

## Assessment of Serum MicroRNA-1281 Expression as a Circulating Biomarker of Diabetic Retinopathy in Patients with Type 2 Diabetes

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### Abstract:

**Background:** Diabetic retinopathy (DR) is a significant complication of type 2 diabetes, often leading to vision impairment or blindness. Early and accurate diagnosis is essential for effective management. Serum microRNAs have shown promise as potential biomarkers for Diabetic Retinopathy (DR). **Methods:** Eighty individuals were categorized into four groups, including healthy controls, diabetic patients without retinopathy (NDR), patients with non-proliferative DR (NPDR), and patients with proliferative DR (PDR). **Results:** Serum miR-1281 levels showed a significant up-regulation in disease group compared with healthy control. The serum miR-1281 expression in healthy control group were (0.957) which were drastically lower than those in non-diabetic retinopathy (NDR) group (1.56), NPDR group (2.992) and PDR group (8.732), respectively with  $P < 0.001$ . The best cutoff of miRNA 1281-fold change for diagnosis of proliferative retinopathy and non-proliferative diabetic retinopathy (NPDR) was  $\geq 3.085$ ,  $\geq 1.598$  respectively with area under curve 0.931, 0.933 sensitivity 90%, 85%, specificity 86.7%, 80%, positive predictive value 69.2%, 68% negative predictive value 96.3%, 91.4%, accuracy 87.5%, 81.7% ( $P < 0.001$ ) respectively. There was a statistically significant positive correlation between miRNA 1281-fold change and each of age, platelet count, fasting blood glucose, HbA1c, fasting insulin, HOMA-IR, urea, creatinine, UACR, total cholesterol, LDL and triglycerides. Among factors significantly correlated to miRNA 1281-fold change among patients with non-proliferative DR, only HOMAIR significantly independently associated with non-proliferative DR (unstandardized  $\beta = 0.428$ ,  $P = 0.005$ ). **Conclusion:** Serum miR-1281 as a potential biomarker could be related to not only occurrence but also progression for DR in patients with T2D. **Keywords:** Diabetic retinopathy; type 2 diabetes; microRNA-1281; biomarker; diagnostic tool.

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## Introduction

Diabetic retinopathy (DR), a leading cause of irreversible blindness in individuals with diabetes, is characterized by progressive microvascular complications resulting from chronic hyperglycemia<sup>[1]</sup>. It is characterized by abnormal retinal blood vessels, which can be proliferative (proliferative diabetic retinopathy, PDR) or non-proliferative, leading to fluid and lipid leakage into the retina (non-proliferative diabetic retinopathy, NPDR). Visual impairment often occurs when this leakage affects the central retina or macula, leading to macular edema (ME)<sup>[2]</sup>.

In the realm of DR research, microRNAs have emerged as promising candidates for advancing our understanding and clinical management. MicroRNAs are short, highly conserved non-coding RNA molecules approximately 22 nucleotides in length that play a crucial role in post-transcriptional gene regulation<sup>[3]</sup>. They are expressed in various cell types, participating in essential biological processes such as cell growth, differentiation, and apoptosis. These microRNAs can be located within introns of coding and noncoding genes, exons, or intergenic regions, with those in introns often being transcribed alongside the host gene and those in exons having their own promoter for autonomous transcription<sup>[4]</sup>. The biogenesis of microRNAs occurs in the nucleus and involves a series of intricate steps. Initially, primary microRNAs (pri-microRNAs) are generated through transcription mediated by RNA polymerase II. Notably, pri-microRNAs contain hairpin structures necessary for recognition by the Drosha/DGCR8 protein complex, where DGCR8 binds to pri-microRNAs and Drosha cleaves them to yield shorter sequences known as pre-microRNAs<sup>[5]</sup>. Pre-microRNAs are then recognized by Exportin-5 and transported to the cytoplasm, where another RNase III

protein called Dicer processes them into shorter double-stranded RNA sequences, containing the mature microRNA<sup>[4]</sup>.

Recently, microRNAs have been implicated in the pathophysiology of various human diseases, including type 2 diabetes and the inflammatory and endothelial dysfunction that can contribute to vascular complications in diabetes<sup>[6]</sup>. As a result, multiple studies in recent years have aimed to identify circulating microRNAs in biofluids that can distinguish diabetic patients with retinopathy from those without retinopathy or healthy controls. Some of these studies have demonstrated remarkable predictive value for the detection of DR<sup>[7]</sup>.

