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**Clinical Research Article**

# Intrafollicular Concentrations of the Oocyte-secreted Factors GDF9 and BMP15 Vary Inversely in Polycystic Ovaries

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## Abstract

**Context:** The oocyte-secreted factors growth differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15) play essential roles in follicle development and oocyte maturation, and aberrant regulation might contribute to the pathogenesis of polycystic ovary syndrome.

**Objective:** Are there measurable differences in concentrations of GDF9, BMP15, and the GDF9/BMP15 heterodimer in small antral follicle fluids from women with and without polycystic ovaries (PCO)?

**Design and Setting:** Follicle fluids (n = 356) were collected from 4- to 11-mm follicles in unstimulated ovaries of 87 women undergoing ovarian tissue cryopreservation for fertility preservation.

**Patients:** Twenty-seven women with PCO were identified and 60 women without PCO-like characteristics (non-PCO women) were matched according to age and follicle size.

**Main outcome measures:** Intrafollicular concentrations of GDF9, BMP15, GDF9/BMP15 heterodimer, anti-Mullerian hormone (AMH), inhibin-A and -B, total inhibin, activin-B and -AB, and follistatin were measured using enzyme-linked immunosorbent assays.

**Results:** The detectability of GDF9, BMP15, and the GDF9/BMP15 heterodimer were 100%, 94.4%, and 91.5%, respectively, and concentrations were significantly negatively correlated with increasing follicle size ($P < 0.0001$). GDF9 was significantly higher in women with PCO (PCO: 4230 ± 189 pg/mL [mean ± SEM], n = 188; non-PCO: 3498 ± 199 pg/mL, n = 168; $P < 0.03$), whereas BMP15 was lower in women with PCO (PCO: 431 ± 40 pg/mL, n = 125; non-PCO: 573 ± 55 pg/mL, n = 109; $P = 0.10$), leading to a significantly higher GDF9:BMP15 ratio in women with PCO ($P < 0.01$). Significant positive associations between BMP15 and AMH, activins, and inhibins in non-PCO women switched to negative associations in women with PCO.

**Conclusions:** Intrafollicular concentrations of GDF9 and BMP15 varied inversely in women with PCO reflecting an aberrant endocrine environment. An increased GDF9:BMP15 ratio may be a new biomarker for PCO.

**Key Words:** PCOS, follicle fluid, antral follicle, BMP15, GDF9, cumulin

**Abbreviations:** AFC, antral follicle count; AMH, anti-Mullerian hormone; AU, arbitrary unit; BMP15, bone morphogenetic protein 15; COC, cumulus-oocyte complex; FF, follicle fluid; GDF9, growth differentiation factor 9; PCO, polycystic ovary; PCOS, polycystic ovary syndrome

The oocyte plays a major role in the regulation of folliculogenesis and modifies its own follicular microenvironment by secretion of paracrine growth factors (1). Growth differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15) act as such paracrine factors secreted by the oocyte to regulate the function of the neighboring granulosa and cumulus cells within the follicular compartment (2). These factors play essential roles in follicle development, oocyte maturation, and ovulation (2-5), and studies have suggested that GDF9 and BMP15 contribute to the pathogenesis of polycystic ovary syndrome (PCOS) (6-10).

PCOS affects 5% to 10% of women of reproductive ages and is considered the most common endocrine disorder characterized by anovulation, ovarian cysts, hyperandrogenism, hirsutism, insulin resistance, obesity, and irregular menstrual bleeding leading to infertility (11-14). Mutations in GDF9 and BMP15 have been associated with PCOS in some studies (15-17); however, the prevalence of these nonsynonymous

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mutations were not significantly different from that in women without PCOS (18), and other studies have reported an absence of such associations (19, 20). Several studies have also reported dysregulated levels and aberrant expression of BMP15 and GDF9 in oocytes, cumulus cells, and granulosa cells from women with polycystic ovaries (PCO) or PCOS compared with controls (6-10, 21, 22); however, results have been contradicting and consistent conclusions are lacking.

GDF9 and BMP15 belong to the TGF-β superfamily, which is the largest family of secreted proteins in mammals, and like all other TGF-β superfamily proteins, they are produced as promature proteins that form dimers and require proteolytic cleavage by furin-like proteases to become active (23, 24). However, GDF9 and BMP15 differ structurally from other TGF-β superfamily ligands because they lack the conserved fourth cysteine residue and form noncovalent dimers to elicit their effects (25, 26). Based on their noncovalent dimer interaction, shared spatiotemporal expression pattern in the oocyte, close structural homology, and coimmunoprecipitation, it has been shown that GDF9 and BMP15 can interact physically ex vivo to form a GDF9:BMP15 heterodimer complex called cumulin (27-30). However, it is unknown exactly how GDF9 and BMP15 are processed and interact in vivo, and which primary bioactive forms are present in biological fluids in humans.

The GDF9 transcript is highly expressed in human oocytes of all follicle stages from primordial, primary, secondary, antral, to preovulatory follicles, and in mature MII oocytes, whereas BMP15 mRNA is expressed in human oocytes from the early secondary stage with increasing expression throughout follicle development to the preovulatory stage, and in mature MII oocytes (31-33). Because of assay limitations protein concentrations of GDF9 and BMP15 in oocytes, follicle fluids (FFs), and serum are less well characterized. Recent studies by Riepsamen and colleagues found low but relative uniform serum concentrations of GDF9 and BMP15 throughout the menstrual cycle in both healthy ovulatory women and infertile women undergoing in vitro fertilization, possibly reflecting the total population of oocytes within a woman's ovaries, similar to anti-Mullerian hormone (AMH) (34, 35). However, no correlations between serum AMH and either GDF9 or BMP15 serum concentrations were found, nor with concentrations of FSH (34).

