

Empagliflozin-Metformin is Associated with Upregulation of *GDF9* and *BMP15* Expression in Cumulus Cells of PCOS Patients Undergoing ICSI: Correlation with Oocyte Quality and Pregnancy Outcomes

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ABSTRACT

Oocyte-secreted factors, including growth differentiation factor 9 (*GDF9*) and bone morphogenetic protein 15 (*BMP15*), play critical roles in follicular development and oocyte maturation. Dysregulation of these factors is implicated in polycystic ovary syndrome (PCOS). This prespecified secondary analysis of a randomized, double-blind, placebo-controlled pilot trial evaluated the effects of empagliflozin-metformin and linagliptin-metformin compared to metformin alone and placebo on *GDF9* and *BMP15* expression in cumulus cells of infertile women with PCOS undergoing intracytoplasmic sperm injection (ICSI), and explored correlations between gene expression, oocyte quality, and pregnancy outcomes. Eighty women with PCOS (20 per arm) received empagliflozin-metformin (5/500 mg), linagliptin-metformin (2.5/500 mg), metformin (500 mg), or matched placebo twice daily for eight weeks prior to ovarian stimulation. Cumulus cells were collected during oocyte retrieval, and *GDF9* and *BMP15* mRNA expression levels were measured using qRT-PCR. Correlations between gene expression and oocyte/embryo outcomes were assessed using Pearson correlation coefficients. Expression of *GDF9* and *BMP15* mRNA was significantly increased in all active treatment groups compared with placebo ($P < 0.001$ for all comparisons), with the empagliflozin-metformin group showing the highest expression of both genes. The fold-change in *GDF9* expression relative to placebo was 4.2 ± 0.8 for empagliflozin-metformin, 2.8 ± 0.6 for linagliptin-metformin, and 1.9 ± 0.4 for metformin. For *BMP15*, the fold-changes were 4.5 ± 0.9 , 3.0 ± 0.7 , and 2.0 ± 0.5 , respectively. Strong correlations were observed between *GDF9/BMP15* expression and improved oocyte and embryo outcomes exclusively in the empagliflozin-metformin group ($r = 0.72-0.82$, $P < 0.001$). In this group, *BMP15* expression was negatively correlated with free androgen index ($r = -0.72$), leptin ($r = -0.75$), and anti-Müllerian hormone ($r = -0.62$), and positively correlated with mature oocytes ($r = 0.72$), grade I embryos ($r = 0.82$), and clinical pregnancy ($rpb = 0.68$, $P < 0.001$). For *GDF9*, the point-biserial correlation with clinical pregnancy was $rpb = 0.64$ ($P = 0.001$). These findings suggest that empagliflozin-metformin is associated with significant upregulation of *GDF9* and *BMP15* expression in cumulus cells, correlating with improved reproductive outcomes. However, these results are exploratory and hypothesis-generating, requiring confirmation in larger mechanistic studies with protein-level validation and live birth as the primary endpoint.

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Introduction

Polycystic ovary syndrome (PCOS) is one of the most common endocrine disorders affecting

women of reproductive age, with a global prevalence ranging from 5% to 15% depending on the diagnostic criteria applied (Moreira *et al.*,



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2023). The syndrome is characterized by hyperandrogenism, chronic oligo-ovulation or anovulation, and polycystic ovary morphology on ultrasound (Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group, 2004). Insulin resistance is a central pathophysiological driver, affecting 50% to 70% of affected women regardless of body weight (Karagül *et al.*, 2018). Hyperinsulinemia stimulates ovarian theca cell androgen production while reducing hepatic synthesis of sex hormone-binding globulin, thereby increasing bioavailable testosterone and exacerbating the hyperandrogenic state (Dumesic *et al.*, 2015). These endocrine and metabolic perturbations disrupt normal folliculogenesis and compromise oocyte quality, leading to impaired oocyte maturation, increased cytoplasmic granularity, vacuolization, and disorganized meiotic spindles that reduce fertilization and embryo development potential (Dumesic *et al.*, 2015).

Among the most critical oocyte-secreted factors are growth differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15), both members of the transforming growth factor-beta (TGF- β) superfamily (Otsuka and Shimasaki, 2002). These paracrine factors are produced exclusively by oocytes and act on surrounding cumulus cells to regulate their function, playing essential roles in follicular development, oocyte maturation, cumulus expansion, and subsequent embryo development. GDF9 and BMP15 function through Smad signaling pathways to regulate granulosa cell proliferation and differentiation, and their coordinated expression is critical for the acquisition of oocyte developmental competence (Karagül *et al.*, 2018). Zhao and colleagues demonstrated significantly decreased *GDF9* expression in cumulus granulosa cells of PCOS patients compared to controls, suggesting that reduced *GDF9* expression may be associated with diminished oocyte developmental potential (Zhao *et al.*, 2010). A recent systematic review and meta-analysis confirmed these findings, reporting consistently reduced *GDF9* and *BMP15* expression in cumulus cells of PCOS patients across multiple studies.

Reduced expression or signaling of GDF9 and BMP15 has been consistently associated with arrested follicular growth and poor oocyte quality

in patients with PCOS (Hassani *et al.*, 2018). Therefore, therapeutic strategies that enhance the expression of these critical factors may provide a targeted approach to improving oocyte quality and fertility outcomes in this challenging patient population (Gode *et al.*, 2011).

