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Linking Biofilm-Producing Bacteria to Inflammation and Tissue Destruction: The Role of MMP-13 and Pro-inflammatory Cytokines in Diabetic Foot Ulcers

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Abstract

The present study sought to evaluate the relationship of pro-inflammatory cytokines (IL-6, TNF-α, and IL-1β), MMP-13, and biofilm-forming bacteria isolates with Wagner grading in diabetic foot ulcers.

In this analytical cross-sectional study, a total of 100 participants were recruited, and patients were divided into four groups (DFU-strong biofilm-forming (S); DFU-weak/non-biofilm-forming (W); Type 2 diabetes mellitus control without foot ulcer (DC); healthy controls (HC)). Bacterial isolates were identified using standard microbiology methods and the VITEK-2 system. The Congo Red Agar and microtiter plate assays were used to evaluate biofilm formation. IL-6, TNF-α, IL-1β, and MMP-13 in serum were detected by ELISA kits. The ulcer severity condition was graded according to the Wagner grading system.

Pseudomonas aeruginosa and *Staphylococcus aureus* were the most frequently isolated among highly biofilm-forming isolates. The levels of IL-6, TNF-α, IL-1β, and MMP-13 were significantly higher in patients with strong biofilm-associated diabetic foot ulcers than those observed in the other groups ($P < 0.001$). Inflammatory cytokines, MMP-13, biofilm strength, and Wagner grade were all significantly positively correlated.

The presence of strong biofilm-forming bacteria isolates was associated with a higher inflammatory response, increased serum MMP-13 levels, and greater diabetic foot ulcer severity. Circulating pro-inflammatory cytokines and MMP-13 may serve as useful biomarkers for assessing ulcer severity and monitoring disease progression. However, further large-scale, multicenter studies are required to validate their potential value and clinical applications in risk stratification and therapeutic guidance.

Keywords: Diabetic Foot Ulcer, MMP-13, Pro-inflammatory Cytokines, Biofilm-forming Bacteria.

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Introduction

Diabetic foot ulcers (DFU) are one of the most severe and frequent complications in patients with diabetes mellitus, contributing as a serious global health problem associated with high morbidity and mortality rates (1). Diabetic foot ulcers are one of the major causes of hospitalization and lower limb amputation in patients worldwide (2). Impaired wound healing due to peripheral neuropathy, vascular insufficiency, and microbial infection is a key contributor to the pathogenesis and progression of diabetic foot ulcers (3). Chronic inflammation and sustained bacterial contamination considerably impede wound healing and cause more severe tissue injury (4).

Diabetic foot ulcers (DFUs) are complex and chronic non-healing wounds, where bacterial colonization, particularly of biofilm-forming cells, is thought to play a key role in their pathogenesis and propagation. Biofilms are organized communities of microorganisms embedded in an organic polymer matrix formed by self-produced extracellular polymeric substances that protect against the actions of host immune and antimicrobial agents (5). In diabetic foot ulcers, biofilm-forming bacteria are believed to cause a chronic infection, antibiotic resistance, inflammation, and delayed wound healing (6). Various bacterial species, including *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*, and *Klebsiella spp*. frequently isolated from diabetic foot infections and were able to form biofilm effectively (7).

The excessive production of pro-inflammatory cytokines, such as interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α), and interleukin-1 beta (IL-1β), is strongly associated with persistent inflammation in diabetic foot ulcers. These cytokines are critical mediators of the inflammatory response in the wound healing process (8). However, a sustained increase of these inflammatory mediators may contribute to persistent tissue damage, impaired angiogenesis, delayed epithelialization, and extracellular matrix degradation (8,9). Previous research has shown that high levels of pro-inflammatory cytokines are linked to poor outcomes in wound healing and more severe diabetic foot infections (8).

Matrix metalloproteinases (MMPs) are zinc-dependent proteolytic enzymes involved in the remodeling of extracellular matrix components during tissue repair. Among these enzymes, MMP-13 has received the most interest due to its consequences in the degradation of collagen and tissue destruction (10). While MMP-13 is essential for normal wound remodeling, this enzyme can be produced excessively and cause over-proteolysis of extracellular matrix proteins, leading to a delay of chronic diabetic ulcer (11). Elevated levels of MMP-13 have been described in other chronic inflammatory diseases and infected diabetic wounds, but its role has not yet been clarified in relation to ulcer progression and tissue damage (12).

