Single-cell expression analysis of BMP15 and GDF9 in mature oocytes and BMPR2 in cumulus cells of women with polycystic ovary syndrome undergoing controlled ovarian hyperstimulation

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Abstract

Purpose To detect expression of bone morphogenetic protein 15 (*BMP15*) and growth differentiation factor 9 (*GDF9*) in oocytes, and their receptor type 2 receptor for BMPs (*BMPR2*) in cumulus cells in women with polycystic ovary syndrome (PCOS) undergoing in vitro fertilization (IVF), and determine if *BMPR2*, *BMP15*, and *GDF9* expression correlate with hyperandrogenism in FF of PCOS patients. **Methods** Prospective case–control study. Eighteen MII-oocytes and their respective cumulus cells were obtained from 18 patients with PCOS, and 48 MII-oocytes and cumulus cells (CCs) from 35 controls, both subjected to controlled ovarian hyperstimulation (COH), and follicular fluid (FF) was collected from small (10–14 mm) and large (>18 mm) follicles. RNeasy Micro Kit (Qiagen®) was used for RNA extraction and gene

expression was quantified in each oocyte individually and in microdissected cumulus cells from cumulus-oocyte complexes retrieved from preovulatory follicles using qRT-PCR. Chemiluminescence and RIA assays were used for hormone assays. **Results** *BMP15* and *GDF9* expression per oocyte was higher among women with PCOS than the control group. A positive correlation was found between *BMPR2* transcripts and hyperandrogenism in FF of PCOS patients. Progesterone values in FF were lower in the PCOS group. **Conclusion** We inferred that *BMP15* and *GDF9* transcript levels increase in mature PCOS oocytes after COH, and might inhibit the progesterone secretion by follicular cells in PCOS follicles, preventing premature luteinization in cumulus cells. *BMPR2* expression in PCOS cumulus cells might be regulated by androgens.

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Capsule Women with PCOS undergoing COH have increased expression of *BMP15* and *GDF9* genes in mature oocytes. *BMPR2* expression in PCOS cumulus cells might be regulated by androgens.

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Introduction

Polycystic ovary syndrome is a heterogeneous endocrine disorder found in 5 % of women of reproductive age, and accounts for about 90–95 % of patients with anovulatory infertility [1]. This syndrome presents defects in primary cellular control mechanisms that result in the expression of chronic anovulation, hyperandrogenism, and polycystic ovaries [2, 3].

Some studies employing DNA microarrays have identified over one thousand genes whose expression was altered in PCOS patients [4–6]. These provide evidence that the genetic abnormality in PCOS affects key mechanisms of follicular development and steroidogenesis, resulting in increased ovarian androgen secretion and anovulatory

infertility due to arrested folliculogenesis. Folliculogenesis, in turn, is under the coordinated regulation of factors from the oocyte itself, as well as from the somatic cells of the ovary, particularly theca and granulosa cells (GCs), which are structural components of the follicle. Thus, a detailed understanding of the molecular mechanisms that govern the follicular development in PCOS women will be needed to improve clinical treatment and increase pregnancy rate.

A variety of different gene-expression assays can be used to measure transcripts levels at a single-cell resolution. qRT-PCR offers higher sensitivity than other RNA assays, and can be applied to tiny amounts of starting material, even the RNA isolated from a single oocyte.

A clinical study indicated that oocyte-derived factors play a role in aberrant folliculogenesis in PCOS [7]. Transgenic deletions and other modifications in mice [8], sheep [9] and humans [10, 11] have provided much of the new information on the mechanisms by which the oocyte affects follicular development [12, 13]. The synergistic process of folliculogenesis is regulated mainly by *GDF9* and *BMP15* as well as their receptors (e.g., *BMPR2*, *TR1*, and *BMPR1B*) [14–17]. These two members of the TGF- β superfamily are coexpressed in human oocytes and play a fundamental role in granulosa cells steroidogenesis, ovulation, oocyte maturation, and embryo development in mammals [11, 18, 19].