Metformin, an insulin-sensitizing biguanide, has been the standard first-line pharmacological treatment for PCOS for decades (Nestler *et al.*, 1998). However, its direct effect on oocyte quality is modest, and gastrointestinal side effects limit its tolerability in up to 20% to 30% of patients (Bouchoucha *et al.*, 2011). Evidence regarding metformin's efficacy in improving in vitro fertilization (IVF) outcomes remains inconsistent, suggesting the need for more effective therapeutic approaches (Hassani *et al.*, 2018).

Recently, combination therapies initially developed for type 2 diabetes mellitus have emerged as promising options for PCOS management. Among these, empagliflozin-metformin and linagliptin-metformin have gained attention for their potential to address both metabolic and reproductive aspects of the syndrome (Sanjari *et al.*, 2025; Javed *et al.*, 2019). Linagliptin, a dipeptidyl peptidase-4 (DPP-4) inhibitor, enhances incretin signaling, thereby improving glucose-dependent insulin secretion and suppressing glucagon release (Gallwitz, 2019). Empagliflozin, a highly selective sodium-glucose cotransporter type 2 (SGLT2) inhibitor, inhibits renal glucose reabsorption, promoting urinary glucose excretion and inducing a net caloric loss of approximately 200-300 kcal/day. This effect promotes lipolysis, reduces body weight, and improves insulin sensitivity (Ferrannini *et al.*, 2014; Zinman *et al.*, 2015). Recent randomized trials have demonstrated that empagliflozin, alone or combined with metformin, significantly improves metabolic and hormonal parameters in PCOS patients (Sharif *et al.*, 2025). A systematic review concluded that SGLT2 inhibitors represent a promising therapeutic approach for improving metabolic and hormonal parameters in PCOS (Kamrul-Hasan *et al.*, 2025). Despite these encouraging metabolic findings, the molecular mechanisms underlying the reproductive benefits of these combination therapies remain poorly understood. To date, no study has comprehensively evaluated the effects of empagliflozin-metformin or linagliptin-

metformin combination therapy on oocyte-specific molecular markers, including *GDF9* and *BMP15*, while simultaneously assessing classical IVF/ICSI outcomes in patients with PCOS. A recent randomized controlled trial by Fakhreddin *et al.* (2026) reported that dapagliflozin-metformin combination therapy was associated with improved oocyte quality and reproductive outcomes in PCOS patients undergoing IVF/ICSI, with concurrent upregulation of *BMP15* and *GDF9* expression. However, the effects of empagliflozin-metformin and linagliptin-metformin on these molecular markers have not been directly compared. The primary metabolic and hormonal outcomes of this trial have been reported previously (Mousavi *et al.*, 2026), and oocyte and embryo outcomes have been reported separately. The present study, a prespecified secondary analysis of the same randomized trial, was designed to evaluate the effects of eight weeks of pretreatment with linagliptin-metformin and empagliflozin-metformin compared with metformin alone and placebo on *GDF9* and *BMP15* expression in cumulus cells of infertile women with PCOS undergoing ICSI. Additionally, we aimed to explore correlations between gene expression and oocyte quality, embryo development, and pregnancy outcomes. We hypothesized that combination therapies would be associated with greater upregulation of *GDF9* and *BMP15* expression compared with metformin monotherapy, and that these molecular changes would correlate with improved oocyte quality and pregnancy outcomes.

Materials and Methods

Study design and participants

This randomized, double-blind, placebo-controlled pilot trial was conducted at the Molood Infertility Clinic affiliated with Zahedan University of Medical Sciences, Iran, from November 2024 to March 2025. The study protocol was approved by the Ethics Committee of Arak University (IR.ARAKU.REC.1403.026), which serves as the primary institutional review board for the corresponding author's research group. The study was conducted at the Molood Infertility Clinic affiliated with Zahedan University of Medical Sciences, which provided clinical facilities and staff. The ethics committee

of Zahedan University of Medical Sciences was informed of the study and granted local institutional approval. The study was registered with the Iranian Registry of Clinical Trials (IRCT20241027063520N1) and conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from all participants.

Women aged 25 to 35 years with a diagnosis of PCOS scheduled for ICSI were assessed for eligibility. Diagnosis was confirmed based on the Rotterdam criteria, which require at least two of the following: 1) oligo-ovulation or anovulation; 2) clinical and/or biochemical signs of hyperandrogenism; and 3) polycystic ovaries on ultrasound (≥ 12 follicles measuring 2- 9 mm in diameter and/or ovarian volume >10 cm³) (Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group, 2004). Exclusion criteria included hypersensitivity to study drugs, congenital adrenal hyperplasia, thyroid disorders, Cushing's syndrome, hyperprolactinemia, androgen-secreting neoplasia, severe liver, kidney, pancreatic diseases, gallstones, type 2 diabetes mellitus, use of medications affecting carbohydrate metabolism within two months prior to enrollment, and recurrent urinary tract infections.

