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ORIGINAL ARTICLE

Assessment of Vascular Endothelial Growth Factor mRNA and miR-200b Levels in Type 2 Diabetes Mellites in Correlation with Clinicopathological and Anatomical Distribution of Diabetic Foot Ulcer

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ABSTRACT

Background: Diabetic foot ulcer (DFU) is one of the most distressing complications in patients with type 2 diabetes mellitus (T2DM). Vascular endothelial growth factor (VEGF) is a strong angiogenic factor associated with wound healing and development. This study aimed to investigate the VEGF mRNA and miR-200b levels in patients with T2DM and to discover their relations with the clinicopathological and anatomical distribution of DFU.

Methods: This case-control study enrolled 70 patients with T2DM and 70 healthy subjects as controls. Physical and neurological examination to assess Wagner classification. RT-PCR was done to assess VEGF mRNA and miR-200b expression levels.

Results: There were significantly higher values of VEGF mRNA levels in patients with DFU (3.13±1.87) compared to patients without DFU (2.41±0.197) and controls (1.07±0.363), with P value <0.001* Additionally, miR-200b levels were significantly higher in patients with DFU (3.48±1.47) compared to patients without DFU (2.94±0.187) and controls (1.13±0.37), with P value <0.001*. The power of VEGF mRNA and miR-200b levels as a diagnostic marker for DFU were projected (2.5 and 3.1, respectively), which yielded a sensitivity of (77.1% and 74.3, respectively) and a specificity of (77.3% and 75.2%), with the AUC at (0.886 and 0.845, respectively). The size of DFU in cm was 3.5±0.421 and the number of ulcers was 2.45±0.433.

Conclusions: The VEGF mRNA and miR-200b levels were significantly higher in patients with T2DM, particularly those with DFU. Consequently, we believed that VEGF mRNA and miR-200b might serve as promising predictive biomarkers for diabetes and DFU.

Keywords: VEGF mRNA; miR-200b; Diabetic foot ulcer; Anatomical distribution; ABI.

INTRODUCTION

Accumulating evidence indicates that diabetic foot ulcers (DFU) are one of the most distressing complications in patients with type 2 diabetes mellitus (T2DM). Indeed, DFU has been considered the most morbid complication of uncontrolled diabetes with long duration [1]. Considering the prevalence of DFU worldwide, about 19% to 34% of patients with diabetes mellitus [2]. Regards the distribution of DFUs in Africans it

is about 7.2%, Asians it is about 5.5% and 3% of Europeans had DFU [3]. In our country Egypt, the distribution of DFUs ranges from 6.1% - 29.3% [4].

It must be noted that DFU leads to amputation in 20% of patients [5]. It has long been established that the pathogenesis of DFU is classified into three types: neuropathic (35%), ischemic (15%), and mixed neuroischemic (50%) [6].

Mounting evidence showed that miRNAs are a cluster of small non-coding RNAs that can target many diverse mRNAs and are involved in several biological processes [7]. It has been demonstrated that the dysregulation of miRNAs often contributes to various skin pathologies [8].

Interesting evidence reveals that vascular endothelial growth factor (VEGF) is a strong angiogenic factor associated with wound healing and development [9]. Importantly, eminent researchers found that VEGF plays a vital role in angiogenesis [9] and endothelial function regulation [11]. Emerging evidence has suggested VEGF is unique for its effects on multiple components of the wound healing cascade, including angiogenesis as well as epithelization, and collagen deposition [12].

Strong evidence proposes that DFU is the main cause of disability in T2DM. It is now widely recognized that complicated DFU leads to amputation and death. Therefore, we indeed for the early prediction of DFU. Thus, this study was designed to investigate VEGF mRNA and miR-200b in Egyptian patients with T2DM and to discover their correlations with clinicopathological and anatomical distribution DFU.

