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Serum GDF9 and BMP15 as potential markers of ovarian function in women with and without polycystic ovary syndrome

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Abstract

Objective: Growth differentiation factor-9 (GDF9) and bone morphogenetic protein-15 (BMP15) are critical paracrine regulators of female fertility and are predominantly expressed by oocytes. However, it is unknown if serum concentrations reflect changes in ovarian function and/or reproductive endocrine disorders. This study aimed to determine if serum GDF9/BMP15 are associated with ovarian, pituitary, oestrogenic, androgenic and metabolic characteristics and the ovarian pathologies, polycystic ovarian morphology (PCOM) and polycystic ovary syndrome (PCOS).

Design: Women aged 21–45 years ($n = 381$) were included from a cross-sectional study at the National University Hospital, Singapore.

Patients: Participants were volunteers and patients with possible PCOS.

Measurements: Anthropometric measurements, transvaginal ultrasound scans and serum sampling were performed and a questionnairecompleted. Serum GDF9 and BMP15 concentrations were matched with menstrual cycle length, ovarian protein and steroid hormone production, pituitary hormone production and metabolic assessments in women with PCOM or PCOS and those with neither (control).

Results: Serum GDF9 and BMP15 were detectable in 40% and 41% of women, respectively and were positively correlated with each other ($r = 0.08$, $p = 0.003$). GDF9, but not BMP15, was positively correlated with ovarian volume ($p = 0.02$) and antral follicle count (AFC) ($p = 0.004$), but not with anti-Müllerian hormone ($p = 0.05$). However, serum GDF9 and BMP15 concentrations were not significantly different between control, PCOM and PCOS women, nor associated with androgenic or metabolic PCOS features. However, the relationship between GDF9 and AFC differed between control, PCOM and PCOS women ($p = 0.02$).

Conclusions: Serum GDF9 and BMP15 concentrations somewhat reflect ovarian but not androgenic or metabolic characteristics of PCOS, with increased GDF9 reflecting high AFC as seen in PCOM/PCOS.

KEYWORDS
AMH, BMP15, GDF9, oocyte-secreted factors, PCOS

1 | INTRODUCTION

Polycystic ovary syndrome (PCOS) is a common endocrine disorder affecting 5%–17% of women of reproductive age and is the major cause of anovulatory infertility and menstrual irregularities.[1] In addition, between 40% and 80% of women with PCOS are obese or overweight and are at risk of insulin resistance and metabolic syndrome. PCOS is characterized by chronic oligo-/anovulation, hyperandrogenism and polycystic ovarian morphology (PCOM), with the recently endorsed Rotterdam criteria requiring at least two of these features for the diagnosis of PCOS.[2] Thus, altered folliculogenesis is a key ovarian feature of PCOS, with signalling between the oocyte and granulosa cells reportedly impaired in women with PCOS and oocyte quality being aberrant (reviewed in[3]). Therefore, understanding how oocyte and ovarian factors are altered in women with PCOS may assist in identifying potential diagnostic targets and improving clinical management.

The oocyte-secreted factors growth differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15), both members of the TGF-β superfamily, are critical for folliculogenesis and oocyte quality.[4] As paracrine growth factors, GDF9 and BMP15 are involved in the regulating the fate of cells within the ovarian follicle, including control of proliferation, metabolism and differentiation of granulosa and cumulus cells,[4] in turn affecting oocyte development and quality.[5] Genetic studies show that gene deletions and inactivating mutations in GDF9 and BMP15 cause sterility and/or subfertility in a range of mammals, while some heterozygous mutations can lead to increased ovulation rate in mono-ovular species.[6,7] Further, it has been postulated that the ratio of GDF9:BMP15 regulates mammalian ovulation rate and fecundity.[8]

GDF9 and BMP15 are potential diagnostic markers of PCOS as they are secreted almost exclusively by the oocyte[8] and their expression may be aberrant in women with PCOS (reviewed in[9]). For example, increased GDF9 and BMP15 gene expression was observed in MII oocytes following ovarian stimulation in women with PCOS compared to controls,[10] and delayed GDF9 (but not BMP15) expression during folliculogenesis was observed in ovarian sections of women with PCOS compared to normally cycling women.[11] However, other studies have shown no difference in these oocyte-secreted factors between women with and without PCOS.[12] To date there is limited knowledge of the diagnostic utility of serum concentrations of GDF9 and BMP15 in women, as validated assays sufficiently sensitive to measure these paracrine growth factors in serum have only recently been developed.[13] Therefore, the aim of this study was to measure GDF9 and BMP15 in serum samples collected from women of reproductive age to determine if these oocyte-secreted factors are associated with ovarian, pituitary, oestrogenic, androgenic and metabolic characteristics, as well as the ovarian pathologies, PCOM and PCOS.

2 | MATERIALS AND METHODS

2.1 | Study population and design

Women aged 21–45 years were recruited at the National University Hospital (NUH), from 2011 to 2016 as previously described.[14] This cross-sectional cohort of 381 women included NUH staff and volunteers from the university community (51%), and cases referred from gynaecological clinics at NUH and KK Women's and Children's Hospital, Singapore (49%). Referred patients were suspected PCOS cases. Exclusion criteria were pregnancy, breast-feeding, menopause (clinically indicated and as assessed by hormone levels), use of any medication influencing reproductive or metabolic function for at least 2 months before the study, hyperprolactinemia (prolactin >1000 mI U/L), thyroid and adrenal diseases, androgen-secreting tumours (on ultrasound scan and assessed clinically), congenital adrenal hyperplasia (clinically indicated and as assessed by hormone levels), established diabetes, severe cardiovascular disease, hysterectomy and/or oophorectomy and women with incomplete data. Ethics approval for the study protocol was obtained from the National Healthcare Group Domain Specific Review Board, Singapore. Informed written consent was obtained from all participants.