Treatment groups and allocation

Of the 118 women assessed, 38 were excluded before randomization. The remaining 80 women were randomly assigned to four equal groups of 20 each: the empagliflozin-metformin group (empagliflozin-metformin 5/500 mg, Abidi, Iran; brand name Synoripa) twice daily, the linagliptin-metformin group (linagliptin-metformin 2.5/500 mg, Al-Hawi, Iran; brand name Melijent-M) twice daily, the metformin group (generic metformin 500 mg twice daily); and placebo group (placebo twice daily). For the remainder of the manuscript, generic names are used consistently. Treatment was initiated two months before ovulation induction and continued until oocyte retrieval. The 8-week treatment duration was selected based on several considerations. First, prior studies have shown that the metabolic effects of SGLT2 inhibitors and DPP-4 inhibitors plateau within 6 to 8 weeks of treatment initiation (Ferrannini *et al.*, 2014; Gallwitz, 2019). Second, this duration balances adequate metabolic

preconditioning with minimizing delays in fertility treatment, which is a critical consideration for infertile couples. Third, a recent randomized trial by Daneshjou *et al.* (2022) demonstrated that 8 weeks of sitagliptin-metformin pretreatment significantly improved fertilization rates and embryo quality in PCOS patients undergoing ICSI, providing a precedent for this duration. Fourth, the 8-week period aligns with the typical waiting time for ovarian stimulation scheduling in our clinic, making it feasible for clinical implementation. During the study, seven participants discontinued due to personal reasons or relocation and were replaced to maintain 20 participants per group for per-protocol analysis.

Randomization, blinding, and sample size

A computer-generated randomization sequence with a block size of 8 was created by an independent statistician. Allocation was concealed using sealed, opaque, sequentially numbered envelopes. Study drugs and matched placebos were packaged identically. All participants, investigators, clinical staff, and laboratory personnel remained blinded throughout the study. This pilot trial was designed to detect a large effect size ($f = 0.4$) in primary metabolic outcomes using one-way ANOVA with 80% power and two-sided $\alpha = 0.05$, requiring 20 participants per group. The study was not powered for molecular outcomes, which are reported as secondary and exploratory.

Ovarian stimulation, oocyte retrieval, and cumulus cell collection

Menstrual cycles were regulated using progesterone (Medrofen®). Ovarian stimulation was conducted following a GnRH antagonist protocol with recombinant FSH (150 IU/day, Gonal-F, Merck Serono). GnRH antagonist (0.25 mg/day, Cetrotide, Merck Serono) was added when follicles reached >12 mm. Final oocyte maturation was triggered with 10000 IU of hCG (Pregnyl, Organon). Oocytes were retrieved transvaginally 36 to 40 hours later.

Cumulus cell collection and processing

Cumulus cells were collected during oocyte retrieval procedures. Following transvaginal ultrasound-guided oocyte retrieval, oocyte-

cumulus complexes were immediately transferred to pre-warmed culture medium (G-IVF™, Vitrolife, Sweden) and maintained at 37°C with 6% CO₂, and 5% O₂. Cumulus cells were mechanically separated from oocyte-cumulus complexes using fine-bore pipettes 2- 3 hours after retrieval, prior to oocyte denudation. Only cumulus cells from oocyte-cumulus complexes containing metaphase II (MII) oocytes were used for analysis to ensure consistency.

To minimize contamination with granulosa cells, cumulus cells were carefully dissected from the outer layers of the cumulus mass using a stereomicroscope (Nikon SMZ745, Japan). Granulosa cells, which typically remain in the follicular fluid aspirate, were not included in the collection. The aspirated follicular fluid was discarded, and only the intact cumulus-oocyte complex was used for cell collection. This approach minimized contamination with mural granulosa cells.

Following collection, cumulus cells were immediately transferred to RNeasy® stabilization solution (Qiagen, USA) and stored at -80°C until RNA extraction. The maximum storage duration was 3 months. RNA was extracted within 3 months of collection.

RNA extraction and qRT-PCR

Total RNA was extracted from cumulus cells using TRIzol (Invitrogen, USA) and treated with DNase I (Fermentas, Germany). RNA integrity and quality were assessed using a NanoDrop 2000 spectrophotometer (Thermo Scientific, USA). Only samples with an A₂₆₀/A₂₈₀ ratio between 1.8 and 2.1 and an A₂₆₀/A₂₃₀ ratio >1.8 were included in the analysis. Additionally, RNA integrity was confirmed by agarose gel electrophoresis (1.5% gel), and samples showing clear 28S and 18S rRNA bands without degradation were used. Samples that did not meet these quality criteria were excluded from analysis ($n = 2$; one from the metformin group and one from the placebo group). RNA concentration was measured and adjusted to 100 ng/μL for all samples prior to cDNA synthesis.

cDNA synthesis was performed using oligo dT primers (Metabion, Germany) and SuperScript II reverse transcriptase (Fermentas). Quantitative real-time PCR (qRT-PCR) was performed using SYBR-Green/ROX qPCR master mix (Thermo

Scientific, USA) with primers specific for *GDF9*, *BMP15*, and *GAPDH* (reference gene). The primer sequences are listed in Table 1. The thermal cycling program consisted of 45 cycles at 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s. Melting curve analysis confirmed amplification specificity. All qPCR experiments followed MIQE guidelines. Relative expression levels were calculated using the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen, 2001).

All samples were analyzed in triplicate. The stability of *GAPDH* as a reference gene was validated using the geNorm algorithm (Vandesompele *et al.*, 2002) and NormFinder (Andersen *et al.*, 2004). The M-value for *GAPDH* was 0.42, which is below the threshold of 0.5, indicating acceptable stability. Additionally, *GAPDH* expression did not differ significantly between treatment groups ($p = 0.412$ by one-way ANOVA).

Table 1. Primer sequences used for qRT-PCR.

