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"SERUM TUMOR NECROSIS FACTOR-A (TNF-A): A POTENTIAL BIOMARKER FOR GRADING AND PROGRESSION OF DIABETIC FOOT ULCERS"

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ABSTRACT

Background: Diabetic Foot Ulcers (DFUs) are a serious complication of diabetes, where inflammation significantly influences disease progression. Tumor Necrosis Factor-α (TNF-α), a key pro-inflammatory cytokine, impairs wound healing via NF-κB activation, apoptosis, and endothelial and fibroblast dysfunction. **Aim:** To investigate the role of serum TNF-α level in individuals with different grades of Diabetic Foot Ulcers. **Methodology:** A case-control study was conducted on 250 participants (125 DFU patients and 125 controls) at a tertiary care center. Serum TNF-α was measured using ELISA. Data were analyzed using the Shapiro-Wilk test and appropriate non-parametric tests.

Results: The mean age of participants was 57.23 ± 10.72 years, with no significant difference in age and sex distribution between groups. DFU patients showed a significant association ($p < 0.001$) with longer duration of diabetes, smoking, family history of diabetes, and other comorbidities. Serum TNF-α levels increased progressively with advancing grades of DFUs. Kruskal-Wallis test with post hoc analysis demonstrated statistically significant differences in TNF-α level across all DFU grades and controls. Elevated TNF-α level were particularly pronounced in higher grades, indicating its association with disease severity. TNF-α also showed a rise peaking at grade 4 and 5 highlighting the link between increased local and systemic inflammation and extreme tissue necrosis.

Conclusion: Serum TNF-α levels correlate strongly with DFU severity and may serve as a useful biomarker for disease progression and a potential therapeutic target in DFU management.

KEYWORDS: Diabetic Foot Ulcer, Tumor Necrosis Factor-α (TNF-α), Inflammation, Biomarker, Wound Healing

INTRODUCTION

Diabetic foot ulcer is recognized as a full-thickness, slow-healing defect of the skin in a diabetic patient, seen typically on the foot, and is more often accompanied with peripheral vascular disease, minor trauma, and neuropathy (1, 2). These ulcers are chronic in nature and when infected or affected with osteomyelitis may lead to lower-extremity amputation (3, 4). DFU has serious clinical significance, with complex consequences for morbidity, mortality, and access to healthcare. Albeit several years of research, DFUs pose to be a constant indelible challenge and are reported to be the leading cause of lower extremity amputation (1).

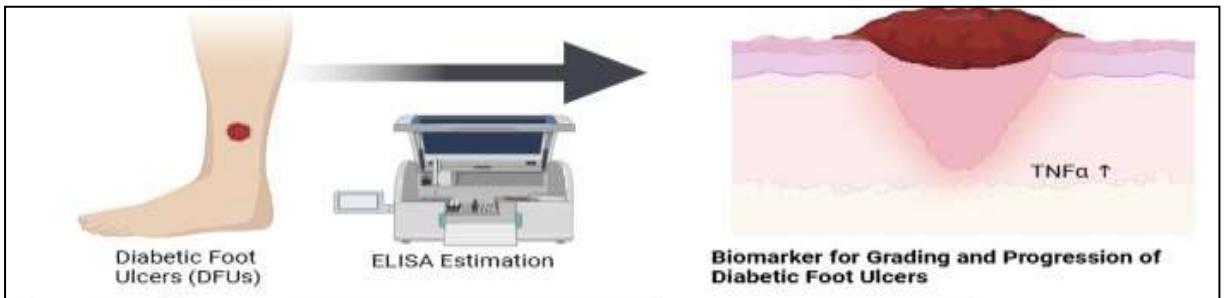
In the worldwide scenario, diabetes mellitus affects a vast population of adults and the constant rise in the prevalence of DFU implies the continuity of the significant burden on healthcare systems (5, 6). An ICMR study conducted in India revealed the vast burden of the disease with 62.4 million people reported with Type 2 DM and 77 million with pre-diabetes (7, 8). The lifetime risk of acquiring a DFU among DM patients ranges from 19% to 35% (9). It is also reported that individuals affected by DFU approximately spend four times more than those not affected by DFU indicating the heavy economic burden borne during treatment (10) Other than direct medical implications there is a strong socioeconomic and psychosocial burden among DFUs. There are substantial expenses towards treatment, hospitalization, readmission, and amputations (11). Studies have also shown that

patients with lower socioeconomic status had faced increased expenditure in DFU care which further increased their burden (6). Inflammation is key component to DFUs pathogenesis, and various inflammatory markers such as tumor necrosis factor alpha (TNF-α) play important roles in the development and progression of these ulcers (12). Tumor Necrosis Factor-α (TNF-α), a pro-inflammatory cytokine primarily produced by macrophages, contributes to impaired wound healing through activation of NF-κB signaling, increased apoptosis, and disruption of endothelial and fibroblast function.[5].Produced mainly by macrophages and also by other immune cells is Tumor Necrosis Factor-α (TNF-α) and which activates NF-κB signalling (13,14). TNF-α is linked to poor wound healing due to increased apoptosis, hampering of endothelial and fibroblast function and amplifies Matrix metalloproteinases (12).

