Correlation between standard blastocyst morphology, euploidy and implantation: an observational study in two centers involving 956 screened blastocysts

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**STUDY QUESTION:** Does conventional blastocyst morphological evaluation correlate with euploidy (as assessed by comprehensive chromosome screening (CCS) of trophectoderm (TE) biopsies) and implantation potential?

**SUMMARY ANSWER:** A moderate relation between blastocyst morphology and CCS data was observed but the ability to implant seems to be mainly determined by the chromosomal complement of preimplantation embryos rather than developmental and morphological parameters conventionally used for blastocyst evaluation.

**WHAT IS KNOWN ALREADY:** Combined with improving methods for cryopreservation and blastocyst culture, TE biopsy and CCS is considered to be a promising approach to select euploid embryos for transfer. Understanding the role of morphology in blastocyst stage preimplantation genetic screening (PGS) cycles may help in further optimizing the cycle management and clinical outcomes.

**STUDY DESIGN, SIZE, DURATION:** This is a multicenter retrospective observational study performed between January 2009 and August 2013. The study includes the data analysis of 956 blastocysts with conclusive CCS results obtained from 213 patients following 223 PGS cycles. Single frozen embryo transfer (FET) cycles of 215 euploid blastocysts were performed where it was possible to track the implantation outcome of each embryo transferred.

**PARTICIPANTS/MATERIALS, SETTING, METHODS:** PGS was offered to infertile patients of advanced maternal age (>35 years) and/or with a history of unsuccessful IVF treatments (more than two failed IVF cycles) and/or previous spontaneous abortion (more than two spontaneous miscarriages). Prior to TE biopsy for CCS, blastocyst morphology was assessed and categorized in four groups (excellent, good, average and poor quality). The developmental rate of each embryo reaching the expanded blastocyst stage was defined according to the day of biopsy post-fertilization. Day 5 and Day 6 biopsied blastocysts were defined as faster and slower growing embryos, respectively. A novel blastocyst biopsy method, not requiring the opening of the zona pellucida at the cleavage stage of embryo development, was used. Linear regression models were used to test the relationship between blastocyst morphology and developmental rate CCS data and FET cycle outcomes of euploid blastocysts.

**MAIN RESULTS AND THE ROLE OF CHANCE:** Among the embryological variables assessed (morphology and developmental rate), only blastocyst morphology was predictive of the CCS data. The euploidy rate was 56.4, 39.1, 42.8 and 25.5% in the excellent, good, average and poor blastocyst morphology groups, respectively. A diagnosis of complex aneuploidy was also associated with blastocyst morphology (P < 0.01) with 6.8, 15.2, 17.4 and 27.5% of excellent, good, average and poor quality embryos, respectively, showing multiple chromosome errors. Faster and slower growing embryos showed a similar aneuploidy rate. Regression logistic analysis showed that none of the parameters...
used for conventional blastocyst evaluation (morphology and developmental rate) was predictive of the implantation potential of euploid embryos. The implantation potential of euploid embryos was the same, despite different morphologies and developmental rates.

LIMITATIONS, REASONS FOR CAUTION: The study is limited by its retrospective nature. A higher sample size or a prospective randomized design could be used in future studies to corroborate the current findings.

WIDER IMPLICATIONS OF THE FINDINGS: This study provides knowledge for a better laboratory and clinical management of blastocyst stage PGS cycles suggesting that the commonly used parameters of blastocyst evaluation are not good enough indicators to improve the selection among euploid embryos. Accordingly, all poor morphology and slower growing expanded blastocysts should be biopsied and similarly considered for FET cycles. This knowledge will be of critical importance to achieve similar cumulative live birth rates in PGS programs compared with conventional IVF, avoiding the potential for exclusion of low quality but viable embryos from the biopsy and transfer procedures. Future research to identify non-invasive biomarkers of reproductive potential may further enhance selection among euploid blastocysts.

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Key words: preimplantation genetic screening / blastocyst biopsy / embryo evaluation / aneuploidies / blastocyst morphology

Introduction

Among the couples seeking assisted reproduction treatment (ART), the female partners are usually of advanced reproductive age and thus at high risk of decreasing implantation and increasing abortion rates owing to the high likelihood of producing chromosomally abnormal embryos (Munné et al., 1993). The weak correlation between conventional methods of embryo evaluation and chromosomal complement has led to the introduction of preimplantation genetic screening (PGS) in IVF cycles as a complementary tool aiming at selecting euploid embryos and improving delivery rate per transfer (Munné et al., 1993).

