Heliyon 10 (2024) e29879

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Research article

Comparing GDF9 in mature follicles and clinical outcomes across different PCOS phenotype

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ARTICLE INFO

Keywords:
PCOS
GDF9
Oocyte competence
PCOS phenotypes
Mature follicles
Blastocyst formation

ABSTRACT

Background: Polycystic ovary syndrome (PCOS) is main cause of anovulatory infertility in women with gestational age. There are currently four distinct phenotypes associated with individualized endocrinology and metabolism. Growth differentiation factor 9 (GDF9) is a candidate as potential biomarker for the assessment of oocyte competence. The effect on oocyte capacity has not been evaluated and analyzed in PCOS phenotypes.

Objective: We aimed to screen the expression levels of GDF9 in mature follicles of women with controlled ovarian hyperstimulation (COS) with different PCOS phenotypes. To determine the correlation between the expression level of GDF9 and oocyte development ability.

Methods: In Part 1, we conducted a retrospective study comparing the clinical outcomes and endocrine characteristics of patients with PCOS according to different subgroups (depending on the presence or absence of the main features of polycystic ovarian morphology (PCOM), hyperandrogenism (HA), and oligo-anovulation (OA)) and non-PCOS control group. We stratified PCOS as phenotype A (n = 29), phenotype B (n = 18) and phenotype D (n = 24). In Part 2, the expression of GDF9 in follicular fluid (FF) and cumulus cells (CCs) were detected by enzyme-linked immunosorbent assay (ELISA) and immunohistochemistry, respectively.

Results: In Part 1, the baseline clinical, hormonal, and ultrasonographic characteristics of the study population were matched with the presence or absence of the cardinal features of each PCOS phenotypes showed a clear difference. Phenotypes A and D had statistically significant associations with blastocyst formation and clinical pregnancy compared with phenotypes B ($p < 0.001$). In Part 2, the levels of GDF9 in FF and CCs for phenotype A and B were significantly were higher than those of phenotype D ($P = 0.019$, $P = 0.0015$, respectively). Multivariate logistic regression analysis showed that GDF9 was an important independent predictor of blastocyst formation ($P < 0.001$). The blastocyst formation rate of phenotype A was higher than that of phenotype B and D ($P < 0.001$). Combining the results of the two parts, GDF9 appears to play a powerful role in the development of embryos into blastocysts.

Conclusions: GDF9 expression varies with different PCOS phenotypes. Phenotype A had higher GDF9 levels and blastocyst formation ability.

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https://doi.org/10.1016/j.heliyon.2024.e29879
Received 27 July 2023; Received in revised form 16 April 2024; Accepted 17 April 2024
Available online 23 April 2024
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1. Background

Polycystic ovary syndrome (PCOS) affects 5 %–20 % of women of reproductive age [1] and is considered the most common endocrine and metabolic disorder. It is characterized by oligo-anovulation (OA), hyperandrogenism (HA), polycystic ovarian morphology (PCOM) (≥12 follicles per ovary, about 2 ± 9 mm in diameter, and/or augmented ovarian volume >10 ml), hirsutism, insulin resistance, obesity, and menstrual irregularity [2]. Therefore, PCOS is multifactorial and heterogeneous with variable phenotypes infertility. Different PCOS phenotypes (A, B, C, D) were diagnosed according to the 2003 Rotterdam criteria [3]. Phenotype A shows all diagnostic features of the syndrome (chronic anovulation, HA, and polycystic ovaries on ultrasound). Type B presents with chronic anovulation and HA but lacks polycystic ovaries on ultrasound. Phenotype C includes women with regular menses but with HA

