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ORIGINAL ARTICLE

The Major role of TNF-α and miR-203 in the Immune Response of Diabetic Foot Ulcer

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ABSTRACT

Background: *Diabetic foot ulcers (DFUs) are a major complications of diabetes mellitus, often complicated by infections particularly with Pseudomonas aeruginosa, a pathogen known to impair wound healing. Tumor necrosis factor-alpha (TNF-α) is a key inflammatory mediator and microRNA-203 (miR-203) has been suggested to play a role in regulating immune responses and inflammation. However, the interactions between Pseudomonas aeruginosa, TNF-α and miR-203 in DFUs remain poorly understood.* ***Objective:*** *This study was designed to explore the correlation between Pseudomonas aeruginosa infection and polymorphism (miR-203 and TNF-α) in a cohort of diabetic patients with foot ulcers.* ***Methodology:*** *The study involved the collection of 210 samples which were categorized based on sex (male and female) and geographic location (Urban and Rural). The following tests were conducted: Complete Blood Count (CBC), C-Reactive Protein (CRP), Erythrocyte Sedimentation Rate (ESR), and HbA1c. Afterward, DNA was extracted from whole blood samples. Immunological assays were performed to measure TNF-α, and miR-203 polymorphisms. Additionally, 70 samples from diabetic foot patients were examined to detect Pseudomonas aeruginosa using biochemical tests, with confirmation achieved through the VITEK2 system compact.* ***Results:*** *A total of 210 diabetic person included 70 healthy control, 70 DM without foot ulcers and 70 DM with foot ulcer through August 2022 to April 2023. In this study TNF-α polymorphism in healthy controls and DM without foot ulcer were (GG and AA(0) and the AG (70), while TNF-α polymorphism in DFU (GG 4 , AA 7 and AG 59) and (G 67 and A 73). The P. value (0.310) in Micro-RNA with healthy control and the P. value (<0.001) in Micro-RNA with DM without foot ulcer and DFUs.* ***Conclusion****: The findings suggest that miR-203 could serve as a potential therapeutic target for modulating inflammation and improving wound healing in DFUs. Additionally, TNF-α may represent a key mediator in the inflammatory pathway that links infection to impaired wound recovery.*

INTRODUCTION

Diabetic foot ulcers are a major complication of diabetes, often leading to chronic infections, delayed healing, and amputations[1]. *P. aeruginosa* is a common and particularly troublesome bacterium due to its ability to form biofilm in DFUs, to Resist antimicrobial treatment and exacerbate chronic inflammation[2]. The Inflammatory response with TNF-α in DFUs is a key driver of impaired wound healing, being a central mediator of this process. The studies have highlighted the potential role of miRNAs, particularly miR-203 in modulating the healing process and regulating inflammatory pathways[3]. MiR-203 has been shown to play a critical role in balancing the response of inflammation in chronic wounds and influence the expression of inflammatory cytokines, including TNF-α This review discover the complex interact between *P. aeruginosa* infection, miR-203[4], and TNF-α in DFUs. *P. aeruginosa* infection may change miR-203 expression, therefore affecting TNF-α levels and exacerbating the chronic inflammation that decreases wound healing[3]. Additionally, improve the resolution of infection, and accelerate healing in diabetic foot ulcers. Understanding the molecular mechanisms linking miR-203, and TNF-α of *P. aeruginosa* could discover the way for new treatment strategies with improving outcomes for diabetic patients with chronic foot wounds[5].

METHODOLOGY

The samples were collected from diabetic foot center which included three groups: The first group (70 samples) of diabetic foot ulcer patients, the second

group includes (70 samples) of diabetic patients only which were examined by specialist physicians and diagnosed and the third group includes (70 samples) of healthy people according to demographic data e.g: (age, gender of the patients, smoking and history of disease).

The consent of the patients or that of their guardians was obtained for each collected sample. All samples were subjected to analysis for molecular polymorphism (TNF-α and micro-RNA-203) by RT-PCR. Specimens were collected during the period between January/ 2022 to June/ 2022 from different anatomical sites including blood (whole blood and serum) and swabs (diabetic foot ulcer) for diagnosis of gram negative bacteria *P. aeruginosa* which were identified according to cultural characters examination, microscopic and biochemical tests which were confirmed by VITEK 2 system compact.

RESULTS

There were 210 participants enrolled in this study and classified into three groups with 70 participants in each, namely, diabetes mellitus with foot ulcer (DM/foot ulcer) group, diabetes mellitus with no foot ulcer (DM/ no foot ulcer) group and the healthy control group. The three groups were almost matched for their demographic characteristics without significant differences in these variables. However, in all the three groups, almost more than 50% of the participants were at age of 60 years and older. Males were relatively dominant they were 55.7%, 54.3% and 58.6% in the DM/foot ulcer, DM/ no foot ulcer and healthy control groups, respectively. Most study participants were of urban origin. Chi square test revealed no significant differences in all these variables with a P. value of > 0.05 as shown in table 1.

