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Exploring the diagnostic potential of micro-RNA-320 and anti-Müllerian hormone in women with polycystic ovary syndrome – a case-control study

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ABSTRACT

Introduction and aim. Polycystic ovary syndrome (PCOS) is a multifactorial endocrine disorder characterized by hormonal imbalance, insulin resistance, and reproductive dysfunction. Due to its heterogeneous clinical presentation, diagnosis remains challenging. MicroRNA-320a-3p and anti-Müllerian hormone (AMH) have recently emerged as promising biomarkers. This study aimed to assess their diagnostic potential in women with PCOS.

Material and methods. A case-control study was performed in 90 women aged 18–40 years, including 45 patients with polycystic ovary syndrome and 45 age- and body mass index-matched healthy controls. Hormonal and metabolic markers were measured using standard immunoassays, and the expression of microRNA-320a-3p was quantified using real-time polymerase chain reaction .

Results. Patients with polycystic ovary syndrome demonstrated significantly higher levels of luteinizing hormone (9.45±6.0 vs 5.09±2.2 mIU/mL), increased luteinizing hormone to follicle-stimulating hormone ratio (1.64±0.87 vs 0.76±0.3), and elevated fasting blood glucose (105.5±14.7 vs 94.3±13.5 mg/dL), all with $p<0.001$. Contrary to expectations, insulin and homeostatic model assessment for insulin resistance values were lower in the polycystic ovary syndrome group, possibly reflecting a predominance of non-obese phenotypes. AMH levels were also reduced (2.27±1.0 vs 3.34±1.1 ng/mL, $p<0.001$). Expression of microRNA-320a-3p was significantly downregulated (0.61±1.27 vs 2.81±5.03-fold, $p=0.0009$). MicroRNA-320a-3p expression correlated positively with luteinizing hormone levels and the luteinizing hormone to

follicle-stimulating hormone ratio, while AMH was associated with insulin resistance. The combined use of both markers improved diagnostic differentiation between groups .

Conclusion. MicroRNA-320a-3p and AMH show promise as diagnostic biomarkers in polycystic ovary syndrome. Their integration with traditional clinical markers may enhance diagnostic accuracy and provide deeper insight into the pathophysiological complexity of the disorder.

Keywords. anti-Müllerian hormone, insulin resistance, microRNA-320, polycystic ovary syndrome

Introduction

Polycystic ovary syndrome (PCOS) is the most common endocrine disorder that impacts approximately 5–10% of women of reproductive age with a global prevalence of about 4-20%. It is characterized by polycystic ovarian morphology, persistent anovulation, and hyperandrogenism. PCOS is a multifactorial condition with complex etiologies involving hormonal, metabolic, and genetic factors, leading to long-term complications such as infertility, cardiovascular diseases, and type 2 diabetes (T2D). Despite advances in understanding its pathophysiology, diagnosing PCOS is still difficult because of its heterogeneity in clinical and biochemical presentations. This necessitates the identification of reliable biomarkers to improve diagnostic precision and provide insights into disease mechanisms.[1,2]

Insulin resistance (IR) is a common physiological characteristic of patients with PCOS, resulting in compensatory hyperinsulinemia, which contributes to hormonal imbalances, hyperandrogenism, and infertility.[3] According to previous research, IR may be crucial to the pathophysiology of PCOS, as it affects over 75% of people with PCOS.[2]

Micro-ribonucleic acid-320 (microRNA-320) has recently emerged as a potential molecular biomarker involved in various metabolic and reproductive disorders. By regulating gene expression, microRNA-320 plays significant roles in glucose metabolism, IR, and ovarian function which are processes intricately linked with PCOS pathogenesis.[4] Similarly, the ovarian reserve marker, anti-Müllerian hormone (AMH), has gained attention for its utility in diagnosing PCOS, reflecting the severity of follicular arrest and dysfunction.[5] Furthermore, metabolic and hormonal indicators such as luteinizing hormone (LH), follicle-stimulating hormone (FSH), insulin and fasting blood glucose (FBG) play essential roles in understanding the endocrine and metabolic disturbances in PCOS. While LH and FSH ratios are traditional diagnostic markers, IR and hyperinsulinemia are metabolic hallmarks of PCOS, which significantly influence disease progression and treatment outcomes.[6,7]

To our knowledge, this is the first study to assess the combined diagnostic performance of microRNA-320a-3p and anti-AMH in patients with PCOS. Unlike many previous studies that have examined either AMH or miRNA-320a-3p individually, this research evaluates both markers together alongside conventional hormonal and metabolic parameters, providing a multi-dimensional diagnostic approach to PCOS. It also

uncovers some unexpected patterns, such as lower levels of AMH and insulin in the PCOS group, which challenge conventional understanding and highlights phenotypic heterogeneity.

Aim

This study aims to evaluate the diagnostic performance of microRNA-320a-3p and AMH in women with PCOS, in comparison with traditional hormonal and metabolic markers. By adopting a case-control design, we investigate the potential of these molecular biomarkers to improve the accuracy of PCOS diagnosis. The study further explores their association with metabolic and endocrine disturbances, with the goal of supporting their future integration into routine clinical assessment and individualized management strategies.

Material and methods

Study design/subjects

A case-control study was conducted at Bent Al-Huda Teaching Hospital in the Thi-Qar governorate of Iraq from September 2024 to December 2024. In this paper, two study groups were employed: The first group included 45 women diagnosed with PCOS according to the revised 2003 diagnostic criteria, which require the presence of 2 out of 3: oligo- or anovulation, presence of polycystic ovaries,

Clinical and/or biochemical manifestations of excess androgen, and the exclusion of other causes, include androgen-secreting tumors, congenital adrenal hyperplasia, and Cushing's syndrome.[8] The second group involved 45 individuals as healthy controls (HCs). All subjects in the study were between 18 and 40 years old.

