Treatment of human embryos with the TGFβ inhibitor SB431542 increases epiblast proliferation and permits successful human embryonic stem cell derivation

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STUDY QUESTION: Is there an effect of the TGFβ inhibitor SB431542 (SB) on the epiblast compartment of human blastocysts, and does it affect subsequent human embryonic stem cell (hESC) derivation?

SUMMARY ANSWER: SB increases the mean number of NANOG-positive cells in the inner cell mass (ICM), and allows for subsequent hESC derivation.

WHAT IS KNOWN ALREADY: It is known that inhibition of TGFβ by SB has a positive effect on mouse ESC self-renewal, while active TGFβ signalling is needed for self-renewal of primed ESC.

STUDY DESIGN, SIZE, DURATION: From December 2011 until March 2012, 263 donated spare embryos were used from patients who had undergone IVF/ICSI in our centre.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Donated human embryos were cultured in the presence of SB or Activin A, and immunocytochemistry was performed on Day 6 blastocysts for NANOG and GATA6. Moreover, blastocysts were used for the derivation of hESC, with or without exposure to SB.

MAIN RESULTS AND THE ROLE OF CHANCE: Immunocytochemistry revealed a significantly higher number of NANOG-positive ICM cells in the SB group compared with the control (12.0 ± 5.9 versus 6.1 ± 4.7), while no difference was observed in the Activin A group compared with other groups (6.7 ± 3.7). The number of GATA6-positive ICM cells did not differ between the SB, Activin A and control group (8.8 ± 4.3, 8.0 ± 4.6 and 7.2 ± 4.0, respectively). Blocking TGFβ signalling did not prevent subsequent hESC line derivation.

LIMITATIONS, REASONS FOR CAUTION: The number of human blastocysts available for this study was too low to reveal if the observed increase in NANOG-positive epiblast cells after exposure to SB affected the efficiency of hESC derivation (12.5% compared with 16.7%).

WIDER IMPLICATIONS OF THE FINDINGS: This work can contribute to the derivation of naive hESC lines in the future.

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Introduction

From mouse experiments, it is becoming increasingly clear that at least two states of pluripotency exist (Hanna et al., 2010; Nichols and Smith, 2011). Naïve mouse embryonic stem cells (mESC) originate from the inner cell mass (ICM) of the preimplantation blastocyst, while primed mouse epiblast stem cells (mEpiSC) are derived from the post-implantation blastocyst. Although human ESCs (hESCs) are derived from the preimplantation embryo, they more closely resemble the post-implantation mEpiSC. Recently, it was shown that the human ICM first develops to a structure which was defined as the post-inner cell mass-intermediate (PICMI), which has undergone X-inactivation in female cells and that is both necessary and sufficient for hESC derivation (O’Leary et al., 2012a,b).

When the first mESC lines were derived (Evans and Kaufman, 1981; Martin, 1981), standard optimized culture conditions (LIF and serum) were yielding stem cells from the 129 mice strain only. Derivation of mESC was far less efficient and mostly unsuccessful in other strains. The 2i condition [combined inhibition of the mitogen-activated protein kinase (MAPK) by PD0325901 and glycogen synthase kinase (GSK)3β pathway by Chir99021] was found to efficiently support the derivation of naïve mESC from non-permissive mice strains (Silva and Smith, 2008; Ying et al., 2008; Nichols and Smith, 2011), as well as from rat embryos (Buehr et al., 2008; Li et al., 2008). Interestingly, these two small molecules have also been found to modulate cell fate during preimplantation development (Nichols et al., 2009).

During the first lineage segregation in embryogenesis, loss of CDX2 results in the segregation of the ICM from the trophectoderm (TE) (Niwa et al., 2005; Ralston and Rossant, 2005; Strumpf et al., 2005; Dietrich and Hiiragi, 2007). The second segregation event allocates the ICM into epiblast (EPI) and hypoblast or primitive endoderm (PE). Initially, the precursor cells of EPI and PE are mixed within the ICM and express NANOG and GATA6, respectively (Chazaud et al., 2006). NANOG expression can be detected in a subpopulation of the ICM cells only (Cauffman et al., 2009), where it is co-expressed with the stemness genes SOX2, SALL4 and OCT4. Hyslop et al. (2005) described NANOG expression in the ICM of expanded blastocysts. Importantly, these NANOG-expressing cells in the ICM are considered as the epiblast compartment and represent the progenitor populations of EScs.

