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Candidate genes for polycystic ovary syndrome are regulated by TGFβ in the bovine foetal ovary

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STUDY QUESTION: Could changes in transforming growth factor β (TGFβ) signalling during foetal ovary development alter the expression of polycystic ovary syndrome (PCOS) candidate genes leading to a predisposition to PCOS?

SUMMARY ANSWER: TGFβ signalling molecules are dynamically expressed during foetal ovary development and TGFβ1 inhibits expression of the androgen receptor (*AR*) and 7 (*INSR*, *C8H9orf3*, *RAD50, ERBB3*, *NEIL2*, *IRF1* and *ZBTB16*) of the 25 PCOS candidate genes in foetal ovarian fibroblasts *in vitro*, whilst increasing expression of the AR cofactor TGFβ-induced transcript 1 (*TGFB111* or Hic5).

WHAT IS KNOWN ALREADY: The ovarian stroma arises from the mesonephros during foetal ovary development. Changes in the morphology of the ovarian stroma are cardinal features of PCOS. The ovary is more fibrous and has more tunica and cortical and subcortical stroma. It is not known why this is and when this arises. PCOS has a foetal origin and perhaps ovarian stroma development is altered during foetal life to determine the formation of a polycystic ovary later in life. PCOS also has a genetic origin with 19 loci containing 25 PCOS candidate genes. In many adult tissues, TGFβ is known to stimulate fibroblast replication and collagen deposition in stroma, though it has the opposite effect in the non-scaring foetal tissues. Our previous studies showed that TGFβ signalling molecules [TGFβs and their receptors, latent TGFβ binding proteins (LTBPs) and fibrillins, which are extracellular matrix proteins that bind LTBPs] are expressed in foetal ovaries. Also, we previously showed that TGFβ1 inhibited expression of *AR* and 3 PCOS candidate genes (*INSR*, *C8H9orf3* and *RAD50*) and stimulated expression of *TGFB111* in cultured foetal ovarian fibroblasts.

STUDY DESIGN, SIZE, DURATION: We used *Bos taurus* for this study as we can ethically collect foetal ovaries from across the full 9-month gestational period. Foetal ovaries (62–276 days, n = 19) from across gestation were collected from pregnant *B. taurus* cows for RNA-sequencing (RNA-seq) analyses. Foetal ovaries from *B. taurus* cows were collected (160–198 days, n = 6) for culture of ovarian fibroblasts.

PARTICIPANTS/MATERIALS, SETTING, METHODS: RNA-seq transcriptome profiling was performed on foetal ovaries and the data on genes involved in TGFβ signalling were extracted. Cells were dispersed from foetal ovaries and fibroblasts cultured and treated with TGFβ1. The effects of TGFβ regulation on the remaining eight PCOS candidate genes not previously studied (*ERBB3*, *MAPRE1*, *FDFT1*, *NEIL2*, *ARL14EP*, *PLGRKT*, *IRF1* and *ZBTB16*) were examined.

MAIN RESULTS AND THE ROLE OF CHANCE: Many TGFβ signalling molecules are expressed in the foetal ovary, and for most, their expression levels increased accross gestation (*LTBP1/2/3/4*, *FBN1*, *TGFB2/3*, *TGFBR2/3* and *TGFB111*), while a few decreased (*FBN3*, *TGFBR3L*, *TGFBI* and *TGFB1*) and others remained relatively constant (*TGFBRAP1*, *TGFBR1* and *FBN2*). TGFβ1 significantly decreased expression of PCOS candidate genes *ERBB3*, *NEIL2*, *IRF1* and *ZBTB16* in cultured foetal ovarian fibroblasts.

LARGE SCALE DATA: The FASTQ files, normalized data and experimental information have been deposited in the Gene Expression Omnibus (GEO) accessible by accession number GSE178450.

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LIMITATIONS, REASONS FOR CAUTION: Regulation of PCOS candidate genes by TGFβ was carried out *in vitro* and further studies *in vivo* are required. This study was carried out in bovine where foetal ovaries from across all of the 9-month gestational period were available, unlike in the human where it is not ethically possible to obtain ovaries from the second half of gestation.

WIDER IMPLICATIONS OF THE FINDINGS: From our current and previous results we speculate that inhibition of TGFβ signalling in the foetal ovary is likely to (i) increase androgen sensitivity by enhancing expression of *AR*, (ii) increase stromal activity by stimulating expression of *COL1A1* and *COL3A1* and (iii) increase the expression of 7 of the 25 PCOS candidate genes. Thus inhibition of TGFβ signalling could be part of the aetiology of PCOS or at least the aetiology of polycystic ovaries.

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Key words: PCOS / stroma TGFβ signalling molecules / ovary development / PCOS candidate genes / regulation / extracellular matrix / RNA-seq / gene expression

Introduction

Polycystic ovary syndrome (PCOS) is a complex reproductive and metabolic disorder with multiple clinical symptoms including psychological consequences (Teede *et al.*, 2010). The syndrome affects around 1 in 10 women, presenting with symptoms associated with excess androgen, reproductive dysfunction and metabolic complications such as insulin resistance, gestational diabetes, central adiposity, obesity, non-alcoholic fatty liver disease, dyslipidaemia and cerebro- and cardiovascular diseases among others (Norman *et al.*, 2007; March *et al.*, 2010; Azziz *et al.*, 2016; Rodgers *et al.*, 2019; Stepto *et al.*, 2019; Berni *et al.*, 2021).

The aetiology of PCOS is not well established. However, a possible foetal predisposition has been observed in numerous studies over the past decades. For example, babies born with congenital adrenal hyperplasia, which causes elevated androgen exposure in foetal life, develop some of the features of PCOS in adulthood (Barnes *et al.*, 1994). Androgen treatment of pregnant animals also produces a phenotype of PCOS features in the offspring (Abbott *et al.*, 2006; Walters, 2016; Tata *et al.*, 2018; Risal *et al.*, 2019; Aflatounian *et al.*, 2020; Stener-Victorin *et al.*, 2020). More so, a low ponderal index (kg/m^3) of children at birth has also been associated with the risk of all three cardinal PCOS symptoms (menstrual dysfunction, hyperandrogenism and polycystic ovaries) in later life (Davies *et al.*, 2012).

