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Original Article

Integrated Bioinformatic Analysis Identifies S100A8/A9 as Novel Mediators Linking Vascular Endothelial Pyroptosis to Immune Dysregulation in Diabetic Foot Ulcer

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Abstract:

Background: Diabetic foot ulcers (DFU) are a severe diabetic complication marked by impaired healing, chronic inflammation, and microvascular dysfunction. Although vascular endothelial cell dysfunction and pyroptosis are implicated in DFU, the key molecular regulators connecting these pathological processes remain elusive.

Methods: An integrative bioinformatic analysis was conducted to identify key drivers. Differentially expressed genes (DEGs) from the DFU dataset GSE80178 were intersected with vascular endothelium and endothelial pyroptosis-related genes from GeneCards. Functional enrichment (GO/KEGG) of the resulting key genes was performed. Expression validation used GSE80178 and an independent dataset (GSE68183). Immune infiltration analysis (CIBERSORT) and upstream transcription factor screening (ChIP-Atlas) were also carried out.

Results: Five core genes (S100A9, S100A8, BNIP3, FSTL1, TACR1) were identified. They were enriched in IL-17 signaling, Legionellosis (involving NLRC4 inflammasome), and Mitophagy. S100A8 and S100A9 were consistently the most upregulated genes in DFU across datasets. Their high expression strongly correlated with a pro-inflammatory immune microenvironment, showing positive associations with monocytes and M0 macrophages, and negative correlations with resting NK and dendritic cells. Furthermore, the transcription factor DUX4 was identified as a potential upstream regulator of S100A9.

Conclusion: This study establishes S100A8 and S100A9 as central hubs in DFU, linking vascular dysfunction and pyroptosis to a sustained pro-inflammatory immune response. The novel identification of DUX4 as an upstream regulator provides deeper mechanistic insight, positioning the S100A8/A9 axis as a promising therapeutic target for breaking the chronic inflammatory cycle in DFU.

Keywords: Diabetic foot ulcer, Diabetes mellitus, S100A8/A9, Orchestrating Vascular Endothelial, Pyroptosis

1. Introduction

As one of the most severe and debilitating chronic complications of diabetes mellitus, diabetic foot ulcer (DFU) affects approximately 19% to 34% of patients during their lifetime [1]. It is the leading cause of non-traumatic lower extremity amputations globally, imposing a tremendous burden on healthcare systems and drastically compromising patients' quality of life [2 - 3]. While the pathogenesis of DFU is multifactorial, involving a complex interplay of peripheral neuropathy, ischemia, and impaired wound healing [4 - 5], the molecular drivers of its persistent inflammation and recalcitrance to

healing remain incompletely defined [6].

Among the various pathological processes, vascular endothelial dysfunction is considered a cornerstone of DFU development and progression [7]. The vascular endothelium, a single cell layer lining the blood vessels, is pivotal in maintaining vascular tone, permeability, angiogenesis, and immune homeostasis [8]. In the hyperglycemic milieu of diabetes, endothelial cells undergo profound dysfunction, characterized by reduced nitric oxide bioavailability, increased oxidative stress, and a pro-inflammatory state, ultimately leading to impaired perfusion and inadequate tissue repair [9 - 10]. Although endothelial apoptosis has been extensively studied, pyroptosis—a more recently characterized, highly inflammatory form of programmed cell death—has emerged as a critical mediator of diabetic complications [11].

Pyroptosis is mediated by gasdermin family proteins, predominantly gasdermin D (GSDMD) [12]. Triggered by inflammasome activation (e.g., NLRP3), it results in cell swelling, plasma membrane pore formation, and the release of pro-inflammatory cytokines including interleukin-1β (IL-1β) and IL-18 [13]. Accumulating evidence now underscores the significant contribution of endothelial pyroptosis to diabetic vascular injury, primarily by amplifying the local inflammatory cascade and exacerbating endothelial dysfunction [14]. However, the specific genetic regulators that connect vascular endothelial pyroptosis to DFU pathogenesis are poorly understood.