Genes	GenBank accession	Oligomer sequences (5'→3')	Products size (bp)
<i>GDF9</i>	NM_005260.5	Forward: 5' ACTGTTCTGGCTCTTCACCC Reverse: 5' GCACACACATTTGACAGCAG	192
<i>BMP15</i>	NM_005448.2	Forward: 5' TAGAGAGAACCGCACCATTG Reverse: 5' CAGGAGAGATTGAAGCGAGT	198
<i>GAPDH</i>	NM_002046.7	Forward: 5' TTTGGTATCGTGAAGGACTC Reverse: 5' CCAGTAGAGGCAGGGATGAT	134

Oocyte and embryo assessment

Oocyte maturity and embryo grading were assessed by an embryologist blinded to treatment allocation. Only metaphase II (MII) oocytes were used for ICSI. Fertilization was confirmed by two pronuclei (2PN) 16- 18 hours after ICSI. Embryo quality was graded on day 3 as follows: grade I (symmetric blastomeres, no fragmentation), grade II (irregular blastomeres, <30% fragmentation), or grade III (irregular blastomeres, >30% fragmentation). Clinical pregnancy was confirmed by serum hCG measurement at 14- 15 days and ultrasound examination at 6 weeks. All assessments followed the Istanbul consensus criteria (Alpha Scientists in Reproductive Medicine & ESHRE Special Interest Group of Embryology, 2011).

Statistical analysis

Data were analyzed using SPSS version 26.0. Normality was assessed using the Shapiro-Wilk test and visual inspection of Q-Q plots. Between-group comparisons for continuous variables were performed using one-way ANOVA, followed by Tukey's post-hoc test (Schulz *et al.*, 2010). For variables that deviated from normality, Spearman's rank correlation was used as a sensitivity analysis; the results were consistent with Pearson correlations. For correlations involving binary outcomes (clinical pregnancy), point-biserial correlation was used as the appropriate statistical method. Additionally,

logistic regression was performed to assess the association between gene expression and clinical pregnancy, adjusting for potential confounders (age, BMI, and HOMA-IR). All statistical tests were two-sided, with $p < 0.05$ considered significant. Given the exploratory nature of the secondary analyses, no adjustment for multiple comparisons was applied.

Results

Participant disposition and baseline characteristics

Of the 118 women assessed for eligibility, 38 were excluded before randomization: 25 did not meet inclusion criteria, 8 declined to participate, and 5 were excluded for other reasons. The remaining 80 participants were randomized into four groups of 20 each. During the study, 7 participants (1 from the placebo group, 2 from the metformin group, 2 from the linagliptin-metformin group, and 2 from the empagliflozin-metformin group) discontinued due to personal reasons or relocation and were replaced. The final analysis included 80 participants (20 per group). Baseline characteristics were comparable across all groups (Table 2), confirming successful randomization. There were no significant differences in mean age, duration of marriage, duration of infertility, BMI, HOMA-IR, or FAI ($p > 0.05$ for all comparisons). The mean age of participants ranged from 27.4 ± 2.7 years in the Linagliptin-Metformin group to 29.1 ± 3.0 years

in the metformin group, with no statistically significant difference among the four groups ($p=0.207$). The duration of marriage ranged from 6.75 ± 1.83 years in the placebo group to 8.15 ± 1.92 years in the Empagliflozin-Metformin group, with no significant difference ($p=0.116$). The duration of infertility ranged from 5.90 ± 1.51 years in the placebo group to 6.50 ± 1.76 years in the Empagliflozin-Metformin group, and the difference was not statistically significant ($p=0.771$). Body mass index (BMI) was similar across all groups, ranging from 27.37 ± 1.35 kg/m² in the placebo group to 28.11 ± 1.54 kg/m² in the metformin group ($p=0.483$). Homeostatic model assessment of insulin resistance (HOMA-IR) values ranged from 4.00 ± 0.72 in the Empagliflozin-Metformin group to 4.56 ± 2.35 in

the metformin group, with no significant difference ($p=0.512$). The free androgen index (FAI) ranged from 3.40 ± 1.34 in the Empagliflozin-metformin group to 3.93 ± 1.32 in the placebo group, and the difference was not statistically significant ($p=0.606$).

Cumulus cell gene expression

Expression of *GDF9* and *BMP15* mRNA was significantly increased in all active treatment groups compared with placebo, $p < 0.001$ for all comparisons (Table 3). The empagliflozin-metformin group showed the highest expression levels of both genes, which were significantly higher than the linagliptin-metformin and metformin groups ($p < 0.001$ for both comparisons).

Table 2. Baseline characteristics of participants by treatment group (n= 20 per group).

Parameter	Placebo	Metformin	Linagliptin-Metformin	Empagliflozin-metformin	p-value*
Age†	28.5± 2.4	29.1± 3.0	27.4± 2.7	28.1± 2.4	0.207
Duration of marriage†	6.75± 1.83	7.10± 1.71	6.80± 2.58	8.15± 1.92	0.116
Duration of infertility†	5.90± 1.51	6.40± 1.78	6.25± 2.46	6.50± 1.76	0.771
BMI (kg/m ²)	27.37± 1.35	28.11± 1.54	27.58± 1.94	27.46± 1.58	0.483
HOMA-IR	4.08± 0.48	4.56± 2.35	4.19± 0.40	4.00± 0.72	0.512
FAI	3.93± 1.32	3.54± 1.52	3.67± 1.57	3.40± 1.34	0.606

*= Data are presented as mean ± SD. P-values from one-way ANOVA; † = Years; Data are presented as mean ± SD. P-values from one-way ANOVA.