Exclusion and inclusion criteria

The study excluded women who had recently used hormonal contraceptives, anti-androgens, or insulin sensitizers (past three months), pregnant or breastfeeding women, those with chronic diseases (diabetes, hypertension, cardiovascular diseases), a history of ovarian surgery, or inadequate sample collection. Inclusion criteria required participants to meet clinical and laboratory PCOS criteria (menstrual irregularities, hyperandrogenism, and ultrasound-confirmed polycystic ovaries), be in the 18–40 age range, possess a body mass index (BMI) ranging from 18.5 to 35, which includes a range of normal to moderately obese individuals, and do not have a history of other endocrine disorders, such as androgens secreted tumors, Cushing's syndrome, or congenital adrenal hyperplasia.

Healthy controls were age-matched (18-40 years) with the PCOS group, had regular menstrual cycles, no history of PCOS, and a BMI within the same range to minimize confounding effects. They did not have any endocrine abnormalities (thyroid disorder, Cushing's syndrome, or congenital adrenal hyperplasia), no history of hormonal medication use, normal fertility status, and a healthy lifestyle.

Sample collection

Five milliliters of peripheral blood were collected from each patient after fasting for 8–12 hours through venipuncture. After collection, the samples were left in gel vacuum tubes and allowed to coagulate at room temperature, and then centrifuged at 3,600 × g for 10 minutes for serum separation. Each sample of serum was divided into two parts, one of which was stored at -80°C until it was time for subsequent analysis, and the other mixed with Trizol (500 µL of serum + 500 µL of Trizol) for miRNA extraction.

Biochemical assays

Serum AMH was measured in nanograms (ng) per mL using an enzyme-linked immunosorbent assay (ELISA) method using an AMH ELISA kit (BT LAB, China). Serum levels of FSH and LH were measured using the AFIAS-10 instrument, which is an automated immunoassay analyzer designed to perform up to 10 parallel tests for various parameters using the AFAIS LH kit and the AFAIS FSH kit, respectively (Boditech, Korea). The test procedure was conducted in accordance with the manufacturer's guidelines. The results are reported in milli-international units (mIU)/mL. Serum insulin was measured in micro-international units (µIU)/mL using an ELISA method with a human insulin ELISA kit (BT LAB, China). The serum levels of FBG were measured using the Mindray BS-230 instrument, a fully automated biochemical analyzer. It operates based on spectrophotometry and biochemical enzymatic reactions using a glucose kit (Mindray, China). According to the manufacturer's guidelines, the test procedure was conducted. The results are reported in milligram (mg)/deciliter (dL). The following equation was used to calculate the homeostatic model assessment of insulin resistance (HOMA-IR).[9]

$$\mathrm{HOMA-IR} = \text{fasting insulin}\ (\mu\mathrm{IU/mL}) \times \mathrm{FBG}\ (\mathrm{mg/dL})/405$$

Molecular detection of microRNA-320a-3p: The molecular assay was conducted in a specialist laboratory in Baghdad, Iraq, following manual guidelines. Total serum RNA was extracted using the Trizol reagent method, and complementary deoxyribonucleic acid (cDNA) was synthesized for miRNA-320a-3p and RNU43 (housekeeping gene) using specific reverse transcriptase (RT) primers (Table 1) and a Thermal Cycler. cDNA concentration and quality (5–7 ng/µL) were measured with a Quantus Fluorometer. Quantification of miRNA-320a-3p and RNU43 was performed by RT-qPCR using specific forward primers and a universal reverse primer (Table 1) on a Mic-qPCR cycler (Fig. 1 and 2).