To our knowledge, there are only two case-control studies that focus on *GDF9* and *BMP15* expression in oocytes and/or CCs of infertile PCOS women undergoing in vitro fertilization and embryo transfer (IVF-ET) [20, 21]. The established role of *GDF9* and *BMP15* in oocyte competence, and recent findings that single-nucleotide polymorphism variants in these genes seem connected with hyperstimulation syndrome and anovulation and infertility in women with PCOS, led us to evaluate the expression of these genes in single mature oocytes and the expression of *BMPR2* in their respective cumulus cells from women with PCOS. We also evaluated gene expression in oocytes between the former and control groups undergoing COH for IVF. In addition, we analyzed the levels of sex steroids present in the follicular fluid of immature and mature follicles, and pregnancy rates of the PCOS patients.

Gene expression profiles of BMP receptors in cumulus cells have only been fragmentally reported, and their results showed that *BMPR2* is hormonally regulated [22–24]. However, evaluation of transcripts of this gene in cumulus cells from PCOS women undergoing COH for IVF has not been thoroughly examined. The TGF β superfamily of ligands interact with heterotetrameric complexes of type 1 and type 2 receptors (*BMPR2*). Thus, *GDF9* activates the type 1 receptor (TGF β RI or activin-like receptor 5 (ALK5) [25] and *BMP15* activates the *BMPR1B* receptor or activin-like receptor 6 (ALK6). Following ligand binding, TGF β RI or *BMPR1B* recruits the *BMPR2* that results in phosphorylation

of the type 1 receptor, which in turn activates the intracellular-signal-mediated (Smad) proteins [16, 26, 27]. Recently, the *BMPR2* has been identified as the receptor that primarily modulates the cooperative effects of *GDF9* and *BMP15* on rat granulosa cells function, with *BMPR2* having the greatest effect on *BMP15* suppression of FSH-induced progesterone production [28]. Furthermore, the type 2 receptor is appointed as the most effective in *BMP15* bioactivity [29].

Because the established role of *GDF9* and *BMP15* in oocyte competence and given the important role of *BMPR2* in modulate the synergistic effects of *GDF9* and *BMP15*, the specific aims of the present study were: 1) to assess the expression of these genes in single mature oocytes and the expression of *BMPR2* in their respective cumulus cells from women with PCOS; 2) to evaluate the gene expression in oocytes between the former and control groups undergoing COH for IVF; and 3) to analyze if sex steroids levels in the follicular fluid of immature and mature follicles correlates with transcript levels of oocyte-secreted factors in MII-oocytes and *BMPR2* in CCs, and pregnancy rates of the PCOS patients.

Material and methods

Patients

The study was approved by the Clinical Ethics Committee and informed written consent was obtained from each of the participants, who donated their surplus oocytes. A total of 365 women received intracytoplasmic sperm injection (ICSI) from December 2007 to February 2009 at the Human Reproduction Division of the Faculty of Medicine of Ribeirão Preto, University of São Paulo. Thirty-five of these women were diagnosed with PCOS (PCOS group), 86 women with regular menstrual cycle and with male factor infertility (according to the World Health Organization [30] criteria for the examination and processing of human semen, with at least 1 % normal spermatozoa) were selected for control group and 244 with other causes of infertility were excluded. Only 18 PCOS women and 35 control women presented surplus oocytes and CCs and fulfilled inclusion criteria and were included in the study.

PCOS was diagnosed according to the Rotterdam criteria [31]. Patients with endometriosis or any other disease that would interfere with the hypothalamus-pituitary-ovary axis were excluded from the study.

The distribution of the sample was homogeneous among the group with PCOS and Control group: age (PCOS=33 $\pm$ 3.31 and Control=33 $\pm$ 4.25), weight (PCOS=67 $\pm$ 10.20 and Control=62.30 $\pm$ 11.13), body mass index (PCOS=26 $\pm$ 7.42 and Control=24.03 $\pm$ 4.92), FSH (PCOS=4.59 $\pm$ 5.37 and

Control=6±2.21), thyroid stimulating hormone (PCOS=1.90±0.85 and Control=1.97±6.88) e Prolactin (PCOS=13.42±14.83 and Control=11.50±7.84). The results were expressed as mean and standard deviation.