PCOS also has a genetic predisposition with large genome-wide association studies and familial microsatellite linkage studies having identified a number of loci associated with PCOS (Legro *et al.*, 1998; Urbanek, 2007; Chen *et al.*, 2011; Goodarzi *et al.*, 2012; Shi *et al.*, 2012; Kosova and Urbanek, 2013; Louwers *et al.*, 2013). There are about 25 genes in or near these loci. We recently identified that PCOS candidate genes in these loci were not differentially expressed in adult human PCOS ovaries (Liu *et al.*, 2020) but were dynamically expressed in developing human and bovine foetal ovaries (Hartanti *et al.*, 2020; Liu *et al.*, 2020). We concluded that expression of PCOS candidate genes in foetal ovaries if perturbed or dysregulated may underpin development of PCOS (Hartanti *et al.*, 2020; Liu *et al.*, 2020).

There are some other unique features of PCOS. PCOS ovaries not only have many antral follicles but they have an expanded and very fibrous stroma (Stein and Leventhal, 1935; Hughesdon, 1982). During foetal development, the ovarian stroma is initially derived from stroma that penetrates into the ovarian primordium from the mesonephros during the first trimester (Hummitzsch *et al.*, 2013, 2015, 2018). It expands the most during early gestation due to high levels of stromal cell replication (Hartanti *et al.*, 2019; Hummitzsch *et al.*, 2019). The causes or consequences of expanded fibrous stroma in PCOS ovaries are still unknown. It is possible that either the stroma is altered in a peculiar way during development of the ovary to enable it to expand and become fibrous in later life or that the adult PCOS ovaries have just became fibrotic due to fibrosis. Fibrosis is usually a wound healing event where death of specialized cells occurs and replaced by stroma and collagen.

Growth of stroma in many organs is usually driven by the growth factor transforming growth factor β (TGFβ) (Hatzirodos *et al.*, 2011). In adult tissues TGFβ stimulates replication and production and deposition of collagen in stroma, particularly in fibrotic tissues (Verrecchia and Mauviel, 2004). In stroma, TGFβ activity is regulated by the extracellular matrix fibrillins (Kielty *et al.*, 2002). Fibrillins achieve this by binding the latent TGFβ binding proteins (LTBPs). There are three fibrillin genes, four LTBPs, three TGFβs and three TGFβ receptors. We previously found that TGFβ signalling molecules (TGFβs, LTBPs, fibrillins and TGFβ receptors) are expressed in foetal ovaries (Hatzirodos *et al.*, 2011, 2019). Their levels of expression were either constant (*TGFB1*, *TGFBR1*), increasing (*TGFB2*, *TGFB3*, *TGFBR2*, *TGFBR3*, *LTBP1*, *LTBP2*, *LTBP3*, *LTBP4*) or decreasing (*FBN2*, *FBN3*) across gestation (Hatzirodos *et al.*, 2011, 2019). We also showed that TGFβ inhibited expression of androgen receptor (*AR*) and 3 PCOS candidate genes (*INSR*, *C8H9orf3* and *RAD50*) and stimulated expression of *TGFB1I1* (transforming growth factor beta 1-induced transcript 1) in cultured foetal ovarian stroma cells (Hartanti *et al.*, 2020). This suggests that TGFβ signalling is dynamic during foetal ovarian development and could be an important part of the aetiology of PCOS,

providing a link between the genetic basis and foetal predisposition to PCOS.

Circulating levels of TGFβ2 were found to be correlated with androgen levels in women (Raja-Khan *et al.*, 2014) and skeletal muscles in adult women with PCOS have higher expression levels of genes controlled by TGFβ and they produce more collagens (Stepto *et al.*, 2020). In adipose tissue of PCOS women, TGFβ1 was identified as the master upstream regulator (Dumesic *et al.*, 2019). In order to further our knowledge of TGFβ and foetal ovary development, we examined the regulation of eight additional PCOS candidate genes and the expression of TGFβ signalling molecules during foetal ovary development using RNA-sequencing (RNA-seq) analysis.

Materials and methods

Ethical approval for the study

Ethical approval was not required for this study, as foetuses and foetal ovaries were scavenged from animals being processed for human consumption and were not owned by the authors or their institutions. As such the University of Adelaide's Animal Ethics Committee only requires notification of this.

Collection of bovine foetal ovaries

For RNA-seq, foetal ovarian pairs across gestation (62–276 days, n = 19) were collected from pregnant *Bos taurus* cows at the abattoir of Midfield Meat International, Warrnambool, Victoria, Australia and were immediately frozen on dry ice on site and later stored in the laboratory at −80°C.

For TGFβ1 treatment, foetuses from pregnant *B. taurus* cows were collected at local abattoirs (Thomas Foods International, Murray Bridge, SA, Australia and Strath Meats, Strathalbyn, SA, Australia) and transported on ice in Hank's balanced-salt solution (HBSS) with calcium and magnesium ($HBSS^{+/+}$) to the laboratory. The gestational ages of all foetuses were estimated from the crown-rump length (CRL) (Russe, 1983). Foetuses with a CRL <8 cm underwent sex determination as previously reported by Hummitzsch *et al.* (2013).

RNA extraction and RNA-seq

Using the Mo Bio Powerlyser 24 (Mo Bio Laboratories Inc., Carlsbad, CA, USA) and 1 ml Trizol® (Thermo Fisher Scientific, Waltham, MA, USA), whole foetal bovine ovaries were homogenized and RNA extracted according to manufacturer's instructions. DNase I (Promega/Thermo Fisher Scientific Australia Pty Ltd, Tullamarine, VIC, Australia) was used to treat all samples. The RNA concentration and quality were then determined using the Experion™ RNA StdSens Analysis kit and the Experion™ Automated Electrophoresis System (Bio-Rad Laboratories Pty., Ltd., Gladesville, NSW, Australia). Of total RNA, 500 ng/50 µl per well (96-well plate) of each sample was used for RNA-seq.

RNA-seq based transcriptome profiling was performed at the SAHMRI Genomics Facility (SAHMRI, Adelaide, SA, Australia). Briefly, single-end poly A-selection mRNA libraries (~35 M reads per sample) were created using the Nugen Universal Plus mRNA-Seq library kit from Tecan (Mannedorf, Switzerland) and sequenced with an Illumina Nextseq 500 using single read 75 bp (v2.0) sequencing chemistry (Illumina Inc., San Diego, CA, USA). Two sequencing runs, with 10 samples per run, were performed and a sample (15/R43t, 135 days gestational age) was used as internal control in both runs.