The purpose of this study was to assess serum microRNA-1281 gene expression as a biomarker for DR in patients with type 2 diabetes.

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## Patients and methods

### Patients:

This case-control study was conducted in the Clinical Pathology Department and Ophthalmology department of Benha University Hospital from 28 February 2022 to 30 June 2024

**Sample size:** The study population comprised a total of eighty patients, and the sample size was determined using G\*power software version 3.1.9.4 with F tests as the test family and a priori power analysis. The input parameters used for sample size calculation included an effect size (f) of 0.48, an alpha ( $\alpha$ ) error level of 0.05, and a power ( $1 - \beta$ ) of 0.8.

Participants were divided into four equal distinct groups: the first group consisted of apparently healthy control individuals (n=20), the second group included diabetic patients without retinopathy (n=20), the third group comprised individuals with non-proliferative DR (n=20), and the fourth group consisted of individuals with proliferative DR (n=20). Ethical considerations were paramount, and informed written consents were obtained from all participants. Furthermore, the

study was officially approved by the ethics committee for research involving human subjects at Benha Faculty of Medicine, with approval granted on 30 February 2022 to 30 June 2024. Approval code: MS 30-2-2022

**Inclusion criteria:** patients diagnosed with DR. The diagnosis of type 2 diabetes followed the American Diabetes Association criteria, which include fasting plasma glucose levels  $\geq 126$  mg/dL (7.0 mmol/L) or 2-hour plasma glucose levels  $\geq 200$  mg/dL (11.1 mmol/L) during the oral glucose tolerance test (OGTT), HbA1C levels  $\geq 6.5\%$  (48 mmol/mol), or the presence of classic symptoms of hyperglycemia<sup>[8]</sup>.

**Exclusion criteria** were renal disease, chronic systemic hypertension, other retinal vascular disease and any diabetic patient who received any interventional treatment modality for the retinal disease.

### Methods

**A) Detailed History Taking:** A detailed history was obtained from each patient, which included personal information such as name, age, gender, and special habits.

Additionally, the present history, encompassing the onset, course, and duration of diabetes, and the family history of diabetes, was recorded.

**B) Full Clinical Examination:** Each patient underwent a full clinical examination. This examination included a general assessment, with specific attention given to blood pressure measurements and the calculation of the body mass index (BMI) using the formula  $BMI = \text{weight (kg)} \div \text{height (m}^2\text{)}$ . The BMI was classified according to international standards, which categorize individuals as underweight ( $< 18.5$  kg/m<sup>2</sup>), within the normal range (18.5 – 24.99 kg/m<sup>2</sup>), overweight ( $\geq 25$  – 29.99 kg/m<sup>2</sup>), or obese ( $\geq 30$  kg/m<sup>2</sup>)<sup>[9]</sup>.

**C) Local Examination for Diabetic Retinopathy:** A local examination for signs of DR was conducted, involving a fundus examination by indirect ophthalmoscopy and auxiliary lenses with a slit lamp. The diagnosis of non-

proliferative DR (NPDR) was based on an ophthalmological examination of the ocular fundus following pupil dilation. The severity of DR was graded based on the International Clinical DR Disease Severity Scale, which categorizes DR into five stages: "no apparent retinopathy," "mild NPDR," "moderate NPDR," "severe NPDR," and "proliferative DR".

Additionally, the presence of macular edema was noted and classified as mild, moderate, or severe based on the distance of exudates and thickening from the center of the fovea.

**D) Imaging:** Fundus Fluorescein Angiography (FFA) was performed for all participants. This involved the administration of sodium fluorescein 10% through an antecubital vein, followed by the capture of macular area photographs with late views 5 minutes after injection using a fundus camera<sup>[10]</sup>.

**E) Laboratory Investigations:** Various laboratory tests were conducted, including:

Complete blood count (CBC), measurement of lipid profile, which included triglycerides, cholesterol, HDL-cholesterol (High-Density Lipoprotein), and LDL\_ cholesterol (Low-Density Lipoprotein), urea and creatinine measurements, fasting blood sugar, insulin, and Homeostatic Model Assessment for Insulin Resistance (HOMA-IR), glycated hemoglobin (HbA1C) assessment, urine Albumin/Creatinine ratio and serum microRNA 1281 expression level determination by real-time PCR.

**Blood and Urine Sampling:** Seven ml of venous blood was drawn from each participant under complete aseptic condition after overnight fasting and divided into 2 parts: 1st part 2 ml was put into EDTA tube for CBC and Glycated Hb measurement, 2nd part 5 ml was put in serum separating tubes and was left for 30 minutes for clotting then centrifuge. One ml of the separating serum was kept at -80°C for assessment of serum microRNA 1281 expression level and the remaining

serum was used for other clinical chemistry tests.

