CLINICAL STUDY

BMP system expression in GCs from polycystic ovary syndrome women and the *in vitro* effects of BMP4, BMP6, and BMP7 on GC steroidogenesis

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Abstract

Background: The bone morphogenetic proteins (BMPs) are growth factors involved in the folliculogenesis. Alteration in their expression may compromise the reproductive process in disease such as the polycystic ovary syndrome (PCOS). This study investigated the expression and role of granulosa cell (GC) BMP from normal cycling and PCOS women.

Methods and results: This prospective study was performed in GCs obtained from 14 patients undergoing IVF: i) six women with normal ovulatory cycles and tubal or male infertility and ii) eight women with PCOS. *BMP2*, *BMP4*, *BMP5*, *BMP6*, *BMP7*, and *BMP8A* and their receptors *BMPRI1A*, *BMPRI1B*, and *BMPRII* were identified by RT-PCR in GCs from normally cycling and PCOS women. *BMP4*, *BMP6*, and *BMP7* expressions were confirmed by immunohistochemistry. Quantitative transcript analysis showed the predominant expression of *BMP6*. In GCs from PCOS women, an overexpression of *BMP6* ($P < 0.01$) and *BMPRI1A* mRNA ($P < 0.05$) was observed. GC culture experiments demonstrated that basal estradiol (E_2) production was threefold higher but FSH-induced E_2 increment was twofold lower in PCOS compared with controls. In PCOS, *BMP6* and *BMP7* exerted a stimulatory effect on basal E_2 production while *BMP4* and *BMP6* inhibited FSH-induced E_2 production. FSH receptor and aromatase expression were not different between both groups.

Conclusion: The BMP system is expressed in human GCs from normal cycling and PCOS women. The BMP may be involved in reproductive abnormalities found in PCOS.

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Introduction

Polycystic ovary syndrome (PCOS) is a common endocrine disease affecting 6–10% of premenopausal women of childbearing age. This clinically heterogeneous disorder appears as the main cause of female infertility (1). The pathophysiology of this ovulatory disorder affecting folliculogenesis is still puzzling. There is evidence for a primary ovarian dysfunction in PCOS reflected by the dramatic increase in the number of small growing follicles at the expense of the dominant follicle selection from the increased pool of selectable follicles (2, 3, 4). Bone morphogenetic proteins (BMPs), which are functionally involved at all stages of folliculogenesis, are multifunctional growth factors belonging to the transforming growth factor β (TGF β) superfamily. Evidence from studies on rodents indicate an autocrine/paracrine role for granulosa-derived BMP6, theca-derived BMP4 and BMP7, and a paracrine

role of oocyte-derived growth differentiation factor 9 (GDF9), BMP15, and BMP6 on granulosa cell (GC) proliferation and on FSH-dependent follicle functions. BMP actions are mediated by their receptors (BMPRs) that share a different degree of affinity for their ligands (5). Unbalanced exposure to BMPs may alter the follicle sensitivity to FSH and thus impair the process of dominant follicle selection. In rodent and mammalian species, GDF9, another member of the BMP family, and BMP15 play a key role on folliculogenesis and female fertility (6, 7, 8, 9, 10). Disruption of follicle development and subsequent infertility were demonstrated in sheep sharing a homozygous mutation of *BMP15* gene (9). In rodent ovary, BMP7 contributes to primordial follicle activation and also to the primordial-to-primary follicle transition (11). Later in the follicle growth process, loss of *BMP6* expression should play a key role for the dominant follicle selection (12). BMP contribution to follicle selection is driven by suppressive

or stimulating effects on FSH action (11, 13, 14). In contrast to rodent, bovine, and ovine species, the role of BMPs in human folliculogenesis has been poorly investigated. BMP6 and BMP7 were shown to stimulate FSH receptor expression in normal human GCs (15, 16). In comparison with healthy ovulatory women, a decrease in *GDF9* expression was demonstrated in women with PCOS (10). In light of these data, disturbances at different stages of folliculogenesis may be due to alterations in the expression pattern of BMP genes in PCOS.

In this study, we have investigated the expression pattern of BMPs including BMP4, BMP6, and BMP7 and their receptors BMPR1A, BMPR1B, and BMPR2 in granulosa luteinizing cells from women affected by PCOS, which were compared with healthy ovulatory women. Moreover, the effects of BMPs on estradiol (E_2) production in cultured GCs from normally cycling and PCOS women were studied.

