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ORIGINAL PAPER

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 disease manifested with hormonal imbalance, insulin resistance (IR), 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 PCOS women.

Material and methods. A case-control research was performed in 90 women aged 18–40 years, including 45 women with PCOS and 45 age- and body mass index-matched healthy individuals. 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. Women with PCOS demonstrated statistically higher concentrations 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 IR values were lower in the PCOS 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 IR. 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) a prevalent endocrine disease among females during their reproductive years (has affected at least 5-10% of women globally), and it has a global prevalence of approximately 4-20%. The features of PCOS include polycystic ovarian morphology, persistent anovulation and hyperandrogenism. PCOS is a multifaceted disorder, with a multitude of

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factors (hormonal, metabolic and genetic) contributing to its development and associated long-term outcomes, including cardiovascular diseases, infertility and type 2 diabetes (T2D). Although the pathophysiological mechanisms associated with PCOS are well-studied, there continues to be a challenge with diagnosing the disorder due to the heterogeneity of clinical findings and laboratory results; therefore, researchers will need to identify new, valid, and reliable biomarkers for improved diagnostic accuracy and the understanding of disease processes.[1,2]

Insulin resistance (IR) is a common physiological finding in patients diagnosed with PCOS, resulting in significant, compensatory hyperinsulinemia, further contributing to hormonal dysregulation, increased hyperandrogenism, and infertility.[3] IR has been noted previously to play a major role in the pathophysiologic processes of PCOS, impacting over 75% of individuals diagnosed with PCOS.[2]

Micro-ribonucleic acid-320 (miRNA-320) has recently gained attention as a proposed molecular marker for various types of reproductive and metabolic disorders. As a regulator of gene expression, miRNA-320 plays an essential role in the regulation of glucose metabolism, IR, and ovarian function, which are all processes involved in the pathophysiology of PCOS.[4] The anti-Müllerian hormone (AMH) has also been investigated as a potential diagnostic marker for PCOS, reflecting the level of follicular arrest and dysfunction in PCOS patients.[5] The other metabolic and hormonal markers that are important in the endocrinology and metabolic profile of PCOS are designed (luteinizing hormone (LH), follicle-stimulating hormone (FSH), insulin, and fasting blood glucose (FBG)), which play critical roles in explaining hormonal and metabolic disturbances in PCOS. The typical hormonal diagnostic markers for PCOS are the LH to FSH ratio, whereas IR and hyperinsulinemia are metabolic features of PCOS that have a significant bearing on the progression of PCOS and the impact of PCOS treatment.[6,7]

To our knowledge, this is the first research determine the combination of diagnostic accuracy of miRNA-320a-3p and anti-AMH in patients with PCOS. Previous studies have primarily aimed on either AMH or miRNA-320a-3p, whereas this current study examines ambidirectionally for hormonal and metabolic parameters to produce an overall assessment of PCOS. It also uncovers some unexpected patterns, such as lower levels of AMH and insulin. which challenge conventional understanding and highlight phenotypic heterogeneity.

Aim

The aim of this research is to compare the performance of microRNA-320a-3p and AMH with traditional hormone and metabolic markers for diagnosing women with PCOS. A case-control design was utilized to ascertain whether these biomarkers provide greater accuracy when used to diagnose women with PCOS. Additionally, the study will identify the relationship of these biomarkers to metabolic and endocrine aberrations, which may facilitate their integration into standard clinical assessment and into personalized management options for women with PCOS.

Material and methods

Study design/subjects

A case-control research 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 incidence of 2 out of 3: oligo- or anovulation ,presence of polycystic ovaries, clinical/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 participants 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 diseases, 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 obtained from each participant after fasting for 8–12 hours through venipuncture. After collection, the samples were left in gel vacuum tubes and allowed to coagulate at room tem-

perature, and then centrifuged at 3,600 × g for 10 minutes for serum separation. Each sample of serum was divided into two aliquot, 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 concentrations 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 protocol was conducted according to the manufacturer's guidelines. The findings are recorded 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). In accordance with the manufacturer's guidelines, the test procedure was conducted. The results are reported in milligram (mg)/deciliter (dL). The following equation was used to detect the homeostatic model assessment of IR (HOMA-IR).[9]

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

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 and a universal reverse primers (Table 1) on a Mic-qPCR cycler (Fig. 1 and 2).