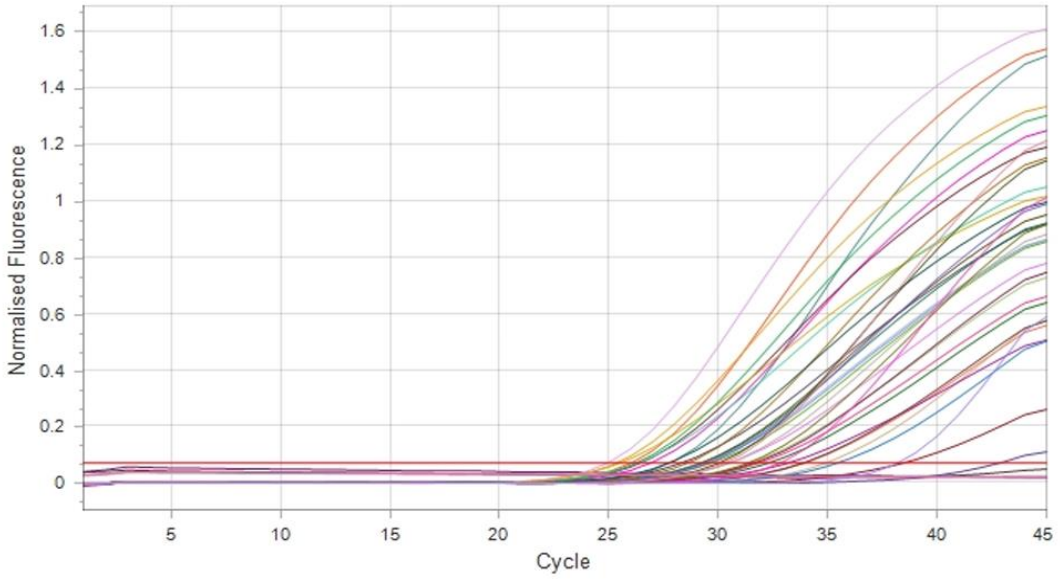

Fig. 1. The step of real-time polymerase chain reaction of microRNA-320a-3p

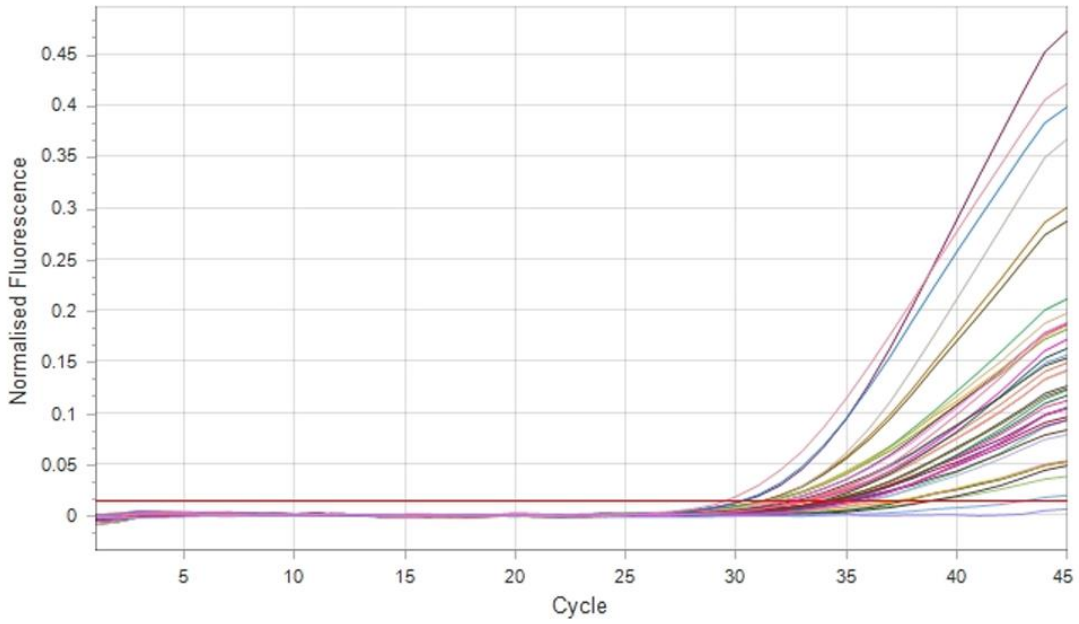

Fig. 2. The step of real-time polymerase chain reaction of RNU43

Gene expression was quantified relatively using the Pfaffl method.[10] For each sample, by using the real-time cycler software, the threshold cycle (CT) was determined. Average values were computed by running each sample twice. The expression data for the chosen genes were normalized using housekeeping genes as a reference. The ΔΔCt method was utilized for data analysis as recommended, with results presented as fold change in gene expression. The following formula was used to calculate the difference (ΔCt) between the CT

values for each target gene and the housekeeping gene for each sample.[11] The normal range for microRNA-320a-3p was determined based on data from the control group.

$$\Delta CT = CTgene - CT\ House\ Keeping\ gene$$

$$\Delta\Delta CT = \Delta CT\ Treated\ or\ Control - Average\ \Delta CT\ Control$$

$$Folding = 2^{-}\Delta\Delta CT$$

Table 1. The study primers[a]

Primers name	Sequence (′5 ——————————3′)	Company	Country	Temperature
microRNA-320a-3pRT*	GTTGGCTCTGGTGCAGGGTCCGAGGT ATTCG CACCAGAGCCAACTTGCCC	Macrogen	South Korea	55 ºC
microRNA-320a-3pF*	GGGAAAAGCTGGGTTGAGA			
RNU43-RT*	GTTGGCTCTGGTGCAGGGTCCGAGGT ATTCG CACCAGAGCCAACAATCAG			
RNU43-F*	GTGAACTTATTGACGGGCG			
Universal reverse*	GTGCAGGGTCCGAGGT			

[a] * – from the National Center for Biotechnology Information Gen-Bank database the miRNA gene cDNA sequences were obtained. Primers for real-time qPCR were designed with a melting temperature range from 58°C to 62°C using primer Premier 3 software, the length of the primers ranging from 18 to 23 nucleotides, and the length of the PCR amplicon ranged between 75 and 150 base pairs

Statistical analysis

Data analysis and visualization were conducted using the statistical package for social science (SPSS) (version 22, IBM, Armonk, NY, USA). The descriptive statistics involved means, frequencies, and standard deviations (SD). Categorical data were compared using the chi-square test. The statistical significance of variations in the means of continuous variables with a normal distribution was assessed using an independent t-test, which was a parametric test. For variables that were not normally distributed, the Mann–Whitney U test, a non-parametric test, was employed. To evaluate the strength and direction of associations between variables, the Spearman correlation coefficient, a non-parametric measure, was used. p-values below 0.05 were regarded as statistically significant.

Results

In the current research, the comparison of insulin levels between the PCOS group and HCs revealed significant differences ($p<0.001$). The mean concentration of insulin was lower in PCOS patients (6.15±2.87 μIU/mL) than in HCs (7.56±2.45 μIU/mL). Regarding HOMA-IR, the mean HOMA-IR

was significantly lower in the group of PCOS patients (1.57±0.66) than the HCs (1.75±0.58) (p=0.0227). While IR is typically more pronounced in PCOS, the current results may reflect a heterogeneous PCOS cohort or the influence of confounding factors such as BMI, physical activity levels, or dietary habits, which were not under the control of this analysis (Table 2).