The advent of high-throughput genomic technologies and public databases now offers an unprecedented avenue to decipher key molecular drivers of complex diseases such as DFU. Herein, we employed an integrative bioinformatics approach combined with clinical validation to identify and characterize hub genes associated with vascular endothelial pyroptosis in DFU. We first sourced genes related to vascular endothelium and endothelial pyroptosis from the GeneCards database. Subsequent differential expression analysis of the GSE80178 dataset, comparing DFU, diabetic, and normal samples, identified a set of DEGs. The intersection of these gene sets yielded five key candidates. Functional enrichment analysis illuminated their potential biological roles. After validating their expression in two independent datasets, we pinpointed S100A8 and S100A9 as hub genes. We further investigated their correlation with immune cell infiltration and ultimately confirmed their elevated expression in DFU patient blood samples via ELISA. Our findings illuminate novel molecular mechanisms in DFU and propose S100A8/A9 as potential biomarkers and therapeutic targets.

2. Materials and Methods

2.1. Data Source and Acquisition

The gene expression profile dataset GSE80178 and GSE68183 were downloaded from the Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo/). GSE80178, based on the GPL17077 platform, was used as the discovery cohort, which included samples from patients with diabetic foot ulcers (DFU), diabetic patients (DM) without foot complications, and healthy controls (Normal). GSE68183, based on the GPL13667 platform, was used as an independent validation cohort. Genes associated with "Vascular Endothelium" and "Endothelial Pyroptosis" were retrieved from the GeneCards database (https://www.genecards.org/). The relevance score cutoff was set above a certain threshold to ensure high-confidence gene sets.

2.2. Identification of Differentially Expressed Genes (DEGs)

The raw data from GSE80178 were preprocessed, including background correction, quantile normalization, and log2 transformation using the limma package in R. Probes were annotated to gene symbols, and those matching multiple genes were removed. For genes corresponding to multiple probes, the average expression value was calculated. Differential expression analysis among the DFU, DM, and Normal groups was performed using the limma package. Genes with an adjusted p-value (Benjamini-Hochberg method) < 0.05 and $|\log2 \text{ fold change (FC)}| > 1$ were considered statistically significant DEGs.

2.3. Screening of Key Genes

Venn diagrams were constructed to identify the overlapping genes among three sets: 1) Vascular Endothelium-related genes, 2) Endothelial Pyroptosis-related genes, and 3) The DEGs identified from the DFU vs. Normal and/or DFU

vs. DM comparisons. The intersecting genes were considered as key genes for subsequent analysis.

2.4. Functional Enrichment Analysis

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses for the key genes were performed using the clusterProfiler package in R. GO terms include biological process (BP), cellular component (CC), and molecular function (MF). Terms with a p-value < 0.05 were considered statistically significant.

2.5. Validation of Key Gene Expression

The expression levels of the key genes were validated in both the GSE80178 (discovery) and GSE68183 (validation) datasets. Box plots were generated to visualize the expression differences among the DFU, DM, and Normal groups. Statistical significance was assessed using t-tests or ANOVA, as appropriate.

2.6. Immune Infiltration Analysis

The relative proportions of 22 types of infiltrating immune cells in the samples from the GSE80178 dataset were estimated using the CIBERSORT algorithm, a deconvolution method for estimating cell type abundances from bulk tissue gene expression profiles. The correlation between the expression levels of the identified hub genes (S100A8 and S100A9) and the abundance of immune cells was evaluated using Spearman's correlation analysis. Results were visualized using correlation heatmaps and lollipop charts.

2.7. Identification of Upstream Transcriptional Regulators

To identify potential upstream transcriptional regulators of the hub gene S100A8 and S100A9, we interrogated the ChIP-Atlas database (http://chip-atlas.org). This platform integrates a vast compendium of publicly available Chromatin Immunoprecipitation sequencing (ChIP-Seq) experiments, which provide direct evidence of protein-DNA interactions.

2.8. Statistical Analysis

For bioinformatic data, statistical analyses were performed as described in respective sections using R software. For clinical data, continuous variables were expressed as mean ± standard deviation (SD) and compared using one-way ANOVA followed by Tukey's post-hoc test for multiple comparisons. The correlation was assessed using Spearman's rank-order correlation. A two-tailed p-value < 0.05 was considered statistically significant. All clinical statistical analyses were performed using SPSS software (version 26.0).