Hence TNF- α play significant role in wound healing among DFU patients. Despite increasing recognition of inflammation in metabolic diseases, data specifically addressing the role of systemic inflammation in diabetic foot ulcers (DFUs) remain limited. And also immune system plays a pivotal role at multiple stages in the development and progression of chronic wounds. Understanding the molecular and inflammatory factors associated with DFU grades is crucial for effective management and prevention of complications. The aim of this study was to investigate the role of serum TNF-α level in individuals with different grades of Diabetic Foot Ulcers which may help in better understanding the pathogenesis of DFU and correlate the values with its severity.

MATERIALS AND METHODS

A total of 125 patients with diabetic foot ulcers (DFUs), comprising 25 patients in each stage, and 125 diabetic patients without foot ulcers (serving as controls and free from foot wounds or any acute or chronic illness) admitted to surgical wards were enrolled in the study. DFUs were classified using the Wagner classification system for diabetic foot ulcers, a widely accepted grading system based on ulcer depth and severity, categorizing wounds from Grade I to Grade V (9); each group included 25 patients. A foot ulcer was defined as a full-thickness skin defect requiring ≥14 days for healing. Cases and controls were matched for age (±3 years), sex, and duration of diabetes. Patients with inflammatory or infectious diseases, autoimmune or rheumatic disorders, malignancies, hematological diseases, pregnancy, lactation, chronic inflammatory conditions, severe renal or hepatic failure, recent venous thromboembolism, or those receiving immunosuppressive therapy were excluded. Following ethical clearance and informed consent, 5 mL of venous blood was collected under aseptic precautions with and without anticoagulant. Samples were allowed to clot at room temperature for 10–20 minutes, centrifuged at 2000–3000 rpm for 20 minutes, and the supernatant was separated; if precipitation occurred, samples were recentrifuged. Serum aliquots were stored at −80°C until analysis. Prior to estimation, samples were thawed, and serum TNF-α level was measured using a quantitative sandwich ELISA method with the GENLISA™ ELISA kit (Krishgen Biosystems, USA). The assay was read at 450 nm using an automated iMark ELISA reader (Bio-Rad Laboratories), with a detectable range of 7.8–500 pg/mLfor serum TNF-α level as specified by the manufacturer.



DFU Grades	Symptoms	TNF-α ng/mL
Controls	Diabetes patients without foot ulcer	2.22±0.14
1	No ulcer High-risk foot	2.24 ± 0.21
2	soft tissue infections deep abscess	2.17 ± 0.13
3	Deep ulcer osteomyelitis	3.90 ± 1.13
4	Localised gangrene, ischemic gangrene	4.70 ± 0.64
5	All gangrene	4.92 ± 0.80

Statistical analysis:

Data was coded in MS Excel and all statistical analysis was carried out. The data was checked for Normal distribution using the Shapiro- Wilk test. The significant difference between two groups (DFU and DWO) of continuous variables was assessed through Student t or Welch t or Mann-Whitney U test depending on assumptions. The summary of categorical variables will be reported in terms of frequencies and percentage.The association between the categories will be assessed using Chi-square test or Fisher's exact test.It is observed that

all the variables do not follow Normality. Thus the statistical difference between the six groups was assessed through Kruskal Walli's test. If null hypothesis is rejected, then post-hoc test (with Bonferroni correction) was carried out for pair-wise comparisons. A p value < 0.05 was considered statistically significant.

RESULTS

The study was conducted on 250 participants including 200 male and 50 female, equally divided into two groups: with 125 in the DFU group and 125 participants in the control group. The mean age and standard deviation of all participants was 57.23±10.72 years. Age and sex distribution showed no significant difference. This indicated that in terms of demographic characteristics the groups were well-matched.

DFU patients had a significantly longer duration of diabetes ($p<0.001$) than the control group, indicating that a longer duration of the disease could be a major risk factor for developing DFUs. Also, DFU patients were likely to use insulin (50.4%) compared to control group (25.6%) [$p<0.001$] which is suggestive of poorly controlled DM in the DFU group.

