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ORIGINAL ARTICE

Diagnostic and prognostic significance levels of tumor necrosis factor (TNF)-α serum and mRNA expression in patients with infected diabetic foot ulcers

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ABSTRACT

Background: Infected diabetic foot ulcer (IDFU) is among the most common complications of Type 2 diabetes mellitus (T2DM), significantly leading to lower extremity amputation. Tumor Necrosis Factor-alpha (TNF-α) is a cytokine with pleiotropic effects on different tissues. This study aimed to investigate TNF-α mRNA and serum levels in Egyptian patients with diabetic foot ulcers in correlation with the risk and severity of IDFU. **Methods**: We enrolled 100 patients with T2DM and 100 healthy subjects. All patients were subjected to thorough history taking, complete clinical and neurological assessment, and foot ulcers were examined for size, site, and duration. The level of TNF-α was measured using an Enzyme-linked immunosorbent assay, and the TNF-α mRNA level was determined by quantitative real-time PCR. **Results:** there were significantly higher values of TNF -α mRNA and serum levels in patients with DFU (4.69±0.97,19.7±6.12, P <0.001* respectively) compared to controls (0.92±0.084,3.57±0.52, P <0.001* respectively). There were significantly higher values of TNF -α mRNA and serum levels in patients with IDFU (4.98±0.52,22.98±5.56, P <0.001* respectively) compared to patients without IDFU (3.8±1.41,9.95±1.42, P <0.001* respectively). Serum and TNF-α mRNA levels were significantly positively correlated with the duration of diabetes, BMI, HbA1c, ESR, and WBC. Linear regression tests revealed that duration of diabetes, BMI, and WBC were the main predictors of serum TNF-α levels, but only ESR was the main predictor of TNF-α mRNA levels, P <0.001*. **Conclusions**: TNF-α mRNA and serum levels are elevated in DFU and IDFU and positively correlate with the risk and severity of IDFU.
Key Words: Infected diabetic foot ulcer; Type 2 diabetes mellitus; Tumor Necrosis Factor-alpha.

INTRODUCTION

Diabetes mellitus is a major global health issue, affecting more than 382 million patients worldwide. Numerous studies conducted in diverse populations have greatly advanced our knowledge about the prevalence of diabetes worldwide [1]. Poorly controlled diabetes can predispose patients to diabetic vascular complications which are a multifactorial condition associated with several risk factors such as HbA1c levels, hypertension, smoking status, and BMI, which also has a genetic component [2].

Even though there are many complications affecting the person with diabetes, none are more devastating than those complications involving the foot [3]. Diabetic foot lesions have significant health and socioeconomic problems conducting adverse effects on the patient's quality of life and economy [4].

Diabetic foot ulcers (DFUs). It has been estimated that 15% of diabetics will develop a DFU in their lifetime [5]. The etiology of DFUs typically reflects trauma superimposed upon peripheral neuropathy and ischemia. Such diabetic foot ulcers commonly become sources of intransigent infection, whereupon they may be termed diabetic foot infections (DFIs). Unfortunately, DFIs can become complicated by osteomyelitis [6].

Accumulating evidence indicates that the management of DFIs is limited to wound care, antibiotics, and amputation [7]. These infections can be difficult to treat and, despite the administration of multiple rounds of antibiotics, prospects of clinical resolution of infection can still be poor and repeated courses of antibiotics risks selecting for antimicrobial resistance [8].

Sequencing studies of complex diseases, like T2D, have demonstrated little success in identifying that proinflammatory cytokines and chemokines are essential for the normal skin wound-healing process. Interestingly, TNF-α was expressed by both polymorphonuclear leukocytes and macrophages in the early phase of wound healing and has expression in the hyperproliferative epithelium at the wound edge [10].

The identification of genes, chemokines, and immune cells involved in DFIs is required for targeting the most relevant pathways in the pathogenesis of DFU and in particular IDFU to prevent limb amputation. To the best of our knowledge, there are no studies in the literature reporting the role of TNF-α mRNA and serum levels in the pathogenesis of IDFU. Thus, we aimed in the current research to explore TNF-α mRNA and serum levels in Egyptian patients with diabetic foot ulcers in correlation with the risk and severity of IDFU.