**Urine samples:** were obtained by mid-stream clean catching early in the morning for assessment of urine albumin and creatinine ratio.

#### **Laboratory Methods:**

**Complete blood count (CBC):** was evaluated using fully automated hematology analyzer (Sysmex XN-L series, Japan).

Clinical chemistry tests (triglyceride-cholesterol-HDL-Cholesterol, LDL-Cholesterol, urea, creatinine and fasting blood sugar): were done using chemical autoanalyzer (Abbott, Architect c 4000 Serial No. 02p24-01 Japan) according to manufacturer's instructions.

#### **Homeostatic model assessment for insulin resistance (HOMA, IR):**

Detection of the insulin level was done by an enzyme-linked immunosorbent assay (ELISA) using Mercodia Ultrasensitive Rat Insulin ELISA Kit lot (10-1251-01) (Mercodia AB, Sylveniusgatan 8A, SE-754 50 Uppsala, Sweden). This assay has a sensitivity of 0.05 ng/mL using a 5 µL sample with CV ≤ 10.0%. HOMA-IR was calculated according to the formula: Fasting insulin (U/ml) Fasting blood sugar (mg/dl)/405<sup>[11]</sup>.

**Glycated hemoglobin (HbA1c):** Blood samples were subjected to quantitative glycohemoglobin determination using immuno Turbidimetry assay using (BioMed-Fast- HBA1C) catalogue no: HBA1C103 supplied by BioMed diagnostics according to the manufacturer's instructions.

**Albumin/creatinine ratio:** Albumin and creatinine were measured using chemical auto analyzer (Abbott, Architect c 4000 Serial No. 02p24-01 Japan) according to manufacturer's instructions.

Alb/Cr ratio is calculated according to this formula: Urine albumin (mg/dl)/urine creatinine (g/dl)<sup>[12]</sup>.

#### **Serum microRNA 1281 expression level by real time PCR:**

**MicroRNA extraction:** Extraction was done by using miRNeasy Mini Kit (Cat. no. 217004) (Qiagen, Hilden, Germany) according to the manufacturer's instruction.

Relative Quantitation of microRNA-1281 by two-step Real Time PCR using SYBR Green (Qiagen QuantiTect SYBR Green PCR Kits, Germany): In this study the 1st step real time PCR was performed using miCURY LNA RT Kit (cat.no.339340). Template RNA was thawed on ice, the reverse transcription master mix had a final volume of 10 µl including (2µl 5x miRCURY LNA Buffer, 1µl 10x miRCURY LNA enzyme Mix, 0.5 µl synthetic RNAspike, 6.5 µl Template RNA + RNase free water).

Template RNA was added to each tube containing reverse transcription master mix. Then mixed gently, briefly centrifuged, and then placed on ice. The tubes were incubated at 37°C for 60 mins. followed by incubation at 95°C for 5 mins in Veriti thermal cycler Applied Biosystem (USA). Un-diluted cDNA was stored in -20 freezer till second step was done.

**Real Time PCR for relative quantitation of miRNA-1281 (2nd step):** PCR was performed on Step One real time PCR, using miRCURY LNA SYBR Green PCR Kit supplied by (QIAGEN, Germany) (cat. No339345) according to manufacturer instructions, for accurate and reproducible results in miRNA quantification by real-time PCR, we normalize the amount of target miRNA by using B-actin as endogenous reference gene.

cDNA was diluted by adding 100 µl of RNase free water to each 10 µl from reverse transcript tube, a reaction mix was prepared according to preparation of the reaction mix as, each reaction was performed in a final volume of 10 µl (5µl 2x SYBER Green MM, 0.05µl ROX Reference dye, 1µl PCR primer MIX, 1µl RNase-free water, 3µl Template c DNA). The reaction mix was mixed thoroughly but gently, and appropriate volumes were dispensed into the plate wells. Then the

plate was seal tightly. Centrifuged for 1 min at 1000 X g at room temperature (15–25°C) to remove bubbles.

Real time PCR was performed on Real time PCR cyclical thermal cyclical (Applied Biosystem, USA). PCR conditions was performed as follows: 2 minutes at 95°C followed by 40 cycles of denaturation at 95°C for 10 seconds then annealing was performed at 56°C for 60 seconds with final extension at 70°C for 30 seconds in which fluorescence was acquired.

