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SHORT COMMUNICATION *Embryology*

A pilot randomized controlled trial of Day 3 single embryo transfer with adjunctive time-lapse selection versus Day 5 single embryo transfer with or without adjunctive time-lapse selection

Daniel J. Kaser[1], Charles L. Bormann[1], Stacey A. Missmer[1,2], Leslie V. Farland[1,2], Elizabeth S. Ginsburg[1], and Catherine Racowsky[1,*]

[1]Division of Reproductive Medicine, Department of Obstetrics, Gynecology and Reproductive Biology, Brigham and Women's Hospital, Harvard Medical School, 75 Francis Street, Boston, MA 02115, USA [2]Department of Epidemiology, Harvard T.H. Chan School of Public Health, Boston, MA 02115, USA

*Correspondence address. Division of Reproductive Medicine, Department of Obstetrics, Gynecology and Reproductive Biology, Brigham and Women's Hospital, Boston, MA, USA. Tel: +1-617-732-5570; Fax: +1-617-975-0825; E-mail: cracowsky@bwh.harvard.edu

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STUDY QUESTION: Compared to D5 selection with conventional morphology (CM), does adjunctive use of the Eeva™ test on D3 or D5 improve the clinical pregnancy rate (CPR) per transfer?

SUMMARY ANSWER: The evidence is insufficient to conclude that adjunctive use of the Eeva™ test on D3 or D5 improves CPR per transfer as compared to D5 selection with CM.

WHAT IS KNOWN ALREADY: Time-lapse imaging is increasingly used for embryo selection, despite there being no class I data to support its clinical application.

STUDY DESIGN, SIZE, DURATION: Pilot randomized controlled trial included 163 patients from August 2014 to February 2016.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Patients up to age 41 years with a planned fresh autologous single embryo transfer (SET), less than four prior oocyte retrievals, and four or more zygotes were blocked according to age (<35, 35–37, 38–40 years) and randomized to one of three study arms: (1) D3 SET + Eeva™, (2) D5 SET + Eeva™ or (3) D5 SET with CM alone. All embryos were cultured in the same time-lapse system under identical conditions. Intention-to-treat (ITT) and as-treated analyses of the primary endpoint (CPR at 7 weeks) and secondary endpoint (ongoing pregnancy rate at 12 weeks) were performed. Multivariate regression analyses adjusted for patient age and ICSI.

MAIN RESULTS AND THE ROLE OF CHANCE: Of 478 eligible patients, 217 consented and 163 were randomized. Demographic characteristics were similar among the three study arms. There were no statistically significant differences in the clinical pregnancy rate or the ongoing pregnancy rate between the study arms for either the ITT or as-treated analyses (CPR ITT: D3 + Eeva™: 41.1% vs. D5 + Eeva™: 38.9% vs. D5 CM: 49.1%).

LIMITATIONS, REASONS FOR CAUTION: This study was designed as a pilot randomized controlled trial and was not powered to detect a statistically significant difference at $\alpha < 0.05$. Importantly, the study was terminated prematurely by the sponsor due to a change in funding priorities, so the sample size is limited and the results should be interpreted with caution due to the role of chance. Furthermore, these findings may not be generalizable to other time-lapse systems.

WIDER IMPLICATIONS OF THE FINDINGS: Our findings do not support the clinical application of these time-lapse markers.

STUDY FUNDING/COMPETING INTEREST(S): This study was funded by Progyny, Inc. There are no competing interests.

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Introduction

Time-lapse imaging (TLI) is increasingly used for embryo selection, despite no high-quality evidence supporting its utility (Kahraman *et al.*, 2013; Rubio *et al.*, 2014; Armstrong *et al.*, 2015; Goodman *et al.*, 2016). Of the three randomized controlled trials (RCTs) available, only one has used the same culture system for the TLI and the control arm (Goodman *et al.*, 2016). Additionally, prior studies have included Day 2 to Day 6 transfers of multiple embryos, both fresh and frozen, and autologous and donor oocytes.