METHODS

The current research was a case-control study conducted on 70 patients with T2DM and 70 healthy subjects as controls. The diagnosis of T2DM was made according to the American Diabetes Association Criteria. Patients with T2DM are classified into 2 groups 35 patients without DFU and 35 patients with DFU. The study groups were matched in gender and age. The Clinical evaluation of DFU was performed including assessment of DFU size, site, and duration. The foot ulcer was diagnosed and classified according to Wagner's Classification as shown in Table 1 in supplementary [13]. The ankle-brachial index (ABI) was calculated. We have assessed the grade of neuropathy by NDS. The exclusion criteria were cancer, autoimmune, and other chronic diseases. Additionally, patients receiving any drugs that affect results were excluded from the study.

Laboratory evaluation was done for the studied participants enrolled from the Departments of Internal Medicine and Tropical Medicine. Testing was done according to operating techniques in Zagazig University Hospital and Medical Microbiology and Immunology laboratories. The study protocol was approved by the Ethical Committee of the Faculty of Medicine, Zagazig University and the reference number was IRB (Ethics number. 10628), and each participant signed a written informed consent document.

Quantitative real-time RT-PCR for assessment of VEGF mRNA and miR-200b

The RNA was obtained from EDTA peripheral blood samples according to the company's directions. The relative expression of miR-200b was calculated using $2^{-\Delta\Delta Ct}$ (Ct, cycle threshold) with U6 sn RNA as the internal reference Regards primer sequence miR-200b Forward,5′-GCGGCTAATACTGCCTGGTAA-3′ reverse,5′-GTGCAGGGTCCGAGGT-3′ U6, forward: 5′- -3′ reverse, 5′-TTCACGAATTTGCGTGTCAT-3′. While Human GAPDH was the housekeeping gene for VEGF mRNA. The following primer pairs were used for VEGF mRNA Forward, 5'-TGCAGATTATGCGGATCAAACC -3', reverse, 5'- TGCATTCACATTTGTTGTGCTGTAG -3', GAPDH; Forward, TGAACGGGAAGCTCACTGG and reverse, TCCACCACCCTGTTGCTGTA.

Statistical analysis: Data was analysed by using SPSS Statistics for Windows, Version 26.0 (IBM SPSS Statistics for Windows, Version 26.0. Armonk, NY: IBMCorp), and $P < 0.05$ was considered statistically significant. the Kolmogorov–Smirnov test method was used to test the normality of the data. Pearson's and Spearman correlation coefficients miR-200b and VEGF mRNA with other studied parameters were done.

RESULTS

A case-control study registered 70 patients with T2DM (35 patients without DFU and 35 patients with DFU) and 70 healthy control groups. Our results indicate clinical parameters, for example, duration of diabetes (years), systolic blood pressure, diastolic blood pressure, and BMI. There were significant differences between studied groups. On the other hand, ABI, value was significantly lower in patients with DFU compared to others. Regarding the anatomical features of DFU, the size of DFU in cm was 3.5±0.421 and the number of ulcers was 2.45±0.433, P<0.001* Table 2.

Comparison between laboratory parameters of patients with T2DM subgroups.

It is interesting to note that metabolic disorders in the form of dyslipidemia, and HbA1C were significantly different between studied groups. Interestingly, renal function was impaired in patients with DFU in comparison with other groups, P<0.001* Table 3.

Clinicopathological features and anatomical distribution of DFU.

There were significant differences between patients with DFU as regards, severity of neuropathy as assessed by Neuropathy Disability Score (NDS) and peripheral vascular disease, P value <0.001* Table 4.

The assessment of anatomical features of DFU revealed that there were significant differences regarding the foot affected by DFU, location of DFU, and severity of DFU according to Wagner classification, P value <0.001* Table 3.

Comparison of VEGF mRNA and miR-200b level in studied groups

To elucidate whether VEGF mRNA and miR-200b levels differ between the studied group, we analyzed the results, and it is interesting to note that there were significantly higher values of VEGF mRNA levels in patients with DFU (3.13±1.87) compared to patients without DFU (2.41±0.197) and controls (1.07±0.363), P value <0.001* figure1. Remarkably, miR-200b levels were significantly higher in patients with DFU (3.48±1.47) compared to patients without DFU (2.94±0.187) and controls (1.13±0.37), P value <0.001* table 2 and figure1.