Intrafollicular concentrations of GDF9 and BMP15 are expected to be higher than in serum. The study by Riepsamen and colleagues showed higher detection rates of GDF9 and BMP15 in FF from preovulatory follicles compared with serum (34), and with a new highly sensitive commercially available ELISA assay our group recently reported high concentrations of GDF9 in human small antral follicles collected in the natural menstrual cycle (36). We also found that women with PCO had significantly reduced intrafollicular concentrations of inhibins and an aberrant regulation of the inhibin-activin-follistatin axis, as well as AMH, which may reflect underlying mechanisms characterizing aberrant follicle growth in PCOS (36).

Using newly developed specific ELISA (Ansh Labs, Webster, TX, USA) to detect native forms of human oocyte-secreted GDF9, BMP15, and the GDF9/BMP15 heterodimer cumulin, the aim of the current study was to measure concentrations of GDF9, BMP15, and the GDF9/BMP15 heterodimer in fluid from 4- to 11-mm small antral follicles obtained from women with or without PCO. Furthermore, these intrafollicular concentrations of GDF9, BMP15, and the GDF9/BMP15 heterodimer were related to the follicle diameter and associated to concentrations of other TGF-β members.

## Materials and Methods

### Study Population

The study included FF obtained from surplus ovarian tissue from unstimulated ovaries of 87 women (aged 16-38 years) undergoing ovarian tissue cryopreservation for fertility preservation at the Laboratory of Reproductive Biology, University Hospital of Copenhagen, Denmark, from 2011 to 2019. The women had 1 ovary laparoscopically removed at various times during their menstrual cycle. The ovarian cortical tissue was isolated and cryopreserved using the slow freezing technique (37). Surplus ovarian material, including antral follicles and FF, was donated for research by patients giving written consent after written and orally conveyed information (ethical approval: H-2-2011-044; Capital Region).

The diagnoses of the patients were breast cancer (n = 44), lymphoma (n = 12), sarcoma (n = 6), brain cancer (n = 10), colorectal cancer (n = 2), cervical cancer (n = 3), leukemia (n = 1), and other diseases including aplastic anemia, Blackfan diamond syndrome, and sclerosis (n = 9).

### Collection of Small Antral Follicle Fluids

Follicle fluids were aspirated from individual small antral follicles visible from the outside of the ovary and located within the surplus medullary tissue. Fluids were gently aspirated with a 23G needle attached to a 1-mL syringe. Based on the aspirated FF volume, the diameter of the follicle was calculated assuming a spherical shape. Included FFs were obtained from follicles with a diameter of 4 to 11 mm. The FF and the granulosa cells were separated by centrifugation at 400*g* for 3 to 5 minutes, and the FF and granulosa cells were snap frozen in liquid nitrogen separately and stored at -80 °C until further analysis.

### Hormone Measurements

ELISAs developed by Ansh Labs were used to measure intrafollicular concentrations of GDF9 (38), BMP15 (39), GDF9/BMP15 heterodimer (40), picoAMH (41), inhibin-A (42), inhibin-B (43), total inhibin (44), activin-B (45), activin AB (46), and follistatin (47). The assays were performed according to the manufacturer's protocol with an appropriate dilution of the FF samples using the supplied assay buffer. Analytical characteristics of the assays for picoAMH, inhibins, activins, and follistatin were described in Kristensen et al (36). Analytical assay characteristics for GDF9, BMP15, and the GDF9/BMP15 heterodimer are described in supplemental Table 1 (48). Samples were diluted 1:5 in the GDF9 calibrator A before measuring in GDF9 ELISA. The standard for the GDF9/BMP15 heterodimer assay had not yet been calibrated into protein concentrations at the time of measurements and is therefore defined in terms of arbitrary units (AU).

Blood samples from the patients were collected before cryopreservation of ovarian tissue and serum levels of AMH, FSH, LH, estradiol, progesterone, and testosterone were measured as routine clinical samples by the clinical biochemical departments at the university hospitals of Copenhagen, Odense, and Aarhus, Denmark.

### Immunofluorescence Analysis

Immunofluorescence analysis was performed to detect GDF9 and BMP15 expression in cumulus-oocyte complexes (COCs) obtained from small antral follicles in PCO. The analysis was performed as described previously (49). In brief, 5-µm tissue sections including the oocyte were deparaffinated in xylene, rehydrated in ethanol followed by antigen retrieval in Tris-egtazic acid (EGTA) buffer (10 mM Tris, 0.5 mM EGTA, pH 9) and blocking in Tris-buffered saline with 1% BSA. Sections were incubated overnight at 4 °C in mouse monoclonal antibodies against GDF9 or BMP15 (generously provided by Ansh Labs) together with rabbit monoclonal antibody against 17β-Hydroxy Steroid Dehydrogenase B1 (HSD17B1, Abcam, Cambridge, UK, catalog no. ab51045) (50) diluted 1:100 in blocking reagent. After incubation with primary antibodies sections were rinsed in Tris-buffered saline with Tween20, incubated 1 hour in appropriate secondary antibody, rinsed, and nuclear DAPI stained (Invitrogen by Thermo Fisher, Roskilde, Denmark, catalog no. D21490) and mounted in Prolong Gold (Invitrogen, catalog no. P36930). Universal negative control serum (BioCare Medical) and antibody dilution buffer was used in place of primary antibody and showed no staining, data published in supplemental Figure 1 (51) and as supplemental data in Mamsen et al (52).

### Statistics

The statistical analysis was performed in R version 3.4.3 using a linear mixed-effect model with random intercept for each woman, and a linear effect of follicle diameter and case (PCO/non-PCO) as well as the interaction between follicle diameter and case as fixed effects. Tests of statistical significance were carried out using Wald tests with a significance level of 0.05. Spearman correlations between the intrafollicular levels of all measured TGF-β members in the non-PCO and PCO groups were performed as well.

