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The Potential Differential Impact of *PRL* and *MIR4760* Gene Expression in Patients with PCOS and Hyperprolactinemia: A Pilot Study

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Abstract

Polycystic ovary syndrome (PCOS) is the most prevalent endocrinopathy affecting reproductive-aged females, in which prolactin (PRL) hormone seems to significantly affect the disease's course. Considering the scarcity of local studies investigating the potential association of *MIR4760* expression in the context of POCS and hyperprolactinemia, the present study aims to assess the potential differential value of *PRL* and *MIR4760* gene expression in hyperprolactinemia and POCS patients. A total of 75 females (age range 14-40 years) were involved in this study (including 25 patients diagnosed with POCS, 25 patients with hyperprolactinemia, and 25 apparently healthy controls). Peripheral blood samples were collected from each of the assessed participants for prolactin serum estimation and RNA extraction. *PRL* and *MIR4760 relative* gene expression was assessed utilizing the qPCR technique, while prolactin serum levels were estimated using the Human PRL (Prolactin) ELISA Kit. The results showed significant elevation (P=0.001) in the expression of *PRL* gene in patients with hyperprolactinemia and PCOS in comparison to healthy controls (expression fold =3.27 and 1.27, respectively). While the expression of *MIR4760* was reduced significantly (P=0.001) in the investigated patients groups (hyperprolactinemia and PCOS) compared to healthy controls. The present study exhibited significantly increased (P≤0.001) prolactin serum levels (P≤0.001) in the PCOS and hyperprolactemia patients in comparison to those of the healthy controls (36.46±4.86, 40.46 ± 15.06, and 19.76±5, respectively). Overall, the present study findings support the assumption that links *PRL* overexpression to PCOS pathogenicity. Additionally, the expression of *PRL* levels seems to be negatively associated with *MIR4760 expression* status in the investigated group of POCS patients.

Keyword: PCOS, hyperprolactinemia, *PRL* expression, *MIR4760*

التأثير التفاضلي المحتمل لتعبير جين *PRL* و *MIR4760* لدى مرضى متلازمة تكيس المبايض وفرط بروﻻكتين الدم: دراسة اولية

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الخلاصة

متلازمة تكيس المبايض (PCOS) هي أكثر اعتلالات الغدد الصماء انتشارًا والتي تصيب الإناث في سن الإنجاب؛ حيث يبدو أن هرمون البرولاكتين (PRL) يؤثر بشكل كبير على مسار المرض. ونظرًا لندرة الدراسات التي تبحث في الارتباط المحتمل بين تعبير *MIR4760* في سياق POCS وفرط برولاكتين الدم، فإن الدراسة الحالية تهدف إلى تقييم تأثيرالاختلافات المحتملة في تعبير جينات *PRL* و*MIR4760* في مريضات فرط برولاكتين الدم وPOCS. تضمنت الدراسة 75 أنثى (تتراوح أعمارهن بين 14 و40 عامًا) بما في ذلك (بما في ذلك 25 مريضة تم تشخيصهن بمتلازمة تكيس المبايض، و25 مريضة بفرط برولاكتين الدم، و25 انثى سليمة في مجموعة السيطرة). جمعت عينات الدم من كل من المشاركات لتقديرمستوى البرولاكتين في مصل الدم واستخلاص الحمض النووي الريبي. وتم تقييم التعبير النسبي للجين *PRL* و*MIR4760* باستخدام تقنية qPCR، في حين تم تقدير مستويات مصل البرولاكتين باستخدام تقنية الـ ELISA. أظهرت النتائج ارتفاعًا معنويا ($P = 0.001$) في التعبير عن جين *PRL* في المرضيات المصابات بفرط برولاكتين الدم ومتلازمة تكيس المبايض بالمقارنة مع مع مجموعة السيطرة (تضاعف التعبير الجيني = 3.27 و 1.27 على التوالي). بينما أظهر التعبير عن *MIR4760* انخفاضًا معنويا ($P = 0.001$) في مجموعات المرضى الذين شملتهم للدراسة (فرط برولاكتين الدم ومتلازمة تكيس المبايض) بالمقارنة مع مجموعة السيطرة. أظهرت الدراسة الحالية زيادة معنوية ($P \leq 0.001$) في مستويات البرولاكتين في مصل الدم ($P \leq 0.001$) لدى مرضى متلازمة تكيس المبايض وفرط برولاكتين الدم مقارنة بمجموعة السيطرة (36.46 ± 4.86، 40.46 ± 15.06، و19.76 ± 5، على التوالي). بشكل عام، تدعم نتائج الدراسة الحالية الافتراض الذي يربط الإفراط في التعبير عن *PRL* بمسببات متلازمة تكيس المبايض. بالإضافة إلى ذلك، يبدو أن التعبير عن مستويات *PRL* مرتبط سلبًا بحالة التعبير عن *MIR4760* في المجموعة التي خضعت للدراسة من مرضى POCS.