Correlations between VEGF mRNA and miR-200b level with other studied parameters

In the DFU group, VEGF mRNA and miR-200b levels were significantly positively correlated with the duration of diabetes, TG, HbA1c, UACR, and ABI, P value <0.01* (Table 4). On the other hand, miR-200b and VEGF mRNA levels were significantly negatively correlated with HDL and eGFR, P value of <0.01* (Table 5).

Regarding VEGF mRNA and miR-200b levels correlations with the clinicopathological and the anatomical distribution of DFU, we have performed Spearman correlation, and we detected a significant positive correlation between VEGF mRNA and miR-200b with NDS, location and severity of DFU, P value of <0.01* (Table 5).

Regarding VEGF mRNA, a linear regression test revealed that among the parameters studied DNS, ABI, UACR and duration of diabetes were the main predictors, P-value <0.05*. However, the severity of DFU, ABI, UACR, and duration of diabetes are the main predictors of miR-200b levels, P value of <0.01* (Table 6).

Based on the ROC curve, the optimal cutoff values of VEGF mRNA and miR-200b levels as a diagnostic marker for T2DM were projected to be (1.52 and 1.55, respectively), which yielded a sensitivity of (88.6% and 88.4, respectively) and a specificity of (88.1% and 86.7%), with the AUC at (0.969 and 0.935, respectively), P value of <0.01*(supplementary Fig. 1).

The ROC curve results regard the optimal cutoff values of VEGF mRNA and miR-200b levels as a diagnostic marker for DFU were projected to be (2.5 and 3.1, respectively), which yielded a sensitivity of (77.1% and 74.3, respectively) and a specificity of (77.3% and 75.2%), with the AUC at (0.886 and 0.845, respectively). P value of <0.01*(Fig. 2).

Table 1: Comparison between Clinical, demographic and anthropometric characteristics of patients with T2DM subgroup.

Variables	Control group (n=70)	Patients without DFU (n=35)	Patients with DFU (n=35)
Age (years)	50.17±8.42	51.5±9.01	52.2±9.58
Gender Male Female	29(41.4%) 41(48.6%)	18(51.4%) 17(48.6%)	21(60%) 14(40%)
Duration of diabetes (years)	-	6.94±1.5	11.07±0.27$^{£}$
Body mass index (kg/m^2)	22.18±1.8	36.18±6.8*	37.13±6.7$^{$}$
Systolic blood pressure	112.4±3.4	137.8± 3.4*	141.8± 9.4$^{$}$
Diastolic blood pressure	73.6±2.3	85.6±9.5*	87.9±10.3$^{$}$
ABI	1.45±0.29	0.9±0.19*	0.6±0.14$^{$}$

miR-200b	1.13±0.37	2.94±0.187*	3.48±1.47$
VEGF mRNA	1.07±0.363	2.41±0.197*	3.13±1.87$
Complication of diabetes			
Retinopathy	-	9 (25.7%)	11(31.4%)
Microalbuminuria	-	10 (28.6%)	12(34.3%)
Stroke	-	3(8.6%)	5 (14.3%)
CHD	-	12(34.3%)	15(42.9%)

DFU, diabetic foot ulcer; CHD, coronary heart disease, ABI; ankle brachial index.
*Significant P values (P < 0.05) when comparing the control group with patients without DFU group.
$ Significant P values (P < 0.05) when comparing the control group with patients in the DFU group.
£ Statistically significant P values (P < 0.05) when comparing patients without DFU group with patients in the DFU group.

Table2: Comparison between laboratory parameters of patients with T2DM subgroups.

Variables	Control group (n=70)	Patients without DFU (n=35)	Patients with DFU (n=35)
TC (mg/dl)	187.3±23.5	210.3±31.9*	234.88±22.1$, £
TG (mg/dl)	141.3±15.9	153.26±21.19*	184.16±25.6$, £
LDL (mg/dl)	104.3±11.2	121.08±30.07*	136.91±20.4$, £
HDL (mg/dl)	55±2.9	41.48±2.87*	38.25±4.63$, £
HbA1C (%)	4.3±1.3	7.38±1.87*	9.78±1.87$, £
eGFR (mL/min)	91.3±8.9	84.37 ±12.5*	58.19±13.2$, £
Serum creatinine (mg/dl)	0.93±0.04	0.87±0.189*	1.21±0.312$, £
UACR (mg/g)	20.3 ±1.6	26.72±2.1*	186±1.4$, £
Serum urea (mg/dL)	21.3 ±4.5	18.3 ±4.6*	27.9±7.18$, £