This pilot study using fresh autologous single embryo transfer (SET) was designed to help address these heterogeneities and to inform the sample size needed for future trials. The culture system (including the type of incubator, TLI system, dishes and medium drop volumes) were identical among study arms. We evaluated the Eeva™ system, a dark field inverted microscope system that pairs uninterrupted group culture with automated image analysis and application of the Eeva™ test. This test assigns a blastocyst prediction rating of High (H), Medium (M) or Low (L) according to the durations of the 2-cell (P2; t3–t2) and 3-cell (P3; t4–t3) stages. While it has been shown to improve blastocyst prediction (Conaghan *et al.*, 2013; Diamond *et al.*, 2015), the Eeva™ test has never been evaluated in an RCT assessing its utility to improve the clinical pregnancy rate (CPR) per transfer.

The purpose of the Eeva™ Pregnancy Pilot Study (PPS) was to determine whether adjunctive use of the Eeva™ test on D3 or D5 improves the CPR per transfer, as compared to D5 selection with conventional morphology (CM) alone.

Material and Methods

Study population, experimental design and randomization scheme

Clinical outcomes following D3 SET with adjunctive TLI versus D5 SET with or without adjunctive TLI were compared (clinicaltrials.gov NCT02218255) at a single site following IRB approval. Enrollment opened in August 2014 and closed in February 2016. All included patients provided informed consent.

All patients aged 18–40 years with a planned fresh SET were screened for eligibility. Exclusion criteria were: more than three prior retrievals without an intervening clinical pregnancy; use of donor oocytes, a gestational carrier, PGD/PGS, or *in-vitro* maturation; and presence of uninterrupted hydrosalpinx or intrauterine adhesions. Patients were likewise excluded if all embryos were frozen due to ovarian hyperstimulation risk prior to randomization, or if they had less than four zygotes and therefore a risk of no blastocyst development.

Subjects were blocked according to age (<35, 35–37, 38–40 years) and randomized 1:1:1 at the fertilization check by an embryologist using computer-generated, random number sequence cards enclosed in opaque, serially numbered envelopes.

All embryos in all three arms were imaged continuously by Eeva™ version 2.2 in the same type of incubator. Only the day of transfer and selection algorithm varied. Embryologists were blinded to the Eeva™ ratings at the CM evaluation (i.e. one embryologist performed CM and a different embryologist reviewed the Eeva™ ratings), and patients and physicians were blinded to the Eeva™ ratings until a negative pregnancy test or the primary endpoint was reached.

A planned interim analysis at 60% recruitment was conducted by an independent Data Safety Monitoring Board; as pre-determined stopping criteria were not met (≥25% difference in CPR between any two arms), recruitment was continued. However, the trial was terminated prematurely in February 2016 by the sponsor due to a change in funding priorities.

Embryo culture and imaging

Immediately following the fertilization check, zygotes were placed in a 12-well Eeva™ dish (one zygote/well), which provides group culture in a 100 µL medium drop (global® total® with HSA; LifeGlobal, CT, USA) overlain with mineral oil. A medium refresh step on D3 was not performed. Eeva™ version 2.2 microscopes were housed within tri-gas box incubators (Penguin AQ-Astec, Steptoe Medical Devices, MA, USA) in 5% O_2, 6–7% CO_2, balanced with N_2. Dark field images were acquired every 5 min from the start of culture until transfer, cryopreservation or discard.

Embryo grading and selection

Selection algorithms are shown in Supplementary Fig. 1. Briefly, the primary screen for all embryos was CM. From a historical cohort of embryos with known implantation status, embryos were assigned a D3 Rank and a D5 Rank (Supplementary Table 1). Within each rank, embryos were further classified with a Priority Score based on implantation rate for each specific combination of cell number, fragmentation and symmetry on D3, or stage, inner cell mass and trophectoderm grade on D5. Next, early TLI markers were annotated manually by an embryologist and embryos exhibiting abnormal cleavage (division of one cell into three cells) or direct cleavage (duration of the 2-cell stage ≤ 5 h) were excluded due to their known detrimental effect on implantation (Rubio *et al.*, 2012; Athayde Wirka *et al.*, 2014). The Eeva™ test was then applied to assign a blastocyst prediction rating of H, M or L according to durations of P2 and P3: Rating H (P2 = 9.33–11.45 h and P3 = 0–1.73 h); Rating M: (if not High and P2 = 9.33–12.65 h and P3 = 0–4 h); or Rating L: (if not High or Medium).

