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ORIGINAL ARTICLE

Non-coding RNA genes modulate PI3K/AKT signaling pathway in polycystic ovary syndrome

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Abstract

Background The PI3K protein kinase B (PI3K/AKT) signaling pathway has crucial roles in insulin signaling and other endocrine disorders. The purpose of this study is to validate the association of PCOS with PI3K/AKT pathway target genes, miRNA486-5p, and miRNA483-5p as well as to evaluate the outcome of metformin on the pathogenesis of PCOS.

Methods This case-controlled study included 3 subject groups: twenty healthy females (control group), twenty PCOS females before treatment, and twenty PCOS females treated with metformin at a dose (500 mg 3 times per day for 3 months). The following gene expressions were assessed by real-time PCR: PI3K, AKT, ERK, GLUT4, miRNA486-5p, and miRNA483-5p in the whole blood.

Results There was a significant decrease in miRNA486-5p and miRNA483-5p in the PCOS group with a significant negative correlation between miRNA486-5p and PI3K and a significant negative correlation between miRNA483-5p and ERK. Metformin treatment resulted in significant elevation of the studied miRNA, significant downregulation of PI3K/AKT target genes, and significant amelioration of the gonadotrophic hormonal imbalance and insulin resistance markers: fasting blood glucose, HBA1C, fasting insulin, and GLUT4 gene expression.

Conclusions miRNA486 and miRNA483 downregulation may contribute to the etiology of PCOS, influence glucose metabolism, and result in IR in PCOS. Metformin's upregulation of those miRNAs affects glucose metabolism by controlling the expression of GLUT4, ameliorates PCOS-related insulin resistance, and improves PCOS-related hormonal imbalance by controlling the PI3K/AKT signaling pathway.

Keywords PCOS · miRNA 486 · miRNA 483 · PI3K/AKT · GLUT 4

Introduction

Polycystic ovary syndrome (PCOS) is a multifactorial endocrine disorder. It is known to be associated with excess androgen, ovarian dysfunction, endocrine disruption, insulin resistance (IR), infertility, obesity, and metabolic disorders of glucose [1]. PCOS is considered the most endocrinal illness in females of reproductive age [2]. It affects approximately 4–18% of all females of childbearing age all over the world [3].

In the presence of compensatory hyperinsulinemia, insulin resistance (IR) is associated with a reduction in liver sex hormone binding globulin (SHBG) production and an increase in ovarian/adrenal production of androgens. Elevated levels of insulin increase the secretion of GnRH, with subsequent disturbance of the action of LH and FSH, development of hyperandrogenism, and ovulatory dysfunction [4]. The important reason for hyperandrogenism in females with PCOS is an upregulated expression of rate-limiting enzymes

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of steroidogenesis (cytochrome p450c17) 3β-hydroxysteroid dehydrogenase (3β-HSD) and 17β-hydroxysteroid dehydrogenase (17βHSD) enzymes in the theca cells of the ovaries [5].

The Phosphoinositide 3-kinase (PI3K) protein family can be split into 3 types (I, II, and III) according to their substrate preference and structure. Class I is the most important one as it has an important effect on many pathological and physiological conditions. At the plasma membrane, PI3K is activated near its substrate. In addition to fibroblast growth factor, vascular endothelial growth factor (VEGF), and insulin, PI3K can be activated by several growth factors [6].

PI3K-AKT signaling pathway stimulation by insulin; activation of insulin receptors, leads to an increase in insulin receptor substrates (IRS), which bind with PI3K which produces phosphoinositide triphosphate (PIP3). PIP3 acts on phosphoinositide-dependent kinase 1 (PDK1), leading to the phosphorylation of the AKT protein. Activated AKT protein affects downstream molecules such as GLUT4; it influences glucose metabolism [7].

miRNAs are the Master Maestro of the human genome. They play a pivotal role in the post-transcriptional modifications in many interacting signaling pathways in different oncological and non-oncological pathological states. miRNA486-5p and miRNA486-3p act as prognostic and diagnostic markers in many diseases such as insulin resistance, hypertension, osteoarthritis, and metabolic syndromes (MS) [8]. In Egyptian males, miRNA486-5p was found to be a prognostic factor for insulin resistance (IR), elevated blood pressure (BP), and PCOS [9].

In addition, several pieces of evidence suggested the involvement of miRNA483 over-expression in some pathological non-oncologic conditions like cardiovascular diseases, DM, obesity, non-alcoholic fatty liver disease (NAFLD), systemic sclerosis, rheumatoid arthritis, and metabolic syndrome [10].

The study aims to determine whether metformin affects gene expression of PI3K, AKT, ERK, GLUT4, miRNA486-5p, and miRNA483-5p as well as to assess the status of insulin resistance and hormonal imbalance associated with PCOS patients.

Method

The study was conducted under the Declaration of Helsinki and approved by the Local Ethics Committee of Cairo University, Faculty of Medicine, and written informed consent from all females (IRB number 284).

In this case-control study, 60 females aged 25 to 35 were recruited from the Obstetrics and Gynecology department at Minia University, during the period from January 2022 to June 2022. The laboratory work was conducted in the Unit of Biochemistry and Molecular Biology, Faculty of Medicine, Cairo University.

PCOS patients were diagnosed according to the revised 2003 consensus on the diagnostic criteria. Long-term health risks associated with PCOS were eligible [11]. There are three criteria for diagnosing PCOS: Oligo-ovulation indoor an-ovulation, clinical indoor biochemical evidence of hyperandrogenism, and polycystic ovarian morphology. The case must meet at least two of the three criteria to be classified as PCOS.

Subjects of the study were subdivided into three groups: Group I: included 20 age-matched females as a control group. Group II: included 20 females in reproductive age with polycystic ovary syndrome without complication, and who did not receive any hormone drugs or oral contraceptives. Group III: included 20 of the PCOS group who were treated with metformin at a dose of 500 mg three times per day for 3 months.

Exclusion criteria included: females in the postmenopausal phase and patients with any condition that causes hyperandrogenism, or hypothyroidism. Female patients with liver, kidney, and heart diseases were also excluded.

The history of all eligible females was taken in detail, including age at the time of examination, disease duration, and types of drugs taken. In addition to measuring weight, height, and body mass index, a thorough clinical examination focused on endocrine gland disease. By local standards, routine laboratory investigations were conducted.