Among these matrix metalloproteinases associated with chronic wound healing, the diabetic foot ulcer has been most widely studied for MMP-2 and MMP-9. In contrast, MMP-13 has received relatively little attention even though it is a potent collagenase and plays a key role in matrix degradation. MMP-13 is sequenced from the cDNA library of healed skin, and shows high binding affinities for types I and III collagens, two major components of scar according to the collagen type composition in wound tissue. Thus, overexpression of MMP-13 may directly lead to tissue injury and impaired repair.

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Moreover, the association between MMP-13 and biofilm-forming bacteria, pro-inflammatory cytokines, and Wagner grading in diabetic foot ulcer patients has not been elucidated yet. Therefore, this suggests investigating the levels of MMP-13 with inflammatory biomarkers and biofilm formation to give a new view regarding the immunopathogenesis and etiology of diabetic foot ulcers (13).

One way in which diabetic foot ulcer severity is commonly assessed, based on the depth of tissue involvement and/or presence of gangrene or osteomyelitis, is the Wagner grading system (14). Higher Wagner grades are typically correlated with increased bacterial load, prolonged inflammatory response, and poorer prognosis. Thus, the relationship between inflammatory mediators, MMP-13 expression, and biofilm-forming bacteria, as well as ulcer severity, should be studied to obtain more information on the immunopathogenesis of a diabetic foot ulcer. While the individual contributions of various inflammatory cytokines, biofilm-forming bacterial isolates, and matrix metalloproteinases in diabetic foot ulcers are increasingly being documented, few studies have investigated assessing their combined association with ulcer severity using Wagner grading. To improve patient prognosis, along with quality of life, the objective of the present study was to analyze an association between pro-inflammatory cytokines (IL-6, TNF-α, and IL-1β), MMP-13 levels, biofilm-forming bacteria isolates, and Wagner grading, and to provide evidence that may support early risk stratification (12).

Materials & Methods

Study Design

This analytical cross-sectional study aimed to evaluate the associations among serum pro-inflammatory cytokines, biofilm-forming bacterial isolates, MMP-13 levels, and Wagner grade in patients with diabetic foot ulcers (DFUs).

Study Setting and Duration

This study was conducted at Tikrit Teaching Hospital, Salah Al-Din Province, Iraq. Patients and control participants were recruited from diabetic clinics and surgical wards. According to predefined exclusion and inclusion criteria, sample collection and laboratory investigations were performed between October 2025 and February 2026.

Study Population

A total of 100 participants were included, divided into four equal groups:

1. Individuals with diabetic foot ulcers who had strong biofilm-forming bacterial isolates (n = 25).
2. Individuals with diabetic foot ulcers who had weak or non-biofilm-forming bacteria (n = 25).
3. Patients with type 2 diabetes mellitus without foot ulcers (n = 25).
4. A control group of 25 people who are apparently healthy control participants.

During the study period, samples were taken from patients who visited surgical wards and diabetic clinics. Data on smoking status were retrieved from the patient interview at enrollment, while clinical variables like hypertension or the duration of diabetes, and HbA1c were obtained from patients' medical records.

Sample Size Justification

The sample size was limited to the number of participants present during that period who met pre-defined inclusion criteria. 100 participants were enrolled, and classified into four equal groups (n = 25) to facilitate comparative

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statistical analysis of inflammatory biomarkers, MMP-13 levels, and biofilm-forming bacterial isolates.

Inclusion Criteria

Participants were eligible for inclusion if they met the following criteria:

1. Aged 40 years or older.
2. Ability and willingness to provide written informed consent.

For diabetic foot ulcer groups:

3. Confirmed diagnosis of type 2 diabetes mellitus.
4. Presence of a clinically diagnosed Diabetic foot ulcer.

For the Diabetic control group:

5. Confirmed diagnosis of type 2 diabetes mellitus without a current Diabetic foot ulcer.

For the healthy control group:

6. Apparently healthy individuals without diabetes mellitus or active foot ulcer.

Exclusion Criteria

- Patients with autoimmune diseases.
- Patients on corticosteroid or immunosuppressive therapy.
- Patients with malignant diseases.
- Patients with severe systemic infection or sepsis.
- Patients who were on antibiotic treatment 72 hours before samples were collected.

Sample Collection

Two samples were collected from each patient with a diabetic foot ulcer:

1) Aseptic wound swab sampling of the ulcer surface for bacteriological analysis.
2) Five milliliters (ml) of venous blood samples were collected in sterile conditions and transferred into gel tubes. To extract serum, blood samples were spun for ten minutes at 3000 rpm after being allowed to clot at room temperature. Serum samples were stored at -20°C until ELISA analysis.