Table 3. *GDF9* and *BMP15* mRNA expression levels in cumulus cells by treatment group (n= 20 per group).

Gene	Placebo	Metformin	Linagliptin-Metformin	Empagliflozin-Metformin	P-value*
<i>GDF9</i>					
Fold-change vs. placebo	1.0± 0.0	1.9± 0.4	2.8± 0.6	4.2± 0.8	< 0.001
Mean difference vs. placebo (95% CI)	Reference	0.9 (0.5, 1.3)†	1.8 (1.4, 2.2)†	3.2 (2.8, 3.6)†	
Mean difference vs. metformin (95% CI)		Reference	0.9 (0.5, 1.3)†	2.3 (1.9, 2.7)†	
<i>BMP15</i>					
Fold-change vs. placebo	1.0± 0.0	2.0± 0.5	3.0± 0.7	4.5± 0.9	< 0.001
Mean difference vs. placebo (95% CI)	Reference	1.0 (0.6, 1.4)†	2.0 (1.6, 2.4)†	3.5 (3.1, 3.9)†	
Mean difference vs. metformin (95% CI)		Reference	1.0 (0.6, 1.4)†	2.5 (2.1, 2.9)†	

*= Data are presented as mean ± SD. p-values from one-way ANOVA; †= $p < 0.001$ for all pairwise comparisons with active treatment groups vs. placebo. Fold-change values are relative to placebo (set as 1.00) and represent relative, not absolute, differences.

GDF9, the fold-change in expression relative to placebo was 4.2 ± 0.8 in the empagliflozin-metformin group, 2.8 ± 0.6 in the linagliptin-metformin group, and 1.9 ± 0.4 in the metformin group. The mean difference in fold-change compared with placebo was 0.9 (95% CI: 0.5 to 1.3) for metformin, 1.8 (95% CI: 1.4 to 2.2) for linagliptin-metformin, and 3.2 (95% CI: 2.8 to 3.6) for empagliflozin-metformin ($p < 0.001$ for all comparisons). Compared with metformin alone, the empagliflozin-metformin group showed a significantly higher fold-change with a mean difference of 2.3 (95% CI: 1.9 to 2.7; $p < 0.001$; Cohen's $d=1.85$), and the linagliptin-metformin group also showed a significantly

higher fold-change with a mean difference of 0.9 (95% CI: 0.5 to 1.3; $p < 0.001$; Cohen's $d=1.12$). *BMP15*, the fold-change in expression relative to placebo was 4.5 ± 0.9 in the empagliflozin-metformin group, 3.0 ± 0.7 in the linagliptin-metformin group, and 2.0 ± 0.5 in the metformin group. The mean difference in *BMP15* expression compared with placebo was 1.0 (95% CI: 0.6 to 1.4) for metformin, 2.0 (95% CI: 1.6 to 2.4) for linagliptin-metformin, and 3.5 (95% CI: 3.1 to 3.9) for empagliflozin-metformin ($p < 0.001$ for all comparisons). Compared with metformin alone, the empagliflozin-metformin group showed a significantly higher *BMP15* expression with a mean difference of 2.5 (95% CI: 2.1 to 2.9;

$p < 0.001$), and the linagliptin-metformin group also showed a significantly higher expression with a mean difference of 1.0 (95% CI: 0.6 to 1.4; $p < 0.001$).

The pattern of gene expression across treatment groups (Fig. 1) demonstrated a clear dose-response relationship. The empagliflozin-metformin combination showing the most pronounced upregulation of both *GDF9* and *BMP15*, followed by linagliptin-metformin, then metformin alone, and finally placebo showing the lowest expression levels.

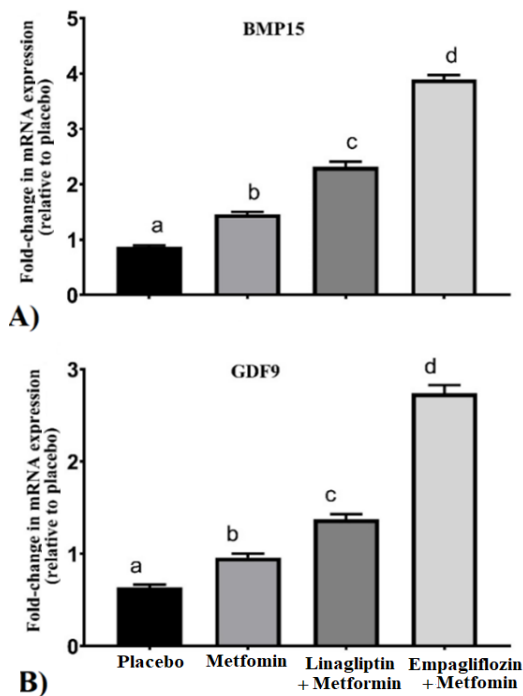


Fig. 1. Effects of metformin, linagliptin-metformin, and empagliflozin-metformin on mRNA expression in cumulus cells from PCOS patients: A) *GDF9*; B) *BMP15*. Data are presented as fold-change relative to placebo (set as 1.00, mean $\pm$ SD). Different lowercase letters (a, b, c, d) indicate significant differences between groups ($p < 0.05$, one-way ANOVA followed by Tukey's post-hoc test). a= significantly different from placebo; b= significantly different from metformin; c= significantly different from linagliptin-metformin; d= significantly different from empagliflozin-metformin. n= 20 per group.