Safety of the biopsy stage and accuracy of the chromosome screening method adopted to perform PGS are critical parameters in the design of a PGS strategy (Northrop et al., 2010; Treff et al., 2010a; Fragouli and Wells 2012; Scott et al., 2013). New comprehensive chromosome screening (CCS) platforms represent a breakthrough in PGS allowing 24-chromosome screening to be performed with high accuracy from single cells (Voullaire et al., 2000; Wells and Delhanty, 2000; Harper and Harton 2010; Treff et al., 2010b; Gutiérrez-Mateo et al., 2011). Furthermore, the parallel advances in blastocyst culture and the introduction of vitrification in the routine management of IVF cycles allow trophectoderm (TE) biopsy to be performed with high efficiency and minimal risks (Schoolcraft et al., 2010; Scott et al., 2012). Biological and clinical evidence of the high efficiency of blastocyst stage PGS are being reported determining a growing clinical application of this strategy worldwide (Fragouli et al., 2008; Schoolcraft et al., 2010; Forman et al., 2012; Yang et al., 2012; Capalbo et al., 2013a, b).

However, as a consequence of the recent introduction of large scale of TE biopsy in PGS programs, there is a lack of knowledge about the relationship between conventional parameters used for blastocyst evaluation (morphology and blastocyst developmental rate), CCS data and the viability of euploid embryos following replacement.

During IVF cycles, the embryonic cohort is asynchronous in development and TE biopsy can equally be performed on Day 5, Day 6 or even Day 7 post-fertilization and on blastocysts of different morphological quality. It is still unknown whether blastocyst morphology and developmental rate relates to the embryo chromosomal constitution. Only one study that attempted to correlate embryo morphology and aneuploidy as determined by CCS on TE biopsies (Alfarawati et al., 2011) showed a weak correlation between aneuploidy and blastocyst morphology. However, the biopsy procedures used in that study used zona opening at the cleavage stage of embryo development to promote TE cell herniation and facilitate the blastocyst biopsy procedure. This method may have introduced interference in embryo development from the cleavage to the blastocyst stage, lowering the reliability of the study when the data are extended to the general population of blastocysts obtained during regular IVF cycles.

Furthermore, no studies have attempted to correlate conventional parameters of blastocyst evaluation, euploid embryo viability in frozen embryo transfer (FET) cycles. It is still unknown whether euploid blastocysts with a different morphology and developmental rate implant at a different rate. This knowledge may be useful to further enhance the selection among euploid embryos.

In this study, the relationship between conventional parameters of blastocyst evaluation, CCS data and implantation potential of euploid blastocysts is investigated. The ultimate aim of this study is to provide knowledge for a better management in the IVF laboratory of blastocyst stage PGS cycles as well as to improve the genetic counseling for patients enrolled in PGS programs.

Materials and Methods

This is a multicenter retrospective observational study including the data analysis of blastocyst stage PGS cycles performed between January 2009 and December 2013 at Reproductive Biology Associates (RBA) in Atlanta, GA, USA and between 2011 and 2013 at GENERA, Center for Reproductive Medicine in Rome, Italy. CCS was offered to infertile patients of advanced maternal age (>35 years) and/or with a history of unsuccessful IVF treatments (>2 failed IVF cycles) and/or previous spontaneous abortion (>2 spontaneous miscarriages; Table I). Both centers performed IVF laboratory procedures and chromosome screening analysis following the same protocols during the study period. The extended observational time period included for RBA allowed more frozen transfers of euploid blastocysts to be performed and included in the data set compared with GENERA where blastocyst stage PGS cycles are performed from 2011 and a considerable number of euploid embryos are still frozen awaiting to be transferred. Furthermore, RBA performed a higher number of double euploid blastocyst transfers (50%; 43/86) compared with GENERA where a single euploid blastocyst transfer policy was adopted in 95% of the cycles (78/82).
Cooper Surgical) with 15% Serum Protein Substitute (Quinn’s Advantage using previously described techniques and instrumentation (Rienzi were then subjected to ICSI, between 36 and 38 h post-hCG administration, separate 35 nuclei. Those displaying two pronuclei were sequentially cultured further in 1998). At 16–18 h post-ICSI, oocytes were assessed for the presence of pro-

midified atmosphere containing 5% O2 and 6% CO2. Expanding and expanded blastocysts underwent biopsy of TE cells and cryopreservation 24 h later on Day 6 or 48 h later on Day 7.