METHODS

This case-control study was conducted on 100 patients with DFU and 100 healthy subjects as a control group, both groups were matched in age and gender. All patients were subjected to thorough history taking, and full clinical and neurological assessment, and foot Ulcers were examined for size, site, and duration. the foot ulcer was diagnosed and classified according to Wagner's Classification and the University of Texas Wound Classification System [11]. DFI was diagnosed according to Demetriou et al., 2013[12].

Laboratory evaluation was done for the studied participants enrolled from the Departments of Internal Medicine and Tropical Medicine. Samples were obtained from wound sites, before starting antibiotic treatment, through biopsy specimens from deep tissues, and, if there was a purulent discharge, specimens were prepared using syringes or swabs. For isolation of aerobic bacteria gathered specimens were cultured on blood, mannitol salt, and MacConkey agar plates (Oxoid Ltd., UK). [13]. The antibiotic susceptibility of the bacteria was determined by the CLSI guidelines [14]. Testing was done according to operating techniques in Zagazig university hospital and medical microbiology and immunology laboratories, as shown in **figure1**. Written informed consent was obtained from all participants and the study was approved by the research ethical committee of the Faculty of Medicine, Zagazig University, and the reference number was IRB (Ethics number. 10628), The work has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for studies involving humans.

Quantitation of TNF-α gene expression: TNF-α

The RNA was extracted from EDTA peripheral blood samples according to the company's instructions. Human GAPDH was the housekeeping gene. The following primer pairs were used: Forward, 5′-CCAGGCAGTCAGATCATCTTC-3′, reverse, 5′-AGCTGCCCCTCAGCTTGA-3′, GAPDH; Forward, TGAACGGGAAGCTCACTGG and

reverse, TCCACCACCCTGTTGCTGTA [16]. The expression level was determined using the $2^{-\Delta\Delta CT}$ method.

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. For descriptive characterization, we used t-tests, frequencies were calculated by $\chi 2$-tests. Pearson's correlation analysis was used to determine the relationship between TNF-α mRNA and its serum levels with other studied parameters was done.

RESULTS

The current research enrolled a total of 100 participants in the case group were compared with a similar number of age and sex-matched participants of the control group as 100 of the patients had DFU (46 males and 54 females) and one hundred were healthy control subjects (43 males and 57 females).

The mean age for controls was (48.18±10.1years) for diabetic patients (51.83±9.9 years). We compared between control and patients with DFU regards metabolic and inflammatory parameters. Regards metabolic parameters for example BMI and HbA1c, there were significant differences between both groups, (p<0.001*). Concerning inflammatory parameters, ESR, WBC and CRP levels were significantly higher in patients with DFU compared to controls, p<0.001* as shown in **Table S1**.

Characteristics of patients with and without IDFU: In order to better evaluation of patients with DFU, we classified patients into two (2) groups based on the severity of DFU, early DFU (less severe) (grade 1 and grade 2) and severe or late DFU (grade 3 and above) using the Wagner classification as shown in **table S2**. There were significantly higher values of BMI, duration of T2DM (years), HbA1c, ESR, and WBC in patients with IDFU compared to patients without IDFU as shown in **table S1**.

Assessment of clinicopathological features of DFU in both studied groups revealed that there were significant differences regards severity and size of DFU between both studied groups of patients with T2DM, P value <0.001* **(Table 1)**.

However, there were non-significant differences regards, CRP, site, and duration of DFU as well as comorbidity including obesity, hypertension, dyslipidemias, and NAFLD, p>0.05.

Distribution of microbiological organisms from the deep tissue samples in patients with IDFU: Among patients with IDFU microbiological examinations of deep tissue samples revealed that 28 patients had staphylococcus aureus, 19 patients had Escherichia Coli,13 patients had Klebsiella, 8 patients had coagulase-negative staphylococci (CoNS), while 6 patients had pseudomonas as shown in **figure 2**.