As shown in Supplementary Fig. S1, selection in the D3 + Eeva™ arm was weighted more heavily on the Eeva™ rating which was designed for application on D3 to predict blastulation (Wong *et al.*, 2010). In contrast, selection in the D5 + Eeva™ was weighted more heavily on CM as the embryo had already blastulated, and the Eeva™ rating was used to distinguish between embryos with a similar morphology.

Outcome measures

Intention-to-treat (ITT) and as-treated analyses of the primary endpoint (clinical pregnancy rate [CPR] i.e. fetal cardiac activity at 7 weeks) and secondary endpoint (ongoing pregnancy rate [OPR] i.e. fetal cardiac activity at 12 weeks) were performed.

Statistical analyses

Crude and adjusted odds ratios (aOR) with 95% confidence intervals (CI) were calculated by unconditional logistic regression. Multivariate regression adjusted a priori for patient age to account for residual confounding after blocked randomization; other covariates were tested as potential confounders and retained if they modified the effect estimate by >10%. The final model adjusted for age and ICSI. Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of the Eeva™ test (High/Medium vs. Low) for predicting CPR were also determined. Fisher's exact test was used for categorical data, and one-way analysis of variance (ANOVA) was used for continuous data. Statistical analyses were performed with SAS® version 9.3 (SAS Institute, Inc., NC, USA). *P*-values <0.05 were considered statistically significant.

Results

Study population

The flow diagram, depicting enrollment, allocation, follow-up and analysis, is shown in Figure 1. Demographic characteristics were similar among the treatment arms (Table I).

Cycle characteristics and Eeva™ ratings

Cycle characteristics were also similar among the treatment arms (Table II). Notably, time-lapse markers were more likely to alter embryo selection for D3 (D3 + Eeva™: 25/49 transfers [51.0%]) than for D5 (D5 + Eeva™: 9/52 transfers [17.3%]) transfers ($P = 0.001$).

Clinical outcomes

ITT and as-treated analyses of CPR and OPR are shown in Table III. Regardless of the analysis performed, neither CPR nor OPR differed significantly among the three groups. As of March 2017, the cumulative clinical pregnancy rates per randomized woman were 73.2% (95% CI 60.3–83.1), 79.6% (95% CI 66.9–88.4) and 75.5% (95% CI 62.3–85.2), for D3 + Eeva™, D5 + Eeva™ and D5 CM alone, respectively (P = 0.73).

Performance characteristics of the Eeva™ test for ongoing pregnancy

There were no statistically significant differences in the performance characteristics of Eeva™ High/Medium for predicting clinical pregnancy on D3 compared to D5 (Supplementary Table S2).