Sample collection

After signing written informed consent: 5 mL venous blood samples were taken from all subjects using the BD Vacutainer system during the 3rd, 4th, and 5th days of the menstrual cycle and at any time for those who had amenorrhea, (NB: two samples were collected from Group III before and after metformin administration). Samples were kept at − 80 °C until the time of analysis of the following parameters:

- Gene expression of PI3K/AKT downstream signaling target genes: extracellular signal-regulated kinase (ERK), serine/threonine kinase 1 (AKT), and GLUT4 in the peripheral blood by real-time PCR.
- Evaluation of miRNA486-5p and miRNA483-5p in peripheral blood by real-time PCR.

Molecular biology techniques

Assessment of expression levels of PI3K, AKT, ERK, GLUT 4, miRNA486-5p, and miRNA483-5p in the whole blood by real-time qRT PCR

RNA extraction RNA was isolated using miRNAs mini kit (Qiagen, Germany, Cat. No. 217004) permitting the manufacturer's recommendations.

Quantitation of isolated RNAs The absorbance of isolated miRNA was measured by Nanodrop® spectrophotometer at 260 nm.

Amplification and quantification of the genes using reverse transcription–polymerase chain reaction (RT–PCR) *Transcript*® Green One-Step qRT-PCR Super Mix kit (Transgenbiotech, China, Cat No. AQ211) was used permitting the manufacturer's recommendations.

Primer selection We obtained the primers for PCR from GenBank RNA sequences cited at http://www.ncbi.nlm.nih.gov/tools/primer-blast. When selecting the ideal primer pair, the following factors were considered: melting temperature (Tm: 60–65 °C), guanine, cytosine content (40–60%),and amplicon length between 90 and 200 bp.

Software version 3.1 of the StepOnePlus Real-Time PCR system (Applied Biosystems, USA) was used to examine gene expression. SYBR® Green (ThermoFisher, USA) was used to measure relative gene expression.

A hardening temperature of 60 °C was adjusted for all primer sets. Real-time PCR was done in 25 µL final volume containing SYBR Green master mix, 900 nmol/L of every PCR primer, and 3 µL of cDNA. Amplification conditions were done based on the manufacturer references: 2 min at 50 °C, 10 min at 95 °C, 40 thermal cycling of 15^{s} denaturation, and 10 min of annealing/and extension at 60 °C.

Calculation of relative quantification (RQ) (relative expression) By employing passive reference dye (ROX) to normalize the fluorescence and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a reference gene, the conventional double delta threshold cycle (ΔΔCt) method for relative quantification (RQ) was employed to calculate the expression of the examined genes. The Ct values of the ref- erence gene and the examined genes were computed using Applied Biosystems Step One Plus software. The analysis of the PCR data included the Ct values of the reference gene (GAPDH), the housekeeping gene, and the target genes. The negative control sample had no template cDNA. All figures were expressed as fold changes in the background levels of the control samples after being normalized to GAPDH.

RQ was calculated according to the following equation:

$$\Delta \text{ Ct} = \text{Ct assessed gene of test sample} - \text{Ct reference gene},$$

$$\Delta \text{ Ct} = \text{Ct assessed gene of control sample} - \text{Ct reference gene},$$

$$\Delta\Delta \text{ Ct} = \Delta \text{ Ct of test sample} - \text{Ct of the control sample},$$

$$\text{RQ} = 2^{-(\Delta\Delta \text{Ct})}.$$

Statistical evaluation

With the help of the statistical program SPSS version 22, data were coded and entered. The mean and standard deviation were used to summarize the data. Chi-square (X2) test results were used to compare gender data. Using a Chi-square test, deviation from Hardy-Weinberg equilibrium (HWE) was evaluated. When comparing more than two groups, analysis of variance (ANOVA) was used, along with multiple comparisons post hoc tests. The Pearson correlation coefficient was used to determine correlations between quantitative variables. A p-value less than 0.05 was regardedas significant.

Result

The present study was conducted on sixty women of matched age with (p-value> 0.05) (Fig. 1A). Participants were further split into three groups; Group I: twenty healthy females as control subjects, Group (II): twenty females with polycystic ovary syndrome and, group (III): twenty PCOS females treated with metformin at a dose (500 mg 3 times per day for 3 months) (Table 1).

Demographic and biochemical data characteristics (Fig. 1; Table 2)

PCOS patients' group and those treated with metformin showed statistically increased BMI, LH, Testosterone, LDL, and TG. Whereas they showed statistically decreased HDL levels matched the normal control group.

Also, the PCOS patients group revealed a statistical increase in FBS, HbA1c, fasting insulin, HOMA-IR, TC, and TG when compared to the normal control and those treated with metformin. (p-value < 0.05). Also, there were significant increases in BMI in both PCOS and those treated with metformin compared to normal control subjects ($p1 < 0.001$) ($p2 = 0.003$), whereas there was no significant difference in BMI between PCOS patients and those treated with metformin ($p3 = 0.3$) (Fig. 1B).

There were significantly higher FBS levels in PCOS patients compared to both the control group and those treated with metformin. ($p1 = 0.007$) ($p3 = 0.015$), Whereas there was no significant difference in FBS between PCOS after treatment with metformin and the control group. ($p2 = 0.9$) (Fig. 1C). Also, there were significantly higher levels of HbA1c among PCOS patients compared to both the control group and those treated with metformin. ($p1 < 0.001$) ($p3 < 0.001$), while there was no significant difference in HbA1c between PCOS after metformin treatment and the control group. ($p2 = 0.6$) (Fig. 1D).