3. Results

3.1. Identification of Key Genes Linked to Vascular Endothelium and Pyroptosis in DFU

Transcriptomic profiling of the GSE80178 dataset revealed a distinct gene expression signature in DFU. Differential expression analysis identified numerous significant changes, visualized by a volcano plot (Figure 1A - C). Unsupervised clustering of these differentially expressed genes (DEGs) clearly distinguished DFU samples from diabetic and normal controls (Figure 1D - F).

To pinpoint key drivers, we intersected the DFU-specific DEGs with genes associated with "Vascular Endothelium" and "Endothelial Pyroptosis" from the GeneCards database. This integrative analysis identified five core genes at the intersection of these pathways: S100A9, S100A8, BNIP3, FSTL1, and TACR1 (Figure 1G). These genes were selected for further investigation as central candidates linking vascular dysfunction and inflammatory cell death to DFU pathogenesis.

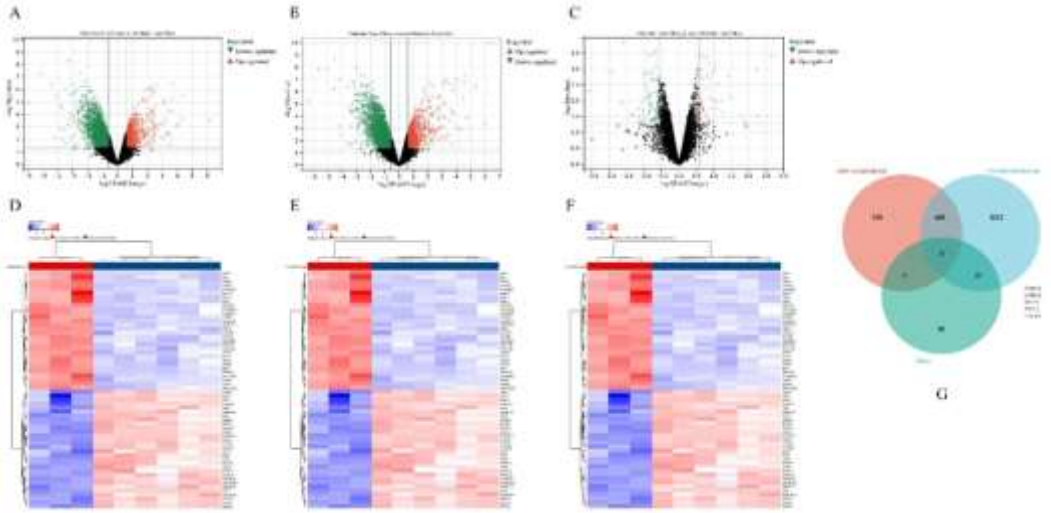

Figure 1, Identification of Differentially Expressed Genes (DEGs) and Core Genes Associated with Vascular Endothelium and Pyroptosis in Diabetic Foot Ulcers (DFU).
(A-C) Volcano plots of differentially expressed genes in DFU versus controls. Significantly up- and down-regulated genes are shown in red and blue, respectively.
(D-F) Hierarchical clustering analysis of DEGs separates DFU samples from diabetic and normal controls.
(G) Venn diagram identifying five core genes (S100A9, S100A8, BNIP3, FSTL1, and TACR1) from the intersection of DFU DEGs with vascular endothelium and pyroptosis-related gene sets.

3.2. Functional Enrichment Analysis Implicates IL-17 Signaling and Ion Homeostasis

To understand the potential biological functions of the five key genes, GO and KEGG enrichment analyses were performed. GO analysis highlighted that these genes are primarily involved in biological processes related to cellular divalent inorganic cation homeostasis and divalent inorganic cation homeostasis (Figure 2A - C), suggesting a crucial role for calcium and other divalent cation regulation in their function. KEGG pathway analysis demonstrated that these genes were significantly enriched in several inflammation and stress-related pathways, most notably the IL-17 signaling pathway, Legionellosis (which involves NLRC4 inflammasome activation and pyroptosis), and Mitophagy - animal (which is linked to BNIP3) (Figure 2E).