## Results

### Patient Demographics and Definitions of Non-PCO and PCO Groups

Women with and without PCO were identified based on ovarian volume, number of aspirated antral follicles, and serum hormone profiles (Table 1). The trimmed ovary was weighed upon arrival to the laboratory and the ovarian volume was determined using the conversion of 1 g of tissue corresponding to 1 mL (53). Women were characterized as having PCO when the ovarian volume exceeded 10 mL and an ovarian PCO appearance was observed with a high number of small antral follicles visible in the periphery of the ovary (Fig. 1A and 1B). Furthermore, the characterization of PCO included aspiration of 7 or more FF. The total number of aspirated FF per ovary was, however, not comparable with a clinical antral follicle count (AFC) by ultrasound. The number of aspirated FF comprised only a fraction of the actual AFC and was used as an indicative parameter of AFC. The morphological findings of PCO were supported by hormone profiles (increased AMH, LH, and LH/FSH ratio), and in total 27 women were included in the PCO group and 60 women without PCO-like characteristics were matched according to age and follicle size (Table 1). The mean age of women without PCO (28.2 ± 5.1 years [± SD]; range, 16-38 years) and with (28.4 ± 5.6 years; range, 16-37 years) was similar. The ovarian volume was significantly

**Table 1.** Patient demographics and clinical information

| | Non-PCO | PCO | *P* |
|---|---|---|---|
| Woman, no. | 60 | 27 | |
| Age in years (mean ± SD), [range] | 28.2 ± 5.1 [16–38] | 28.4 ± 5.6 [16–37] | NS |
| Ovarian volume in mL (mean ± SD), [range] | 6.9 ± 1.9 [2.4–9.5] | 13.8 ± 2.9 [10.0–20.9] | <0.001 |
| Median number of FF collected per woman, [range] | 4 [1–7] | 10 [7–14] | <0.001 |
| Serum AMH in pmol/L (mean ± SEM) | 14.7 ± 1.2 | 43.2 ± 4.3 | <0.001 |
| Serum FSH in IU/L (mean ± SEM) | 6.0 ± 0.4 | 5.1 ± 0.4 | NS |
| Serum LH in IU/L (mean ± SEM) | 6.5 ± 0.6 | 9.6 ± 1.2 | < 0.02 |
| LH/FSH ratio (mean ± SEM) | 1.1 ± 0.1 | 2.1 ± 0.2 | P < 0.001 |
| Follicle diameter, mm | 3.9-10.7 | 4.2-10.7 | |
| Total no. of FF analyzed | 170 | 189 | |
| Median no. of FF analyzed per woman, [range] | 2 [1-7] | 8 [2-12] | |
| Diagnosis, no. | Breast cancer (32) Lymphoma (8) Sarcoma (2) Brain cancer (8) Cervical cancer (1) Colorectal cancer (1) Leukemia (1) Others (7) | Breast cancer (12) Lymphoma (4) Sarcoma (4) Brain cancer (2) Cervical cancer (2) Colorectal cancer (1) Others (2) | |

Abbreviations: AMH, anti-Mullerian hormone; FF, follicle fluid; NS, not significant; PCO, polycystic ovary.

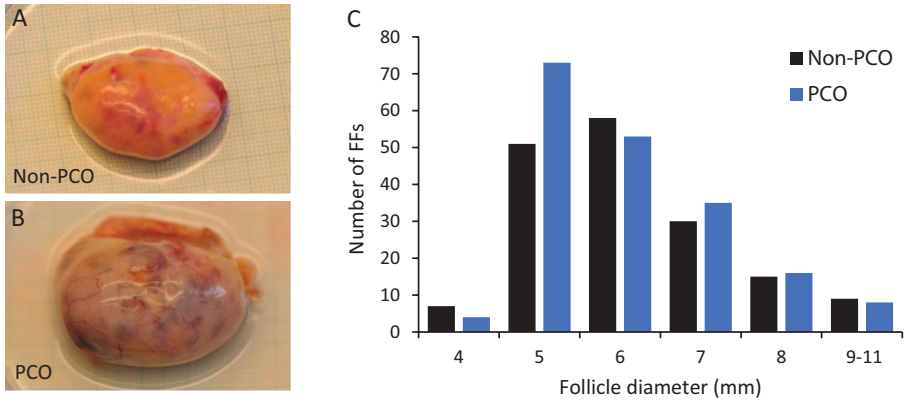


**Figure 1. Distribution of analyzed follicle fluids from women with non-PCO and PCO in relation to follicle diameter.** (A) Ovary from a woman representing the non-PCO group. (B) Ovary from a woman representing the PCO group. (C) Number of follicle fluids (FFs) analyzed according to follicle diameter (4–11 mm) in the non-PCO and PCO groups.

higher in the PCO group (13.8 ± 2.9 mL; range, 10-20.9 mL) compared with the non-PCO (6.9 ± 1.9 mL; range, 2.4-9.5 mL) (Table 1). The median number of collected FF per woman was also significantly higher in the PCO group (median of 10 FF) compared with the non-PCO group (median, 4 FF). Clinical hormone data showed that the PCO group had significantly higher serum AMH concentrations (43.2 ± 4.3 pmol/L) compared with non-PCO (14.7 ± 1.2 pmol/L) (Table 1). Serum FSH concentrations were similar in the 2 groups, whereas LH concentrations were significantly higher in the PCO group (9.6 ± 1.2 IU/L) compared with non-PCO women (6.5 ± 0.6 IU/L), and consequently the LH/FSH ratio was significantly higher in PCO group (2.1 ± 0.2) compared with the non-PCO group (1.1 ± 0.1) (Table 1). Serum concentrations of estradiol, progesterone, and testosterone were not statistically significantly different in the 2 groups (data not shown). Clinical information regarding menstrual cycle or body mass index was not available, and a clinical diagnosis of PCOS according to the Rotterdam criteria was rarely possible in the current setting as the women were referred for fertility preservation with the primary aim of preserving follicles within a very limited time frame.