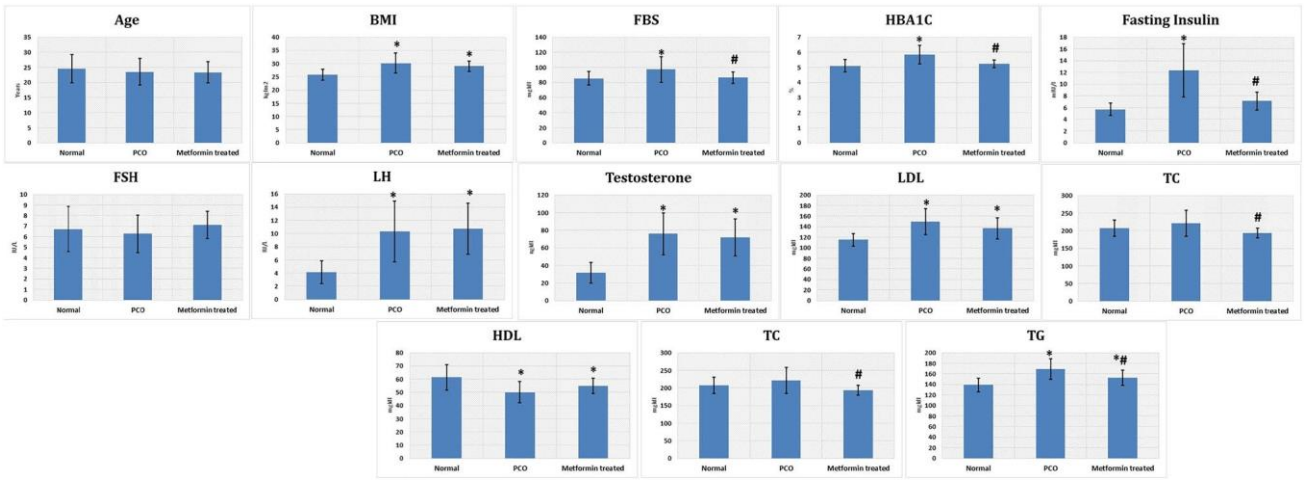


Fig. 1 Age, BMI, fasting blood sugar, HbA1c mean levels, fasting insulin, FSH, LH, testosterone, LDL, HDL, TC, TG, and HOMA mean levels among different studied groups. Data were expressed as Mean ± SD p-valuables < 0.05 was significant. (*) Denotes significant difference between PCOS patients versus control subjects. (#) Denotes a significant difference between metformin-treated patients versus PCOS patients

Table 1 The primer sequences of the studied genes and miRNAs

Gene symbol	Primer sequence from 5′–3′
PI3K NM_006219.3 https://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=nucleotide&id=1698173417	Forward: 5′-TTGGAATAGTAGCAGGCGGC-3′
	Reverse: 5′-CGCCCAGATGTCAAGGATGT-3′
ERK D31661.1 https://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=nucleotide&id=495677	Forward: 5′-AAGAGATGGATGTGGGTTCCA-3′
	Reverse: 5′-GGTCCGTAGCCAGTTGTTCT-3′
Serine/Threonine kinase 1 (AKT1), NM_001382431.1 https://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=nucleotide&id=1838745030	Forward: 5′-CCGAAGACGGGAGCAGG-3′
	Reverse: 5′-ATGGAAAGCAGGCCAGACTC-3′
GLUT 4 M91463.1 https://www.ncbi.nlm.nih.gov/tools/primer-blast/primertool.cgi?ctg_time=1668587361&job_key=ZG67v2BSbfpKwP3F8KXZ94q-yMWnrdPYpg	Forward: 5′-CCCTCAGAAGGTGATTGAACAG-3′
	Reverse: 5′-AGAGATGATACCAATGAGGAAGG-3′
MiRNA486-5p doi: https://doi.org/10.1042/BSR20200392	Forward: 5′-GGCAGCTCAGTACAGGATAAA-3′′
	Reverse: 5′- CGGGGCAGCUCAGUACAGGAT-3′
MiRNA483-5p doi: https://doi.org/10.12659/MSM.897301	Forward: 5′-ACACTCCAGCTGGGTCCAACATTG TCTTTA G-3′
	Reverse: 5′-TGGTGTCGTGGAGTCG-3′
GAPDH doi: https://doi.org/10.12659/MSM.897301	Forward: 5′-GAAGGTGAAGGTCGGAGTC-3′
	Reverse: 5′-GAAGATGGTGATGGGATTG-3′

F Forward primer, *R* Reverse primer

Fasting insulin levels in PCOS patients showed significantly higher levels as compared to both the control group and those treated with metformin. (p1 < 0.001) (p3 < 0.001), whereas there was no significant difference in Fasting insulin between PCOS after metformin treatment and the control group. (p2 = 0.3) (Fig. 1E). LH levels in both PCOS patients and those treated with metformin showed a significant increase as compared to the control group. (p1 < 0.001) (p2 < 0.001). There was no significant difference in LH between PCOS patients and those treated with metformin. (p3 = 0.9). FSH levels showed no significant differences among the three studied groups (p values > 0.05) (Fig. 1F and G). Moreover, testosterone levels in both PCOS patients and those treated with metformin showed significant elevation as compared to the control group. (p1 < 0.001) (p2 < 0.001). There was no significant difference in testosterone between PCOS patients and those treated with metformin. (p3 = 0.7) (Fig. 1H). Significantly high levels of LDL were found in PCOS patients and those treated with metformin as compared to the control group. (p1 < 0.001) (p2 = 0.006), whereas there were no significant differences between LDL levels in PCOS patients and those treated with metformin. (p3 = 0.07) (Fig. 1I). Significantly

Table 2 Mean values ± SD of some demographic and biochemical data among the studied groups

Groups/demographic and biochemical data	Normal	PCOS	Metformin treated	p1 value	p2 value	p3 value
Age	24.6 ± 4.65	23.55 ± 4.47	23.3 ± 3.51	0.64	0.6	0.9
BMI	25.74 ± 2.06	30.21 ± 3.79	29.01 ± 2.01	< 0.001	0.003	0.3
FBS (mg\dl)	85.48 ± 9.08	97.15 ± 17.26	86.46 ± 7.57	0.007	0.9	0.015
HBA1c %	5.1 ± 0.41	5.85 ± 0.61	5.24 ± 0.25	< 0.001	0.6	< 0.001
Fasting insulin (mlU\l)	5.71 ± 1.04	12.34 ± 4.57	7.12 ± 1.52	< 0.001	0.3	< 0.001
FSH (IU\L)	6.72 ± 2.14	6.27 ± 1.78	7.12 ± 1.28	0.6	0.7	0.2
LH (IU\L)	4.14 ± 1.75	10.31 ± 4.65	10.69 ± 3.88	< 0.001	< 0.001	0.9
Testosterone (ng\dl)	31.58 ± 11.62	75.89 ± 23.98	71.67 ± 21.03	< 0.001	< 0.001	0.7
LDL (mg\dl)	115.33 ± 11.85	149.59 ± 25.17	136.7 ± 20.41	< 0.001	0.006	0.07
HDL (mg\dl)	61.32 ± 9.83	50.1 ± 8.11	55.01 ± 5.74	< 0.001	0.04	0.07
TC (mg\dl)	206.75 ± 23.09	220.99 ± 37.56	192.85 ± 13.77	0.2	0.3	0.003
TG (mg\dl)	138.96 ± 12.66	169.25 ±19.19	152.42 ± 14.21	< 0.001	0.03	0.001
HOMA-IR	1.2 ± 0.19	3.09 ± 1.68	1.53 ± 0.4	< 0.001	0.6	< 0.001