Materials and methods

Patients

This prospective study concerned women undergoing IVF, women with anovulatory PCOS ($n=8$), and women with healthy ovulatory function undergoing IVF for tubal and/or male infertility ($n=6$). Their age ranged between 20 and 35 years. According to Rotterdam criteria 2003, the diagnosis of PCOS was based on the association of two out of three of the following criteria: i) ovulatory dysfunction, oligomenorrhea, or amenorrhea; ii) more than 12 follicles in the 2–9 mm range in each ovary at ultrasonography and/or an ovarian volume >10 ml; and iii) clinical and/or biological hyperandrogenism. Exclusion criteria for control women were: an age >35 years; a history of menstrual disturbance, hirsutism, or endometriosis; high prolactin or androgen serum levels; FSH >10 IU/l at day 3 after menstrual bleeding; and PCOS ovarian pattern at ultrasonography.

All patients gave informed consent before their inclusion in the study. This study was approved by the Institutional Review Board of the University Hospital of Caen. This work is supported by Program Hospitalier de Recherche Clinique (PHRC), France.

Stimulation protocol

Follicular growth was stimulated by a s.c. injection of recombinant FSH (rFSH; Gonal-F; Merck-Serono) at 150–225 IU/day on the second day of the menses induced by progesterone (Duphaston; Abbott). Follicular growth was monitored by serum E_2 level and transvaginal ultrasonography. A GnRH antagonist (Cetrotide; Merck-Serono) was administered i.m. at dose 0.25 mg/day (multiple dose protocol) starting from the day when the dominant follicles reached a mean diameter 14 mm and/or when peripheral E_2 level

reached 700 pg/ml and was repeated until the day of i.m. administration of human chorionic gonadotropin (hCG). hCG (5000 IU i.m.) was administered when at least three follicles reached a mean diameter of 18 mm. Oocyte retrieval was performed 36 h later under transvaginal ultrasound guidance.

Isolation of GCs

After oocyte retrieval, all follicular fluids from each patient were pooled and stored in a tube. Cells were collected by centrifugation for 10 min at 300 *g*. The cell pellet was resuspended in 1 ml minimal essential medium (PAN Biotech, Aidenbach, Germany), supplemented with 100 IU/ml penicillin, 100 µg/ml streptomycin, and 2 µg/ml amphotericin B. The suspension overlaid on 3 ml Ficoll (PAN Biotech) was centrifuged at 600 *g* for 5 min. Granulosa–luteinized cells were aspirated from the interface, washed with medium, and counted on a hemacytometer. One part of the cells was used for culture and the rest were stored at -80°C until mRNA and proteins analysis.

RNA extraction and RT

Total mRNA was extracted from GCs using the kit Tri Reagent (Sigma–Aldrich). For each sample, RT was performed in a total volume of 40 µl with 1 µg RNA, 0.2 µg oligo-dT as primers in the presence of dNTP (500 µg; Promega), RNasin (20 IU; Promega), and Moloney murine leukemia virus reverse transcriptase (200 IU; Promega) for 1 h at 42°C .

RT-PCR analysis

The cDNA obtained was used to evaluate the BMPs' mRNA profile expression in GCs. Amplification was performed at 94°C for 45 s for denaturing, at 60°C for 45 s for annealing, and at 72°C for 1 min for extinction with 1.5 IU Taq DNA polymerase (Promega) in a buffer containing 200 mmol/l dNTP, 1.5 mmol/l MgCl_2 , and 25 pmol of each primer (Eurogentec, Liege, Belgium) in a total volume of 50 µl. Thirty-three cycles of PCR for FSH receptor and actin were performed. Primers used in supplemental data are summarized in Table 1.

Real-time RT-PCR analysis

Evaluation of gene expression levels was achieved by real-time quantitative PCR using SYBR Green. Real-time PCR was performed with 3 µl appropriate cDNA using GoTaq qPCR Master Mix (Promega) according to the manufacturer's instruction; forward- and reverse-specific primers for human BMP4, BMP6, BMP7, BMPR1A, BMPR1B, and BMPR2 (Supplementary Table 1, see section on supplementary data given at the end of this article) in a Stratagene Mx 3005P (Agilent

Table 1 Clinical parameters in normo-ovulatory controls and patients with PCOS. Values are median with 10th–90th percentiles (in parentheses).