Table 1: Demographic characteristics of the studied groups

Variable	Category	Group			P. value*
		DM/foot ulcer (n=70)	DM/ no foot ulcer (n=70)	Healthy control (n=70)	
		N %	N %	N %	
Age (year)	40 – 49	14 (20%)	13 (18.6%)	16 (22.9%)	0.873
	50 – 59	18 (25.7%)	21 (30%)	23 (32.9%)	
	60 – 69	23 (32.9%)	25 (35.7%)	19 (27.1%)	
	≥ 70	15 (21.4%)	11 (15.7%)	12 (17.1%)	
Sex	Male	39 (55.7%)	38 (54.3%)	41 (58.6%)	0.909
	Female	31 (44.3%)	32 (45.7%)	29 (41.4%)	
Residence	Urban	51 (72.9%)	54 (77.1%)	57 (81.4%)	0.504
	Rural	19 (27.1%)	16 (22.9%)	13 (18.6%)	

*Chi-square test used in all comparisons

The frequency of TNF-α Genotyping among the studied groups showed a significantly higher frequency of GG and AA in diabetic patients with foot ulcer where 5.7% and 10% of those patients had these two genotypes respectively compared to none of diabetic patients with no foot ulcer and healthy controls. Relatively lower frequency (84.3%) of AG genotypes compared to 100% in each of the other two groups, (P. value <0.001) was detected. The distribution of the singly genotyping G and A showed no significant differences among the three groups, (P. value > 0.05). These findings are shown in (table 2) and (Fig. 2).

Table 2: Comparison of TNF-α Genotyping (Rs2275913) among the studied groups

TNF-α Genotyping	DM / foot ulcer (n=70)	DM no foot ulcer (n=70)	Healthy control (n=70)	P. value
	N %	N %	N %	
GG	4 (5.7%)	0 (0%)	0 (0%)	<0.001*
AA	7 (10%)	0 (0%)	0 (0%)	
AG	59 (84.3%)	70 (100%)	70 (100%)	
Total	70 (100%)	70 (100%)	70 (100%)	
G	67 (47.9%)	70 (50%)	70 (50%)	0.917 **
A	73 (52.1%)	70 (50%)	70 (50%)	
Total	140 (100%)	140 (100%)	140 (100%)	

*Fisher's Exact Test used in comparison
**Chi-square test used in comparison

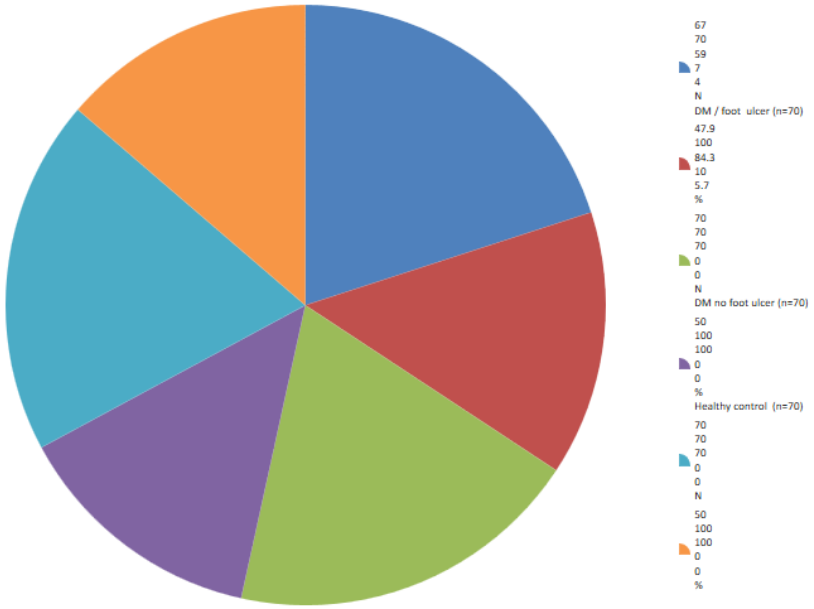

Fig. 2: Comparison of TNF-α Genotyping (Rs2275913) among the studied groups

Comparison of MiRNA 203 among the studied groups is shown in Table 3. Patients with DM /DFUs had much higher mean MiRNA203 level than the DM no foot ulcer group and controls group: 6.0781, 1.6068 and 1.4523 Respectively, ANOVA was Significant at (P. value <0.001). The median level was also significantly higher in DM / foot ulcer than the other two groups. Moreover, the post-hoc (LSD) testing showed no significant difference between DM patients with no foot ulcer and control with, (P. value >0.05), while LSD testing showed that DM / foot ulcer group when compared with the other two groups, the differences were statistically significant in both cases (P. value <0.05).

