starting to herniate through the zona; and (6) a hatched blastocyst, in which the blastocyst has completely escaped from the zona. For blastocysts with EH stage ≥3, ICM grade and TE grade were evaluated. The ICM was assessed as one of the following: (A) tightly packed, many cells; (B) loosely grouped, several cells; and (C) very few cells. The TE was assessed as one of the following: (A) many cells forming a cohesive epithelium; (B) few cells forming a loose epithelium; and (C) very few, large cells. To harmonize intra- and interclinic scoring of the morphology parameters, a common prestudy training session was held with the responsible embryologist(s) from each of the participating centres. All embryologists had also to pass an online scoring test provided by Fertaid (www.fertaid.com) to be able to participate in the trial. In addition, an atlas with representative pictures of all morphology parameters was prepared as a visual aid and distributed to all embryologists before the start of the trial to further ensure standardized assessments. Examples of blastocyst grading included in the atlas are shown in **Figure 1**.

Clinical outcome

A serum βHCG test was performed 13–15 days after blastocyst transfer. Clinical and ongoing pregnancy was confirmed by transvaginal ultrasound 5–6 and 10–11 weeks, respectively, after transfer. Early pregnancy loss was defined as a pregnancy loss occurring between the positive βHCG test and ongoing pregnancy, and late pregnancy loss as a pregnancy loss occurring after confirmed ongoing pregnancy. All patients with an established ongoing pregnancy were followed until delivery.

Statistical analysis

Continuous data were presented as median values with interquartile range (IQR) with differences between groups tested using Wilcoxon's test. Categorical data were presented as frequencies and percentages accompanied with *P*-values based on the likelihood ratio chi-squared test. All reported *P*-values were two-sided. A P-value < 0.05 was considered significant. No adjustment for multiplicity was applied. The interdependency between the blastocyst grade parameters was evaluated using pairwise chi-squared tests. Assessment of the relationship between clinical outcome (positive βHCG, early pregnancy loss, clinical and ongoing pregnancy, and live birth) and the individual grading parameters and potential confounding parameters were based on logistic regression. The following strategy was applied: each parameter was analysed using simple logistic regression to identify potential candidates for the multiple logistic regression modelling. Parameters identified as significant in the simple logistic regression were entered in a multiple logistic regression model of live birth rate. Stepwise backward elimination was then performed: i.e. the least significant parameter was removed (eliminated) and the model was then refitted. This process was repeated until all remaining variables were significant. Based on the final model, the predicted probabilities of live birth accompanied with 95% Wald confidence limits were reported.

Results

A total of 618 women (HP-HMG $n = 304$; recombinant FSH $n = 314$) underwent a compulsory single-blastocyst transfer on day 5 after oocyte retrieval. For this investigation, data from all patients were integrated as no differences were observed between HP-HMG and recombinant FSH groups on baseline characteristics, end-of-stimulation ovarian response, number of blastocysts available on day 5, distribution of EH stage, ICM and TE for transferred blastocysts and live birth rates. **Table 1** shows the composite grading of the transferred blastocysts. The three morphology parameters were pairwise positively associated ($P < 0.001$), with EH stage 5 more frequently associated with ICM and TE grades AA, stage 4 with grades AA or BB and stage 3 with grades BB or CC. No blastocysts with EH stage 6 were observed for any of the patients.

Treatment outcome according to blastocyst quality

There were no differences between the patients who achieved a live birth ($n = 200$, 32%) and those who did not ($n = 418$, 68%) concerning patient characteristics, except for a significantly lower serum progesterone concentration ($P = 0.047$) and a higher serum LH concentration ($P = 0.028$) at the end of stimulation in patients with live birth (**Table 2**). The patients who achieved a live birth had