2.2 | Ovarian characteristics

Menstrual cycle regularity and length were assessed. Participants reported the average length of their menstrual cycles since age 16, and whether their current menstrual cycle length was regular. For analysis, normal cycles were defined as those with regular cycles of 25–34 days and abnormal cycles were those with irregular cycles and/or an average menstrual cycle length <25 or ≥35 days.

Ovarian volume and antral follicle count (AFC) were measured with transvaginal ultrasound in real time using a Voluson E8 machine with a 6–12 MHz transducer, as described previously.[15] To calculate ovarian volume, the longest medial axis of the ovary was determined, the length and height of both ovaries measured and the probe rotated to measure width. For each ovary, the ovarian volume was calculated using the formula for the volume of a prolate ellipsoid (0.523 x length x height x width). For analysis, the average ovarian volume of the left and right ovaries was used for analysis. To calculate AFC, all visible antral follicles were counted from the left and right ovaries and the average of these used for analysis. To minimize the endocrine effects of emergent dominant follicles, women were excluded if they presented with at least one ovarian cyst/follicle >10 mm, including those with a repeat evaluation in their subsequent cycle.

2.3 | Reproductive endocrinology

Fasting blood samples were taken between menstrual Days 2–5, between 7:30 and 8:30 AM and reproductive hormones measured in serum, as previously described.[14,15] Anti-Müllerian hormone (AMH) was measured using the Gen II ELISA (Beckman Coulter Inc.), following the recommended premix procedure.[16] FSH, LH, prolactin and sex hormone binding globulin (SHBG) were measured by sandwich immunoassay (Beckman Coulter Inc.). Estrone, estradiol, testosterone and dehydroepiandrosterone-sulphate (DHEAS) were measured by competitive-binding immunoassays (Beckman Coulter Inc.). Dihydrotestosterone (DHT) and androstenedione (ADT) were measured by ELISA (IBL International; GmbH). Assay performance characteristics were determined contemporaneously as previously described.[15] Free androgen index was calculated as the ratio of total testosterone to SHBG, expressed as a percentage. Hirsutism was assessed using the modified Ferriman–Gallwey (mFG) score. Androgen receptor bioactivity was measured ex-vivo using a reporter gene assay, as described previously.[17]

2.4 | Metabolic and anthropometric characteristics

Fasting insulin, glucose and lipids, including triglycerides, cholesterol, high-density lipoprotein and low-density lipoprotein were determined using Beckman Coulter analysers, as described previously.[14,15] As an indicator of insulin resistance, the homoeostatic model assessment for insulin resistance was also calculated [(glucose (mmol/L) x insulin (ml U/ml))/22.5].[18]

Height, weight and waist circumference were measured and body mass index (BMI; ratio of height and weight, expressed as kg/m^2) and waist-to-hip ratio [WHR = weight (cm)/height (cm)] calculated, as previously described for this cohort.[19]

2.5 | PCOS and PCOM

Women were classified as PCOS based on the Rotterdam criteria requiring at least two of the following features: chronic oligo-/anovulation, hyperandrogenism and PCOM.[20] Oligo-/anovulation was defined as having an average menstrual cycle length ≥35 days,[15] and PCOM was defined as having ≥22 follicles in either ovary, and/or ovarian volume ≥8.44 ml in either ovary. These parameters are based on a published study by Indran et al.,[21] where it was concluded that AFC ≥22 follicles and ovarian volume ≥8.44 ml best predicted oligomenorrhoea and were used as threshold values for PCOS criteria. Hyperandrogenism was defined as having either biochemical or clinical hyperandrogenism.[22] Biochemical hyperandrogenism was any of the following: ADT ≥13.7 nmol/L, DHEAS ≥8.30 µmol/L or testosterone ≥1.89 nmol/L.[21] Clinical hyperandrogenism was a mFG ≥5 based on thresholds previously established in a Chinese population, where the incidence of hirsutism is low (21%).[23]

2.6 | GDF9 and BMP15 ELISAs

Serum concentrations of GDF9 were determined using a commercially available GDF9 ELISA (AL-176; Lot #041619; Ansh Laboratory), as described previously.[24] This ELISA uses antibodies directed at the mature region of human GDF9 and cross-reacts with mature and promature GDF9.[25] The GDF9 ELISA sensitivity was 15.4 pg/ml, with intra-assay variation of 2.4% and inter-assay variation of 6.9%. Serum concentrations of BMP15 were determined using a BMP15 ELISA previously developed and validated for detecting BMP15 in human sera.[13] Recombinant promature human BMP15 protein used as reference preparation was supplied by A/Professor Craig Harrison (Monash University). The BMP15 assay sensitivity was 23 pg/ml, with intra-assay variation was 3.7% and inter-assay variation, 8.7%. For GDF9 and BMP15, samples below the limit of quantification (LOQ) were assigned the sensitivity of the assay (15.4 and 23.0 pg/ml for GDF9 and BMP15, respectively), except where concentrations were derived based on modelling using a maximum likelihood method (see below).

2.7 | Statistical analysis

Statistical analyses were performed using GraphPad Prism version 8.3.1 (GraphPad Software) and R version 4.0.4.[26] Concentrations of GDF9 and BMP15 below the LOQ led to the presence of left-censored observations. GDF9 and BMP15 data were initially stratified into undetectable (≤LOQ) and detectable (>LOQ) groups and associations with participant characteristics were analysed using a Mann–Whitney test for continuous data and a χ^2 test for categorical data. To account for multiple comparisons, the Holm–Sidak method was used to calculate adjusted p values, where $p < 0.05$ was considered significant.