Fasting blood sugar levels demonstrated a statistically significant elevation in the PCOS group (105.46±14.7 mg/dL) compared to the HCs group (94.3±13.51 mg/dL), with a p-value of 0.0003, supporting the presence of impaired glucose metabolism in PCOS. Luteinizing hormone levels were markedly higher in the group with PCOS (9.45±6.0 mIU/mL) than the controls (5.09±2.2 mIU/mL), having a difference that is statistically highly significant (p<0.001), consistent with the well-established association between elevated LH and the pathophysiology of PCOS. In contrast, levels of FSH were lower in the PCOS group (5.8±2.13 mIU/mL) than in controls (6.89±2.02 mIU/mL), and the mean difference reached statistical significance (p=0.0145) (Table 2).

Significantly, the LH/FSH ratio was higher in the PCOS group (1.64±0.87) than in the HCs (0.76±0.3), with p-values <0.001, reinforcing the LH/FSH ratio diagnostic value in distinguishing PCOS. Regarding AMH, unexpectedly, the concentration of mean was lower in the PCOS group (2.27±1 ng/mL) in comparison with the HCs (3.34±1.12 ng/mL), with significant differences (p<0.001). This finding contrasts with the traditional view of elevated AMH in PCOS and may reflect phenotypic heterogeneity within the PCOS population or methodological differences, suggesting the need for further investigation (Table 2).

The expression levels of microRNA-320a-3p demonstrated significant differentiation between groups. The PCOS group exhibited a markedly lower mean expression (0.61±1.27 folds) compared to HCs (2.81±5.03 folds) (p=0.0009). This suggests that microRNA-320a-3p could contribute to the pathophysiology of PCOS and may be used as a molecular biomarker to identify diseases or monitor progression (Table 2).

Table 2. Biochemical and molecular parameters comparison between PCOS patients and healthy controls groups (Mann-Whitney U test and independent t-test)

Parameters	PCOS Group	Healthy Control	p
	Mean±SD	Mean±SD	
Insulin (µIU/mL)	6.15±2.87	7.56±2.45	<0.001
HOMA-IR	1.57±0.66	1.75±0.58	0.0227
FBG (mg/dL)	105.46±14.7	94.3±13.51	0.0003
LH (mIU/mL)	9.45±6.0	5.09±2.2	<0.001
FSH (mIU/mL)	5.8±2.13	6.89±2.02	0.0145
LH/FSH Ratio	1.64±0.87	0.76±0.3	<0.001
AMH (ng/mL)	2.27±1	3.34±1.12	<0.001
microRNA-320a-3p Folds	0.61±1.27	2.81±5.03	0.0009

Table 3 presents the distribution of IR (measured by HOMA-IR) in relation to microRNA-320a-3p expression levels among PCOS women and HCs. The correlation between HOMA-IR and expression levels of microRNA-320a-3p was evaluated in both the PCOS and HCs groups. Among individuals with PCOS, a statistically significant positive relationship was noted between HOMA-IR values and microRNA-320a-3p expression levels ($r=0.51$, $p<0.001$). Specifically, participants with high microRNA-320a-3p expression demonstrated a markedly higher mean HOMA-IR (1.59) compared to those with low microRNA-320a-3p expression, who exhibited a mean HOMA-IR of 1.44. Notably, 2/2 (100%) of PCOS participants with high microRNA-320a-3p expression exhibited normal HOMA-IR compared to participants with low microRNA-320a-3p expression, 13/15 (86%), yet the subgroup with high microRNA-320a-3p expression still showed a disproportionately elevated HOMA-IR level, pointing to a possible role of this microRNA in IR among patients with PCOS. In contrast, although a trend toward increased HOMA-IR level was noted in HCs individuals with high microRNA-320a-3p expression (mean=1.68) relative to those with low microRNA-320a-3p expression (mean=1.63), the correlation failed to achieve statistical significance ($r=0.24$, $p=0.10$). This may reflect physiological variability in insulin sensitivity among healthy individuals or the absence of underlying endocrine-metabolic disturbances characteristic of PCOS.

Table 3. Correlation of HOMA-IR and microRNA-320a-3p expression levels in PCOS and HCs (Spearman's correlation analysis, Mann-Whitney U test, independent t-test, and Chi-square test)

Biomarkers			HOMA-IR						r	p
			Normal (<2)		High (≥2)		Total			
			FR (%)	Mean	FR (%)	Mean	FR (%)	Mean		
microRNA-320a-3p Folds	PCOS	Low (n=15)	13 (86.7)	1.27	2 (13.3)	2.56	15 (100)	1.44	0.51	0.00
		Normal (n=3)	3 (100)	1.55	0 (0)	0	3 (100)	1.55		
		High (n=2)	2 (100)	1.59	0 (0)	0	2 (100)	1.59		
		Total (n=20)	18 (90)	1.35	2 (10)	2.56	20 (100)	1.47		
	HCs	Low (n=7)	7 (100)	1.63	0 (0)	0	7 (100)	1.63	0.24	0.10
		Normal (n=7)	4 (57.1)	1.71	3 (42.9)	2.87	7 (100)	2.21		
		High (n=6)	4 (66.7)	1.45	2 (33.3)	2.14	6 (100)	1.68		
		Total (n=20)	15 (75)	1.6	5 (25)	2.58	20 (100)	1.85		

Table 4 presents the association between levels of HOMA-IR and AMH in both the PCOS and HCs groups. Among the PCOS group, AMH levels demonstrated a positive correlation with HOMA-IR values, revealing a significant correlation coefficient statistically ($r=0.38$, $p=0.01$). Participants with high AMH exhibited a substantially higher mean HOMA-IR concentration (3.44) than those with normal AMH, with a mean

