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Effects of Liraglutide on Leptin Promoter Methylation in Ovarian Granulosa Cells of Obese Polycystic Ovary Syndrome Patients

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Serum leptin (LEP) is elevated in polycystic ovary syndrome (PCOS) patients, especially in obese PCOS patients, which may link to the etiology and development of PCOS. Obesity adversely affects female fertility, and most PCOS patients are obese. Liraglutide, a glucagon-like peptide-1 (GLP-1) analog, regulates adipokine production, causes weight loss, and regulates ovarian physiology to improve or manage reproductive status, thus ameliorating obesity and PCOS. This study investigated the impact of liraglutide on *LEP* promoter methylation levels in ovarian granulosa cells of obese PCOS patients to seek possible potential targets for the clinical treatment. This prospective observational study enrolled 348 PCOS patients with strong fertility desire in our hospital during March 2020-January 2022 who were planned to undergo in vitro fertilization and embryo transfer. Based on the inclusion and exclusion criteria, 207 eligible patients (72 non-obese and 135 obese PCOS patients, 23-37 year-old) were enrolled. Obese PCOS patients [body mass index (BMI) ≥ 25 kg/m^2] received liraglutide treatment. Obese PCOS patients exhibited elevated BMI, fasting insulin, fasting blood glucose, HOMA-IR, triglyceride, estradiol, and testosterone levels and reduced high-density lipoprotein cholesterol, luteinizing hormone (LH), LH/follicle-stimulating hormone ratio, and LEP promoter methylation. Liraglutide increases *LEP* promoter methylation, decreases LEP levels, and affects sex hormone secretion, providing a reference for the investigation of the mechanism of liraglutide in obese PCOS patients. Additionally, weight and fat loss, decreased serum and follicular fluid LEP levels, and increased LEP promoter methylation levels in ovarian granulosa cells may be crucial strategies for treating obese PCOS patients.

Keywords: leptin; liraglutide; methylation; obesity; polycystic ovary syndrome
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Introduction

Polycystic ovary syndrome (PCOS) is a prevalent endocrine disorder characterized by polycystic ovaries, anovulation, and hirsutism. Often accompanied by obesity, dyslipidemia, and insulin resistance, PCOS poses significant risks for metabolic and cardiovascular complications, including metabolic syndrome and diabetes (Meier 2018). The clinical manifestations of PCOS primarily involve metabolic and ovarian dysfunctions (Nylander et al. 2017). However, estimating its global incidence precisely is challenging due to variations in definition, phenotype diversity, and factors such as ethnicity, age, and diagnostic criteria (Bednarz et al. 2022). Consequently, PCOS prevalence varies widely, ranging from 2.2% to 26% across different studies (Tehrani et al. 2011). While the exact etiology of PCOS remains elusive, emerging evidence suggests that it is a multifaceted condition influenced by genetic, environmental, and epigenetic factors, including lifestyle and dietary habits (Escobar-Morreale 2018). Thus, understanding the underlying mechanisms of PCOS is crucial for developing effective therapeutic interventions.

Liraglutide, a glucagon-like peptide-1 (GLP-1) receptor agonist, is commonly prescribed for individuals with type 2 diabetes mellitus, pre-diabetes, and obesity. Notably, liraglutide has been shown to exert significant effects in reducing body mass index (BMI), abdominal circumference, and body weight in obese and overweight PCOS women (Papaetis et al. 2020). Obesity affects up to 78% of women with PCOS and significantly impacts their quality

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of life (Kahal et al. 2019). In preclinical studies, liraglutide has been found to modulate adipokine expression during adipogenesis, reduce the number of cystic follicles, and mitigate obesity and PCOS-related symptoms in mice (Singh et al. 2019). Furthermore, liraglutide has been associated with decreased free testosterone levels and substantial weight loss, thereby improving ovarian function in overweight PCOS women (Nylander et al. 2017).

Leptin (LEP) is a peptide hormone primarily secreted by adipose tissues, playing a crucial role in regulating energy balance and adipose tissue mass. Recessive mutations in the *LEP* gene have been linked to severe obesity, underscoring the genetic underpinnings of obesity (Friedman 2009). LEP is implicated in various physiological processes, including obesity, hypertension, insulin resistance, and aberrant lipid metabolism, making it a significant biomarker for diagnosing and prognosticating PCOS (Cui et al. 2017; Seth et al. 2021). LEP is significantly elevated in the serum of PCOS patients, especially in obese PCOS patients (Peng et al. 2022), and is involved in the apoptosis of granulosa cells in PCOS (Wang et al. 2020). An elevated serum LEP is significantly correlated with PCOS, which may be of great significance in PCOS-related metabolic disorders (Peng et al. 2022). The pathological changes of these hormones, such as increased insulin, decreased growth hormone, enhanced ghrelin, and LEP resistance, lead to an increase in the prevalence of diabetes and obesity in PCOS women (Yang and Chen 2024). A high LEP level may be associated with insulin resistance, metabolic disorders, and infertility in PCOS, which may be related to the etiology and development of PCOS (Zheng et al. 2017). Besides, the emerging field of epigenetics has shed light on the role of DNA methylation in regulating gene expression in adipose tissues during obesity, with evidence suggesting a strong association between human LEP tissue distribution and promoter methylation status (Marchi et al. 2011). Methylation patterns of specific CpG sites have been shown to modulate *LEP* gene expression during adipocyte differentiation (Yokomori et al. 2002). Given these findings, this study hypothesizes that liraglutide may mitigate obesity and PCOS symptoms by modulating methylation levels of the *LEP* promoter in ovarian granulosa cells and regulating LEP levels in obese PCOS patients. Therefore, the present study aims to investigate the impact of liraglutide on LEP levels and promoter methylation levels in obese PCOS patients.