A subset of findings were further analysed using GDF9 and BMP15 data as continuous data, accounting for left censoring as previously described.[13] Comparisons between categorical characteristics and hormone concentrations were performed by using censored regression models[27] assuming a log-normal distribution for GDF9 and BMP15 concentrations, which were confirmed to be appropriate based on Q–Q plots.[28] Associations between hormone concentrations and continuous characteristics, as well as between pairs of hormone concentrations, were estimated using the NADA R package,[29] which produces a version of Kendall's tau correlation coefficient that allows for doubly censored data,[30] as well as its estimated asymptotic standard error. This was used to produce 95% confidence intervals (CI) for the correlation estimates and perform global Wald tests for interaction analysis of differences in correlations between groups (p(int)). A significance level of 0.05 was used.

The ratio of GDF9 to BMP15 was determined using data from participants with both hormone concentrations above their respective LOQs. Associations between GDF9:BMP15 and participant characteristics were examined using the Spearman's correlation for continuous data and the Kruskal–Wallis test for categorical characteristics. To account for multiple comparisons, the Holm–Sidak

method was used to calculate adjusted p values, where <0.05 was considered significant.

3 | RESULTS

Of the 381 women analysed, GDF9 was detectable in 40% of samples and BMP15 was detectable in 41% of samples, varying 360- and 95-fold between women, respectively. To investigate associations with ovarian, endocrine and metabolic characteristics, women were initially stratified into undetectable (≤LOQ) and detectable (>LOQ) groups for GDF9 and BMP15 (Table 1).

3.1 | Ovarian characteristics

All ovarian characteristics (regular menstrual cycles, ovarian volume, AFC and AMH) showed differences ($p < 0.01$) between the undetectable and detectable GDF9 groups, with a significant association between GDF9 and AFC following adjustment for multiple comparisons ($p = 0.012$) (Table 1).

The proportion of women with normal menstrual cycles (regular and 25–34 days in length) was higher (70%) in women with undetectable GDF9, compared to detectable GDF9 (55%; Table 1). A similar pattern was observed for undetectable (68%) versus detectable (57%) BMP15. To investigate this further, using the continuous measurements for GDF9 and BMP15 and accounting for left censoring, and stratifying women per cycle duration, there were lower mean GDF9 concentrations in women with normal menstrual cycle lengths (<35 days) compared to women with cycles >35 days and/or irregular cycles ($p = 0.06$; Figure 1A). As expected, there was also strong evidence of a difference in mean AMH ($p < 0.001$; Figure 1C) between normal and irregular cycles, but the corresponding difference in BMP15 was nonsignificant (Figure 1B).

Ovarian volume, AFC and AMH were all lower in women with undetectable GDF9 compared to those with GDF9 > LOQ, and similarly for undetectable BMP15 which was associated with lower ovarian volume and AFC (Table 1). To investigate this further, analysis of the continuous measurements (Figure 2) demonstrated evidence of a positive correlation of GDF9 with ovarian volume ($p = 0.02$; Figure 2A) and with AFC ($p = 0.004$; Figure 2B), as well as marginally significant evidence of a positive correlation with AMH ($p = 0.05$; Figure 2C). The corresponding correlations between BMP15 and ovarian volume, AFC and AMH, were not statistically significant ($p = 0.06$, $p = 0.09$ and $p = 0.37$, respectively; see Figure 2D–F).

3.2 | PCOM and PCOS

Correlation analyses between GDF9 and BMP15 and the ovarian characteristics (ovarian volume, AFC and AMH) were compared between women with PCOS ($n = 129$), with PCOM only ($n = 58$) and women with neither PCOM nor PCOS, termed control ($n = 192$) (Table 2 and Supporting Information: Figure 1). A test for differences in these correlations showed no differences between the control, PCOM and PCOS groups in terms of their respective correlations with ovarian volume or AMH (p(int) nonsignificant; 0.20 and 0.14, respectively). However, for GDF9 and AFC, there was a significant difference between the correlations in control, PCOM and PCOS women (p(int) = 0.02), with a significant positive correlation between GDF9 and AFC in the PCOM group ($p = 0.004$), a positive trend in the PCOS group ($p = 0.05$), whereas no relationship in the control group ($p = .9$). This indicated that the associations between GDF9 and AFC differ in women with PCOM/PCOS compared to the control group. By contrast, there was no evidence of associations for BMP15 and ovarian volume/AFC/AMH between the control, PCOM and PCOS groups. Analysis of the mean concentrations of GDF9 or BMP15 were not significantly different between control, PCOM and PCOS groups (Table 3). AMH was significantly different between the groups ($p < 0.001$).

3.3 | Pituitary, oestrogenic and androgenic characteristics

Pituitary, oestrogenic and androgenic characteristics were analysed relative to GDF9 and BMP15 (Table 1). There were no significant differences observed between undetectable and detectable groups for GDF9 or BMP15 when p values were adjusted for multiple comparisons.

3.4 | Metabolic characteristics

Of the 10 metabolic characteristics assessed relative to GDF9 and BMP15 (Table 1), none were significantly different between the undetectable and detectable GDF9 or BMP15 groups when p values were adjusted for multiple comparisons (Table 1).