RNA-seq data analysis using Partek flow

The raw data containing FASTQ files were uploaded to Partek Flow® Software, version 8.0 (Partek Incorporated, St. Louis, MO, USA). All samples underwent a pre-alignment quality assessment and showed Phred Quality Scores larger than 30. The reads were aligned and annotated to the bovine genome ARS-UCD1.2 (bosTau9; https://www.ncbi.nlm.nih.gov/assembly/GCF_002263795.1/) using STAR 2.7.3a aligner (>97% alignment rate for all samples) and Partek E/M, respectively. The FASTQ files, normalised data and experimental information have been deposited in the Gene Expression Omnibus (GEO) (Barrett *et al.*, 2009), accessible by accession number GSE178450. The expression of TGFβs, LTBPs, fibrillins and TGFβ receptors were analysed using Partek Flow® Software (version 8.0). Scatter plots showing the expression patterns for fibrillins, LTBPs, TGFβs and TGFβ receptors across gestation were generated using GraphPad Prism version 8 (GraphPad Software Inc., La Jolla, CA, USA). Pearson's correlation of the genes with gestational age as well as with each other were further analysed.

Treatment of bovine foetal fibroblasts with TGFβ1

Foetal fibroblasts (160–198 days, n = 6) were cultured and treated with 5 ng/ml or 20 ng/ml TGFβ1 (R&D Systems) as previously described by Bastian *et al.* (2016). Briefly, bovine foetal fibroblasts were seeded at 30 000 cells/well in 24-well plates in DMEM/F12 medium containing 5% (v/v) foetal calf serum, 100 IU/ml penicillin, 0.01% streptomycin sulfate (GIBCO, Carlsbad, CA, USA, 15140122) and 0.1% fungizone at 38.5°C and 5% CO_2. At 60–70% confluency, cells were treated with 5 ng/ml or 20 ng/ml of TGFβ1 for 18 h in DMEM/F12 medium containing 1% foetal calf serum and then harvested and stored at −80°C for analysis.

RNA isolation, cDNA synthesis and quantitative real-time PCR

Bovine foetal fibroblasts were homogenized in 1 ml Trizol® (Thermo Fisher Scientific, Waltham, MA, USA) using the Mo Bio Powerlyser 24 (Mo Bio Laboratories Inc., Carlsbad, CA, USA) and RNA extracted according to manufacturer's instructions as reported previously (Bastian *et al.*, 2016). Complementary DNA was then synthesized from 200 ng of DNase-treated RNA using random hexamers (Sigma, Adelaide, SA, Australia) and 200 U Superscript Reverse Transcriptase III (Thermo Fisher Scientific, Waltham, MA, USA) as previously described (Hartanti *et al.*, 2020). Based on available RNA sequences in NCBI, PCR primers for eight newly discovered PCOS candidate genes (*ERBB3*, *MAPRE1*, *FDFT1*, *NEIL2*, *ARL14EP*, *PLGRKT*, *IRF1* and *ZBTB16*) were designed to span introns using Primer3 plus and Net primer software (PREMIER Biosoft Palo Alto, CA, USA) and primers are listed in Table I. Primer combinations were tested as previously described (Hummitzsch *et al.*, 2013; Liu *et al.*, 2020). The amplification conditions were 95°C for 15 s, then 60°C for 60 s for 40 cycles using Rotor

Table I List of genes and primers used for qRT-PCR.

Gene name	Gene symbol	Primers (5′→3′) (F = forward, R = reverse)	Accession number	Size (bp)
Ribosomal protein L32	*RPL32*	F: GCCATCAGAATCACCAATCC R: AAATGTGCACACGAGCTGTC	NM_001034783.2	73
Peptidylprolyl isomerase A (cyclophilin A)	*PPIA*	F: CTGGCATCTTGTCCATGGCAAA R: CCACAGTCAGCAATGGTGATCTTC	NM_178320.2	202
ADP ribosylation factor like GTPase 14 effector protein	*ARL14EP*	F: ACCTGGTTGGAAGCTTTGTC R: TTCTGCCGGTCTTCAGAATC	NM_001031761.3	78
Erb-B2 receptor tyrosine kinase 3	*ERBB3*	F: TGGTCATGGTCAAGTGTTGG R: CATCCTGGTGAACTCATTGG	NM_001103105.1	80
Farnesyl-diphosphate farnesyltransferase 1	*FDFT1*	F: CAAGGAAAAGGACCGACAAG R: ACGCGCTTATCCAGAAACTC	NM_001013004.1	144
Interferon regulatory factor 1	*IRF1*	F: AAGGATGCCTGTCTGTTTCG R: CAATATCTGGCAGCGAGTTC	NM_001191261.2	127
Microtubule associated protein RP/EB family member 1	*MAPRE1*	F: AGGCCCATTACAACACACAG R: TTCAGCTGCTTCGTCATCTC	NM_001075334.2	102
Nei like DNA glycosylase 2	*NEIL2*	F: CGAAGAAGGCAAACAAGAGG R: AAGAGAAGCGCCATGTCATC	NM_001013003.1	117
Plasminogen receptor with a C-terminal lysine	*PLGRKT*	F: TCCCGACTTCAGTTGGAAAG R: ACCAAGCAATCTGCATAGCC	NM_001034426.2	79
Zinc finger and BTB domain containing 16	*ZBTB16*	F: CACTCAGCGGGTGCCAAAG R: TTCCCACACAGCAGACAGAAG	NM_001037476.2	131

Gene 6000 cycler (Q series, Qiagen GmbH, Hilden, Germany). Ct values were determined using Rotor Gene 6000 software at a threshold of 0.05 normalized fluorescent unit. Gene expression values were determined using $2^{-\Delta Ct}$ method and ribosomal protein L32 (*RPL32*) and peptidylprolyl isomerase A (*PPIA*) were used as housekeeping genes. The fold change of gene expression in foetal fibroblasts treated with the two different concentrations of TGFβ1 were presented as $2^{-\Delta\Delta Ct}$ data with the same housekeeping genes in $2^{-\Delta Ct}$ method.