Lifestyle factors also differed significantly difference between the DFU and control groups. The DFU group showed higher prevalence for smoking ($p<0.001$) and alcohol consumption ($p = 0.008$), highlighting the possibility of behavioral factors contributing to the development of DFUs. Family history of diabetes was significantly higher ($p<0.001$) in the control group compared to DFU group, indicating that genetic predisposition may not be the primary determining factor for DFU development (Table 1). TNF-α levels were assessed for all participants based on the Wagner scale ranging from Grade I (early-stage ulcers) to Grade V (extensive gangrene). The markers evaluated provided an insight on the inflammation levels and ability of tissue to repair. TNF-α also showed a rise peaking at grade 4 and 5 highlighting the link between increased local and systemic inflammation and extreme tissue necrosis. The distribution of TNF-α across DFU grades are represented in Table 2.

The distribution of TNF-α varied significantly from normality in grades III ($p = 0.010$), IV and V ($p < 0.001$), whereas control, grade I, and grade II groups showed normal distribution. The skewness of distribution, particularly in the higher grades of ulcer indicates heightened response to tissue necrosis seen in the higher ulcer grades.The differences between TNF-α across the five grades of DFUs was assessed using Kruskal-Wallis test as the data was not normally distributed. This test assesses the significance in difference of one group when compared to the other groups without assuming normality. The test revealed marked significant difference ($p<0.001$) for biomarker across DFU grades implying that inflammation markers TNF-α differ significantly across DFU grades. This is represented in Table 3.

TNF-α:

The levels of TNF-α were found to be relatively low in controls, grade I and grade II and the interquartile ranges were narrow. Pairwise comparisons were carried out which revealed non-significant results in the early grades of DFUs which indicated low response of inflammation in individuals having no ulcers or low-grade ulcers whereas in advanced ulcer stages heterogenous inflammatory responses were seen. The box-plot for TNF-α shows sharp increase in median and interquartile range at moderate ulcer levels suggesting escalating tissue necrosis and inflammation. The box-plot shows findings similar to that represented in table 3 which represent the pairwise comparisons across the DFU grades. This is represented in the graph 1 below

Table 1: Baseline variables among the Study Subjects .

Variables		Total Sample (N=250) (%)	DFU (n=125) (%)	DWO (n=125) (%)	p value
Age		57.23±10.72	57.30±11.01	57.17±10.47	0.965
Sex Male/Female		200/50 (80.0%/20.0%)	100/25 (80.0%/20.0%)	100 (80.0%/20.0%)	1.00
Duration of DM(months)		372.54±303.46	117.81±89.34	125.28±69.43	<0.001***
Smoking History	Current	42(16.8%)	7(5.6%)	35(28.0%)	<0.001***
	Never	114(45.6%)	71(56.8%)	43(34.4%)	
	Past	94(37.6%)	47(37.6%)	47(37.6%)	
Alcohol History	Current	41(16.4%)	10(8.0%)	31(24.8%)	0.008**
	Never	131(52.4%)	76(60.8%)	55 (44.0%)	
	Past	78(31.2%)	39(31.2%)	39(31.25%)	
Drug History	Insulin	95(38.0%)	63(50.4%)	32(25.6%)	<0.001***
	Tablet	85(34.0%)	17(13.6%)	68(54.4%)	
	Insulin & tablet	70(28.0%)	45(36.0%)	25(20%)	
History of Hypertension Yes/No		152/66 (70%/30%)	89/36 (71.2%/28.8%)	63/40 (61.2%/38.8%)	<0.001***
Family history of diabetes Yes/No		145/105 (58.0%/42.0%)	28/77 (38.4%/61.6%)	97/28 (77.6%/22.45)	<0.001***

DFU- Diabetic foot ulcer, DWO: Diabetic patient without foot ulcer, * p<0.05, **p<0.01, ***p<0.001