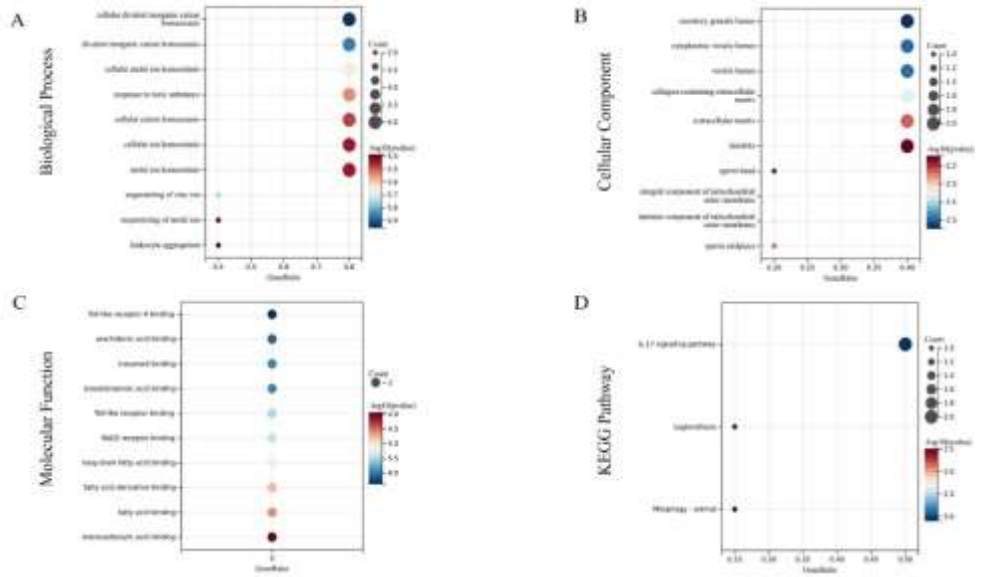

Figure 2. Functional Enrichment Analysis of the Five Key Genes in DFU.

(A-C) Gene Ontology (GO) enrichment analysis of the five key genes. The bar plots show their significant involvement in biological processes related to divalent inorganic cation homeostasis. (D) Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis. The five key genes are significantly enriched in inflammation and stress-related pathways, including the IL-17 signaling pathway, Legionellosis, and Mitophagy - animal.

3.3. S100A8 and S100A9 are Consistently and Highly Upregulated in DFU

The expression patterns of the five key genes were examined in both the GSE80178 and GSE68183 datasets. In GSE80178, S100A8 and S100A9 exhibited the most dramatic and significant upregulation in the DFU group compared to both the DM and Normal groups ($p < 0.001$) (Figure 3A - B). The other genes (BNIP3, FSTL1, TACR1) showed less consistent or significant changes (Figure 3C - E). This striking overexpression of S100A8 and S100A9 was successfully replicated in the independent validation dataset GSE68183. Based on their consistent and prominent dysregulation across datasets, S100A8 and S100A9 were designated as the hub genes for DFU.

