Association Between Morphologic Grading and Implantation Rate of Euploid Blastocyst

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Blastocyst morphologic grading, Day of trophectoderm biopsy, Euploid, Single frozen-thawed embryo transfer, Implantation rate.
Abstract

Objective To determine the association between the morphologic grading and implantation rate of euploid blastocysts in single frozen-thawed embryo transfer (SET) cycles.

Design: Retrospective cohort study.

Setting: Single university-based fertility center

Patient(s): Women aged 25–40 years undergoing euploid SET from January 2017 to December 2019 were identified.

Main Outcomes Measure(s): Implantation rate (IR)

Result(s): A total of 271 euploid SET cycles were included. The cycles were divided into three groups based on their morphologic grading before cryopreservation: good-quality (n = 58), average-quality (n = 88) and poor-quality blastocysts (n = 125). Good-quality blastocysts yielded statistically significantly higher implantation rates than poor-quality embryos (79.31% vs. 48%; adjusted odds ratio [OR] 3.269; 95% confidence interval [CI] 1.518–7.040). The OR remained significant after adjusting for maternal age, body mass index (BMI), type of infertility, basal follicle-stimulating hormone (FSH), AMH (anti-Müllerian hormone), peak endometrial thickness, type of SET cycle and day of blastocyst biopsy. According to post hoc analysis of age and the day of trophectoderm (TE) biopsy, the implantation rates in women aged < 35 years were higher after good-quality blastocyst transfer than poor-quality blastocyst transfer (80.39% vs. 48.62%, P = 0.003). Logistic regression analyses that adjusted for these variables identified higher implantation rates (OR 3.478, 95% CI 1.530–7.908) for good-quality blastocysts than for blastocysts that underwent poor-quality cycles, but not in women aged ≥ 35 years (71.43% vs. 62.50%, P = 0.402) (Table 2). The implantation rates were higher among women with good-quality blastocysts on both day 5 and day 6 of TE biopsy than among those with poor-quality blastocysts (day 5, 80.43% vs. 55.77%, P = 0.017; day 6, 75.00% vs. 42.47%, P = 0.074). Day 5 euploid blastocysts had no significant difference in implantation potential compared with similarly graded day 6 euploid blastocysts.

Conclusions: Blastocyst morphologic grading was associated with implantation rate for euploid embryo transfers after adjustment for potential confounders. These findings suggest that evaluating
blastocyst morphology is critical when selecting the best euploid blastocyst.

**Objective**
The primary goal of assisted reproductive technology (ART) is to identify an embryo with a high implantation potential to achieve a single healthy live birth\(^1\). During the last few years, concerted efforts have focused on reducing multiple pregnancy after ART by restricting the number of embryos to transfer\(^2\). Therefore, one crucial step in an ART program is the selection of the embryo with the highest implantation potential\(^3\). Different strategies can be considered to meet this objective. One strategy involves culturing to blastocyst stage, thereby allowing the self-selection of embryos capable of proceeding to blastulation, together with microscopic evaluation of morphologic criteria associated with improved viability, such as trophectoderm, inner cell mass and blastocoel expansion. Another strategy involves using preimplantation genetic testing for aneuploidy (PGT-A), mainly to reduce the likelihood of transferring an aneuploid embryo, which represents the leading cause of failed implantation, miscarriage and disordered embryo development\(^4\).

PGT-A was originally applied as an embryo selection technique in women of advanced maternal age, those with recurrent pregnancy loss or patients with recurrent in vitro fertilization (IVF) failure\(^5\).

Next-generation sequencing (NGS), which is the newest platform for PGT-A, performs high-throughput and high-resolution sequencing by synthesis. NGS is capable of generating large amounts of DNA sequence information both rapidly and at a low cost per base. Sequencing-based technologies are more costeffective and tend to have greater sensitivity than most other screening approaches\(^6\).