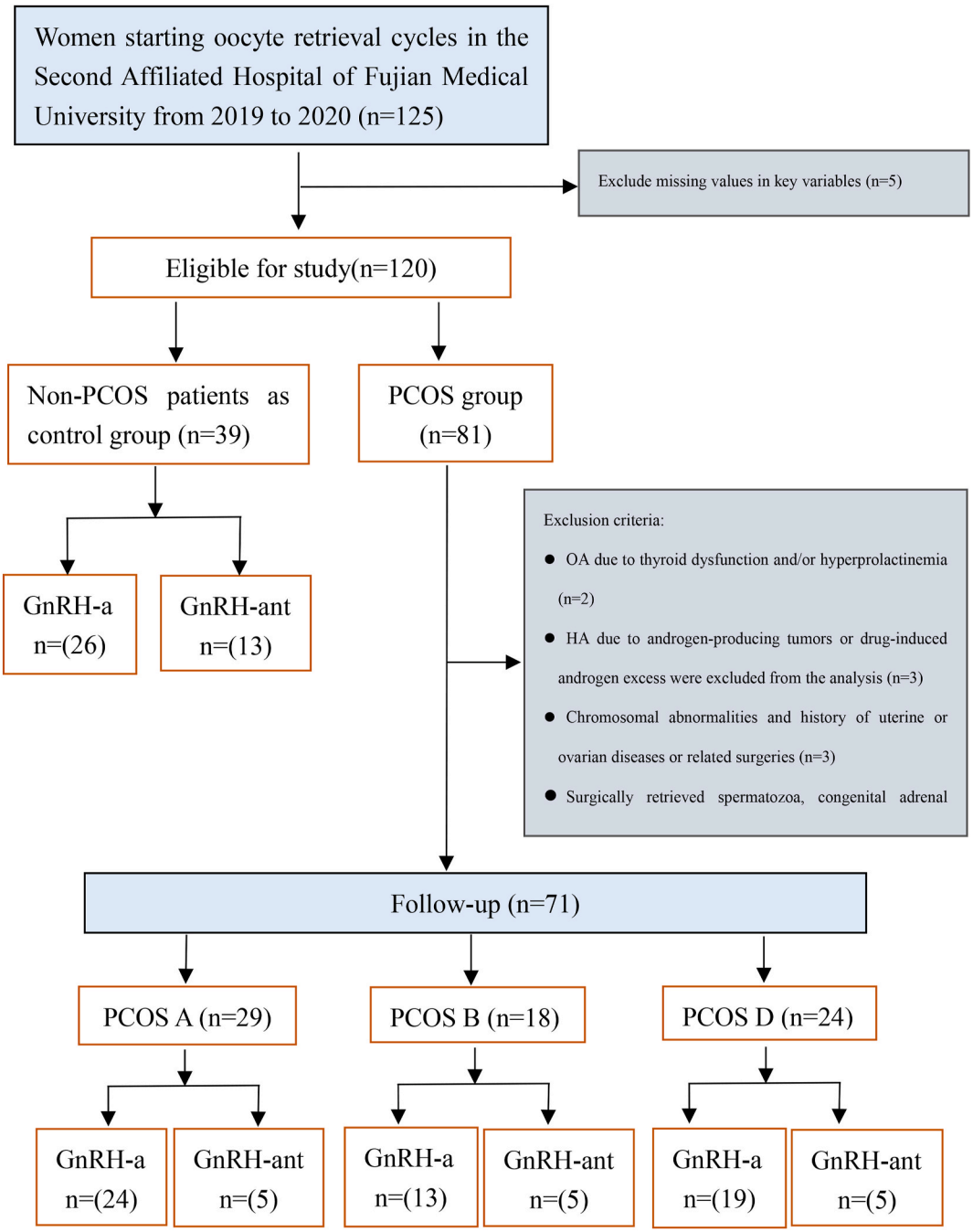

Fig. 1. Flow chart of patients and experimental design.

and polycystic ovaries, while phenotype D included women with irregular menstruation and polycystic ovaries ultrasonography but with no evidence of HA.

The effect of chronic HA on PCOS negatively affects the physiological androgen waning that occurs with the progression of follicle growth. Ovulation induction is often used to intervene in anovulatory PCOS; However, many women still fail to conceive and turn to assisted reproductive technology (ART). PCOS oocytes may also be of poor quality due to both intra-ovarian and extra-ovarian factors. PCOS is considered to be a systemic disease that requires multidisciplinary treatment. Therefore, more detailed studies of PCOS patients through phenotypic analysis are warranted to better monitor oocyte quality [4–6].

Numerous studies have shown that changes in biomolecular expression play an important role in oocyte development in PCOS [7]. Variations in biological molecules may be reflected in the FF composition, thereby affecting the microenvironment of oocyte growth [8]. The concentration of PCOS biomolecules in FF provides information about potential biomarkers of oocyte competence. In addition, perturbed fluid physiology has a synergistic effect on abnormal follicular genesis and disordered oogenesis in PCOS [9]. GDF9 is known to be an oocyte-specific paracrine factor. Both oocytes and CCs express GDF9, which is exchanged through gap junctions [10,11]. Studies have shown that increased GDF9 levels in FF are significantly correlated with oocyte maturation and embryo quality [12,13], suggesting a potential relationship between GDF9 levels and oocyte competence. GDF9 was an important biomarker for predicting oocyte development potential [14]. The FF and CCs are by-products of *in vitro* fertilization (IVF)/intracytoplasmic sperm injection (ICSI) which can reflect the ovarian microenvironment to a certain extent and directly reflect the oocyte metabolism and quality [15]. Therefore, understanding the exact relationship between the presence of GDF9 and oocyte volume in follicular development microenvironments with different PCOS phenotypes may help identify potential diagnostic targets and improve clinical management.

Based on these issues, this study aims to investigate the relationship between human oocyte volume and PCOS phenotype. It would be interesting to understand the reproductive potential of oocytes from women with different types of PCOS to determine whether oocyte abnormalities are associated with PCOS-related infertility. In this study, we detected the expression levels of GDF9 in FF and CCs in dominant follicle. In this study, we determined whether the expression of GDF9 varied with the phenotype of PCOS, and observed the correlation between GDF9 levels and oocyte developmental potential.