Controlled ovarian hyperstimulation protocol

A gonadotropin releasing hormone (GnRH) agonist (Synarel, Zodiac) was used for IVF and followed the standard long downregulation protocol. Recombinant follicle stimulating hormone (rFSH), with the usual starting dose of 100 to 200 IU/day, was used to induce follicular growth. A dose of 10,000 IU of human chorionic gonadotropin (hCG) (Serono) was administrated when at least two follicles >17 mm in diameter were observed. Oocytes were retrieved after 36 h, under transvaginal ultrasound guidance.

Collection of surplus mature oocytes and cumulus cells

Follicles were divided into two groups according to follicular maturity: large preovulatory follicles (>18 mm) or small follicles (10–14 mm). Cumulus-oocyte complexes used to analyze BMP15, GDF9, and BMPR2 expression were obtained from the large preovulatory follicles (>18 mm) to avoid any stage interference. Surplus morphologically normal MII-oocytes were recruited from patients undergoing routine ICSI. Briefly, oocytes were aspirated 36 h after hCG injection and the ICSI procedure was performed 4–6 h later. Seven mature oocytes were selected for ICSI and remaining fully grown oocytes at metaphase II (MI) were donated for experiment. It must be noted that the all oocytes used for experiment were in good morphology and submitted to PolScope evaluation for meiotic spindle and confirmation of MII-stage according to the routine standard criteria. After microdissection of COCs, hyaluronidase and mechanical methods were used to denude the oocytes in order to remove any CCs remaining.

On egg collection day, each sample of cumulus cells was collected individually and processed for gene expression analysis. The procedure was initiated 40 h post-hCG administration. CCs were mechanically separated from the oocyte by microdissection using two needles, washed in HTF medium (Irvine Scientific, CA, USA) to remove any blood and cell residue caused by puncture, then individually placed in cryotubes and stored in liquid nitrogen. The corresponding follicular fluid was collected and stored at -20 °C. Eighteen mature oocytes and their dissociated CCs were obtained from 18 large follicles, from 18 PCOS patients. From 35 control patients, 48 mature oocytes and 56 CCs from 56 large follicles were obtained. Cumulus cells after oocyte microdissection, without surplus mature oocytes, were obtained from 8 patients of the control group.

Real-time PCR analysis

GDF9, *BMP15*, and *BMPR2* expression at the mRNA level were determined by quantitative real-time reverse-transcriptase polymerase chain reaction (qRT-PCR) analysis. Total RNA from PCOS ($n=18$ oocytes; $n=18$ CCs) and control patients ($n=48$ oocytes; $n=56$ CCs) was isolated in a single assay from individual oocytes and dissociated CCs using the RNeasy Micro Kit (Qiagen, Valencia, CA, USA) following the manufacturer's protocol. cDNA synthesis was performed with the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA). Relative mRNA expression levels of the studied genes were quantified using real-time PCR analysis with the ABI PRISM 7700 Sequence Detection System (Applied Biosystems Inc., Foster City, CA). Amplification of specific sequences was performed using on-demand Taq-Man probes: *BMP15* (Hs00193763_ml), *GDF9* (Hs00193364_ml), *BMPR2* (Hs00176148_ml), and *ACTB* (Hs99999903_ml) (Applied Biosystems). The PCR program consisted of denaturing at 94 °C for 15 s, annealing at 55 °C for 30 s, and extension at 72 °C for 45 s. Samples were amplified for 36 cycles. All samples were analyzed in triplicate, using *ACTB* as the reference gene. The $2^{-\Delta\Delta CT}$ equation was used to calculate the relative expression of *ACTB* samples [32]. Cycle threshold (Ct) means of the control group (women with infertility due to male factor) sample was used as a calibrator.

Follicular fluid and hormone evaluation

Follicular fluid was individually aspirated from small and large follicles from each patient and banked at the time of egg retrieval. FF samples were divided into four groups: G1 (35 samples of FF from a small follicle in the control group), G2 (18 samples of FF from a small follicle in the PCOS group), G3 (35 samples of FF from a large follicle in the control group), and G4 (18 samples of FF from a large follicle in the PCOS group). FF was cleared by centrifugation at 500 x g for 10 min at 4 °C, aliquoted into 5-mL Falcon tubes, and stored at -80 °C until analysis. Dilutions were performed before estradiol (1:500), progesterone (1:1000), testosterone (1:5 and 1:10 for large and small follicles, respectively), and androstenedione (1:5) measurements. Estradiol and progesterone were determined using chemiluminescence assays by DPC 2000 Immulite (Diagnostic Products Corporation Los Angeles, CA, USA). The intra-assay error was 81.8, and the coefficients of variation (CV) were estradiol 3.78 % and progesterone 2.95 %. Testosterone and androstenedione levels were measured in duplicate by radioimmunoassay (RIA) using a Tri-Carb 2100TR scintillator (Packard Instrument Company, IL,