Comparison of serum TNF- α(pg/ml) and TNF-α mRNA level in studied groups: There were significantly higher values of serum TNF-α levels in patients with DFU (19.7±6.12) compared to controls (3.57±0.52), P value <0.001* **(Table 1)**. Interestingly TNF -α mRNA levels were significantly higher in patients with DFU (4.69±0.97) compared to controls (0.92±0.084), with P value <0.001* as shown in **Table S1**.

Comparison of serum TNF- α(pg/ml) and TNF-α mRNA level in patients with T2DM: There were significantly higher values of serum TNF- α levels in patients with IDFU (22.98±5.56) compared to patients without IDFU (9.95±1.42), P value <0.001* **(Table 1)**. Interestingly TNF -α mRNA levels were significantly higher in patients with IDFU (4.98±0.52) compared to patients without IDFU (3.8±1.41), P value <0.001* **(Table 1)**.

Correlation between serum and expression levels of TNF-α with other studied parameters: In the IDFU group, (*n*=75), serum TNF-α levels were significantly positively correlated with duration of diabetes, BMI, HbA1c, ESR, and WBC. P-value <0.01*. Concerning TNF-α, there was a significantly positive correlation with the duration of

diabetes, BMI, HbA1c, ESR, and WBC P value <0.001* as shown in **table S3**.

Linear regression analysis In the IDFU group: Among the studied parameters duration of diabetes [odds= -0.234 (95% CI = -1.085--0.189)], BMI odds= 0.269 (95% CI = 0.747-2.046)], and WBC [odds= 0.031 (95% CI = -0.251-0.419)] were the main predictors of serum TNF-α levels, P-value <0.01*. Nonetheless, only ESR odds= 0.599 (95% CI = 00.062-0.169)]was the main predictor of TNF-α mRNA levels, P value <0.001* as shown in **table S4**.

The accuracy of serum and expression levels of TNF-α for discriminating patients with DFU from the control group: Concerning TNF- α(pg/ml) serum levels, the AUC was 0.988 (95% CI = 0.972-1.000) with sensitivity = 99%, specificity = 98 %, and the cutoff values was (6.35), **(Figure 3a)**.

Regarding TNF-α mRNA, the AUC was 0.992 (95% CI = 0.978–1.000) with sensitivity = 99 %, specificity = 94 %, and the cutoff values was (0.991), **(Figure 3b)**.

The accuracy of serum and expression levels of TNF-α for distinguishing patients with IDFU from others without IDFU: Concerning TNF- α(pg/ml) serum levels, the AUC was 0.980 (95% CI = 0.651-1.000) with sensitivity = 96%, specificity = 99 %, and the cutoff values was (13.11), **(Figure 4a)**.

Regarding TNF-α mRNA, the AUC was 0.782 (95% CI = 0.989–0.912) with sensitivity = 93.3%, specificity = 72.3 %, and the cutoff values were (4.101), **(Figure 4b)**.

Table 1: Clinicopathological and laboratory parameters of patients with and without IDFU.

Variables	Diabetic patients without IDFU, (n=25)	Diabetic patients with IDFU, (n=75)	*P value*
BMI	28.6±2.91	32.8±4.62	<0.001*
Duration of T2DM (years)	9.62±2.29	12.14±2.1	<0.001*
Duration of DFU			
<3 months	20(80%)	66(88%)	0.330
≥3 months	5(20%)	9(12%)	0.330
Severity of DFU			
Severe	9 (36%)	50(66.7%)	<0.001*
Non-severe	16(64%)	25(33.3%)	<0.001*
Size of DFU			<0.001*
<4 cm	19 (76%)	70 (93.3%)	<0.05*
≥4 cm	6 (24%)	5 (6.7%)	<0.05*
Site of ulcer			
Dorsal	3(12%)	9 (12%)	0.622
Plantar	22(88%)	66 (88%)	0.622
Comorbidity **Obesity** **Hypertension** **Dyslipidemias** **NAFLD**	 19(76%) 18(72%) 17(68%) 20(80%)	 54(72%) 53(70.6%) 52(69.3%) 53(72.6%)	0.456 0.558 0.543 0.352
HbA1c (%)	8.95±1.72	9.63±1.613	<0.001*
CRP (mg/dl)	12.9±1.45	13.26± 1.915	0.318
ESR	35.9±6.56	54.9±14.56	<0.001*
WBC	8.25±1.63	13.27± 1.73	<0.001*
TNF-α mRNA expression level	3.8±1.41	4.98±0.52	<0.001*
TNF- α(pg/ml)	9.95±1.42	22.98±5.56	<0.001*