HOMA-IR of 1.47. Although all PCOS individuals with high AMH levels (100%) fell within the high HOMA-IR range compared to those with normal AMH levels (11.9%), the marked increase in AMH among those with elevated IR highlights a potential link between ovarian reserve markers and metabolic dysfunction in PCOS. Similarly, in the HCs group, a significant positive correlation between AMH and HOMA-IR levels was observed (r=0.36, p=0.02). The mean HOMA-IR level among HC individuals with high AMH was 2.28, compared to 1.6 in those with a normal AMH level. Notably, nearly half (45.5%) of the HC participants in the high AMH subgroup demonstrated elevated HOMA-IR, suggesting that even in the absence of clinical PCOS, higher AMH levels may be linked to underlying IR.

Table 4. Correlation of HOMA-IR and AMH levels in PCOS and HCs (Spearman's correlation analysis, Mann-Whitney U test, independent t-test, and Chi-square test)

Biomarkers			HOMA-IR						r	p
			Normal (<2)		High (≥2)		Total			
			FR (%)	Mean	FR (%)	Mean	FR (%)	Mean		
AMH (ng/mL)	PCOS Group	Low (n=1)	0 (0)	0	1 (100)	2.24	1 (100)	2.24	0.38	0.01
		Normal (n=42)	37 (88.1)	1.33	5 (11.9)	2.52	42 (100)	1.47		
		High (n=2)	0 (0)	0	2 (100)	3.44	2 (100)	3.44		
		Total (n=45)	37 (82.2)	1.33	8 (17.8)	2.71	45 (100)	1.57		
	HCs	Low (n=0)	0 (0)	0	0 (0)	0	0 (0)	0	0.36	0.02
		Normal (n=34)	30 (88.2)	1.52	4 (11.8)	2.19	34 (100)	1.6		
		High (n=11)	6 (54.5)	1.57	5 (45.5)	2.98	11 (100)	2.28		
		Total (n=45)	36 (80)	1.53	9 (20)	2.63	45 (100)	1.75		

Table 5 explores the relationship between microRNA-320a-3p expression levels and several metabolic and reproductive parameters in both PCOS (G1) and HC (G2) groups. Among patients with PCOS, a significant association was found between the expression of microRNA-320a-3p and the LH/FSH ratio (p=0.007), as well as serum LH levels (p=0.029). Specifically, those with high miRNA expression had a notably elevated LH/FSH ratio (3.16±2.31) and LH concentration (18.80±13.73 mIU/mL), suggesting a potential role of this miRNA in modulating gonadotropin imbalance characteristic of PCOS. Conversely, there were no statistically significant correlations found between the levels of microRNA-320a-3p and FBG, insulin, FSH, or AMH, indicating that the miRNA may not be primarily involved in glucose metabolism. No significant relationships were observed between level of microRNA-320a-3p and any of the tested parameters in the control group. These results support a disease-specific regulatory function of microRNA-320a-3p in reproductive hormone imbalance in PCOS but not in healthy individuals.

Table 5. Association between microRNA-320a-3p expression, hormonal and metabolic parameters in patients with PCOS and healthy control groups (Mann-Whitney U test, independent t-test)*

Variables/Groups (Mean±SD)		microRNA-320a-3p Folds			p
		Low (<1)	Normal (1-2)	High (>2)	
FBS (mg/dL)	G1	100.46±13.62	126.20±27.50	106.50±1.70	0.523
	G2	98.43±8.07	98.49±13.60	92.87±22	0.713
LH/FSH ratio	G1	2.23±0.53	2.20±0.49	3.16±2.31	0.007
	G2	0.70±0.13	0.54±0.18	0.65±0.19	0.728
Insulin (µIU/mL)	G1	5.89±2.61	5.23±1.99	6.06±1.56	0.928
	G2	6.78±1.08	9.20±3.47	7.27±2.09	0.742
LH (mIU/mL)	G1	13.50±4.84	14.89±3.36	18.80±13.73	0.029
	G2	5.25±1.06	3.88±1.46	4.53±1.51	0.540
FSH (mIU/mL)	G1	6.16±1.87	6.95±2.05	5.97±0.02	0.877
	G2	7.69±1.86	7.28±2.03	7.01±1.00	0.615
AMH (ng/mL)	G1	2.16±1.15	2.39±0.38	2.18±0.88	0.982
	G2	3.12±0.68	3.35±1.96	3.28±0.70	0.791

* G1 – polycystic ovary syndrome patient group, G2 – healthy control group

Table 6 evaluates the association between various biochemical and hormonal parameters and serum levels of AMH in both study groups. Among the patients with PCOS, levels of AMH were significantly associated with FBG (p=0.006), where individuals with high AMH levels exhibited the lowest FBG (88.8±3.82 mg/dL) compared to those with normal (105.98±14.65 mg/dL) and low AMH levels (117 mg/dL). This negative correlation between FBG and AMH in PCOS-afflicted women suggests a possible protective role of AMH against hyperglycemia. Additionally, the correlation between insulin concentration and AMH levels was found to be statistically significant (p=0.010), with the highest insulin values noted in those with high AMH levels (15.7±1.4 µIU/mL), indicating potential compensatory hyperinsulinemia or a complex interaction between ovarian reserve markers and insulin signaling pathways. Although trends were observable, no significant associations were noted with LH,

LH/FSH ratio, or FSH (p>0.05). None of the associations between AMH and the studied parameters reached statistical significance in the HCs group, which may reflect a more stable metabolic and endocrine environment in non-PCOS individuals. Taken together, these findings emphasize the dual endocrine and metabolic relevance of AMH in PCOS pathophysiology and support its candidacy as a multidimensional biomarker.