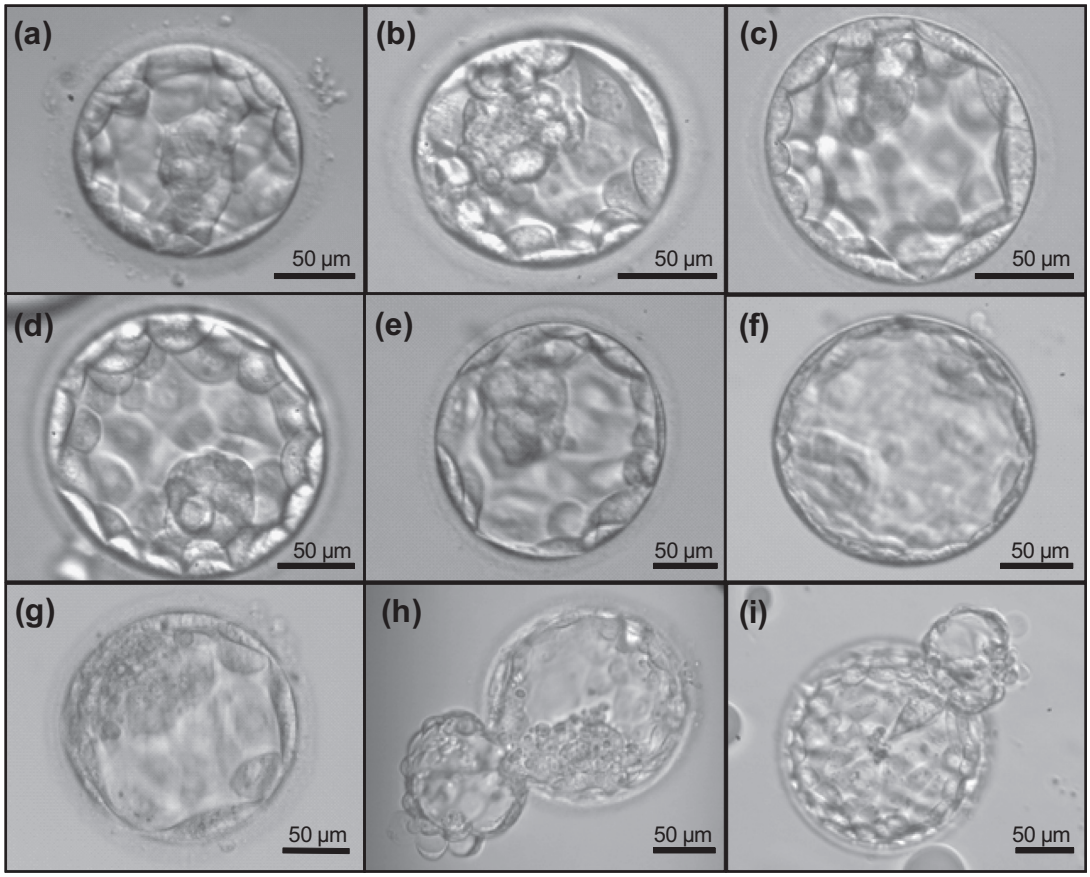

Figure 1 Examples of blastocyst grading: (a) 3AA blastocyst; (b) 3AB blastocyst; (c) 3BA blastocyst; (d) 4AA blastocyst; (e) 4AB blastocyst; (f) 4BA blastocyst; (g) 4CC blastocyst; (h) 5AA blastocyst; (i) 5CA blastocyst. For details of the EH stages and ICM and TE grades, see Materials and methods. Bars = 50 µm.

Table 1 Distribution of composite morphology parameters of transferred blastocysts on day 5 post insemination.

EH stage	n	*ICM and TE grades*								
		AA	*AB*	*AC*	*BA*	*BB*	*BC*	*CA*	*CB*	*CC*
6	0	–	–	–	–	–	–	–	–	–
5	152	80 (53)	28 (18)	0 (0)	15 (10)	22 (14)	5 (3)	0 (0)	0 (0)	2 (1)
4	255	101 (40)	38 (15)	1 (<1)	20 (8)	63 (25)	7 (3)	3 (1)	12 (5)	10 (4)
3	106	9 (8)	10 (9)	3 (3)	6 (6)	37 (35)	9 (8)	1 (1)	7 (7)	24 (23)
2[a]	52	–	–	–	–	–	–	–	–	–
1[a]	53	–	–	–	–	–	–	–	–	–

Values are *n* (%).
EH = expansion and hatching; ICM = inner cell mass; TE = trophectoderm.
[a]ICM and TE grades were not evaluated.

significantly more blastocysts available on day 5: median (IQR) 3 (2—5) versus 2 (1—4); $P < 0.001$. The relative distribution of individual scores for each morphology parameter for the transferred blastocysts was significantly different between the patients with a live birth and those with no live birth ($P < 0.001$ for each parameter). For patients with a live birth, a larger proportion of the transferred blastocysts were of EH stages 4 or higher (87% versus 56%), ICM grade A (62% versus 47%) and TE grade A (55% versus 40%) compared with the patients with no live birth (**Table 2**).

In the simple logistic regression analysis, increasing blastocyst EH stage was positively associated with positive βHCG, clinical pregnancy, ongoing pregnancy and live birth ($P < 0.001$ for each). Female age, body mass index, primary cause of infertility, type of gonadotrophin preparation and serum concentrations of FSH, LH, oestradiol and

Table 2 Patient and morphology characteristics of transferred blastocysts by live birth status.