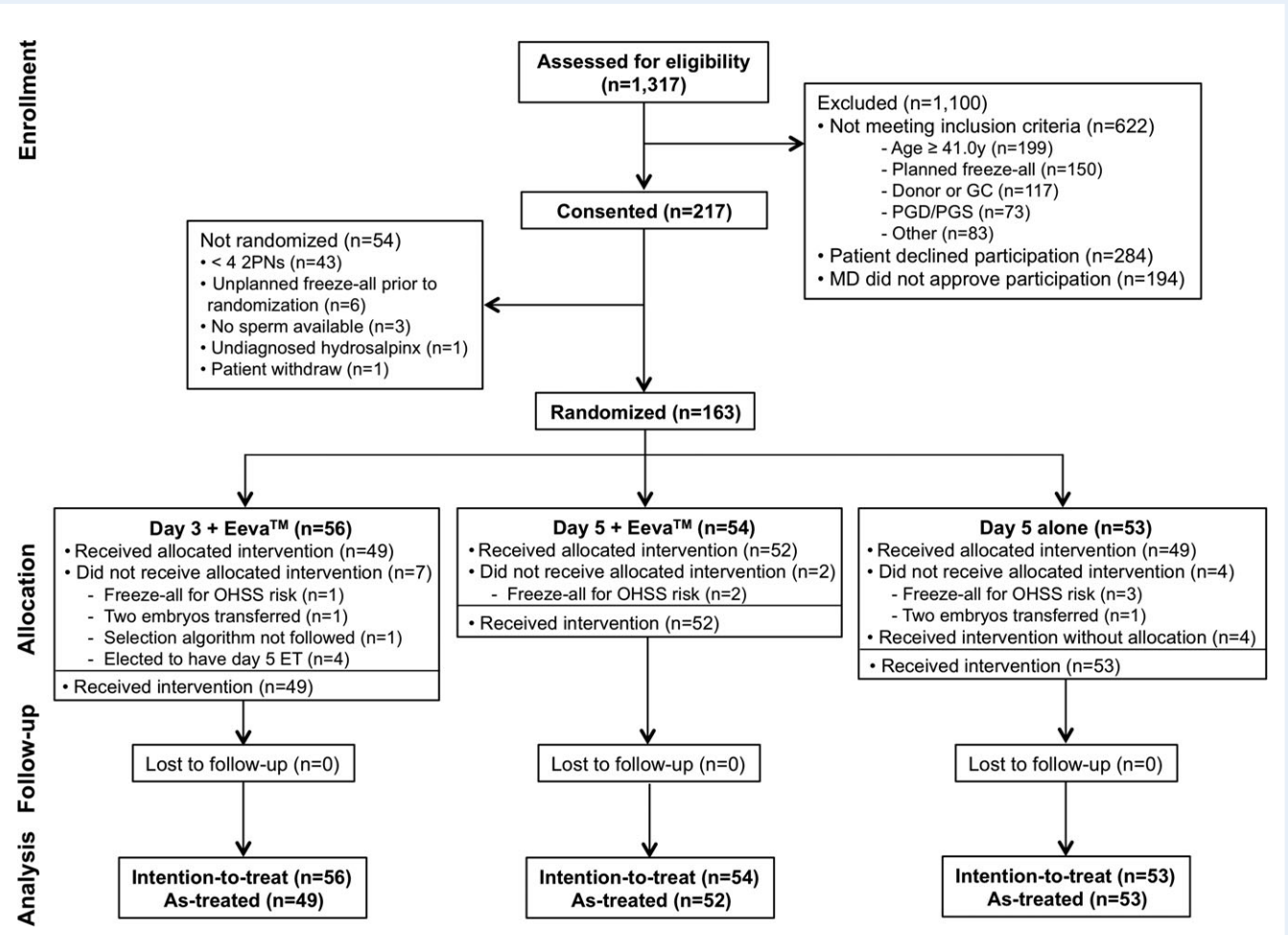

Figure 1 Flow diagram of Eeva™ Pregnancy Pilot Study (PPS) population. GC, gestational carrier; MD, medical doctor; PNs, proncuclear embryos; OHSS, ovarian hyperstimulation syndrome; ET, embryo transfer.

Table I Demographic characteristics of Eeva™ PPS population.

	Day 3 + Eeva™ ($n = 56$)	Day 5 + Eeva™ ($n = 54$)	Day 5 alone ($n = 53$)
Age (y)	34.6 ± 3.1	33.7 ± 3.4	34.1 ± 3.1
Race/ethnicity			
Asian	10 (17.9)	10 (18.5)	9 (17.0)
Black	1 (1.8)	3 (5.6)	0
Hispanic	2 (18.2)	5 (9.3)	4 (7.6)
White	40 (71.4)	33 (61.1)	39 (73.6)
Other	3 (5.4)	3 (5.6)	1 (1.9)
Body mass index (kg/m^2)	26.0 ± 6.9	25.5 ± 6.1	25.5 ± 6.5
Nulligravid	31 (55.4)	39 (72.2)	31 (58.5)
Parous	11 (19.6)	9 (16.7)	14 (26.4)
Primary infertility diagnosis			
Anovulation	8 (14.3)	10 (18.5)	7 (13.2)
Diminished ovarian reserve	5 (8.9)	2 (3.7)	4 (7.6)
Endometriosis	2 (3.6)	3 (5.6)	4 (7.6)
Male	13 (23.2)	12 (22.2)	18 (34.0)
Tubal	3 (5.4)	2 (3.7)	5 (9.4)
Unknown	19 (33.9)	19 (35.2)	13 (24.5)
Uterine	1 (1.8)	3 (5.6)	0
Other	5 (8.9)	3 (5.6)	2 (3.8)
Day 3 FSH (IU/mL)	7.0 ± 2.1	6.9 ± 1.8	7.8 ± 5.8
AMH (ng/mL)	4.6 ± 3.7	4.3 ± 3.0	5.0 ± 4.7
No. prior IVF cycles			
0	46 (82.1)	48 (88.9)	41 (77.4)
≥1	10 (17.9)	6 (11.1)	12 (22.6)

Values represent *n* (%) or mean ± standard deviation.

