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ASSOCIATION OF MOLECULAR BIOMARKERS WITH DISEASE SEVERITY IN DIABETIC FOOT ULCERS: A CASE CONTROL STUDY

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ABSTRACT

Introduction: Diabetic foot ulcers (DFUs) represent one of the most serious chronic complications of diabetes mellitus and are a major cause of increased morbidity, recurrent hospital admissions, and lower-limb amputations. Persistent inflammation plays a central role in the development and progression of DFUs. Identifying inflammatory molecular biomarkers associated with ulcer severity may facilitate early diagnosis, prognostic assessment, and improved clinical management.

Aim: To determine the serum levels of the inflammatory biomarkers tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), and high-sensitivity C-reactive protein (hs-CRP) in diabetic patients with and without foot ulcers, and to investigate their association with disease severity across different Wagner grades of diabetic foot ulcers.

Methods: A hospital-based case-control study was conducted involving 250 participants, including 125 patients with diabetic foot ulcers and 125 diabetic patients without foot ulcers. Demographic, clinical, and biochemical characteristics were retrieved from electronic medical records. Diabetic foot ulcers were graded using the Wagner classification system. Serum TNF-α, IL-6, and hs-CRP levels were quantified using enzyme-linked immunosorbent assay (ELISA). Comparative statistical analyses were performed between groups, and correlations between biomarker levels and Wagner grades were evaluated.

Results: Serum TNF-α, IL-6, and hs-CRP concentrations were significantly higher in patients with diabetic foot ulcers than in diabetic controls without foot ulcers ($P < 0.001$). Moreover, the levels of all three inflammatory biomarkers showed a significant stepwise increase with increasing Wagner grades. Positive correlations were observed between biomarker concentrations and ulcer severity ($P < 0.001$), suggesting that systemic inflammation intensifies with disease progression.

Conclusion: Elevated serum TNF-α, IL-6, and hs-CRP levels are strongly associated with increasing severity of diabetic foot ulcers. These inflammatory biomarkers may serve as useful adjunctive indicators for disease severity assessment, risk stratification, and individualized management of patients with diabetic foot ulcers.

KEYWORDS: Diabetic foot ulcer; inflammation; TNF-α; IL-6; hs-CRP; molecular biomarkers; Wagner grade; ELISA.

INTRODUCTION

Diabetic foot ulcers (DFUs) represent one of the most serious chronic complications of diabetes mellitus and are a major cause of increased morbidity, recurrent hospital admissions, and lower-limb amputations. It is estimated that 15–25% of diabetic patients will develop a chronic foot ulcer during their lifetime [1, 2]. Despite global advancements in preventive strategies and wound care, DFUs continue to account for a large proportion of lower limb amputations [3]. The primary risk factors for foot ulcers are diabetic neuropathy and peripheral arterial disease. Diabetic Foot ulcer is increasingly recognized not only as a metabolic disorder but also as a state of chronic low-grade inflammation. A complex interplay exists between inflammatory mediators and metabolic dysfunction, contributing significantly to cardiovascular risk. Emerging evidence suggests that hormones, cytokines, and various inflammatory markers interact in a dynamic network, further complicating disease progression and systemic effects [4, 5].

Despite increasing recognition of inflammation in metabolic diseases, data specifically addressing the role of systemic inflammation in diabetic foot ulcers (DFUs) remain limited. Chronic low-grade immune activation is a known risk factor for the development of type 2 diabetes [6], and it is also implicated in both macrovascular complications such as myocardial infarction and stroke, and microvascular complications like neuropathy and nephropathy [7]. Key