Table I Baseline characteristic of patients involved in the study and clinical outcomes of blastocyst frozen embryo transfer cycles with known outcome for implantation

<table>
<thead>
<tr>
<th>Indication</th>
<th>IVF center 1 (GENERA)</th>
<th>IVF center 2 (RBA)</th>
<th>Combined data</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of patients</td>
<td>134</td>
<td>79</td>
<td>213</td>
<td></td>
</tr>
<tr>
<td>No. of PGS cycles</td>
<td>140</td>
<td>83</td>
<td>223</td>
<td></td>
</tr>
<tr>
<td>Female age (years) (95% CI)</td>
<td>38.8 (38.1–39.4)</td>
<td>36.1 (35.2–37.0)</td>
<td>37.8 (37.3–38.6)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Indication</td>
<td></td>
<td></td>
<td></td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>AMA (%)</td>
<td>96 (71.6)</td>
<td>36 (45.6)</td>
<td>132 (62.0)</td>
<td></td>
</tr>
<tr>
<td>AMA and RPL (%)</td>
<td>12 (9.0)</td>
<td>18 (22.8)</td>
<td>30 (14.1)</td>
<td></td>
</tr>
<tr>
<td>RPL (%)</td>
<td>12 (9.0)</td>
<td>16 (20.2)</td>
<td>28 (13.1)</td>
<td></td>
</tr>
<tr>
<td>AMA and RIF (%)</td>
<td>2 (5.2)</td>
<td>4 (5.1)</td>
<td>11 (5.2)</td>
<td></td>
</tr>
<tr>
<td>RIF (%)</td>
<td>7 (5.2)</td>
<td>5 (6.3)</td>
<td>12 (5.6)</td>
<td></td>
</tr>
<tr>
<td>No. of embryo analyzed (mean per cycle; SD)</td>
<td>490 (3.4, 1.9)</td>
<td>666 (5.8, 3.7)</td>
<td>956</td>
<td></td>
</tr>
<tr>
<td>Aneuploidy rate (N; 95% CI)</td>
<td>61% (299; 56.5–65.4)</td>
<td>49.8% (232; 45.1–54.4)</td>
<td>55.5% (531; 52.3–58.7)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Mean number of euploid embryos per cycle</td>
<td>1.3 ± 1.1</td>
<td>2.9 ± 2.1</td>
<td>1.9 ± 1.8</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>No. of euploid blastocysts transferred (% of total euploid)</td>
<td>86 (45.0)</td>
<td>129 (55.1)</td>
<td>215 (50.6)</td>
<td></td>
</tr>
<tr>
<td>Pregnancy rate (N; 95% CI)</td>
<td>57.3% (47/82; 39.9–62.4)</td>
<td>75.6% (65/86; 65.1–84.2)</td>
<td>66.7% (112/168; 59.0–73.7)</td>
<td>0.01</td>
</tr>
<tr>
<td>% biochemical pregnancy (N; 95% CI)</td>
<td>6.4 (3; 5.1–6.5)</td>
<td>12.3 (8; 5.5–22.8)</td>
<td>9.8 (11; 5.0–16.9)</td>
<td>NS</td>
</tr>
<tr>
<td>% miscarriages (N; 95% CI)</td>
<td>4.2 (2; 0.5–14.5)</td>
<td>9.2 (6; 3.5–19.0)</td>
<td>7.1 (8; 3.1–13.6)</td>
<td>NS</td>
</tr>
<tr>
<td>Clinical pregnancy rate (N; 95% CI)</td>
<td>51.2% (42/82; 45.9–68.2)</td>
<td>59.3% (51/86; 48.2–69.8)</td>
<td>55.4% (93/168; 47.5–63.0)</td>
<td>NS</td>
</tr>
<tr>
<td>bOngoing implantation rate (N; 95% CI)</td>
<td>48.8% (42; 37.9–59.9)</td>
<td>49.6% (64; 40.7–58.5)</td>
<td>49.3% (106; 42.4–56.2)</td>
<td>NS</td>
</tr>
</tbody>
</table>