Data were expressed as Mean ± SD, and p-value < 0.05 was significant

P1 value: comparison between PCOS and normal control; P2 value: comparison between metformin-treated and normal control; P3 value: comparison between PCOS and metformin-treated

decreased HDL levels were found in PCOS patients and in those treated with metformin as compared to the control group. (p1 < 0.001) (p2 = 0.04), while there was no significant difference in HDL levels between PCOS patients and those treated with metformin. (p3 = 0.07) (Fig. 1J). Significantly decreased TC levels were found in patients treated with metformin as compared to the PCOS group. (p3 = 0.003), whereas there was no significant difference in TC level between PCOS patients and those treated with metformin as compared to the control group (p1 = 0.2) (p2 = 0.3) (Fig. 1K). There was a significant increase in TG levels in both PCOS patients and those treated with metformin in comparison to the control group. (p1 < 0.001) (p2 = 0.03), while there was a significant decrease in TG level after metformin treatment as compared to PCOS patients (p3 = 0.001) (Fig. 1L).

There was a significant increase in HOMA in PCOS patients in comparison to both the control group and those treated with metformin. (p1 < 0.001) (p3 < 0.001), while there was no significant difference in HOMA between PCOS after metformin treatment and the control group. (p2 = 0.6) (Fig. 1M).

Expression levels of PI3K, AKT, ERK, Glut 4, miRNA-486-5p, and miRNA483-5p genes

There was a significant decrease in miRNA 486 level in PCOS patients and those treated with metformin as compared to the control group. (p1 < 0.001) (p2 < 0.001). Whereas there was a significant increase in miRNA 486 level after metformin treatment as compared to PCOS patients. (p3 < 0.001). There was a significant decrease in miRNA 483 levels in PCOS patients and in those treated with metformin as compared to the control group. (p1 < 0.001) (p2 < 0.001). While miRNA 483 was significantly increased after metformin treatment as compared to PCOS patients. (p3 < 0.001). Significant increases in AKT levels were found in PCOS patients and in those treated with metformin as compared to the control group. (p1 < 0.001) (p2 < 0.001). Whereas miRNA 483 was significantly decreased after metformin treatment compared to PCOS patients. (p3 < 0.001). There was a significant increase in PI3K levels in PCOS patients and in those treated with metformin as compared to the control group. (p1 < 0.001) (p2 < 0.001). While PI3K level was significantly decreased after metformin treatment as compared to PCOS patients. (p3 < 0.001). There was a significant decrease in ERK levels in PCOS patients and in those treated with metformin compared to the control group. (p1 < 0.001) (p2 < 0.001), a significant decrease in GLUT4 levels in PCOS patients and in those treated with metformin compared to the control group. (p1 < 0.001) (p2 < 0.001). While GLUT4 was significantly increased after metformin treatment compared to PCOS patients. (p3 < 0.001). (Table 3; Fig. 2).

Correlation between miRNA 486 &PI3K and AKT among the studied groups

Results of the study showed a significant negative correlation between miRNA 486 and PI3K in all the studied groups (p < 0.0001) and a significant negative correlation between

miRNA 486 and AKT in all the studied groups ($p < 0.0001$). (Table 4; Figs. 3 and 4).

Correlation between miRNA 483&GLUT4 among the studied groups

Results of the study showed a significant positive correlation between miRNA 483 and GLUT4 among all the studied groups, $p < 0.0001$ (Table 5) (Fig. 5).

Discussion

Hyperandrogenism and ovarian abnormalities are two features of polycystic ovary syndrome (PCOS), which is caused by a malfunction in the hypothalamic-pituitary-ovarian axis [12]. Clinically, insulin resistance and hyperandrogenism are the primary causes of reproductive and metabolic problems in women with PCOS [13].