The 2 groups do not reflect the prevalence of PCO-like phenotypes within the entire cohort of patients undergoing ovarian tissue cryopreservation because they were explicitly selected according to specific phenotypes for this study.

## Sample Details

In total, 356 FF were collected and analyzed from 87 women. Of these samples, 187 were included in our previous study (36), which did not include measurements of BMP15 and the GDF9/BMP15 heterodimer. In the current study a total of 170 and 189 FF were included in the non-PCO and PCO groups, respectively. The diameters of the follicles ranged from 3.9 to 10.7 mm and were similar in both groups (Table 1; Fig. 1C). The median number of analyzed FF per woman was higher in the PCO group (n = 8 FF/woman; range, 2-12) compared with non-PCO (n = 2 FF/woman; range, 1-7) (Table 1).

## GDF9 and BMP15 Expressed in the Oocytes of COCs

Immunofluorescent analysis showed localization of GDF9 and BMP15 in both the oocyte and cumulus cells of COCs from women with PCO (Fig. 2). Staining for HSD17B1 showed cumulus-specific localization in the COCs (Fig. 2).

## Detectability of GDF9, BMP15, and the GDF9/BMP15 Heterodimer

A total of 356 samples were analyzed for GDF9, which was detectable in all samples. A total of 248 samples were analyzed for BMP15; BMP15 was detectable in 234 of the samples, resulting in an overall detectability of 94.4%. A total of 342 samples were analyzed for the GDF9/BMP15 heterodimer, and the heterodimer was detectable in 313 of the samples resulting in an overall detectability of 91.5%. BMP15 and the GDF9/BMP15 heterodimer were measured in fewer samples compared to GDF9 because of insufficient sample material.

## Intrafollicular Concentrations of GDF9, BMP15, and the GDF9/BMP15 Heterodimer

Overall, the concentration of GDF9 (3884 ± 138 pg/mL; mean ± SEM) was approximately 8-fold higher than BMP15 (497 ± 33 pg/mL) based on the standards developed for each of the assays (Table 2). The concentrations of GDF9, BMP15, and the GDF9/BMP15 heterodimer were significantly negatively correlated with increasing follicle size ($P < 0.0001$ for all 3 proteins) (Fig. 3A, 3B, and 3C).

Concentrations of GDF9 were significantly higher ($P < 0.03$) in the PCO group (4230 ± 189 pg/mL; n = 188) compared with the non-PCO group (3498 ± 199 pg/mL; n = 168), whereas no significant differences in BMP15 concentrations were found between the PCO group (431 ± 40 pg/mL; n = 125) and the non-PCO group (573 ± 55 pg/mL; n = 109), but BMP15 concentrations were overall lower in the PCO group (Table 2; Fig. 3A and 3B). No significant differences in the concentrations of the GDF9/BMP15 heterodimer were found between the PCO group (473 ± 29 AU/mL; n = 167) and the non-PCO group (483 ± 37 AU/mL; n = 147) (Table 2).

Intrafollicular concentrations of GDF9 and BMP15 in relation to follicle size grouped according to diameter clearly showed a consistently higher concentration of GDF9 in the PCO group compared to non-PCO (Fig. 3D). In contrast, intrafollicular concentrations of BMP15 were consistently lower in the PCO group compared with the non-PCO group in the smallest follicles until follicle selection (ie, diameters of around 7-8 mm), whereas levels were similar in 9- to 10-mm follicles (Fig. 3E). No clear tendencies were observed between the non-PCO and PCO groups for concentrations of the GDF9/BMP15 heterodimer according to follicle diameters (Fig. 3F).

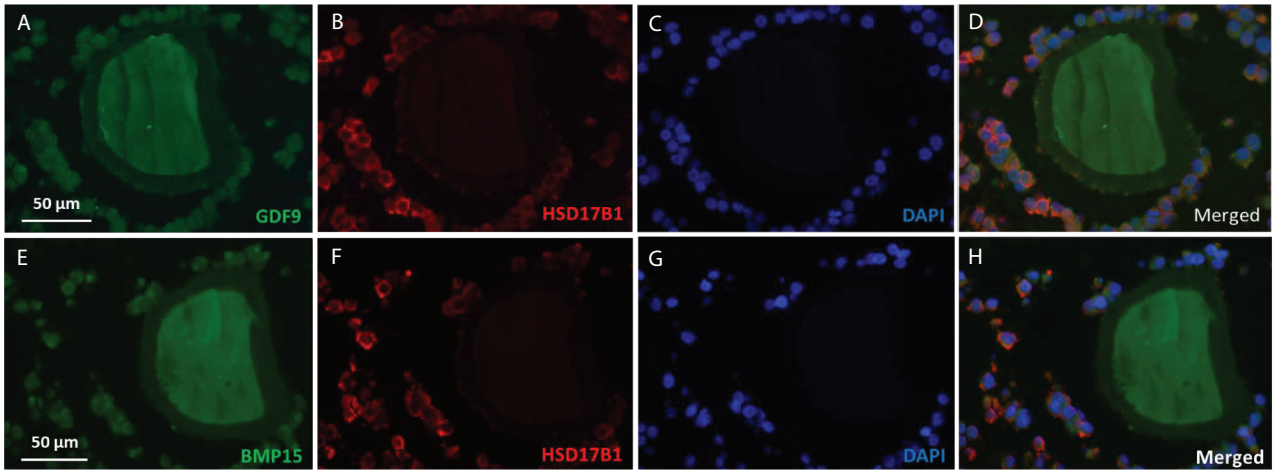


**Figure 2. GDF9 and BMP15 expressed in human oocytes.** Immunofluorescent analysis showing localization of (A-D) GDF9 and (E-H) BMP15 in the oocyte and cumulus cells of COCs from women with PCO. (A + E) GDF9/BMP15; (B + F) HSD17B1 showing cumulus-specific localization in the COCs; (C + G) DAPI nuclear staining; (D + H): merged images.