Discussion

This pilot study revealed no statistically significant increase in CPR per transfer following adjunctive use of the Eeva™ test on D3 or D5, as compared to D5 selection with CM alone. Although this study was not powered to detect a significant difference at $\alpha < 0.05$, the higher CPR in the D5 CM alone arm suggests that use of early time-lapse markers may not improve embryo selection.

Previous RCTs have shown a non-significant difference in CPR with time-lapse selection (Kahraman *et al.*, 2013; Rubio *et al.*, 2014; Goodman *et al.*, 2016). Prospective, non-randomized studies offer conflicting results (Adamson *et al.*, 2016; Kieslinger *et al.*, 2016). Further investigation is necessary to draw firm conclusions regarding clinical utility of adjunctive TLI, as no RCT to date has shown an improvement in CPR.

To our knowledge, this is the first study reporting performance characteristics of TLI in predicting clinical pregnancy. Remarkably, the PPV and NPV of Eeva-H on D3 are similar to that of CM alone (Racowsky *et al.*, 2009), which may reflect the importance of CM in our selection algorithms and/or suggest that these markers do not improve performance. Of note, these values were derived from a highly selected population of embryos (good-quality and selected for transfer) and were limited by relatively few transfers of Eeva-L embryos.

Strengths of this study include: (1) identical time-lapse system and culture conditions for the study and control arms; (2) SET, which obviates the need to restrict analysis to transfers with known implantation data; (3) liberal inclusion criteria; (4) randomization to the day of embryo transfer; and (5) blinding of the embryologist, physician and patient.

Nevertheless, this study has limitations. First, this RCT was designed as a pilot study to inform sample sizes needed in future studies, as outlined in the trial registration. Accordingly, based on the CPR we observed in the ITT analysis for D3 + Eeva™ versus D5 CM alone (41.1% vs. 49.1%), 606 patients would need to be randomized to each arm to show superiority of CM ($\alpha = 0.05$, $\beta = 0.20$). Second, the study was terminated prematurely by the sponsor due to a change in funding priorities. The resulting limited sample size may have increased the likelihood of Type II error. Likewise, the sample size may have resulted in uneven clustering of certain demographic characteristics I (e.g. nulligravidity or number of prior IVF cycles). Third, the design did not include a fourth arm of D3 with only CM selection, as there was concern that such inclusion would impede recruitment due to prevalence of D5 transfers in the United States. Fourth, the Eeva™ test was not designed for embryo selection on D5 and the Eeva™ ratings modified D5 embryo selection in a minority of cases due to our selection algorithm;

Table II Cycle characteristics of Eeva™ PPS population.

	Day 3 + Eeva™ ($n = 56$)	Day 5 + Eeva™ ($n = 54$)	Day 5 alone ($n = 53$)	*P*-value[a]
Type of stimulation protocol				
GnRH antagonist	48 (85.7)	42 (77.8)	44 (83.0)	
Luteal GnRH agonist	6 (10.7)	6 (11.1)	6 (11.3)	0.95, 0.82
Flare/Patch	1 (1.8)	4 (7.4)	2 (3.8)	
OI to IVF conversion	1 (1.8)	2 (3.7)	1 (1.9)	
Days of stimulation	12.1 (2.5)	12.4 (2.5)	12.1 (1.7)	0.94, 0.48
Peak E2 (pg/mL)	1884.5 (768.0)	1804.1 (628.1)	1901.0 (707.1)	0.91, 0.46
Trigger type				
hCG	40 (71.4)	39 (72.2)	37 (69.8)	0.85, 0.83
Dual hCG/GnRH agonist	16 (28.6)	15 (27.8)	16 (30.2)	
No. oocytes	16.4 (8.1)	14.9 (6.5)	18.1 (9.4)	0.33, 0.05
% MII	81.0 (13.3)	79.5 (14.9)	76.9 (16.2)	0.14, 0.39
ICSI	20 (35.7)	17 (31.5)	31 (58.5)	0.02, 0.01
No. 2PNs	10.2 (5.3)	9.1 (4.4)	10.5 (6.3)	0.78, 0.20
No. blastocysts frozen	3.8 (3.2)	3.9 (3.4)	3.5 (3.7)	0.82, 0.56
Eeva™-High transferred	42 (75.0)	34 (63.0)	33 (62.3)	0.21, 0.94
Eeva™-Medium transferred	10 (17.8)	14 (25.9)	9 (17.0)	0.90, 0.35
Eeva™-Low transferred	3 (5.4)	4 (7.4)	8 (15.1)	0.06, 0.11