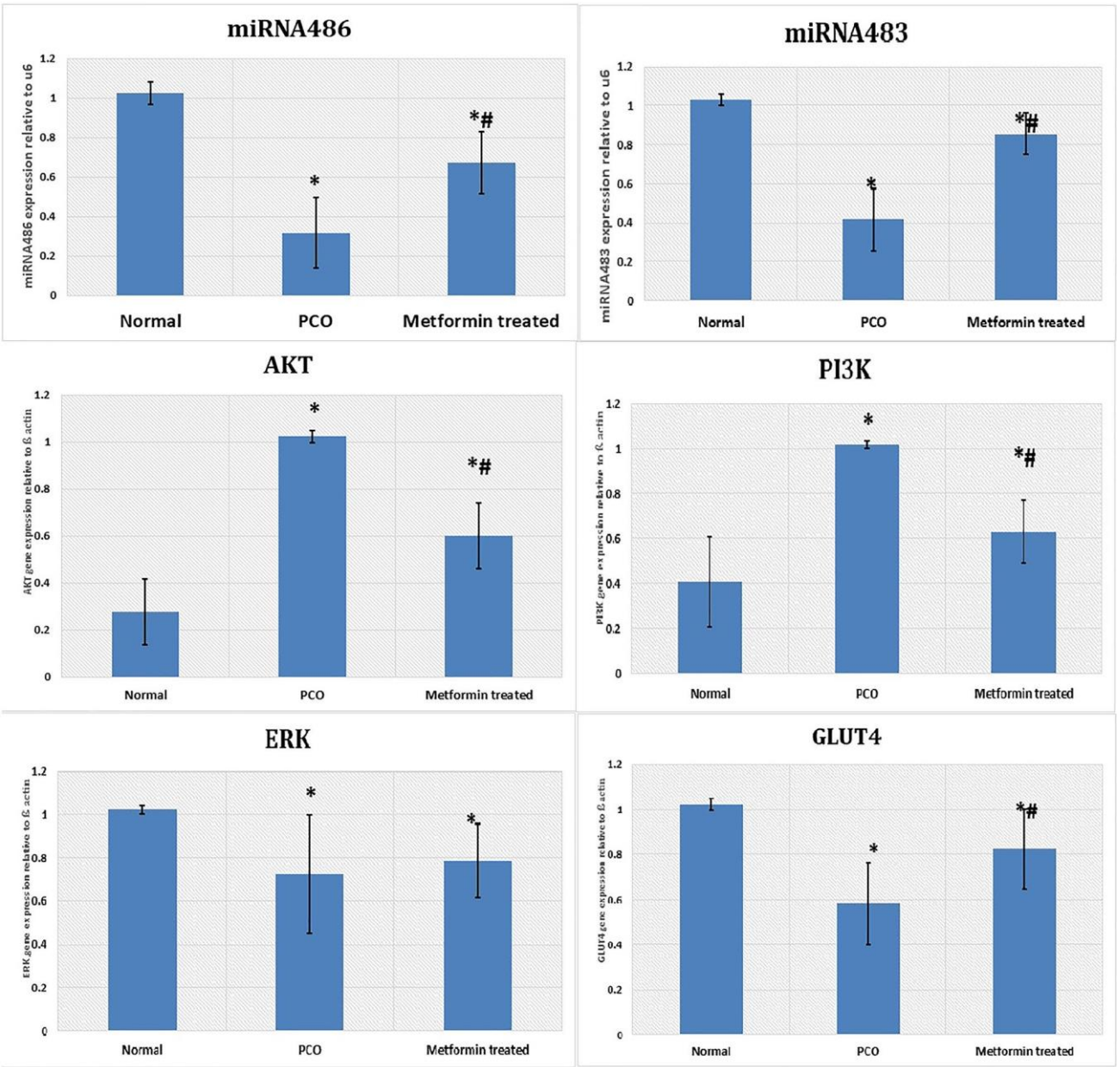


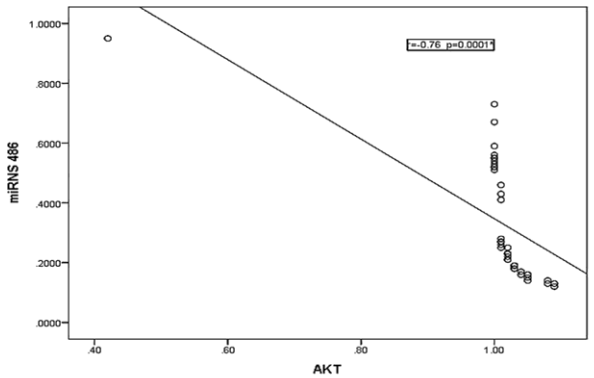
Fig. 2 Levels of PI3K, AKT, ERK, Glut 4, miR-486-5p, and miR-483-5p genes by real-time PCR among different studied groups.Data were expressed as Mean ± SD, and p-value < 0.05 was significant. (*) Denotes significant difference between PCOS patients versus control subjects. (#) Denotes a significant difference between metformin-treated patients versus PCOS patients

Table 3 Levels of PI3K, AKT, ERK, Glut 4, miR-486-5p, and miR-483-5p genes by real-time PCR among different studied groups

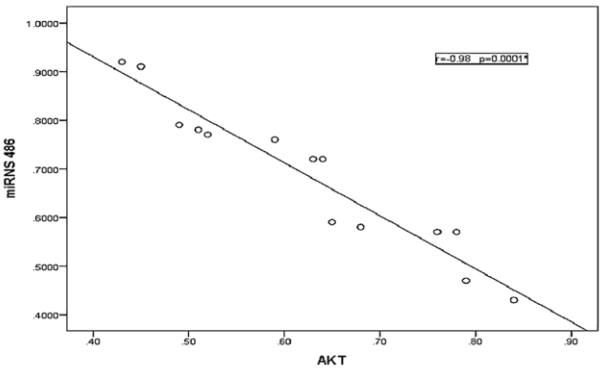
Groups/genes	Normal	PCOS	Metformin treated	p1 value	p2 value	p3 value
MIR486	1.03 ± 0.06	0.32 ± 0.18	0.67 ± 0.16	< 0.001	< 0.001	< 0.001
MIR483	1.03 ± 0.03	0.41 ± 0.16	0.85 ± 0.11	< 0.001	< 0.001	< 0.001
AKT	0.28 ± 0.14	1.02 ± 0.03	0.6 ± 0.14	< 0.001	< 0.001	< 0.001
PI3K	0.41 ± 0.2	1.02 ± 0.02	0.63 ± 0.14	< 0.001	< 0.001	< 0.001
ERK	1.02 ± 0.02	0.72 ± 0.27	0.78 ± 0.17	< 0.001	0.002	0.5
GLUT4	1.02 ± 0.02	0.37 ± 0.25	0.83 ± 0.18	< 0.001	< 0.001	< 0.001

Data were expressed as Mean ± SD, and the pp-value<0.05 was significant

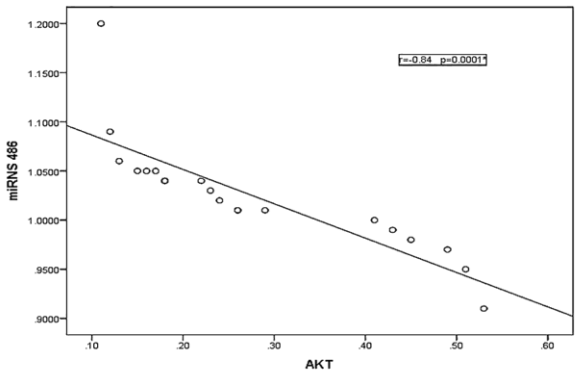
P1 value: comparison between PCOS and normal control; P2 value: comparison between metformin-treated and normal control; P3 value: comparison between PCOS and metformin-treated



Correlation between miRNA 486 & AKT in PCOS group.