3.5 | Age and live birth count

There were no significant differences between undetectable and detectable GDF9 or BMP15 groups and either age or live birth count when p values were adjusted for multiple comparisons (Table 1). Correlation analyses with age were performed, as ovarian reserve is known to decline with age. The correlation coefficients for GDF9 ($r = -0.05$; $p = 0.10$) and BMP15 ($r = -0.05$; $p = 0.12$) were not statistically significant, whilst AMH showed evidence of a significant decline ($r = -0.26$, $p < 0.001$). Tests (p(int)) for differences between control, PCOM and PCOS groups in terms of their respective relationships between age and either GDF9, BMP15 or AMH were nonsignificant (see Supporting Information: Figure 2).

TABLE 1 Ovarian, endocrine and metabolic characteristics associated with polycystic ovary syndrome (PCOS) comparing women with undetectable and detectable serum concentrations of GDF9 and BMP15

		GDF9				BMP15			
Characteristics	All women	Undetectable (≤15.4 pg/ml)	Detectable (>15.4 pg/ml)	*p* value	Adjusted *p* value	Undetectable (≤23 pg/ml)	Detectable (>23 pg/ml)	*p* value	Adjusted *p* value
N	381	227	154			223	157		
Participant									
Age (years)	31.4 ± 5.0	31.9 ± 5.1	30.7 ± 4.7	**0.016**	0.380	31.8 ± 5.0	30.9 ± 4.9	0.104	0.963
No. live births	0.6 ± 0.9	0.57 ± 0.92	0.53 ± 0.91	0.635	1.000	0.57 ± 0.94	0.53 ± 0.88	0.690	1.000
Ovarian									
Regular menstrual cycles,^ *n* (%)	243 (64%)	158 (70%)	85 (55%)	**0.004**	0.116	152 (68%)	90 (57%)	**0.031**	0.605
Ovarian volume[a]	6.04 ± 2.96	5.69 ± 2.64	6.54 ± 3.32	**0.006**	0.164	5.77 ± 2.82	6.42 ± 3.12	**0.035**	0.658
AFC[a]	18.8 ± 12.4	17.0 ± 10.1	21.6 ± 14.9	**0.0004**	**0.012**	17.7 ± 11.6	20.4 ± 13.5	**0.041**	0.718
AMH (pmol/L)	48.2 ± 36.6	44.0 ± 33.4	54.3 ± 40.3	**0.008**	0.207	46.1 ± 35.6	51.1 ± 38.0	0.201	0.999
Pituitary									
FSH (IU/L)	7.43 ± 2.50	7.57 ± 2.68	7.22 ± 2.20	0.185	0.998	7.52 ± 2.72	7.31 ± 2.17	0.424	1.000
LH (IU/L)	5.91 ± 4.33	5.67 ± 4.09	6.26 ± 4.65	0.207	0.999	5.50 ± 3.73	6.50 ± 5.05	**0.033**	0.634
Prolactin (ml U/L)	275.7 ± 137.6	266.5 ± 123.9	289.1 ± 155.2	0.116	0.975	278.7 ± 141.6	270.2 ± 131.9	0.554	1.000
Oestrogenic									
Estrone (E1, pg/ml)	127.2 ± 48.7	124.0 ± 40.2	132.5 ± 60.3	0.164	0.995	123.0 ± 45.4	132.6 ± 52.8	0.110	0.970
Estradiol (E2, nmol/L)	178.1 ± 65.2	177.0 ± 67.4	179.8 ± 62.0	0.689	1.000	175.0 ± 57.9	182.8 ± 74.6	0.249	1.000
Androgenic									
Testosterone (nmol/L)	1.43 ± 0.74	1.37 ± 0.71	1.52 ± 0.78	0.055	0.819	1.37 ± 0.70	1.51 ± 0.77	0.086	0.932
DHT (nmol/L)	1.41 ± 1.00	1.33 ± 0.85	1.52 ± 1.19	0.067	0.876	1.46 ± 1.08	1.33 ± 0.87	0.215	0.999
Androstenedione (ADT) (nmol/L)	8.56 ± 6.31	8.02 ± 4.35	9.35 ± 8.35	**0.045**	0.749	7.74 ± 4.11	9.75 ± 8.40	**0.002**	0.068
DHEAS (μmol/L)	5.48 ± 2.29	5.35 ± 2.32	5.67 ± 2.24	0.178	0.997	5.36 ± 2.35	5.64 ± 2.20	0.236	1.000
SHBG (nmol/L)	53.7 ± 30.4	56.2 ± 32.2	50.1 ± 27.3	0.053	0.808	54.4 ± 28.9	52.9 ± 32.6	0.617	1.000
Free androgen index (FAI)	4.07 ± 4.37	3.89 ± 4.51	4.34 ± 4.17	0.330	1.000	3.85 ± 4.27	4.33 ± 4.47	0.293	1.000
mFG score (1–9)	1.65 ± 2.67	1.56 ± 2.16	1.77 ± 3.28	0.454	1.000	1.66 ± 2.82	1.62 ± 2.44	0.882	1.000
Androgen receptor activity	162.5 ± 108.4	152.8 ± 98.1	177.8 ± 121.9	**0.036**	0.671	153.4 ± 101.7	175.7 ± 117.0	0.060	0.842
Metabolic									
BMI	23.7 ± 5.3	23.3 ± 4.4	24.4 ± 6.25	**0.040**	0.707	23.8 ± 5.34	23.7 ± 5.1	0.964	1.000
WHR	0.79 ± 0.06	0.78 ± 0.06	0.79 ± 0.06	0.191	0.998	0.78 ± 0.06	0.79 ± 0.06	0.127	0.983
Insulin (mu/L)	8.95 ± 8.78	8.67 ± 8.38	9.37 ± 9.35	0.445	1.000	8.36 ± 7.94	9.77 ± 9.84	0.123	0.981
Glucose (mmol/L)	4.76 ± 0.61	4.78 ± 0.67	4.74 ± 0.51	0.541	1.000	4.79 ± 0.68	4.72 ± 0.50	0.286	1.000
Triglycerides (mmol/L)	1.02 ± 0.73	0.97 ± 0.47	1.10 ± 0.99	0.076	0.908	0.99 ± 0.79	1.07 ± 0.64	0.307	1.000
Cholesterol (mmol/L)	4.83 ± 0.86	4.77 ± 0.75	4.91 ± 0.99	0.114	0.973	4.80 ± 0.87	4.87 ± 0.84	0.483	1.000