Table 6. Association between levels of AMH, hormonal and metabolic parameters in patients with PCOS and healthy control groups (Mann-Whitney U test, independent t-test)

Variables/Groups (Mean±SD)		AMH (ng/mL)			p
		Low (<1)	Normal (1-4)	High (>4)	
FBS (mg/dL)	G1	117±0.0	105.98±14.65	88.8±3.82	0.006
	G2	0	95.7±14.8	89.94±7.47	0.590
LH/FSH ratio	G1	0.92±0	1.65±0.89	1.84±0.130	0.561
	G2	0	0.74±0.296	0.85±0.31	0.680
Insulin (µIU/mL)	G1	7.76±0	5.66±2.01	15.7±1.4	0.010
	G2	0	6.81±1.43	9.88±3.42	0.067
LH (mIU/mL)	G1	1.69±0	9.71±6.07	7.95±1.90	0.581
	G2	0	5.22±2.28	4.69±1.96	0.776
FSH (mIU/mL)	G1	1.84±0	5.97±2.08	4.37±1.34	0.082
	G2	0	7.24±1.87	5.83±2.18	0.444

Discussion

This study aimed to evaluate and compare key molecular, biochemical, and hormonal parameters in PCOS patients diagnosis and matched HCs. The results highlight multiple significant differences, reflecting the complex and heterogeneous nature of PCOS, which encompasses metabolic dysfunction, hormonal imbalances, and emerging molecular signatures such as altered microRNA expression. Insulin resistance is recognized as a key pathogenic characteristic of PCOS, often associated with hyperinsulinemia and compensatory metabolic changes. Interestingly, our research showed that the mean serum levels of insulin were significantly lower in the group of PCOS patients than in HCs. This finding diverges from most studies reporting elevated insulin levels in PCOS due to systemic IR.[12] One explanation could be the inclusion of lean or non-obese PCOS phenotypes, which are typically less IR.[13] Additionally, the study did not control for confounders such as BMI, physical activity, or dietary habits, which significantly influence insulin dynamics.[14]

Although the frequency of IR (as identified by elevation of HOMA-IR) showed no significant difference between groups, the mean of HOMA-IR was surprisingly lower in PCOS participants than in controls. This contradicts previous studies indicating higher HOMA-IR in PCOS women.[15] However, as Daan et al.[16] and others have observed, the expression of IR in PCOS can vary based on age, BMI, and ethnic background. It is also possible that HOMA-IR underrepresents tissue-specific IR (e.g., ovarian or hepatic), which could still be present in PCOS despite lower systemic levels.[17]

Our findings showed significantly elevated FBG levels in the PCOS group, consistent with prior research documenting impaired glucose tolerance and increased risk for T2D in PCOS women.[18] Moran et al.[19]

demonstrated that PCOS increases the risk of metabolic syndrome and glucose dysregulation even in normal-weight individuals. These results underscore the importance of metabolic monitoring in all PCOS phenotypes, regardless of body habitus.

The elevated LH levels and LH/FSH ratios observed in our PCOS cohort reflect well-established features of the disorder. In PCOS, increased gonadotropin-releasing hormone (GnRH) pulse frequency leads to preferential LH secretion, enhancing androgen production, and contributing to ovulatory dysfunction.[20] Our finding that over 50% of PCOS patients exhibited high LH levels and a significantly higher LH/FSH ratio than controls aligns with studies by Azziz et al.[21] and Rosenfield and Ehrmann,[22] further validating these parameters as useful diagnostic indicators. In contrast, FSH levels were modestly but significantly lower in PCOS patients. Although the frequency distribution across FSH categories was not significant, the mean value difference may reflect disrupted granulosa cell function and impaired follicular development, as previously described.[23]

Unexpectedly, PCOS patients had lower mean AMH levels than controls, contradicting the conventional understanding of AMH elevation in PCOS due to an increase in small antral follicles.[24] Discrepancy of this could be due to heterogeneity in PCOS phenotypes, assay differences, or diminished ovarian reserve in a subset of patients.[25] Ahmad et al.[26] reported similar variability in AMH among different PCOS subtypes, suggesting it should not be used in isolation for diagnosis. This result aligns with a study conducted by Hmood et al.,[27] who discovered that anovulatory women with polycystic ovaries had a relative lack of AMH in their transitional and primordial follicles in the ovaries. This could be an influencing factor that leads to the abnormal early follicle development seen in PCOS.

Recent research has emphasized the role of non-coding RNAs, including miRNAs, in regulating PCOS-related pathways. Our study found a markedly reduced level of microRNA-320a-3p expression in the PCOS patient group compared to controls. This is consistent with findings by Chen et al.,[28] who reported that microRNA-320a modulates insulin signaling and granulosa cell proliferation. Downregulation of this miRNA may therefore contribute to follicular arrest in PCOS.[29] These findings support the growing interest in microRNA-320a-3p as a potential biomarker for disease diagnosis or progression monitoring.