Materials and Methods

Ethics statement

The study was authorized by the academic ethics committee of Shanxi Fenyang Hospital (approved number: 2023079). All procedures strictly followed the code of Declaration of Helsinki. All subjects involved were fully informed of the study objective and signed informed consent before sampling.

Study subjects

This study is a prospective observational study. A total of 348 PCOS patients with strong fertility desire who were diagnosed with PCOS at Shanxi Fenyang Hospital between March 2020 and January 2022 and planned to undergo in vitro fertilization (IVF) and embryo transfer were randomly selected for this study. Among them, 68 patients declined participation, 46 did not meet the inclusion criteria, 11 withdrew during the study, 4 had incomplete clinical data, and 12 experienced gastrointestinal intolerance or other reasons precluding continued treatment according to the protocol (e.g., pregnancy). Ultimately, 207 eligible patients aged 23-37 years were enrolled in the study. The diagnosis of PCOS was based on the Rotterdam Consensus on Diagnostic Criteria for PCOS (Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group 2004), which requires the presence of at least two of the following criteria: (1) oligo-ovulation or anovulation, (2) biochemical and/or clinical signs of hyperandrogenism, and (3) polycystic ovaries observed via vaginal ultrasound (≥ 12 follicles measuring 2-9 mm in diameter in each ovary or ovarian volume of 10 cm^2 or more). Of these participants, 72 were classified as non-obese (BMI < 25 kg/m^2), with a mean age of 27.51 ± 4.62 years, and 135 were classified as obese (BMI ≥ 25 kg/m^2), with a mean age of 28.16 ± 4.12 years. The obese PCOS patients received subcutaneous injections of liraglutide (S20110046, Novo Nordisk, Tianjin, China) once daily. The initial dose was 0.6 mg/day for one week, followed by an increase to 1.2 mg/day for another week based on gastrointestinal tolerance. The final dose was adjusted to 1.8 mg/day, and the treatment duration was six months (Kahal et al. 2019). Participants did not alter their original dietary patterns during the study.

Inclusion criteria were as follows: 1. Age between 18 and 40 years; 2. Diagnosis of PCOS according to the Rotterdam Consensus on Diagnostic Criteria for PCOS (Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group 2004); 3. No relevant treatment within 3 months before enrollment; 4. Good cognitive and comprehension abilities; 5. Planned to undergo IVF and embryo transfer after treatment.

Exclusion criteria were as follows (Salamun et al. 2018): 1. Severe cardiovascular, pulmonary, hepatic, or renal dysfunction, or history of cancer (including medullary thyroid cancer) or pancreatitis, diabetes, or hyperthyroidism; 2. Women planning pregnancy; 3. Contraindications to study drugs; 4. Previous use of hormonal therapy or weight-loss medications during the study period; 5. Endocrine-related diseases such as adrenal gland and pituitary microadenoma; 6. Mental disorders and communication disorders; 7. Severe allergies; 8. Severe blood and immune system diseases.

Blood sample collection

Participants fasted for at least 8 hours and remained seated for 30 minutes before blood collection. Fasting

peripheral venous blood (5 mL) was then drawn and centrifuged at 3,000 rpm for 15 minutes to separate the serum, which was stored at 4°C in a refrigerator. On the same day, serum hormones and biochemical indicators were assessed, and a portion of the serum was aliquoted and preserved at −20°C for subsequent analysis.

Data collection and index detection

Baseline data, including age, weight, BMI, and insulin resistance indexes, were collected before and after treatment in both non-obese and obese PCOS patients.

Serum levels of low-density lipoprotein cholesterol (LDL), high-density lipoprotein cholesterol (HDL), and triglycerides (TG) were measured using a Hitachi 7600 automatic biochemical analyzer. Fasting blood glucose (FBG) was measured using the glucose oxidase method. Follicle-stimulating hormone (FSH), serum estradiol (E2), testosterone (T), luteinizing hormone (LH), and fasting insulin (FINS) were quantified using a Roche Cobas-e-602 automatic chemiluminescence immunoanalyzer. Insulin resistance was assessed using the homeostatic model assessment of insulin resistance (HOMA-IR) formula: FINS (mU/L) × FBG (mmol/L)/22.5 (Cao et al. 2023).