However, even euploid embryos may fail to implant. The causes of failure warrant investigation. Embryo quality has always been considered an important predictor of successful implantation and pregnancy. Standard morphologic evaluation has been the most widely adopted approach to embryo selection, and remains the most common strategy\(^7\). Gardner and Schoolcraft’s morphologic parameter scheme entails expansion grades together with the individual evaluation of the inner cell mass (ICM) and trophectoderm. On this scale, blastocysts with an ICM-trophectoderm quality graded below “BB” are considered of a poor morphology. A higher overall euploid blastocyst quality
correlates strongly with optimal pregnancy outcomes. Thus, trophectoderm and ICM morphologic grades are likely to be effective supplementary parameters to consider during embryo selection\[^8\]. Moreover, in a study by Mohamad evaluating the role of blastocyst development rate in euploid embryo selection, day 5 euploid blastocysts had higher implantation rates and live birth rates compared with similarly graded day 6 blastocysts\[^9\].

Several aspects that could affect clinical embryo selection in euploid blastocysts should be considered. Therefore, we sought to determine whether the most important consideration is blastocyst morphologic grading or blastocyst development. The aim of this study was to assess the prognostic value of blastocyst morphologic grading on the implantation competence in PGT-A with NGS.

**Materials And Methods**

**Study Design**

The Third Affiliated Hospital of Zhengzhou University’s medical institutional review board approved this study. All patients attempting conception through intracytoplasmic sperm injection (ICSI) utilizing PGT-A at the Third Affiliated Hospital of Zhengzhou University Center for Reproductive Medicine from January 2017 to December 2019 were included. The inclusion criteria included advanced reproductive age (> 38 years) a history of unsuccessful IVF attempts (≥ 3) or miscarriages (≥ 3). Embryos that reached the blastocyst stage were biopsied and then vitrified to allow time for NGS analysis. All patients meeting the study criteria underwent SET of euploid blastocysts. The two exclusion criteria were donor cycles and preimplantation genetic testing for monogenetic/single gene defects (PGT-M).

**Ovarian Stimulation Protocol**

The GnRH antagonist protocol was preferentially used. The physician adjusted the starting dose according to the patient’s age, body mass index (BMI) and ovarian reserve. Ovarian follicle development was monitored based on serum estradiol and transvaginal ultrasonographic measurements. The GnRH antagonist (Cetrotide, 0.25 mg, Merck-Serono) was started on day 6 of stimulation. Oocytes were retrieved transvaginally 33–36 hours after the GnRH agonist (0.2 mg Dophereline, Ipsen Pharma Biotech) was administered, which was done when at least 40% of follicles had reached or exceeded an average diameter of 18 mm as determined by ultrasound. The follicles
were aspirated using a single-lumen needle attached to a syringe under transvaginal ultrasound guidance.

Laboratory Protocol

The oocytes were then inseminated via ICSI approximately 4 hours after retrieval. Embryos were placed into the incubator (K-MINC-1000, Cook, United States) and cultured at 6% CO₂, 5% O₂ and 37 °C. G-1™ plus (Vitrolife, Sweden) was used to culture the embryos from the pronucleate stage to day 3, followed by G-2™ plus (Vitrolife, Sweden) from day 3 to the blastocyst stage. Laser-assisted trophectoderm (TE) biopsy was performed at the blastocyst stage on day 5 or 6. An opening of 6–9 mm was made in the zona pellucida with the use of a Saturn laser system (Research Instruments, Singapore), and 3–5 herniated TE cells were aspirated and separated from the blastocyst by applying multiple laser pulses. Harvested TE cells were washed, placed in 0.2-mL PCR tubes containing 5 µL phosphate-buffered saline solution (PBS) and stored at –20 °C until further use. The biopsied embryos were screened for aneuploidy utilizing a targeted NGS-based PGT-A platform, as described in Zimmerman et al. After biopsy, the blastocyst vitrification was performed using Cryotop® (Kitazato Corporation, Shizuoka, Japan), as described previously.

Next-generation Sequencing

NGS technology (Thermo Fisher Scientific, United States) was used to analyze the TE biopsy samples. Whole genome amplification (WGA) was performed using SurePlex (Bluegnome, United Kingdom). Following WGA, the library preparation consisted of incorporating individual barcodes for the amplified DNA of each embryo. After isothermal amplification and enrichment, sequencing was performed in a 316 or a 318 chip using a Personal Genome Machine sequencer (Thermo Fisher Scientific, United States). Aneuploidy data analysis was performed using Ion Reporter software (Thermo Fisher Scientific), using the low-coverage whole-genome workflow. The blastocysts received a diagnosis of euploid, aneuploid or chimera.