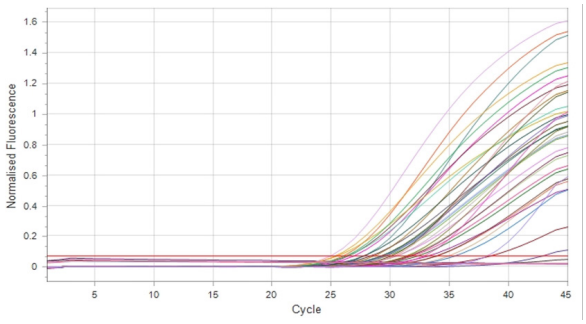

Fig. 1. The step of real-time polymerase chain reaction of microRNA-320a-3p

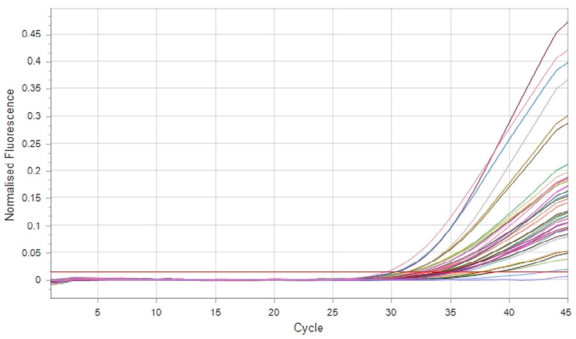

Fig. 2. The step of real-time polymerase chain reaction of RNU43

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.

Table 1. The study primers[a]

Primers name	Sequence ('5——————————3')	Company	Country	Temperature
microRNA-320a-3pRT*	GTTGGCTCTGGTGCAGGGTCCGAGGTATTCG CACCAGAGCCAACTTGCCC	Macrogen	South Korea	55 °C
microRNA-320a-3pF*	GGGAAAAGCTGGGTTGAGA			
RNU43-RT*	GTTGGCTCTGGTGCAGGGTCCGAGGTATTCG 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.

ΔCT = CTgene – CT House Keeping gene
ΔΔCT = ΔCT Treated or Control – Average ΔCT Control
Folding = 2^{-}ΔΔCT

Statistical analysis

Data analysis and visualization were employed using the statistical package for social science (SPSS) (version 22, IBM, Armonk, NY, USA). The descriptive methods involved means, frequencies, and standard deviations (SD). Categorical variables were compared using the chi-square test. The statistical differences of variations in the means of continuous variables with a normal distribution was calculated 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. The Spearman correlation coefficient, which is a non-parametric test, was calculated to determine the strength and direction of the correlations between variables. A p-value <0.05 was determined to be statistically valuable.

Results

In the current research, the differences in insulin levels between the PCOS and HC cohorts were statistically significant (p<0.001), where the mean insulin concentration was depleted in the PCOS women (6.15±2.87 μIU/mL) than HCs (7.56±2.45 μIU/mL). Regarding HOMA-IR, the mean HOMA-IR was statistically lower in the 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) than 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 concentrations were statistically elevated in the PCOS women (9.45±6.0 mIU/mL) than the controls (5.09±2.2 mIU/mL), having a difference that is statistically significant (p<0.001), consistent with the well-established association between elevated LH and the pathophysiology of PCOS. In contrast, concentrations of FSH were lower in the PCOS patients (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 elevated in the women with PCOS (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. For AMH, unexpectedly, the mean concentration was lower in the PCOS patients (2.27±1 ng/mL) than in HCs (3.34±1.12 ng/mL) with a statistical difference (p<0.001). This finding is opposite to the traditionally accepted view of increased AMH levels associated with PCOS and may indicate that there are phenotypic differences within the PCOS population, or it may be the result of methodological differences, so the findings require further research (Table 2).

There was also a large difference in microRNA-320a-3p expression levels between groups. The mean expression for microRNA-320a-3p in the PCOS group was markedly lower (0.61±1.27 folds) when 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 genetic biomarker to detect 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 folds of microRNA-320a-3p was evaluated in both the PCOS and HCs groups. Among individuals with PCOS, a statistically valuable 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. On the other hand, even though there appears to be an increasing trend toward higher HOMA-IR levels in HCs with high expression levels of microRNA-320a-3p (mean=1.68) relative to those with low microRNA-320a-3p expres-

sion (mean=1.63), the relationship was not statistically valuable (r=0.23, p=0.10). This may indicate potential physiologic variability in insulin sensitivity between healthy individuals, and that this variability is not necessarily a result of underlying endocrine/metabolic disturbances, as is commonly seen in PCOS.