USA). RIA sensitivity was less than 10 ng/dl, and the CV was 9.6 % for androstenedione and 9.6 % for testosterone. Serum human chorionic gonadotropin- β (β -hCG) was determined by chemiluminescence at 14 days after embryonic transfer. High or low results were repeated with appropriate dilution.

Statistical analysis

Differences in *GDF9*, *BMP15*, and *BMPR2* mRNA levels between PCOS and control groups were determined by analysis of variance (ANOVA) followed by the Mann–Whitney test. Data regarding sex steroids in FF were analyzed with an unpaired *t*-test. ANOVA was used to compare data between the four groups, followed by the Neuman–Keuls test. Fisher's exact test was used to analyze the pregnancy rate. Correlation was determined by the method of Pearson (Corr, SAS 2003). Data are reported as the median $\pm$ SD, and significance was set at $p < 0.05$.

Results

General conditions and IVF outcomes

Table 1 summarizes the number of cumulus oocyte complexes (COCs) retrieved, injected oocytes, fertilization and implantation rates, and pregnancy rates per oocyte retrieval in the PCOS and control groups. Pregnancy rate did not differ between the PCOS (9/18; 50 %) and control groups (13/35; 37 %) ($p = 0.39$).

GDF9 and *BMP15* expression in mature oocytes between PCOS and control patients

Eighteen surplus MII-stage oocytes were collected from 18 patients with PCOS and 48 MII-stage oocytes from 56 normal controls (surplus oocytes were not obtained from 8 patients of the control group). The transcript relative abundance (RA) of *GDF9* and *BMP15* genes per oocyte, determined by real-time PCR, is shown in Fig. 1.

Relative abundance of *BMP15* transcripts was significantly higher in MII-oocytes from women with PCOS compared to control (median = 5.95 vs. 0.59, respectively; $p = 0.006$). *GDF9* mRNA abundance was also higher in women with PCOS compared to control (median = 2.70 vs. 0.63, respectively; $p = 0.0006$) (Fig. 1).

BMPR2 expression in cumulus cells between PCOS and control patients

Cumulus cells were collected from mature oocytes (COCs) from 18 patients with PCOS and 56 healthy controls. No

Table 1 Results of fertilization treatment of PCOS and control group

Group	total oocytes	MI stage (%)	MI stage (%)	Injected (%)	Fertilized (%)	Cleaved (%)	Embryos (%)	Transferred embryos	Pregnancy rates
PCOS	8,76 $\pm$ 5,23	5,97 $\pm$ 3,68 (74,1)	2,06 $\pm$ 2,37 (25,9)	5,65 $\pm$ 2,17 (64,5)	4,29 $\pm$ 1,77 (75,9)	3,53 $\pm$ 1,46 (62,47)	3,35 $\pm$ 1,53 (59,29)	2 $\pm$ 0,49	9/18 (50 %) ^a
Control	8,06 $\pm$ 4,54	7,53 $\pm$ 4,06 (85,95)	1,13 $\pm$ 1,49 (14,05)	4,66 $\pm$ 2,27 (57,81)	3,66 $\pm$ 1,84 (78,54)	3,20 $\pm$ 1,65 (68,66)	3,06 $\pm$ 1,49 (65,66)	2 $\pm$ 0,64	13/35 (37 %) ^a

^a Data was reported as mean and $\pm$ SD

Fig. 1 Graphic of *BMP15* and *GDF9* expression in single mature oocytes and *BMPR2* in cumulus cells of PCOS and control groups. The line crossing the middle represents the median value

significant difference was detected in *BMPR2* expression between PCOS and control patients (median 0.74 vs. 0.70, respectively; $p=0.76$).