Values represent *n* (%) or mean (standard deviation); first *P*-value compares Day 3 + Eeva™ vs. Day 5 + CM; second *P*-value compares Day 3 + Eeva™ vs. Day 5 + Eeva™.
[a]Fisher's exact or one-way analysis of variance.

Table III Intention-to-treat and as-treated analyses of clinical and ongoing pregnancy rates according to transfer day and selection method.

	Day 3 + Eeva™ ($n = 56$)	Day 5 + Eeva™ ($n = 54$)	Day 5 alone ($n = 53$)
Intention-to-treat			
Clinical pregnancy[a]	23 (41.1)	21 (38.9)	26 (49.1)
Crude OR	0.72 (0.34–1.54)	0.66 (0.31–1.42)	1.00 (Referent)
AOR[b]	0.84 (0.38–1.84)	0.71 (0.32–1.57)	1.00 (Referent)
Ongoing pregnancy[c]	21 (37.5)	18 (33.3)	25 (47.2)
Crude OR	0.67 (0.31–1.44)	0.56 (0.26–1.22)	1.00 (Referent)
AOR	0.76 (0.34–1.66)	0.60 (0.26–1.33)	1.00 (Referent)
	Day 3 + Eeva™ ($n = 49$)	**Day 5 + Eeva™ ($n = 52$)**	**Day 5 alone ($n = 53$)**
As-treated			
Clinical pregnancy	21 (42.9)	21 (40.4)	27 (50.9)
Crude OR	0.72 (0.33–1.58)	0.65 (0.30–1.41)	1.00 (Referent)
AOR	0.79 (0.35–1.76)	0.66 (0.30–1.47)	1.00 (Referent)
Ongoing pregnancy	19 (38.8)	18 (34.6)	26 (49.1)
Crude OR	0.66 (0.30–1.45)	0.55 (0.25–1.20)	1.00 (Referent)
AOR	0.70 (0.32–1.57)	0.56 (0.25–1.25)	1.00 (Referent)

Values represent *n* (%) or OR (95% CI).
[a]Clinical pregnancy defined as fetal cardiac activity at ≥7 weeks' gestation.
[b]Adjusted odds ratio; adjusted for patient age and ICSI.
[c]Ongoing pregnancy defined as fetal cardiac activity at ≥12 weeks' gestation.

so our D5 data should be interpreted with caution due to the possible contribution of chance. Fifth, the cumulative pregnancy data likewise should be interpreted cautiously, as randomization was broken after the first transfer, and all embryos were cryopreserved at the blastocyst stage. Sixth, our selection algorithm only included four time-lapse markers, so the findings are not generalizable to other markers.

Conclusion

In summary, this pilot study does not provide sufficient evidence to conclude that adjunctive use of the Eeva™ test on D3 or D5 improves the CPR per transfer as compared to D5 selection with CM alone. This study, when taken in context with the three other RCTs that showed no significant improvement in CPR with TLI selection (1–3), calls into question the clinical application of this technology. Until further research demonstrates a definitive improvement in clinical outcomes using TLI, we believe that this selection strategy should remain experimental.

Supplementary data

Supplementary data are available at *Human Reproduction* online.

Authors' roles

D.J.K. contributed to the study design and implementation and to data acquisition, analysis and interpretation, and drafted the manuscript. C.L.B. contributed to the study design and implementation and to data acquisition, and critically revised the manuscript. S.A.M. contributed to the study design and to data analysis and interpretation, and critically revised the manuscript. L.V.F. contributed to data analysis and interpretation and critically revised the manuscript. E.S.G. contributed to the study conception and design and to data interpretation, and critically revised the manuscript. C.R. contributed to the study conception, design and implementation and to data acquisition and interpretation, and critically revised the manuscript and served as guarantor for accuracy of the final manuscript. All authors provided final approval of the version to be published.

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Conflict of interest

None declared.

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