#### Data analysis

The expression level of the miRNA was reported as  $\Delta C_t$  value, the fold change in the expression level of the miRNA was calculated (fold change =  $2^{-\Delta\Delta C_t}$ ) to define the relative quantitative levels of individual miRNA.  $\Delta\Delta C_t$  = tumour (CtmiRNA - Ct RNU6B) - mean normal (CtmiRNA - CtRNU6B) <sup>[13]</sup>.

#### Statistical analysis

Statistical Package for the Social Sciences (SPSS) 28.0 for windows SPSS Inc., Chicago, IL, USA) was used. The normality of distribution for the analyzed variables were tested using Kolmogorov-Smirnov test assuming normality at  $P > 0.05$ . The collected data were summarized in terms of median and Inter Quartile Range (IQR) as appropriate for nonparametric data and mean  $\pm$  Standard Deviation (SD) as appropriate for parametric data and Frequency and distribution for qualitative data, the statistical significance of the difference between the treated groups and the control group was evaluated using Kruskal Wallis test as followed by Mann Whitney test for comparison between 2 groups appropriate for nonparametric data, One way ANOVA with Bonferroni post HOC test as appropriate for parametric data, Inter-group comparison of categorical data was performed by using chi square test ( $\chi^2$ -value). Sensitivity, specificity values and

Area under the curve (AUC) of miRNA predictability of proliferative and non proliferative diabetic retinopathy were calculated using ROC Curve (Receiver Operating Characteristic). A P value less than 0.05 was considered significant. A P value less than 0.01 was considered highly significant. A P value more than 0.05 was considered non-significant.

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## Results

Demographic data and laboratory parameters between studied groups was demonstrated according to Table 1 Serum of miRNA-1281 expression profile in the studied group. Serum miR-1281 expression profile in the studied group showed a significant up-regulation in disease group compared with healthy control. The serum miR-1281 expression in healthy control group were (0.957) which were drastically lower than those in NDR group (1.56), NPDR group (2.992) and PDR group (8.732), respectively with  $P < 0.001$ . Table 2 Evaluation of diagnostic accuracy of serum miRNA1281 expression in proliferative diabetic retinopathy and non-diabetic DR.

Receiver operating characteristics curve showed that the best cutoff of miRNA 1281-fold change in evaluation of the diagnostic accuracy of serum miR-1281 expression in proliferative retinopathy and non-proliferative retinopathy was  $\geq 3.085$ ,  $\geq 1.598$  respectively with area under curve 0.931, 0.933 sensitivity 90%, 85% specificity 86.7%, 80% positive predictive value 69.2%, 68% negative predictive value 96.3%, 91.4%, accuracy 87.5%, 81.7% ( $P < 0.001$ ) respectively. Figure 1 Correlation between serum mi RNA1281 expression and studied parameters among studied groups.