Characteristic	*No live birth* (n = *418*)	*Live birth* (n = *200*)	P-*value*[b]
Baseline			
Female age (years)	31 (29–33)	31 (29–33)	NS
Body mass index (kg/m^2)	21.8 (20.3–23.6)	22.0 (20.4–23.5)	NS
Primary cause of infertility			NS
Unexplained infertility	38	40	
Mild male factor	62	60	
No. of previous ovarian stimulation cycles	0 (0–1)	0 (0–0)	NS
Day 1 (before start of stimulation)			
Antral follicle count	15 (12–18)	15 (12–19)	NS
Anti-Müllerian hormone (pmol/l)	23 (13–38)	26 (14–38)	NS
LH (IU/l)	5.8 (4.6–7.4)	5.9 (4.7–7.6)	NS
FSH (IU/l)	6.9 (6.1–8.2)	7.0 (6.2–8.1)	NS
Endometrial thickness (mm)	3 (2–5)	3 (3–5)	NS
End of stimulation			
Oestradiol (nmol/l)	6.6 (4.6–9.5)	6.2 (4.6–9.4)	NS
Progesterone (nmol/l)	2.6 (1.9–3.5)	2.4 (1.8–3.4)	0.047
LH (IU/l)	1.8 (1.0–2.8)	2.0 (1.2–3.2)	0.028
Endometrial thickness (mm)	11 (10–12)	11 (9–12)	NS
Total gonadotrophin dose (IU)	1350 (1200–1500)	1350 (1200–1500)	NS
No. of oocytes retrieved	9 (6–13)	9 (6–14)	NS
Blastocysts available on day 5	2 (1–4)	3 (2–5)	<0.001
EH stage			<0.001
5	82 (20)	70 (35)	
4	152 (36)	103 (52)	
3	88 (21)	18 (9)	
2	46 (11)	6 (3)	
1	50 (12)	3 (2)	
ICM grade[a]			<0.001
A	151 (47)	119 (62)	
B	124 (39)	60 (31)	
C	47 (15)	12 (6)	
TE grade[a]			<0.001
A	130 (40)	105 (55)	
B	143 (44)	74 (39)	
C	49 (15)	12 (6)	

Values are median (interquartile range), % or *n* (%).
EH = expansion and hatching; ICM = inner cell mass; TE = trophectoderm.
[a]For blastocysts of EH stage 3-5.
[b]Wilcoxon's test (continuous data) or the likelihood ratio chi-squared test (categorical data).

progesterone at end of stimulation were not significantly associated with any parameter of treatment outcome. **Table 3** displays the observed positive βHCG, clinical pregnancy, ongoing pregnancy and live birth rates as well as the early pregnancy loss rates according to the individual grades of the three blastocyst morphology parameters. The live birth rate increased from 6% for transfer of EH stage 1 blastocysts to 46% for EH stage 5 blastocysts. For blastocysts with EH stages 3–5, the ICM grade had a significant impact on the likelihood of achieving a positive βHCG ($P = 0.004$), clinical pregnancy ($P = 0.002$), ongoing pregnancy ($P < 0.001$) and live birth ($P < 0.001$). Likewise, the TE grade showed significant predictive value for positive βHCG ($P = 0.002$), clinical pregnancy ($P = 0.003$), ongoing pregnancy ($P = 0.002$) and live birth ($P < 0.001$). The live birth rate increased from 20% for ICM grade C to 44% with

Table 3 Clinical outcome by morphology characteristics of transferred blastocysts on day 5 post insemination.