Table 2: The Wagner-ulcer classification system with five different DFU grades

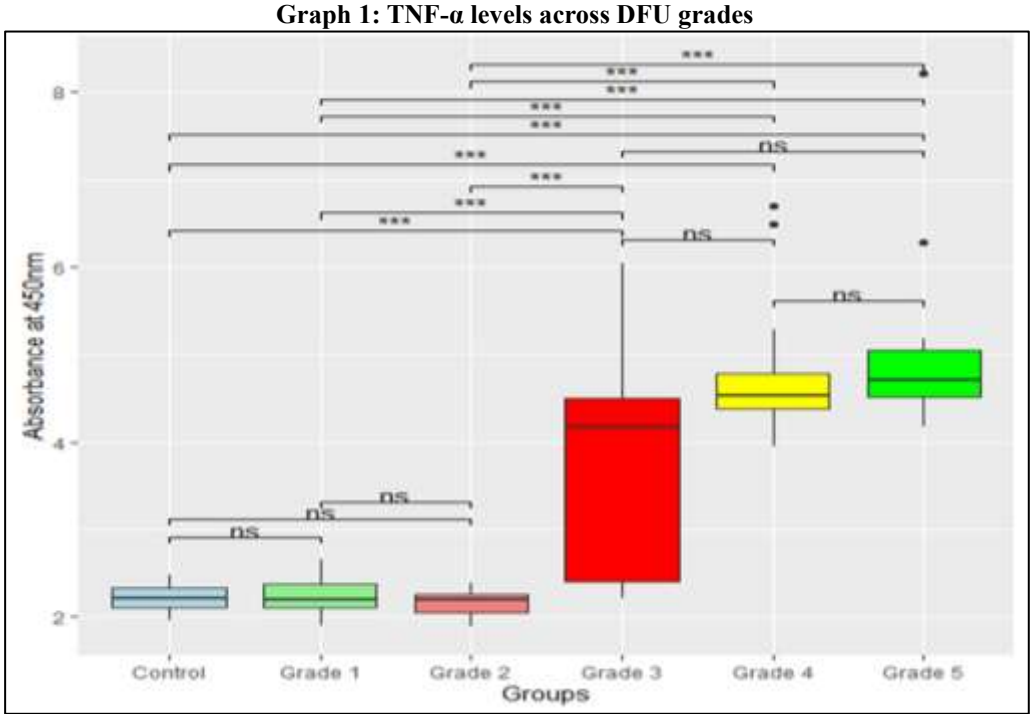
DFU Grades	Symptoms	TNF-α ng/mL Mean ± SD
Controls	No ulcer in a high-risk foot	2.22 ± 0.14
1	Shallow ulcer in the foot, no infection, neuropathic ulcer	2.24 ± 0.21
2	More deep ulcers, often combined with soft tissue infections, on- osteomyelitis or deep abscess	2.17 ± 0.13
3	Deep ulcer, abscess or osteomyelitis	3.90 ± 1.13
4	Localised gangrene, ischemic gangrene	4.70 ± 0.64
5	All gangrene	4.92 ± 0.80

DFU: Diabetic Foot Ulcer, 1: Early-stage ulcer with localized tissue damage and minor infection, 2: Moderate ulcer with deeper tissue involvement and visible wound expansion, 3: Severe ulcer with extensive tissue necrosis, exposed underlying structures, and infection, 4:Advanced stage characterized by widespread gangrene and tissue destruction, 5 :Extensive ulceration with significant necrosis and risk of limb loss.

Table 3: Summary of results on distribution of the biomarkers with Shapiro-Wilk test:

			Shapiro Wilk	
	Group	**Mean±SD**	**W**	**p**
TNF-α	Control	2.22±0.142	0.967	0.566
	Grade 1	2.25±0.219	0.924	0.065
	Grade 2	2.17±0.136	0.949	0.240
	Grade 3	3.90±1.136	0.888	**0.010**
	Grade 4	4.71±0.643	0.748	**<0 .001**
	Grade 5	4.93±0.806	0.643	**< 0.001**

Graph 1: TNF-α levels across DFU grades



DISCUSSION

Diabetic Foot Ulcers (DFUs) are among the most debilitating complications of diabetes mellitus and are associated with prolonged hospitalization, recurrent infections, and increased risk of lower-limb amputation (1). Chronic inflammation plays a central role in the pathogenesis and progression of DFUs, with pro-inflammatory cytokines contributing significantly to impaired wound healing(12). In the present study, serum TNF-α level were evaluated across different Wagner grades of DFUs to determine their association with ulcer severity and disease progression. TNF-α is a key pro-inflammatory cytokine produced mainly by activated macrophages, neutrophils, and T lymphocytes. It plays an important role in the inflammatory cascade by activating NF-κB signaling pathways,

increasing oxidative stress, stimulating matrix metalloproteinases, and promoting apoptosis of endothelial cells and fibroblasts. These mechanisms impair angiogenesis, collagen synthesis, and tissue remodeling, ultimately delaying wound healing (13, 14). Elevated TNF-α levels observed in advanced ulcer grades in this study may therefore reflect persistent inflammatory activity and ongoing tissue injury.