The transforming growth factor (TGF)b/Activin/Nodal pathway also plays an important role in the maintenance of primed EScs (Beattie et al., 2005; Xu et al., 2008; Hassan et al., 2012; Gu et al., 2012). In undifferentiated hESC and mEpiSC, TGFβ signaling is intracellularly transmitted by the phosphorylation of SMAD2/3, which can directly induce the expression of NANOG, thereby promoting their self-renewal (Yamanaka et al., 2010). Inhibition of TGFβ/Activin receptors by the small molecule SB431542 (SB), which inhibits the type I receptors ALK4/5/7, reduces SMAD2/3 phosphorylation and induces differentiation of these primed ESCs (Inman et al., 2002). In mESCs, the role of different members of the TGFβ superfamily is still a subject of controversy. Nevertheless, most data are supporting the idea that inhibition of TGFβ has a positive effect on self-renewal of mESCs, while TGFβ signalling is needed in order to stimulate self-renewal of primed ESC (Maherali and Hochedlinger, 2009; Galvin et al., 2010; Hassani et al., 2012).

As the small molecules 2i and SB can support naïve mESC pluripotency by activating naïve and repressing primed pathways, we hypothesised that similar to 2i, SB may also have a beneficial effect on pluripotent epi-blast proliferation in humans. Therefore, we set out to investigate if inhibition of the TGFβ pathway by SB increases the NANOG-positive epiblast cell number in human blastocysts, similar to the 2i condition. Furthermore, we explored whether SB had an effect on the derivation of hESC, and used this information to reflect on possible routes for naïve hESC derivation.

Materials and Methods

Ethical aspects

Institutional Review Board approval was obtained from the Ethical Committee, Ghent University Hospital (2009/281) and the Belgian Federal Ethical Committee on Embryo Research (Adv-030). In order to donate embryos for this study, patients signed informed consent before they started their IVF/ICSI cycle.

Embryo source

To test the effect of the TGFβ inhibitor SB and activator Activin A on embryos, we used 169 fresh embryos, spare following embryo transfer, that did not meet the criteria of the IVF laboratory for cryopreservation due to high fragmentation (≥35%) on Day 2 or 3 of development (day of oocyte retrieval being Day 0), multinucleated blastomeres on Day 2, delayed development (<5 blastomeres on Day 3 or a ≤1 blastomere increase from Days 2 to 3) or abnormal fertilization (1 or 3 pronuclei) on Day 1. Only embryos that contained at least four blastomeres on Day 3 and showed <50% fragmentation were included into the study. Although these embryos are of poor quality, we have previously shown that they are still very useful for research purposes (O’Leary et al., 2013).

Thawing of dimethyl sulfoxide frozen embryos

For the derivation experiments, 94 dimethyl sulfoxide (DMSO) frozen embryos were used. The thawing of these DMSO frozen embryos was performed by exposure to sequential sucrose solutions. Solution 1 contained 1 M sucrose in human tubal fluid (HTF) supplemented with 5% human serum albumin (HSA), solution 2 contained 0.5 M sucrose in HTF plus 5% HSA, solution 3 contained a mixture of 0.5 M sucrose and HTF only and solution 4 contained HTF only. Straws were removed from liquid nitrogen and kept at room temperature for 30 s, followed by exposure to ice water for 25 s. The embryos were expelled from the straw and quickly moved to solution 1 for 10 min at room temperature followed by solutions 2, 3 and 4 each for 10 min at room temperature. Finally, the embryos were moved to a
fresh drop of solution 4 at 37 °C for 5 min before they were cultured under 6% CO₂ and 5% O₂.