Statistical analysis

All statistical analyses were carried out using Microsoft Office Excel 365 (Microsoft Redmond, WA, USA) and IBM SPSS Statistics for Windows, version 25 (IBM Corp., Armonk, NY, USA). Scatter plots and columns were made using GraphPad Prism version 8.00 (GraphPad Software Inc., La Jolla, CA, USA). One-way ANOVA with Dunnett's *post hoc* tests were conducted to compare the difference between the control and the TGFβ1 treatments of foetal fibroblasts.

Results

Expression of TGFβs, LTBPs, fibrillins, TGFβ receptors other molecules involved in TGFβ signalling

The expression of the TGFβ signalling molecules in foetal ovaries showed dynamic differences across gestation. The expression levels of most TGFβ signalling molecules significantly correlated with gestational age either positively (*LTBP1*, *LTBP2*, *LTBP3*, *LTBP4*, *FBN1*, *TGFB2*, *TGFB3*, *TGFBR2*, *TGFBR3* and *TGFB111*) or negatively (*FBN3*, *TGFBR3L*, *TGFBI* and *TGFB1*), while few showed no significant correlation with gestational age (*TGFBRAP1*, *TGFBR1* and *FBN2*). Also, almost all the TGFβ signalling molecules, with the major exception being *TGFBRAP1*, correlated either positively or negatively with each other (Table II), consistent with there being a regulated network.

The expression of all four *LTBP* genes increased across gestation (Fig. 1D–G). *LTBP3* and *LTBP4* expression levels plateaued at the second half of gestation or at the start of the third trimester, respectively. *FBN1* expression (Fig. 1A) increased across gestation and *FBN3* decreased significantly across gestation (Fig. 1C). *FBN2* also increased slightly across gestation (Fig. 1B).

Notably, *TGFB1* expression decreased across gestation until about the end of the second trimester and gradually increased during the third semester. The expression patterns of *TGFB2* and *TGFB3* increased across gestation (Fig. 2B and C). Although *TGFB3* expression increased across gestation, the expression levels were variable in the third trimester (Fig. 2C). The levels of *TGFBR1* expression increased gradually until about the end of the second semester and decreased during the third semester. *TGFBR2* and *TGFBR3* expression both increased across gestation without any decline in the third trimester (Fig. 2E and F).

The expression of other molecules involved in TGFB signalling such as *TGFBRAP1* (transforming growth factor beta receptor associated protein 1), *TGFBR3L* (transforming growth factor beta receptor 3 like), *TGFBI* (transforming growth factor beta-induced protein or βig-H3) and *TGFB111* were also studied. While *TGFBR3L* and *TGFBI* expression

Table II Pearson's correlation coefficients (R) between TGFβ signalling molecules mRNA expression levels and gestational age (62–276 days, n = 19) in bovine foetal ovaries.

	Mean Age	*FBN3*	*TGFBR3L*	*TGFBI*	*TGFB1*	*TGFBRAP1*	*TGFBR1*	*FBN2*	*TGFB3*	*LTBP4*	*LTBP1*	*LTBP3*	*FBN1*	*LTBP2*	*TGFBR3*	*TGFB2*	*TGFB1I1*	*TGFBR2*
FBN3	-0.90[d]																	
TGFBR3L	-0.86[d]	0.87[d]																
TGFBI	-0.78[d]	0.82[d]	0.72[c]															
TGFB1	-0.66[b]	0.52[a]	0.45	0.71[c]														
TGFBRAP1	0.36	-0.58[b]	-0.48[a]	-0.60[b]	0.01													
TGFBR1	0.41	-0.43	-0.31	-0.52[a]	-0.80[d]	-0.04												
FBN2	0.41	-0.38	-0.41	-0.50[a]	-0.61[b]	0.12	0.66[b]											
TGFB3	0.52[a]	-0.49[a]	-0.37	-0.51[a]	-0.57[a]	-0.05	0.47[a]	0.08										
LTBP4	0.53[a]	-0.53[a]	-0.36	-0.66[b]	-0.70[c]	0.13	0.63[b]	0.38	0.82[d]									
LTBP1	0.61[b]	-0.57[a]	-0.52[a]	-0.59[b]	-0.68[b]	0.05	0.72[c]	0.61[b]	0.61[b]	0.66[b]								
LTBP3	0.78[d]	-0.79[d]	-0.58[b]	-0.73[c]	-0.74[c]	0.28	0.65[b]	0.37	0.79[d]	0.87[d]	0.66[b]							
FBN1	0.89[d]	-0.77[c]	-0.76[c]	-0.71[c]	-0.77[c]	0.17	0.66[b]	0.70[c]	0.49[a]	0.54[a]	0.82[d]	0.71[c]						
LTBP2	0.90[d]	-0.76[c]	-0.81[d]	-0.65[b]	-0.53[a]	0.34	0.23	0.54[a]	0.22	0.26	0.44	0.52[a]	0.83[d]					
TGFBR3	0.91[d]	-0.77[c]	-0.78[d]	-0.69[b]	-0.71[c]	0.14	0.54[a]	0.46[a]	0.69[b]	0.62[b]	0.78[d]	0.77[c]	0.93[d]	0.77[b]				
TGFB2	0.93[d]	-0.80[d]	-0.80[d]	-0.72[c]	-0.76[c]	0.2	0.60[b]	0.63[b]	0.51[a]	0.54[a]	0.74[c]	0.74[c]	0.98[d]	0.86[d]	0.95[d]			
TGFB1I1	0.95[d]	-0.82[d]	-0.83[d]	-0.75[c]	-0.64[b]	0.34	0.3	0.48[a]	0.46[a]	0.52[a]	0.55[a]	0.71[c]	0.84[d]	0.92[d]	0.86[d]	0.90[d]		
TGFBR2	0.97[d]	-0.90[d]	-0.84[d]	-0.85[d]	-0.74[c]	0.41	0.54[a]	0.50[a]	0.563[a]	0.64[b]	0.65[b]	0.84[d]	0.89[d]	0.85[d]	0.91[d]	0.94[d]	0.92[d]	

Positive and negative correlations are marked in pink and blue, respectively. The colour intensity corresponds with the strength of the correlation. *P*-values: [a]<0.05; [b]<0.01; [c]<0.001; [d]<0.0001.

decreased across gestation (Fig. 3B and C), expression of *TGFB1I1* increased across gestation (Fig. 3D). Expression of *TGFBRAP1* was relatively constant across gestation (Fig 3A).