2. Materials and methods

2.1. Part 1

2.1.1. Study population and sampling

This study was conducted in the Second Affiliated Hospital of Fujian Medical University. Approved by the Ethics Committee of the Second Affiliated Hospital of Fujian Medical University (Ref:2019-222) with written informed consent from all patients. All participants were fully informed and provided written informed consent materials for public publication.

In the primary cohort, 125 patients who visited and received IVF/ICSI assisted pregnancies between 2019 and 2020 were assigned to each group. An independent cohort of 71 patients thoroughly followed up were assigned to the PCOS group and used to validate the model. The criteria of control group was referred to Anne-Laure Barbotin' research [16]. There were 39 non-PCOS patients with normal ovarian morphology, regular menstrual cycle, pathological infertility of female tubal or male infertility. To ensure a proper evaluation of the association between GDF9 expression and PCOS phenotypes, we restricted our analyses to strict cases selection criteria, including inclusion criteria and exclusion criteria. More details were shown in study diagram Fig. 1.

2.1.2. Inclusion criteria

Inclusion criteria are as follows: (1) Women aged 18–38 undergoing IVF/ICSI; (2) patients undergoing controlled ovarian stimulation treatment with the GnRH-a long protocol and GnRH-ant protocol; (3) Diagnosis of polycystic ovary syndrome according to Rotterdam criteria (Rotterdam ESHRE/ASRM Sponsored PCOS Consensus Workshop Group 2004) and so fulfilled at least two of the following three criteria: oligo- and/or anovulation, hyper-androgenism and polycystic ovary. PCOS patients were categorized as : phenotype A, B, C and D. In our study, all patients sought treatment for irregular menstruation, which contributed to the lack of PCOS type C patients. Notably, the ovulatory phenotype C demonstrated a relatively high likelihood of spontaneous pregnancy, which were excluded in the study.

2.1.3. Exclusion criteria

The exclusion criteria were as follows: (1) women with OA due to thyroid dysfunction and/or hyperprolactinemia; (2) HA due to androgen-producing tumors or drug-induced androgen excess were excluded from the analysis; (3) Chromosomal abnormalities and history of uterine or ovarian diseases or related surgeries; (4) surgically retrieved spermatozoa; (5) congenital adrenal hyperplasia, androgen secreting tumors of Cushing syndrome.

2.1.4. Ovarian stimulation

Based on clinical data such as infertility cause, age, follicle-stimulating hormone (FSH) levels, and antral follicle count (AFC), patients were selected for a personalized *in vitro* fertilization (IVF) protocal. The main use of follicular phase long acting gonadotrophin releasing hormone (GnRH) inhibitor protocal and GnRH antagonist protocal. After ovarian stimulation with gonadotropin -releasing hormone agonist (Serono, Geneva, Switzerlang) and recombinant FSH (Serono, Geneva, Switzerlang). Three or more follicles reached 17 mm diameter, and then 6000–10000 IU of human chorinic gonadotropin (hCG, Lizhu Inc., Zhuhai, China) was then administered to

Table 1
Characteristics and clinical outcome of patients according to PCOS phenotypes [$\bar{x} \pm s$, M(P_{25}, P_{75})].

Item	Control Group	PCOS A	PCOS B	PCOS D	F/x^2	P
No. of cycles	39	29	18	24		
Age (year)	31.38 ± 4.28	29.83 ± 2.73	29.83 ± 3.91	30.29 ± 3.20	1.328	0.269*
BMI (kg/m^2)	22.87 ± 2.63	23.53 ± 3.67	27.19 ± 3.07[a]	22.10 ± 2.85[c]	10.99	0.000 < 0.001*
AFC	15 (9,20)	29 (24,31)[a]	17 (15,18)[b]	27 (24.25,34.5)[c,d]	68.502	0.000 < 0.001**
SerumT (ng/ml)	0.31 (0.27,0.34)	0.66 (0.57,0.76)[a]	0.82 (0.72,0.93)[e]	0.31 (0.21,0.38)[c,f]	78.179	0.000 < 0.001**
Dosage of Gn used (IU)	2486.54 ± 137.63	2317.24 ± 159.60	3175.00 ± 202.5	1949.48 ± 175.46[c]	7.228	0.000 < 0.001*
E2 on hCG injection day (u mol/L)	4531.51 ± 490.25	6592.79 ± 568.53	4331.17 ± 721.63	5838.38 ± 624.95	3.388	0.021*
No. of follicles ≧14 mm	10.54 ± 4.24	14.69 ± 4.63[a]	12.17 ± 4.32	15.37 ± 6.29[c]	6.57	0.000 < 0.001*
No. of oocytes retrieved	15.54 ± 6.00	21.14 ± 4.20[a]	14.22 ± 5.05[b]	18.38 ± 7.66	6.989	0.000 < 0.001*
Blastocyst formation rate (%)	46.6 (232/498)	60.8 (318/523)[a]	44.4 (87/196)[g]	55.5 (207/373)[c,f]	20.75	0.000 < 0.001***
Clinical pregnancy rate (%)	56.9 (33/58)	70.7 (29/41)[a]	48.3 (14/29)[g]	64.7 (22/34)	4.176	0.243***
Type of fertilization (%)					1.056	0.798***
IVF	79.5 (31/39)	82.8 (24/29)	72.2 (13/18)	75.0 (18/24)		
ICSI	20.5 (8/39)	17.2 (5/29)	27.8 (5/18)	25.0 (6/24)		
Ovarian stimulation protocol					2.559	0.479***
Follicular phase long-acting GnRHagnonist protocol	66.7 (26/29)	82.8 (24/29)	72.2 (13/18)	79.2 (19/24)		
GnRH antagonist protocol	33.3 (13/39)	17.2 (5/29)	27.8 (5/18)	20.8 (5/24)		