inflammatory markers including interleukin-6 (IL-6), high-sensitivity C-reactive protein (hs-CRP), and tumor necrosis factor-alpha (TNF-α) have shown associations with insulin resistance and obesity [8].
The immune system plays a pivotal role at multiple stages in the development and progression of chronic wounds. Similar to its involvement in the early stages of type- 2 diabetes and cardiovascular disease, immune activation may also precede the onset of DFUs. Pro- inflammatory pathways are integral to the wound healing process; any dysregulation in immune function may disrupt tissue repair and homeostasis. This can contribute to the persistence of non-healing, chronic wounds that characterize diabetic foot syndrome. (6) Because of the limited treatment availability and serious consequences, diabetic foot syndrome is becoming an important issue. An inflammatory cascade through increased expression of proinflammatory cytokines exist in diabetic foot, whereas no study, to our knowledge, evaluated the role of TNF-α, IL- 6 and Hs-CRP and immune-inflammatory biomarkers such as inflammatory cytokines in patients with different grades of DFU .
Based on this rationale, our study aimed to evaluate the circulating levels of IL-6, hs-CRP, and TNF-α in individual with diabetic foot ulcers compared to diabetic patients without foot complications in different stages which may help in better understanding the pathogenesis of DFU and correlate the values with its severity.

METHODS

Study Population and Sample Collection

A total of 250 participants were enrolled in this hospital-based case-control study, comprising 125 patients with diabetic foot ulcers (DFU) and 125 diabetic patients without foot ulcers (DWO) admitted to the Department of Surgery. The DFU group included 25 patients in each Wagner grade (Grades I–V), while the DWO group consisted of diabetic patients without active or previous foot ulcers and without any acute or chronic inflammatory conditions.
Diabetic foot ulcers were classified according to the Wagner classification system, one of the most widely accepted grading systems for diabetic foot lesions, which categorizes ulcers from Grade 0 to Grade V based on ulcer depth, extent of tissue involvement, and severity. Patients were graded at the time of hospital admission, and an equal number of participants (n = 25) were included in each Wagner grade. A diabetic foot ulcer was defined as a full-thickness skin lesion requiring at least 14 days for complete healing. The DWO group was matched with the DFU group for age and sex.
Patients with inflammatory or infectious diseases, autoimmune or rheumatological disorders, malignancies, hematological diseases, chronic inflammatory conditions, severe hepatic or renal failure, recent venous thromboembolism, pregnancy, lactation, or those receiving immunosuppressive or immunomodulatory therapy were excluded from the study.
Following written informed consent and approval from the Institutional Ethics Committee, approximately 5 mL of venous blood was collected from each participant under aseptic conditions. Blood samples were allowed to clot at room temperature for 20–30 minutes and were subsequently centrifuged at 2,000–3,000 rpm for 20 minutes to separate the serum. The serum was carefully aliquoted into sterile cryovials. Samples showing any visible precipitate were re-centrifuged before aliquoting. All serum aliquots were stored at −80°C until analysis of biochemical and inflammatory biomarkers.

Statistical Analysis

Data was coded in MS Excel and all statistical analysis was carried out using R software (V 4.5.1). 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 as applicable. 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. A p value < 0.05 was considered statistically significant.

RESULTS

A total of 125 DFU patients and 125 DWO patients, admitted to surgery were selected. Age and sex were matched between the two groups. The subjects' ages ranged from 34 to 60 years. Among DFU patients, 25 individuals were enrolled for each Wagner stage (grades 1 to 5) to ensure balanced representation across the spectrum of ulcer severity. The baseline demographic and clinical characteristics are detailed in Table 1.The mean age was similar between DFU (57.30 ± 11.01 years) and DWO (57.17 ± 10.47 years) groups ($p = 0.965$), with both groups having the same male-to-female ratio (80% male, 20% female).The duration of diabetes was significantly longer in DFU patients (117.81 ± 89.34 months) compared to DWO (125.28 ± 69.43 months), ($p < 0.001$).A significantly higher number of DFU patients had a history of smoking, alcohol consumption, insulin usage, and hypertension. A positive family history of diabetes was significantly more prevalent in the DWO ($p < 0.001$), suggesting genetic factors alone may not predict DFU development.. Table 2 shows a progressive increase in inflammatory markers was observed across DFU grades: IL-6 rose from 0.05 ± 0.00 pg/mL in Grade 1 to 2.66 ± 0.39 pg/mL in Grade 5. hs-CRP increased from 0.14 ± 0.04 µg/mL (Grade 1) to 0.68 ± 0.24 µg/mL (Grade 5).TNF-α increased from 2.24 ± 0.21 pg/mL in Grade 1 to 4.92 ± 0.80 pg/mL in Grade 5.Compared to DWO, who had significantly lower values (IL-6: 0.06 ± 0.01 pg/mL, hs-CRP: 0.15 ± 0.03 µg/mL, TNF-α: 2.22 ± 0.14 pg/mL), DFU patients showed statistically significant elevations in all three markers ($p < 0.001$).The correlation between inflammatory marker levels and Wagner grades was strong and statistically significant ($P = 0.001$), indicating increasing systemic inflammation with ulcer severity.