**Table 1:** Comparison between the studied groups regarding their demographic characters and laboratory data

Study groups	Group I N.=20		Group II N.=20		Group III N.=20		Group IV N.=20		$F/\chi^2$	P value
	Mean	SD	Mean	SD	Mean	SD	Mean	SD		
Age (years)	50.05	9.29	50.35	10.85	61.6	9.61	71.2	7.03	1. 23.776	2. <0.001**
LSD	P <sub>1</sub> 0.919		P <sub>2</sub> <0.001**		P <sub>3</sub> 0.002*		P <sub>4</sub> <0.001**		3. P <sub>5</sub> <0.001**	4. P <sub>6</sub> <0.001**
Sex										
Female	12	60%	15	75%	15	75%	8	40%	7.04	
Male	8	40%	5	25%	5	25%	12	60%		0.071
Hemoglobin (g/dl)	13.76 ± 1.45		13.41 ± 1.67		12.86 ± 1.71		12.14 ± 1.61		3.823	0.013*
LSD	P <sub>1</sub> 0.495		P <sub>2</sub> 0.289		P <sub>3</sub> 0.16		P <sub>4</sub> 0.084		P <sub>5</sub> 0.002*	P <sub>6</sub> 0.015*
PLT (10 <sup>3</sup> /mm <sup>3</sup> )	288.92±30.99		279.54±12.05		277.79±14.54		278.79±14.42		1.672	0.179
	Median (IQR)		Median (IQR)		Median (IQR)		Median (IQR)		KW	P
TLC (10 <sup>3</sup> /mm <sup>3</sup> )	9.7 (5.5 – 16.3)		8.1 (5.6 – 12.2)		7.7 (4.8 – 12.3)		7.85 (5.6 – 12.5)		0.809	0.847
HbA1c (%)	5.32 ± 0.6		6.0 ± 0.66		6.7 ± 0.71		7.06 ± 1.1		18.953	<0.001**
LSD	P <sub>1</sub> 0.008*		P <sub>2</sub> 0.007*		P <sub>3</sub> 0.15		P <sub>4</sub> <0.001**		P <sub>5</sub> <0.001**	P <sub>6</sub> <0.001**
	Median (IQR)		Median (IQR)		Median (IQR)		Median (IQR)		KW	P
Fasting blood sugar(mg/dl)	99.5 (87 – 107)		130.5(122 -139)		154 (126.75 - 179)		170.5 (127.75 – 193.5)		41.027	<0.001**
Pairwise	P <sub>1</sub> <0.001**		P <sub>2</sub> 0.153		P <sub>3</sub> 0.395		P <sub>4</sub> <0.001**		P <sub>5</sub> <0.001**	P <sub>6</sub> 0.023*
Fasting insulin (mIU/L)	4.2 (3.78 – 7.38)		5.05 (4 – 5.65)		19.5 (3.83 – 25)		17.85 (8.45 – 25)		29.104	<0.001**
Pairwise	P <sub>1</sub> 0.376		P <sub>2</sub> 0.002*		P <sub>3</sub> 0.086		P <sub>4</sub> 0.024*		P <sub>5</sub> <0.001**	P <sub>6</sub> <0.001**
HOMA-IR (μIU/mL)	1.35 (1 – 1.7)		3.7 (3.23 – 4.5)		6.5 (1.43 – 9.25)		7.6 (3.13 – 10.2)		35.63	<0.001**
Pairwise	P <sub>1</sub> <0.001**		P <sub>2</sub> 0.408		P <sub>3</sub> 0.175		P <sub>4</sub> <0.001**		P <sub>5</sub> <0.001**	P <sub>6</sub> 0.029*
Total cholesterol (mg/dL)	179 (150 – 207.5)		189.5 (164 – 200.75)		232 (200.75 – 286.75)		238 (205.75 -283.75)		15.208	0.002*
Pairwise	P <sub>1</sub> 0.57		P <sub>2</sub> 0.027*		P <sub>3</sub> 0.676		P <sub>4</sub> 0.005*		P <sub>5</sub> 0.001**	P <sub>6</sub> 0.008*
HDL cholesterol (mg/dL)	58.5 (49 – 70)		66 (60 – 73.5)		63.5 (44.25 – 71.75)		52 (43.5 – 89.25)		2.065	0.559
LDL cholesterol (mg/dL)	100 (75.25 – 121.5)		104 (87.75 -122.25)		146 (113.75 – 192.5)		141.5 (116.25– 185.75)		16.918	0.001**
Pairwise	P <sub>1</sub> 0.479		P <sub>2</sub> 0.018*		P <sub>3</sub> 0.785		P <sub>4</sub> 0.002*		P <sub>5</sub> 0.001**	P <sub>6</sub> 0.008*
Triglycerides (mg/dL)	95.5(59.25- 155.25)		99 (85.75 – 150)		157.5 (100 237.75)		168 (108.5 – 227.5)		10.016	0.018*
Pairwise	P <sub>1</sub> 0.593		P <sub>2</sub> 0.076		P <sub>3</sub> 0.757		P <sub>4</sub> 0.021*		P <sub>5</sub> 0.009*	P <sub>6</sub> 0.037*
Urea(mg/dL)	22.1 ± 6.37		17.67 ± 4.88		34 ± 13.39		43.75 ± 14.52		24.535	<0.001**
LSD	P <sub>1</sub> 0.193		P <sub>2</sub> <0.001**		P <sub>3</sub> 0.005*		P <sub>4</sub> <0.001**		P <sub>5</sub> 0.001**	P <sub>6</sub> 0.001**
Creatinine(mg/dL)	0.82 ± 0.15		0.85 ± 0.18		1.32 ± 0.3		1.34 ± 0.29		28.927	<0.001**
LSD	P <sub>1</sub> 0.693		P <sub>2</sub> <0.001**		P <sub>3</sub> 0.742		P <sub>4</sub> <0.001**		P <sub>5</sub> 0.001**	P <sub>6</sub> 0.001**
	Median (IQR)		Median (IQR)		Median (IQR)		Median (IQR)		KW	P
Urine albumin/ creatinine ratio (mg/g)	27 (20 – 28.75)		24 (20 – 28)		37.5 (29.25 – 45.75)		89 (46.25 – 150)		52.372	<0.001**
Pairwise	P <sub>1</sub> 0.87		P <sub>2</sub> <0.001**		P <sub>3</sub> 0.021*		P <sub>4</sub> <0.001**		P <sub>5</sub> <0.001**	P <sub>6</sub> <0.001**