Note: BMI: body mass index; T: testosterone; AFC: antral follicle count; AMH: anti-Müllerian hormone; FSH: follicle-stimulating hormone; LH: luteinizing hormone; E_2: estrogen; PRL: prolactin; Gn: gonadotropin; non-normal distributions are described statistically by median (IQR) *One-way ANOVA statistical analysis; ** Kruskal Wallis rank sum test; ***Pearson chi-square test.

[a] $P < 0.01$, compared with Control Group.
[b] $P < 0.01$, compared with PCOS A.
[c] $P < 0.01$, compared with PCOS B.
[d] $P < 0.01$, compared with Control Group.
[e] $P < 0.01$, compared with Control Group.
[f] $P < 0.01$, compared with PCOS A.
[g] $P < 0.01$, compared with PCOS A.

trigger final maturation. Oocytes were retrieved from under transvaginal ultrasonography-guided follicular aspiration was performed approximately 36 h after the hCG injection.

2.1.5. Embryo quality assessment and reproductive outcome

Embryo morphology was evaluated at 3, 5 and 6 days after oocyte retrieval. Blastocysts were scored according to the Gardner grading system [17]and recorded on the base of the expansion stage, inner cell mass and trophectoderm. Embryo vitrification was performed via a Cyrotop carrier system combined with DMSO-EG-S as cryoprotectants. Embryo thawing was operated in a sequential manner when cyrotop was transferred into dilution solution. Four to five weeks after embryo transfer, the gestational sac was observed by ultrasound scan to determine clinical pregnancy. And clinical pregnancy rate was calculated on a per transfer cycle.

2.1.6. Statistical analysis

The Statistical Package for Social Sciences (SPSS 22.0) and Graphpad Prism version 5 was used for statistical analysis. For normally distributed data sets, the quantitative variables are expressed as mean ± standard deviation, and comparisons between two groups were performed with the One-way ANOVA statistical analysis for parametric conditions. Continuous variables with non-normal distribution were represented via median [interquartile range (IQR)], and Kruskal–Wallis rank sum test allows comparisons for nonparametric conditions among the four groups. Comparison of the proportion of cases was evaluated by Pearson chi-square test between groups. To determine the independent effect of GDF9 on blastocyst formation and clinical pregnancy, a Binary Logistic Regression analysis was used after adjustment for well-established, pre-specified confounding factors including BMI, serum HA, AFC, dosage of Gn used, PCOS phenotypes. In the design, the dependent variable is dichotomous (blastocyst formation and clinical pregnancy), and the independent variables are dichotomous variables (serum HA), continuous variables (BMI, AFC, dosage of Gn used) and ordered multicategorical variables (PCOS phenotypes). In categorical variables (PCOS phenotypes), We designed individually PCOS phenotype A, B, D and analyze the risk of blastocyst formation and clinical pregnancy compared to control groups. The threshold for statistical significance was set to $p < 0.05$.

2.2. Part 2

2.2.1. Human FF and oocyte-cumulus complex collection

The FF was obtained from the first aspirated follicle which contains a single CCs. FF samples were chilled on ice and then centrifuged at 4 °C for 10 min at 300 g; and then 1 ml clear supernatant was transferred to a microfuge tube and stored at −80 °C for subsequent GDF9 assessment. The CC of each oocyte retrieved from the single follicle was mechanically removed with a 16-gauge microdissection needle [18]. The CCs were prepared on cell slides.

2.2.2. Detection of GDF9 levels in human FF

The FF was diluted 1:5 in phosphase-buffered saline (PBS) and measured using a human GDF9 ELISA Kit (Elabscience, Wuhan, China) according to the manufacture's instructions. The absorbance value at 450 nm was measured with an automatic microplate reader. The FF concentration was calculated using a standard curve.