**Table 2.** Intrafollicular concentrations of GDF9, BMP15, and the GDF9/BMP15 heterodimer in small antral follicle fluids from non-PCO and PCO

| | All samples (n = 356) | Non-PCO n = 168 | PCO n = 188 | *P* |
|---|---|---|---|---|
| GDF9, pg/mL | 3884 ± 138 | 3498 ± 199 | 4230 ± 189 | *P = 0.03* |
| BMP15, pg/mL[a] | 497 ± 33 | 573 ± 55 | 431 ± 40 | *P = 0.10* |
| GDF9/BMP15 heterodimer, AU/mL[b] | 477 ± 23 | 483 ± 37 | 473 ± 29 | *P = 0.88* |

Values are mean ± SEM. *P* values show significant differences between non-PCO and PCO.
Abbreviations: BMP15, bone morphogenetic protein 15; GDF9, growth differentiation factor 9; PCO, polycystic ovary.
[a]234 follicle fluids with detectable measurements (109 non-PCO and 125 PCO).
[b]313 follicle fluids with detectable measurements (147 non-PCO and 167 PCO).

## Follicle Content of GDF9, BMP15, and GDF9/BMP15 Heterodimer

When concentrations of GDF9 were analyzed as follicle content (volume × concentration), the content of GDF9 was similar in follicles with diameters of 4 to 5 mm in the PCO and non-PCO groups but markedly higher in the PCO group compared with non-PCO in follicles with diameters exceeding 6 mm (Fig. 3G). The follicle content of GDF9 increased with increasing follicle diameter and peaked in 8-mm follicles (Fig. 3G). The follicle content of BMP15 was similar and stabile between the PCO and non-PCO groups in follicles with diameters of 4 to 6 mm, but the BMP15 content peaked around follicle selection in 7- to 8-mm follicles in the non-PCO group, whereas it remained low with increasing follicle diameter in the PCO group (Fig. 3H). The follicle content of the GDF9/BMP15 heterodimer according to increasing follicle diameter was similar to BMP15 but the peak in the non-PCO group appeared a little bit later (Fig. 3I).

## An Increased GDF9:BMP15 Ratio in PCO Follicle Fluids

The inverse relationship in intrafollicular concentrations of GDF9 and BMP15 resulted in a significantly higher GDF9:BMP15 ratio in the PCO group compared with the non-PCO group (non-PCO: 12 ± 1 [mean ± SEM]; PCO: 18 ± 2; $P < 0.01$) (Fig. 4A). Further analysis showed that the GDF9:BMP15 ratio increased with increasing follicle diameter when calculated according to follicle content and was consistently higher in the PCO group at all follicle diameters compared with the non-PCO group (Fig. 4B).

## Intrafollicular Correlations Between TGF-β Growth Factors in non-PCO/PCO

A significant positive correlation between GDF9 and BMP15 was found in both PCO and non-PCO, with the most pronounced association and significance in the non-PCO group ($r = 0.52$, $P < 0.001$) compared with the PCO group ($r = 0.36$, $P < 0.05$) (supplemental Table 2 (54)). The GDF9/BMP15 heterodimer was significantly positively correlated with GDF9 and BMP15 in both PCO and non-PCO (supplemental Table 2 (54)).

To elucidate on the potential associations between the oocyte-secreted proteins and other TGF-β growth factors, Spearman correlations were also performed for GDF9, BMP15, and the GDF9/BMP15 heterodimer, and AMH, inhibins, activins and follistatin (supplemental Table 2 (54)). Significant negative correlations were found between GDF9 and the inhibins (inhibin-A, inhibin-B, and total inhibin), whereas significant positive correlations were found for GDF9 and the activins (activin-A and -B) and follistatin, which was consistent in both non-PCO and PCO (supplemental Table 2 (54)). The correlations between the GDF9/BMP15 heterodimer and the inhibins, activins, and follistatin were similar to that of GDF9 showing a tendency to more significant negative correlations with the inhibins in the PCO group compared with the non-PCO group (supplemental Table 2 (54)). Interestingly, Spearman correlations