Bacterial Isolation and Identification

Each swab sample was plated onto nutrient agar, MacConkey agar, and blood agar. After inoculation, plates were aerobically incubated at 37°C for 24 to 48 hours. Bacterial isolates were characterized by their colony morphology, Gram staining properties, and biochemical tests. The final identification was performed by VITEK-2 Compact System (bioMérieux, France). If more than one bacterial species was recovered from the same ulcer, only the predominant bacterial isolate was retained to avoid non-uniformity amongst patients in this comparative analysis, and to maintain one microbiological observation per patient.

Detection of Biofilm Formation

1. Qualitative Method (Congo Red Agar)

Congo Red Agar (CRA) was employed to detect biofilm formation phenotypically. Positive biofilm production was defined as dry crystalline black colonies (15).

2. Quantitative Method (Microtiter Plate Assay)

The ability to form biofilms was assessed quantitatively using the tissue culture microtiter plate assay. Based on optical density readings from the microtiter plate experiment, biofilm categorization was carried out using the criteria outlined by Stepanović et al (16). The bacterial isolates were categorized based on optical density readings as strong biofilm-forming, moderate

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biofilm-forming, and weak/non-biofilm-forming. Patients with strong biofilm-forming bacterial isolates were compared to patients with weak/non-strong biofilm-forming isolates for statistical analysis, and the detailed distribution of strong, moderate, and weak biofilm-forming bacterial isolates is reported separately (Fig 1).

Pro-inflammatory cytokine and MMP-13 measurement

According to the manufacturers' instructions, commercially available enzyme-linked immunosorbent assay (ELISA) kits were used to quantify serum levels of MMP-13 (Boster Biological Technology, Fremont, CA, USA), IL-1β, TNF-α, and IL-6 (R&D Systems, Minneapolis, MN, USA). TNF-α, IL-6, and IL-1β had detection limits of >0.7 pg/ml, 3.5 pg/ml, and 1.6 pg/ml, respectively.

Wagner Grading System

The Wagner classification system was used to evaluate the severity of diabetic foot ulcers. using the Wagner classification system (14).

- Wagner Grade 0: pre-ulcerative lesion or a deformity.
- Wagner Grade 1: Superficial ulcer
- Wagner Grade 2: without abscess or osteomyelitis, a deep ulcer extends to the joint capsule, a ligament, bone, tendon, or deep fascia.
- Wagner Grade 3: Osteomyelitis or a deep ulcer with an abscess
- Wagner Grade 4: Partial gangrene of the foot
- Wagner Grade 5: gangrene involving the entire foot.

Statistical Analysis

The data were subsequently analyzed using the Statistical Package for Social Sciences (SPSS) version 26. Result expressed as mean ± standard deviation (SD). The normality data was evaluated using the Shapiro-Wilk test. Furthermore, Data between groups were compared using one-way analysis of variance (ANOVA) and Tukey's post hoc test for multiple comparisons. Associations between cytokine levels, MMP-13, and biofilm production and Wagner grading were assessed by using Pearson correlation analysis. P-value < 0.05 was measured as significant.

Results:

Shown in Table 1, compared with the other groups, patients with strong biofilm-forming bacterial isolate diabetic foot ulcers had significantly increased age (62.4 ± 8.2), duration of diabetes (14.2 ± 4.5), HbA1c levels (9.4 ± 1.3), Wagner grade (3.8 ± 0.7), and ulcer duration (8.7 ± 2.1) (P < 0.05). Moreover, smokers (52%) and patients with hypertension (64%) were more frequently identified in those with strong biofilm-associated ulcers. There was no significant difference regarding gender distribution between study groups (P = 0.622). Essentially, patients with strong biofilm-forming bacterial isolates exhibited poorer glycemic control, longer diabetes duration, and greater ulcer severity.

The isolates of bacteria and their biofilm-forming ability are shown in Figure 1 in patients with diabetic foot ulcers. *P. aeruginosa* was the most common pathogen isolated (24%) and had the highest prevalence of strong biofilm-forming potential at 83.3%, followed by *S.aureus* (22%) with 63.6% strong biofilm-forming. Escherichia coli and *Klebsiella* spp. also indicate considerable biofilm-forming capacities. In summary, 62% of the bacterial isolates were strong biofilm-forming, and just 14% showed weak or no biofilm formation, revealing a high rate of biofilm-forming bacterial isolates in diabetic foot ulcer isolates.