Collection of ovarian granulosa cells

The extraction of ovarian granulosa cells followed previously established methods (Matsubara et al. 2000; Wei et al. 2023). Ovulation induction in obese patients with PCOS was initiated using an antagonist regimen before and after treatment, followed by gonadotropin injection on the 3rd day of menstruation. When the diameter of the dominant follicle reached 14 mm, patients received daily subcutaneous injections of 0.25 mg gonadotropin-releasing hormone antagonist (Baxter, Germany) until 2 days before oocyte retrieval. From the 2nd to the 3rd day of the menstrual cycle, patients received daily injections of 150-300 U of human menopausal gonadotropin (HMG). Ovulation was triggered by intramuscular injection of 5,000-7,000 U of human chorionic gonadotropin (LIVZON, Zhuhai, Guangdong, China) once the diameter of the dominant follicle reached 18-20 mm, and oocytes were retrieved 36 hours later. Following oocyte retrieval, follicular fluid was collected, centrifuged at 250 × *g* for 10 minutes at room temperature, and stored at −80°C. Luteinized granulosa cell precipitates were collected, suspended in phosphate buffer saline (PBS), layered over lymphocyte separation solution at a volume ratio of 1:1, and centrifuged at 450 × *g* for 30 minutes to isolate granulosa cells. Any remaining red blood cells were removed using a red blood cell lysate, and the granulosa cells were washed with PBS and frozen at −80°C.

Detection of LEP levels in serum and follicular fluid

LEP levels in serum and follicular fluid were assessed using an enzyme-linked immunosorbent assay (ELISA) kit (Solarbio, Beijing, China) following the manufacturer's instructions. Briefly, standard and sample wells were prepared, and different concentrations of standards (50 μL) were added to the standard wells. Samples to be tested (10 μL) were added to the sample wells along with a sample diluent (40 μL). Horseradish peroxidase-labeled detection antibody (100 μL) was added to each well except the blank well. The reaction wells were sealed and incubated in a 37°C water bath or incubator for 60 minutes. After incubation, the wells were washed, substrate A and substrate B (50 μL each) were added, and the samples were incubated at 37°C for 15 minutes in the dark. Finally, the terminal solution (50 μL) was added, and the optical density of each well was measured at 450 nm within 15 minutes.

Detection of LEP promoter methylation in granulosa cells

Frozen granulosa cells were thawed at room temperature, and genomic DNA was extracted using a DNA extraction kit (Takara, Tokyo, Japan) according to the manufacturer's instructions. The extracted genomic DNA was treated with bisulfite using the EZDNA methylation gold label kit (Zymo Research, Orange, CA, USA). *LEP* promoter methylation levels were determined by quantitative real-time methylation-specific polymerase chain reaction (qMSP). The *LEP* promoter sequence was obtained from the UCSC database (http://genome.ucsc.edu/): CAGTGGG GACCAGAATAGGCCTGGGTTCCTAGCCCATTGCTAT TCCTTACCAGCTGTGGATTCTAAGGAAAGTCATTTA ACCTCGCTGGACCTTAGATTCCTCATCCCTGAAGCC CAAGGGTAAAACAAAACAAAACAAAACAAAACA AACCAACCCATCATGTAAAGCGGGGAACTACAAAC GATACAGGTGAAACATGCCTACCACACCACTCACA GGCTATGATGACAAAAACGTGGCTACATCTGGGAC CACCCCCCAACCCCCACTTTGTACGTAGGAAATAC GGAGTTGAGGATGGAGACCCACAGTATGTCCAGAG TGTCCCCAAAGGCCACAGTGCCCGCCTGGAGCCCT CCAGAGAGCGTGCACTCCCTGGGGTGCCAGCCAG AGACAACTTGCCCTGAGGCTTGGAACTCGATTCTC CGCGTGCCAGAGAAGGGGTGGGACTTCAGAACCC CCAACCCCGCAATCTGGGTCGGGGAGCCTGGCGCA CTGCGGGCCGCTCCCTCTAACCCTGGGCTTCCCTG GCGTCCAGGGCCGTCGGGGCCGAGTCCCGATTCGC TCCCACCCCGAAGCCGCGCCAGGACCAACGAGGG CGCAGCCGTATGCCCCAGCCCGCTCCGCGGAGCCC CTCACAGCCACCCCCGCCCCGACCGCGCCCCGCGC GGCTCGAAGCACCTTCCCAAGGGGCTGGTCCTTGC GCCATAGTCGCGCCGGAGCCTCTGGAGGGACATCA AGGATTTCTCGCTCCTACCAGCCACCCCCAAATTTT TGGGAGGTACCCAAGGGTGCGCGCGTGGCTCCTG GCGCGCCGAGGCCCTCCCTCGAGGCCCCGCGAGG TGCACACTGCGGGCCCAGGGCTAGCAGCCGCCCG GCACGTCGCTACCCTGAGGGGCGGGGCGGGAGCT GGCGCTAGAAATGCGCCGGGGCCTGCGGGGCAGT TGCGCAAGTTGTGATCGGGCCGCTATAAGAGGGGC GGGCAGGCATGGAGCCCCGTAGGAATCGCAGCGC CAGCGGTTGCAAGGTAAGGCCCCGGCGCGCTCCT. Methylated (M) or unmethylated (U) DNA primer sequences (Set 1-5) were designed using the website (http://

Table 1. Primer sequence.