F One Way ANOVA test / $\chi^2$  Chi square test p<sub>1</sub> difference between groups I and II p<sub>2</sub> difference between groups II and III p<sub>3</sub> difference between groups III and IV p<sub>4</sub> difference between groups I and III p<sub>5</sub> difference between I and IV p<sub>6</sub> difference between groups II and IV  
 \*\*P≤0.001 is statistically highly significant IQR interquartile range KW Kruskal Wallis test.

**Table 2:** Comparison between the studied groups regarding miRNA 1281-fold change.

Fold change	Group I Median (IQR)	Group II Median (IQR)	Group III Median (IQR)	Group IV Median (IQR)	KW	p
<b>miRNA 1281</b>	0.957 (0.911 – 1.089)	1.56 (1.34 – 1.668)	2.992 (2.47 – 6.374)	8.732 (7.28 – 9.09)	<b>64.505</b>	<0.001**
Pairwise	P1 0.002*	P2 0.014*	P3 0.045*	P4 <0.001**	P5 <0.001**	P6 <0.001**

p1 difference between groups I and II p2 difference between groups II and III p3 difference between groups III and IV p4 difference between groups I and III p5 difference between I and IV p6 difference between groups II and IV \*\*P≤0.001 is statistically highly significant IQR interquartile range KW Kruskal Wallis test

There was significant positive correlations between miRNA 1281 expression and studied parameters among studied groups including age (r =0.623, P<0.001), platelet count (r =0.342, P=0.002) fasting blood glucose (r =0.642, P<0.001) HbA1c (r =0.607, P<0.001) fasting insulin (r =0.62, P<0.001) HOMA-IR (r= 0.767, P<0.001) urea (r=0.608, P<0.001) creatinine (r=0.608, P<0.001) UACR (r=0.565, P<0.001) total cholesterol (r=0.29, P=0.009). While, there was no statistically significant correlation between miRNA 1281-fold change and either TLC nor HDL

cholesterol, LDL cholesterol nor triglycerides. Table 3

After controlling for hemoglobin A1c, there is still significant positive correlation between miRNA 1281-fold change and age, platelet count, fasting blood glucose, fasting insulin, HOMA-IR, urea, creatinine, UACR, and total cholesterol. Table 4

Regression analysis revealed that among factors significantly correlated to miRNA 1281-fold change in patients with non-proliferative DR, only HOMAIR was significantly independently associated with DR (unstandardized  $\beta$ =0.428, P=0.005).

**Table 3:** Correlation between miRNA 1281 fold change and the studied parameters among the diseased participants.

	r	p
<b>Age (year)</b>	<b>0.623</b>	<0.001**
<b>Hemoglobin (g/dl)</b>	<b>-0.289</b>	0.009*
<b>TLC (10<sup>3</sup>/mm<sup>3</sup>)</b>	-0.097	0.392
<b>Platelet count (10<sup>3</sup>/mm<sup>3</sup>)</b>	<b>0.342</b>	0.002*
<b>Fasting blood glucose (mg/dl)</b>	<b>0.642</b>	<0.001**
<b>HbA1c (%)</b>	<b>0.607</b>	<0.001**
<b>Fasting insulin</b>	<b>0.62</b>	<0.001**
<b>HOMA-IR (μIU/mL)</b>	<b>0.767</b>	<0.001**
<b>Urea (mg/dL)</b>	<b>0.608</b>	<0.001**
<b>Creatinine (mg/dL)</b>	<b>0.608</b>	<0.001**
<b>UACR (mg/g)</b>	<b>0.565</b>	<0.001**
<b>Cholesterol (mg/dL)</b>	<b>0.29</b>	0.009*
<b>HDL cholesterol (mg/dL)</b>	-0.059	0.602
<b>LDL cholesterol (mg/dL)</b>	<b>0.327</b>	0.007*
<b>Triglycerides (mg/dL)</b>	<b>0.253</b>	0.024*