Correlation analysis

Correlation analyses between gene expression and clinical parameters are presented in Table 4. Given the exploratory nature of these analyses, no

Table 4. Exploratory correlation analysis between gene expression and clinical parameters by treatment group

adjustments for multiple comparisons were made; therefore, the results should be interpreted with appropriate caution.

In the empagliflozin-metformin group, *BMP15* expression showed moderate to strong correlations with several metabolic and reproductive parameters. *BMP15* expression was negatively correlated with free androgen index (FAI; $r = -0.72$, $p < 0.001$), leptin ($r = -0.75$, $P < 0.001$), and anti-Müllerian hormone (AMH; $r = -0.62$, $p = 0.003$). These negative correlations indicate that higher *BMP15* expression was associated with greater reductions in these parameters. In the context of PCOS, where AMH is typically elevated and contributes to follicular arrest and anovulation, a reduction in AMH is considered a favorable outcome, reflecting a shift toward more normal follicular dynamics. *BMP15* expression was positively correlated with the number of mature oocytes (MII; $r = 0.72$, $p < 0.001$), the number of grade I embryos ($r = 0.82$, $p < 0.001$), and for the binary outcome of clinical pregnancy, point-biserial correlation showed a significant association with *BMP15* expression ($r_{pb} = 0.68$, $p < 0.001$). Logistic regression confirmed these associations after adjusting for age, BMI, and HOMA-IR (*BMP15*: OR=1.85, 95% CI: 1.12-3.05, $p = 0.016$).

GDF9 expression in the empagliflozin-metformin group showed similar correlations. *GDF9* expression was negatively correlated with FAI ($r = -0.69$, $p = 0.001$), leptin ($r = -0.73$, $p < 0.001$), and AMH ($r = -0.58$, $p = 0.007$). *GDF9* expression was positively correlated with the number of mature oocytes (MII; $r = 0.68$, $p = 0.001$), the number of grade I embryos ($r = 0.79$, $p < 0.001$), and point-biserial correlation showed a significant association with clinical pregnancy ($r_{pb} = 0.64$, $p = 0.001$). Logistic regression confirmed this association (OR=1.72, 95% CI: 1.05-2.82, $p = 0.031$).

In the linagliptin-metformin group, similar but generally weaker correlations were observed. *BMP15* expression showed a moderate negative correlation with HOMA-IR ($r = -0.52$, $p = 0.018$), leptin ($r = -0.61$, $p = 0.004$), and AMH ($r = -0.48$, $p = 0.032$), and positive correlations with mature oocytes ($r = 0.58$, $p = 0.007$), grade I embryos ($r = 0.65$, $p = 0.002$), and clinical pregnancy ($r_{pb} = 0.55$, $p = 0.011$).

Parameters	Groups	BMP15	p-value	GDF9	p-value
HOMA-IR	Placebo	-0.15	0.532	-0.12	0.621
	Metformin	-0.38	0.098	-0.35	0.128
	Linagliptin-Metformin	-0.52	0.018*	-0.48	0.032*
	Empagliflozin-Metformin	-0.65	0.002†	-0.61	0.004†
FAI	Placebo	-0.18	0.445	-0.16	0.502
	Metformin	-0.42	0.065	-0.39	0.087
	Linagliptin-Metformin	-0.58	0.007†	-0.55	0.011*
	Empagliflozin-Metformin	-0.72	<0.001‡	-0.68	0.001†
Leptin	Placebo	-0.22	0.351	-0.19	0.423
	Metformin	-0.45	0.047*	-0.41	0.072
	Linagliptin-Metformin	-0.61	0.004†	-0.58	0.007†
	Empagliflozin-Metformin	-0.75	<0.001‡	-0.71	<0.001‡
AMH	Placebo	-0.25	0.287	-0.22	0.351
	Metformin	-0.35	0.128	-0.32	0.167
	Linagliptin-Metformin	-0.48	0.032*	-0.45	0.046*
	Empagliflozin-Metformin	-0.62	0.003†	-0.58	0.007†
Mature Oocytes (MII)	Placebo	0.28	0.234	0.25	0.287
	Metformin	0.45	0.047*	0.42	0.065
	Linagliptin-Metformin	0.58	0.007†	0.55	0.011*
	Empagliflozin-Metformin	0.72	<0.001‡	0.68	0.001‡
Grade I Embryos	Placebo	0.35	0.128	0.32	0.167
	Metformin	0.52	0.018*	0.49	0.028*
	Linagliptin-Metformin	0.65	0.002†	0.62	0.003†
	Empagliflozin-Metformin	0.82	<0.001‡	0.79	<0.001‡
Clinical Pregnancy	Placebo	0.20	0.398	0.18	0.445
	Metformin	0.38	0.098	0.35	0.128
	Linagliptin-Metformin	0.55	0.011*	0.52	0.018*
	Empagliflozin-Metformin	0.68	<0.001‡	0.64	<0.001‡

Data are presented as Pearson correlation coefficients (r) for continuous variables and point-biserial correlation coefficients (rpb) for the binary outcome of clinical pregnancy. These analyses are exploratory and hypothesis-generating; no adjustment for multiple comparisons was applied. * = $p < 0.05$; † = $p < 0.01$; ‡ = $p < 0.001$. Negative correlations indicate that greater parameter reduction is associated with higher gene expression. Positive correlations indicate that higher gene expression is associated with improved outcomes.