1.Introduction:

Polycystic ovary syndrome (PCOS) is a widespread endocrinopathy affecting females of reproductive age. Global estimates have highlighted that up to 20 % of women in the aforementioned age category suffer from this devastating health issue. It is also estimated that approximately 70% of the affected women went undiagnosed worldwide[1]. Genetically, PCOS can run in families with ethnic variations in how it manifests itself and how it affects women[2]. It could start during adolescence; however, symptoms may fluctuate over time. PCOS is marked by increased androgen levels, cysts in the ovaries, hormonal imbalances, and irregular periods. Due to irregular periods, which are usually associated with an absence of ovulation, it is difficult for women with PCOS to become pregnant; that is why PCOS is a major cause of infertility[3]. Chronic PCOS conditions are not curable; nevertheless, switching to a healthy lifestyle could improve some of the PCOS-associated symptoms, along with the use of medications and fertility treatments. PCOS is known for its connection with a diverse spectrum of long-term health problems that influence women physically and emotionally[4]. Although the rationales behind PCOS are not clearly known, a family history of this syndrome or type 2 diabetes may confer a higher risk. Additionally, PCOS is manifested by the overproduction of male hormones from the ovaries or adrenal glands, leading to the growth of cysts on the ovaries. Usually, these cysts represent small malformed egg follicles that do not progress to ovulation. Irregular menstrual periods, infertility, excess hair growth, weight gain, acne, and thickened skin patches are the main PCOS-associated symptoms [5,6]. Women with PCOS may have multiple small ovarian follicles and are at risk of metabolic syndrome, sleep apnea, and mood disorders[7].

PCOS and hyperprolactinemia (HPRL) are the two most common etiologies of anovulation in women. Thus, it is more likely that hyperprolactinemia and PCOS are independent disorders.

Altered dopamine turnover could result in hyperprolactinemia. As elevated androgen levels can occur with hyperprolactinemia, it has to be ruled out in order to establish the diagnosis of PCOS. Additionally, POCS is believed to be an etiology of hyperprolactinemia, which is a highly common hypothalamic pituitary axis endocrine disorder [8]. Interestingly, up to 37% of POCS cases suffer from hyperprolactinemia [9]. PRL elevated levels could be due to the contribution of abnormal secretion of many sex hormones, including estradiol (E2), luteinizing hormone (LH), and total testosterone [10, 11]. Several lines of evidence have highlighted the crucial role of prolactin (PRL) in PCOS manifestation. PRL levels were mostly significantly elevated at PCOS presentation and are considered part of the diagnostic components of this syndrome [12]. In this regard, it is thought that PCOS-related human prolactin (hPRL) could be attributed to hypothyroidism, pregnancy, and drugs that might increase PRL. However, more research on this topic needs to be undertaken before the association between PRL levels and PCOS is more clearly understood [13, 14].

MicroRNAs (miRNAs) are small non-coding RNAs (19-23 nucleotides) that are considered powerful transcription regulators (either promoting or suppressing/silencing). miRNAs have the potential to interfere with key cellular processes *via* reacting with a broad spectrum of target genes under physiological and pathological conditions [15]. The human genome has approximately 2500 different miRNAs that collectively govern the transcription activity of more than one-third of known genes [16]. These miRNAs are involved in the regulation of pathways and cellular processes related to normal physiological activities [17], and regulate normal cell homeostasis and hematopoiesis [18, 19]. Aberrant expression of miRNA is associated with several human diseases, including different types of solid tumors and hematological malignancies [20]. *MIR4760* maps to the 21q22.2 genomic region, and its expression dysregulation has been shown to be associated with a number of health issues [21]. *MIR4760* is also overexpressed in triple negative breast cancer (TNBC), which is an aggressive form that is accounting for up to 20% of breast malignancies [22, 23]. MIR4760 involved in the expression regulation of *SIRT2* gene, which is normally expressed in a number of reproductive system's origins [24]. Furthermore, ***MIR4760*** is up-regulated in cases with intracerebral hemorrhage [25]. It is also associated with *CFTR* gene, which is involved in cystic fibrosis, that negatively influences women's fertility by reducing ovarian reserve, which may contribute to sub-fertility [26].