The present study provides novel insights into the association between microRNA-320a-3p expression and IR, measured using HOMA-IR, in PCOS women compared to HCs. Among the PCOS group, our findings revealed a positive significant association between HOMA-IR and microRNA-320a-3p expression, while the association was non-significant in the HCs group. These findings indicate a possible involvement of microRNA-320a-3p in the pathophysiology of IR, specifically among PCOS patients (Table 3). Our results agree with previous research that has implicated microRNA-320a in metabolic disorders, particularly in regulating signaling pathways for insulin. For example, a study by Luo et al.[30] showed that microRNA-320a expression was positively associated with markers of IR, including HOMA-IR and fasting insulin levels. Similarly, Vogt et al.[31] reported that microRNA-320a targets several key genes involved in insulin signaling,

such as insulin receptor substrate 1 (IRS1) and phosphoinositide 3-kinase (PI3K), and its overexpression was linked to impaired insulin sensitivity in ovarian granulosa cells, further supporting its mechanistic involvement in PCOS-associated IR. In contrast, other studies have reported conflicting results regarding the role of microRNA-320a in metabolic regulation. For instance, Du et al.[32] discovered that the levels of circulating microRNA-320 were downregulated in patients with T2D and speculated that its loss may contribute to IR via the dysregulation of insulin-responsive genes. This discrepancy could reflect tissue-specific or context-dependent roles of microRNA-320a, whereby its expression may differ between insulin-sensitive tissues (e.g., muscle, adipose) and the systemic circulation or ovaries in PCOS. Notably, in our HCs group, although individuals with high microRNA-320a-3p expression had slightly higher mean HOMA-IR levels, the association failed to achieve statistical significance (Table 3). This may highlight the absence of underlying endocrine or inflammatory disturbances in healthy individuals that are known to amplify miRNA dysregulation in PCOS. It is possible that microRNA-320a-3p exerts a more pronounced effect on insulin metabolism in the presence of hormonal imbalances and chronic low-grade inflammation typical of PCOS, as previously suggested by Cirillo et al.[33] Taken together, our findings reinforce the growing body of evidence that microRNA-320a-3p could potentially act as a promising biomarker for IR in PCOS. Its expression appears to be more tightly linked to metabolic dysfunction in pathological contexts than in physiological conditions. However, future studies with larger cohorts and mechanistic in *vitro* models are warranted to further elucidate the causative role of microRNA-320a-3p in IR and explore its therapeutic potential.

The positive correlation observed between levels of AMH and HOMA-IR in both the PCOS and HCs groups in our research (Table 4) aligns with an emerging body of evidence suggesting that AMH, beyond its recognized function as an indicator of ovarian reserve, may be implicated in metabolic dysregulation. In the PCOS cohort, a statistically significant correlation and notably higher HOMA-IR values among those with elevated AMH support previous findings by Pigny et al.,[34] who reported elevated levels of AMH in women with PCOS, often associated with hyperandrogenism and IR, suggesting that AMH could play a role in the pathophysiological mechanisms linking ovarian dysfunction and metabolic issues abnormalities. Moreover, the finding that 100% of PCOS individuals with high AMH fell within the high HOMA-IR category underscores the potential role of AMH as a surrogate marker for metabolic risk stratification in PCOS patients. This supports the work of Jun et al.,[35] who proposed AMH as a predictor not only of reproductive outcomes but also of metabolic profiles in PCOS women. Interestingly, our study also found a notable positive association between HOMA-IR and AMH in the HCs group, with 45.5% of participants with high AMH levels demonstrating elevated HOMA-IR. This novel observation suggests that the association between IR and AMH may extend beyond clinically diagnosed PCOS. While limited, some previous studies provide preliminary support for this notion. For instance, Capuzzo et al.[36] observed that higher AMH levels in normo-ovulatory women could be associated with subtle endocrine or metabolic irregularities. Furthermore, Teede et al.[37] reported that IR may precede the onset of overt PCOS in women with elevated AMH, suggesting a

potential preclinical metabolic phenotype. However, not all studies have reported a significant association between AMH and IR. Simoes-Pereira et al.,[38] for example, found no consistent correlation in non-PCOS populations, highlighting the potential influence of age, BMI, and assay variability. Therefore, differences in sample characteristics, inclusion criteria, and analytical methods may partly explain the observed discrepancies. Taken together, our findings emphasize a broader metabolic relevance of AMH, reinforcing the hypothesis that AMH may serve not only as a reproductive biomarker but also as a metabolic indicator. This has significant clinical implications, particularly for early detection and preventive strategies in women at risk of developing PCOS or related metabolic disorders. Future longitudinal studies are warranted to further elucidate the causal relationships and underlying mechanisms linking AMH with IR across diverse populations.

The present study reveals a significant association between microRNA-320a-3p expression and both LH/FSH ratio and serum levels of LH in PCOS women, but not in HCs (Table 5). This supports the hypothesis that microRNA-320a-3p may play a disease-specific regulatory role in gonadotropin imbalance, a hallmark of PCOS pathogenesis. These results align with new evidence suggesting that microRNA-320 family members are involved in regulating reproductive hormones and ovarian function. For instance, Cirillo et al.[33] reported that microRNA-320a levels were dysregulated in the granulosa cells in PCOS patients, and this dysregulation was linked to impaired folliculogenesis and altered LH signaling pathways. Similarly, Zhang et al.[39] demonstrated that microRNA-320a targets the pathways of runt-related transcription factor 2 and mitogen-activated protein kinase, both of which contribute to gonadotropin responsiveness and ovarian function steroidogenesis. The current study's observation of elevated LH/FSH ratios and serum LH levels in individuals with high microRNA-320a-3p expression further supports a mechanistic link between this miRNA and hypothalamic-pituitary-gonadal axis dysregulation. Notably, the LH/FSH ratio is widely recognized as an important diagnostic and pathophysiological marker in PCOS, contributing to anovulation and hyperandrogenism.[22] The miRNA's selective association with reproductive parameters, but not metabolic indices (e.g., FBG, insulin, AMH), suggests a functional specificity of microRNA-320a-3p in neuroendocrine signaling rather than in metabolic homeostasis. Moreover, the lack of association with metabolic indicators in our cohort aligns with previous studies that questioned the role of microRNA-320a in IR or glucose metabolism among PCOS patients.[40] Overall, this study contributes novel evidence to the field by highlighting a distinct regulatory association between microRNA-320a-3p and gonadotropin imbalance in PCOS. These results support the potential utility of microRNA-320a-3p as a biomarker for PCOS-related neuroendocrine dysfunction, though additional studies are necessary to confirm causality and clarify mechanistic pathways.