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Table 1: Demographic and Clinical Characteristics of Study Groups

Variables	Strong Biofilm DFU (n=25)	Weak/non-biofilm DFU (n=25)	Diabetic without DFU (n=25)	Healthy Control (n=25)	P-value
Age (Years)	62.4 ± 8.2	58.7 ± 7.9	56.9 ± 6.8	54.3 ± 5.7	0.041
Gender	17/8	15/10	12/13	13/12	0.622
Duration of diabetes (Years)	14.2 ± 4.5	11.8 ± 3.9	10.5 ± 3.1	-----	0.032
HbA1c (%)	9.4 ± 1.3	8.6 ± 1.1	7.8 ± 0.9	5.2 ± 0.4	<0.001
Wagner grade	3.8 ± 0.7	2.4 ± 0.6	-----	-----	<0.001
Ulcer duration (weeks)	8.7 ± 2.1	5.1 ± 1.8	-----	-----	0.003
Smoking (%)	52%	40%	36%	20%	0.048
Hypertension (%)	64%	56%	48%	16%	0.015

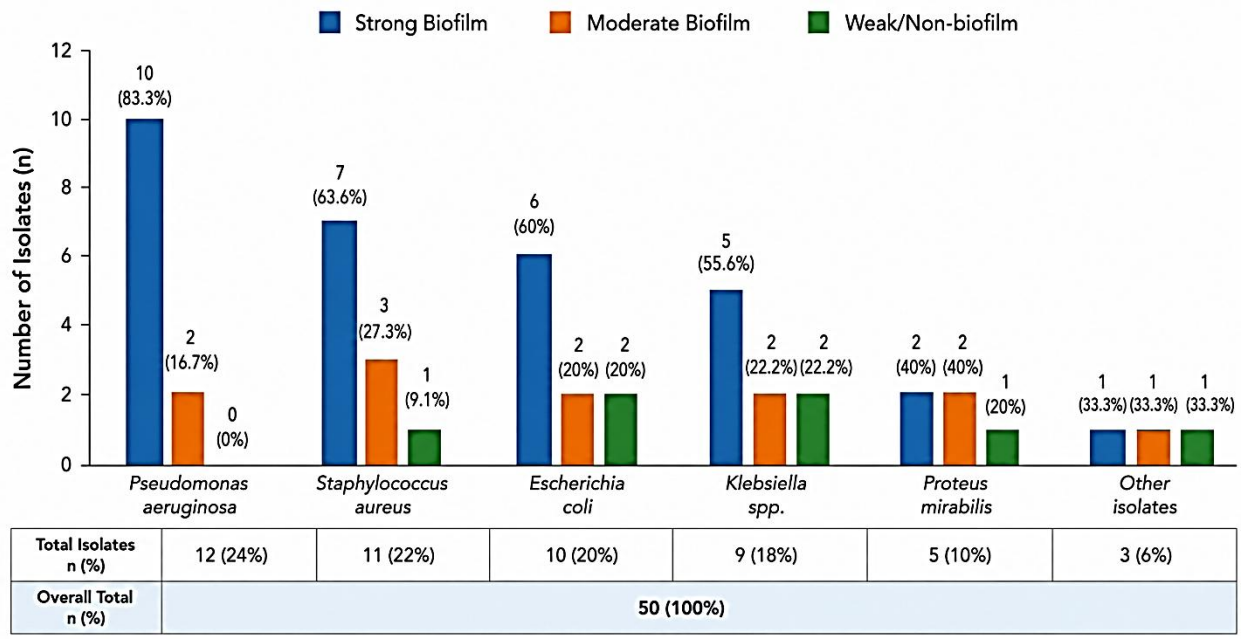

Figure 1. The distribution of bacterial isolates among people with diabetic foot ulcers according to their capacity to form biofilms

The levels of pro-inflammatory cytokines and MMP-13 in serum were shown as illustrated in Figures 2 and 3. The levels of TNF-α, IL-1β, and IL-6 were significantly higher in DFU patients than in diabetic patients without ulcer or healthy controls ($P < 0.001$). The highest concentrations of the biomarkers were observed in patients with a strong biofilm-forming bacterial isolate diabetic foot ulcer (TNF-α: 146.7 pg/ml, IL-1β: 112.9 pg/ml, IL -6: 182.4 pg/ml, and MMP-13: 1024.6 pg/ml), whereas the lowest concentrations were observed in the healthy control patients. Patients with weak/non-biofilm diabetic foot ulcers showed intermediate biomarker levels (IL-6: 121.8 pg/ml, TNF-α: 98.4 pg/ml, IL-1β: 76.2 pg/ml, and MMP-13: 742.8 pg/ml). These findings indicate that biofilm-forming bacterial isolate capacity is associated with higher inflammatory biomarkers and increased MMP-13 levels.