Table 3. Correlation of microRNA-320a-3p expression and HOMA-IR concentrations 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 displays the association between HOMA-IR values and AMH levels for both groups (PCOS and HCs). For females diagnosed with PCOS, there was a positive relationship between AMH concentrations and HOMA-IR concentrations, with a statistically significant correlation coefficient of 0.38 (P = 0.01). Women with high AMH had much higher average HOMA-IR concentrations (3.44) than women with normal AMH (1.47). Although all PCOS individuals with high AMH levels (100%) fell within the high HOMA-IR range than those with normal AMH levels (11.9%), this difference indicates that an increase in AMH concentrations correlates with an elevation in IR in patients diagnosed with PCOS and suggests the possibility of a relationship between ovarian reserve and metabolic dysfunction in patients with PCOS. For the HCs women, there was also a positive relationship between AMH and HOMA-IR, with a correlation coefficient of 0.36 (P = 0.02). The mean HOMA-IR for women in the HCs group with high AMH concentrations was 2.28, whereas women in the HCs group with normal AMH levels had a mean HOMA-IR value of 1.6. 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 5 explores the relationship between microRNA-320a-3p folds levels and several metabolic and reproductive parameters in both PCOS (G1) and HC (G2) groups. Among patients with PCOS, a statistical relationship was reported between the expression of microRNA-320a-3p and the LH/FSH ratio (p=0.007), as well as serum LH concentrations (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 valuable 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 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. 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 relationship between insulin and AMH concentrations was statistically valuable (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. There were no statistically significant relationships between AMH and LH, the LH/FSH ratio, or FSH (P > 0.05). Therefore, as there were no statistically valuable associations between the parameters studied and AMH in HCs females, this suggests that the metabolic and endocrine environments in HCs females are generally more stable than those of females with PCOS. Combining these results emphasizes the dual endocrine and metabolic relevance of AMH in PCOS pathophysiology and supports its candidacy as a multidimensional biomarker.

Table 6. Correlation between levels of AMH, metabolic and hormonal parameters in 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

The aims of this study was to assess and compare significant molecular, biochemical, and hormonal variables of PCOS patients versus matched control groups. In studying these two populations, we identified a number of statistically valuable differences between both groups, which are indicative of the heterogeneous and multifactorial nature of PCOS. Many features of PCOS include metabolic derangements (IR), hormonal disturbances, and emerging novel molecular patterns (microRNAs). At this time, IR has been established as a primary pathogenic feature associated with PCOS with respect to hyperinsulinemia and additional concurrent metabolic changes associated with hyperinsulinemia and additional metabolic adaptations. In our study population, we also found that there were statistically depleted mean serum insulin concentrations in the PCOS patient population as than the HCs. Our findings contrast with the majority of previous research that indicates elevated concentrations of serum insulin in PCOS as a consequence of systemic IR.[12] A potential explanation for this difference may be due to the inclusion of non-obese and lean PCOS phenotypes that do not demonstrate high levels of IR.[13] Furthermore, confounding variables such as BMI, physical activity, and nutrition were not controlled in this research, and all have been shown to considerably impact insulin dynamics.[14]

Although the frequency of IR (as identified by elevation of HOMA-IR) showed no statistical 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 elevates 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 elevations in serum LH and ratios of LH to FSH seen in the PCOS population studied are consistent with multiple previously reported features of PCOS. In PCOS, the elevated frequency of the LH pulsatile release of gonadotropin-releasing hormone (GnRH) leads to a predominant secretion of LH and a subsequent increase in androgen production, which are contributing factors to the ovulatory dysfunction seen in PCOS.[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 concentrations were modestly but significantly depleted 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 a small