Steroid concentration in follicular fluid of small and large follicles between PCOS and control patients

Estradiol concentrations did not differ between the study and control groups or between the small and large follicles (Table 2). Progesterone values from the small and large follicles were lower in the PCOS group ($8,095 \pm 4,151$ ng/mL) compared to the control ($9,824 \pm 3,128$ ng/mL) ($p=0.03$) (Table 2).

Testosterone levels differed between the small and large follicles in the control group ($p<0.001$) and in the large follicles between PCOS and control groups ($p<0.001$). Small follicles showed higher testosterone levels than the large follicles, irrespective of the group ($p<0.0001$) (Table 2). Androstenedione levels did not differ between the two study groups or between the small and large follicles (Table 2). Linear regression analysis showed a positive correlation between mRNA expression of *BMPR2* in PCOS

cumulus cells and testosterone levels ($r=0.29$, $p=0.04$) and androstenedione ($r=0.55$, $p=0.03$) in FF from preovulatory follicles. There was no significant association between *BMP15* and *GDF9* expression with sex steroid levels in FF from the large follicles.

Discussion

The study of PCOS and its molecular dynamics in patients with this disease is an important step to improve clinical treatment and increase pregnancy rate. Oocyte-secreted factors, namely *BMP15* and *GDF9*, play a crucial role in follicular growth and oocyte quality [19, 33], which are strongly defective in PCOS patients ([5, 10, 34]. Herein we detected the expression of *BMP15* and *GDF9* in single mature oocytes and *BMPR2* in the CCs of women with PCOS undergoing ovarian stimulation for IVF. We also evaluated sex steroid concentrations in FF and their correlation with *BMP15*, *GDF9*, and *BMPR2* mRNA.

We found that *GDF9* and *BMP15* expression was higher in mature oocytes from PCOS patients than in those from the control group, with both patient groups undergoing COH for IVF. Consistent with our findings, a study by Zhao and colleagues [21] revealed increased expression of *GDF9* in GV-stage oocytes ($n=12$) and *BMP15* in MI-stage oocytes ($n=10$) in women with PCOS after COH, suggesting that exogenous rFSH use may correct dysfunction in *BMP15* and *GDF9* expression in PCOS oocytes. However, these authors used an immunocytochemistry method and evaluated *BMP15* and *GDF9* expression at the protein level in immature-stage oocytes, which differs the approach we used. Another study in an animal model suggests that oocyte treatment with rFSH increased mRNA levels of oocyte-secreted factors during follicular development in parallel with oocyte maturation [35]. Thus, it is possible that over-expression of these paracrine oocyte-derived factors in PCOS oocytes relate to ovarian hyperstimulation syndrome susceptibility for patients with PCOS and exogenous gonadotropin treatment [36]. Indeed, a recent study has provided evidence to associate three linked markers within the *BMP15* gene with high follicle production in woman undergoing rFSH stimulation [37], suggesting a potential role of rFSH stimulation on *BMP15* gene expression. Nevertheless, further study is needed to clarify the hypothesis that *GDF9* and/or *BMP15* are up-regulated in oocytes from PCOS patients undergoing COH for IVF. Highlighting the gene expression profiles of PCOS oocytes, previous microarray analyses have demonstrated that more than 80 % of differentially expressed genes in PCOS oocytes had increased mRNA abundance compared with oocytes from normal women, and indicated that androgens may play a role in this process [5]. Our results are in striking

Table 2 Sexual steroids levels in follicular fluid from women with Polycystic Ovary Syndrome (PCOS) and control group

	G1	G2	G3	G4
Estradiol (pg/mL)	393,640.15±211,418.36	427,771.79±197,817.94	406,582.06±223,111.99	503,091.43±161,769.48
Progesterone (ng/mL)	8,779.71±2,996.54 ^a	7,596.29±3,519.94 ^b	10,571.18±2,684.75	9,562.86±4,511.64
Testosterone (ng/dL)	325.97±122.64 ^c	416.43±111.47	207.47±97.05 ^d	336.57±120.30
Androstenedione (ng/dL)	547.44±291.38	485.209±261.23	454.97±206.44	512.36±299.45

Data was reported as mean ± SD. G1 (small control), G2 (small PCOS), G3 (large control) and G4 (large PCOS)