**Embryo scoring and culture**

Fresh Day 3 spare embryos cultured from the zygote stage in Cook Cleavage medium (Cook Ireland LTD, Limerick, Ireland, www.cookmedical.com) at 37 °C, 5% O₂ and 6% CO₂ (balance N₂) were collected daily from the IVF laboratory. After inclusion into the study, they were cultured in Cook Blastocyst medium (Cook Ireland Ltd, Limerick, Ireland, www.cookmedical.com) for another 3 days. The small molecule SB431542 (Tocris Bioscience, Bristol, UK) and the growth factor Activin A (R&D Systems, Abingdon, UK) were used to supplement the culture medium in this study. SB431542 inhibits the type I receptors ALK4/5/7 of the TGFβ pathway, while Activin A is a TGFβ superfamily ligand, known to activate the pathway.

Embryos were randomized using QuickCalc across the following study groups based on their blastomere number on Day 3 of development: (i) 10 μM SB431542, (ii) 50 ng/ml Activin A and (iii) control. Morphological evaluation of the embryos was performed on Days 4, 5 and 6 of development. On Day 6, blastocysts were scored at a fixed time interval using the grading system taken from Stephenson et al. (2006). ICM grades A and B are given to large and distinct ICMs, with grade A being more compact and B having larger, less compact cells. Grade C is used for small ICMs, grade D for degenerating ICMs, and grade E when no apparent ICM is visible. For the scoring of the TE, grades A, B or C correspond to good, moderate and poor quality, respectively. Blastocyst expansion was scored from 1 (no expansion of overall size) to 6 (fully hatched from zona pellucida).

**Immunocytochemistry**

Blastocysts and hESCs were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 20 min (room temperature) or overnight (4 °C). They were rinsed and stored in PBS (4 °C) until staining. Blastocysts were permeabilized in PBS + 0.1% Triton X-100 for 10 min, moved to PBS + 0.5% Triton X-100 for 20 min, rinsed in PBS and eventually blocked overnight in PBS + 0.05% Tween-20 + 1% bovine serum albumin (BSA) (blocking solution). Primary antibodies applied were goat anti-NANOG (1:200, AF1997, R&D Systems, Oxon, UK) and rabbit anti-GATA6 (1:200, SC-9055, Santa Cruz Biotechnology, Inc., Heidelberg, Germany). Blastocysts were treated with primary antibodies in blocking solution for 48 h at 4 °C. Following this incubation, embryos were rinsed in PBS, and treated with secondary antibodies in blocking solution for 48 h at 4 °C. Secondary antibodies were the same as applied for the blastocysts (see above).

**HESC derivation**

The protocol used in this study for ESC derivation was recently reported by O’Leary et al. (2011, 2012a,b, 2013). In summary, Day 6 (D6) blastocysts with both good and poor quality ICMs were exposed to pre-warmed Acidic Tyrode’s (Sigma, Bornem, Belgium, T1788) to remove their zona pellucida. After washing, the blastocysts were plated in individual culture dishes with a nearly confluent feeder layer of mitomycin-C (Sigma, M4287) treated CDI mouse embryonic fibroblasts (MEFs). The culture environment from blastocyst stage to hESC level consisted of 37 °C, 6% CO₂ and 5% O₂ in standard hESC culture medium composed of Knockout Dulbecco’s Modified Eagle Medium (Invitrogen, Merelbeke, Belgium, 10829-018), 20% Knockout Serum Replacement (KO-SR, Invitrogen, 10828-028), 1% non-essential amino acids (Invitrogen, 11140-035), 0.1 mM L-glutamine (Invitrogen, 25030-024), 1% penicillin/streptomycin (Invitrogen, 15140-122), 0.1 mM beta-mercaptoethanol (Sigma, M3148) and 4 ng/ml basic fibroblast growth factor (bFGF, Invitrogen, 13256-029). This standard medium was applied for the control and the + SB/−SB condition, while the +SB/+SB condition required additional supplementation with 10 μM SB431542. Observation and refreshment was done daily in order to observe areas of presumed ICM-derived cells (post-ICM-intermediates or PICMs) or emerging colonies (O’Leary et al., 2012a,b). Once hESC outgrowths emerged, they were mechanically passaged until stable colonies could safely be transferred to a tissue culture flask. These hESC were expanded with 1 mg/ml collagenase type IV (Invitrogen, 17104-019) every 5–6 days with daily media refreshment.