Primer type	Primer sequence
M	Forward: 5'-TTTATTTTGTACGTAGGAAATACGG-3'
	Reverse: 5'-GCTCTCTAAAAAACTCCAAACGA-3'
U	Forward: 5'-TTTATTTTGTATGTAGGAAATATGG-3'
	Reverse: 5'-CACACTCTCTAAAAAACTCCAAACA-3'

M: methylated; U: unmethylated.

www.urogene.org/cgi-bin/methprimer/methprimer.cgi). Set 1 primers were selected for detection. Primer sequences are shown in Table 1.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 8.01 (GraphPad Software Inc., San Diego, CA, USA). Data were tested for normal distribution using the Shapiro-Wilk test and expressed as mean ± standard deviation. The *t*-test was used for comparisons between groups, and one-way analysis of variance (ANOVA) was applied for comparisons among groups, followed by Tukey's multiple comparisons test. Pearson correlation analysis was used for correlation analysis. A *P-value* < 0.05 was considered statistically significant.

Results

Comparison of clinical baseline characteristics between non-obese and obese PCOS patients before treatment

A total of 72 non-obese PCOS patients and 135 obese PCOS patients were enrolled. Analysis of clinical baseline data revealed that compared to the non-obese PCOS group, the obese PCOS group exhibited elevated BMI, FINS, FBG, HOMA-IR, TG, E2, and T levels, while HDL, LH, and LH/FSH levels were diminished (all $P < 0.05$). There were no significant differences in age, LDL, or FSH levels between the two groups (all $P > 0.05$) (Table 2).

Correlation between LEP levels in serum and follicular fluid and serum E2, T, and LH/FSH in obese PCOS patients

Serum LEP levels were found to be increased in obese PCOS patients (Peng et al. 2022). Additionally, obesity also influences the secretion of sex hormones and LH in women (Maheshwari et al. 2007). Therefore, this study further explored the correlation between LEP levels in serum and follicular fluid and serum E2, T, and LH/FSH in obese PCOS patients. Compared to non-obese PCOS patients, LEP levels in serum and follicular fluid were both intensified in obese PCOS patients (Fig. 1A,B, all $P < 0.01$). Pearson correlation analysis demonstrated that LEP levels in serum and follicular fluid of obese PCOS patients were positively correlated with serum E2 and T levels (r = 0.7553, 0.7498 and 0.7710, 0.7839) and negatively correlated with serum LH/FSH (r = −0.7267, −0.6957) (Fig. 1C,D, all $P < 0.0001$).

Table 2. Comparison of clinical baseline characteristics between non obese and obese PCOS patients before treatment.

Characteristics	Non-obese PCOS group (N = 72)	Obese PCOS group (N = 135)	*P* value
Age (year)	27.51 ± 4.62	28.16 ± 4.12	0.3015
BMI (kg/m^2)	21.07 ± 1.31	29.07 ± 1.56	< 0.0001
FINS (mU/L)	12.36 ± 1.85	19.36 ± 2.01	< 0.0001
FBG (mmol/L)	5.86 ± 1.16	6.24 ± 1.18	0.0275
HOMA-IR	3.26 ± 0.84	5.43 ± 1.21	< 0.0001
TG (mmol/L)	1.02 ± 0.94	1.93 ± 1.05	< 0.0001
LDL (mmol/L)	2.42 ± 0.96	2.36 ± 0.71	0.6103
HDL (mmol/L)	1.67 ± 0.54	1.22 ± 0.26	< 0.0001
E2 (pg/mL)	68.01 ± 8.54	124.80 ± 12.26	< 0.0001
T (ng/mL)	63.26 ± 7.12	93.47 ± 8.98	< 0.0001
LH (mIU/mL)	15.76 ± 4.24	8.60 ± 2.25	< 0.0001
FSH (mIU/mL)	5.84 ± 1.48	5.46 ± 1.53	0.0867
LH/FSH	2.64 ± 0.72	1.60 ± 0.69	< 0.0001

The Shapiro-Wilk test was used to verify the normal distribution, and the data were expressed as mean ± standard deviation. The unpaired *t* test was used for comparisons between two groups. PCOS, polycystic ovary syndrome; BMI, body mass index; FINS, fasting insulin; FBG, fasting blood glucose; HOMA-IR, insulin resistance index; TG, triglyceride; LDL, low-density lipoprotein cholesterol; HDL, high-density lipoprotein cholesterol; E2, serum estradiol; T, testosterone; LH, luteinizing hormone; FSH, follicle-stimulating hormone.