Embryo Transfer And Clinical Outcomes

Euploid SET was performed after preparation via hormone replacement treatment (HRT) or during a natural cycle. In the natural frozen embryo transfer (FET) cycle, women underwent monitoring by
regular vaginal ultrasound combined with urine luteinizing hormone (LH) measurement to observe the development of the dominant follicle and endometrium from the 10th day of the menstrual cycle until ovulation. One euploid blastocyst was transferred 5 days after ovulation. In the HRT FET cycles, women received escalating doses of estradiol valerate (4 mg daily from the 3rd day of the menstrual cycle for 7 days, then 6 mg daily for 5 days). Endometrial thickness and pattern were monitored by vaginal ultrasound, and when the endometrial thickness was ≥ 7 mm, vaginal progesterone 900 mg/d (Crinone, Merck Serono, Switzerland) was provided for luteal support. On day 6 of progesterone administration, a single vitrified euploid blastocyst was selected for transfer based on morphologic grading. All of the transfer procedures were directed by ultrasound guidance as previously described[12].

Statistical analysis
The primary outcome measure for the study was implantation rate, defined as the proportion of transferred embryos whose implantation resulted in intrauterine gestational sacs visualized by transvaginal ultrasound. Early spontaneous abortion was considered to be a pregnancy that did not progress after a fetal heartbeat was seen on scan before 12 weeks of gestation.

Patients were divided into three groups according to the morphologic grading of euploid blastocysts: good-quality (AA, AB, BA), average-quality (BB) or poor-quality (AC, BC). The outcomes and the baseline demographic characteristics were compared among the three groups.

Chi-square and Fisher’s exact tests were used to compare the categorical variables. The continuous variables were expressed as mean ± standard deviation (SD) and were tested for normality. Student’s t test was used for parametric data. Odds ratios (OR) with 95% confidence intervals (CI) were calculated and adjusted for maternal age, BMI, type of infertility, basal FSH, AMH, peak endometrial thickness, type of SET cycle and day of TE biopsy. Planned subgroup analyses were conducted by age at oocyte retrieval (range: 20–34 and 35–40 years) and the day of blastocyst biopsy (day 5 and day 6). P < 0.05 was considered statistically significant. All of the data analyses were performed with SPSS software version 22.0 (IBM, United States).

Results
Baseline Characteristics
A total of 271 frozen euploid blastocysts after transfer were included. The numbers of cycles classified into the three groups based on morphologic grading were as follows: good-quality blastocysts (n = 58), average-quality blastocysts (n = 90), poor-quality blastocysts (n = 123). The demographic parameters of patients in these groups are summarized in Table 1. There were no differences in age, BMI, type of infertility, basal FSH, AMH, peak endometrial thickness or type of SET cycle between the three groups. Day 5 blastocysts that were transferred were more likely to be of good quality and less likely to be classified as poor quality (P < 0.001).

| Parameter | Good-quality (n = 58) | Average-quality (n = 90) | Poor-quality (n = 123) | P value |
|-----------|----------------------|--------------------------|------------------------|---------|
| Female age (y) | 30.95 ± 3.50 | 31.64 ± 3.81 | 30.79 ± 3.78 | 0.253 |
| Male age (y) | 31.67 ± 3.76 | 32.49 ± 4.25 | 31.80 ± 4.54 | 0.417 |
| Female BMI (kg/m^2) | 24.11 ± 3.42 | 23.96 ± 3.00 | 24.04 ± 2.79 | 0.952 |
| Duration of infertility (y) | 2.20 ± 1.95 | 2.68 ± 2.13 | 2.45 ± 1.98 | 0.364 |
| Type of infertility | | | | 0.607 |
| Primary infertility | 17 (29.3%) | 31 (35.2%) | 46 (36.8%) | |
| Secondary infertility | 41 (70.7%) | 57 (64.8%) | 79 (73.2%) | |
| Basal FSH (IU/L) | 6.18 ± 2.55 | 6.58 ± 2.39 | 6.08 ± 2.32 | 0.314 |
| AMH (ng/mL) | 4.86 ± 4.01 | 4.60 ± 3.03 | 4.58 ± 3.09 | 0.854 |
| Peak endometrial thickness (mm) | 8.82 ± 1.52 | 9.05 ± 1.59 | 8.96 ± 1.56 | 0.687 |
| Day of TE biopsy | | | | 0.000 |
| D5 (%) | 46 (79.3%) | 53 (60.2%) | 52 (41.6%) | |
| D6 (%) | 12 (20.7%) | 35 (39.8%) | 73 (58.4%) | |