TC, total cholesterol; TG, triglycerides; LDL, low-density lipoprotein; HDL, high-density lipoprotein-cholesterol HbA1c, hemoglobin A1c; eGFR, estimated glomerular filtration rate.
; UACR, urine albumin-creatinine ratio.
*Significant P values (P < 0.05) when comparing the control group with patients without DFU group.
$ Significant P values (P < 0.05) when comparing the control group with patients in the DFU group.
£ Statistically significant P values (P < 0.05) when comparing patients without DFU group with patients in the DFU group.

Table 3: Clinicopathological characteristics and anatomical distribution of diabetic foot ulcer

Characteristics	DFU group(n=35) Number (%)	χ^2	P value
Neuropathy (NDS):			
Absent 0-2	2	10.60	0.014
Mild 3-5	7		
Moderate 6-8	15		
Sever 9-10	11		
Peripheral Vascular Disease:			
No disease	2	31.269	0.000
Claudication	23		
Gangrene	5		
DVT	5		

Foot affected by DFU: Right Left Both	 19 12 4	9.657	0.008
Location of DFU: **Dorsum** No Yes **Plantar** No Yes **Heel** No Yes **Toes** No Yes	 25 10 22 13 9 26 28 7	 6.249 2.314 8.257 12.60	 0.011 0.128 0.004 0.000
Severity of DFU (Wagner classification) Grade 1 Grade 2 Grade 3 Grade 4 Grade 5	 1 9 15 10 0	11.514	0.009

NDS, Neuropathy Disability Score; ABI: ankle brachial index; BMI: body mass index, Hba1C: glycosylated hemoglobin, DVT: deep venous thrombosis, * Significant P value (P < 0.05).

Table 4: Correlations between VEGF mRNA and miR-200b with other parameters studied in Patients with DFU.

Variables	miR-200b		VEGF mRNA	
	r	p	r	p
Duration of diabetes (years)	0.611	<0.001*	0.416	<0.001*
BMI	0.042	0.732	0.009	0.994
TC	0.223	0.064	0.220	0.067
TG	0.451	<0.001*	0.551	<0.001*
LDL	0.198	0.100	0.085	0.484
HDL	-0.398	<0.001	-0.567	<0.001*
HbA1c	0.429	<0.001*	0.629	<0.001*
eGFR	-0.470	<0.001*	-0.373	<0.001*
UACR	0.657	<0.001*	0.438	<0.001*
ABI	-0.651	<0.001*	-0.622	<0.001*
Severity of DFU	0.792	<0.001*	0.744	<0.001*
NDS	0.813	0.100	0.770	0.484
Location of DFU	0.790	<0.001*	0.758	<0.001*

* Significant P value *(P* < 0.05).

Table 5: linear regression analyses in DFU to test the influence of the main independent variables against miR-200b and VEGF mRNA levels (dependent variable).

Model		Unstandardized Coefficients		Standardized Coefficients	t	P value	95.0% C.I	
		B	Std. Error	Beta			Lower Bound	Upper Bound
VEGF mRNA	(Constant)	1.306	0.207		6.298	0.000	0.891	1.720
	DNS	0.168	0.075	0.372	2.228	<0.05*	0.017	0.318
	Severity of DFU	0.101	0.109	0.096	0.934	0.354	-0.116	00.319
	Location of DFU	0.003	0.115	0.003	0.027	0.978	-0.226	0.232
	ABI	0.799	0.129	0.251	6.218	<0.001*	.542	1.056
	HbA1c	-0.032	0.018	-0.090	-1.767	0.082	-00.068	0.004
	UACR	0.005	0.001	0.521	6.598	<0.001*	0.004	.007
	Duration of diabetes	0.073	0.019	0.236	3.893	<0.001*	0.035	0.110
miR-200b	(Constant)	1.859	0.369		5.032	0.000	1.120	2.598
	DNS	0.165	0.134	0.345	1.231	0.223	-0.103	0.433
	Severity of DFU	0.409	0.193	.366	2.114	<0.05*	0.022	0.796
	Location of DFU	-0.321	0.204	-0.329	-1.572	0.121	-0.729	0.087
	ABI	0.772	0.229	0.229	3.372	<0.001*	0.314	1.230
	HbA1c	-0.033	.032	-0.088	-1.031	0.306	-0.098	0.031
	UACR	0.006	00.001	0.538	4.060	<0.001*	.003	0.008
	Duration of diabetes	0.074	0.033	0.225	2.211	<0.001*	0.007	0.141

* Significant P value ($P < 0.05$).