Correlation between miRNA 486 & AKT after metformin treatment

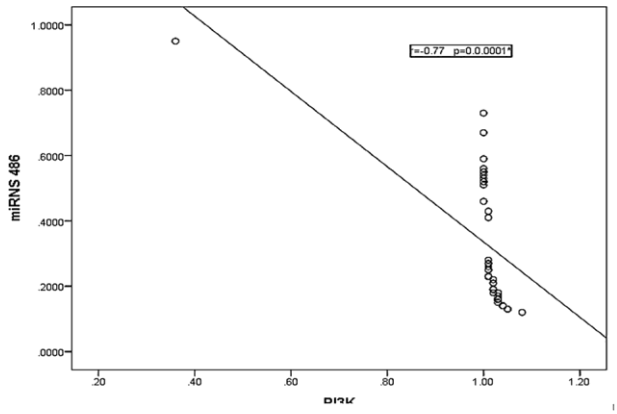


Correlation between miRNA 486 & AKT in the control group

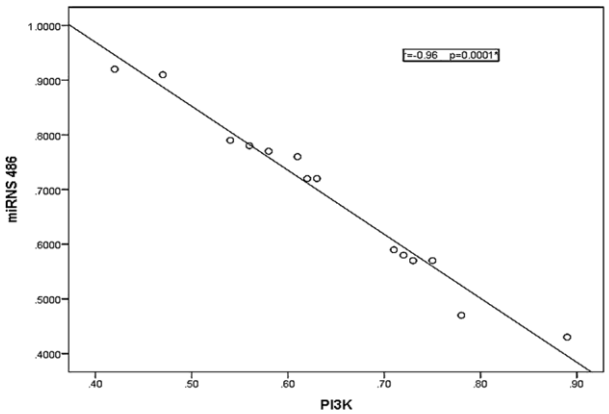
Fig. 3 Shows a significant inverted correlation between AKT and miRNA 486 in the studied groups. Data were expressed as Mean ± SD, and p-value < 0.05 was significant. (*) Denotes significant difference between PCOS patients versus control subjects. (#) Denotes a significant difference between metformin-treated patients versus PCOS patients

In the present study, we compared 3 groups (group 1 as a control; group 2 as PCOS patients, and group 3 as PCOS patients after metformin treatment for 3 months). Studied parameters included: PI3K/AKT pathway target genes; GLUT 4 and miRNA486, and miRNA483.

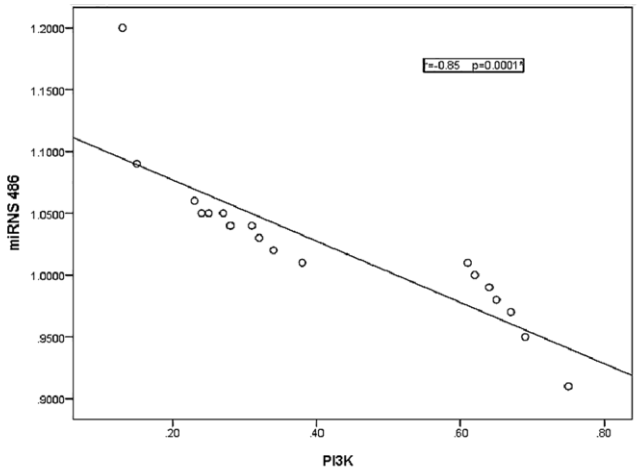
In the present study, PCOS women's BMI and LH levels significantly increased when in comparison to the healthy control subjects (p 0.001). Our findings were in line with a previous study which discovered that PCOS patients' BMI was significantly increased when compared to the control



Correlation between miRNS 486 &PI3K in PCOS group.



Correlation between miRNS 486 &PI3K after metformin treatment.



Correlation between miRNA 486 &PI3K in the control group.

Fig. 4 Shows a significant inverted correlation between PI3K and miRNA 486 in the studied groups. Data were expressed as Mean ± SD, and p-value < 0.05 was significant. (*) Denotes significant difference between PCOS patients versus control subjects. (#) Denotes a significant difference between metformin-treated patients versus PCOS patients

Table 4 Correlation between miRNS 486 & PI3K and AKT among the studied groups

	miRNS 486 r(p)		
	Normal	PCO	Metformin treated
PI3K	− 0.85 (0.0001*)	− 0.77 (0.0001*)	− 0.96 (0.0001*)
AKT	− 0.84 (0.0001*)	− 0.76 (0.0001*)	− 0.98 (0.0001*)

group. In contrast, they showed that there were no appreciable variations in LH levels between the research groups [14].

When compared to the normal control group and those receiving metformin treatment, the PCOS patient group in the current study demonstrated statistically significant increases in FBS, HbA1c, fasting insulin, HOMA-IR TC, TG, LDL, and testosterone while there was a significant decrease in HDL levels (p-value 0.05). In the same vein. A recent study discovered that individuals with PCOS had significantly higher levels of (BMI), (T), (FBG), and (INS) of fasting insulin than did healthy controls (p0.05) [15]. A previous study confirmed our findings, they discovered that the PCOS group had considerably lower levels of HDL, FSH, and E2 than the controls while significantly higher levels of BMI, fasting insulin, HOMA-IR, LDL, TG, TC, testosterone, and LH were present [16].

Furthermore, our findings are confirmed by a previous study that showed a statistically significant difference in BMI, FBS, HbA1c, fasting insulin, HOMA-IR TC, TG, and LDL in PCOS women as compared to the control group. The aforementioned study included 67 women, comprising 32 with PCOS and 35 age-matched controls. (0.05 p-value) [17].

On the other hand, Jalilian et al. [14] found that there was no significant difference between the study groups in terms