2.2.3. Immunohistochemical staining

The distribution of GDF9 in CCs was detected using immunohistochemical staining following the manufacturer's instructions (BOSTER Biological Technology Co. Ltd, Wuhan, China). Briefly, the CCs cell slices were fixed with 4 % paraformaldehyde in phosphate-buffered saline for 15 min, then immersed in 3 % hydrogen peroxide to block endogenous peroxidase activity, and subsequently blocked in goat serum for 1 h. The slices were then incubated overnight at 4 °C with primary antibodies rabbit anti-GDF9 (GDF9, Abcam, USA, 1:100), followed by incubation with biotin-labeled anti-rabbit secondary antibody for half an hour. Finally, the slides were incubated with the peroxidase substrate DAB at room temperature until the desired staining intensity was achieved, lightly counter stained with hematoxylin, and covered with glass coverslips. Signals were examined and photographed using a microscope (Leica MZ16FA, Germany). Immunoreactivity was quantified using Image J 6.0, and 3–5 fields were randomly selected from each slide to determine the mean optical density (MOD).

3. Results

3.1. Part 1: participant characteristics

Table 1 showed the basic clinical characteristics of different PCOS phenotype groups. Overall, 71 PCOS patients and 39 control individuals were included. Of these, 29 out of 71 (40.8 %) patients had PCOS phenotype A, 18 out of 71 (25.6 %) had phenotype B, 24 out of 71 (33.8 %) had phenotype D. Of note. There were significant differences between the three PCOS phenotypes and control group in BMI and higher for PCOS phenotype B. ($P < 0.001$). Serum HA levels in phenotype A and B groups were significantly higher than those in phenotype D. Phenotypes A and D patients had higher AFC than phenotypes B. According to the ovarian stimulation cycle characteristics, the total dosage of Gn used was significantly higher for phenotype B than for phenotypes A and D. However, the number of follicles ≧14 mm, number of oocytes retrieved for phenotypes A and D was significantly higher than for phenotype B ($p < 0.001$). Finally, phenotypes A and D were associated with a statistically significantly greater blastocyst formation and clinical pregnancy than phenotypes B ($p < 0.001$); There are no statistically differences between the groups in age, E2 on HCG injection day, type of fertilization, Ovarian stimulation protocol.

Overall, 71 patients with PCOS and 39 controls were included in this study. Among the PCOS patients, 29 out of 71 (40.8 %)

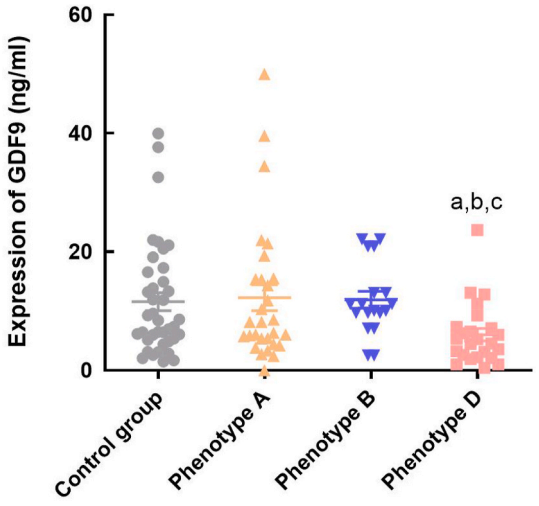

Fig. 2. Distribution of GDF9 in FF from the three PCOS phenotypes and control group. Data are presented as mean ± SD. $^{d}P < 0.01$, compared with Control Group; $^{a}P < 0.01$, compared with Control Group; $^{b}P < 0.05$, compared with PCOS A; $^{c}P < 0.05$, compared with PCOS B.

patients had PCOS phenotype A, 18 out of 71 (25.6 %) had phenotype B, and 24 out of 71 (33.8 %) had phenotype D. Notably, significant differences were observed between the three PCOS phenotypes and the control group in terms of BMI, which was higher in PCOS phenotype B. ($P < 0.001$). Serum HA levels were significantly higher in phenotypes A and B than in phenotype D. Patients with phenotype A and D had more AFC than patients with phenotype B. According to the ovarian stimulation cycle characteristics, the total dosage of Gn used was significantly higher for phenotype B than for phenotypes A and D. However, the number of follicles ≧14 mm and the number of oocytes retrieved for phenotypes A and D were significantly more than those for phenotype B ($P < 0.001$); The characteristics of PCOS subgroups matched with the presence or absence of the cardinal features of each PCOS phenotypes. Phenotypes A and D were associated with a statistically significantly greater blastocyst formation and clinical pregnancy than phenotypes B ($P < 0.001$); No statistically significant differences were observed between the groups in terms of age, E_2 on hCG injection day, type of fertilization, and Ovarian stimulation protocol.

3.2. Part 2: GDF9 in the FF of PCOS patients

Fig. 2 summarizes the GDF9 levels in the FF of all participants, and the expression of GDF9 in the three PCOS phenotypes and control groups was compared. The median GDF9 levels in FF was 7.35 ng/ml, (interquartile range 4.47–13.49 ng/ml). We observed that the level of GDF9 in phenotype D was significantly reduced compared to the control group, while there was no statistical difference in the other groups.