To the best of our knowledge, no previous research has addressed the potential association of *MIR4760* expression in the context of POCS and hyperprolactinemia. Thus, the present pilot study is sought to assess the expression of *MIR4760* in relation to prolactin (*PRL*) in hyperprolactinemia and POCS Iraqi patients.

2. Methodology

Subjects

A total of 75 females (age range 14-40 years) were investigated in the present study, including 25 patients diagnosed with POCS, 25 patients with hyperprolactinemia, and 25 apparently healthy controls. Approximately three ml of whole blood was collected from each of the assessed participants for prolactin serum estimation and RNA extraction. Subjects' recruiting was done at Kamal Al- Samaraea Hospital during the period from 2023 to 224. The present study was approved by the Scientific and Ethical Committee of the Iraqi Center for Cancer and Medical Genetics Research, Mustansiriyah University, Baghdad, Iraq (Ref. No. 358-23/2/2024).

RNA extraction, concentration, and purity measurement

Total RNA was extracted directly from the whole blood sample using Easy Pure® Blood RNA Kit (cat no. ER401) (TransGen Biotech Company, China) according to the protocol

provided by the manufacturer. The Thermo Fisher Scientific (USA) NanoDrop was used to estimate the extracted RNA purity and its concentration in the collected samples, to ensure their suitability for further assessment by RT-qPCR. The range of concentration of RNA in the samples was 60-140 ng/µl. The purity was measured at two wavelengths with an A260/A280 ratio, and the absorbance of the samples was read at approximately 2.0, suggesting that the purity of the RNA samples.

Synthesis of cDNA

The protocol in One-Step gDNA Removal and cDNA Synthesis Super Mix was EasyScript® was utilized for the synthesis of cDNA. The components needed for cDNA synthesis were reaction mix, random primer, attached oligo dT, genomic DNA eliminator, RNase-free water, E-mix reverse transcriptase, and the final amount of total RNA was added according to the manufacturer's instructions. Three consecutive thermal cycler steps were adapted for this conversion, including 25℃ for 10 min, 42℃ for 15 min, and finally inactivating the enzyme at 85℃ for 5 sec.

Gene expression analysis (primers sequence, qPCR reaction components, and conditions)

For *PRL* and *MIR4760* relative gene expression evaluation, primer sets were designed for the current study, and the specific sequences were synthesized by Alpha DNA Company-Canada, in lyophilized form. The forward primer sequence for the target gene (*PRL*) is `5-ACTTCTTCCCTTGCCACCCC-3`, while its reverse sequence is `5-CCTCCGGGGCTTCTTGCATA-3`. Forward and reverse primers' sequences for the calibrator's (*GAPDH*) are `5-GAAATCCCATCACCATCTTCCAGG-3` and `5-GAGCCCCAGCCTTCTCCATG-3`, respectively. With respect to the *MIR4760* expression, the forward and reverse primers' sequences are `5-TTTAGATTGAACATGAAGTTAG-3` and `5-GCGAGCACAGAATTAATACGAC-3`, respectively, were used to simplify the sequence of interest, in addition to the adaptation of universal miRNA (Forward-`5-CAGGTCCAGTTTTTTTTTTTTTTVN-3`) and miR-universal (Reverse-`5-GCGAGCACAGAATTAATACGAC-3`) primers as a calibrator for the relative miRNA expression estimation.

According to the manufacturer's instructions, the procedure was done in a final reaction volume of 20 µl. The reaction components included 10 µl of master mix SYBR Green, 3µl of cDNA, 1µl of forward primer, 1µl of reverse primer, and 5µl nuclease-free water. The qPCR reaction conditions included the initial denaturation stage (94℃/60 sec). Forty cycles with 94℃/5 sec for the denaturation, 58℃/15 sec annealing, 72℃/20 sec for the extension step. This was followed by one dissociation cycle at 95℃.

Prolactin serum level estimation

Elabscience® Human PRL (Prolactin) ELISA Kit was used to estimate the serum level of prolactin. Following the manufacturer's instructions, the investigated samples and the provided kit's standards are added to ELISA microplate wells coated with a specific antibody. This was followed by the addition of biotinylated detection antibody specific for Human PRL and HRP conjugate. Following the incubation, any unconjugated components were washed away. Upon the addition of substrate solution, only wells containing Human PRL, biotinylated detection antibody and Avidin-HRP conjugate looked blue. The reaction then terminated through the addition of the stop solution, which turns the color yellow. Ultimately, the optical density (OD) was measured via an adapted spectrophotometer at 450 ± 2 nm wavelength. The OD value is proportional to the concentration of Human PRL. The standard curve was adapted to estimate Human PRL in the samples' concentration.