^a $p=.03$, G1 + G3 vs. G2 + G4

^b $p=.004$, G2 vs. G3

^c $p<.001$, G1 vs. G3

^d $p<.001$, G3 vs. G4

contrast with a recent study employing Nested-PCR analysis of oocyte pools ($n=7$), in which a lower expression of *GDF9* and *BMP15* genes was observed in stimulated mature PCOS oocytes [20]. The reasons for these discrepancies are unknown, but could be attributed to stage-dependent differences or oocyte quality, follicular diameter, or sensitivity and specificity of the PCR analysis (Nested-PCR vs. Real-Time PCR in the present study). Another explanation could be differences between PCOS patients recruited for the study. In women with PCOS, patients carrying the heterozygous genotype for *BMP15* gene present an advantage for fecundity, with a protective effect against anovulation and infertility [11]. In a recent published work, the same *BMP15* markers associated with anovulation and infertility in PCOS women are associated with high follicle production in patients undergoing COH [37]. It is also possible that the differences between the two studies in sample size and RNA extraction techniques (Trizol vs. RNeasy Micro kit used in the present study) from a pool of oocytes vs. individual oocytes might yield different results. To eliminate any stage-dependent differences in our study, analyses between PCOS ($n=18$) and control ($n=35$) oocytes were conducted with good quality MII-stage oocytes, all collected from pre-ovulatory follicles (>18 mm) and characterized by PolScope evaluation avoiding interference of intermediate stages of meiosis, such as Telophase I. Also, gene expression profile was conducted in single oocytes and expressed as median to increase the reliability of data obtained. Nevertheless, care should be taken when comparing studies, especially when it concerns methodological differences. Another study found a decreased level of *GDF9* mRNA in developing PCOS oocytes compared with normal, with no differences in *BMP15* expression, but in non-stimulated patients [7]. Thus, *BMP15* and *GDF9* expression in PCOS oocytes remains controversial and the impact of these oocyte-derived factors on oocyte maturation, as well as embryo and pregnancy outcomes, requires further elucidation in PCOS

patients. The molecular mechanisms underlying human ovarian pathologies such as premature ovarian failure or PCOS have been extensively studied, and *BMP15* and *GDF9* are good candidate genes since these two oocyte-secreted proteins have been identified as potential biomarkers of ovarian reserve [16]. *BMP15*, *GDF9*, and their receptors have been identified already in primordial follicles from various mammalian species, including humans [7, 27].

Even though the roles of *GDF9* and *BMPs* during PCOS folliculogenesis have been investigated, gene expression profiles of their receptors in GCs are still unknown. Each ligand of the TGF- β superfamily is known to signal through specific receptor complexes composed of type I and type II serine–threonine kinase receptors in the GC membrane, and *BMPR2* has been identified as one of the potential type II receptors involved in *BMP15* and *GDF9* signaling [16, 27, 38]. In the present study, mRNA expression of *BMPR2* was detected in cumulus cells from PCOS and control patients, but showed no significant differences between them. However, a positive correlation was observed between mRNA expression of *BMPR2* in PCOS cumulus cells and levels of testosterone and androstenedione in FF from pre-ovulatory follicles. In fact, several studies have shown that *BMP* receptors are hormonally regulated [22–24]. PCOS-related hyperandrogenism can exert complex effects on the ovarian follicle. Data from microarray studies indicate that androgen might play a role in up-regulate gene expression in PCOS oocytes [5] and androgen treatment in female rhesus monkeys increases mRNA expression of FSH and IGF-1 receptors in granulosa cells and follicle primordial oocytes [39]. In bovine granulosa cells, estrogen used alone and in combination with FSH has been shown to up-regulate *BMPR2* mRNA expression [22, 24]. Supporting this finding, a study using a human granulosa-like tumor cell line showed that FSH up-regulated *BMPR2* in GCs [23]. Our results indicate the likelihood that *BMPR2* expression might be up-regulated by rFSH alone or in combination with steroids in PCOS cumulus cells. However, mRNA

expression and functional properties of this receptor were not determined in cumulus cells from women with PCOS and remain unclear. Further experiments are being conducted in our laboratory to examine androgen receptor expression in PCOS oocytes and its companion CC and testing the hypothesis that BMPR2 expression is regulated by excess androgens. Inversely, *BMP15* and *GDF9* expression did not correlate with hyperandrogenism in FF of PCOS patients or with other steroid levels in FF from both patient groups. As expected, and regardless of follicular size, testosterone levels were higher in patients with PCOS compared to control a result that is compatible with hyperandrogenism in women with PCOS. High androgen levels and a high testosterone/estradiol ratio have been reported in PCOS patients [40]. Similarly, PCOS follicles have elevated androgen levels before and after GnRH analogue/rhFSH therapy for IVF [41].