antral follicles increase.[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 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 folds in the PCOS patient group than the controls. This is parallel with findings by Chen et al.,[28] who found 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 research provides novel insights into the relationship between microRNA-320a-3p folds and IR, measured using HOMA-IR, in PCOS women than the HCs. In our PCOS group, we found a statistically valuable positive relationship between microRNA-320a-3p folds and the HOMA-IR score. This was not the case for participants in the HC group. Thus, microRNA-320a-3p may have some type of role in the pathogenesis of IR for people diagnosed with PCOS (Table 3). Our results agree with previous research that has implicated microRNA-320a in metabolic disorders, particularly in regulating signaling pathways for insulin. Luo et al.[30] showed that microRNA-320a expression was positively associated with markers of IR, including HOMA-IR and fasting insulin concentration and 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 recorded conflicting findings 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 marker for IR in PCOS. Its expression appears to be more tightly linked to metabolic dysfunction in pathological contexts than in physiological conditions. Further study is needed with larger sample sizes and appropriate in *vitro* models to better clarify the direct relationship between microRNA-320a-3p and IR, as well as to investigate the potential for therapeutics. In current study, we also reported a positive relationship between AMH concentrations and HOMA-IR scores for both the PCOS and HCs groups (Table 4). This finding matches with other studies suggesting that AMH is also involved in metabolic problems aside from being an indicator of ovarian reserve. Additionally, we found there to be a statistically significant positive relationship between higher AMH concentrations and higher HOMA-IR scores in the PCOS cohort, which confirms previous observations made by Pigny et al.[34] results from those researchers indicated that women diagnosed with PCOS have high concentration of AMH and often have hyperandrogenism and IR, which provides evidence that AMH may help link ovarian dysfunction with metabolic abnormalities. In addition, it is interesting to note that 100% of PCOS individuals with high AMH awere found to belong to the category of high HOMA-IR, which suggests that AMH may be used as an indicator of metabolic risk stratification in patients with PCOS. This finding corroborates the findings of Jun et al.,[35] who postulated that AMH might be used as a predictor of reproductive and metabolic profiles in women with PCOS. In this same vein, high HOMA-IR was also significantly correlated with high AMH in the HC sample, with 45.5% of high AMH subjects showing high HOMA-IR levels. This observation provides preliminary evidence that the relationship between IR and AMH may not be restricted to women with PCOS, and while still limited, other studies suggest this possibility as well. Capuzzo et al.[36] noted that among normoovulatory women, persons with high AMH may exhibit subtle endocrine or metabolic abnormalities. Teede et al.[37] reported that IR could appear before symptoms of PCOS show in women with higher levels of AMH, which indicates the possibility of a preclinical metabolic phenotype. However, there are some

studies that do not support a clear relationship between AMH and IR. For instance, Simoes-Pereira et al.[38] did not report a linear correlation between AMH and IR among non-PCOS groups based on the individuals' ages, BMI, and differences in how AMH was measured. So the differences in the sample population's characteristics, how participants were chosen to be part of a study, and the methodology used by each study to analyze data might account for some of the differences found when reading these studies. Thus, our findings support the idea that AMH has an even bigger role than just being a biological marker of reproduction by suggesting that AMH could be linked as an indicator for metabolic health. This finding also has far-reaching clinical applications concerning how to recognize and prevent the development of PCOS and other metabolic diseases that are related to PCOS. To better understand the significance of our current findings, further long-term studies will need to examine the causal pathways and mechanisms linking AMH to IR in many different populations.

In this investigation, microRNA 320a-3p expression is statistically correlated with both the LH/FSH ratio and LH serum concentrations in patient's diagnosed with PCOS but not with HC (Tables 5). This implies that microRNA 320a-3p is likely to play an important disease-specific regulatory role in the imbalance of gonadotropins which is characteristic of the development of PCOS. 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 women, 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 relationship between microRNA-320a-3p and gonadotropin imbalance in PCOS. The findings of this study speculate that microRNA-320a-3p could be used as a biomarker for neuroendocrine dysregulation associated with PCOS; however, further studies will need to be conducted to establish causality and elaborate on the mechanisms of action.

The current work has also demonstrated an inverse correlation between FBG and AMH concentrations in women diagnosed with PCOS. This result is in line with other investigations indicating a role for both endocrine and metabolic pathways through which AMH influences the development of PCOS. Women having the highest amount of AMH available had significantly decreased concentrations of FBG than did women having lower amounts of AMH, indicating a potential protective mechanism by AMH from the effects of hyperglycemia (Table 6). Results from our study were consistent with those reported in the cohort of González et al.,[41] who found a negative relationship between elevated AMH concentrations in PCOS patients of reproductive age and FBS. However, our findings differed from the study by Md Muslim et al.,[42] which recorded no statistically significant associations between AMH and either FBG or IR in PCOS subjects. The differences observed may be due to different BMI distributions, AMH cut-offs used by the studies which classified study participants into their respective levels of AMH, or assay variability within different research settings. The observation of elevated levels of insulin concentration among women who have high levels of AMH raises the possibility of compensatory hyperinsulinemia. Similar observations were also found by Guo et al.,[43] who proposed a paradoxical relationship between AMH and FBG, stating that while AMH may be associated with low FBG, AMH may also be positively correlated with hyperinsulinemia due to impaired action of insulin at the cellular level. This evidence supports the concept that the markers of ovarian reserve and metabolic markers may have a complex and potentially bidirectional interaction.