Statistical Analysis

The expression fold change of the genes of interest is calculated based on the 2-ΔΔCq (Livak) method [27]. The Statistical Analysis System- SAS (2018) program was utilized to distinguish the effect of the studied parameters between the different groups. T-Test was used to assess the significant differences between the levels' mean. Pearson correlation coefficients were calculated using GraphPad Prism version 8 to evaluate the relationship between assessed parameters. A cut-off of <0.05 was considered for significant differences.

3. Results

PRL gene expression has the potential to distinguish PCOS and hyperprolactinemia from healthy controls

This study examined the expression status of *PRL* gene and *MIR4760* to elucidate their association with PCOS and hyperprolactinemia. Gene expression analysis results have demonstrated significant elevation (P=0.001) in the expression of *PRL* gene in patients with hyperprolactinemia and PCOS compared to healthy controls (Table1). The expression level of *PRL* gene showed to retain a discrimination potential between hyperprolactinemia and PCOS patients from healthy controls (expression fold =3.27 and 1.27, respectively, Figure 1). Such findings could be utilized in the design of management approaches for both hyperprolactinemia and PCOS based on the differential levels of *PRL* expression.

Table1. *PRL* relative gene expression fold change in the investigated cases with PCOS and hyperprolactemia in comparison to healthy controls

Groups Studied	Mean Ct (PLR)	Mean Ct (GAPDH)	ΔCt	2^{-}ΔCt	Fold change	P value (t-Test)
PCOS	19.01	19.35	-0.33	1.26	1.72	
Hyperprolactemia	18.26	19.51	-1.26	2.39	3.27	0.001
Healthy controls	19.21	18.76	0.45	0.73	1	

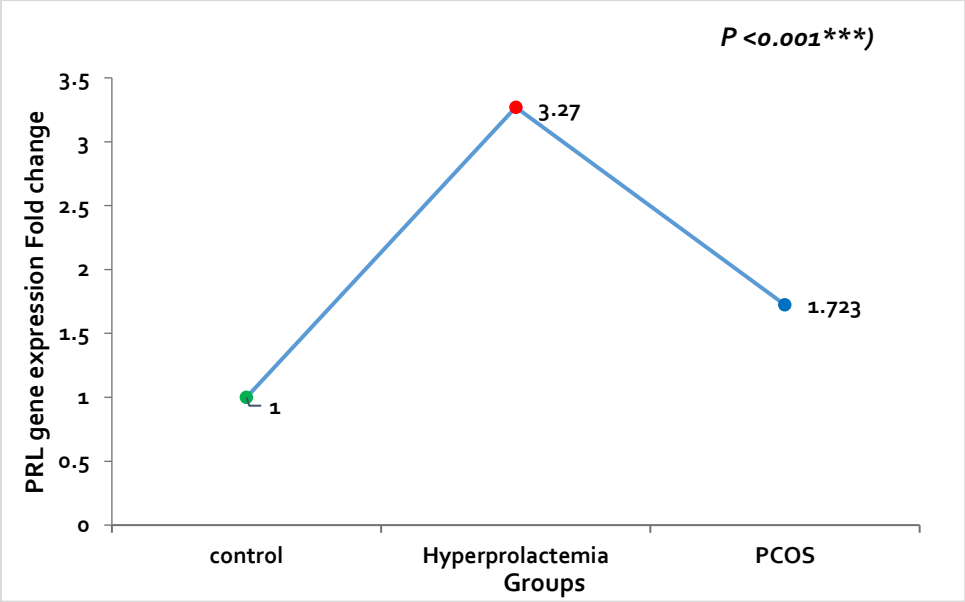

Figure1. Folds change in the *PRL* gene expression among hyperprolactinemia, PCOS, and healthy controls

MIR4760 is downregulated in both hyperprolactinemia and PCOS cases

The findings of the present study have highlighted the upregulation *PLR* gene expression in both hyperprolactinemia and PCOS cases. However, this is not the case with respect to the

expression status of *MIR4760* where its expression was shown to be reduced significantly (P=0.001) in the investigated patients groups (hyperprolactinemia and PCOS) in comparison to healthy controls (Table 2).