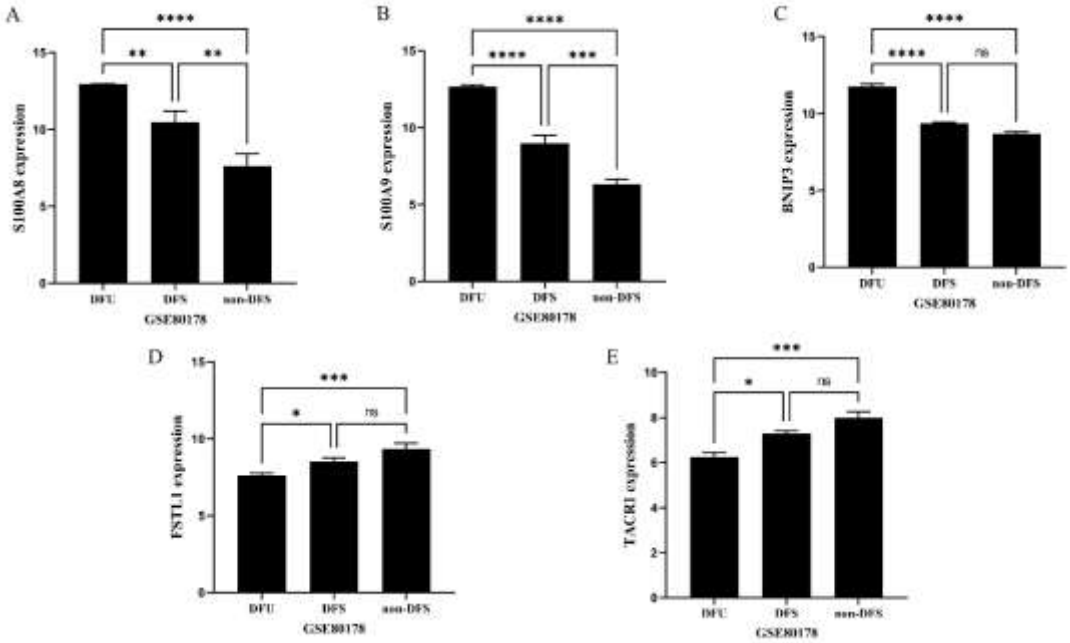

Figure 3. S100A8 and S100A9 are highly and consistently upregulated in DFU.
(A-B) In the GSE80178 dataset, S100A8 (A) and S100A9 (B) are significantly upregulated in DFU compared to diabetic (DM) and normal skin.
(C-D) Expression changes of the other three candidate genes (BNIP3, FSTL1, TACR1) in the GSE80178 dataset, which showed less consistent or significant alterations.
Note: *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001.

3.4. S100A8 and S100A9 Expression is Correlated with an Activated Pro-inflammatory Immune Microenvironment

To investigate the relationship between the hub genes and the immune landscape in DFU, we performed a comprehensive immune infiltration analysis using the CIBERSORT algorithm on the GSE80178 dataset. Spearman correlation analysis revealed a distinct pattern of immune cell associations for S100A8 and S100A9 (Figure 4).

The expression of both hub genes showed a strong and highly significant positive correlation with the abundance of Monocytes (S100A8: $r = 0.84$, $p < 0.001$; S100A9: $r = 0.70$, $p < 0.01$) and M0 Macrophages (S100A8: $r = 0.66$, $p < 0.05$; S100A9: $r = 0.61$, $p < 0.05$). Additionally, S100A9 expression was positively correlated with Activated NK cells ($r = 0.59$, $p < 0.05$).

Conversely, significant negative correlations were observed between both S100A8/A9 and immune cells typically associated with a resting or regulatory state. These included Resting NK cells (S100A8: r = -0.77, p < 0.001; S100A9: r = -0.75, p < 0.001), Resting Dendritic cells (S100A8: r = -0.72, p < 0.01; S100A9: r = -0.66, p < 0.05), and Resting CD4+ Memory T cells (S100A8: r = -0.66, p < 0.05).

These results suggest that elevated S100A8 and S100A9 expression is associated with an immune microenvironment in DFU that is skewed towards innate immune activation (enriched for monocytes and M0 macrophages) and away from immune quiescence (depleted of resting NK and dendritic cells).