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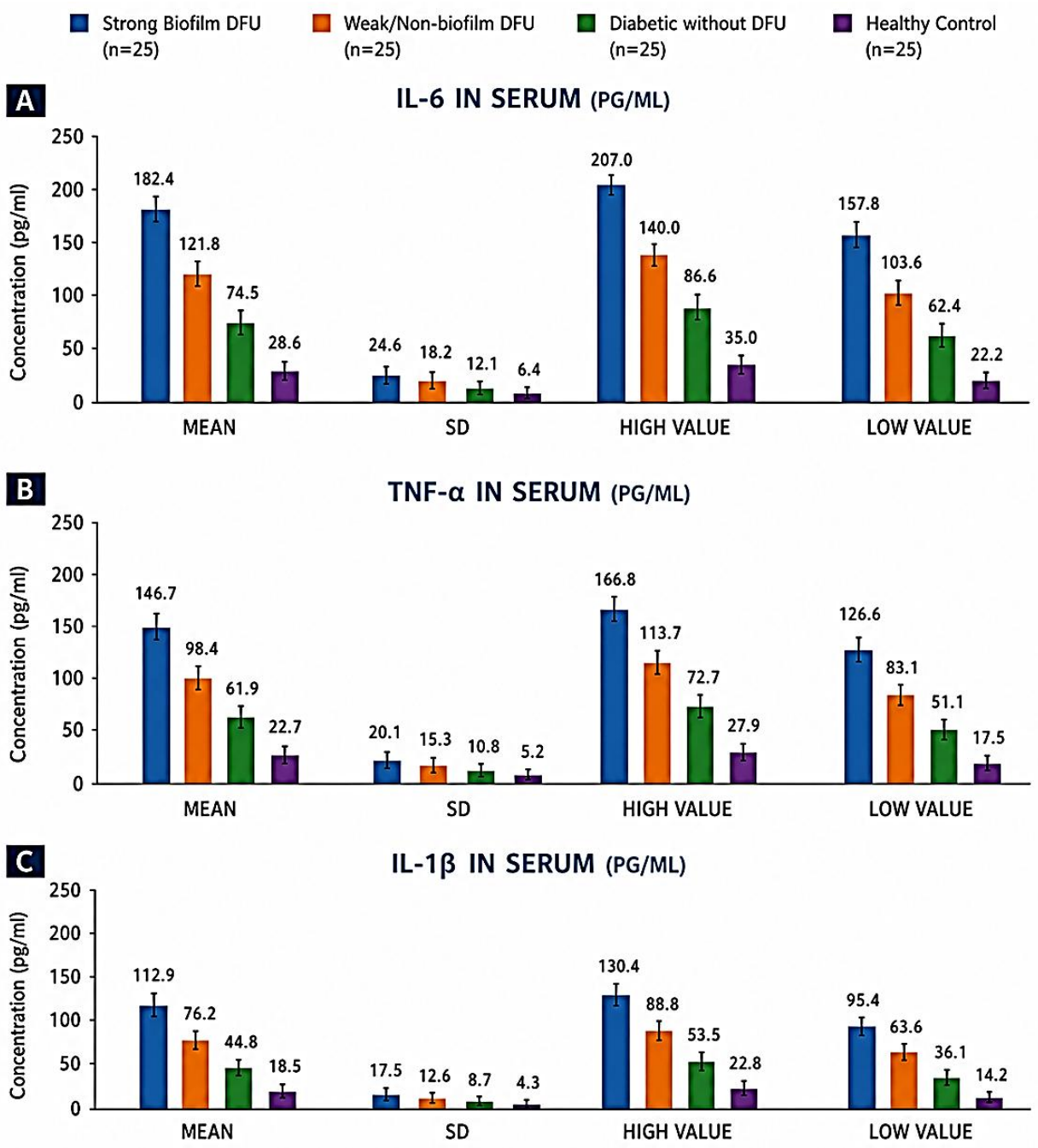

Figure 2: Serum pro-inflammatory cytokine levels in study groups. Concentration of (A) IL-6, (B) TNF-α, and (C) IL-1β. Data are denoted as mean ± SD. Compared with other subject groups, strong biofilm-forming bacterial isolates from diabetic foot ulcer patients had the highest levels of cytokines ($P < 0.001$).