	Controls	PCOS patients	P value
<i>n</i>	6	8	
Age (years)	29.5 (26–33)	29.5 (23–32)	0.85
BMI (kg/m ²)	21.7 (18–23)	25.4 (19–41)	0.1
rFSH totals (IU)	1275 (1065–3690)	1350 (930–1470)	0.57
Number of follicles > 14 mm	9 (4–12)	10 (3–19)	0.4
Number of follicles < 14 mm	12 (1–16)	18 (3–30)	0.08

Technologies, Santa Clara, CA, USA). The PCR protocol used an initial denaturing step at 95 °C for 3 min followed by 40 cycles at 95 °C for 15 s and annealing at 61 °C for 1 min. Results were analyzed using Mxpro v4.10 Software (Stratagene). Relative gene expression was calculated as a ratio of each target concentration to housekeeping gene actin concentration. The identity of all PCR amplicons was confirmed by sequencing.

Immunohistochemistry

Cells were mounted and fixed in 4% paraformaldehyde on slides precoated with polylysine. All the primary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and included exclusively those proven suitable for immunohistochemistry by the manufacturer. The primary antibodies included goat polyclonal antibodies against BMP4, BMP6, BMP7, BMPRI1A, BMPRI2, and aromatase (Supplementary Table 2, see section on supplementary data given at the end of this article). The secondary antibodies used were coupled with FITC for BMP7 and with Texas red for aromatase. Control staining was performed on adjacent serial sections and consisted in replacing the primary antibody with PBS–BSA 3%. Slides were mounted with mounting medium with DAPI (Santa Cruz Biotechnology). Images were captured using a confocal microscope (Olympus Fluo View FV 1000).

Primary cell culture

GCs were cultured in a 24-well plate with 0.5 ml medium (Milieu Essential Minimum de Eagle, Dutcher, Brumath, France) supplemented with SVF at 37 °C in an atmosphere of 5% CO₂ in air. After 72-h culture, 10^{−7} M androstenedione, a substrate for P450 aromatase, was added to the media. GCs were cultured in the presence or absence of 500 mUI/ml FSH and in the presence or absence of BMP4, BMP6, or BMP7. BMP4, BMP6, and BMP7 were purchased from Sigma–Aldrich Co. Ltd. The dose of BMP used was 50 ng/ml as described previously in rat GCs (17). After 48 h of culture, the supernatant of culture media was collected and stored at −20 °C until assay for E₂.

Assessment of E₂ production

The level of E₂ was measured in the 10 µl culture media by a RIA (P.A.R.I.S antibodies). The within-assay coefficient of variation (CV) was 5%, the between-assay CV was 10%, and sensitivity of estrogen assay was 60 pg/ml.

Statistical analysis

Because values from any parameter were not normally distributed, all comparisons between PCOS and control were performed using the nonparametric Mann–Whitney *U* test. Univariate analysis of correlation between expressions of the different parameters was performed with the nonparametric Spearman's test (Sigma Stat. V3.5, SYSTAT, Chicago, IL, USA).

Results

Clinical details: Patients

The main clinical parameters from healthy ovulatory women and from patients with PCOS are summarized in Table 1. No differences were found between both groups for age, BMI, and the total dose of rFSH received by each patient. Small follicles were more numerous in PCOS ovaries but this difference was not significant.

BMPs and BMP receptor gene expression in human GCs from normal cycling women

BMP2, BMP4, BMP5, BMP6, BMP7, and BMP8A mRNA expressions were detected in GCs using PCR techniques (Fig. 1). No signal was found for *GDF9* and *BMP15* (data not shown). These results were confirmed for BMP4, BMP6, and BMP7 by immunohistochemistry (Fig. 2). Co-localization of aromatase and BMP7 confirmed that BMP7 was actually synthesized by human GCs (Fig. 2).