Blastocyst grade component	*Blastocysts transferred*	*Positive βHCG*		*Clinical pregnancy*		*Ongoing pregnancy*		*Live birth*		*Early pregnancy loss*	
	n *(%)*	n *(%)*	P-value	n *(%)*	P-value	n *(%)*	P-value	n *(%)*	P-value	n *(%)*	P-value
EH stage											
All data (*n* = 618)			<0.001		<0.001		<0.001		<0.001		NS
5	152 (25)	93 (61)		76 (50)		72 (47)		70 (46)		21 (23)	
4	255 (41)	139 (55)		115 (45)		106 (42)		103 (40)		33 (24)	
3	106 (17)	27 (25)		21 (20)		19 (18)		18 (17)		8 (30)	
2	52 (8)	13 (25)		6 (12)		6 (12)		6 (12)		7 (54)	
1	53 (9)	5 (9)		3 (6)		3 (6)		3 (6)		2 (40)	
ICM grade											
All data (*n* = 513)			0.004		0.002		<0.001		<0.001		0.033
A	270 (53)	150 (56)		130 (48)		123 (46)		119 (44)		27 (18)	
B	184 (36)	90 (49)		66 (36)		61 (33)		60 (33)		29 (32)	
C	59 (12)	19 (32)		16 (27)		13 (22)		12 (20)		6 (32)	
Blastocysts with EH stage 4 or 5 (n = 407)			NS		NS		NS		NS		NS
A	248 (61)	144 (58)		124 (50)		117 (47)		115 (46)		27 (19)	
B	132 (32)	73 (55)		54 (41)		49 (37)		48 (36)		24 (33)	
C	27 (7)	15 (56)		13 (48)		12 (44)		11 (41)		3 (20)	
TE grade											
All data (*n* = 513)			0.002		0.003		0.002		<0.001		NS
A	235 (46)	135 (57)		112 (48)		107 (46)		105 (45)		28 (21)	
B	217 (42)	104 (48)		85 (39)		76 (35)		74 (34)		28 (27)	
C	61 (12)	20 (33)		15 (25)		14 (23)		12 (20)		6 (30)	
Blastocysts with EH stage 4 or 5 (n = 407)			NS		NS		NS		NS		NS
A	219 (54)	131 (60)		110 (50)		104 (47)		103 (47)		27 (21)	
B	163 (40)	88 (54)		73 (45)		65 (40)		64 (39)		23 (26)	
C	25 (6)	13 (52)		9 (36)		9 (36)		7 (28)		4 (31)	

Simple logistic regression.
EH = expansion and hatching; ICM = inner cell mass; TE = trophectoderm.

grade A, and from 20% for TE grade C to 45% with grade A. When restricting the evaluation to blastocysts with EH stages 4 and 5, there were no significant associations between the outcome parameters and ICM grade or TE grade. Of the three quality parameters, only the ICM grade was found to be significantly ($P = 0.033$) associated with early pregnancy loss. This association did not remain significant when restricting the analysis to blastocysts with EH stages 4 and 5. In total, there were only six pregnancy losses after confirmed ongoing pregnancy in the study and no evaluation of the association between blastocyst morphology and late pregnancy loss was performed.

Prediction of live birth rate

Only the blastocyst quality parameters were included in the multiple logistic regression analysis of live birth rate, as no confounding parameters were significantly associated with live birth rate in the simple logistic regression analyses. Stages 4 and 5 of blastocyst EH were combined in the prediction model because there was no significant difference with respect to live birth rate between these two stages. After stepwise backward elimination, the minimal model simply included blastocyst EH stage, as the predictive power ($P = 0.002$) of this parameter overruled those of the other two parameters. However, because of the strong significance of ICM and TE grade observed in the simple logistic regression, these were reintroduced in the final model.

Table 4 provides the estimated probabilities of achieving a live birth depending on the composite quality score of the blastocyst. As examples for the model, a patient with transfer of a blastocyst with a score of 4—5AA, 4—5BB or 3CC is predicted to have a probability of achieving a live birth of 47%, 38% or 12%, respectively.

Table 4 Predicted live birth rate by composite morphology classification of transferred blastocysts.

EH stage	*ICM grade*	*TE grade*	*Live birth rate*
4 or 5	A	A	47 (41—54)
	A	B	43 (33—53)
	A	C	37 (20—58)
	B	A	42 (32—53)
	B	B	38 (30—47)
	B	C	32 (17—51)
	C	A	36 (19—56)
	C	B	32 (17—51)
	C	C	27 (14—45)
3	A	A	24 (15—38)
	A	B	21 (12—34)
	A	C	17 (8—35)
	B	A	21 (12—34)
	B	B	18 (11—28)
	B	C	15 (7—29)
	C	A	17 (7—34)
	C	B	14 (6—29)
	C	C	12 (5—23)
2	—	—	12 (5—23)
1	—	—	6 (2—16)

Values are % (95% CI). Predicted live birth rate based on the multiple logistic regression model.
EH = expansion and hatching; ICM = inner cell mass; TE = trophectoderm.

Discussion

The present study in a single-blastocyst setting showed that high scores of blastocyst EH stage, ICM grade and TE grade were all significantly associated with increased pregnancy and live birth rates after fresh transfers. The relevance of blastocyst EH stage has been previously documented, as higher implantation rates were obtained with transfer of expanded blastocysts compared with non-expanded blastocysts (della Ragione et al., 2007; Kresowik et al., 2012; Racowsky et al., 2003; Wilson et al., 2004) and with hatching blastocysts compared with non-hatching blastocysts (Balaban et al., 2000; Yoon et al., 2001). Previous studies have also reported that an ICM tightly packed with many cells contributes to vital implantation or live birth rate (Kovacic et al., 2004; Richter et al., 2001). In the present study, ICM was positively associated with βHCG, clinical and ongoing pregnancy and live birth rates; interestingly, the percentage of early pregnancy loss after transfer of a blastocyst with ICM grade A was about half of that after transfer of a blastocyst with ICM grade B or C suggesting that a large ICM increases the probability of maintaining the pregnancy beyond the initial positive βHCG finding, reflecting a higher viability of the initial implantation.