Table 3: Comparison of MiRNA -203 (fold change) among the studied group

MiRNA -203(fold change)		Group			P. value
		DM / foot ulcer (n=70)	DM no foot ulcer (n=70)	Healthy control (n=70)	
Mean		6.0781	1.6068	1.4523	<0.001*
Standard deviation		1.5123	0.2966	0.2601	
Median		6.326	1.612	1.413	<0.001**
Inter-quartile range	Lower border	5.159	1.371	1.265	
	Upper border	7.329	1.804	1.606	
*ANOVA test and Post-Hoc (LSD) analysis					
DM/foot ulcer vs. DM no foot ulcer		<0.001			
DM/foot ulcer vs. healthy control		<0.001			
DM no foot ulcer vs. healthy control		0.310			

ANOVA: Analysis of variances, LSD: least significant difference
**Median test used to compare medians

DISCUSSION

The demographic characteristics of the studied groups reveal several notable observations. The age distribution indicates that the majority of participants across all groups were 60 years and older, with no significant differences between groups. This finding aligns with existing literature highlighting age as a significant factor in diabetes development and complications, including foot ulcers. Advanced age is associated with increased insulin resistance and cumulative exposure to hyperglycemia, contributing to microvascular and macrovascular complications such as diabetic foot ulcers. Some reports documented a higher prevalence of diabetes-related complications in older populations, consistent with the present findings [6,7]. The male predominance observed in our study is supported by some research indicating higher diabetes prevalence in men. A meta-analysis demonstrated a slightly greater risk of diabetes-related complications among males due

to hormonal differences, particularly testosterone levels, and lifestyle factors such as smoking and physical inactivity[8]. However, other studies have suggested a higher prevalence of diabetes in women in specific populations due to disparities in healthcare access and cultural influences[9]. These findings suggest that gender-related risks for diabetes and its complications may vary based on regional and socioeconomic contexts. The urban predominance of participants is consistent with studies indicating a higher prevalence of diabetes in urban areas. Urbanization is associated with lifestyle changes, including reduced physical activity, increased consumption of processed foods, and higher rates of obesity, which are major risk factors for diabetes [6]. However, the growing burden of diabetes in rural areas due to limited healthcare in the structure, highlights the need for targeted public health interventions[7]. The lack of significant differences in demographic variables across the groups confirms the comparability of these cohorts, reducing potential confounding factors and increasing the reliability of the findings related to diabetes complications. These results support the generalizability of the study, particularly in populations with similar demographic structures.

Tumor Necrosis Factor-alpha (TNF-α) is a pro-inflammatory cytokine implicated in the pathogenesis of chronic inflammation and various complications associated with diabetes, including diabetic foot ulcers (DFUs). The current study demonstrates a significant association between specific TNF-α genotype (GG and AA) and diabetic patients with foot ulcers, in contrast to diabetic patients without foot ulcers and healthy controls. These findings emphasize the potential genetic predisposition to DFU development influenced by TNF-α polymorphisms. The presence of AA and GG genotypes in 10% and 5.7% of DFUs patients with DM, respectively and their absence in DM patients without foot ulcers and controls group, highlights an important genetic divergence. This divergence may contribute to increased susceptibility to inflammation and impaired wound healing in individuals with these genotypes. The AG genotypes, predominant in the DM patients without and DFUs and control group persons 100% appears to confer a protective effect against the inflammatory pathways leading to DFUs. The significantly lower frequency of the AG genotype in DFU patients further supports this hypothesis. The lack of significant differences in the alleles genotypes may modulate the risk of DFUs, individual alleles don't have the same impact. This observation aligns with previous studies indicating that specific TNF-α genotypes, rather than individual alleles, influence the healing processes in chronic conditions and inflammatory response such as DFUs[10]. The role of TNF-α in DFU pathogenesis can be explained by its regulatory effects on chronic inflammation, angiogenesis, and tissue remodeling. Elevated TNF-α levels have been linked to increased apoptosis, reduced endothelial cell function, and impaired fibroblast activity, all of which compromise wound healing in DM Individuals [11].

Genetic polymorphisms such as GG and AA could lead to differential TNF-α expression, exacerbating the chronic inflammatory environment and hindering the resolution of wounds. Furthermore, environmental and metabolic factors, including hyperglycemia, oxidative stress, and microbial infections, likely interact with these genotypes to amplify the risk of DFU development. For example, the GG genotype has been associated with higher TNF-α transcription and secretion, leading to prolonged inflammation in response to tissue injury [12]. On the other hand, the AA genotype may enhance susceptibility to extracellular matrix degradation and angiogenic imbalance, which are critical in the progression of DFUs [13].