Note: Values are presented as mean ± standard deviation (SD) or number (%). BMI = body mass index, AMH = anti-Mullerian hormone, FSH = follicle-stimulating hormone, TE = trophectoderm

**Primary Outcomes**

Good-quality blastocysts yielded a statistically significantly higher implantation rate than poor-quality blastocysts (79.31% vs. 48.00%; P < 0.001; Fig. 1). The OR remained significant after adjusting for maternal age, BMI, type of infertility, peak endometrial thickness, basal FSH, AMH, type of FET cycle and day of TE biopsy (adjusted OR 3.269; 95% CI, 1.518–7.040). There were no differences in implantation rate between the average- and poor-quality blastocysts (64.77% vs. 48.00%, P = 0.069). There were no differences in early spontaneous abortion rate between the three groups (P = 0.414).

**Secondary Analyses**

Planned subgroup analyses by age and the day of TE biopsy were conducted. The relationship between morphologic grading and implantation rates appeared to differ by a woman’s age. Higher implantation rates were found after good-quality than poor-quality blastocyst transfer, but only in women aged < 35 years (80.39% vs. 48.62%, P = 0.003). Logistic regression analyses that adjusted
for these variables confirmed higher implantation rates (OR 3.478, 95% CI 1.530–7.908) for the good-quality blastocysts than for those that underwent poor-quality cycles, but not in women aged ≥ 35 years (71.43% vs. 62.50%, P = 0.402) (Table 2).

### Table 2

| Parameter         | Category | No. of cycles | Implantation rate | P value | Adjusted P value |
|-------------------|----------|---------------|-------------------|---------|------------------|
| **Age**           | Good     | 51            | 80.39% (41/51)    | 0.000   | 0.003            |
|                   | Average  | 69            | 63.77% (44/69)    | 0.049   | 0.144            |
|                   | Poor     | 109           | 48.62% (53/109)   | Reference | Reference |
| **35–40**         | Good     | 7             | 71.43% (5/7)      | 0.232   | 0.693            |
|                   | Average  | 19            | 68.42% (13/19)    | 0.146   | 0.624            |
|                   | Poor     | 16            | 43.75% (7/16)     | Reference | Reference |
| **Day of TE biopsy** | Good     | 46            | 80.43% (37/46)    | 0.011   | 0.017            |
|                   | Average  | 53            | 71.70% (38/53)    | 0.091   | 0.104            |
|                   | Poor     | 52            | 55.77% (29/52)    | Reference | Reference |
| **D5**            | Good     | 12            | 75.00% (9/12)     | 0.047   | 0.074            |
|                   | Average  | 35            | 54.29% (19/35)    | 0.250   | 0.283            |
|                   | Poor     | 73            | 42.47% (31/73)    | Reference | Reference |

| Adjusted for male/female age, BMI, type of infertility, basal FSH, AMH, peak endometrial thickness, type of SET cycle among the three groups. OR, odds ratio; CI, confidence interval. *P < 0.05 was considered statistically significant. |

Subsequent subgroup analysis did not reveal significant effect modification by the day of TE biopsy.

On the same day as TE biopsy, morphologic grading was associated with the implantation rates of euploid blastocysts. Specifically, the implantation rates were higher among women with good-quality blastocysts on both day 5 and day 6 of TE biopsy than those with poor-quality blastocysts (day 5, 80.43% vs. 55.77%, P = 0.011; day 6, 75.00% vs. 42.47%, P = 0.047). However, when adjusted for the above-mentioned variables, the differences in implantation rates on day 6 (P = 0.074) were no longer apparent.

Finally, to further assess the effects of blastocyst morphologic grading and the day of TE biopsy on implantation rate, we stratified the data by the day of TE biopsy, as shown in Fig. 2. In every morphologic grading group, there was no significant difference in implantation potential between similarly graded euploid blastocysts from day 5 and day 6.