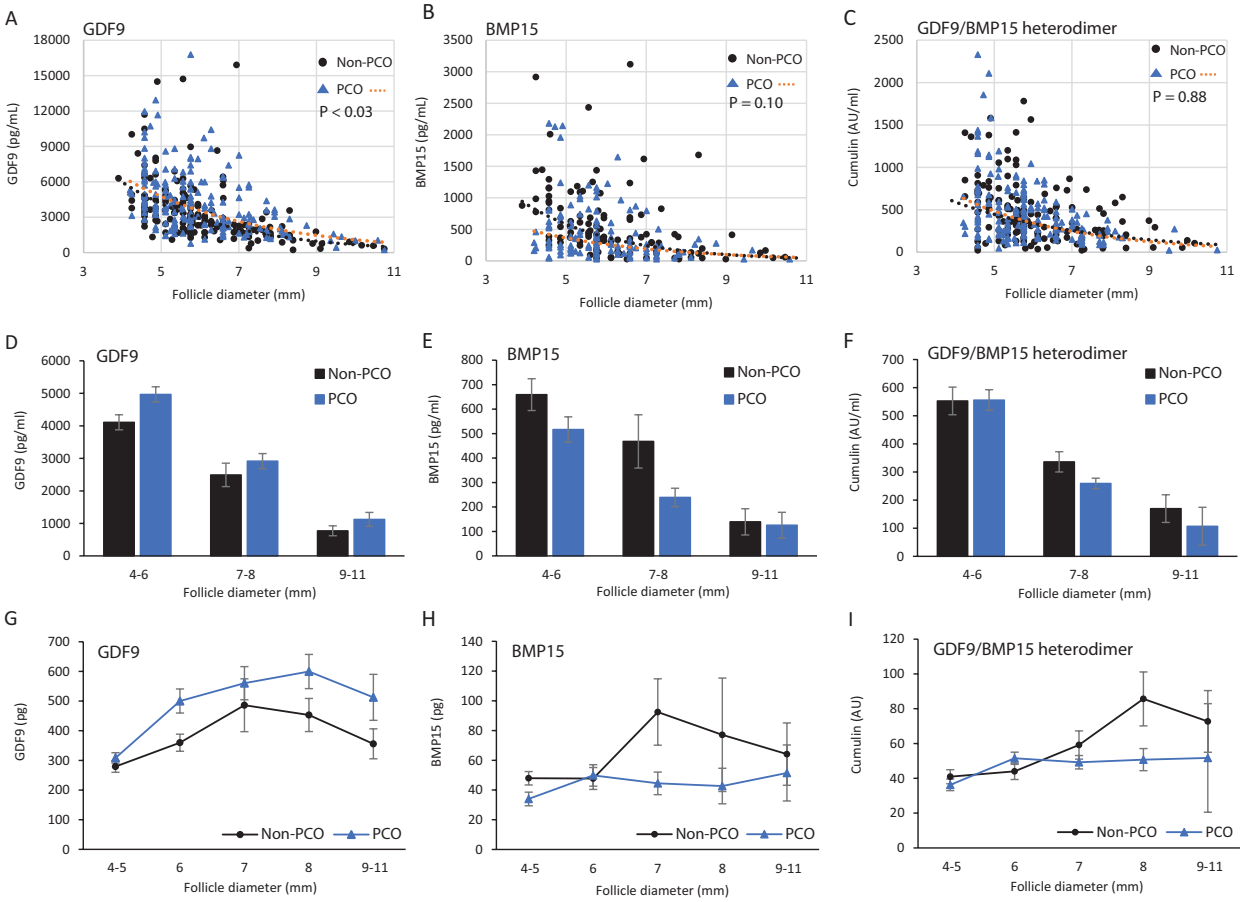


**Figure 3. Intrafollicular concentrations and content of GDF9, BMP15, and the GDF9/BMP15 heterodimer in non-PCO and PCO.** (A-C) Scatter plots and linear regression analysis showing intrafollicular concentrations of (A) GDF9, (B) BMP15, and the (C) GDF9/BMP15 heterodimer in relation to follicle diameters in non-PCO and PCO groups. (D-F) Intrafollicular concentrations of GDF9, BMP15, and the GDF9/BMP15 heterodimer in small antral follicles grouped by follicle diameter and non-PCO/PCO. Number of follicle fluids analyzed according to follicle diameters (non-PCO/PCO); GDF9 (D): 4-6 mm (n = 114/127), 7-8 mm (n = 45/51), 9-11 mm (n = 8/8); BMP15 (E): 4-6 mm (n = 70/88), 7-8 mm (n = 33/33), 9-11 mm (n = 6/4); GDF9/BMP15 heterodimer (F): 4-6 mm (n = 104/121), 7-8 mm (n = 37/42), 9-11 mm (n = 6/3). (G-I) Follicle content of GDF9 (G), BMP15 (H), and the GDF9/BMP15 heterodimer (I) calculated by follicle volume and concentration according to follicle diameters. Values are mean ± SEM.

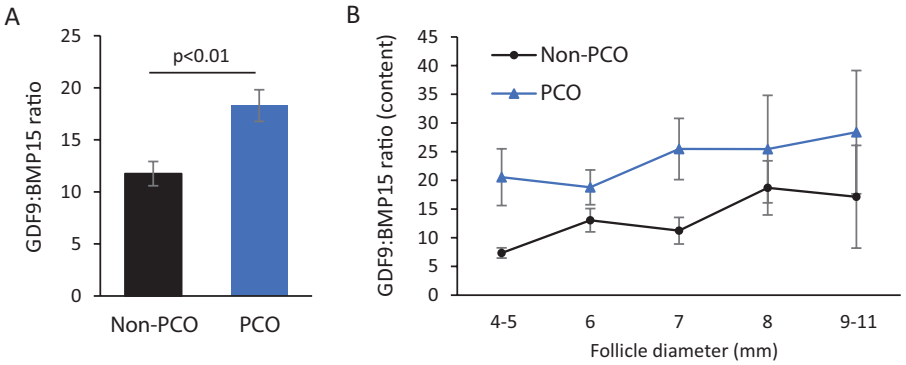


**Figure 4. An increased GDF9:BMP15 ratio in PCO follicle fluids.** (A) The GDF9:BMP15 ratio in the non-PCO group (n = 109) and the PCO group (n = 122). A significantly increased GDF9:BMP15 ratio was found in the PCO group compared with the non-PCO group ($P < 0.01$). (B) The GDF9:BMP15 ratio based on intrafollicular content of GDF9 and BMP15 and depicted according to follicle diameter.