Table 1: Demographic and baseline data of the Subjects

Variables	DFU	DWO	p value

Age	57.30±11.01	57.17±10.47	0.965b
Sex n (%) Male/Female	100/25(80.0%/20.0)	100(80.0%/20.0%)	1.00a
Duration of DM(months)	117.81±89.34	125.28±69.43	<0.001***b
History of Hypertension Yes/No	89/36(71.2%/28.8%)	63/40(61.2%/38.8%)	<0.001***a
Family history of diabetes Yes/No	28/77(38.4%/61.6%)	97/28(77.6%/22.45)	<0.001***a

a Chi-square test was performed and bMann-Whitney U test .Level of significance: * $p<0.05$,***$p<0.001$ $p>0.05$ non significant (ns).

Table 2: The Wagner-ulcer classification system with 5 different DFU grades

DFU Grades	Symptoms	IL-6 pg/mL	hs-CRP µg/mL	TNF-α pg/mL
Controls	Diabetes patients without foot ulcer	0.06±0.01	0.15±0.03	2.22±0.14
1	No ulcer High-risk foot	0.05 ± 0.22	0.14 ± 0.04	2.24 ± 0.21
2	More deep ulcers, with soft tissue infections deep abscess	0.32 ± 0.47	0.29 ± 0.17	2.17 ± 0.13
3	Deep ulcer, abscess with osteomyelitis	0.91 ± 0.59	0.35 ± 0.13	3.90 ± 1.13
4	Localised gangrene, ischemic gangrene	1.64 ± 0.64	0.45 ± 0.21	4.70 ± 0.64
5	All gangrene	2.66 ± 0.39	0.68 ± 0.24	4.92 ± 0.80

DISCUSSION

Infected DFU is a common cause of morbidity, ultimately leading to dreaded complications like gangrene and amputations (10). Inflammation is a key component in the pathogenesis of DFUs, and various inflammatory markers such as IL-6, hs-CRP and TNF-α, have been implicated in the development and progression of these ulcers (11).

Diabetic foot wounds are marked by a persistent and dysregulated inflammatory phase, an enhanced production and release of proinflammatory cytokines causes disturbance the balance between proinflammatory and anti-inflammatory macrophages (12). Cytokines are small proteins, soluble in the serum and interacting with cell surface receptors. They serve as a communication network between cells. By interaction with specific transmembrane receptor, they activate signal cascade, which result in cellular response (13). These cytokines may mediate the synthesis of acute phase proteins which are able to initiate and support inflammatory process in the vascular wall(14). Interleukins (ILs), produced by blood monocytes and tissue macrophages, are a group of cytokines that are critical important for the function of the immune system – both innate and adaptive. When the body is infected, ILs is secreted onto target cells and bind to the signals on the surface that activate the target cells, altering cell behavior. ILs levels would be elevated in patients with DFU along with the development of insulin resistance, abnormal healing and decreased with the healing of ulcers (15).