The lack of association between AMH and gonadotropins (LH and FSH) found in our study was inconsistent with other studies, including Lie Fong et al.,[44] who found some evidence of a correlation between LH and AMH in a smaller and more homogenous population of women diagnosed with PCOS. It is likely that differences in sample sizes and to what extent the sample was representative of the larger population explain these discrepancies. The lack of association between AMH and gonadotropins in our analysis could be attributed to the insulin-dependent disruption of the hypothalamic-pituitary-ovarian axis, which may separate the typical interactions of gonadotropins and ovarian follicular activity.

In addition to supporting the concept that AMH has many influences (e.g., metabolic and hormonal) not yet captured by standard laboratory measurements, the lack of correlation between AMH and metabolic or hormonal markers in the control group indicates that AMH has a greater range of influences (e.g., metabolic and hormonal) in the dysregulated metabolic-endocrine environment of women with PCOS than in women without menstrual irregularities.

There are various limitations within this research study. One limitation is that the sample size was very low (n=90), restricting its applicability to larger populations and decreasing the overall power of the outcome variables. Additionally, the research was conducted at only one location (Iraq), which could introduce geographic/ethnic bias that is not present in other countries. Finally, because of the case/control design of this study, there is no way to determine how levels of biomarkers evolve over time, or due to treatment/intervention. Therefore, it would be beneficial to include a larger number of microRNAs in addition to microRNA-320a-3p; this might provide additional or more accurate information for diagnostic purposes.

The unexpectedly low HOMA-IR measurement values that were recorded in the PCOS group need to be interpreted with caution. These results may be indicative of the predominant presence of a lean or non-obese phenotype within the population of patients with PCOS along with other uncontrolled confounding factors, including BMI, dietary consumption and level of exercise. In addition, the HOMA-IR calculation provides an estimation of systemic insulin IR but does not assess tissue-specific IR (eg, ovarian IR or hepatic IR), and therefore it is possible that tissue-specific IR continues to exist despite the measurement of lower systemic IR.

Conclusion

This research demonstrates that microRNA-320a-3p and AMH have diagnostic value for women with PCOS, adding to understanding of both the physiological and clinical implications of these factors. Expression of microRNA-320a-3p is statistically decreased in PCOS women, and there are changes in the amount of AMH produced by the body. The correlation between microRNA-320a-3p and IR (HOMA-IR), LH, and the LH/FSH ratio indicates that these biomarkers represent disturbances in both metabolism and the neuroendocrine system, which are inherent to PCOS.

The different patterns of expression observed between patients with PCOS and HC suggest that microRNA-320a-3p and AMH may be useful as additional diagnostic markers alongside traditional biochemical and hormonal parameters. Furthermore, the relationship between these markers supports that there is a multi-dimensional role for them in defining PCOS phenotype heterogeneity. In addition to serving as a diagnostic biomarker for PCOS, microRNA-320a-3p has the potential to be used as a therapeutic target in the future, due to its function in regulating metabolic and reproductive pathways involved in the pathophysiology of PCOS. Therefore, large-scale, multi-center, longitudinal studies are needed to confirm the observations of this study; however, this work highlights the potential value of using molecular biomarkers as part of the diagnostic framework for PCOS, thereby facilitating earlier diagnosis, improving risk stratification, and eventually providing a foundation for developing individualized management approaches.

Declarations

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Author contributions

Conceptualization, K.R.H., H.A.A.K; 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 affirm there is no conflict of interest.

Data availability

The data used in the research and/or the methodology are available from the corresponding author by request.

Ethics approval

Conducting this study project was approved by the ethics committee of the training and human development unit of the Ministry of Health in Iraq via the Helsinki Declaration on september 3, 2024, as it was part of my master's thesis (Approval No: 197/2024).

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