Pregnancy rate, presence of gestational sac in Week 7 after transfer; Clinical pregnancy rate, beyond 20 weeks of gestation; AMA, advanced maternal age; RPL, recurrent pregnancy loss; RIF, repeated IVF failures; PGS, preimplantation genetic screening; CI, confidence interval; GENERA, Genera, Center for Reproductive Medicine, Italy; RBA, Reproductive Biology Associates, Atlanta, GA, USA.

*P-values for statistical comparison between the two IVF centers.

**Ongoing implantation rate is defined as number of fetuses with heart activity beyond 20 weeks of gestation per transferred embryo. Ongoing implantation was higher than pregnancy rate in IVF center 2 because 13 twin pregnancies were obtained.

However, these differences did not affect the results because the analysis of conventional morphology in predicting blastocyst developmental ability was only focused on implantation rate. Furthermore, all the logistic regression models used were corrected for IVF center, ruling out any potential for confounding factors between the two centers from the statistical analysis.

Approval for the study was obtained from the local institutional review board, and patients were asked to provide written informed consent to the anonymous use of clinical data for statistical evaluation and research purposes.

Laboratory procedures and blastocyst grading

Controlled ovarian stimulation, oocyte collection and denudation were performed as previously described (Capalbo et al., 2013a). Metaphase II oocytes were then subjected to ICSI, between 36 and 38 h post-hCG administration, using previously described techniques and instrumentation (Rienzi et al., 1998). At 16–18 h post-ICSI, oocytes were assessed for the presence of pronuclei. Those displaying two pronuclei were sequentially cultured further in separate 35 µl microdrops (Sage) up to blastocyst stage (Day 5/6) in a humidified atmosphere containing 5% O2 and 6% CO2. Expanding and expanded blastocysts underwent biopsy of TE cells and cryopreservation on Day 5. Cavitating morulas were transferred to a fresh individual 35 µl drop of blastocyst medium (Quinn’s Advantage® Blastocyst Medium, Cooper Surgical) with 15% Serum Protein Substitute (Quinn’s Advantage® Serum Protein Substitute, Cooper Surgical) and biopsy was attempted 24 h later on Day 6 or 48 h later on Day 7.

Blastocyst quality was assessed immediately before TE biopsy, defined according to the criteria presented by Gardner and Schoolcraft (1999) and categorized in four groups: excellent, group 1 (≥3AA); good, group 2 (3,4,5,6, AB and BA); average, group 3 (3,4,5,6 BB, AC and CA); poor, group 4 (≤3BB) based on inner cell mass (ICM) and TE quality score. Furthermore, individual ICM and TE scores were recorded. The TE was assigned one of the following grades: A: many cells organized in epithelium; B: several cells organized in loose epithelium; or C: few large cells. The ICM was assigned one of the following grades: A: numerous tightly packed cells; B: several and loosely packed cells; or C: very few cells. Day 5 biopsied blastocysts were defined as ‘faster growing’ embryos and Day 6 biopsied blastocysts as ‘slower growing’ embryos. Embryos reaching the blastocyst stage on Day 7 were also included for TE biopsy.

All embryo grading was reviewed in real-time by two senior embryologists for verification and consistency.

Biopsied blastocysts were vitrified according to the protocol described by Nagy et al. (2008). Euploid blastocysts were selected for transfer based on morphological score, and were warmed and cultured at 37°C (6% CO2 and 5% O2) until transfer. Only cycles with single or double embryo transfers were included. In case of double embryo transfers, only cycles with 2 or 0 fetal heart beats were included. Endometrial preparation and transfer procedures were performed as previously described (Ubaldi et al., 2010). According to Farquharson et al. (2005) the absence of an identifiable pregnancy on ultrasound examination was named ‘Biocellular pregnancy loss’. Clinical pregnancy was defined as the presence of gestational sac in Week 7 after transfer. A miscarriage between Week 7 and Week 20 was defined as an
abortion. The ongoing implantation rate was defined as number of fetuses with heart activity beyond 20 weeks of gestation per transferred embryo.