**Discussion**

To summarize, this study described the correlations between blastocyst morphologic grades and their implantation rates. Our findings indicated that good-quality euploid blastocysts had higher implantation rates than poor-quality blastocysts, although this increase was only apparent for patients under 35 years old. On the same day as TE biopsy, morphologic grading was associated with
the implantation rates of euploid blastocysts. Day 5 euploid blastocysts showed no significant
difference in implantation potential compared with similarly graded day 6 euploid blastocysts. Unlike
the implantation rates, however, the early spontaneous abortion rate was not correlated with
blastocyst grading.
In this study, TE biopsy was performed at the blastocyst stage and NGS-based PGT-A was conducted
to select euploid blastocysts for single vitrified-warmed blastocyst transfer. NGS has grown in
popularity due to its ability to identify unbalanced translocations, segmental aneuploidies, some
triploidies[^6] and lower levels of mosaicism than other techniques[^13]. Some research has found
improved pregnancy outcomes and reduced spontaneous abortion rates with NGS due to the
exclusion of these abnormal embryos[^14]. However, the findings are not unanimous. In a multicenter
randomized clinical trial[^15] that performed PGT-A versus morphology as a selection criterion for
single frozen-thawed embryo transfer in good-prognosis patients, PGT-A did not improve overall
pregnancy outcomes for the cohort of women, as analyzed per embryo transfer or per intention to
treat. It is possible that the detrimental effect of the biopsy pre-vitrification on the embryo viability
may outweigh the benefit of PGT-A[^16].
Despite the advances made with PGT-A, a considerable proportion of euploid embryos still fail to
implant due to as yet unknown etiologies. In our study, the priority for euploid blastocyst selection for
transfer was based on blastocyst morphology. Our data confirm that good-quality euploid blastocysts
were correlated with higher implantation rates than poor-quality blastocysts, which partially explains
some of the failed euploid cycles. Our data are consistent with previously reported data by Irani et al.,
who demonstrated that excellent-quality embryos yielded a statistically significantly higher ongoing
pregnancy rate than poor-quality embryos (84.2% vs. 35.8%; adjusted OR, 11.0; 95% CI, 3.8–32.1)
and average-quality embryos (84.2% vs. 55.8%; adjusted OR, 4.8; 95% CI, 1.7–13.3)[^17]. The
difference is that whereas the present study excluded the potential effects of embryo-embryo
interactions by only analyzing single-blastocyst cases, Irani et al. analyzed cycles in which either one
or two blastocysts of the same overall quality were transferred. Blastocyst quality is assessed based
[^6]:
[^13]:
[^14]:
[^15]:
[^16]:
[^17]:
on blastocoel expansion, appearance of TE and appearance of ICM. TE grade has been shown to be a useful predictor of ongoing pregnancy rate for euploid embryos, and the critical role of the TE in mediating correct embryo implantation has been well established\(^1\).

Preventing miscarriage and maximizing the chance of implantation by SET are of utmost importance in euploid blastocysts. Here, we provide evidence that the early spontaneous abortion rate is not correlated with blastocyst grading. Barash et al. demonstrated an association of euploidy rates with morphologic characteristics of blastocysts. Embryos with an advanced degree of blastocyst expansion and well formed ICM/TE had higher chances of being euploid ($\chi^2 = 10.73, P < 0.05$ and $\chi^2 = 4.34, P < 0.05$, respectively)\(^{18}\). Elsewhere, poor-quality blastocysts showed a higher miscarriage rate per clinical pregnancy (36.4%) than non-poor-quality blastocysts (13.9%) after euploid SET\(^{19}\). However, once the risk of miscarriage is overcome, poor-quality blastocysts go to term apparently without greater obstetrical or perinatal risks\(^{20}\).