showed that significant positive correlations between BMP15 and AMH ($r = 0.13$, $P < 0.03$), inhibins (inhibin-B: $r = 0.20$, $P < 0.01$; inhibin-A: $r = 0.03$, $P > 0.05$; total inhibin: $r = 0.12$, $P > 0.05$), activins (activin-B: $r = 0.28$, $P < 0.001$; activin-AB: $r = 0.17$, $P < 0.05$), and follistatin ($r = 0.33$, $P < 0.001$) in the non-PCO women switched to negative correlations in the PCO women (AMH: $r = -0.27$, $P > 0.05$; inhibin-B: $r = -0.45$, $P < 0.02$; inhibin-A: $r = -0.37$, $P < 0.05$; total inhibin: $r = -0.44$, $P < 0.02$; activin-B: $r = -0.11$, $P > 0.05$; activin-AB: $r = -0.31$, $P > 0.05$; follistatin: $r = -0.20$, $P > 0.05$) (supplemental Table 2

(54)). Furthermore, the significant positive correlation between GDF9 and AMH in the non-PCO group ($r = 0.39$, $P < 0.001$) also switched to a nonsignificant correlation in the PCO group ($r = 0.01$, $P > 0.05$). No changes in the correlations between the GDF9/BMP15 heterodimer and AMH were observed between the non-PCO group ($r = 0.27$, $P < 0.001$) and the PCO group ($r = 0.31$, $P < 0.001$) (supplemental Table 2 (54)).

Highly significant correlations between all three inhibins (inhibin-B, inhibin-A, total inhibin) and activin-B were observed in the non-PCO group, which switched to nonsignificant correlations in the PCO group (supplemental Table 3 (55)). In contrast, no significant correlations were found between inhibins and activin-AB in the non-PCO group, whereas strong significant correlations were found in the PCO (supplemental Table 3 (55)). Follistatin showed significant, strong correlations with activin-B and activin-AB in both groups, but the correlations between follistatin and the inhibins switched from significant negative correlations in non-PCO women to positive or nonsignificant correlations in the PCO group (supplemental Table 3 (55)). These correlations between the inhibins, activins, and follistatin in non-PCO and PCO corroborates our previous findings in Kristensen et al (36).

## Discussion

This is the first study to perform quantitative measurements of GDF9, BMP15, and the GDF9/BMP15 heterodimer proteins in a large sample set including 356 FF from human small antral follicles collected during the natural cycle. The concentrations of GDF9 and BMP15 were present in surprisingly high concentrations given that only the oocyte itself secretes these growth factors, which enforces the view that the oocyte actively influences the local environment within the follicle. In addition, it is likely that accumulation of these proteins takes place within small antral follicles. The intrafollicular concentrations of GDF9 were approximately 8-fold higher than BMP15, and GDF9 and BMP15 concentrations varied inversely in FF from women with PCO, which resulted in a significantly higher GDF9:BMP15 ratio in the PCO group compared with the non-PCO group. Furthermore, the GDF9/BMP15 heterodimer was measured for the first time in biological fluids and intrafollicular concentrations were similar to BMP15 and between the PCO and non-PCO groups.

Oocyte-specific proteins such as GDF9 and BMP15 are ideal candidates as potential diagnostic biomarkers of oocyte quality and function. However, quantification of these proteins in biological fluids has proven difficult, primarily because of the lack of specific monoclonal antibodies available and the atypical structural features of the BMP15 and GDF9 proteins. Only a few studies to date have quantified GDF9 and BMP15 in human serum and FF using either in-house developed ELISAs or a commercially available ELISA for GDF9 (34-36, 56). However, serum concentrations of GDF9 and BMP15 have not yet proven useful as diagnostic biomarkers of female fecundity or oocyte quality as the current assays show low detectability and high variability between patients (34, 35). Recently, Riepsamen and colleagues measured GDF9 and BMP15 in preovulatory FF from 138 women undergoing in vitro fertilization and found that BMP15 and GDF9 were detectable in 76% and 60% of samples, respectively, compared with a detectability of 67% and 29% in serum (34). In our current study, we found higher detectability in fluids from small antral follicles with the GDF9, BMP15, and GDF9/BMP15 heterodimer being measured in 100%, 94.4%, and 91.5% of all samples, respectively. The higher detectability of GDF9 and BMP15 in small antral follicles compared with preovulatory follicles is in line with the significant negative correlations that we found between the concentrations of GDF9 and BMP15 in relation to increasing follicle size in the current study. Thus, the highest intrafollicular concentrations of GDF9 and BMP15 appeared to be found in small follicles prior to follicle selection. Interestingly, our data also show that small antral follicles contained GDF9 in abundance compared with BMP15, indicating that GDF9 exerts functions on its own during follicle development because the vast majority of GDF9 will not be trapped in the heterodimer complex. It is also noticeable that BMP15 in the non-PCO group appeared to peak just around follicle selection at 7 to 8 mm, enforcing that this particular developmental stage is an important landmark for the hormonal regulation of human follicles.

Several studies in mammalian species have shown improved oocyte competence when the culture medium was supplemented with recombinant GDF9 and/or recombinant BMP15 (30, 57-59). In 2001, GDF9/BMP15 double knockout mice studies by Yan and colleagues showed that GDF9 and BMP15 cooperate, indicating redundant or synergistic biological actions of GDF9 and BMP15 (60). However, to date, it is still unknown whether this cooperation is generated by GDF9 and BMP15 homodimers separately or by the GDF9/BMP15 heterodimer. Interestingly, in vitro studies have shown that the recombinant human GDF9/BMP15 heterodimer potently activates Smad2/3 signalling in mammalian granulosa cells and improve embryo development during oocyte in vitro maturation compared to human GDF9 and BMP15 homodimers (29, 30, 59). However, our current findings indicate similar intrafollicular concentrations of the GDF9/BMP15 heterodimer and BMP15 in small antral follicles, suggesting that only a minor proportion of the total GDF9 would potentially form a heterodimer with BMP15 in vivo, and that GDF9 performs actions on its own as a homodimer. Moreover, our findings showed no differences in the intrafollicular concentrations of the GDF9/BMP15 heterodimer between the women with and without PCO. The follicle content of the GDF9/BMP15 heterodimer in relation to follicle diameter also followed the same pattern of expression as BMP15 in both non-PCO and PCO samples. Thus, a role for the GDF9/BMP15 heterodimer in the abnormalities in follicle growth in PCOS appears to be questionable based on our results.