ESR, erythrocyte sedimentation rate; HbA1c, glycated haemoglobin; WBC, white blood cell; CRP, C-reactive protein; TNF-α, tumor necrosis factor -alpha * Significant P value ($P < 0.05$).

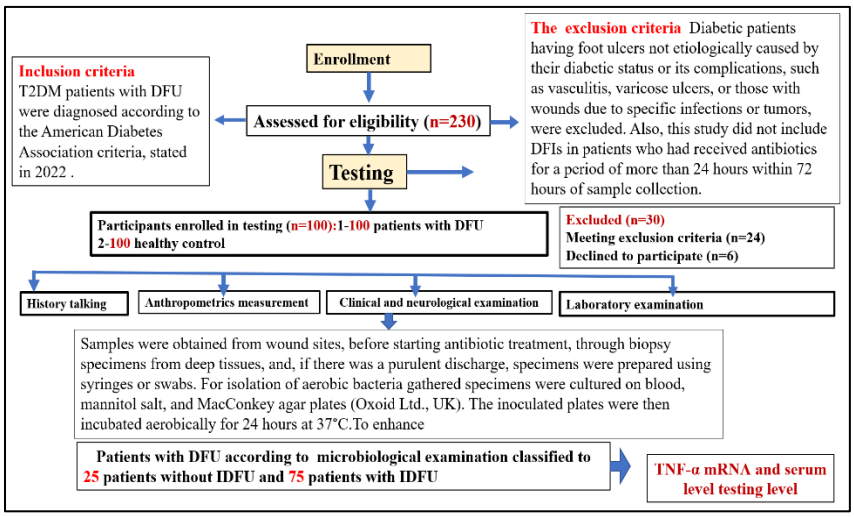

Figure 1: Flowchart of the study.

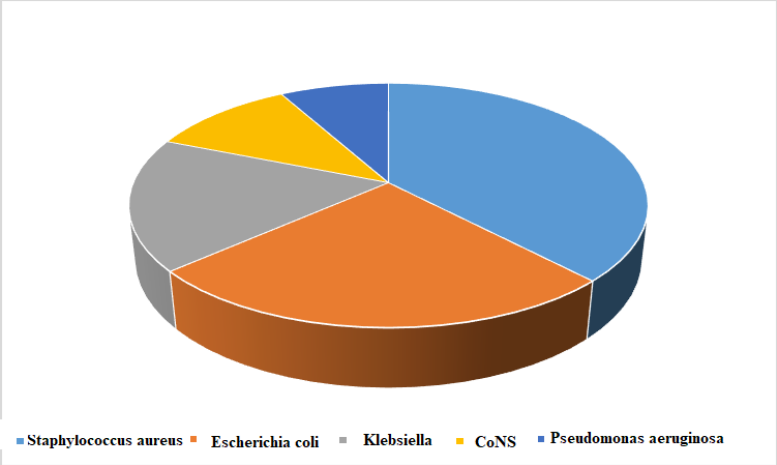

Figure 2: Distribution microbiological organisms from the deep tissue samples in patients with IDFU.