3.3. GDF9 expression in CCs

The role of GDF9 in follicles was explored in CCs. Considering oocytes were used to culture and transfer embryos, the corresponding CCs were used to analyze the expression of GDF9. Immunohistochemical staining of CCs from the three PCOS phenotypes and the control group is shown in Fig. 3. GDF9-positive cells, stained with brown cytoplasm, were detected in the CCs (Fig. 3a). Phenotype D had fewer GDF9-positive locations in the CCs than phenotypes A and B (Fig. 3a). The staining intensity for GDF9 in phenotype A (0.2592 ± 0.01505) and phenotype B (0.2407 ± 0.02748) were significantly higher than that in the control group (0.1388 ± 0.008261) (Fig. 3b). Interestingly, the GDF9 expression in phenotype D (0.1566 ± 0.007416) was similar to that in the control group. This suggests that the expression of GDF9 in different PCOS phenotypes is complex, diverse, and may be influenced by other factors, such as BMI and testosterone levels.

Analysis of multiple factors affecting blastocyst formation and clinical pregnancy in the three PCOS phenotypes.

Risk factors associated with blastocyst formation and pregnancy outcomes were investigated using logistic regression analysis (Table 2). Blastocyst formation rate was chosen as the dependent factor. BMI, serum HA, AFC, Dosage of Gn and GDF9 (categorical variables), and PCOS phenotypes were chosen as independent factors. The findings revealed that: (1) GDF9 might be a significant independent prognosticator for blastocyst formation, while it had no significant predictive value for clinical pregnancy when adjusted for BMI, serum HA, AFC, dosage of Gn used, and PCOS phenotypes; (2) serum HA had a markedly negative influence on blastocyst formation (OR = 0.321, 95 % CI: 0.232–0.443); and (3) the phenotype A group had a 3.347 times higher odds of blastocyst formation than the control group.

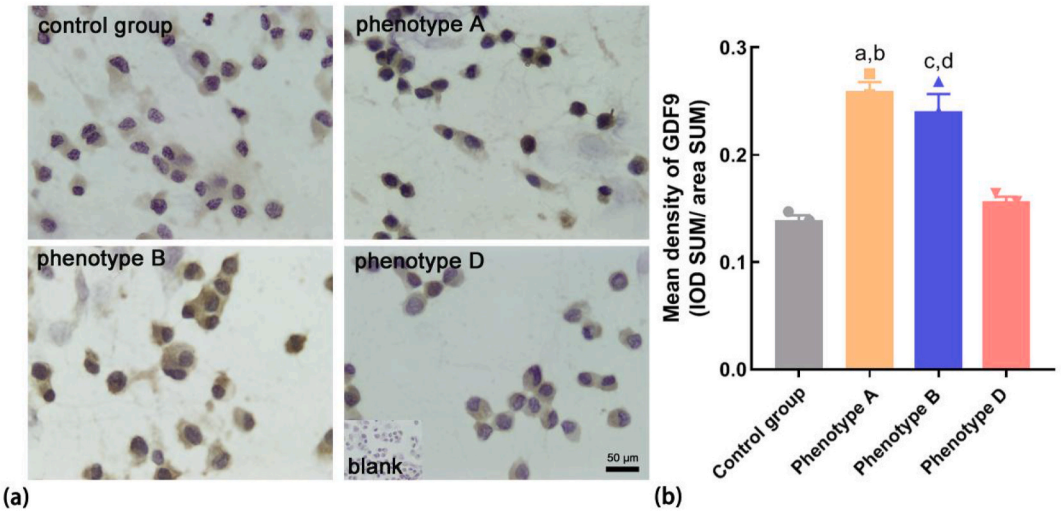

Fig. 3. Expression of GDF9 in the CCs. (a) Immunohistochemical staining of GDF9 expressed in the control group, phenotype A, phenotype B and phenotype D. (Original magnification: × 400, bars, 50 μm). (b) Mean density of GDF9 in CCs of the three PCOS phenotypes and control group, $^{a}P < 0.01$, compared with Control Group; $^{b}P < 0.01$, compared with PCOS D; $^{c}P < 0.01$, compared with Control Group; $^{d}P < 0.01$, compared with PCOS D.