The current study identified a notable negative relationship between serum levels of FBG and AMH in PCOS women, aligning with emerging evidence that supports a dual endocrine and metabolic role for AMH in the pathophysiology of PCOS. Women with high levels of exhibited significantly lower FBG values, suggesting

a possible protective role of AMH against hyperglycemia (Table 6). These results are in agreement with González et al.,[41] who noted that elevated levels of AMH had a negative relationship with FBS in a cohort of reproductive-aged PCOS patients. However, our data contrast with results from Md Muslim et al.,[42] where there was no reported significant relationship between AMH and FBG or IR in patients with PCOS. This discrepancy could stem from differing BMI distributions, AMH stratification thresholds, or assay variability across studies. Interestingly, although our study found significantly higher insulin concentrations in women with high AMH levels – suggestive of compensatory hyperinsulinemia. Guo et al.[43] had also noted this paradoxical association, and suggested that while AMH might be inversely associated with FBG, it could simultaneously be linked to hyperinsulinemia due to impaired insulin action at the cellular level. Such findings highlight the complex and potentially bidirectional relationship between ovarian reserve markers and metabolic parameters.

In contrast to these metabolic associations, we found no notable correlation between AMH and gonadotropins, including LH, FSH, or the LH/FSH ratio. This finding diverges from studies like Lie Fong et al.,[44] demonstrating a modest but significant correlation between LH and AMH in patients with PCOS. However, their study included a smaller and more homogeneous population, which may explain the differing outcomes. The lack of association in our data might also reflect the influence of insulin-mediated disruption of the hypothalamic-pituitary-ovarian axis, which could uncouple traditional gonadotropin regulation from ovarian follicle dynamics. Among the control cohort, the absence of significant correlations between AMH and metabolic or hormonal parameters supports the notion that AMH's multidimensional behavior is more pronounced in the dysregulated metabolic-endocrine milieu of PCOS, rather than in normo-ovulatory women.

This study has a number of limitations that should be noted. First, the size of the sample was relatively small (n=90),which may limit the generalizability of the results to broader populations and affect the statistical power. Second, research was carried out in Iraq at a single center, potentially introducing geographic and ethnic biases that may not reflect the diversity seen in other regions. Additionally, the study's case-control design did not include longitudinal follow-up, which limits the capacity to assess how levels of biomarkers change dynamically over time or in response to interventions. Furthermore, the study focused solely on microRNA-320a-3p, whereas a broader panel of microRNAs may yield complementary or more robust diagnostic insights.

The unexpectedly lower HOMA-IR values in the PCOS group should be interpreted cautiously. This result may reflect the predominance of lean or non-obese PCOS phenotypes, along with uncontrolled confounders such as BMI, diet, and physical activity levels. Additionally, HOMA-IR primarily estimates systemic IR and may not reflect tissue-specific resistance (e.g., ovarian or hepatic), which could still be present despite lower systemic values.

Conclusion

The present study highlights the diagnostic utility of microRNA-320a-3p and AMH in women with PCOS, providing novel insights into their pathophysiological and clinical relevance. Significantly reduced expression of microRNA-320a-3p and altered AMH levels in PCOS patients, along with their associations with IR (HOMA-IR), LH, and LH/FSH ratio, suggest that these biomarkers reflect both metabolic and neuroendocrine disturbances intrinsic to PCOS.

Importantly, the differential patterns observed between PCOS patients and HC support the potential incorporation of microRNA-320a-3p and AMH as complementary diagnostic markers alongside traditional biochemical and hormonal parameters. The observed associations also reinforce the multidimensional role of these markers in capturing the heterogeneous nature of the PCOS phenotypes. Moreover, microRNA-320a-3p, in addition to its diagnostic potential, may also serve as a future therapeutic target, considering its regulatory role in both metabolic and reproductive pathways involved in PCOS pathophysiology. While further large-scale, multi-center, longitudinal studies are required to validate these results, the research underlines the possible clinical value of combining molecular biomarkers into the diagnostic framework of PCOS to enhance early detection, improve risk stratification, and ultimately guide personalized management strategies.

Declarations

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Author contributions

Conceptualization, H.A.A.K. and K.R.H.; Methodology, Z.M.T.; Software, H.A.A.K..; Validation, Z.M.T., K.R.H. and H.A.A.K.; Formal Analysis, H.A.A.K.; Investigation, Z.M.T.; Resources, K.R.H.; Data Curation, Z.M.T.; Writing – Original Draft Preparation, Z.M.T.; Writing – Review & Editing, H.A.A.K.; Visualization, H.A.A.K.; Supervision, K.R.H.; Project Administration, K.R.H.; Funding Acquisition, Z.M.T..

Conflicts of interest

The authors declare no conflict of interest.

Data availability

The datasets provided and/or examined in the current investigation, along with methodological details, are available upon reasonable request from the corresponding author.

Ethics approval

The research was carried out in line with the principles outlined in the Helsinki Declaration and received approval from the Ethics Committee of the Training and Human Development Unit, Thi-Qar Health Department, Ministry of Health, Iraq (Approval No. 197/2024) on September 3, 2024, as (part of the master's thesis).

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