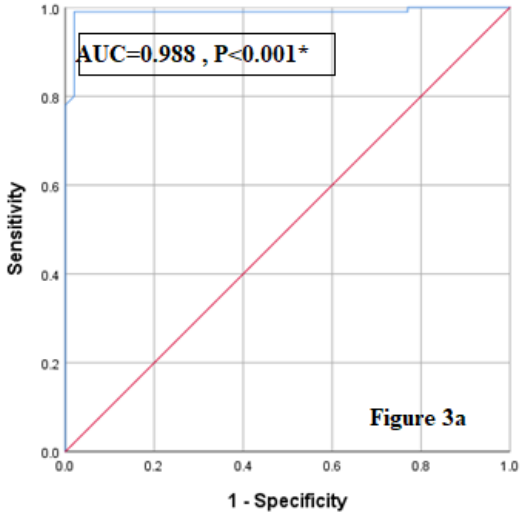

Figure 3a: ROC curve of serum TNF- α(pg/ml) level for prediction of patients with DFU among the studied groups.

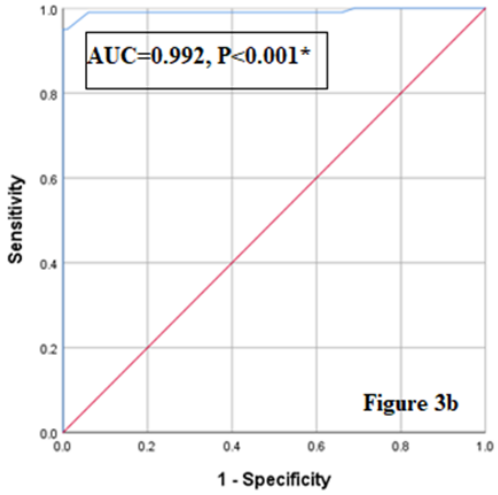

Figure 3b: ROC curve of serum expression levels of TNF-α for prediction of patients with DFU among studied groups.

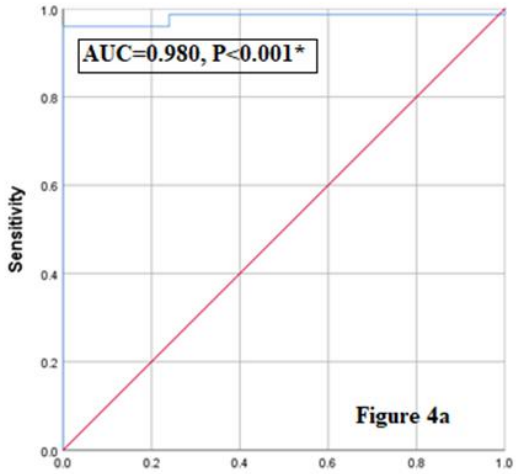

Figure 4a: ROC curve of serum TNF- α (pg/ml) level for distinguishing patients with IDFU from others without IDFU.

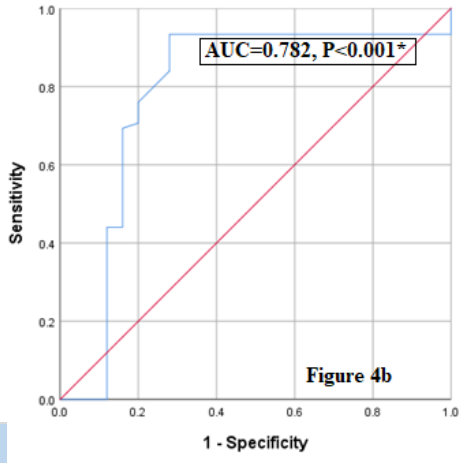

Figure 4b: ROC curve of serum expression levels of TNF-α for distinguishing patients with IDFU from others without IDFU.

DISCUSSION

Mounting evidence indicates that in diabetes, the deregulation of glucose metabolism is associated with long-term degenerative effects. Interestingly, the microvascular complication of diabetes includes retinopathy, nephropathy, and peripheral neuropathy [17].

The current research enrolled a total of 100 participants in the case group compared with a similar number of age and sex-matched participants in the control group. Similar results were detected in another Egyptian study [18] as detected that age and gender were not correlated with DFUs. Additionally, similar results were obtained by Al Kafrawy et al who observed that age and sex were not associated with DFUs [19].