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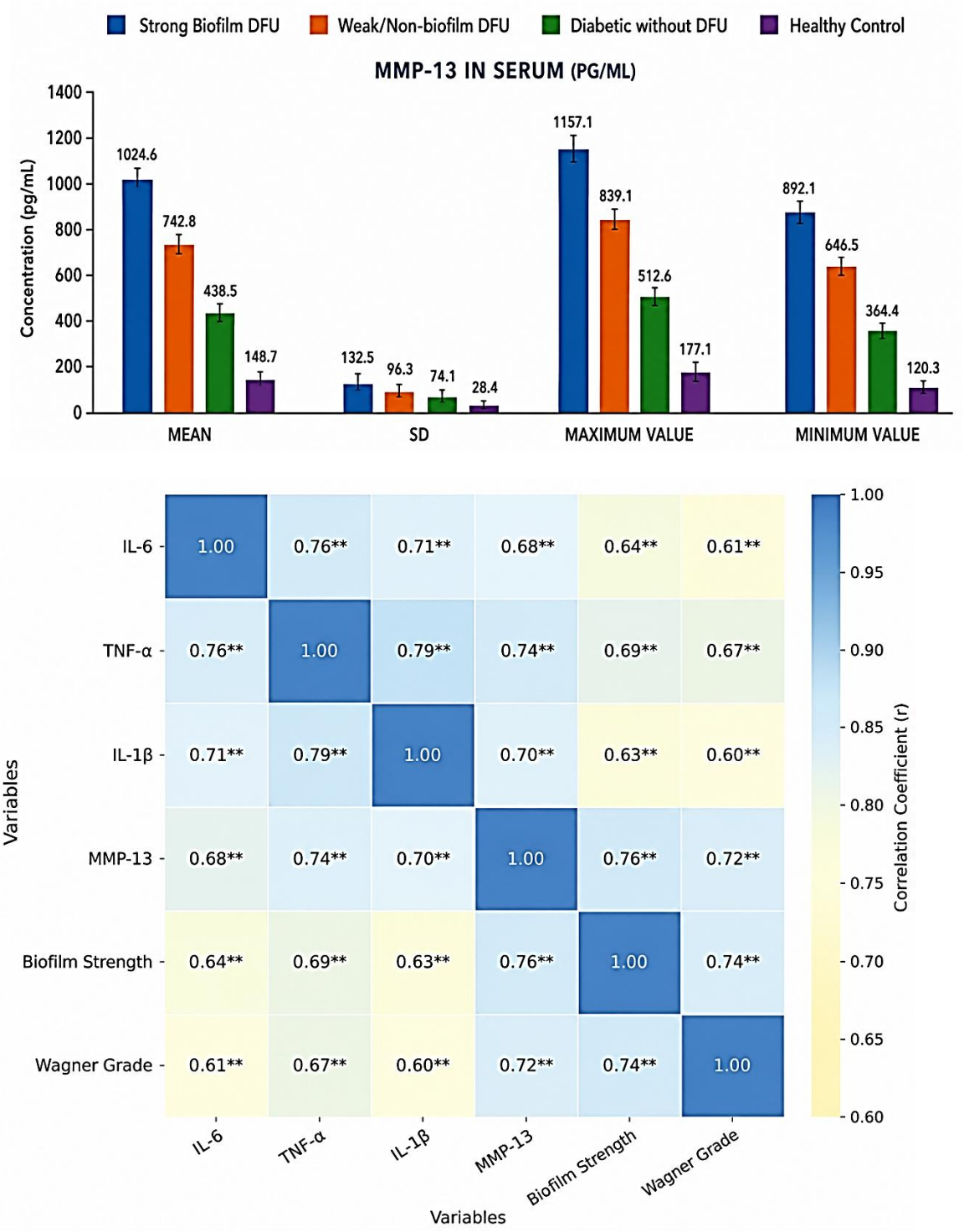

Figure (4): Correlation heatmap of various serum cytokines, including IL-6, TNF-α, IL-1β, and MMP-13 levels and biofilm-forming bacterial isolate capacity, Wagner grade, for evaluating diabetic foot ulcer patients (n = 50). Correlation coefficients (r) are shown in each cell and were obtained using Pearson (PCC). ** indicate statistical significance at $P < 0.01$.