In the current study, progesterone levels in PCOS patients were reduced in FF if compared to controls. In contrast, studies using isolated theca cells (TCs) obtained from the ovaries of women with PCOS have demonstrated that androgen and progesterone biosynthesis is increased, resulting from increased expression of the cholesterol-side-chain cleavage enzyme (P450_{scc}), 3 β -hydroxysteroid dehydrogenase type II (HSD3B2), and *CYP17* [42]. In this study, the reduced progesterone levels observed in FF of PCOS patients undergoing COH may be explained by inhibition of progesterone synthesis in GCs and TCs by *GDF9* and *BMP15*, which are potent inhibitors of luteinization in follicular cells [27, 43]. Previous studies showed that *GDF9* could inhibit FSH-stimulated progesterone secretion synthesis in human GCs and TCs [44]. Similarly, *BMP15* inhibits the production of progesterone induced by 8-bromo-AMPC in follicular cells [45]. BMP15 has been shown to activate the Smad 1/5/8 pathway through BMPR2 and ALK6 [29], with BMPR2 having the greatest effect on BMP15 suppression of FSH-induced progesterone production [28]. Moreover, a recent study reports the co-operative effects of GDF9 and BMP15, modulated through BMPR2, on inhibition of FSH-stimulated progesterone production [28] that reinforces our results.

Estradiol levels in FF did not differ between groups. PCOS patients undergoing IVF commonly demonstrate elevated estradiol levels as rFSH stimulates aromatase activity in GCs, which in turn may correct estrogen levels in FF [46]. When interpreting estradiol concentrations observed in PCOS patients, it is also important to consider the possibility that GDF9 and BMP15 participate in the aromatization process with FSH.

A comparison between the two patient groups reveals no significant differences for pregnancy rates. Although PCOS patients are typically characterized by increased numbers of oocytes retrieved during IVF, these oocytes are often of poor quality, leading to lower fertilization, cleavage, and

implantation rates, and a higher miscarriage rate [47]. Therefore, it can be inferred that increased *BMP15* and *GDF9* expression in patients with PCOS undergoing ovarian stimulation for IVF might be associated with pregnancy rates similar to the control group, since these genes are potent promoters of cumulus cell expansion [48], oocyte maturation, and quality [49, 50]. Furthermore, we observed that estradiol levels in PCOS patients were similar to those in controls, which can contribute to improved IVF outcomes, since high estradiol levels in PCOS patients may be detrimental to oocyte maturation and embryonic development [51]. Supporting this hypothesis, Wood and colleagues [5] reported that a number of the genes whose transcript abundance is altered in PCOS oocytes, contain putative nuclear receptor binding sites, indicating that pharmacological interventions might correct molecular defects in PCOS oocytes and improve pregnancy outcome.

Finally, our results need to be refined by determining the potential role of these ligands and their receptors in the pathogenesis of PCOS using a larger sample size of oocytes and CCs, and how alterations in *BMP15* and *GDF9* expression might be implicated in IVF outcomes. Our data suggest that in PCOS patients undergoing COH for IVF, terminally differentiated follicles remain hyperandrogenic and contain meiotically-competent (metaphase II) oocytes with increased expression of the *BMP15* and *GDF9* genes. The present data also indicate that progesterone levels were reduced in FF of patients with PCOS despite COH, suggesting that *GDF9* and *BMP15* acts alone or through co-operative effects that might inhibit the secretion of progesterone by follicular cells, potentially preventing premature luteinization in PCOS cumulus cells through BMPR2. Nonetheless, for a better understanding of the roles of GDF-9 and BMP-15 and its receptors in PCOS-stimulated cumulus-oocyte complexes from women undergoing IVF, and about pregnancy outcomes, an exhaustive analysis would be required.

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