In contrast, a study conducted on Iranian patients detected that male sex was a risk factor for DFU [20]. on the other hand, a Saudi study observed that female sex is a risk factor for DFU [21]. These differences could be related to variances in the study participants and methodology used.

Regards the distribution of microbiological organisms obtained from the deep tissue samples in patients with IDFU, 28 patients had staphylococcus aureus, 19 patients had Escherichia Coli, 13 patients had Klebsiella, 8 patients had coagulase-negative staphylococci (CoNS), while 6 patients had pseudomonas.

Similar to the current results, in the study conducted by Hefni et al about 40% of DFI were polymicrobial. P. aeruginosa and S. aureus were the most identified Gram-negative and Gram-positive microorganisms [22]. however Strong evidence from interesting studies reported that gram-negative bacilli are the most predominant pathogens in DFIs [23,24].

In this study, we analyzed patients with DFU (n=100). The majority of diabetic patients in the current study have IDFU (n=75). In our study, we found significantly higher serum and TNF-α mRNA levels. In the current study, there is no statistically significant difference between the two groups regarding sex.

Indeed, it has recently been reasoned that TNF-α increases the cytokine which can initiate and support the inflammatory process in the vascular wall leading to the upregulation of cellular genes involved in immune inflammation which will stimulate the synthesis of matrix metalloprotease (MMP) which causes degradation of matrix proteins and growth factors that will make wound healing process becomes improper [25].

In the current, we assess the inflammatory markers for example, CRP, ESR, WBC, and serum and TNF-α mRNA levels in patients with DFU (n=100). The majority of diabetic patients with DFU in the current study have IDFU (n=75). One of the most important findings in this study is that CRP, ESR, and WBC as well as serum and TNF-α mRNA levels were significantly higher in patients with DFU. Additionally, serum and TNF-α mRNA levels were significantly positively correlated with the duration of diabetes, BMI, HbA1c, ESR, and WBC. Interestingly, duration of diabetes, BMI, and WBC were the main predictors of serum TNF-α levels, but only ESR was the main predictor of TNF-α mRNA levels. Similarly, Siqueira, et al conducted their experimental study to assess the role of TNF-α in wound healing among mice with diabetes and they observed that diabetic wounds had increased TNF-α [26].

Accumulating evidence indicates that DFU increases apoptosis and decreases fibroblast proliferation of fibroblasts and inflammatory reactions are elongated, with a proven presence of neutrophil granulocytes in large quantities in the wound which will stimulate the synthesis of matrix metalloprotease (MMP) which causes degradation of matrix proteins and growth factors that will make wound healing process becomes disconnected and uncoordinated [27].

Similarly, Lipsky et al found higher levels of CRP, ESR, and WBC in patients with IDFU compared to non-infected ulcers [28]. Furthermore, Majeed et al confirmed the role of

inflammatory markers in differentiating infected DFUs from non-infected ones [29].

To assess the diagnostic power of both serum and TNF-α mRNA levels in discriminating DFU from the control group we performed ROC tests, and we detected that TNF- α serum levels had a sensitivity of 99%, specificity of 98 %. Considering TNF-α mRNA, the sensitivity was 99%, and the specificity was 94%. For distinguishing patients with IDFU from others without IDFU, TNF- α, sensitivity was 96%, and specificity was 99%. Regarding TNF-α mRNA, the AUC was 0.782 (95% CI = 0.989–0.912) with a sensitivity was 93.3%, specificity was 72.3 %, thus, they could be used as diagnostic markers of DFU in particular IDFU.

Conclusions: The current research results find that TNF-α mRNA and serum levels are significantly higher in DFU and IDFU and positively correlated with the risk and severity of IDFU; the duration of diabetes, BMI, HbA1c, ESR, and WBC. These findings highlight the potential of TNF-α mRNA and serum levels as biomarkers for DFU and IDFU.

The strength of the current study: This study has several unique strengths. It is the first Egyptian study ever published aiming to investigate whether TNF-α mRNA and serum levels could be used as diagnostic markers of DFU and IDFU. The diagnosis of DFU and IDFU is based on microbiological, laboratory in addition to clinical and neurological examinations. The limitation of our study is that it included only Egyptians, and therefore, it remains unclear whether our findings apply to other ethnic groups.