These results under score the importance of genetic screening for TNF-α polymorphism in DM patients as a potential tool for identifying patients at risk for DFUs. Moreover, targeted therapeutic approaches object at modulating TNF-α activity may offer promising strategies for preventing and managing DFUs in genetically predisposed individuals.

P. aeruginosa, a common and highly virulent pathogen in diabetic foot infections. This bacteria possess a broad virulence factors, including exotoxin a, elastases, and LPS, which can directly stimulate the production of TNF-α and other pro-inflammatory cytokines. Chronic exposure to high levels of TNF-α due to persistent infections with *P. aeruginosa* contributes to chronic inflammation, delayed wound healing, and tissue destruction[14]. *P. aeruginosa* forms biofilms that prevent it from the immune system and antibiotics. TNF-α production by biofilm components such as alginate and pyocyanin, may exacerbate the chronicity of infection in susceptible genotypes while persistent infection with *P. aeruginosa* in DFUs may lead to immune boosters. TNF-α dysregulation, particularly in patients with GG or AA genotypes, may obstructs the resolution of inflammation perpetuating bacterial survival. The overexpression of TNF-α contributes to the degradation of proteins extracellular matrix and impairs angiogenesis, both of which are critical for wound healing. The presence of *P. aeruginosa* improves this detrimental effect through the secretion of proteases and toxins [15].

In our work the expression of micro-RNA-203 is significantly elevated in patients with DFUs (DM/foot ulcer group) compared to both DM patients without foot ulcers (DM no foot ulcer group) and healthy controls. This finding suggests a strong association between increase MiRNA 203 levels and the pathophysiological processes underlying DFUs. The biomarker increases in micro-RNA-203 levels in the DM/foot ulcer group could be attributed to the increase the inflammatory response and tissue damage which in these patients.

DFUs are often complicated by chronic infections, impaired wound healing, and immune dysregulation[11]. All of which contribute to the altered expression of MiRNAs 203, emerging evidence indicates that specific MIRNAs plays a crucial role in regulating key pathways such as angiogenesis, inflammation, and cell proliferation, which are disrupted in DFUs [15]. *P. aeruginosa* is a frequent pathogen in DFUs infections and is associated with poor healing and increased morbidity. Its ability to form biofilms and produce a wide range of virulence factors, such as elastases, exotoxins, and quorum-sensing molecules, exacerbates tissue damage and inhibits immune clearance[16]. These virulence mechanisms can trigger the upregulation of certain MiRNAs, acting as biomarkers of infection severity and immune response. For instance, some studies have shown that specific MiRNAs are upregulated in response to infections caused by *P. aeruginosa*, reflecting their role in modulating the host's inflammatory and immune responses [17,18]. The lack of significant difference in MiRNA levels between the (DM/ no foot ulcer group) and healthy controls is also noteworthy. This suggests that MiRNA dysregulation is not a generalized feature of diabetes but is likely associated with specific complications, such as infections and chronic ulcers. The elevated MiRNA levels in diabetic foot ulcer patients may, therefore, serve as a diagnostic biomarker for infection severity and disease progression. These findings emphasize the potential of MiRNA-203 as biomarker for early detection and monitoring of diabetic foot infections, particularly those involving *P. aeruginosa.* Future studies should explore the specific MiRNA-203 signatures associated with *P. aeruginosa* infections and their role in predicting outucome and tailoring therapeutic interventions. Moreover, targeting MiRNA-203 pathways could offer a novel therapeutic approach to mitigate the effects of chronic infections and enhance wound healing in diabetic DFUs [19].

CONCLUSION

Our study under scores the significant roles of miR-203 and TNF-α in the immune response to diabetic foot ulcers infected with *P. aeruginosa*. MiR-203 appears to modulate inflammation and immune cell function, while TNF-α plays a pivotal role in driving the inflammatory response to infection. Together, these factors contribute to the chronicity and delayed healing of diabetic foot ulcers. Targeting miR-203 and TNF-α may offer potential therapeutic strategies to improve immune response and promote wound healing in diabetic patients with *P. aeruginosa* infections. Further investigations are necessary to fully understand their interactions and therapeutic potential.

Assignment: All participants provided informed consent for inclusion in the study and were assured that their information would be used solely for the purposes of this research and treated confidentially.

Ethical Approval: The procedures followed in this study were in accordance with the Regulations of the relevant clinical research ethics committee and aligned with the Code of Ethics of the World Medical Association (Declaration of Helsinki). Additionally, each participant received a comprehensive overview of the project

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