(Continues)

TABLE 1 (Continued)

Characteristics	All women	GDF9 Undetectable (≤15.4 pg/ml)	GDF9 Detectable (>15.4 pg/ml)	GDF9 *p* value	GDF9 Adjusted *p* value	BMP15 Undetectable (≤23 pg/ml)	BMP15 Detectable (>23 pg/ml)	BMP15 *p* value	BMP15 Adjusted *p* value
HDL (mmol/L)	1.47 ± 0.36	1.49 ± 0.36	1.45 ± 0.34	0.297	1.000	1.47 ± 0.36	1.48 ± 0.35	0.720	1.000
LDL (mmol/L)	2.90 ± 0.79	2.82 ± 0.70	3.02 ± 0.90	**0.017**	0.401	2.90 ± 0.83	2.91 ± 0.74	0.887	1.000
Cholesterol: HDL ratio	3.45 ± 1.00	3.38 ± 0.99	3.55 ± 1.02	0.108	0.967	3.45 ± 1.02	3.45 ± 0.98	0.998	1.000
HOMA-IR	1.94 ± 2.06	1.86 ± 1.80	2.04 ± 2.39	0.403	1.000	1.81 ± 1.75	2.12 ± 2.43	0.145	0.991
Ovarian pathologies									
PCOM,[b] *n* (%)	187 (49%)	101 (44%)	86 (56%)	**0.030**	0.594	99 (44%)	88 (56%)	**0.025**	0.535
PCOS, *n* (%)	130 (34%)	71 (31%)	59 (38%)	0.155	0.994	70 (31%)	60 (38%)	0.167	0.996

Note: Data are mean ± SD, with comparisons of undetectable and detectable groups analysed by Mann–Whitney for continuous data, and proportional data are *n* (%) analysed by χ^2 test. To account for multiple comparisons, the Holm–Sidak method was used to calculate adjusted *p* values, where <0.05 was considered significant (highlighted in bold). ^25–34 days.

Abbreviations: AFC, antral follicle count; AMH, anti-Müllerian hormone; BMI, body mass index; BMP15, bone morphogenetic protein-15; DHEAS, dehydroepiandrosterone-sulphate; DHT, dihydrotestosterone; FSH, follicle stimulating hormone; GDF9, growth differentiation factor-9; HDL, high-density lipoprotein; HOMA-IR, homoeostatic model assessment for insulin resistance; LDL, low-density lipoprotein; LH, luteinizing hormone; mFG, modified Ferriman–Gallwey; PCOM, polycystic ovarian morphology; PCOS, polycystic ovary syndrome; SHBG, sex hormone-binding globulin; WHR, waist to hip ratio.

[a]Averaged from left and right ovaries.

[b]All PCOM: ≥22 follicles in either ovary, and/or ovarian volume ≥8.44 ml in either ovary (including PCOS).

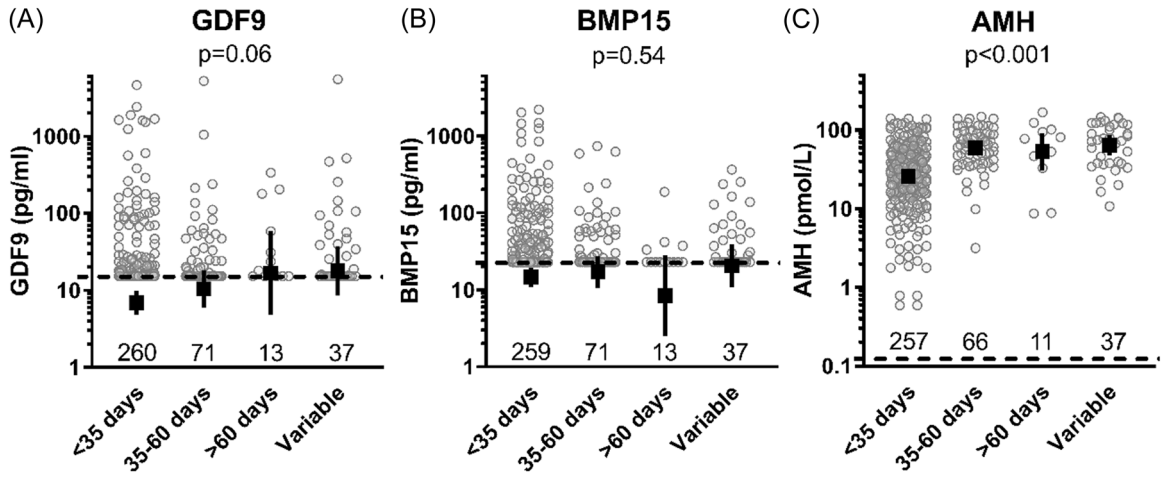

FIGURE 1 Menstrual cycle length relative to serum biomarker concentrations of (A) GDF9, (B) BMP15 and (C) AMH. Dashed horizontal lines indicate the limit of quantification of the assays, black squares with bars represent the estimated geometric mean ±95% CI, and numbers above the *x*-axis show the number of women in each group. AMH, anti-Müllerian hormone; BMP15, bone morphogenetic protein-15; CI, confidence intervals; GDF9, growth differentiation factor-9.