Table 2. *MIR4760* relative gene expression fold change in the investigated cases with PCOS and hyperprolactinemia in comparison to healthy controls

Groups Studied	Mean Ct (*MIR4760*)	Mean Ct (GAPDH)	ΔCt	$2^{-}\Delta Ct$	Fold change	P value t-Test
PCOS	23.25	17.26	5.99	0.016	0.69	
Hyperprolactinemia	23.21	17.40	5.81	0.018	0.78	0.001
Healthy controls	22.80	17.35	5.45	0.023	1	

Although both PCOS and hyperprolactinemia patients exhibited downregulation of *MIR4760* levels, PCOS cases seem to have relatively lower mean expression level of *MIR4760* , by approximately 12%, than those with hyperprolactinemia (Figure 2). This lower expression of the transcription regulator (*MIR4760)* might affect the transcription activity of genes and pathways implicated in regulating ovarian estrogen biosynthesis by amplifying the FSH-stimulated signal via the nuclear soluble adenylyl cyclase.

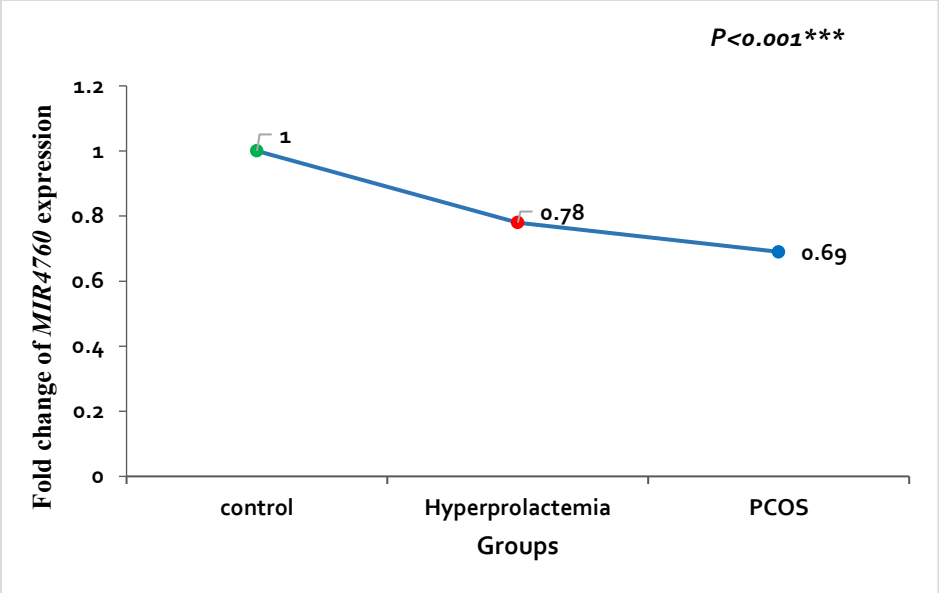

Figure 2: Fold change of *MIR4760* expression in the different investigated groups (Healthy controls, hyperprolactinemia, and PCOS)

The results of the present study exhibited a significant increase (P≤0.001) in the serum prolactin level in the PCOS and hyperprolactemia patients in comparison to that of the healthy controls (Figure 3). Interestingly, serum prolactin mean level was approximately two folds higher than healthy controls (40.46±15.06 *vs.* 19.76±5, respectively). This is quite consistent with the findings obtained through the analysis of *PLC* gene expression in the present study. Similarly, serum prolactin mean levels were significantly elevated in PCOS patients compared to healthy controls (36.46±4.86 *vs.* 19.76±5, respectively). However, although hyperprolactemia patients have relatively higher serum prolactin mean levels than those with PCOS (40.46±15.06 *vs.* 36.46±4.86, respectively), these differences do not reach a significant level (P>0.05).