**Oil Red O staining**

The Oil Red O solution (Janssen Chimica, Geel, Belgium) was prepared in 99% isopropanol. Blastocyst outgrowths were rinsed with PBS, fixed with neutral-buffered formol, rinsed with water and incubated for 5 min with 60% isopropanol. After aspiration of the isopropanol, the cells were incubated with the filtered Oil Red working solution for 10 min and rinsed with water.

**Alkaline phosphatase live staining**

The alkaline phosphatase (AP) live staining was performed using 1X AP Live Stain working solution (Invitrogen, A14353), diluted in DMEM/F-12 (Invitrogen, 10565-018). Stem cells were first rinsed with pre-warmed DMEM/F-12 and then incubated with the 1X AP working solution for 30 min at 37 °C. Afterwards, the AP live stain was removed and cells were washed with DMEM/F-12. After addition of fresh DMEM/F-12, pictures were taken within 30–90 min of staining (Olympus 1X71, Olympus, Aartselaar, Belgium). Following visualization, DMEM/F-12 was replaced with fresh hESC medium, and the cells were maintained in culture.

**Karyotyping of hESC lines**

Nearly confluent culture flasks of hESCs at early passages were blocked in metaphase by colcemid (1:100 in salt solution; Karyomax, Invitrogen, 15210-057), harvested with 0.25% trypsin-EDTA (Invitrogen, 25200-056), treated with a hypotonic solution of KCl (Sigma, T-3038) and fixed in a 3:1 methanol/acetic acid solution (Merck, Overijse, Belgium, 1.0609.1000; Sigma, A 6283). G-banding was used to analyse the metaphase spreads (Meisner and Johnson, 2008). Ten metaphases were checked for numerical aberrations and three metaphases were completely karyotyped.
The final values are described as fold change expression values compared with controls. All Ct values were normalized against the housekeeping genes and means.

Mix with ROX (BioRad, 172-5850), depending on the gene assay. Which showed a detrimental effect on cell growth. HESC colonies renewal capacity of the colonies after 3 days of culture, in contrast to SB, morphology and the expression level of OCT4, NANOG and SOX2.

Results
The effect of SB and Activin A on hESC
In the first experiments, we wanted to confirm the published effects of SB and Activin A on established hESC, before applying these small molecules to embryos. Therefore, we cultured the UGent2 hESC line for 8 days in the presence of standard hESC medium, supplemented with 10 μM SB or 50 ng/ml Activin A, and we determined the effects on both the morphology and the expression level of OCT4, NANOG and SOX2. Overall, we noticed a positive effect of Activin A on the survival and self-renewal capacity of the colonies after 3 days of culture, in contrast to SB, which showed a detrimental effect on cell growth. HESC colonies cultured in the presence of SB did not proliferate well and showed early signs of differentiation at their periphery (Fig. 1A). RT-qPCR for the pluripotency markers NANOG, OCT4 and SOX2 confirmed these observations after 8 days of culture (Fig. 1B). Expression of OCT4, NANOG and SOX2 was significantly lower in the SB condition compared with the control. In the Activin A condition, OCT4 and NANOG were significantly more highly expressed, while SOX2 showed a similar expression level to the control cells.

NANOG expression increases after inhibition of the TGFβ pathway
After the functionality of SB and Activin A was demonstrated at the hESC level, we applied the same concentrations at the embryo level. Day 3 embryos were cultured in the presence of 10 μM SB or 50 ng/ml Activin A until developmental Day 6. Blastocyst rates were similar in the different groups (Table I), and there were no differences observed in blastocyst quality over the different groups.

Immunocytochemistry revealed a significantly higher number (mean ± SD) of NANOG-positive ICM cells in the SB group compared with the control (12.0 ± 5.9 versus 6.1 ± 4.7), while no difference was observed in the Activin A group compared with other groups (6.7 ± 3.7). The number of GATA6-positive ICM cells did not differ between the SB, Activin A and control groups (8.8 ± 4.3, 8.0 ± 4.6 and 7.2 ± 4.0, respectively) (Table I and Fig. 2).