Our results showed that intrafollicular concentrations of GDF9 were significantly higher in women with PCO, whereas BMP15 concentrations were lower in women with PCO. The increased protein concentrations of GDF9 in FF from women with PCO are in line with 2 previous studies showing higher expression of *GDF9*/GDF9 at both mRNA and protein level in oocytes from PCOS patients compared with a control group undergoing controlled ovarian stimulation (7, 10). In striking contrast, another study found that the expression of *GDF9* mRNA was lower in stimulated mature oocytes from women with PCOS compared with women without PCO (8). The discrepancies in the published data are unknown but may be attributed to stage-dependent differences or oocyte quality, follicle diameter, or sensitivity and specificity of the quantitative PCR analysis (10). Interestingly, these previous studies also found a concomitant higher or lower expression of *BMP15*/BMP15 in the oocytes from PCOS patients compared with controls, which contrasts with our results. Our

findings are the only data showing an inverse relationship between GDF9 and BMP15 in women with PCO. Thus, the expression dynamics of GD9 and BMP15 in human follicles remains controversial, but current findings support the concept that any changes in especially GDF9 protein levels can disrupt ovarian function and female fertility (61). Further studies assessing the expression of GDF9 and BMP15 in FF and corresponding oocytes and cumulus or granulosa cells in women with PCO/PCOS during the natural cycle are needed to reveal the complex interplay between the oocyte and the follicle compartment in ovarian pathologies like PCOS.

That intrafollicular concentrations of GDF9 and BMP15 varied inversely in women with PCO compared to women without PCO resulted in a significantly increased intrafollicular GDF9:BMP15 ratio in women with PCO in our study. The GDF9:BMP15 ratio is known to play an important role in mammalian ovulation rate and fecundity by mechanisms involving GDF9 and BMP15 regulation of LH receptor function on granulosa cells (62, 63). Interestingly, the studies by Riepsamen and colleagues suggested an increased GDF9:BMP15 ratio in serum with increasing oocyte numbers in women without PCOS, but not in women with PCOS (34). Thus, it can be speculated whether the GDF9:BMP15 ratio could be developed as a potential biomarker and diagnostic tool in women with and without PCOS. However, with the low detectability of GDF9 and BMP15 in serum further research is needed using even more sensitive assays to reveal whether these essential oocyte-secreted growth factors could prove useful in a clinical context.

A number of recent studies have shown that GDF9 and BMP15 modulate FSH-induced secretion of a number of granulosa and cumulus cell-secreted proteins and hormones including other TGF-β members (26, 64-67), which suggests that the oocyte itself is actively engaged in the control of follicle development. It is therefore noticeable that the associations between BMP15 and the concentrations of other TGF-β members show positive associations in the non-PCO group that turn into negative associations in the PCO group. This reflects aberrant regulation of follicle growth in the PCO group, but this study is unable to pinpoint the underlying mechanisms. We also found that the significant positive associations between both GDF9 and BMP15 and AMH in the non-PCO group were nonexisting in the PCO group. Studies in mice and human granulosa and cumulus cells have shown that GDF9 and BMP15 induce AMH expression (66, 68-71). Interestingly, studies also showed that FSH inhibits the GDF9/BMP15-induced AMH expression (66, 71). It may be hypothesized that the fine-tuned interactions of growth factors with FSH in the control of granulosa and cumulus cell functions is disturbed in the PCO group. Collectively, alterations in the associations between GDF9, BMP15, and other TGF-β members in our study probably reflect aberrant regulation of these TGF-β members in women with PCO, and our findings complement those of previous studies implicating members of the TGF-β superfamily in the pathophysiology of PCOS (21, 36, 72-75).

A limitation to the current study is that women with PCO were not diagnosed clinically with PCOS according to the Rotterdam criteria. However, these data were not recorded nor required in management of these women for whom the priority was fertility preservation. Selected patients in the PCO group had an average ovarian volume of 14 mL, a high number of small antral follicles, high serum concentrations of AMH and LH, and a high LH/FSH ratio, which collectively indicate a potential PCO-like phenotype. Furthermore, the data set was not large enough to analyze the within-patient variation of the growth factors at specific follicle diameters nor the potential impact that a cancer diagnosis may have on baseline ovarian hormone levels. Finally, only a limited number of samples from small antral follicles larger than 8 mm in diameter were available for analysis and results obtained in these larger antral follicles should be considered with caution.

## Conclusion

Concentrations of oocyte-secreted factors GDF9 and BMP15 varied inversely in small antral FF from PCO and an increased intrafollicular GDF9:BMP15 ratio could be a biomarker for PCO. However, measurements in circulation are required to determine whether an increased GDF9/BMP15 ratio could have clinical potential as a biomarker for PCO or PCOS. Further, our results indicate that the bioactive forms of GDF9 and BMP15 in vivo probably act as homodimers and the role of a GDF9/BMP15 heterodimer needs further clarification. Collectively, our findings provide new perspectives for understanding the pathogenesis of PCOS.

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## Conflict of Interest

The authors assert they have no conflicts of interest.

## Disclosures

The authors have no conflicts of interest and nothing to disclose. A.K. and B.K. are employed by Ansh Labs. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Data Availability

The datasets generated during the current study are not publicly available but are available from the corresponding author on reasonable request.

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