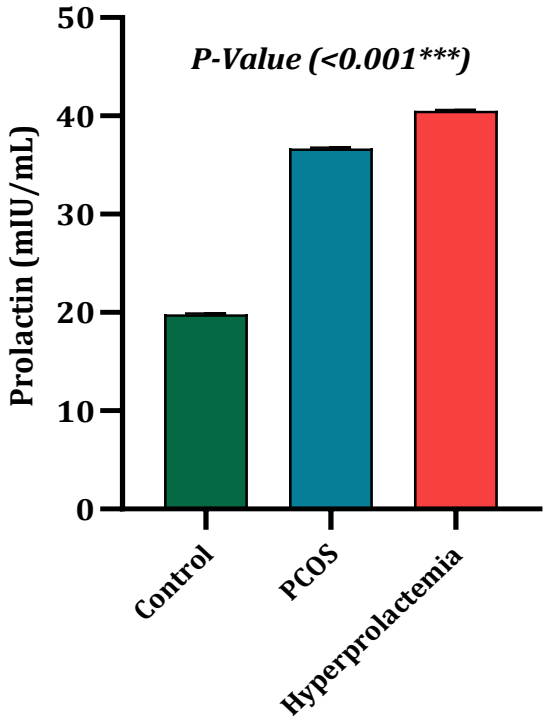

Figure 3: Prolactin serum levels among the studied PCOS and hyperprolactinemia in comparison to healthy controls.

Correlation between PLR and miRNA expression (with other studied parameters) in POCS and hyperprolactinemia cases

Notably, the expression levels of *PRL* were negatively correlated with those of *MIR4760* expression (r=-0.8, P= 0.001, Figure 4). Accordingly, downregulation of *MIR4760* expression seems to confer a selective advantage for *PRL* overexpression, contributing to POCS pathogenesis. However, the other analyzed corrections were not statistically significant.

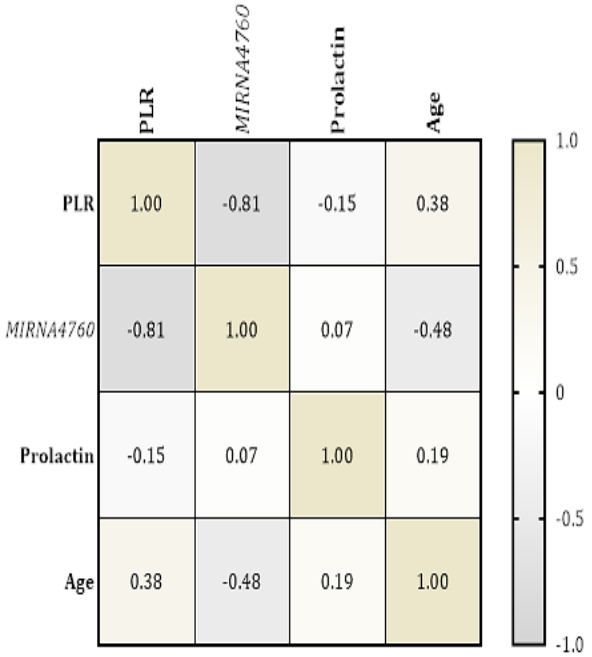

Figure 4: Correlation between the investigated parameters in the POCS group With respect to the hyperprolactinemia, age showed to be negatively correlated with *MIR4760* expression (r=-0.66, P=0.019, Figure 5). This suggests that the very low expression levels of *MIR4760 are* associated with increased hyperprolactinemia patients' age.

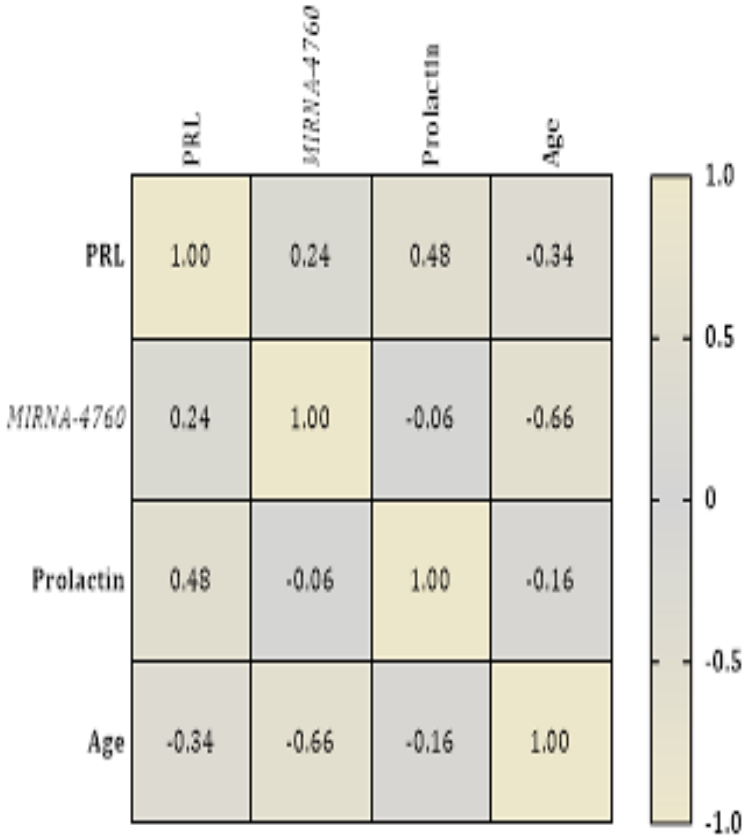

Figure 5: Correlation between the assessed parameters in the hyperprolactemia group, where *MIR4760* expression negatively correlated with age (P=0.019)