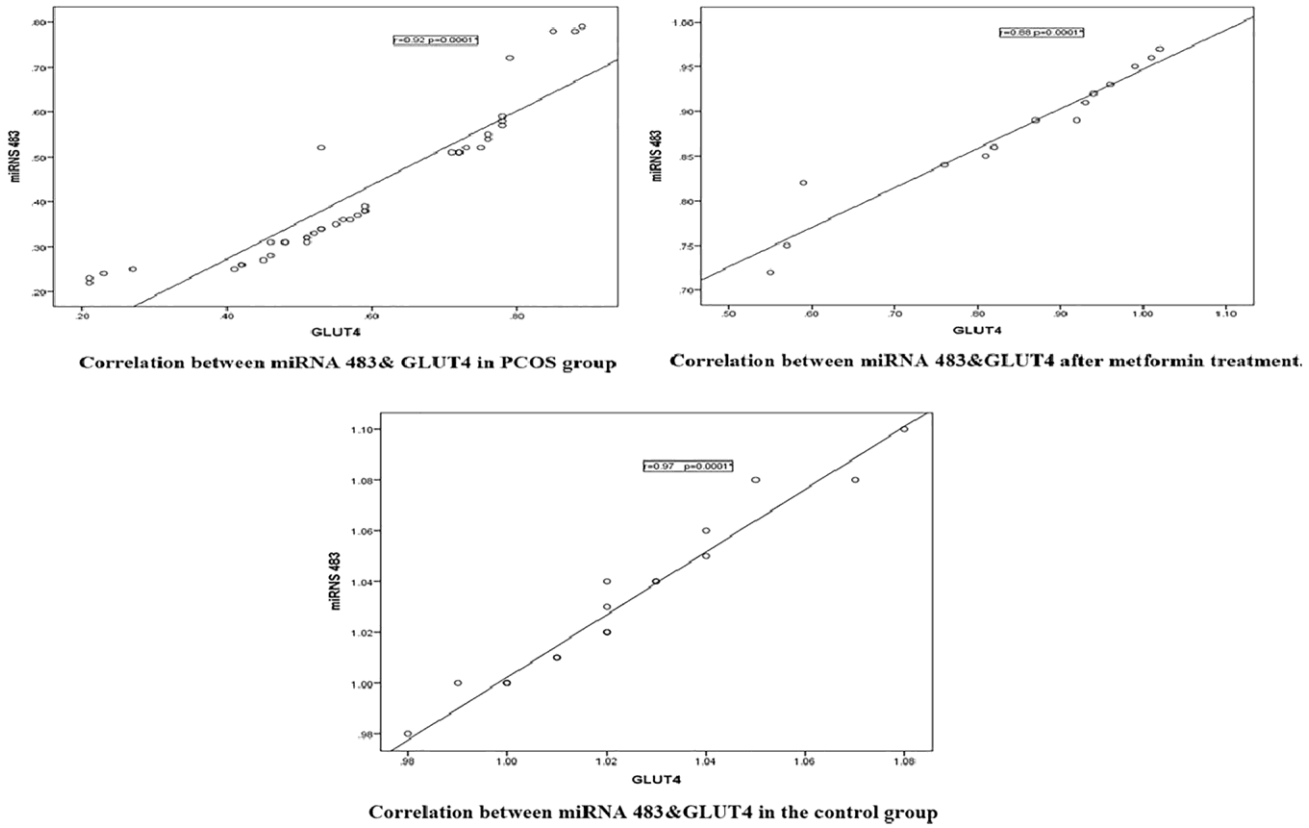


Fig. 5 Shows a significant direct correlation between miRNA 483 and GLUT4 among the studied groups. Data were expressed as Mean ± SD, and p-value < 0.05 was significant. (*) Denotes significant difference between PCOS patients versus control subjects. (#) Denotes a significant difference between metformin-treated patients versus PCOS patients

Table 5 Correlation between miRNS 483 & GLUT4 among the studied groups

	miRNS 483r(p)		
	Normal	PCOS	Metformin treated
GLUT4	0.97 (0.0001*)	0.92 (0.0001*)	0.88 (0.0001*)

of mean fasting blood sugar (FBS), FSH, and fasting insulin levels (40 women with PCOS and 36 healthy women).

By preventing gluconeogenesis and adipogenesis, metformin can lower the amount of glucose produced by the liver, increase the insulin sensitivity of peripheral tissues, and reduce obesity and metabolic diseases as well. Numerous studies have demonstrated that metformin can help women with PCOS conceive by regulating menstrual cycles, restoring ovulation, and even correcting menstrual patterns [18].

After treating PCOS women with metformin (500 mg three times daily for three months), there was an improvement in some biochemical markers as demonstrated by the significantly lower levels of fasting insulin, HOMA-IR, TC, TG, and FBS in the current study. (p-value 0.05), but there were no significant differences in levels of testosterone, HDL, LDL, LH, or BMI.

These results are consistent with a previous study that examined the impact of metformin therapy on PCOS patients over 12 weeks and discovered that parameters related to lipid metabolism (LDL and HDL) were similar in PCOS patients before and after metformin therapy, while glucose and insulin levels tended to drop [19]. Whereas there were no statistically significant variations in FBS and fasting insulin, the HOMA-IR value was significantly lower after treatment with a drop of 0.5 points (p 0.05), and there was a significant drop in both TG and TC (p 0.05) [19].

On the other hand, Guan et al. [20] found that overweight women with polycystic ovarian syndrome who used metformin saw significant improvements in their endocrine and metabolic indicators, such as testosterone, FSH, LH, and LDL. The secretory indices of fasting insulin, HOMA-IR, HDL, TC, TG, and FBS were not affected by metformin, though.

As miRNA 483-5p and miRNA 486-5p target mediators of insulin-like growth factor (IGF) signaling, including IGF-I receptor (IGF1R) and PI3K regulatory subunit 1 (alpha) (PIK3R1), and are observed to be reduced in plasma

of diabetic patients, several miRNAs play an important role in the pathogenesis of PCOS. [21].

These miRNAs are involved in the death of human primary T-helper cells through apoptosis. Apoptotic cell death has also been linked to PCOS, which may explain PCOS' subfertility and abnormal follicular development [22]. However, roughly 27% of miRNA-486's verified target genes are associated with insulin sensitivity in PCOS [23].

MiRNA483 and miRNA486 showed significantly downregulated in PCOS patients compared to the control group in the current study but significantly upregulated after metformin treatment. (p 0.001). Whereas gene expressions of PI3K/AKT downstream signalling pathway molecules were significantly upregulated in PCOS patients compared to the control group, with significant downregulation after metformin treatment (p 0.001). These findings suggest the role of miRNA486 and miRNA483 in the regulation of these genes and affect the insulin signalling mechanism and PCOS pathogenesis.

Finally, we found that GLUT 4 gene expression was downregulated in the PCOS group compared to the control group (p 0.001) with significant upregulation after metformin treatment.