When including all three morphology parameters in a logistic regression model of live birth rate, only the EH stage remained as a significant independent predictor. However, because of the strong significance of ICM and TE grade observed in the simple logistic regression, a composite classification consisting of all three morphology parameters were used in the model for prediction of live birth. Thus, transfer of a blastocyst with a high stage (4 or 5) of EH and high grades of ICM and TE (i.e. AA) was estimated to result in approximately twice as high chance of obtaining a live birth compared with transfer of low grades of ICM and TE (i.e. CC). It should be noted that the actual predicted probabilities of obtaining a live birth could not be discriminated in the present data set in which the 95% confidence intervals overlapped.

The finding in the present study that the EH stage is the most important parameter when selecting a blastocyst for transfer is in contrast with some recent retrospective cohort studies suggesting the TE grade to have the strongest predictive power for treatment outcome in fresh transfers (Ahlström et al., 2011; Hill et al., 2013) and frozen—thawed transfers (Honnma et al., 2012). Although these studies also used the grading system of Gardner and Schoolcraft (1999), differences in the relative distribution of blastocysts with different morphology grades between the studies may contribute to explain the apparently discrepant relative importance of blastocyst morphology parameters: the vast majority of blastocysts included were of good or excellent quality, with very few (5% or less) blastocysts with C grades for ICM or TE; however, the data set in present study was derived from a clinical trial in which single-embryo transfer was mandatory irrespective of the blastocyst quality and therefore included significant numbers of blastocysts with EH stages 1—5 as well as ICM and TE grades of A, B and C. Furthermore, all data in the present study were derived from only one fresh cycle per patient. The results were also less influenced by confounding parameters, as the study cohort consisted of good-prognosis patients with a narrow age range and who were prospectively managed in a harmonized and standardized manner, i.e. all patients underwent a similar ovarian stimulation protocol and all oocytes were fertilized by ICSI and cultured to a similar time point on day 5 (120 ± 2 h). On the other hand, an inherent problem with the blastocyst grading system introduced by Gardner and Schoolcraft (1999) is the loose definitions used of the ICM and TE morphology. Despite written definitions and a visual aid atlas as well as common training sessions, it cannot be ruled out that the blastocyst scoring was still to some extent a subjective assessment.

In the present study, ICM and TE A and B grades were more frequently associated with higher blastocyst EH stages. Also, the qualities of ICM and TE were highly associated, i.e. AA, BB or CC grades occurred more frequently than other possible combinations. These observations may indicate interdependency between developmental stage, ICM and TE of the blastocyst, increasing the complexity when interpreting the actual impact of the individual factors. A better understanding of the correlation and potential interlink between these three morphology parameters may lead to a more consensus-oriented position in the selection of the best blastocyst for transfer. As discussed by Ahlström et al. (2011), the extent of blastocyst expansion is related to the number and cohesiveness of the TE cells, which would prevent leakage of the blastocoele liquid and sodium ions. Thus, a good TE grade may reflect that the blastocyst is efficiently pumping ions into the cavity and inducing

osmotic accumulation of water in the cells resulting in higher blastocyst expansion (Ahlström et al., 2011). In other words, a fully expanded blastocyst by definition requires a functional TE, and this functionality may be more dependent on the molecular quality of TE cells than on their quantity and cohesiveness.

Finally, other quantitative aspects of blastocyst expansion, ICM and TE not currently evaluated may play a role in blastocyst implantation. In this respect, other measures of ICM such as size, shape and fragmentation, or measures of cell number, blastocyst diameter and blastulation timing, have been reported to be associated with implantation potential and viability (Richter et al., 2001; Shapiro et al., 2008). Further research is needed to evaluate whether additional blastocyst parameters, or embryo development parameters at earlier time points, could contribute to provide a more precise prediction model of live birth based on single-blastocyst transfer in fresh cycles.

In conclusion, the EH stage should be considered first among the three morphology parameters when selecting a blastocyst for transfer, as this parameter has the highest predictive value of live birth. At any blastocyst EH stage, additional consideration should be given to both ICM and TE grade. Transfer of a blastocyst with ICM grade A may reduce the risk of an early pregnancy loss.

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