PRL and MIR4760 expression pattern in POCS and hyperprolactinemia in relation to patients age groups

Detailed analysis of the expression levels of *PRL* and *MIR4760* showed a distinct pattern in POCS and hyperprolactinemia patients, who stratified into two different age groups (14-25 years and 26- 35 years). The results illustrated in Figure 6 showed significantly lower ($P< 0.05$) expression levels of *PRL* in younger POCS patients (14-25 years) than those categorized with the older age-group (Expression fold change: 1.246 *vs.* 2.145, respectively, Figure 1). However, this is not the case with respect to the *MIR4760* expression status, where its levels were significantly higher ($P< 0.05$) in younger-age POCS patients compared to those aged older (0.423 vs. 0.236, respectively, Figure 2). These findings suggest that continuous reduction of *MIR4760* expression over age might be needed for PCOS development among patients from different age groups. While the increase of *PRL* expression seems to be required for PCOS progression.

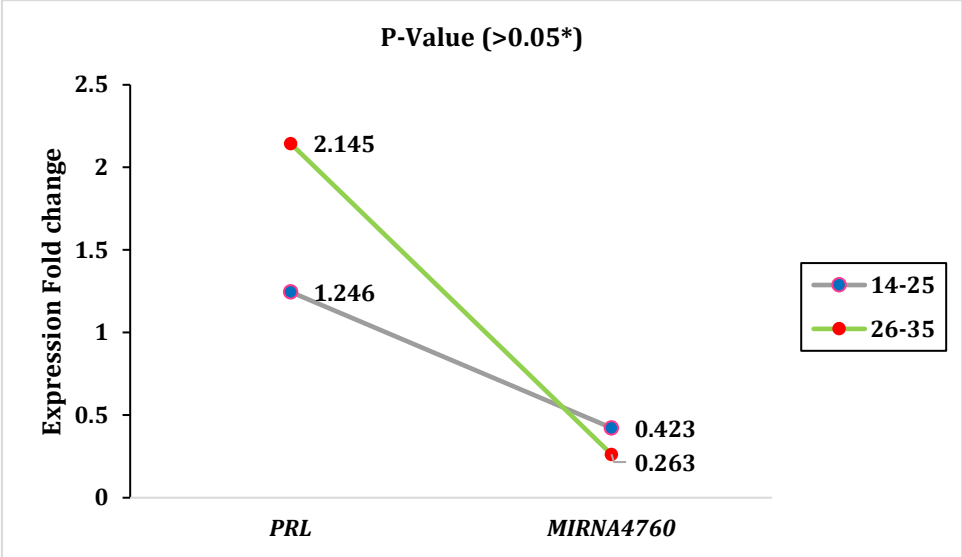

Figure 6: The expression levels of *PRL* and *MIR4760 in* POCS patients according to their age status

In respect to expression patterns of *PRL* and *MIR4760 in* hyperprolactinemia according to patients age groups (25-35 years and 26-45 years), Younger patients group showed to have relatively higher expression of both *PRL* and *MIR4760* than those in the older age group (3.662 vs. 2.829 and 1.06 vs. 0.4931, respectively, P<0.05, Figure 7). These results indicate the potential role of increased *PRL* expression in the hyperprolactinemia phenotype, which mainly relies on *PRL* overexpression. While the manifestation of hyperprolactinemia phenotype seems to be dependent on *MIR4760* reduced expression in older patients.