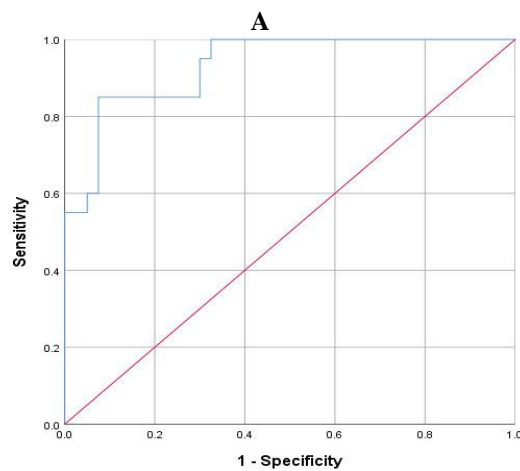
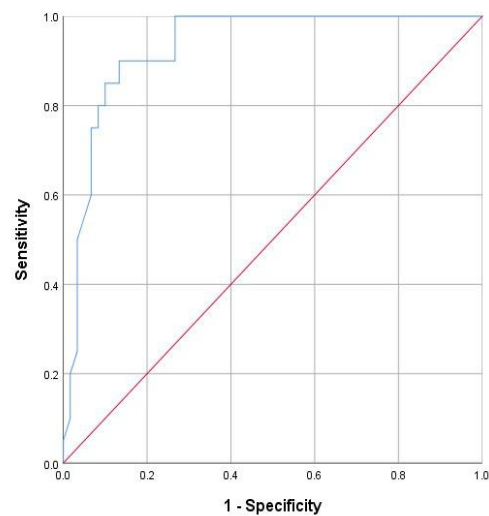
r Spearman rank correlation coefficient \*\*P≤0.001 is statistically highly significant. \*P<0.05 is statically significant

**Table 4:** Multivariable regression analysis for predicting independent variables affecting miRNA-1281 in group NPDR

HbA1c (%)			r	p		
			0.519	<0.001**		
	Unstandardized Coefficients	Standardized Coefficients	t	p	95.0% Confidence Interval	
	β	Std. Error	Beta		Lower	Upper
(Constant)	1.594	0.932		1.710	0.104	-0.364 3.552
HOMA-IR (μIU/mL)	0.428	0.132	0.606	3.233	0.005*	0.150 0.706

r Partial correlation coefficient \*P&lt;0.05 is statistically significant \*\*P≤0.001 is statistically

\*P&lt;0.05 is statistically significant

**Figure 1:** ROC curve showing performance of miRNA 1281-fold change in diagnosis of (A) proliferative diabetic retinopathy, (B) non-proliferative

## Discussion

Chronic hyperglycemia in both type 1 and type 2 diabetes is associated with the development of long-term microvascular and macrovascular complications [14]. DR is one of the leading causes of vision loss.

It is frequently the first microvascular complication to appear, and the risk of developing DR is directly related to the duration of diabetes and to the level of metabolic control [15].



MiRNAs are an evolutionarily conserved class of short non-coding single stranded RNAs, which recently have been implicated in the pathophysiology of many human diseases, including type 2 diabetes, as well as in the inflammatory and endothelial dysfunction that can trigger the development of vascular complications in diabetes <sup>[16]</sup>.

The application of circulating miRNAs as biomarkers for DR has gained much attention, particularly, in aberrant angiogenic growth of retinal endothelial cells, by adversely affecting the levels of vascular endothelial growth factor A, a secreted mitogen which seems to play a role in DR, by inducing neovascularization and vascular permeability of retinal vessels <sup>[17]</sup>.

In the current study, serum miR-1281 expression profile in the studied group showed a significant up-regulation in disease group compared with healthy control.

In Greco et al. study after comprehensive serum miRNA 1281 profiling and validation of individual miRNAs, 5 circulating miRNAs appeared to be highly up-regulated in patients with DR, compared to diabetic patients without DR. In particular, one of them, miR-1281, was the most up-regulated and seemed to be more specifically related to DR, displaying the strongest sensitivity and specificity in detecting this microvascular complication of diabetes <sup>[17]</sup>.

Serum miRNA is apparently steady both in vivo and in vitro circumstances with multiple external conditions such as pH, temperature and pressure. Generally, DR was characterized by microvascular structural and functional abnormalities, involving occlusion and leakage of retinal vessels, resulting in macular edema in the non-proliferative phase and angiogenesis and to tufts of highly permeable vessels in the proliferative phase. As a consequence, specific miRNAs could be selectively excessively secreted into the circulatory system through destroyed blood-retinal

barrier via small membrane vesicles, such as exosomes, that are released into the extracellular environment. Recently, there is growing evidence that the expression patterns and levels of specific miRNAs could reveal altered physiological and pathological conditions in both the microvascular and macrovascular complications of patients with T2D, and circulating miRNA is widely recognized to be as an attractive novel biomarker <sup>[18]</sup>.