Regulation of PCOS candidate genes in foetal fibroblast

We also examined the effects of TGFβ1 on the PCOS genes in cultured foetal fibroblasts. Treatment with TGFβ1 had an effect on half of the eight genes (*ERBB3*, *MAPRE1*, *FDFT1*, *NEIL2*, *ARL14EP*, *PLGRKT*, *IRF1* and *ZBTB16*) studied. Significantly, TGFβ1 (20 ng/ml) decreased the expression of *ERBB3* (Fig. 4A), *NEIL2*, *IRF1* and *ZBTB16* (Fig. 4D, G and H), with the latter three also decreased by treatment with 5 ng/ml TGFβ1.

Discussion

In this study, we explored the expression of TGFβ signalling molecules (TGFβs, LTBPs, fibrillins and TGFβ receptors and other associated proteins) in bovine foetal ovaries across gestation using RNA-seq analysis. We also analysed the effects of TGFβ1 in regulating expression of PCOS candidate genes in cultured bovine foetal ovarian fibroblasts. Our results indicate that TGFβ signalling pathways operating in the foetal ovary should be considered contenders for involvement in at least some aspects of the aetiology of PCOS, especially the development of polycystic ovaries.

The current theories on the aetiology of PCOS strongly implicate androgens or androgen signalling during foetal development (Abbott *et al.*, 2006; Walters, 2016; Tata *et al.*, 2018; Risal *et al.*, 2019; Aflatounian *et al.*, 2020; Stener-Victorin *et al.*, 2020) and in particular androgen signalling in the brain (Cox *et al.*, 2020). The question has remained what would stimulate or initiate enhanced androgen signalling *in vivo*. As far as we are aware, there are no environmental androgens, if anything, many are anti-androgens. Recently, it has been suggested that AMH, which is elevated in PCOS women during pregnancy by about 2-fold, could elevate the levels of androgens and hence initiate the PCOS phenotype in their offspring (Tata *et al.*, 2018). However, AMH and androgens do not alter expression of any of the PCOS candidate genes in cultured foetal fibroblasts (Hartanti *et al.*, 2020; Liu *et al.*, unpublished results), but in contrast, TGFβ1 alters the expression of 7 out of 25 PCOS candidate genes as shown previously (Hartanti *et al.*, 2020) and in this study. Importantly, TGFβ also regulates the expression of *AR* and one of its cofactors, *TGFβ1I1* (Hartanti *et al.*, 2020). Members of the TGFβ signalling pathways are operative before and when the AR is expressed in the foetal ovary. The concept that TGFβ is involved in PCOS not only links the foetal and genetic predispositions to PCOS but it specifically links to the ovarian morphology of PCOS (Hatzirodos *et al.*, 2011). In adult tissues in general, TGFβ stimulates stromal fibroblast replication and collagen deposition but in foetal tissues, which do not scar in wound healing, it has the opposite effect (Rolfe *et al.*, 2007; Rolfe and Grobbelaar, 2012). This has also been demonstrated in the foetal ovarian fibroblasts where TGFβ inhibited *COL1A1* and *COL3A1* expression (Liu *et al.*, unpublished results). TGFβ also inhibited expression of *AR*. Thus, we speculate that inhibition of TGFβ signalling in the foetal ovary is likely to increase androgen sensitivity and stromal activity and thus this mechanism could be part of the aetiology of PCOS. It is also possible that such mechanisms act in other organs affected by PCOS such as skeletal muscle where in PCOS women expression levels of genes controlled by TGFβ are elevated, including collagens (Stepto *et al.*, 2020).