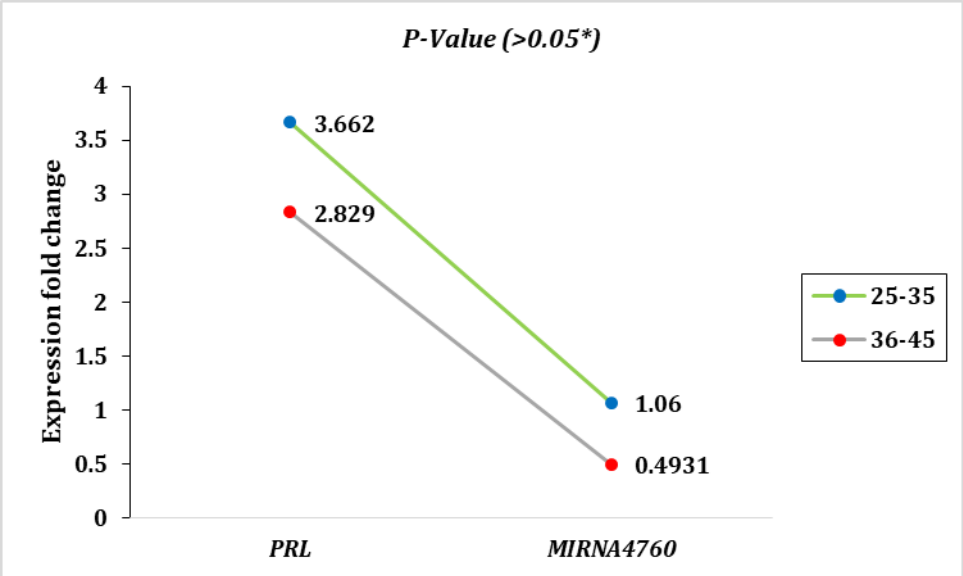

Figure 7: The expression levels of *PRL* and *MIR4760 in* hyperprolactinemia patients based on their age status

4. Discussion

The debate about the involvement of aberrant gene expression of key cellular components and regulatory elements, e.g. miRNAs, in the PCOS pathogenesis has gained fresh prominence. This is mainly due to the potential impact of molecular genetic events in triggering such health issues and the inconsistency of the reported findings. Notably, normal activity of both *PRL* and *miRNAs* is required for healthy reproduction system functions[28, 29].

In the present study, women with PCOS and hyperprolactinemia exhibited dysregulated *PRL* gene expression, where its levels were much higher than those who did not have the disease. Higher *PRL* levels could negatively affect ovarian functions and trigger serous infertility-associated health issues. This is mainly because this hormone binds to specific prolactin receptors of target genes that regulate proliferation and cell survival [30]. PRL receptors are widely present in different organs and tissues, including the mammary gland, genital tract, central nervous system, adrenal cortex, bone, pancreas, and lymph glands [31]. Pituitary prolactin receptors are probably involved in the auto-regulation of the hormone secretion. Although a number of studies have highlighted the association of elevated *PRL* levels with PCOS, the precise mechanism of its effect on the disease course remains to be elucidated. More research is needed to better understand when PRL level modulations could be utilized for PCOS management to improve patients' outcomes. The aberrant level of PRL could constitute the foundation for personalized treatment of both PCOS and hyperprolactinemia patients [10, 32]. However, with a small sample size, caution must be applied, as the findings might not be transferable to the clinical setting, and large-scale studies are required to determine exactly how PRL could be used for patients' stratification.

On the other hand, *MIR4760* expression was reduced significantly (P=0.001) in both PCOS and hyperprolactinemia patients in comparison to healthy controls. This potentially negatively affects *PRL* expression in the investigated groups of patients. If such an association could be confirmed by larger cohort studies, this may open a new venue for both PCOS and hyperprolactinemia management. Considering the transcription activity regulation of *MIR4760* [33-35], functional studies could be conducted to investigate the impact of inducing *MIR4760* re-/ overexpression on PCOS and hyperprolactinemia pathogenesis. In this regard, targeted therapies could be designed for the assessment of *MIR4760* expression manipulations on the levels of *PRL* expression in the context of PCOS, hyperprolactinemia, and other related health issues. Additionally, epigenetic modifications (including DNA methylation and histone modifications) might have the potential to alter the expression of key cellular components[36], including *PRL* and *MIR4760* expression in the context of PCOS *MIR4760* .

5. Conclusion

Overall, the data reported here appear to support the assumption that links *PRL* overexpression to PCOS pathogenicity. Additionally, the expression of *PRL* levels has seemed to be negatively associated with *MIR4760* expression status in the investigated groups of POCS and hyperprolactinemia patients. Large-scale studies are recommended to elucidate the therapeutic potential of such an association in the context of both POCS and hyperprolactinemia patients.

Conflict of interest: The authors state that they have absolutely no conflicts of interest.

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