The activin receptor-like kinases 2 (*ALK2* (*ACVR1*)), *ALK5*, *BMPRI1A*, *BMPRI1B*, and *BMPRI2* receptors mRNA were also detected in GCs (Fig. 1) and the

Figure 1 mRNA detection of *BMP4*, *BMP8A*, *BMP2*, *BMP5*, *BMP6*, *BMP7*, *ALK2*, *BMPRI1A*, *ALK5*, *BMPRI1B* and *BMPRI2* in granulosa cells by RT-PCR. MW, Molecular Weight markers 100 pb DNA Ladder (Promega).

Figure 2 Cells were immunostained with FITC (green) for BMP4 (C), BMP6 (D), BMPRI (E), BMPRII (F), BMP7 (G) and nuclei were counterstained with DAPI (blue) and examined using confocal microscopy. Cells were immunostained for both aromatase (Texas red) and BMP7 (FITC) (H). The controls for FITC (A) and Texas red (B) staining were realized without primary antibodies. Full colour version of this figure available via <http://dx.doi.org/10.1530/EJE-12-0891>

presence of proteins BMPRI and BMPRII was confirmed by immunohistochemistry (Fig. 2).

Differential expression level of BMP4, BMP6, and BMP7 and their receptors in GCs from normal cycling women

All BMP expression levels were expressed as the ratio of BMP mRNA to actin mRNA. We observed different expression levels of the different BMPs and their

receptors in GCs from normal cycling women. BMP6 was predominantly expressed in comparison with BMP4 and BMP7 (233 ± 11 vs 16 ± 4 and 2 ± 1 respectively, $P < 0.01$). The BMPRII receptor was predominantly expressed (1570 ± 318) compared with the BMPRII (460 ± 110) (Fig. 3).

Expression level of BMPs and their receptors in GCs from PCOS women

BMP6 expression determined by quantitative RT-PCR was higher in GCs from PCOS women compared with healthy women ($P = 0.01$; Fig. 4B). BMPRII expression was also higher in GCs from PCOS women compared with healthy women ($P < 0.05$; Fig. 4D). Otherwise, no significant differences were observed between both groups for BMP4, BMP7, BMPRII, and BMPRII expression levels (Fig. 4A, C, E and F). The BMP6 mRNA and BMPRII mRNA levels were strongly and positively correlated in healthy women and PCOS women (Spearman's coefficient $R = 0.752$, $P < 0.01$). Moreover, no significant difference between healthy women and PCOS women was found in the mRNA expression level for aromatase gene (7221 ± 4664 vs $15\,546 \pm 2734$), FSH receptor (70.7 ± 81.9 vs 50.6 ± 66.5), and in the progesterone production by GCs (164.9 ± 116.8 pg/50 000 cells vs 226.3 ± 176.3 pg/50 000 cells).

Effect of BMPs on basal and FSH-stimulated estrogen production in cultured GCs from healthy ovulatory and PCOS women

Basal E_2 production by cultured GCs was threefold higher in PCOS women compared with control women (108 ± 22.4 vs 380 ± 45.9 pg/50 000 cells respectively, $P < 0.01$; Fig. 5). Although absolute E_2 production levels after FSH stimulation were similar in both groups, the FSH-induced E_2 increase from baseline was weaker in cells from PCOS compared with healthy women (1.5- vs 3-fold increase respectively; Fig. 5). In cultured cells obtained from normally cycling women, BMP6 and BMP7 but not BMP4 decreased basal E_2 production by GCs from healthy women whereas BMPs did not affect basal E_2 production in PCOS women (Fig. 6A). FSH-stimulated E_2 production by GCs from control ovary was not affected by BMP, whereas in PCOS ovary a dramatic decrease in FSH-stimulated E_2 production was observed in the presence of BMP4 and BMP6 (Fig. 6B).

Discussion

This study is the first to explore the expression pattern of the BMP family in human GCs. For this purpose, we screened mRNA expression of physiologically relevant BMPs and their membrane receptors in human GCs from healthy ovulatory women and from anovulatory PCOS women.

Figure 3 Expression levels of BMPs and their receptors in granulosa cells from women. mRNA expression is quantified with actin as housekeeping gene. Data expressed as the ratio of BMP mRNA to actin mRNA are the mean $\pm$ S.E.M. of six healthy patients. BMP values are compared with BMP4 value and BMP receptors values are compared with BMPR1A. * $P < 0.05$ and ** $P < 0.01$.