Recommendations: Further intervention studies should be done on large numbers and patients from different ethnicity to evaluate the role of TNF-α inhibitors in the treatment of DFU and IDFU.

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DECELERATION OF INTEREST

The authors report no conflicts of interest.

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Table S1: Clinical, anthropometric and laboratory characteristics of the studied groups.

Variables	Control group (*n* =100)	Patients with DFU, (*n* =100)	*P* value
BMI	23.4±0.74	31.79±4.62	<0.001*
HbA1c (%)	4.97±0.52	9.46±3.58	<0.001*
ESR	13.4±2.74	49.41±12.74	<0.001*
WBC	4.41±2.74	12.01±4.7	<0.001*
CRP (mg/dl)	5.77±1.55	13.18±1.81	<0.001*
TNF-α mRNA expression	0.92±0.084	4.69±0.97	<0.001*
TNF- α(ng/ml)	3.57±0.52	19.7±6.12	<0.001*

BMI, body mass index; ESR, erythrocyte sedimentation rate; HbA1c, glycated haemoglobin; WBC, white blood cell; CRP, C-reactive protein; TNF-α, tumor necrosis factor -alpha * Significant P value *(P* < 0.05).

Table S2: Severity of Foot Ulcer (Wagner Scale) (n=100).

Parameter	Values (Grade 0–5)	Diabetic patients without IDFU, (n=25), n (%)	Diabetic patients with IDFU, (n=75), n (%)	
Severity of Ulcer	No ulcer but foot at risk (Grade 0)	0(0%)	0(0%)	**χ2=7.503 P =0.057**
	Superficial ulcers (Grade 1)	5(20%)	9(12%)	
	Deep ulcers (Grade 2)	11(44%)	16(21.3%)	
	Abscessed Deep ulcers (Grade 3)	7(28%)	36(48%)	
	Limited gangrene (Grade 4)	2(8%)	14(18.7%)	
	Extensive gangrene (Grade 5)	0(0%)	0(0%)	

Table S3: Correlations between TNF-α mRNA and serum levels with other studied parameters in patients with IDFU.

Variables	TNF-α mRNA		TNF- α(ng/ml)	
	r	p	r	p
Duration of diabetes	0.435	<0.001*	0.324	<0.001*
BMI	0.411	<0.001*	0.117	0.247
HbA1c	0.427	<0.001*	0.427	<0.001*
CRP	0.112	0.266	0.096	0.343
ESR	0.598	<0.001*	0.322	<0.001*
WBC	0.443	<0.001*	0.398	<0.001*
Duration of DFU	0.205	<0.001*	0.532	<0.001*

Table S4: Linear regression analyses to test the influence of the main independent variables against TNF-α mRNA and serum levels in patients with IDFU.

Model		Unstandardized Coefficients		Standardized Coefficients	t	P value	95% C.I.	
		B	S.E	Beta			Lower Bound	Upper Bound
TNF- α	(Constant)	-4.020	2.799		-1.436	0.154	-9.577	1.537
	Duration of diabetes	-0.637	0.226	-0.234	-2.821	<0.001*	-1.085	-0.189
	BMI	1.396	0.327	0.269	4.267	<0.001*	0.747	2.046
	WBC	0.084	0.169	0.031	0.500	0.618	-0.251	0.419
	ESR	1.943	0.201	0.823	9.664	<0.001*	1.544	2.343
TNF-α mRNA	(Constant)	2.720	0.763		3.563	<0.001*	1.204	4.235
	Duration of diabetes	0.067	0.067	0.127	0.994	0.323	-00.067	0.200
	BMI	0.006	0.049	0.011	0.117	0.907	-0.091	0.102
	WBC	-.0110	0.081	-0.241	-1.361	0.177	-0.270	0.050
	ESR	0.115	0.027	0.599	4.267	<0.001*	00.062	0.169

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