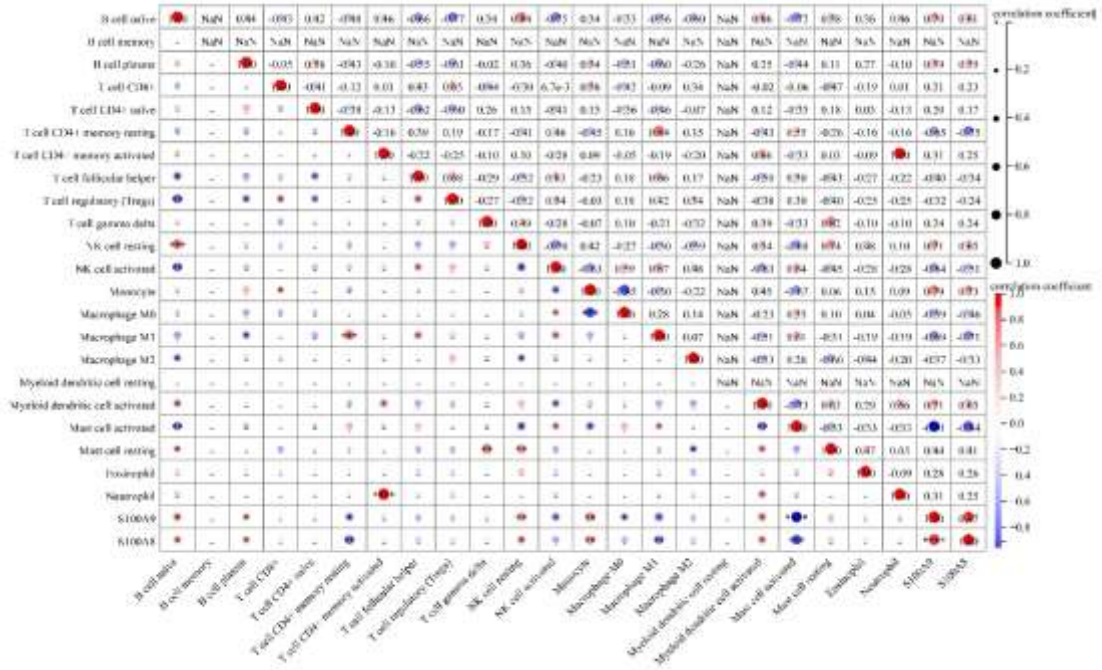

Figure 4. Correlation of S100A8/A9 Expression with Immune Cell Infiltration in DFU. Immune infiltration analysis using CIBERSORT reveals significant correlations between S100A8/A9 expression levels and the abundance of specific immune cell populations. S100A8 and S100A9 show strong positive correlations with pro-inflammatory innate immune cells (e.g., Monocytes, M0 Macrophages) and significant negative correlations with immune cells in a resting state (e.g., Resting NK cells, Resting Dendritic cells). Statistical significance is indicated as *p < 0.05, **p < 0.01, ***p < 0.001.

3.5. DUX4 is Identified as a Potential Upstream Regulator of S100A9

To explore upstream regulatory mechanisms, we sought to analyze transcription factor binding to the promoters of the hub genes, S100A8 and S100A9, using ChIP-Seq data from the ChIP-Atlas database. However, no significant ChIP-Seq records were found for the S100A8 promoter, precluding its analysis. Consequently, we focused on the S100A9 promoter. This analysis identified significant binding of the transcription factor DUX4 in monocytes (Supplementary Table 1). Subsequent correlation analysis in the GSE80178 dataset revealed a significant positive relationship between DUX4 and S100A9 expression (ρ = 0.678, p = 0.015, Figure 5).

Supplementary Table 1, Transcription factor binding profile at the S100A9 promoter from ChIP-Atlas analysis.

Target_genes	S100A9|Average	SRX245458 51|Monocyt	SRX245458 53|Monocyt	SRX24545855|Monocytes	SRX24545857|Monocytes	SRX24545858|Monocytes	SRX24545860|Monocytes	SRX24545862|Monocytes	SRX24545864|Monocytes

		es	es						
DUX4	1006.625	1155	1243	1216	461	1035	903	1080	960
FRG2C	414.5	510	361	629	213	439	351	440	373
CD300LB	332.75	0	586	0	404	0	742	0	930
CD300C	332.75	0	586	0	404	0	742	0	930
ART1	220.75	306	252	305	187	383	333	0	0
FRG2	169.5	241	100	213	0	182	183	260	177
PHRF1	119	0	0	308	213	222	209	0	0
CCDC200	45.375	0	0	0	0	0	0	222	141
LOC10272 4770	44.625	0	0	0	0	0	0	357	0
DGCR6	44.625	0	0	0	0	0	0	357	0
COL23A1	30.25	0	0	0	0	0	0	0	242
USP17L22	21.75	174	0	0	0	0	0	0	0
USP17L21	21.75	174	0	0	0	0	0	0	0
USP17L20	21.75	174	0	0	0	0	0	0	0
USP17L12	21.75	174	0	0	0	0	0	0	0
USP17L5	18.25	0	0	0	0	0	0	0	146
USP17L30	18.25	0	0	0	0	0	0	0	146
USP17L29	18.25	0	0	0	0	0	0	0	146
USP17L28	18.25	0	0	0	0	0	0	0	146
USP17L27	18.25	0	0	0	0	0	0	0	146
USP17L26	18.25	0	0	0	0	0	0	0	146
USP17L25	18.25	0	0	0	0	0	0	0	146
USP17L24	18.25	0	0	0	0	0	0	0	146
EPC1	9.375	0	75	0	0	0	0	0	0