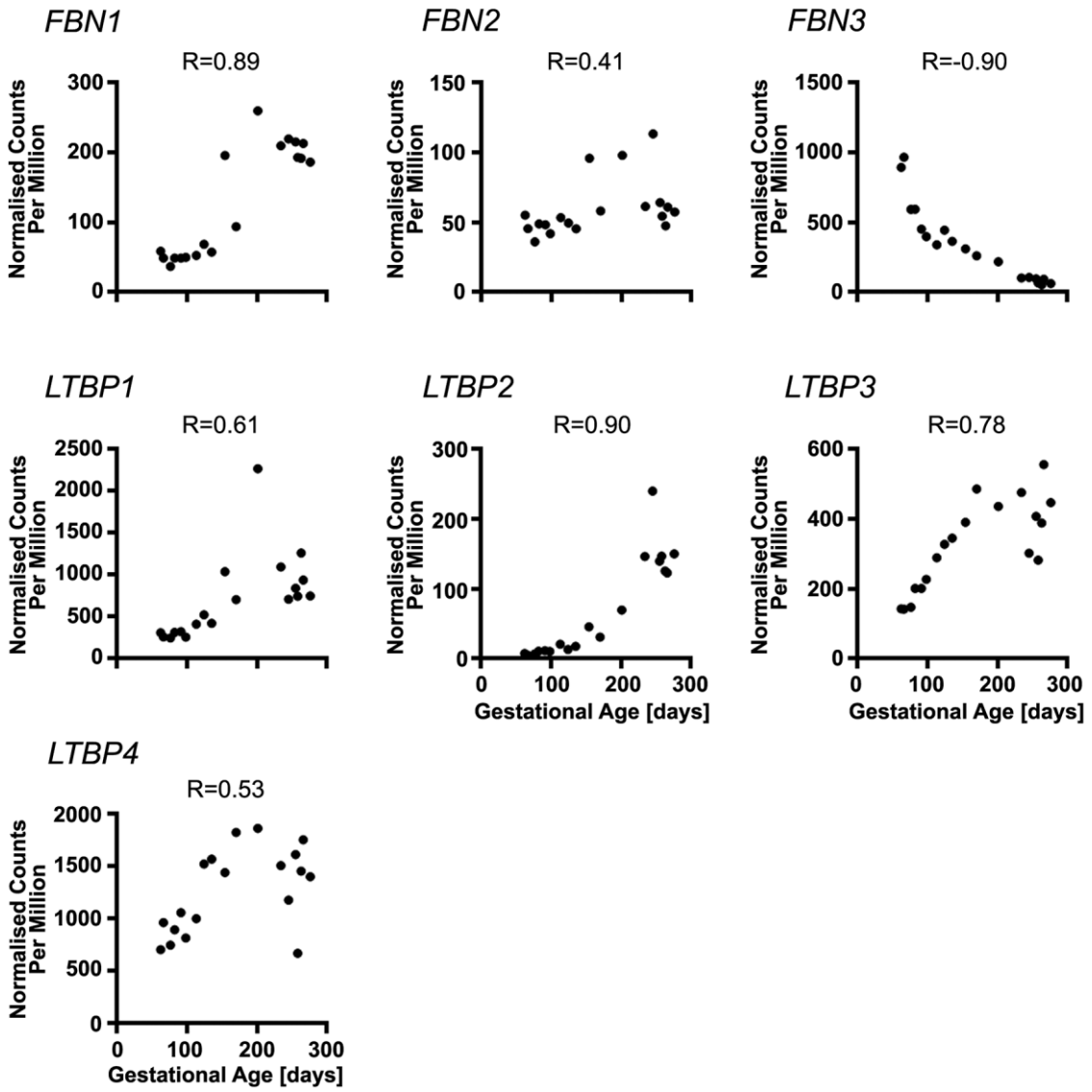

Figure 1. **Scatter plots showing the expression patterns of *FBN*s and *LTBP*s across gestation.**

In this study, we found that most of TGFβ signalling molecules significantly correlated negatively or positively with gestational age similar to our previous studies of bovine foetal ovaries using qRT-PCR (Hatzirodos *et al.*, 2011, 2019; Hartanti *et al.*, 2019). The expression of TGFβ signalling molecules in foetal and adult ovaries have also been studied in various species now including human, goat, sheep, pig and cattle (Chegini and Flanders, 1992; Nilsson *et al.*, 2003; Sriperumbudur *et al.*, 2010; Hatzirodos *et al.*, 2011, 2019; Rodrigues *et al.*, 2014; Hartanti *et al.*, 2019). Stromal growth factors play significant roles in embryonic gonadal development via extracellular matrix contributing significantly to cell growth, differentiation and development (Ingman and Robertson, 2002; Memon *et al.*, 2008). TGFβ signalling molecules are also known to contribute significantly to ovarian function in later life including follicle development (Oliver, 2016), granulosa proliferation (Dodson and Schomberg, 1987), differentiation of thecal-interstitial cells (Magoffin *et al.*, 1989) and antral follicle growth and follicle selection (Knight and Glister, 2006), among others.

The relationship between TGFβ and androgen signalling in general is complex as their regulation could be negative or positive depending on the various signals or environmental conditions (Qi *et al.*, 2008). Although TGFβ is positively regulated by androgen in certain cancer cell lines (Rosas *et al.*, 2021), TGFβ is known to inhibit androgen through interaction with Smad3 in different cells including thecal-interstitial cells, prostate and prostate epithelial cells (Kyprianou and Isaacs, 1988, 1989; Magoffin *et al.*, 1989; Chipuk *et al.*, 2002). Furthermore, studies focused on TGFβ1 deficiency in reproduction have received significant attention and have been reviewed in detail by Ingman and Robertson (2009). TGFβ1 is a ligand of the TGFβ superfamily, which is essential in development and cell differentiation (Wu and Hill, 2009; Zinski *et al.*, 2018). In adult bovine ovaries, TGFβ1

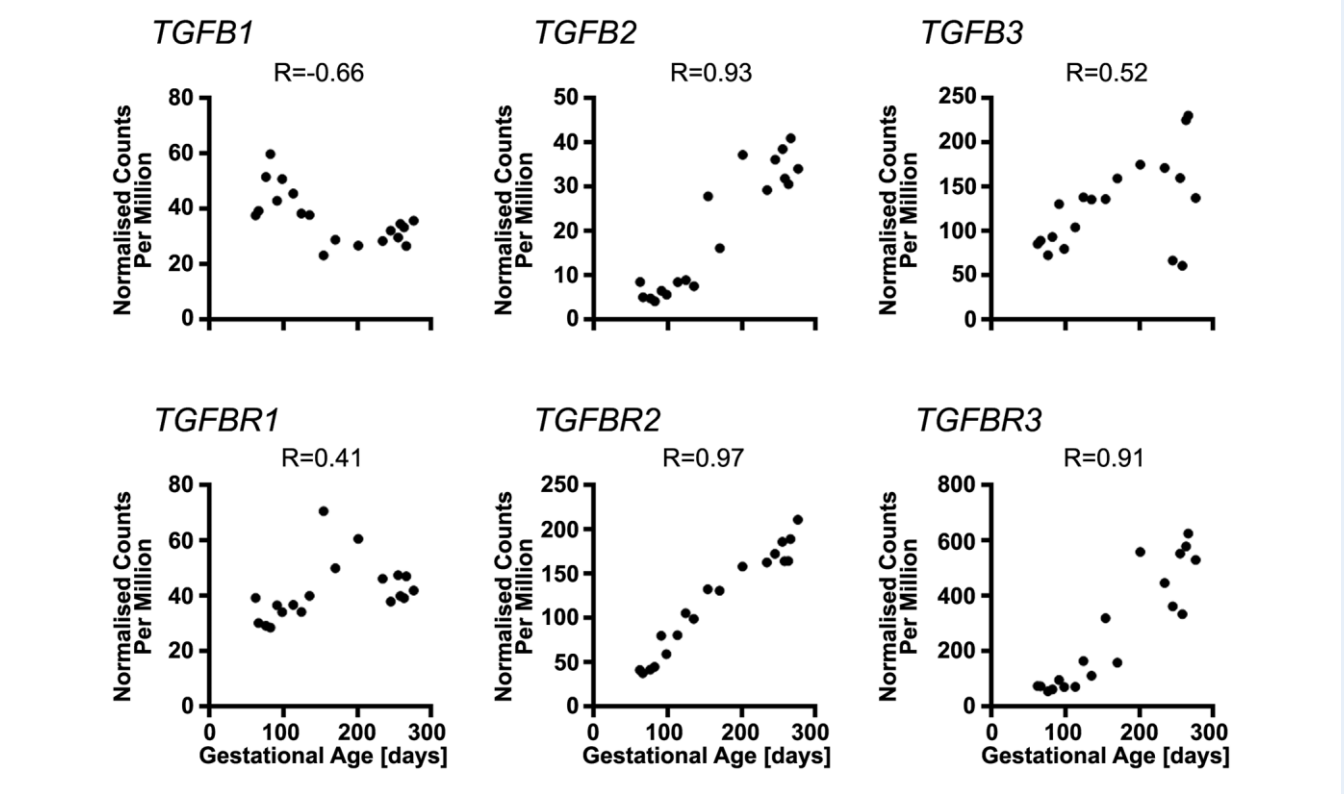

Figure 2. Scatter plots showing the expression patterns of *TGFBs* and *TGFBRs* across gestation.