**Blastocyst biopsy procedure**

On Day 5, 6 or 7 of embryo development expanding and expanded blastocysts with or without herniating cells underwent TE biopsy. However, the main difference with previous reports describing a blastocyst biopsy procedure (Schoolcraft et al., 2010; Alfarawati et al., 2011) is that opening of the zona pellucida at the cleavage stage of embryo development was not used in the present blastocyst stage biopsy program.

Therefore, most of the embryos (93.8%) that underwent TE biopsy were in the expanding or expanded stage of blastocyst development without herniating cells. All the biopsy procedures were performed on the heated stage of a Nikon IX-70 microscope, equipped with micromanipulation tools, in dishes prepared with three droplets of 10 μl HEPES-buffered medium (Sage) overlaid with pre-equilibrated mineral oil. A diode laser (Research Instruments, Cornwall TR11 4TA, UK) was used to assist the opening of a 10–20 μm hole in the zona pellucida. The biopsy procedure performed on a good quality blastocyst is detailed in Fig.1. The blastocyst is positioned on the holding pipette to give a clear view of the ICM at 7 o’clock (i.e. away from the biopsy pipette) under high power magnification (Fig. 1A). An opening in the embryo zona is made with a series of laser pulses, working inwards from the outer surface of the zona taking care to avoid damaging the embryo. As soon as the aperture is wide enough to accommodate the passage of several TE cells (~10 μm), the biopsy pipette is carefully pressed against the zona, gently expelling medium through the breach to release the TE cells from the internal surface of the zona (Fig. 1B and C, respectively). This step helps to avoid blastocyst collapse during the subsequent TE cell separation. Once the TE is detached from the internal surface of the zona, 3–10 cells are aspirated into the biopsy pipette with gentle suction (Fig. 1D). The laser was positioned to the thinnest part of the aspirated cells and several laser pulses were directed at the junctions between cells to disconnect the aspirated cells from the body of the embryo. In the meanwhile a moderate suction was applied to the biopsy pipette to stretch the target cells, helping the separation from the body of the embryo (Fig. 1D). Figure 1E shows the blastocyst following the TE biopsy procedure. Five to ten TE cells are aspirated into the TE biopsy pipette and released into the biopsy drop (Fig. 1F).

**Array CGH analysis and embryo classification**

All TE biopsies were washed in sterile phosphate-buffered saline (PBS) solution in a laminar flow cabinet to avoid any contamination of the sample, placed in microcentrifuge tubes containing 2 μl PBS and then processed for array comparative genome hybridization (aCGH) analysis according to the 24 sure protocol (BlueGnome). Visualization and reporting of aneuploidy were performed using the Bluefuse Software (BlueGnome) on a per chromosome basis as previously described (Capalbo et al., 2013a). The copy number and segmental calls were based on the deviation from the acceptable

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**Figure 1**  
Trophectoderm biopsy protocol for human expanded blastocysts at the cleavage stage of embryo development. This figure shows the clinical protocol used to biopsy good quality human blastocysts on Day 5, Day 6 or Day 7 of development to obtain multicellular samples for DNA amplification and genetic testing. The protocol is suitable for both hatching and non-hatching blastocysts and requires no breaching of the zona pellucida during cleavage stages or the period of culture prior to biopsy. (A) Expanded good quality human blastocyst positioned on the holding pipette to give a clear view of the ICM at 7 o’clock, ×20 magnification. (B and C) Biopsy pipette expelling medium through the breach in order to release the TE cells from the internal surface of the zona, ×40 and ×20 magnification, respectively. (D) TE cells are aspirated into the biopsy pipette with gentle suction and several laser pulses are directed at the junctions between cells to disconnect the aspirated cells from the body of the embryo, ×40 magnification. (E) Blastocyst after TE biopsy, ×20 magnification. (F) TE biopsy cell sample released into the biopsy drop, ×20 magnification.
thresholds, as follows: monosomy of $-0.48$ and lower, euploid 0 and trisomy $+0.38$ and higher. This is based on a standard log2 ratio with X-separation of $+0.48$. Positive (3-cell samples of normal male fibroblast cell lines) and negative controls (empty biopsy medium) were included in every run. The latest genome build used for analysis was hg18. To investigate whether different degrees of chromosomal errors may result in a different developmental behavior of the embryos, the aneuploid aCGH results were further separated as single or double aneuploid, and as complex aneuploid when more than two chromosome errors were observed in the TE cell samples.