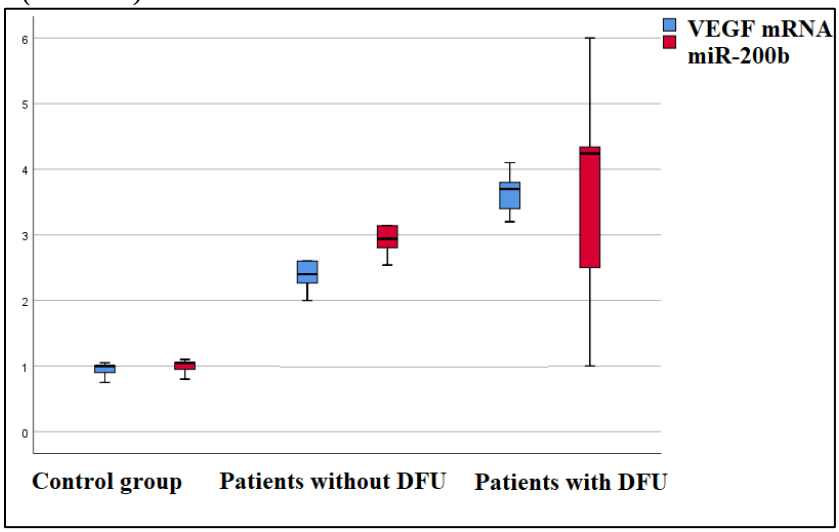

Figure 1: Comparison of VEGF mRNA and miR-200b level in studied groups

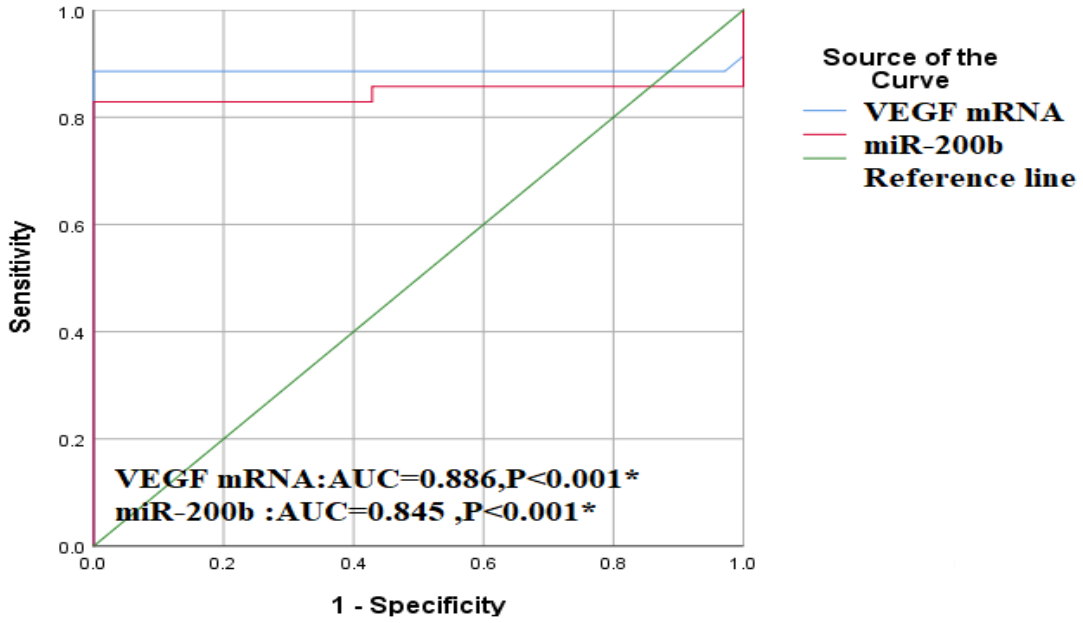

Figure 2: The accuracy of VEGF mRNA and miR-200b level for distinguishing patients with DFU from others without DFU.