GDF9 expression in the linagliptin-metformin group showed a moderate negative correlation with HOMA-IR ($r = -0.48$, $p = 0.032$), FAI ($r = -0.55$, $p = 0.011$), and leptin ($r = -0.58$, $p = 0.007$), and positive correlations with mature oocytes ($r = 0.55$, $p = 0.011$), grade I embryos ($r = 0.62$, $p = 0.003$), and clinical pregnancy (rpb = 0.52 , $p = 0.018$). In the metformin group, the correlations were weaker and often did not reach statistical significance.

BMP15 expression showed a moderate negative correlation with leptin ($r = -0.45$, $p = 0.047$) and positive correlations with mature oocytes ($r = 0.45$, $p = 0.047$) and grade I embryos ($r = 0.52$, $p = 0.018$). *GDF9* expression in the metformin group showed a positive correlation with grade I embryos ($r = 0.49$, $p = 0.028$). No significant correlations were observed in the placebo group for any parameter.

Exploratory analyses were performed to assess correlations between baseline demographic characteristics (age, duration of marriage, and duration of infertility) and the main outcomes (*GDF9* and *BMP15* expression, MII oocytes, grade I embryos, and clinical pregnancy). No significant correlations were observed between age and *GDF9* expression ($r = -0.12$, $p = 0.312$), *BMP15* expression ($r = -0.15$, $p = 0.208$), MII oocytes ($r = -0.18$, $p = 0.132$), grade I embryos ($r = -0.14$, $p = 0.245$), or clinical pregnancy (rpb = -0.10 , $p = 0.398$). Similarly, no significant correlations were found between duration of marriage or duration of infertility and any of the main outcomes (all $p > 0.05$). These findings suggest that the observed effects of treatment on gene expression and reproductive outcomes were independent of baseline demographic characteristics.

It is critical to emphasize that these correlations are exploratory and do not imply causation. The observed associations should be viewed as hypothesis-generating, and any mechanistic interpretations require confirmation in dedicated studies. The gradient of correlation strength across treatment groups, with the strongest effects observed in the Empagliflozin-Metformin group and the weakest in the placebo group, suggests a potential dose-response relationship between metabolic improvement and gene expression changes. The correlation between gene expression and clinical pregnancy, while statistically significant, should be interpreted with caution. The sample size of 20 participants per group limits the stability of these estimates, and the point-biserial correlation, while appropriate for binary outcomes, may still overestimate the true association. Future studies with larger sample sizes are needed to confirm these findings.

Discussion

This secondary analysis examined the effects of eight weeks of treatment with empagliflozin-metformin on *GDF9* and *BMP15* expression in cumulus cells of women with PCOS undergoing ICSI. The findings are exploratory and hypothesis-generating, requiring confirmation in larger mechanistic studies.

Metformin remains first-line treatment for PCOS due to its proven efficacy against insulin resistance and hyperandrogenism (Sanjari *et al.*, 2025). However, its limited direct effects on oocyte quality justify the development of more targeted combination therapies (Hassani *et al.*, 2018). Empagliflozin, a highly selective SGLT2 inhibitor, improves obesity-related problems, glucose tolerance, and insulin resistance (Ferrannini *et al.*, 2014; Zinman *et al.*, 2015). This study was associated with significantly increased expression of *GDF9* and *BMP15* in cumulus cells, with fold-changes of 4.2 ± 0.8 and 4.5 ± 0.9 , respectively, compared with placebo. These findings suggest that the improved metabolic environment may be associated with oocyte paracrine signaling through upregulation of these critical factors.

To our knowledge, this is the first study to show that empagliflozin-metformin combination therapy is associated with upregulation of *GDF9* and *BMP15* expression in cumulus cells of PCOS

patients undergoing ICSI. While previous studies have investigated the metabolic effects of SGLT2 inhibitors in PCOS (Javed *et al.*, 2019; Sharif *et al.*, 2025), and a recent trial by Fakhreddin *et al.* (2026) reported that dapagliflozin-metformin was associated with upregulation of *BMP15* and *GDF9* expression, none have directly compared empagliflozin-metformin with linagliptin-metformin and correlated these molecular changes with reproductive outcomes. Our findings provide a novel mechanistic link between metabolic optimization and improved oocyte quality, offering a potential explanation for the reproductive benefits of empagliflozin-metformin in this challenging patient population. Furthermore, the comparison with linagliptin-metformin and metformin alone provides valuable insight into the relative efficacy of different combination strategies, which may inform future clinical decision-making.

The mechanistic pathway linking empagliflozin-metformin treatment to *GDF9/BMP15* upregulation may involve multiple converging mechanisms. First, empagliflozin-induced glucosuria and caloric loss lead to reduced visceral adiposity and improved insulin sensitivity, which in turn reduces hyperinsulinemia. Since hyperinsulinemia directly suppresses oocyte paracrine signaling, its reduction may relieve inhibitory signals on *GDF9* and *BMP15* expression (Dumesic *et al.*, 2015). Second, empagliflozin has been shown to activate AMPK in peripheral tissues, potentially enhancing mitochondrial function and reducing oxidative stress in cumulus cells, creating a more favorable environment for the production of oocyte-secreted factors (Hawley *et al.*, 2016). Third, the reduction in leptin levels observed in our study may contribute to improved oocyte quality, as leptin has been shown to impair oocyte maturation and steroidogenesis (Garruti *et al.*, 2014). Fourth, the decrease in AMH levels may reflect a shift in follicular dynamics toward a less crowded, more functionally active ovarian environment, facilitating better maturation of individual oocytes (Garg and Tal, 2016).