**Statistical analysis**

Continuous data and categorical variables are presented as mean and percentage frequency with 95% confidence interval (CI), respectively. Fisher’s exact test was used to assess differences between categorical variables. A bivariate logistic regression model was used at the beginning of the analysis to assess the relationship between female age and aneuploidy in the multicenter database. Logistic regression analysis adjusted for female age and IVF center was used to assess the relationship between variables of conventional blastocyst evaluation (developmental grade and morphology) with CCS results and clinical outcomes of euploid FET cycles. Statistical significance was set at $P < 0.05$. All statistical analyses were performed using R version 2.8.0 (The R Foundation for Statistical Computing).

**Results**

This study includes the data analysis of 956 blastocysts with conclusive CCS results obtained from 213 patients following 223 PGS cycles. The mean female age of this patient population was 37.8 (95% CI 37.3–38.6; range 26–44) at the time of egg collection. Basal and cycle characteristics of the patient population as well as clinical outcomes obtained up to the time this paper was written are reported in Table I. In the original PGS cycles 983 embryos were biopsied and 27 gave no result (2.7%), due to failed amplification or due to poor quality results. These cases were determined to be inconclusive and not included in this analysis. The overall aneuploidy rate of the embryonic population included was 55.5% (531/956; 95% CI 52.3–58.7). A total of 857 chromosomal abnormalities were detected in the aneuploid embryos (Supplementary data, Table SI), with monosomies and trisomies represented almost equally (Fig.2). A small percentage (5.5%) of these errors were a consequence of chromosome breakage, resulting in partial losses or gains of chromosomal material, most of which affected the larger chromosomes (chromosomes 1–11, Fig.2). Within the complex aneuploid embryo group, a mean number of 3.5 ($\pm$ 1.2, range 3–5) chromosomal errors were found. As expected, advancing female age was significantly related to aneuploidy (odds ratio (OR) = 1.12, 95% CI 1.08–1.15, $P < 0.01$). A higher aneuploidy rate was observed in IVF center 1 compared with IVF center 2 as a consequence of a significantly different mean female age in the patient population included in the two centers. More embryos of poor quality were selected for biopsy and CCS at IVF center 1 compared with IVF center 2 (121/490 and 32/466, respectively). FET cycles of 215 euploid blastocysts were performed in 134 patients where it was possible to track the implantation outcome of all embryos transferred (Table I). One hundred and twenty-five embryos implanted (125/215; 58.1%; 95% CI 52.2–64.8) and 106 (106/215; 49.3%; 95% CI...
42.4–56.2) resulted in ongoing pregnancies or deliveries. Eleven biochemical pregnancies and eight miscarriages were recorded. Clinical outcomes following PGS and FET cycles did not differ between the two centers involved in the study (Table I).

**Blastocyst morphology and aneuploidy screening data**

Among the embryological variables of blastocyst evaluation assessed (embryo quality, day of biopsy, and ICM and TE scores) the logistic regression analysis adjusted for the IVF center and female age showed that only blastocyst morphology was predictive of the CCS data (Supplementary data, Table SII).

Embryos with the highest morphological scores showed a higher euploidy rate compared with lower quality embryos \( (P = 0.01; \text{Fig. 3}) \). The euploidy rate was 56.4, 39.1, 42.8 and 25.5% in the excellent, good, average and poor blastocyst morphology groups, respectively. A diagnosis of complex aneuploidy was also associated with blastocyst morphology \( (P < 0.01) \) with 6.8, 15.2, 17.4 and 27.5% of excellent, good, average and poor quality embryos showing multiple chromosome errors. ICM and TE scores were also independently associated with aneuploidy screening data (Supplementary data, Table SIII).