The findings of the present study demonstrated a significant progressive rise in serum TNF-α levels with increasing DFU severity. Patients with advanced Wagner grades (Grades III–V) exhibited markedly elevated TNF-α levels compared to controls and early-stage ulcers. These findings support the hypothesis that systemic inflammation intensifies with worsening tissue destruction, infection, ischemia, and necrosis associated with advanced DFUs. The Kruskal–Wallis analysis further confirmed statistically significant differences in TNF-α levels across all groups ($p < 0.001$), indicating a strong association between TNF-α and ulcer progression. The result in present study shows peaking of TNF-α levels at higher DFU grades. Tissue necrosis, angiogenesis inhibition are stimulated by TNF-α which is a pro-inflammatory cytokine. Higher levels also interrupt deposition of collagen, fibroblast proliferation along with delayed wound healing and similar results are presented in existing literature (15, 16 , 17).

TNF-α distribution was close to near normal in early grades but varied in later grades of DFU. This result can be attributed to secondary infections or inflammatory responses which are commonly seen in advanced foot ulcer patients (18). Thus, localized necrosis of tissues and bacterial inflammation in increased ulcer severity can result in increased levels of TNF-α which indirectly may result in skewed distribution (19).

Kruskal-Wallis test along with post hoc pairwise analyses revealed significant differences among all four biomarkers and grades of DFUs indicating that the levels of these biomarkers differ implicitly based on ulcer severity and not arbitrarily distributed across the Wagner grades. Similar results were seen in earlier studies (20, 21).

In the present study, TNF-α levels remained relatively low in controls and early ulcer grades (Grade I and II), suggesting that mild or superficial ulcers may not trigger a strong systemic inflammatory response. However, a sharp increase was observed from Grade III onwards, corresponding to deeper ulcers, abscess formation, osteomyelitis, gangrene, and extensive tissue necrosis. This pattern indicates that TNF-α may serve as a marker of worsening inflammation and tissue destruction in DFUs. The widened interquartile range and non-normal distribution in Grades III–V further suggest heterogeneity in inflammatory responses among patients with severe ulcers, likely influenced by infection severity, ischemia, and associated comorbid conditions.

These findings are consistent with previous studies that reported elevated TNF-α level in chronic diabetic wounds and severe DFUs. Previous investigators have demonstrated that increased TNF-α expression is associated with delayed wound closure, impaired granulation tissue formation, and enhanced proteolytic activity in diabetic wounds (22). Studies have also shown that chronic hyperglycemia promotes sustained cytokine release and macrophage activation, thereby maintaining a prolonged inflammatory state. The current findings reinforce the role of TNF-α as an important mediator in the pathophysiology of diabetic wound progression (23).

The present study highlights the potential utility of serum TNF-α as a biomarker for grading and monitoring DFUs. Since TNF-α level increased progressively with ulcer severity, measurement of this cytokine may help clinicians identify patients at risk for rapid progression, severe infection, or gangrene. Early identification of high-risk patients may facilitate timely intervention and improved management strategies. Furthermore, TNF-α may represent a potential therapeutic target in DFU treatment. Anti-inflammatory therapies targeting TNF-α pathways could possibly reduce chronic inflammation and improve wound healing outcomes, although further clinical trials are required to establish their effectiveness and safety in diabetic wound management.

Overall, the present study demonstrates that serum TNF-α level are significantly elevated in patients with advanced diabetic foot ulcers and correlate strongly with ulcer severity. These findings emphasize the importance of inflammation in DFU pathogenesis and support the potential role of TNF-α as a biomarker for disease progression and therapeutic monitoring.

CONCLUSION

Increased serum TNF-α level is strongly associated with the severity of Diabetic Foot Ulcers, highlighting its role in inflammation-mediated impaired wound healing. TNF-α may serve as a potential biomarker for assessing disease progression and could be a target for therapeutic intervention in DFU management. Monitoring these inflammatory markers may aid in early detection, assessment of severity, and development of targeted therapies to improve outcomes in DFU patients.

Limitations:

There are certain limitations to be acknowledged in this study. First, the study was conducted in an institutional setting and in single geographic location. This hampers the generalizability of the results as characteristics of the patients, access to healthcare and the disease profile may vary across different healthcare settings and geographic regions. A study conducted on a multicentre level, involving distinct populations can ensure increased external validity and permit wider application of the results

Second, since this is a cross-sectional design, definite causality cannot be established between biomarkers and DFU progression. Even though strong associations are observed between IGF-1, inflammatory markers and DFU

severity, temporal relations could not be established. Longitudinal studies are needed to assess biomarker dynamics over time to verify causality.

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