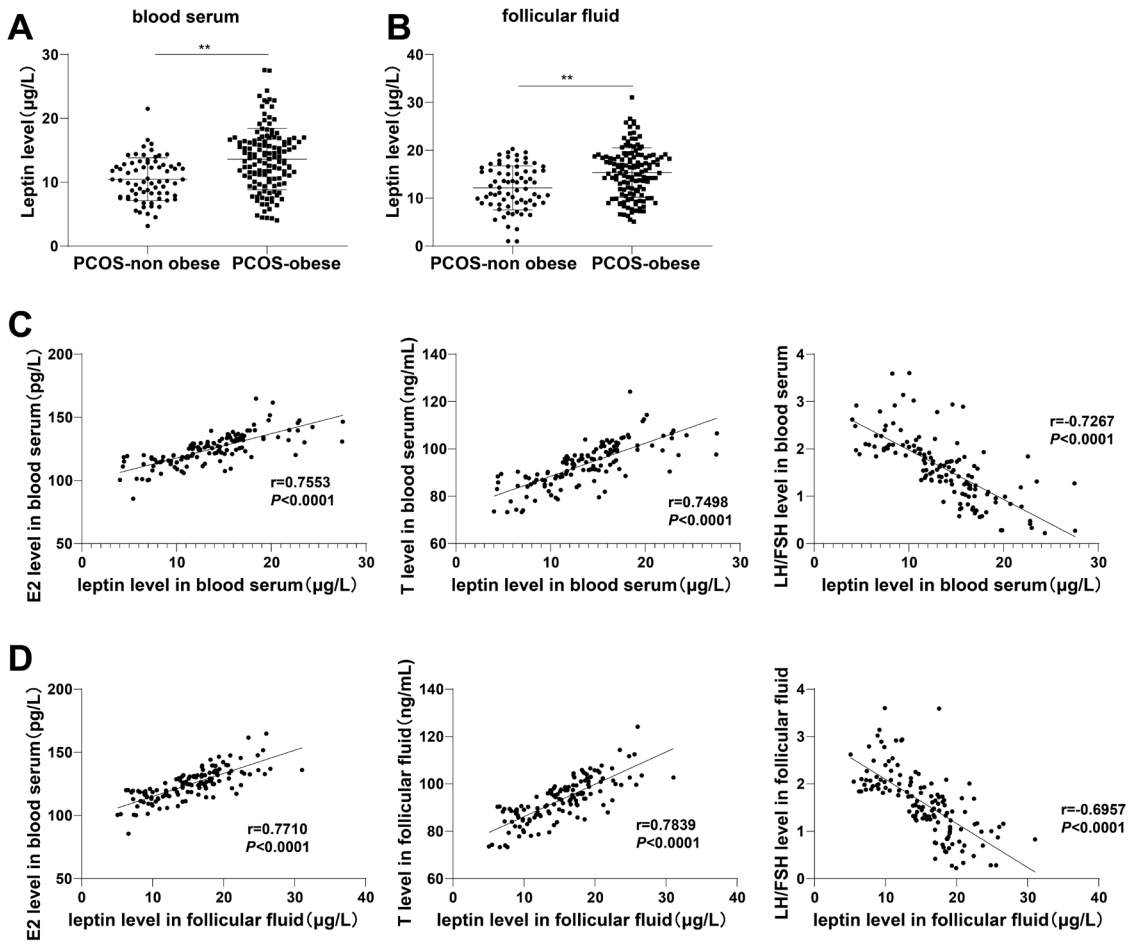

Fig. 1. LEP levels in serum and follicular fluid of obese PCOS patients and their correlation with serum E2, T, and LH/FSH. (A,B) Serum and follicular fluid LEP levels were assessed by ELISA in non-obese PCOS patients (N = 72) and obese PCOS patients (N = 135); (C) The correlation between serum LEP level and serum E2, T, and LH/FSH levels in obese PCOS patients was analyzed by Pearson correlation analysis; (D) The correlation between follicular fluid LEP level and serum E2, T and LH/FSH levels in obese PCOS patients was analyzed by Pearson correlation analysis. Non-paired *t*-test was applied for comparison between groups in panels A,B. $^{**}P < 0.01$.

Table 3. Comparison of clinical baseline characteristics of obese PCOS patients before and after liraglutide treatment.