Similar to the literature, in this study we found that DFW had significantly elevated serum levels of IL-6, hs-CRP, and TNF-α compared to DWO. Importantly, these inflammatory markers showed a positive correlation with Wagner grading, indicating that systemic inflammation increases with the severity of foot ulceration. In patients with DFU in comparison with DWO, a higher plasma level of IL-6 could be linked to foot ulcer pathogenesis by micro-vascular and inflammatory mechanisms (16). Scheller et al (2021)', studied on the pro- and anti-inflammatory properties of the cytokine interleukin-6. It is a review article and concluded that IL-6 is involved not only in the activation of the immune system but also in regenerative processes as well as in the regulation of metabolism, in the maintenance of bone homeostasis, and in many neural functions (16).

C-reactive protein (CRP), a hepatic-originated plasma protein, is a key element of any inflammatory reaction. As a result of tissue damage or infection, the plasma levels of CRP may rapidly increase to more than 1000-folds above normal values, therefore it is widely used a monitor marker in the acute phase, responding to tissue damage, infection and inflammation in routine clinic. Increased hs-CRP levels, a general marker of systemic inflammation, further underscore the pro-inflammatory state in patients with advanced DFUs. We found that DFU had significantly elevated serum levels of hs-CRP compared to DWO. Studies have shown that elevated CRP levels are associated with diabetes .CRP, IL-1β, IL-6 and TNF-α level were higher in diabetic patient without foot lesion (PC) when Compared to Healthy Controls, and its level was higher in patient with Diabetic Foot Lesion when compared to PC(17). Elevated TNF-α and IL-6 levels are

known to interfere with angiogenesis, collagen synthesis, and fibroblast activity, all of which are essential for proper wound repair. (18)

TNF-α may act as local intensification signals in pathological processes associated with chronic inflammation (19).A high dose of TNF α induces the increase of apoptosis, a mechanism of physiological cell death, in vitro. In this study, we found that DFU had significantly elevated serum levels of TNF-α compared to DWO. Previous studies have observed significant increased plasma levels of TNF α in DFU patient as compared with DWO, indicating immune inflammatory activation in such patients. (20)

Our findings are consistent with previous research highlighting the role of chronic inflammation in the pathogenesis of diabetic complications, particularly impaired wound healing. Our study not only confirms these associations but also demonstrates a graded increase of these markers across Wagner stages, adding strength to their potential use as clinical biomarkers. A study on ssystematic review and meta-analysis on the efficacy of inflammatory markers in diagnosing infected diabetic foot ulcers and diabetic foot osteomyelitis concluded that CRP is the ideal marker for diagnosing grade 2 DFU from non-infected DFU and CRP is the best markers for diagnosing grade 2 and grade 3 DFU(21).

In addition to inflammation, we observed that patients with DFUs had higher HbA1c levels, indicating poor glycaemic control. This finding is in line with the understanding that hyperglycaemia impairs immune function and delays wound healing, further contributing to ulcer severity. Lifestyle factors like smoking and alcohol use were also more prevalent in the DFU group, reinforcing the need for comprehensive patient education and lifestyle modification. All of these elements and molecular markers play an important role in extending the healing of diabetic ulcerated wounds and can be used as diagnostic tools by researchers to detect patients suffering from DFU.

CONCLUSION

This study emphasizes the significant role of systemic inflammation in the pathogenesis and progression of diabetic foot ulcers, as evidenced by markedly elevated serum levels of TNF-α, IL-6, and hs-CRP. These inflammatory biomarkers demonstrated a strong positive correlation with increasing Wagner grades, suggesting their value as reliable indicators of disease severity. Assessment of these biomarkers may aid in the early detection of patients at high risk, enhance diagnostic and prognostic precision, and facilitate effective risk stratification. Moreover, interventions targeting inflammatory pathways may improve wound healing, slow disease progression, and reduce the incidence of serious complications, including lower-limb amputation, among patients with diabetic foot ulcers.

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