MiR-1281, that targets and binds to substantial genes and key factors of insulin/insulin-like signaling pathway, has been previously verified to be related to metabolic syndrome, diabetes and its complications. Furthermore, under hyperglycemic conditions, significantly up-regulated miR-1281 could induce cell proliferation, migration and apoptosis, lead to facilitate retinal vascular endothelial dysfunction, promote retinal vascular hyperplasia and occlusion to pursue retinal ischemia as well hypoxia, and expedite retinal neovascularization. Consequently, aberrantly increased miR-1281 can be detected in serum of patients with T2D, then gradually increased with the severity of DR which could be attributed to exacerbated blood-retinal barrier breakdown to aggravate casual retinal vascular damage and dysfunction exposed to hyperglycemic environment. Therefore, it has been implied that miR-1281 may be involved in angiogenesis, suggesting a new theory for DR in patients with T2D <sup>[10]</sup>.

In our study, for diagnosing proliferative DR, an optimal cutoff value of  $\geq 3.085$  was established, demonstrating robust diagnostic capability with a high area under the curve (AUC) of 0.931. At this cutoff, sensitivity and specificity were notably high at 90% and 86.7%, respectively, emphasizing its effectiveness as a diagnostic marker. Similarly, for non-proliferative DR, an optimal cutoff of  $\geq 1.598$  was determined, presenting strong diagnostic ability with an AUC of 0.933. The sensitivity and specificity at this

cutoff were 85% and 80%, respectively. These results underscore the potential of miRNA 1281-fold change as a diagnostic marker for both proliferative and non-proliferative DR, with statistically significant findings.

The current study investigated the correlation between miRNA 1281-fold change and a range of parameters. Notable significant positive correlations were discovered, establishing a connection between miRNA 1281-fold change and key health indicators. These included age, platelet count, fasting blood glucose, HbA1c, fasting insulin, HOMA-IR, urea, creatinine, UACR, total cholesterol, LDL, and triglycerides. Interestingly, there was an absence of statistically significant correlations between miRNA 1281-fold change and total leukocyte count (TLC) or HDL cholesterol in the studied participants, underlining specific associations between miRNA 1281 and these health markers.

Supporting these findings, Sacks et al. reported in their study that triglycerides was significantly and independently associated with diabetic microvascular disease <sup>[19]</sup>. Moreover, consistent with existing evidence on diabetic complications, Toth et al. highlighted significant independent associations between LDL-cholesterol, triglycerides, and non-HDL - cholesterol with risks for microvascular complications among patients with type 2 diabetes mellitus (T2DM). HDL- cholesterol showed no significant differences between groups. These insights reinforce the critical importance of considering lipid profiles in understanding and addressing diabetic complications <sup>[20]</sup>.

Finally, a linear stepwise regression analysis was conducted in non-proliferative retinopathy patient group, focusing on factors significantly correlated with miRNA 1281-fold change. The results highlighted HOMA-IR as the singular significant independent association. HOMA-IR exhibited a

substantial positive association with miRNA 1281-fold change in this patient group, underscoring its potential as an independent predictor for miRNA 1281-fold change in non-proliferative DR. These findings collectively offer crucial insights into the nuanced correlations and potential predictive factors associated with miRNA 1281-fold change in the context of DR.

In the context of correlations, Greco et al. conducted a Spearman univariate correlation analysis, revealing associations between certain miRNAs and gender, BMI, and DR. Specifically, miR-1281 expression showed a strong correlation with DR, suggesting its specificity for this diabetic complication. This association was further validated through multiple linear regression analysis, solidifying the potential of miR-1281 as a specific marker for DR, considering covariates like age, sex, BMI, and duration of diabetes <sup>[17]</sup>.

Finally, the current cross-sectional study implies that miR-1281 as a potential biomarker could be related to occurrence and progression of DR in patients with T2D. Nevertheless, a prospective, high-quality, large sample, long-term clinical trial coincided with experimental research is warranted to be further investigated.

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## Conclusion

Serum miR-1281 as a potential biomarker could be related to not only occurrence but also progression for DR in patients with T2D. However, a prospective clinical trial is warranted.

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## Author contribution

Authors contributed equally in the study.

## Conflicts of interest

No conflicts of interest

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