Characteristics	Obese PCOS patients before treatment (N = 135)	Obese PCOS patients after treatment (N = 135)	*P* value
BMI (kg/m^2)	29.07 ± 1.56	24.65 ± 1.53	< 0.0001
FINS (mU/L)	19.36 ± 2.01	15.84 ± 2.12	< 0.0001
FBG (mmol/L)	6.24 ± 1.18	6.02 ± 1.15	0.122
HOMA-IR	5.43 ± 1.21	4.87 ± 1.18	0.0001
TG (mmol/L)	1.93 ± 1.05	1.54 ± 1.04	0.0024
LDL (mmol/L)	2.36 ± 0.71	2.40 ± 0.90	0.6855
HDL (mmol/L)	1.22 ± 0.26	1.40 ± 0.50	0.0003
E2 (pg/mL)	124.80 ± 12.26	96.85 ± 11.89	< 0.0001
T (ng/mL)	93.47 ± 8.98	79.85 ± 9.15	< 0.0001
LH (mIU/mL)	8.60 ± 2.25	12.68 ± 4.36	< 0.0001
FSH (mIU/mL)	5.46 ± 1.53	5.69 ± 1.50	0.2134
LH/FSH	1.60 ± 0.69	2.28 ± 0.70	< 0.0001

The Shapiro-Wilk test was used to verify the normal distribution, and the data were expressed as mean ± standard deviation. The paired t test was used for comparisons between two groups. PCOS, polycystic ovary syndrome; BMI, body mass index; FINS, fasting insulin; FBG, fasting blood glucose; HOMA-IR, insulin resistance index; TG, triglyceride; LDL, low-density lipoprotein cholesterol; HDL, high-density lipoprotein cholesterol; E2, serum estradiol; T, testosterone; LH, luteinizing hormone; FSH, follicle-stimulating hormone.

Comparison of clinical baseline characteristics of obese PCOS patients before and after liraglutide treatment

After 6 months of liraglutide treatment in 135 obese PCOS patients, BMI, FINS, HOMA-IR, TG, E2, and T levels were reduced (all $P < 0.05$), while HDL, LH, and LH/FSH levels were increased (all $P < 0.05$). However, FBG, LDL, and FSH levels did not show statistically significant changes (all $P > 0.05$) (Table 3).

Liraglutide reduced LEP levels in serum and follicular fluid of obese PCOS patients

To investigate the effects of liraglutide on LEP levels in obese PCOS patients, LEP levels in serum and follicular fluid of 135 obese PCOS patients after liraglutide treatment were measured using ELISA. Following liraglutide treatment, LEP levels in serum and follicular fluid of obese PCOS patients were reduced (Fig. 2A,B, all $P < 0.01$), indicating that liraglutide effectively lowered LEP levels in serum and follicular fluid of obese PCOS patients.

Liraglutide enhanced LEP promoter methylation in ovarian granulosa cells of obese PCOS patients

To further investigate the impact of liraglutide on *LEP* promoter methylation in ovarian granulosa cells of obese

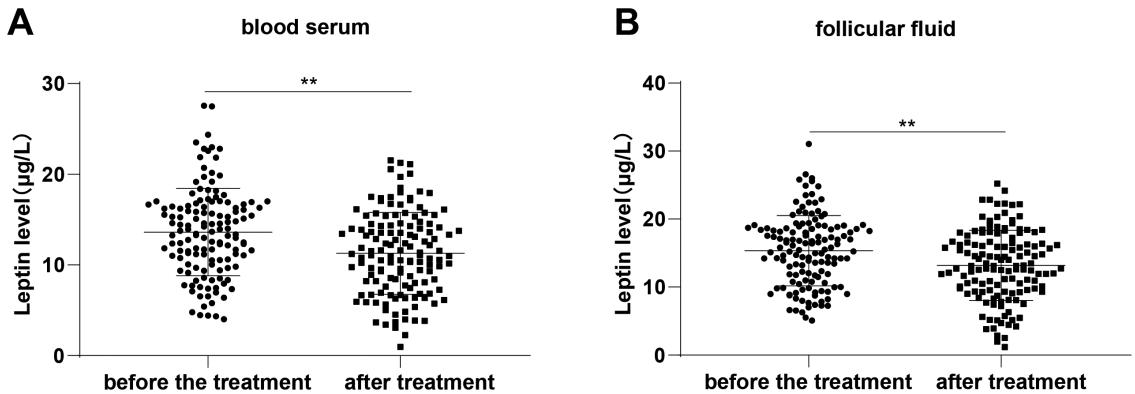

Fig. 2. Liraglutide reduced LEP levels in serum and follicular fluid of obese PCOS patients.
(A) Serum LEP level in obese PCOS patients before and after liraglutide treatment was determined by ELISA; (B) Follicular fluid LEP level of obese PCOS patients before and after liraglutide treatment was assessed by ELISA. N = 135. The paired *t*-test was adopted for comparison between groups. $^{**}P < 0.01$.