3.6 | GDF9 and BMP15

A positive correlation was observed between serum concentrations of GDF9 and BMP15 for all women ($p = 0.003$; Table 4 and Supporting Information: Figure 3). A test for differences between the correlation coefficients for the control, PCOM and PCOS groups (*p*(int)) showed no evidence of differences between correlation coefficients.

Of the 318 women, 78 (20%) had both GDF9 and BMP15 serum concentrations above their respective LOQs. In this subset, the GDF9:BMP15 ratios ranged from 0.015 to 82.25, with a median of 0.83 (95% CI: 0.57–1.14) and mean ± SD of 4.84 ± 13.72. There was no evidence of a difference in (median; 95% CI) GDF9:BMP15 ratios between control, PCOM and PCOS groups (see Table 3). GDF9:BMP15 ratios were also analysed relative to participant

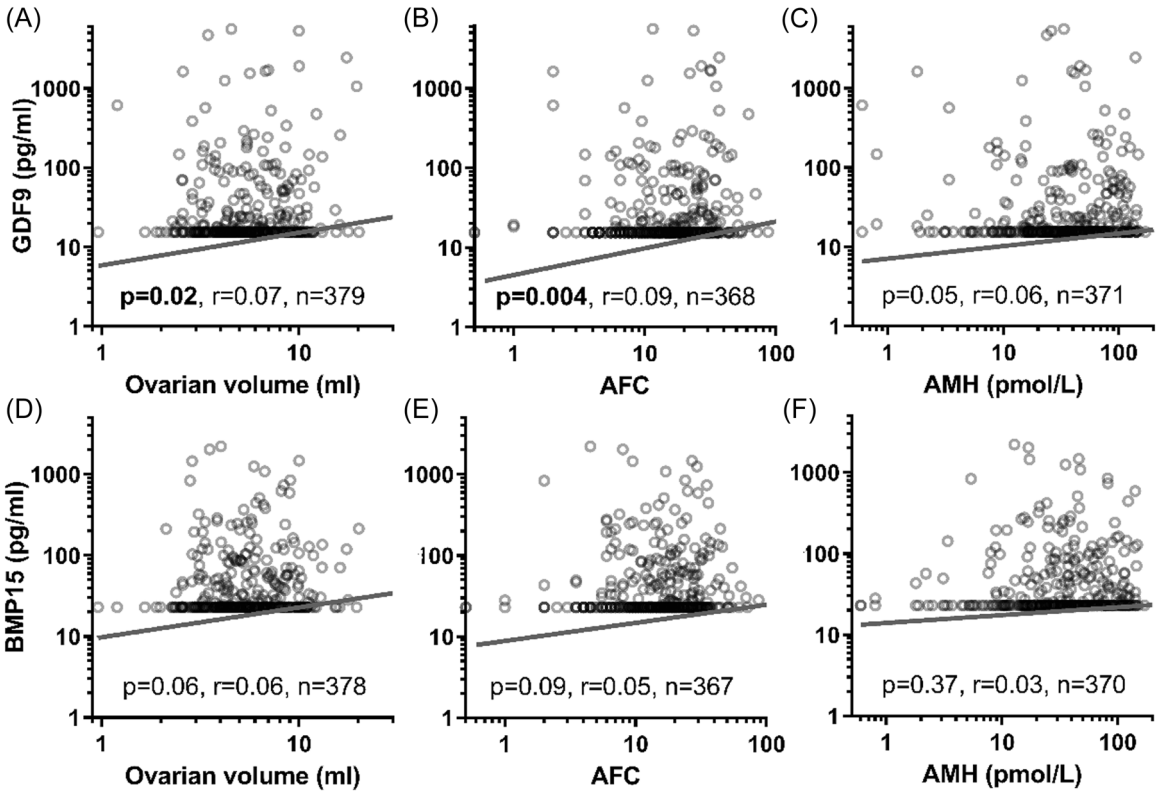

FIGURE 2 Relationship between serum biomarker concentrations and ovarian characteristics for all women. (A–C) GDF9 and (D–F) BMP15 concentrations relative to ovarian volume (A, D), antral follicle count (AFC) (B, E) and AMH (C, F) for all women (open circles), with regression lines shown. The limit of quantification of the assays was 15.4 pg/ml for GDF9 and 23 pg/ml for BMP15. Ovarian volume and AFC are averaged from the left and right ovaries. AMH, anti-Müllerian hormone; BMP15, bone morphogenetic protein-15; GDF9, growth differentiation factor-9.

characteristics and did not significantly correlate with age, live birth count, or with any of the ovarian, pituitary, oestrogenic, androgenic and metabolic characteristics when *p* values were adjusted for multiple comparisons (see Supporting Information: Table 1), except evidence of a significant positive correlation with triglycerides ($r = 0.373$, $p = 0.022$).