BMPs are the largest subfamily from the TGF β superfamily. BMPs were initially identified in bone and cartilage (18, 19, 20) and thereafter in other tissues including the ovary (12, 21). These proteins were shown to regulate several key biological processes in the ovary including cell proliferation, differentiation, apoptosis, and steroidogenesis. Alterations in the BMPs system may be involved in human folliculogenesis disorders such as PCOS that represents the more prevalent endocrine disturbance in women of reproductive age.

We describe for the first time the expression pattern of the BMP in human granulosa–luteinized cells using a RT-PCR approach. We demonstrate that BMP2, BMP4, BMP5, BMP6, BMP7, and BMP8A and their BMPR1A, BMPR1B, and BMPR2 receptors are expressed in ovarian GCs from healthy ovulatory women.

Several of our data may be highlighted: first, BMP4 and BMP7 are expressed by human GCs, in contrast to rodents that exclusively express these proteins in theca cells (21). BMP7 synthesis by GCs was confirmed by immunohistochemistry experiments showing the co-localization of BMP7 and aromatase in GCs (22). Such results illustrate the species specificity of the expression pattern of BMP proteins. We were not able to identify any expression of *GDF9* and *BMP15* in GCs by RT-PCR. Our data do not confirm previous reports showing an expression of *GDF9* in human GCs by an approach using nonspecific immunohistochemistry techniques (23). However, these discrepancies may be explained by the effect of the stimulation protocol used.

The study was also designed to investigate the influence of PCOS on the BMP expression. BMP6 and BMPR1A expression levels were higher in GCs from PCOS compared with normal healthy women. The difference in the number of small follicles in PCOS compared with ovary from healthy women may not fully explain BMP6 and BMPR1A overexpression as a

progesterone production of similar magnitude by GCs from PCOS and control follicles suggests the same degree of luteinization between both groups.

Our study is the first to report an overexpression of BMPs in PCOS. This result was independent of the IVF stimulation protocols as the same protocol was used in the two groups. There is evidence that PCOS involves a primary ovarian dysfunction (2) characterized by a two- to threefold increase in follicle number together with an arrest of growing follicles at the 2–5 mm stage (3), thus explaining the impairment of dominant follicle selection, a hallmark of PCOS pathogenesis. The mechanism of such follicular arrest in PCOS is not fully explained. Studies of the influence of growth factors such as insulin-like growth factor 1 (24), TGF β , and anti-Müllerian hormone (AMH) (25) on the follicle growth disturbance in PCOS were realized. Moreover, early appearance of LH receptor in GCs may drive the premature luteinization of GCs observed in PCOS (26).

Our data suggest that the BMP system may be a new protagonist involved in the etiology of the PCOS. BMP6 overproduction by GCs from PCOS ovary may be involved in the antral follicle accumulation and the anovulatory pattern observed in PCOS. In rodents, BMP6 inhibits FSH action on GCs by suppressing adenylate cyclase activity and by reducing progesterone production (14), which is essential for ovulation (27, 28, 29). The drop in BMP6 expression in GCs may be permissive at the time of dominant follicle

Figure 4 Box-and-whisker plots showing the BMP4 (A), BMP6 (B), BMP7 (C), BMPR1A (D), BMPR1B (E), and BMPR2 (F) mRNA levels (ratio of BMP mRNA to actin mRNA) in granulosa cells from eight PCOS patients and granulosa cells from six healthy patients. Horizontal small bars represent the 10th–90th percentile range and the boxes indicate the 25th–75th percentile range. The horizontal line in each box corresponds to the median. * $P < 0.05$.