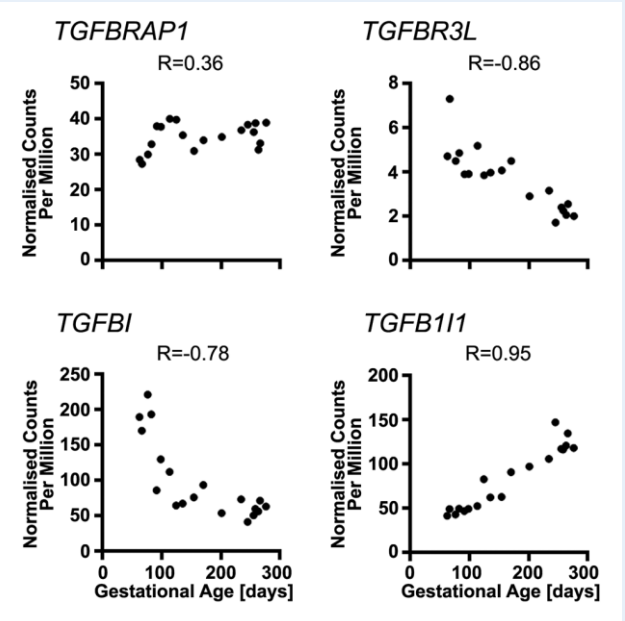

Figure 3. Scatter plots showing the expression patterns of other molecules involved in TGFβ signalling across gestation.

protein was detected in the granulosa cells of early pre-antral and early antral follicles (1–2 cm) but was not observed after these stages (Nilsson *et al.*, 2003). *TGFB1* null mice have perturbed hypothalamic–pituitary–gonadal axis function, which results in reduction in LH levels, further resulting in reduced serum androstenedione and testosterone production in males and oestrous cycle abnormalities in female mice (Ingman *et al.*, 2006; Ingman and Robertson, 2007). Notably, oocyte developmental incompetence due to a TGFβ1 deficient follicular environment has also been associated with the early embryo arrest in these mice (Ingman *et al.*, 2006; Ingman and Robertson, 2009). These observations further affirm the significance of TGFβ1 in ovarian functions and their possible association with PCOS.

Dysregulation of TGFβ signalling during foetal ovary development could involve a number of different members of the pathways and could vary across gestation. *FBN3*, *TGFBR3L*, *TGFBI* and *TGFB1* were highly expressed at the early stages of foetal ovary development and their levels significantly dropped as the ovary developed. It is during the early stages of ovarian development that stroma expansion predominantly occurs (Hartanti *et al.*, 2019). Thus, these genes are mostly expressed when the stroma of the mesonephros, containing fibroblasts, fibres and capillaries, penetrates into the gonadal ridge resulting in the formation of the ovigerous cords containing the gonadal ridge epithelial like (GREL) cells and oogonia. *TGFBR3L* was recently shown to bind inhibin B and null mice were found to have elevated levels of