Table 2
Analysis of multiple factors affecting blastocyst formation and clinical pregnancy rate in the PCOS phenotypes.

	blastocyst formation		clinical pregnancy	
	OR (95%CI)	P	OR (95%CI)	P
GDF9 (ng/ml)	0.991 (0.986–0.995)	<0.001	1.001 (0.958–1.046)	0.956
BMI(kg/m^2)	0.936 (0.922–0.950)	<0.001	0.851 (0.744–0.973)	0.018
Serum T (ng/ml)	0.321 (0.232–0.443)	<0.001	0.253 (1.119–5.72)	0.042
AFC	0.995 (0.993–0.997)	<0.001	1.066 (0.996–1.141)	0.064
Dosage of Gn used (IU)	1.000 (1.000–1.000)	0.261	1.001 (1.000–1.001)	0.020
PCOS phenotypes				
Control group	1.00		1.00	
Phenotype A	3.347 (2.862–3.914)	<0.001	0.356 (0.070–1.898)	0.231
Phenotype B	1.507 (1.214–1.870)	<0.001	0.205 (0.029–1.462)	0.114
PhenotypeD	1.475 (1.318–1.650)	<0.001	0.960 (0.267–3.456)	0.960

OR: odds ratio; CI: confidence interval.

4. Discussion

The specific features of PCOS include OA, HA, and PCOM. These cardinal features, either individually or in combination. The incidence and severity of PCOS varied with different phenotypes, and classified as phenotypes A, B, C, or D. In addition to aggravating the severity of PCOS [19], this mutation also affects the reproductive potential of PCOS patients [20]. In the present study, we evaluated the changes in intraovarian GDF9 levels in patients with PCOS who underwent IVF treatment. Over the past 10–15 years, increasing evidence shown that GDF9 is a key regulator of follicular genesis and promotes important bidirectional communication between oocytes and somatic cells through cross-regional projection [21,22]. We observed differences in GDF9 expression among oocytes with different phenotypes. The median GDF9 level in FF was 7.35 ng/ml, which is suitable for embryo development. However, FF GDF9 levels in phenotype D were markedly lower than those in the other phenotypes. Moreover, consistent with FF findings, CCs stained positive for GDF9. In this study, phenotypes A and B showed more GDF9-positive staining in CCs than phenotype D. Our study corroborated previous reports indicating the presence of GDF9 in human FF, accompanied by its expression in human CCs. Bidirectional communication between oocytes and CCs favors the normal follicular balance and development [23]. We found that compared to phenotype D, both FF and histological inspection of GDF9 protein levels in phenotypes A and B were increased. GDF9 is involved in regulating CCs gene expression, a broad range of CCs functions, glycolysis in CCs, and amino acid uptake and transport in oocytes [24]. We speculated that the oocyte and embryo qualities were different for various PCOS phenotypes.

For PCOS patients undergoing assisted reproductive therapy (ART), the best protocol for controlling ovarian hyperstimulation (COH) is controversial. The current study shows that the GnRH-a long-term protocal has better pregnancy outcomes [25]. Actually, there are relatively limited studies comparing the outcomes of different COH protocols in patients with different PCOS phenotypes. The likely reason that long-acting GnRH-a regimens are superior to other regimens is that follicular long-acting GnRH-a regimens use higher doses of GnRH-a for a longer duration [26]. Jie Qiao and her companions [27] recommended initial FSH dose should be individualized for IVF/ICSI PCOS patients receiving the GnRH antagonist protocol. This dose helped to obtain an optimal number of oocytes and minimized the risk of ovarian hyperstimulation syndrome (OHSS).

In combination with the observation of clinical characteristics and treatment outcomes of patients with PCOS phenotypes, phenotypes A and D were associated with significantly greater blastocyst formation and clinical pregnancy than phenotype B. It has been suggested that women with PCOS, as an intermediate group of metabolic dysfunction, show similar or better outcomes during IVF cycles than those with normal ovulation [28]. A sufficient amount of GDF9 promotes granulosa cells to produce a receptor effect on FSH and E2 in favor of blastocyst formation [29]. Phenotypes A and D appear to have similar blastocyst formation and clinical pregnancies.

However, to adjust for blastocyst formation and clinical pregnancy of PCOS phenotypes, we designed and analyzed the risk of specified confounding factors, including BMI, serum HA, AFC, and Gn dosage. In this study, PCOS phenotypes treated with HA had more GDF9 than their normoandrogenic counterparts. Androgens are essential in early folliculogenesis and preovulatory follicular stage [30]. GDF9 plays an important role in the process of follicular development, such as the recruitment of primordial follicles, ovulation, and even in corpus luteum formation [31–33]. They may have a synergistic effect on folliculogenesis and embryonic development by coordinating fluid physiology, specifically in subgroups of obese women with HA [34]. Likewise, the impact of HA on oocyte quality is subject to debate. Similarly, the effect of HA on oocyte quality is debatable. Androgens are involved in folliculogenesis, and a hyperandrogenic environment leads to abnormal folliculogenesis, premature activation of follicles, mitochondrial abnormalities, and failure of meiotic progression to MII. Furthermore, HA induces the premature luteinization of granulosa cells, which prevents their progression to physiological atresia. Recent reports have shown that phenotypes A and B are associated with a greater risk of adverse outcomes in pregnancy [35], and PCOS phenotypes with HA are associated with lower cumulative live birth rates when compared with their normoandrogenic counterparts. There is increasing evidence regarding the effects of increased BMI and HA on IVF outcomes, which may be related to the pathogenesis of PCOS [36–38]. Dyslipidemia plays a potential role in fertility failure by inducing oxidative stress [39]. Previous research even mentioned [40] that the modest increase in serum FSH levels by rFSH administration during COS is inversely correlated with the decrease in serum AMH levels that precedes the emergence of a dominant follicle. This hypothesis is reinforced by our finding that for phenotype with PCOM during IVF, the number of follicles ≧14 mm and