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The correlation of pro-inflammatory cytokines, MMP-13, biofilm formation, and Wagner grading in diabetic foot ulcer patients is shown in Figure 4. Correlation between all studied variables was found to be positive, $P \leq 0.01$. Furthermore, MMP-13 showed strong positive correlations with biofilm strength and Wagner grade ($r = 0.76$; $r = 0.72$, respectively), suggesting a potential link between ulcer progression, inflammation, and chronic infection. Regarding biofilm strength, moreover, both IL-6 and TNF-α, as well as IL-1β, were positively correlated with each other and with ulcer severity. These findings indicate that inflammatory biomarkers and biofilm are closely related to diabetic foot ulcers.

Discussion:

Microbiological results showed two of the most common bacterial isolates, *P. aeruginosa* and *S. aureus*, with a strong biofilm-forming bacterial isolate (Figure 1). This is consistent with the results of previous studies reporting that *P. aeruginosa* and *S. aureus* are among the most frequently linked to biofilm formation isolated in DFUs (17). Banu et al.'s investigation found that 46% of DFU isolates formed biofilms, of which 26.5% were *P. aeruginosa* and 10.5% were E. coli (7). According to Mottola et al., *Pseudomonas* strains produced biofilms at the highest rate, followed by *Acinetobacter, Corynebacterium, Enterococcus*, and *Staphylococcus* (18). Furthermore, Vatan et al. and Maity et al. confirmed that A. baumannii (65%), *P. aeruginosa* (52%), and *Klebsiella spp*. (40%) were the most prevalent biofilm-associated bacteria in DFUs (19,20). However, differential bacterial distributions among bacterial isolates have been reported depending on the geographic area. As an example, Proteus mirabilis was isolated in a study performed in Ouargla, southern Algeria, as the most common Gram-negative pathogen in diabetic foot infections (21). These differences may be associated with differences in patient populations, healthcare settings, antibiotic usage patterns, or regional microbial ecology.

Furthermore, a few studies link DFUs to biofilm development. Zubair et al. reported that biofilm-forming bacteria isolates were present in 77.1% of DFU patients, and that the presence of a biofilm was associated with gender, neuropathy, osteomyelitis, ulcer length, ulcer grade, necrotizing ulcer, and ulcer size [20]. Malik et al. found that the same risk variables identified by Zubair et al. were involved in the overall biofilm-forming infection rate among DFUs, which was 67.9% (22,23). According to Vatan et al., 34% of the 339 wound isolates produced biofilms overall (19). These results support prior reports that indicate biofilm-forming bacterial isolates are more commonly isolated from chronic diabetic wounds and contribute to chronic infection and impaired wound healing (5,6). Additionally, bacterial adherence and biofilm formation are facilitated by the necrotic tissue and detritus found in DFUs (24). Biofilms are markedly more resistant to eradication than planktonic bacteria (25-27). Indeed, biofilm development is considered a major contributor to that traditional antimicrobial agents fail to treat wound infections (28,29).

The present study evaluated the associations among pro-inflammatory cytokines, MMP-13, biofilm-forming organisms, and Wagner grading in diabetic foot ulcers. Increased levels of both IL6, TNF-a, IL1β, and MMP-13 in diabetic foot ulcer patients were also shown if the infection was associated with strong biofilm-forming bacteria (Figures 2 and 3).

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Furthermore, strong positive associations were found among inflammatory biomarkers, biofilm formation, and ulcer grade (Figure 4), which highlights that chronic inflammation plays a significant role in diabetic foot ulcer development.

Patients with diabetic foot ulcers had significantly higher levels of IL-6, TNF-α, and IL-1β than both healthy controls and diabetes patients without ulcers. Patients infected with strong biofilm-forming bacterial isolates had the greatest amounts of cytokines. Chronic inflammation, increased leukocyte recruitment, and disruption of normal tissue repair mechanisms are all contributing outcomes of persistent production of these cytokines. Pro-inflammatory cytokines and cytotoxic chemicals, including TNF-α, IL-1, IL-6, IL-8, and nitric oxide (NO), are released when macrophages are activated (1). Biofilms possess the ability to avoid effective host immune clearance and simultaneously promote a chronic inflammatory microenvironment, so they contribute to sustained inflammation and delayed wound healing (30,31). Similar results have been reported in chronic diabetic wounds, where tissue damage and delayed healing are caused by increased inflammatory responses (8,9).