DISCUSSION

An increasing number of studies have shown that DFU is one of the main reasons for morbidity and mortality among patients with T2DM and it is well established that treatment of DFU is costly [14]. Currently, several evidence exist, demonstrating that screening for DFU may meaningfully improve the morbidity and mortality of patients with DFU. Previously, it was shown that there are 2 major causes of DFU: diabetic neuropathy and PVD [15].

The results of the current research observed that patients with DFU had statistically significant higher values of metabolic disorders in the form of dyslipidaemia, Hb A1c. Interestingly, renal function was impaired in patients with DFU in comparison with other groups .

Similar results were detected in Parchman and his colleagues' study as they found that poor glycaemic control was associated with DFU [15]. Interesting studies have also demonstrated that PAD is one of the key causes of DFU [16,17]. In-depth studies have found that neuropathy is an important cause of DFU [18].

Based on clinicopathological characteristics and anatomical distribution of diabetic foot ulcers, the current research found that there were significant differences regards the severity of neuropathy, PVD, foot affected by DFU, location of DFU, and severity of DFU. In this context, Vahwere *et al* proposed that most DFUs were located on the right foot and more specifically in the plantar area of the foot [19].

Despite the absence of a clear mechanism of endothelial dysfunction's role in DFU pathogenesis, there is ongoing progress toward VEGF inducing endothelial cell growth within the collagen matrix [20]. Mounting evidence showed that the epigenetic dysregulation induced by "metabolic memory" affects several markers, such as miRNAs implicated in diabetic ulcer healing [21].

we aimed in the current research to explore the VEGF mRNA and miR-200b levels in T2DM and to explore their relations with the clinicopathological and anatomical distribution of DFU. Remarkably, the results presented here are pioneering as this study executes a sturdy estimation of VEGF mRNA and miR-200b levels in patients with T2DM.

The most important finding in the current research is that there were significantly higher values of VEGF mRNA and miR-200b levels in patients with DFU compared to patients without DFU and controls. Additionally, VEGF mRNA and miR-200b levels were significantly positively correlated with the duration of diabetes, TG, Hb A1c, UACR, and ABI. Regards correlations with clinicopathological and anatomical distribution DFU, we have perceived a significant positive correlation between VEGF mRNA and miR-200b with NDS, location, and severity of DFU.

For further evaluation of these findings, we performed a linear regression test and we observed that among the studied parameters DNS, ABI, UACR, and duration of diabetes were the main predictors of VEGF mRNA. Nevertheless, the severity of DFU, ABI, UACR, and duration of diabetes are the main predictors of miR-200b levels.

An interesting study conducted by Dangwal *et a.l* observed that T2DM patients with both peripheral arterial disease and DFU had higher values of miR-200b. Nevertheless, they found that T2DM patients with PAD alone without wound had similar levels of miR-200b compared to T2DM patients. Thus, they suggested that inflammation and angiogenesis in wound healing are associated with high levels of miR-200b [22].

This evidence is supported by Cappellari *et al.* who reported that despite higher levels of pro-angiogenic factors in T2DM, angiogenesis is impaired in DFU. This could be attributed to decreased expression levels of pro-angiogenic which makes them unresponsive to the angiogenic factors [23]. Concomitantly, Ott *et al.* detected impairment of eosinophils and neutrophils in T2DM and they explained their findings that elevated levels of glycated products lead to inflammation and endothelial dysfunction which contribute to impairment of eosinophils and neutrophil's function [24].

Regarding the role of VEGF in the pathogenesis of diabetic ulcers, Del Cuore *et al.* detected higher levels of VEGF in patients with DFU. They conducted their study to evaluate the impact of epigenetic changes on miRNA and proangiogenic molecules (e.g., ENOS, VEGF, and HIF-1alpha) in diabetic patients with or without DFU and they found that HIF-1alpha regulates several target genes, particularly VEGF [25]. These findings are consistent with other studies [26].