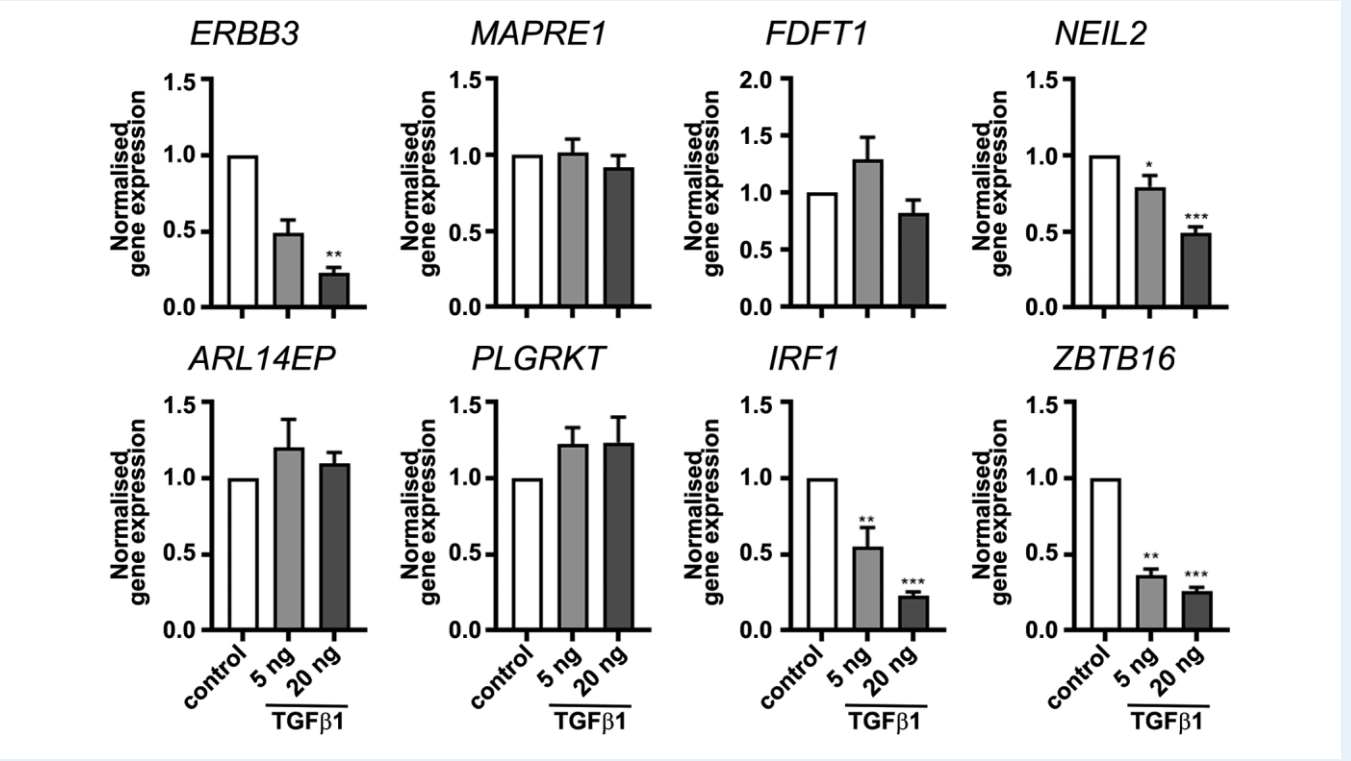

Figure 4. Normalized gene expression of eight newly studied polycystic ovary syndrome (PCOS) candidate genes in cultured foetal fibroblasts treated with transforming growth factor β-1 (TGFβ1). Fibroblasts from foetal ovaries (160–198 days, n = 6) were cultured in the presence of 5 or 20 ng/ml of TGFβ1 for 18 h. Fold changes of gene expression to the control groups are presented as mean ± SEM (normalized to *PPIA* and *RPL32*). Significant differences among groups were determined by one-way ANOVA with Dunnett's *post hoc* test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

FSH, antral follicles and corpora lutea (Brûlé *et al.*, 2021). *TGFBI*, previously named Beta Ig-H3 (*βig-H3*), has been studied over the past two decades. *TGFBI* is a secreted extracellular matrix protein, expressed in collagen-rich tissues in response to TGFβ. *TGFBI* is known to play significant roles in numerous physiological processes such as morphogenesis, extracellular matrix interactions, cell adhesion/migration and angiogenesis required for development; however, its functional contribution is poorly understood (Ferguson *et al.*, 2003; Thapa *et al.*, 2007). *TGFBI* polymorphisms have also been associated with levels of insulin and BMI (Park *et al.*, 2005) and its roles in physiological and pathological conditions including diabetes and tumourigenesis have been reviewed (Ween *et al.*, 2012). Recent studies have also shown that *TGFBI* regulates adipose angiogenesis and stimulates the angiogenic capacity of endothelial cells (Lee *et al.*, 2021).

During the later stages of ovary development, the expression of TGFβ signalling molecules such as *TGFB2*, *TGFB3*, *LTBP1*, *LTBP2*, *LTBP3*, *LTBP4*, *FBN1*, *TGFBR2*, *TGFBR3* and *TGFB111* increases. During this stage of ovary development, folliculogenesis and ovarian steroidogenesis occur and stroma beneath the surface epithelial basal lamina develops into the tunica albuginea in human and bovine ovaries (Hummitzsch *et al.*, 2013, 2019; Heeren *et al.*, 2015). Activation of some primordial follicles occurs leading to their development into primary and preantral follicles. LTBPs, whose genes are expressed the most during the third trimester, are well known for their ability to bind to FBN1 and FBN2 to modulate TGFβ availability. They also act as structural components of extracellular matrix (Todorovic *et al.*, 2005). Notably in adult bovine ovaries, TGFβ2 and TGFβ3 levels increase as follicles develop into large antral stage, while TGFβ1 levels decrease during this stage (Nilsson *et al.*, 2003). *TGFB1* and *TGFBR3* were downregulated in cumulus cells isolated from matured metaphase II oocytes of PCOS women (Haouzi *et al.*, 2012). *TGFBR1* and *TGFBR3* expression were dysregulated in ovaries of cows with cystic ovarian disease (Matiller *et al.*, 2019). It is unclear if these dysregulations are of foetal origin given that *TGFBR1* is expressed more in foetal fibroblasts than adult fibroblasts of the ovary (Liu *et al.*, unpublished results).

TGFB111, also known as hic-5, is a transcription factor and a co-activator of the AR (Fujimoto *et al.*, 1999). It regulates proliferation and apoptosis via Wnt/β-catenin signal pathway (Sha *et al.*, 2020) and suppresses cell migration and invasion by inhibition of the TGFβ pathway and epithelial-mesenchymal transition (Ruan *et al.*, 2020). Also, expression of *TGFB111* is increased in the presence of TGFβ1 (Hartanti *et al.*, 2020). *TGFB111* was also among the TGFβ signalling molecules downregulated in cumulus cells isolated from matured metaphase II oocytes of PCOS women (Haouzi *et al.*, 2012).

In conclusion, we consider the following data support the hypothesis that altered TGFβ signalling could be involved in the foetal predisposition to PCOS or at least in the development of polycystic ovaries: (i) TGFβ is linked to the development of fibrous stroma, which is a

hallmark of polycystic ovaries, (ii) in foetal ovarian fibroblasts TGFβ1 can regulate seven genes genetically associated with PCOS, (iii) in foetal ovarian fibroblasts TGFβ1 can alter the expression of *AR* and an AR cofactor and androgen signalling has been shown to be very likely involved in the foetal development of an adult PCOS phenotype, (iv) in foetal ovarian fibroblasts TGFβ1 can regulate expression of *COL1A1* and *COL3A1* thus regulating collagen synthesis and (v) many of the components of TGFβ signalling are dynamically expressed in foetal ovaries across gestation as are the PCOS candidate genes. The implications of these findings for the function of other organ systems associated with PCOS, like the skeletal muscle, should also be explored.

Data availability

The FASTQ files, normalised data and experimental information have been deposited in the Gene Expression Omnibus (GEO) accessible by accession number GSE178450. Data used to create Figs 1-4 can be found at FigShare.

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Authors' roles

R.J.R. and K.H. initiated the conception and design of the study. R.A., M.L., N.A.B., M.D.H. and K.H. acquired the data. R.A. and M.L. conducted the statistical analysis. R.A., M.L., K.H., H.F.I-R. and R.J.R. interpreted the data. R.A., M.L. and R.J.R. drafted the manuscript. K.H., H.F.I-R., R.A.A. and R.J.R. supervised and conducted critical revision of manuscript. All authors read and approved the final version to be published.

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Conflict of interest

The authors of this manuscript have nothing to declare and no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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