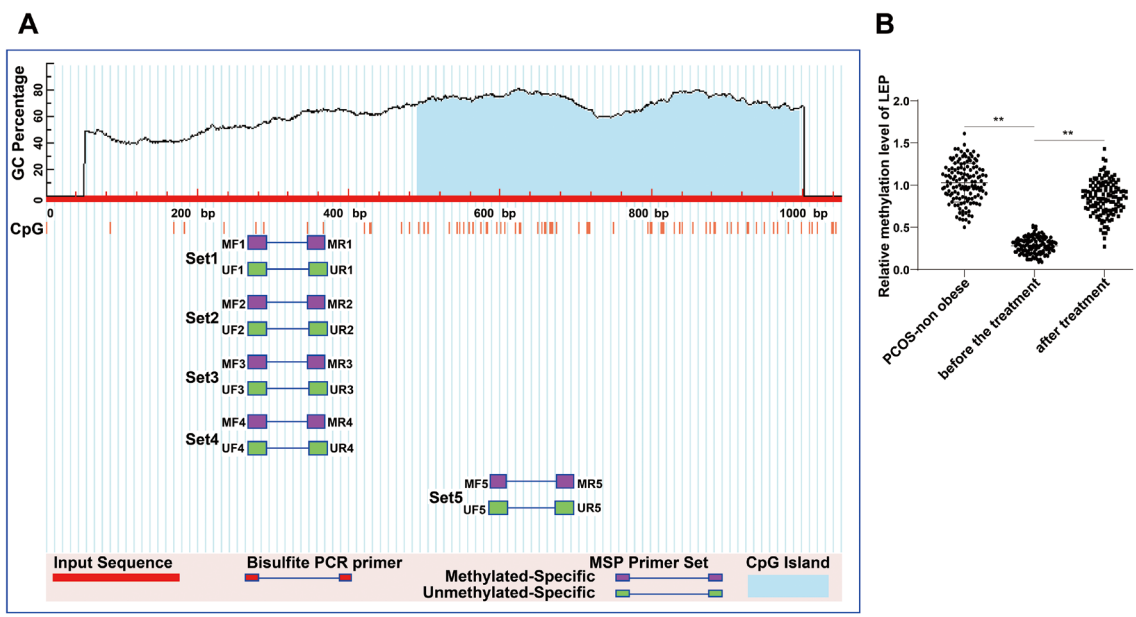

Fig. 3. Liraglutide enhanced *LEP* promoter methylation in ovarian granulosa cells of obese PCOS patients.
(A) CpG island methylation in the *LEP* promoter region was predicted and analyzed using the website (http://www.urogene.org/cgi-bin/methprimer/methprimer.cgi). Primers for qMSP were designed, and Set 1-5 represented five groups of methylation-specific PCR primers; (B) *LEP* promoter methylation level in ovarian granulosa cells of non-obese PCOS patients (N = 72) and obese PCOS patients (N = 135) before and after liraglutide treatment was measured by qMSP. One-way ANOVA was employed for analysis among groups, followed by Tukey's multiple comparisons test. $^{**}P < 0.01$.

PCOS patients, CpG island methylation in the *LEP* promoter region was predicted and analyzed using the website (http://www.urogene.org/cgi-bin/methprimer/methprimer.cgi) (Fig. 3A). Subsequently, the methylation levels of the *LEP* promoter in ovarian granulosa cells of obese and non-obese PCOS patients before and after liraglutide treatment were determined by qMSP. Compared with non-obese PCOS patients, the *LEP* promoter methylation level in ovarian granulosa cells of obese PCOS patients was suppressed, while the level in ovarian granulosa cells of obese PCOS patients was strengthened following liraglutide treatment (Fig. 3B, $P < 0.01$). These results indicate that liraglutide increased *LEP* gene promoter methylation levels in ovarian granulosa cells of obese PCOS patients.

Discussion

PCOS is a prevalent endocrine disorder affecting reproductive-aged women worldwide, characterized by reproductive, metabolic, and hormonal abnormalities (Barthelmess and Naz 2014; Lizneva et al. 2016). Evidence has highlighted the significant role of liraglutide in the management of PCOS (Ge et al. 2022). This study revealed that liraglutide increased *LEP* promoter methylation levels in ovarian granulosa cells, reduced LEP levels in serum and follicular fluid, and influenced sex hormone secretion in obese PCOS patients.