Although aneuploidy is the most significant determinant of cycle outcome in the older population, an age-related decline in implantation occurs in euploid embryos, supporting the view that factors other than chromosome segregation errors play a role in age-related fertility decline\(^{21}\). During a 5-year study period, Cimadomo collected various data to outline the clinical significance of poor-quality blastocysts during an ICSI cycle with PGT-A. The only feature that showed an association with the prevalence of poor-quality blastocysts was the maternal age at oocyte retrieval\(^{19}\). These results contradict previous findings in which the differences in outcomes based on age were nonsignificant after adjusting for embryo morphology\(^{22}\). After controlling for maternal age, here we provide evidence that good-quality euploid blastocysts have a higher chance of implanting than poor-quality blastocysts in women under 35 years; however, we found no differences in implantation rate based on blastocyst morphology in women over 35. The most likely explanation is that for women aged 35 or older, the most important feature associated with the euploid blastocyst implantation rate is the maternal age at oocyte retrieval rather than blastocyst morphology, which highlights the competence
of poor-quality euploid embryos in women of advanced maternal age. This conjecture is supported by Gonzalez's finding that in women of advanced maternal age following euploid SET after PGT-A via NGS, clinical outcomes were not significantly affected by the embryo morphology\textsuperscript{18}. Our study demonstrated that good-quality euploid blastocysts yield higher implantation rates than same-day poor-quality blastocysts. Both day 5 and day 6 euploid blastocysts with good quality showed increased implantation ability, and no significant difference in implantation potential was found between similarly graded blastocysts from days 5 and 6. Shapiro et al.\textsuperscript{23} found that day 5 blastocysts were associated with higher clinical pregnancy rates than their day 6 counterparts in fresh cycles, but had similar outcomes in FET cycles. While day 5 and day 6 embryos may have similar implantation potentials, the difference in the success rates observed in fresh cycles is essentially related to the suboptimal embryo-endometrial synchrony of day 6 blastocysts. Contradicting our findings, Irani et al., in a study correlating blastocyst development rate with pregnancy outcomes after euploid SET, showed that day 5 euploid blastocysts yielded higher implantation rates and live birth rates than similarly graded day 6 blastocysts\textsuperscript{9}. Blastocyst grading and the speed of embryo development to the blastocyst stage may reflect the metabolic health of the embryo. Irani et al. confirmed that day 5 blastocysts that were transferred were more likely to be of good quality and less likely to be classified as poor quality, while the higher implantation rate of day 5 blastocysts was mainly due to there being more good-quality blastocysts on day 5 than day 6. In any event, morphologic grading is correlated with implantation potential, which is why it was chosen as the main criterion used to select the embryos for transfer in this study.

In this regard, it is vital to provide patients with specific counseling focused on the evidence reported in the literature so they can make an informed choice.

This study has several strengths. First, it was specifically designed to find a strategy for selecting the best euploid embryo in patients who have multiple euploid embryos. Second, the chosen age range allowed the results to be clearly stratified by age. Third, we evaluated the role of the blastocyst morphology along with blastocyst development in euploid embryo selection. Fourth, as this study
involved single euploid blastocyst transfer, all of the embryos were subjected to the same NGS platform protocols with uniform analysis of the PGT-A results. Our embryologists also used a consistent scoring system consisting of a number of standardized transfer parameters.

This study also has several limitations. First, being a retrospective study with a relatively small sample size and data collection from a single center, some bias was inevitable. Second, implantation rate, not live birth rate, was the primary outcome. Third, patients over 40 years of age were not included because of the risk of not having access to euploid blastocyst transplantation. This may affect the applicability of the clinical outcomes to older patients with different quality blastocysts. Fourth, although blastocyst morphology and blastocyst development were both explored, the study was not capable of identifying specifically which embryos were most able to be implanted successfully.

Conclusions
This study confirms that embryo morphologic grading is correlated with implantation potential. If multiple embryos are euploid, morphology should be the main criterion used to select an embryo for transfer. However, the association between morphologic grading of blastocyst quality and implantation potential appeared to only hold in younger, not older women. On the same day as TE biopsy, morphologic grading was associated with the implantation rates of euploid blastocysts. Day 5 euploid blastocysts showed no significant difference in implantation potential compared with similarly graded day 6 euploid blastocysts.

Declarations

**Ethics approval and consent to participate**

The study was approved by the Institutional Review Board and Ethics Committee of the Third Affiliated Hospital of Zhengzhou University.

**Consent for publication**

All authors approved the final manuscript for publication.

**Availability of data and materials**

The datasets used and/or analysed during the current study are available from the corresponding
author on reasonable request.

**Competing interests**

The authors declare that they have no competing interests.

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**Authors’ contributions**

Designer of the study: HL, SHC. Data acquisition and analysis: NL,YCZ,DYH. Draft of the manuscript and interpretation: HL, Revision of the manuscript: SHC,YCG. All authors approved the final manuscript.

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Figures