4 | DISCUSSION

The possible clinical significance of these oocyte-secreted factors as biomarkers is yet to be established, despite their well-recognized and described central role in regulation of granulosa cell differentiation and control of fecundity. In this study, elevated serum GDF9 was associated with increased ovarian volume, AFC and to a lesser extent AMH, as well as with irregular menstrual cycle length, supporting the notion of serum GDF9 being of follicular origin and a potential marker of ovarian function and ovarian reserve. These results complement recent evidence showing increased serum GDF9 with increased numbers of oocytes retrieved during IVF.[13]

While previous studies have reported aberrant mRNA expression of these oocyte-secreted factors in women with PCOS,[10,11] in the current study neither serum GDF9 nor BMP15 were predictors of PCOS. In addition, the relationships between GDF9 and BMP15 and ovarian characteristics were not significantly different between control, PCOM and PCOS groups, except for that between GDF9 and AFC. Furthermore, neither GDF9 nor BMP15 were associated with the androgenic or metabolic characteristics of PCOS. Taken together, these results suggest that serum concentrations of the oocyte secreted factors reflect the number of follicles present/ovarian status rather than PCOS pathology. Supporting this, a study by Kristensen et al.[31] measured GDF9 concentrations in the follicular fluid of small antral follicles and observed no difference in GDF9 concentrations between women with and without PCOM. The authors concluded that the production of GDF9 is not altered within individual follicles in women with PCOM compared to controls.

It is of interest that the relationship between GDF9 and AFC demonstrated opposing relationships between the control group and women with PCOM/PCOS. These were mutually exclusive groups, with the PCOM group having no cases of PCOS. However, due to the recruitment strategy, almost all (98%) of the women with PCOS had polycystic ovaries, and given the lack of association with androgenic characteristics, this is likely indicative of the higher number of follicles present producing GDF9, rather than a pathological abnormality.

Serum GDF9 and BMP15 were positively correlated with each other, but GDF9 was a stronger predictor of ovarian characteristics than BMP15. In humans, there is evidence that GDF9 is expressed throughout folliculogenesis, increasing from primordial through to

TABLE 2 Correlations between GDF9 and BMP15 serum biomarker concentrations and ovarian characteristics in women with and without PCOM and/or PCOS

	GDF9				BMP15			
	n	*r* (95% CI)	*p*	*p* (int)	*n*	*r* (95% CI)	*p*	*p* (int)
Ovarian volume								
Control	192	−0.02 (−0.10 to 0.06)	0.65		191	0.01 (−0.07 to 0.10)	0.73	
PCOM	58	0.11 (−0.05 to 0.28)	0.17	0.20	58	0.08 (−0.09 to 0.24)	0.37	0.81
PCOS	129	0.08 (−0.03 to 0.18)	0.15		129	0.02 (−0.09 to 0.13)	0.71	
AFC								
Control	192	−0.01 (−0.09 to 0.08)	0.90		191	0.00 (−0.08 to 0.08)	0.94	
PCOM	57	0.24 (0.08–0.40)	**0.004**	**0.02**	57	0.03 (−0.14 to 0.19)	0.74	0.96
PCOS	121	0.11 (0.00–0.22)	0.05		121	0.01 (−0.10 to 0.12)	0.85	
AMH								
Control	189	−0.03 (−0.11 to 0.05)	0.46		188	0.03 (−0.06 to 0.11)	0.54	
PCOM	59	0.03 (−0.13 to 0.19)	0.75	0.14	59	−0.10 (−0.27 to 0.06)	0.21	0.37
PCOS	123	0.11 (0.00–0.21)	0.06		123	0.00 (−0.11 to 0.11)	1.00	

Note: Data shown are the estimated Kendall's tau correlation coefficient (*r*) and 95% CI with associated p value for each slope. The *p*(int) term is the result from a global Wald interaction test for differences in correlations between the mutually exclusive groups of control, PCOM and PCOS. *p* values <0.05 were considered significant (highlighted in bold). Ovarian volume and AFC are averaged from the left and right ovaries (see Supporting Information Material for corresponding figures).

Abbreviations: AFC, antral follicle count; AMH, anti-Müllerian hormone; BMP15, bone morphogenetic protein-15; CI, confidence intervals; GDF9, growth differentiation factor-9; PCOM, polycystic ovarian morphology; PCOS, polycystic ovary syndrome.

TABLE 3 GDF9, BMP15 and AMH mean serum concentrations and GDF9:BMP15 ratios in control, PCOM and PCOS groups

	Control (*n* = 192)	PCOM (*n* = 58)	PCOS (*n* = 129)	*p* Value
GDF9 pg/ml, mean (95% CI)	6.46 (4.32–9.67)	9.93 (5.34–18.49)	11.30 (7.33–17.43)	0.11
BMP15 pg/ml, mean (95% CI)	12.79 (9.14–17.91)	17.56 (10.44–29.52)	18.30 (12.71–26.33)	0.26
AMH pg/ml, mean (95% CI)	2.58 (2.30–2.88)	6.09 (4.99–7.45)	10.11 (8.80–11.61)	**<0.001**
GDF9:BMP15 ratio (95% CI)[+]	(*n* = 33)	(*n* = 15)	(*n* = 30)	
	0.90 (0.52–2.01)	0.48 (0.26–0.82)	1.08 (0.56–1.92)	0.25

Note: Estimated geometric mean pg/ml (95% CI). +analysed by Kruskal–Wallis; samples with both GDF9 and BMP15 serum concentrations above their respective limits of quantification. *p* values <0.05 were considered significant (highlighted in bold).