Derivation of hESC lines after embryo culture in SB431542
In the following experiments, we set out to investigate if the higher number of NANOG-positive cells in the ICMs from SB-cultured embryos had an effect on subsequent hESC derivation. Frozen embryos were thawed and cultured in the presence or absence of SB from Days 3 to 6 of development. After this initial embryo culture, Day 6 blastocysts were plated in standard hESC medium or hESC medium supplemented with SB. The experimental set-up was as follows: (1) 6 control blastocysts, all of which were plated in standard hESC medium (−SB/−SB); 15 SB blastocysts: 7 were plated in SB supplemented hESC medium (+SB/+SB) (2), whereas 8 were plated in control hESC medium (−SB/−SB) (3) (Table II). There were no differences in blastocyst quality observed between the different groups, and each included similar amounts of good and poor quality ICMs.

PICMI formation was observed in 2/6 plated control blastocysts; from one PICMI, hESC colonies could be obtained. However, this new hESC population differentiated after two more passages. Of the seven SB-treated blastocysts that were plated in SB medium (+SB/+SB), two developed into PICMIs, but no stem cells could be derived. Curiously, lipid vesicles appeared in these cultures after several passages, as determined by Oil Red O staining. The oil was produced by the MEFs and not by the embryonic cells, as addition of SB to MEFs in the absence of a human embryo also resulted in the production of lipid vesicles. Moreover, in this condition, we observed a slightly different PICMI morphology, with a less defined region of hESC precursor cells (Fig. 3).

The +SB/− SB condition resulted in the formation of one PICMI (out of 8 blastocysts), which gave rise to a new ESC line that could be kept in culture for at least 30 passages (UGent12-1). This newly established hESC line originated from a blastocyst with an ICM and TE of very good quality (scored as A, according to Stephenson et al., 2006).
Pluripotency of UGent 12-1 was confirmed by immunocytochemistry for OCT4 and NANOG and by demonstrating high levels of AP. G-banding revealed a normal XY karyotype.

Discussion

In the current study, we examined whether inhibition of the primed TGFβ pathway has similar effects on the human embryo as the 2i condition, known to support naive pluripotency. Based on the earlier results of 2i application to human embryos (Van der Jeught et al., 2013), we expected that inhibition of TGFβ would have similar effects on the expression of NANOG in the ICM. Furthermore, the effect on subsequent hESC derivation was explored.

Substantial evidence suggests that the pluripotent status of primed hESC relies on Activin A signalling and that inhibition of the TGFβ pathway promotes hESC differentiation (Vallier et al., 2005; Xiao et al., 2006; Saha et al., 2008). We plated blastocysts that were cultured in the presence of SB, and accordingly succeeded in the establishment of a new hESC line. This line was derived in standard hESC medium and the colonies showed a normal morphology, consisting of flattened cells with a high nucleus-to-cytoplasm ratio, typical for the primed pluripotent state.

A limitation of the study is that the number of human blastocysts available was too low to reveal if the observed increase in NANOG-positive epiblast cells would affect the efficiency of hESC derivation (12.5% compared with 16.7%). An increase in derivation efficiency could be expected, as the NANOG-positive ICM cells are known to be the progenitors of ESC. Moreover, it should be noted, that we are working with human spare embryos, and our results may therefore be influenced by their inherent abnormalities. In this respect, we previously showed that embryos with multiple poor-quality traits were unable to generate hESC lines, and that maternal age influences the ability of good quality ICMs to generate hESCs (O’Leary et al. 2011, 2012a,b). However, these parameters were taken into account here.

Our data further support the importance of TGFβ signalling in the maintenance of hESC pluripotency. In order to investigate the role of this signalling pathway with respect to the second lineage commitment during early human embryo lineage commitment, we supplemented...
sparing embryos with 10 μM SB and 50 ng/ml Activin A. Beattie et al. (2005) compared the effects of 5, 50 and 100 ng/ml Activin A on hESC, and concluded that 50 ng/ml is the optimal concentration to maintain hESC in the undifferentiated state. SB was applied at 10 μM, as Laping et al. (2002) described that SB can be used up to 10 μM without any unwanted side effects. The functionality of these concentrations was tested on hESC before applying them to human embryos, and confirmed similar effects to the studies mentioned above.