Of the 956 biopsied blastocysts, 639 reached the expanding or expanded stage on Day 5 following fertilization (66.8%), 294 on Day 6 (30.8%) and 23 on Day 7 (2.4%) of embryo development. Blastocyst development rate was negatively correlated with increasing female aging (OR = 1.17, 95% CI 1.13–1.23; \( P < 0.01 \)). The mean female age among Day 5, 6 and 7 blastocysts was 36.1, 38.5 and 39.4 years, respectively. However, faster growing embryos (Day 5 blastocysts) showed a similar euploidy rate (46.6% 298/639; 95% CI 42.7–50.6) compared with slower growing ones (40.1%, 127/317, 95% CI 34.6–45.7; \( \text{Fig. 3} \)).

**Implantation potential of euploid blastocysts according to blastocyst morphology and stage of development**

The logistic regression analysis adjusted for IVF center and female age showed that morphology and developmental rate were not predictive of the developmental potential of euploid embryos following FET cycles (Supplementary data, Table SIV).

Poor and average quality euploid embryos yielded the same ongoing implantation rate (7/13, 53.8% and 26/60, 43.3%, respectively) compared with blastocysts evaluated as of excellent and good morphological quality (54/110 49.1% and 19/32 59.4%, respectively, \( \text{Fig. 4} \)). ICM and TE grade were also not related to the implantation outcomes of euploid embryos \( (P = 0.7 \text{ and } P = 0.8, \text{respectively}) \).

The developmental potential of euploid embryos reaching the blastocyst stage at different days post fertilization was also similar with 48.8% (82/168; 95% CI 41.0–56.6) and 51.2% (22/43; 95% CI 35.5–66.7)
of Day 5 and Day 6 blastocysts, respectively, yielding an ongoing or term pregnancy (Fig. 4). Furthermore, of the four Day 7 euploid embryos transferred, 50% yielded an ongoing implantation.

**Discussion**

In this multicenter study it was possible to accurately assess the relationship between conventional parameters used for conventional blastocyst evaluation and CCS data, as well as their role as an additional tool to predict the implantation potential of euploid embryos.

A straightforward point for the purpose of the study relied on the strategy of blastocyst biopsy used during the PGS cycles. Contrary to previous approaches proposed for blastocyst biopsy (Schoolcraft et al., 2010; Alfarawati et al., 2011), no laser-assisted breach in the zona pellucida was performed and a conventional embryo culture system up to the expanded blastocyst stage was conducted. This aspect helped to avoid any interference on blastocyst development as well as associated stress due to warming during the laser shooting at the cleavage stage. Furthermore, almost all embryos were biopsied at the same expanded stage of development, with morphology and day of biopsy post fertilization being the only differences among the blastocysts analyzed. All these clues together provided a more representative picture of the relationship between conventional blastocyst evaluation, CCS data and viability. In addition, according to the Italian legislation, all developing embryos were considered as viable irrespective of their quality and were included for aneuploidy screening. This aspect offered a powerful study model to gain significant information about aneuploidy screening outcomes and implantation potential of euploid embryos of particularly low quality and developmental grade.

Using a large sample size, this analysis was able to confirm the moderate association between aneuploidy and several distinct features of blastocyst morphology reported in an earlier study (Alfarawati et al., 2011). Increased aneuploidy rate among blastocysts with poor morphologic scores and a greater likelihood of euploidy for embryos with good scores was observed. However, this association was weak with a significant proportion of aneuploid embryos capable of achieving the highest morphologic scores (52% of excellent and good quality blastocysts). Accordingly, traditional morphology-based selection could not be relied on to significantly increase the likelihood of transferring chromosomally normal embryos in the absence of PGS.

Another embryological parameter analyzed in this study was the timing of embryo development to the blastocyst stage. Embryos reaching the expanded blastocyst stage on Day 6 showed a similar risk of being aneuploid as faster growing ones. Furthermore, even if based on a small sample size, Day 7 blastocysts present the same aneuploidy rate. This evidence suggests that the timing of blastocyst formation is not linked to or affected by chromosomal abnormalities. Eventhough we have used a different blastocyst biopsy procedure and development grading system, these data are in good agreement with two recent publications showing that delayed blastulation was not associated with increased aneuploidy rates (Alfarawati et al., 2011; Kroener et al., 2012).