Abbreviations: AMH, anti-Müllerian hormone; BMP15, bone morphogenetic protein-15; CI, confidence intervals; GDF9, growth differentiation factor-9; PCOM, polycystic ovarian morphology; PCOS, polycystic ovary syndrome.

preovulatory oocytes, whilst BMP15 is primarily derived from oocytes of small antral follicles (>75 um) increasing through to preovulatory oocytes.[32–34] Therefore, GDF9 may be more reflective of the ovarian reserve than BMP15. Further, BMP15 may be more reflective of the later-stage follicles than GDF9, with our previous study demonstrating elevated serum BMP15 mid-cycle following the LH surge.[24] It is also possible that there are extra-ovarian sources of these growth factors. A study using proteomic analysis detected GDF9 and BMP15 in human oocytes, but also reported low abundance BMP15, but not GDF9, in blood.[35] An extra-ovarian source of BMP15 may account for the lack of association between serum BMP15 and ovarian characteristics. One challenge for this area of research is that GDF9 and BMP15 are paracrine growth factors, not hormones, ostensibly expressed by just one cell, and as such have low abundance in serum, in the pg/ml range, as compared to AMH which is in the 2–6.8 ng/ml range.[36] Hence, this study was limited by the low detection rate of the GDF9 and BMP15 ELISAs, which in addition to being attributable to the very low levels of the proteins in circulation, may also be due to certain circulating form/s (e.g., pro-forms, processed mature domains) of either GDF9 or BMP15 not being detected by the current assays, as the native forms of these molecules are yet to be identified.

The association between GDF9 and ovarian reserve markers (ovarian volume, AFC and menstrual cycle regularity, as well as

TABLE 4 Correlations between serum biomarker concentrations of GDF9 and BMP15 in women with and without PCOM and/or PCOS, and all women combined

	GDF9			
	n	*r* (95% CI)	*p*	*p* (int)
BMP15				
Control	191	0.11 (0.04–0.17)	**0.002**	
PCOM	59	0.09 (−0.06 to 0.24)	0.23	0.39
PCOS	130	0.02 (−0.07 to 0.12)	0.63	
All women	380	0.08 (0.03–0.13)	**0.003**	n/a

Note: Data shown are the estimated Kendall's tau correlation coefficient (*r*) and 95% CI with associated *p* value for each slope. The *p*(int) term is the result from a global Wald interaction test for differences in correlations between the mutually exclusive groups of control, PCOM and PCOS (see Supporting Information Material for corresponding figure). *p* values <0.05 were considered significant (highlighted in bold).

Abbreviations: BMP15, bone morphogenetic protein-15; CI, confidence intervals; GDF9, growth differentiation factor-9; PCOM, polycystic ovarian morphology; PCOS, polycystic ovary syndrome.

weakly with AMH) was not reflected in comparisons with other markers of ovarian ageing; FSH, estradiol and age. These findings agree with previously published results showing no association with estradiol, FSH and age,[13] despite the two studies using different GDF9 ELISAs and geographically distinct study cohorts, and may be due to the relatively young age of the cohort (21–45 years).

One limitation of this study was the referral bias. PCOS patients can be divided into four phenotypes (A–D) based on the presence of the Rotterdam diagnostic criteria: phenotype A: hyperandrogenism (HA) + ovulatory disorder (OD) + PCOM; phenotype B: HA + OD; phenotype C: HA + PCOM; and phenotype D: OD + PCOM.[37] Referral bias is known to result in an overrepresentation of the complete PCOS phenotype A, and an increased rate of menstrual dysfunction.[38] In the current Singaporean cohort of predominantly Chinese ethnicity (70%), the prevalence of phenotypes A–D was 38%, 2%, 29% and 31%, respectively. In comparison, a cohort of 833 Chinese women reported phenotypes A–D of 29%, 19%, 37% and 15%, respectively, despite having a higher threshold mFG score (>6).[39] Therefore, this Singaporean cohort was predominantly women with PCOM, with a lower prevalence of hyperandrogenism. The non-hyperandrogenic PCOS phenotype (D) has the least endocrine and metabolic dysfunction and the lowest prevalence of metabolic syndrome.[37] However, referral PCOS patients are reported to have greater body mass indices, higher hirsutism scores and androgen levels when compared with unselected cohorts,[40] which did not seem to be the case in our cohort. Further, Singaporean women with PCOS and high BMI tend to be in the overweight rather than obese range.[19]

In summary, GDF9 and BMP15, as unique products of the oocyte and key regulators of female fertility, are hypothesized to reflect reproductive potential. They are therefore potential diagnostic tools to assess oocyte quantity/quality and/or ovarian pathologies. Current studies suggest that while GDF9, in particular, may be a marker of potential ovarian function and ovarian reserve, it is not a predictor of PCOS, and is not associated with the pituitary, oestrogenic, androgenic or metabolic features of PCOS. Clinical application of these biomarkers requires development of more sensitive assays, and characterization of the native forms of these proteins and their bioactivity in human serum.

AUTHOR CONTRIBUTIONS

Angelique H. Riepsamen, David M. Robertson, Robert B. Gilchrist, William L. Ledger and Eu-Leong Yong designed the study and secured funding for the project. Inthrani R. Indran and Eu-Leong Yong recruited the clinical cohort and acquired clinical data and samples. Angelique H. Riepsamen conducted GDF9 and BMP15 sample analysis. Angelique H. Riepsamen, Mark W. Donoghoe, Inthrani R. Indran, Leah Hechtman, David M. Robertson and Eu-Leong Yong analysed the data, with advanced statistical modelling and analysis provided by Mark W. Donoghoe. All authors provided data interpretation and manuscript preparation/revision.

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CONFLICTS OF INTEREST

A. H. R., D. M. R., R. B. G. and W. L. L. are inventors on a patent relevant to this work. The remaining authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Data underlying this article are available in the Dryad Digital Repository, at https://doi.org/10.5061/dryad.pc866t1pp, or are available are available from the corresponding author on reasonable request.

ETHICS STATEMENT

Ethics approval for the study protocol was obtained from the National Healthcare Group Domain Specific Review Board,

Singapore. Informed written consent was obtained from all participants. All participants providing written informed consent.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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