Our results show that inhibition of the type I receptors ALK4/5/7 by SB leads to a significant increase in the mean number of NANOG-positive cells in the ICM, but does not affect the mean number of GATA6-positive ICM cells. Activation of TGFβ signalling using Activin A had no effect on this. We conclude that inhibition of TGFβ signalling using SB can increase the number of NANOG-positive epiblast cells in the human blastocyst, similar to dual inhibition of MAPK and GSK3β in the 2i condition (Van der Jeught et al., 2013). The increase in NANOG-positive cells should be explained as a result of increased proliferation of the NANOG-positive epiblast compartment, rather than resulting from a preferential promotion of the epiblast lineage during ICM segregation. These findings confirm our hypothesis that 2i and SB produce a similar effect on NANOG at the blastocyst level. To investigate whether the observed increase in NANOG-positive cells in the ICM had an effect on stem cell derivation, SB cultured blastocysts were

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**Table II** The effect of the TGFβ inhibitor SB431542 (SB) on blastocyst and PICMI formation, and hESC derivation.

<table>
<thead>
<tr>
<th>Conditions, a (n)</th>
<th>Blastocyst formation, n (%)</th>
<th>PICMI formation, n (% of blastocysts)</th>
<th>hESC derivation, n (% of blastocysts)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Control (26)</td>
<td>6 (23)</td>
<td>2 (33)</td>
<td>1 (17)</td>
</tr>
<tr>
<td>2. +SB/−SB (38)</td>
<td>7 (18)</td>
<td>2 (29)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>3. +SB/−SB (30)</td>
<td>8 (27)</td>
<td>1 (13)</td>
<td>1 (13)</td>
</tr>
</tbody>
</table>

*aControl: standard embryo culture and derivation medium, no SB was applied; +SB/−SB: embryo culture in SB-supplemented medium, hESC derivation in SB-supplemented hESC medium; +SB/−SB: embryo culture in SB-supplemented medium, hESC derivation in standard hESC medium; PICMI, post-inner cell mass intermediate.*
plated in both standard and SB-supplemented hESC medium. When cultured in standard derivation medium (+ SB/− SB), a well-defined PICMI structure was observed that gave rise to a new hESC line (UGent 12-1). The two PICMI outgrowths that originated from the SB blastocysts in SB-supplemented medium (+ SB/+ SB) were rather atypical, and had a less distinct outline. One possible hypothesis for this difference in morphology between these outgrowths is that the cells cultured in the presence of SB were partly driven towards a naïve-like pluripotent state, by inhibition of the ALK4/5/7 receptors during derivation. In this + SB/− SB condition, lipid droplets appeared after several passages, but no hESC were derived. It is known that TGFβ/SMAD3 signalling plays an important role in adipocyte formation in humans. Deregression of this pathway by SB could explain this observed phenomenon (Tsuru et al., 2011; Yadav et al., 2011).

The derivation of naïve hESC is of great interest, because they show superior levels of pluripotency and can be cultured as single cells in bulk for future clinical applications. Future research will focus on the derivation of naïve hESC, by exposing the cells to N2B27 medium supplemented with multiple small molecules directed at preventing the cells from differentiation. To our knowledge, only four studies have investigated, both directly and indirectly, the derivation of naïve ESC directly from human preimplantation embryos (Lengner et al., 2010; Roode et al., 2012; Kuijk et al., 2012; Van der Jeught et al., 2013), but these did not meet with success. In our study, the presence of a partly naïve signalling condition may have resulted in an atypical PICMI, unable to produce hESCs.

Conclusion

To our knowledge, this study is the first to describe that SB-mediated inhibition of TGFβ signalling through ALK4/5/7 leads to an increased expression of NANOG in the human ICM. Moreover, we succeeded in the derivation of an hESC line from an embryo that was cultured in the presence of SB. As previous work revealed a similar increase in NANOG after embryo culture in 2i, with subsequent ESC derivation, and since both inhibitory conditions are known to sustain the naïve ESC condition, these small molecules may therefore serve as good candidates for naïve hESC derivation.

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Authors’ roles

M.J.: study design, execution, manuscript drafting. B.H. and T.D.: study design, manuscript drafting, critical discussion, final approval of the manuscript. T.L.: study design, critical discussion, N.R.: contribution in execution. N.R.: critical discussion. S.M.C.S.L.: critical discussion. D.D.: final approval of the manuscript. P.S.: study design, critical discussion and final approval of the manuscript.

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Conflicts of interest

None declared.

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