oocytes retrieved were obviously increased. Spontaneously, this is because once CCs have received sufficient FSH for a sufficient time, the imbalance between the effects of FSH and AMH on the control of aromatase expression is corrected, leading to the clearing of the excess AMH and increasing the content of E_2 within the CCs.

Indeed, decreased blastocyst formation was confirmed in phenotype D after logistic regression analysis controlling for all potential confounders. Furthermore, our findings suggest that phenotype A, which is considered the most severe phenotype, is not associated with poor reproductive outcomes. Instead, it contributes beneficially to oocyte competence, as evidenced by the highest blastocyst formation after adjusting for BMI, serum HA, AFC, and Gn dosage. These data demonstrated the important functions of GDF9 in oocyte development. Among PCOS with different phenotypes, GDF9 may be the most suitable biomarker for phenotype A. Blastocyst formation and clinical pregnancy rates did not increase, accompanied by increased GDF9 expression. After adjusting for BMI, serum HA, AFC, Gn dose and PCOS phenotype, GDF9 as an independent predictor of blastocyst formation had no significant predictive value for clinical pregnancy. And the phenotype A group had a 3.347 times higher odds of blastocyst formation compared to the control group. Researchers have shown that biomarkers in FF should focus on subgroups of patients with good characteristics based on phenotype comparison [41]. On the other hand, Qiao et al. found that the metabolic syndrome of PCOS, rather than other phenotypes, was a predictor of IVF clinical outcomes [42]. GDF9 may be a suitable biomarker for PCOS with different phenotypes, which may have important implications for advancing clinical management strategies for PCOS. We also verified that HA had a markedly negative effects on blastocyst formation in our analysis results and phenotype B had the least blastocyst formation. A report about the association of hyperandrogenism and patients with different PCOS phenotypes undergoing IVF/ICSI indicated the adverse pregnancy outcomes [43].

5. Conclusions

Various morphological parameters are used to evaluate oocyte quality and predict embryonic development. The evidence presented in our study supports the role of GDF9 produced by oocytes and CCs as a predictive biomarker of oocyte competence and blastocyst formation in different PCOS phenotypes, particularly phenotype A. Our study prospectively examined the effect of GDF9 on embryo development and clinical outcomes in different PCOS phenotypes by incorporating clinically relevant characteristics such as BMI, serum HA, AFC, and Gn dosage.

However, this study had some limitations. First, we are aware that the participants number in our study is the relatively small because of the strict inclusion criteria applied. This study lacked patients with PCOS phenotype C, which could introduce an enrollment bias in a clinical setting. So further studies are warranted to confirm our founding. Second, The current study is a single-center design, which might have caused selection bias. Thus, we would collect data from other medical centers to further validate. In addition, due to the retrospective nature of this cohort study, the choice of COH protocol for patients was determined based on factors such as their AMH level and age, and was not randomly assigned. Therefore, this conclusion needs to be verified by more randomized controlled trials (RCTs) and prospective studies.

Funding

This study was supported by the Science and Technology Projects of Quanzhou [grant numbers: 2019N085S and 2021N016S], Startup Fund for Scientific Research, Fujian Medical University [grant number: 2018QH1100], the Natural Science Foundation of Fujian Province [grant number: 2022J01778] and Second Affiliated Hospital of Fujian Medical University PhD Nursery Project [grant number BS202108].

Availability of data and materials

The data that support the study are available upon reasonable request to the corresponding author.

Data availability statement

The Data of this study will be made available on request.

Ethics approval and consent to participate

The study was approved by the Ethical Committee of the Second Affiliated Hospital of Fujian Medical University and written informed consent was obtained from all patients. (Ethics Number: 2019-222). All participants signed the informed consent form before any study-specific procedures were performed.

CRediT authorship contribution statement

Jingjing Cai: Writing – original draft, Funding acquisition, Conceptualization. **Xiangmin Luo:** Writing – original draft, Investigation, Data curation. **Zhengyao Wang:** Software, Resources, Investigation. **Zixuan Chen:** Software, Resources, Project administration. **Donghong Huang:** Visualization, Supervision, Software. **Hui Cao:** Resources, Project administration, Methodology. **Jing Chen:** Validation, Resources, Project administration. **Jinxiang Wu:** Writing – review & editing, Software, Resources, Funding

acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors would like to thank the patients for their participation in this study.

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