Reduced signaling of these oocyte-secreted factors is associated with arrested follicular growth and poor oocyte quality (Karagül *et al.*, 2018; Fountas *et al.*, 2024). *GDF9* and *BMP15* are essential for follicular maturation and oocyte

competence, acting through paracrine mechanisms to regulate cumulus cell function (Gode *et al.*, 2011). The observed upregulation may explain the improved oocyte and embryo outcomes reported separately.

The reduction in AMH observed in the empagliflozin-metformin group is particularly noteworthy. Elevated AMH, a hallmark of PCOS, is thought to contribute to anovulation by attenuating FSH sensitivity and impairing dominant follicle selection (Garg and Tal, 2016). In the context of PCOS, where AMH is typically elevated, a reduction in AMH is considered a favorable outcome, reflecting a shift toward more normal follicular dynamics. The negative correlation between *BMP15* expression and AMH suggests that the upregulation of *BMP15* may be associated with a 'normalization' of the follicular pool, potentially allowing for better maturation of individual oocytes.

The correlation analysis revealed a gradient of correlation strength across treatment groups, with the strongest effects in the empagliflozin-metformin group. Increased *GDF9/BMP15* expression showed strong negative correlations with FAI and leptin and strong positive correlations with embryo quality and clinical pregnancy rate. This suggests a potential positive reinforcement cycle: the combination treatment may create a favorable metabolic-hormonal environment that may be associated with increased *BMP15* and *GDF9* expression, further improving oocyte maturation and quality.

The lack of significant correlations between age, duration of marriage, or duration of infertility and the main outcomes is noteworthy. This may be due to the relatively narrow age range (25- 35 years) and the homogeneous nature of our study population. Previous studies have reported that age is a significant predictor of oocyte quality and IVF outcomes, particularly in women over 35 years (Anderson *et al.*, 2025). However, in our cohort of younger women with PCOS, age did not appear to be a major determinant of the response to metabolic preconditioning. Future studies with larger sample sizes and broader age ranges are needed to confirm these findings.

Recent studies have provided additional insights into the role of SGLT2 inhibitors in PCOS. A 2025 systematic review by Kamrul-Hasan *et al.* concluded that SGLT2 inhibitors, alone or

combined with metformin, represent a promising therapeutic approach for improving metabolic and hormonal parameters in PCOS. Another recent study by Rakic *et al.* (2024) reported that empagliflozin was associated with improved ovarian histological parameters in a preclinical rat model of PCOS, providing further support for its potential reproductive benefits. Our findings extend these observations by showing, for the first time, that empagliflozin-metformin is associated with upregulation of *GDF9* and *BMP15* expression in cumulus cells of PCOS patients undergoing ICSI (Rakic *et al.*, 2024).

Several limitations must be acknowledged. First, this pilot study was powered for metabolic endpoints, not molecular outcomes. Second, correlational analyses were not adjusted for multiple comparisons. Third, qPCR was performed on cumulus cells rather than oocytes directly. Fourth, the eight-week study duration may not capture full effects on gene expression. Fifth, the absence of protein-level validation is a limitation. Finally, generalizability is limited by the single-center design. Consistent with our previous trials (Daneshjou *et al.*, 2022; Fakhreddin *et al.*, 2026), *GAPDH* was used as the reference gene. However, we acknowledge that *GAPDH* expression can be influenced by metabolic status and metformin treatment. Although the M-value (0.42) and between-group comparison ($p=0.412$) suggested acceptable stability, the use of a single reference gene remains a limitation. Future studies should validate and use multiple reference genes (*e.g.*, *HPRT1*, *RPLP0*) to enhance the robustness of gene expression analyses.

Conclusion

This secondary analysis found that combination therapy with Empagliflozin-metformin was associated with significant upregulation of *GDF9* and *BMP15* expression in cumulus cells of infertile women with PCOS undergoing ICSI. These molecular changes correlated with improved oocyte quality and pregnancy outcomes. However, these findings are exploratory and hypothesis-generating, not confirmatory. The lack of protein-level validation, the single-center design, and the use of a single reference gene (*GAPDH*) are important limitations. Larger, adequately powered

mechanistic studies with protein-level validation and multiple reference genes are needed to confirm these findings.

Data availability

The deidentified participant-level dataset, statistical analysis code, and qRT-PCR raw data are available from the corresponding author upon reasonable request.

Conflict of interests

The authors declare no conflict of interest.

Author contributions

MSM: Conceptualization, methodology, validation, formal analysis, investigation, writing—original draft, visualization, project administration; MM (Corresponding): Conceptualization, methodology, formal analysis, writing—original draft, supervision, funding acquisition; FF: Conceptualization, methodology, investigation, writing—review and editing, supervision; ES: Investigation, resources, writing—review and editing. All authors reviewed and approved the final manuscript. Author contributions follow the CRediT (Contributor Roles Taxonomy) classification.

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