PCOS presents as an endocrine disorder in reproductive-aged women, contributing to metabolic, hormonal, and reproductive irregularities (Faghfoori et al. 2017). It encompasses ovarian dysfunction, hyperandrogenism, polycystic ovarian morphology, and metabolic disturbances such as dyslipidemia and insulin resistance (Wang et al. 2019). Disrupted sex hormone secretion underpins the pathogenesis of PCOS (Zheng et al. 2020). Obesity is closely associated with impaired reproductive function and alterations in sex hormones and LH secretion (Maheshwari et al. 2007). This study's findings indicated elevated levels of BMI, FINS, FBG, HOMA-IR, TG, E2, and T, alongside reduced levels of HDL, LH, and LH/FSH in obese PCOS patients. Previous studies have underscored hyperleptinemia as a key contributor to metabolic dysregulation, suggesting that lowering plasma LEP levels could enhance hypothalamic sensitivity to LEP and improve insulin sensitivity (Zhao et al. 2019). LEP plays a pivotal role in adipocytokines and insulin signaling (Osinski et al. 2020), with the insulin-LEP axis crucial for metabolic disorders linked to obesity (Umbayev et al. 2023). Additionally, PCOS is characterized by dysfunction in the hypothalamic-pituitary-ovarian axis, marked by increased gonadotropin-releasing hormone (GnRH) pulse amplitude, resulting in elevated LH secretion and reduced FSH secretion, contributing to a common clinical presentation of LH/FSH ratio > 2.5 in PCOS patients (Burt Solorzano et al. 2012). Notably, the functional status of the growth hormone (GH) axis differs significantly between obese and non-obese PCOS women. Studies have shown that GH can enhance LH pulse release (Roth et al. 2014), while elevated insulin levels in PCOS patients can lead to feedback repression of GH secretion via the insulin-like growth factor (IGF) system (Stubbs et al. 2013). This study revealed significantly higher levels of LEP, insulin, and insulin resistance indices in obese PCOS patients compared to non-obese PCOS patients, alongside notably lower LH and LH/FSH levels. The observed negative correlation between LEP and LH/FSH may be attributed to the elevated LEP levels in obese PCOS patients, exacerbating insulin resistance, hyperinsulinemia, and insulin metabolism disorders, thereby eliciting negative feedback on the IGF system and ultimately resulting in decreased GH levels (Kasa-Vubu et al. 2010). Moreover, androgens, as steroid hormones, accumulate primarily in adipose tissues, leading to elevated androgen levels in obese individuals (Comim et al. 2013), with adipose tissue aromatase activity catalyzing the conversion of testosterone (T) into estradiol (E2) (Genchi et al. 2022). Insulin resistance and hyperinsulinemia are key mechanisms driving androgen elevation in PCOS patients (Dean et al. 2013). This study revealed a significant positive correlation between serum LEP levels and androgens T and E2 in obese PCOS patients, likely attributed to insulin resistance and hyperinsulinemia resulting from high LEP levels in obese PCOS patients, with the subsequent increase in androgens T in PCOS patients further converting into E2.

LEP exerts significant effects on the development and progression of obesity (Cui et al. 2017). Recent studies have reported notably elevated serum LEP levels in overweight/obese PCOS patients (Peng et al. 2022). The heightened levels of LEP in the serum and follicular fluid of obese PCOS patients may impact patient fertility through alterations in serum sex hormones (Abdulslam Abdullah et al. 2022). Moreover, liraglutide has emerged as a promising treatment option for PCOS (Siamashvili and Davis 2021). Liraglutide treatment is associated with reductions in BMI and increases in LH levels and insulin sensitivity in PCOS patients (Jensterle et al. 2015). This study's findings align with previous reports, indicating that following liraglutide treatment, BMI, FINS, HOMA-IR, TG, E2, and T levels were reduced, while HDL, LH, and LH/FSH levels were increased, and LEP levels in serum and follicular fluid were decreased in obese patients with PCOS (Frossing et al. 2018).

Methylation within the *LEP* promoter region plays a pivotal role in regulating LEP expression (Marchi et al. 2011). Specific CpG site methylation and methylation-sensitive proteins contribute to *LEP* gene expression during adipocyte differentiation (Yokomori et al. 2002). This study, for the first time, identified suppressed methylation levels within the *LEP* promoter in ovarian granulosa cells of obese PCOS patients. Importantly, liraglutide treatment was found to reduce LEP levels in the serum and follicular fluid of obese PCOS patients. These findings suggest that decreasing LEP levels in serum and follicular fluid may represent a crucial therapeutic strategy for managing obese

PCOS patients.

While this study provides valuable insights, it is important to acknowledge its limitations. The sample size included in this study was relatively small, and there were some limitations in data collection. Furthermore, the specific molecular mechanism underlying how liraglutide affects *LEP* promoter methylation levels remains unclear, and the improvements of fertility and embryo quality in PCOS patients mediated by liraglutide have not been explored in depth, which are the limitations of this study.

Moving forward, it is essential to expand the number of subjects in future studies and conduct more in-depth research to elucidate the internal molecular mechanism by which liraglutide influences *LEP* promoter methylation in obese PCOS patients. Additionally, further studies are needed to explore the effects of liraglutide treatment on patient fertility and embryo quality.

In summary, this study highlights the therapeutic potential of liraglutide in obese PCOS patients by revealing its role in decreasing LEP levels in serum and follicular fluid, increasing *LEP* promoter methylation levels in ovarian granulosa cells, inhibiting serum and follicular fluid LEP levels, and affecting sex hormone secretion. These findings contribute to the understanding of the mechanisms underlying the action of liraglutide in obese PCOS patients. Moreover, strategies such as weight loss, reduction of LEP levels in serum and follicular fluid, and enhancement of *LEP* promoter methylation levels in ovarian granulosa cells may be crucial in the comprehensive management of obese PCOS patients. Further research in this area holds promise for improving therapeutic outcomes and enhancing the quality of life for patients with this complex condition.

Author Contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Xiaoxia Hao and Wenhong Lu. The first draft of the manuscript was written by Lina Su and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Conflict of Interest

The authors declare no conflict of interest.

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