For further evaluation of our interesting findings, we analyzed our results by linear regression test which revealed that among the studied parameters duration of diabetes, HbA1c, and ABI were the main predictors of VEGF mRNA. While, duration of diabetes, UACR, and ABI are the main predictors of miR-200b levels. To evaluate the diagnostic power of VEGF mRNA and miR-200b levels, we used the ROC curve. Based on its results, the AUC was 0.969 with sensitivity = 88.6%, specificity = 88.1 %, and the cutoff values were (1.52). While miR-200b level, the AUC was 0.935 with sensitivity = 88.4 %, specificity = 86.7 %, and the cutoff values were (1.55).

This study has several unique strengths. To date, according to our information, no study has evaluated the role of VEGF mRNA and miR-200b as predictor markers of DFU among Egyptian patients with T2DM. Our study also has a few potential limitations. The research was conducted on Egyptians only, and therefore, it remains unclear whether our findings apply to other ethnic groups.

CONCLUSIONS

High levels of VEGF mRNA and miR-200b were found in patients with T2DM particularly patients with DFU. Even more importantly, VEGF mRNA and miR-200b levels were significantly correlated with the duration of diabetes, TG, HbA1c, UACR, ABI. NDS, location, and severity of DFU. Thus, early prediction of DFU among T2DM decreases morbidity and mortality from DFU and its complications.

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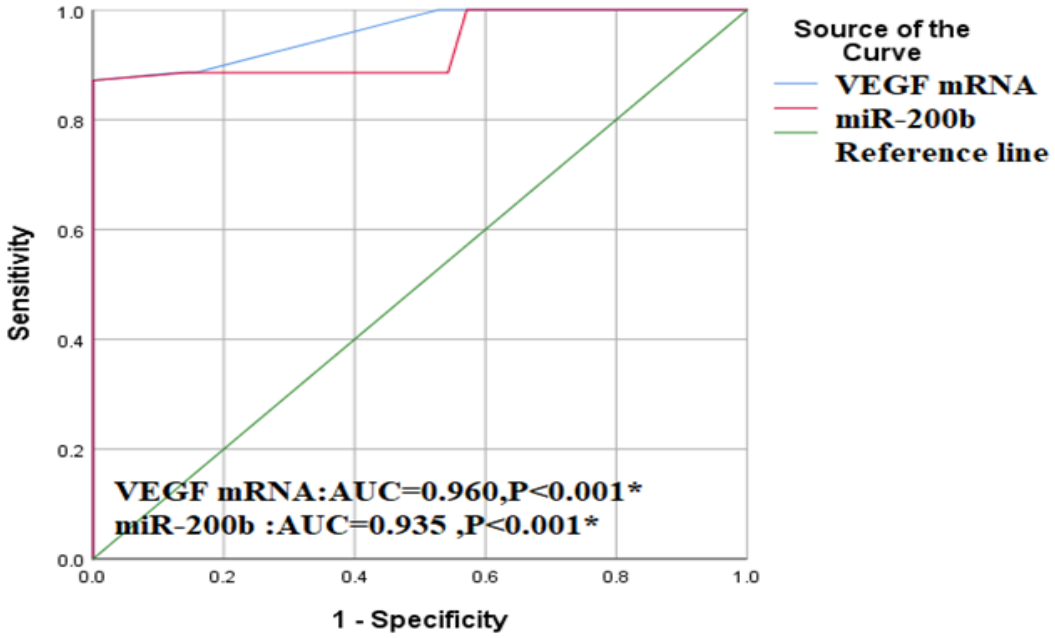
Supplementary table 1: Wagner's classification of diabetic foot ulcers.

Wagner's Classification	
Grade 0	Skin intact but bony deformities lead to "foot at risk"
Grade 1	Superficial ulcer
Grade 2	Deeper, full thickness extension
Grade 3	Deep abscess formation or osteomyelitis
Grade 4	Partial Gangrene of forefoot
Grade 5	Extensive Gangrene



Supplementary figure 1: The accuracy of VEGF mRNA and miR-200b level for discriminating patients with T2DM from the control group by ROC curve.

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