The examination of the FET outcomes of blastocysts only provided a powerful study model to investigate the predictive role of conventional embryological evaluation when the prevailing confounding factor of chromosomal abnormalities is excluded from the analysis. Interestingly, in this data analysis blastocyst morphology and developmental rate were not associated with embryo viability. Lower-quality euploid embryos yielded the same ongoing implantation rate (45.2%) compared with blastocysts evaluated as of excellent and good morphological quality (51.4%). These findings also agree well with a recent study showing that excellent clinical outcomes are obtained when CCS-based selection was different than morphology-based selection (Forman et al., 2013). Thus, morphology seems not to be an additional parameter to consider when multiple euploid embryos are available for transfer. As a consequence, the recognized association between conventional evaluation of blastocyst morphology and embryo viability (Heitmann et al., 2013) can be mainly ascribed to the observed relationship between morphology and aneuploidy.

The implantation potential of euploid embryos reaching the blastocyst stage at different days post fertilization was also similar in this study suggesting that a delay in development is not related to viability. The extended delay in the development of preimplantation embryos has been commonly assumed to be a sign of poor quality (Shapiro et al., 2001; Utsunomiya et al., 2004; Barrenetxea et al., 2005). However, several biases can be identified behind this belief, including the endometrial factor for fresh embryo transfer (Shapiro et al., 2008). Furthermore, a sample bias in FET cycles could be identified where slower growing embryos are transferred in patients who did not get pregnant after Day 5 blastocyst and are considered to be of poorer prognosis. Indeed, a recent meta-analysis comparing Day 6 and Day 5 frozen blastocysts showed similar live birth rates when the analysis was restricted to studies comparing only Day 6 and Day 5 embryos of similar morphology and thus presumably of similar aneuploidy rate (Sunkara et al., 2010). In addition, Kovalevsky and colleagues recently showed that ongoing pregnancy rates were not significantly different from Day 7 and Day 5 blastocysts in FET cycles, with 27% of Day 7 transferred blastocyst capable of achieving an ongoing pregnancy (Kovalevsky et al., 2013). Thus, provided that the expanded stage is reached, it is reasonable to conclude that slower growing euploid embryos retain a clinically important chance of implantation and should be considered for biopsy and embryo transfer in PGS cycles.

In conclusion, this study provides knowledge for a better laboratory and clinical management of blastocyst stage PGS cycles. The association between blastocyst morphology and aneuploidy explains the higher implantation potential of good quality embryos reported during conventional IVF cycles. The relationship between morphology and aneuploidy screening data suggests that when PGS is not available, blastocyst morphology should be used to slightly reduce the risk of transferring aneuploid embryos. However, traditional morphology-based selection cannot be used as an alternative to PGS to minimize the risk of transferring chromosomally abnormal embryos. In addition, the commonly used parameters of blastocyst evaluation are not good indicators to improve the selection among euploid embryos. Thus, provided that the expanded stage is reached, all poor morphology and slower growing embryos have to be biopsied and similarly considered for FET cycles. This knowledge will be of critical importance to achieve similar cumulative live birth rates in PGS programs compared with conventional IVF avoiding the potential for exclusion of low quality but viable embryos from the biopsy and transfer procedures. It is expected that the more the genetic and molecular features of embryo development are characterized, the more the role of traditional morphology-based selection will be replaced in IVF. Future research to identify non-invasive biomarkers of reproductive potential may further enhance selection among euploid blastocysts.

In addition, a new clinical protocol for TE biopsy suitable for both hatching and non-hatching blastocysts and requiring no breaching of...
the zona pellucida during the cleavage stage period of culture was presented. By using this method, interference with embryo development and extra stress due to long exposure to a suboptimal environment, as well as the potential dangerous effect of warming following laser shooting at the cleavage stage of development, can be avoided.

**Supplementary data**

Supplementary data are available at http://humrep.oxfordjournals.org/.

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**Authors’ roles**

A.C. conceived the study. A.C., R.M., G.W. and T.E. performed the embryo laboratory procedures. A.C. and D.C. performed the data collection and statistic analysis. Z.P.N., F.M.U. and L.R. provided a critical discussion of the data and manuscript.

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**Conflict of interest**

None declared.

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