These findings support the findings of Han et al. [24] that the expression of miRNA486-5p was much lower in PCOS tissues than in normal tissues, suggesting that miRNA486-5p may prevent the proliferation of ovarian granulosa cells, hence preventing the onset of PCOS. Additionally, Butler et al. [25] demonstrated that the expression of miRNA486-5p in PCOS serum was considerably lower than that of the control group ($p < 0.05$) and related to the pathways of reproductive disorders but not with anti-mullerian hormone (AMH) or metabolic parameters.

In contrast, a prior investigation by Zhao et al. [26] using a rat model of polycystic ovarian syndrome revealed that the PCOS model had considerably greater levels of miRNA-486 expression.

Additionally, our findings supported the findings of Zhao et al. [27] that PCOS patients' cumulus cells dramatically downregulate the expression of miRNA483-5p and miRNA-486-5p (p 0.001). In PCOS cumulus cells, IGF2 (the miRNA483 host gene) expression was dramatically downregulated (P 0.001). These findings suggested that miRNA483 may be crucial in lowering insulin resistance and that downregulated miRNA486-5p may boost cumulus cell proliferation through the activation of PI3K/AKT. Xu et al. [28] show that miRNA483 can control Notch3/MAPK3 expression and progesterone levels in PCOS patients' cumulus GCs and follicular fluid.

MiRNA483 was considerably downregulated in the lesioned ovarian cortex of PCOS patients, according to previous research that agreed with our findings [29].

According to these findings, miRNA483 is a PCOS suppressor that inhibits cell proliferation by targeting IGF1 and is involved in insulin-induced cell proliferation. As a result, miRNA 483 offers a potential substitute for PCOS diagnosis and treatment.

Since we discovered that miRNA483 and miRNA486 expression was considerably upregulated after metformin administration, our work is the first to demonstrate a link between metformin treatment and (miRNA483 and miRNA486 expression) among PCOS. (p 0.05). Cheng et al., [30] demonstrated that metformin can prevent the proliferation of breast cancer cells by blocking the miRNA483-3p/METTL3/m6A/p21 pathway, which was reported to be increased by metformin.

Furthermore, Fujita et al. [31] suggest that metformin treatments lead to the upregulation of certain miRNAs and the downregulation of others. The authors reported that metformin has a significant effect on visceral preadipocyte differentiation, subsequently insulin resistance.

Previous research has demonstrated that endometrial cancer and insulin resistance are significantly affected when the PI3K-AKT signaling pathway is activated in PCOS women [7].

In the current study, we found that PI3K and AKT were upregulated in the PCOS group in comparison to the control (p 0.001) and downregulated in the PCOS group after metformin administration in comparison to PCOS women before treatment (p 0.001).

Our findings agree with Yang et al., [32] who discovered that PCOS mice have considerably higher levels of pAKT/AKT expression compared to the control group (p 0.01).

The expression and phosphorylation of AKT and ERK1/2 were found to be significantly higher in PCOS endometrium tissues compared to controls (p 0.05) in a study in another study that investigated the relationship between activation of the Akt and ERK1/2 signaling pathways and endometrium malignant transformation in polycystic ovary syndrome [33]. Additionally, PCOS patients with endometrial hyperplasia and cancer had significantly greater levels of p-AKT ($p = 0.018$) and p-ERK1/2 ($p = 0.035$) expression than those with normal endometrium tissues.

Metformin has been shown to restore the cellular metabolic sensors AMPK, p38MAPK, and PI3K/AKT, which are responsible for insulin sensitivity and glucose absorption [34]. Metformin is believed to increase insulin sensitivity and glucose absorption by cells via activating AMPKs and PI3K/AKT within the cell signaling pathway.

In terms of GLUT4 gene expression, we discovered that it was considerably upregulated following metformin treatment in PCOS women but dramatically downregulated in the control group (p 0.001).

Older research suggested a direct mechanism by which metformin could reduce insulin resistance in muscle cells by

reducing Histone Deacetylase 5 (HDAC5) connection with the glucose transporter type 4 (GLUT4) gene, leading to enhanced GLUT4 expression in human primary myotubes [35].

In the same vein, Morley et al. [36] claim that Metformin has been demonstrated to raise GLUT-4 protein and mRNA levels in soleus muscle from diabetic rats (caused by streptozotocin).

Previous findings linked overexpression of miR-93 with the reduced expression of GLUT4 and poor glucose transmembrane transport in PCOS patients [37] confirmed our findings. Another study indicated that miR-33b-5p could play a role in the inhibition of GLUT4 synthesis, leading to PCOS IR [38].

Therefore, we can surmise that miRNA486 and miRNA483 downregulation may contribute to the etiology of PCOS, influence glucose metabolism, and result in IR in PCOS. Metformin's upregulation of those miRNAs affects glucose metabolism by controlling the expression of GLUT4, ameliorates PCOS-related insulin resistance, and improves PCOS-related hormonal imbalance by controlling the PI3K/AKT signaling pathway.

It is necessary to conduct an additional study on the molecular signaling pathways of miRNA (miRNA-486-5p and miRNA-483-5p) and PCOS in humans. Future research is required to determine the impact of miRNA (miRNA-486-5p and miRNA-483-5p) overexpression on the expression of the downstream target genes for PI3K/AKT signaling, including GLTU4, ERK, AKT, and serine/threonine kinase 1.

Conclusion

miRNA486 and miRNA483 downregulation observed in the study may contribute to the etiology of PCOS, influence glucose metabolism, and result in IR in PCOS. Metformin's upregulation of those miRNAs affects glucose metabolism by controlling the expression of GLUT4, ameliorates PCOS-related insulin resistance, and improves PCOS-related hormonal imbalance possibly by controlling the PI3K/AKT signaling pathway.

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Author contributions HSO participated in the debate and wrote the initial manuscript, figure legends, text descriptions of the histology observations, and interpretations. OA performed biochemical and gene investigations, analysis, and interpretation, while MS took part in the manuscript's writing and data search. MG took part in the biochemical analysis, connected the research data, and produced the paper's final draft. HF and EM gathered information from the literature and carried out the morphometric analysis, investigation, and result correlation. The final draft of the work was approved by all authors.

Data availability The data used and/or analyzed during this study are available from the corresponding author upon reasonable request.

Declarations

Conflict of interest The authors have no conflicts of interest or other disclosures to report.

Ethical approval The study was designed and conducted according to ethical norms approved by the Local Ethics Committee of Cairo University, Faculty of Medicine, and written informed consent from all females (IRB number (MD-284-2020).

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