The significant increase of IL-1β in the current study further confirms an ongoing driver of excessive inflammation affecting diabetic foot ulcer development. IL-1β is an important early mediator of the innate immune response, produced mainly by activated macrophages, and is released rapidly after tissue injury or microbial challenge. Chronic elevation of this cytokine may contribute to chronic inflammation, tissue destruction, and impaired wound healing (32,33). Similar findings from earlier research indicate that non-healing wounds continue to sustain a detrimental microenvironment by failing to transition into a restorative state and maintaining a chronic inflammatory state (34). Hence, IL-1β may serve as a potential biomarker to detect diabetic foot ulcers with hyper-inflammatory features.

Among patients with biofilm-associated diabetic foot ulcers with the highest levels, MMP-13 was one of the most significantly elevated findings in the current study. MMPs are involved in extracellular matrix remodeling during wound healing, but excessive MMP activity can lead to aberrant collagen degradation, which impairs tissue regeneration (35). Matrix metalloproteinase (MMP) activity is tightly regulated to enable extracellular matrix remodeling during wound healing. In chronic wounds, persistent inflammation tips the scale and leads to uncontrolled protease activity in healing-deficient tissue. Along with the activation of neutrophils and macrophages that additionally support this inflammatory microenvironment by multiple productions of inflammatory cytokines and proteases (36). Multiple studies have reported the stimulating effect of proinflammatory cytokines (TNF-α and IL-1β) on matrix metalloproteinases expression, which contributes to tissue destruction and the development of ulcers (10). Accordingly, the elevated MMP-13 levels identified in this study would likely represent increased extracellular matrix degradation in chronic diabetic wounds.

MMP-13, inflammatory cytokines, biofilm strength, and Wagner grading had a strong positive correlation according to the correlation analysis. Notably, MMP-13 had particularly strong correlations with both biofilm formation and ulcer severity, suggesting it could serve as a potential biomarker of chronic inflammation, degradation of extracellular matrix, and disease progression in diabetic foot ulcers. These

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findings support our own hypothesis that biofilm-mediated inflammation may contribute to proteolytic activity and progression of diabetic foot ulcer categories. Similar findings have been indicated for matrix metalloproteinases of chronic wounds (11,37).

These findings also support a positive association between Wagner grading, which correlates closely with inflammatory biomarkers, and imply that both inflammation-related burden and extracellular matrix disintegration rise with advancing ulcer severity. High-grade Wagner scores were associated with increased inflammatory cytokines and MMP-13 in tissue, suggesting a greater inflammatory burden and more advanced ulcer severity.

Overall, the clinical significance of our findings lies in the fact that TNF-α, IL-1β, IL-6, and especially MMP-13 may serve as biomarkers for ulcer severity evaluation and disease monitoring. Additionally, these findings may provide a rationale for future studies investigating biofilm-forming bacterial isolates and inflammation-targeted therapeutic strategies.

Study Limitations

Several limitations must be considered when interpreting these findings. First, the study was conducted at a single center; therefore, it may not be generalizable to other populations and healthcare systems. Second, the relatively small sample size may have reduced the statistical power to detect weaker associations among the study variables. Third, multivariate statistical analyses were not performed to adjust for potential confounding factors, including age, duration of diabetes, glycemic control, smoking status, and hypertension. Therefore, the individual role of each variable could not be determined. Fourth, assessment of biofilm formation was based solely on phenotypic methods, and molecular characterization of biofilm-associated genes or molecular confirmation of biofilm production was not done.

In addition, a formal sample size calculation or statistical power analysis was not conducted before the recruitment of participants. The present findings provide preliminary data for better designing future multicenter studies with larger sample sizes, combined molecular strategies, and adequate multivariate statistical analyses that would confirm and further expand current results.

Conclusion

The present study revealed significant connections between serum concentrations of IL-6, TNF-α, IL-1β, MMP-13, and Wagner grade as well as biofilm-forming bacterial isolates in patients with diabetic foot ulcers. These observations indicate that these inflammatory biomarkers may be clinically relevant in measuring disease severity. Nonetheless, given the cross-sectional nature of this study, no potential interpretations can be drawn from observed associations. Much larger multicenter prospective longitudinal studies are needed to confirm these findings and assess the incremental value of these biomarkers for both clinical risk stratification and therapeutic decision-making.

Ethical Approval: The Middle Technical University, Balad Technical Institute, Iraq's Research Ethics Committee examined and accepted the study protocol (Approval No.: 1015, Date: September 10, 2025). Before sample collection and data collection, each subject provided written informed consent. Every procedure was carried out in compliance with the Declaration of Helsinki's ethical standards.

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