Figure 5 Estrogen production by granulosa cells in six healthy women and eight PCOS women. After 72 h of culture, granulosa cells were cultured with 10^{-7} M androstenedione and in the presence or absence of 500 mUI/ml FSH. Results are expressed as mean of ratio of the control value (a, b and c represent a significant difference ($P < 0.05$)).

selection, and in PCOS women, *BMP6* overexpression may impair the process of selection. Indeed, in rat GC, *BMP6* was shown to inhibit FSH-dependent gene expression of LH receptors (14). Based on these findings, we hypothesized that the early appearance of LH receptor in preantral follicles of PCOS women (24) should be FSH-independent and therefore a *BMP6*-independent event. In opposition to rodent models, *BMP6* increases E_2 , androstenedione, and inhibin production in sheep (30). Taken together, these data suggest that an increase in *BMP6* expression may explain some features related to anovulation and the arrest of dominant follicle selection observed in PCOS women. An *in vitro* study by Shi *et al.* (15) found that FSH receptor, inhibin, activin subunits, and AMH were overexpressed by human GCs cultured in the presence of *BMP6*. In contrast with these results, we observed in this study no difference in FSH receptor expression between normal and PCOS GCs, which overexpressed *BMP6*. Other authors found that PCOS GCs originating from subsets of small and large follicles overexpressed FSH receptors in comparison with control cells, a finding of which significance is clouded by the larger FSH dose in the control group which may induce downregulation of FSH receptor (31). The difference observed in these studies may be explained by the stimulation protocol used.

BMP2, *BMP4*, and AMH bind *BMPR1A* with different degrees of affinity, which results in triggering the intracellular phosphorylation of Smad 1/5/8 and thus induces a signaling pathway responsible for BMP effects. *BMPR1A* mediates AMH effect on Smad 1 activation and P450 side-chain cleavage enzyme in Sertoli cell line (SMAT-1) (32). *BMP4* modulates FSH signaling in a way that inhibits basal and FSH-stimulated progesterone synthesis in the sheep (33). The enhanced expression of *BMPR1A* in PCOS GCs may contribute to

amplify the effect of *BMP4* and AMH in GCs. The increased expression of *BMP6* and *BMPR1A* observed in this study may act synergistically in the pathogenesis of anovulation in PCOS women, as suggested by the positive correlation observed between *BMP6* and *BMPR1A* overexpression in PCOS GCs.

We also performed the first functional studies based on the effect of BMPs on steroidogenesis in human cultured GC. In contrast with GCs from normal cycling women, in PCOS GCs, *BMP4* or *BMP6* induced a dramatic decrease in FSH-stimulated estrogen production while estrogen basal production was not affected. The overproduction of *BMP6* by PCOS GCs may not explain such an inhibitory effect, as *BMP4* and *BMP7* also exerted such an inhibitory effects despite lack of overexpression of these proteins in PCOS GCs.

These results suggest that *BMP6* overexpression in PCOS GCs may exert an inhibitory effect on FSH-stimulated E_2 production and so may induce some degree of resistance to FSH in PCOS women (34). This contrasts with the absence of such an effect in

Figure 6 Effect of BMP on (A) basal and (B) stimulated estrogen production in cultured granulosa cells in six healthy and eight PCOS women. After 72-h culture, granulosa cells were cultured with 10^{-7} M androstenedione, in the presence or absence of 500 mUI/ml FSH and in the presence or absence of 50 ng/ml *BMP4*, *BMP6*, or *BMP7*. Results are expressed as a ratio of the control value without BMP in healthy granulosa cells (a and b represent a significant difference ($P < 0.05$)).

GCs from normal cycling women and in rodent studies (11, 14). This inhibitory effect of BMP6 could affect aromatase activity as the levels of FSH receptor and aromatase mRNA were not modified in PCOS cells.

In the presence of FSH and BMP in cell culture medium, E₂ production level from PCOS GCs is similar to the unstimulated basal E₂ production level, highlighting the inhibitory effects of BMP on FSH-dependent steroidogenesis. In our study conditions, the basal E₂ level is threefold higher in PCOS compared with normal GCs as described previously by Mason *et al.* (35), suggesting that BMP effects are mediated mainly by FSH and that enhanced E₂ basal production in PCOS GCs is driven by another trigger independently from BMP.

In conclusion, the present data raise the question on the involvement of the BMP system in the pathogenesis of PCOS. The different expression patterns of BMP proteins observed in PCOS compared with normal ovaries suggest that gonadotropin stimulation of PCOS ovaries did not fully reverse the folliculogenesis disturbance present in PCOS, i.e. alterations of expression patterns of BMP proteins that may be involved in follicle development disruption. Further studies are needed to explore the mechanism involved in BMP overexpression in PCOS ovary and to study the signaling pathways involved in the BMP actions on human GCs.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/EJE-12-0891>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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