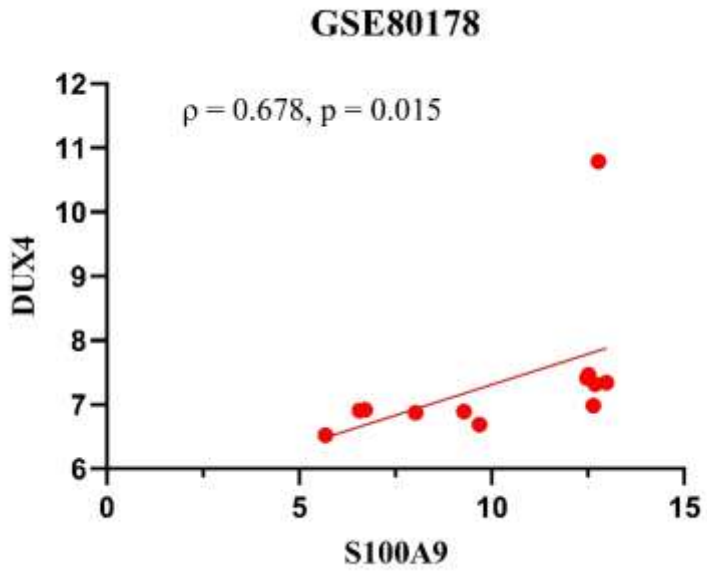

Figure 5. DUX4 expression is positively correlated with S100A9 in DFU.
Scatter plot showing the significant positive correlation between DUX4 and S100A9 mRNA expression levels in the GSE80178 dataset. Each point represents an individual sample. Spearman's correlation coefficient (ρ) and the p-value are indicated.

4. Discussion

This study integrates bioinformatic analysis with clinical validation to elucidate the role of vascular

endothelial pyroptosis-related genes in DFU. Our principal finding is the identification of S100A8 and S100A9 as hub genes that are robustly overexpressed in DFU and are intricately linked to dysregulated immune responses.

The initial screening yielded five key genes at the intersection of vascular biology, pyroptosis, and DFU-specific gene expression. The functional enrichment of these genes provided critical insights into potential mechanisms [15]. The significant enrichment in the IL-17 signaling pathway is particularly noteworthy. IL-17 is a potent pro-inflammatory cytokine implicated in the pathogenesis of psoriasis and other chronic inflammatory diseases [16 - 17]. Its role in DFU is gaining attention, as it can promote neutrophil recruitment and activation, and impair epithelial barrier function [18]. S100A8/A9 are well-established mediators and amplifiers of IL-17-driven inflammation, forming a self-perpetuating vicious cycle that sustains chronic inflammation and tissue damage in the diabetic foot [19-20]. This pathogenic role is strongly supported by our transcriptomic findings, which identified a consistent and dramatic upregulation of S100A8 and S100A9 across two independent DFU datasets. Functionally, these proteins dimerize to form calprotectin—a major damage-associated molecular pattern (DAMP) released predominantly by neutrophils and other myeloid cells. Upon release, calprotectin activates signaling through Toll-like receptor 4 (TLR4) and the receptor for advanced glycation end products (RAGE), leading to NF-κB activation and further amplification of pro-inflammatory responses [21 - 22], thereby fueling the chronicity of DFU pathology.

Our immune infiltration analysis provided crucial mechanistic insights by revealing that S100A8/A9 expression strongly correlates with an activated innate immune landscape, showing significant positive correlations with pro-inflammatory monocytes and M0 macrophages, and concurrent negative correlations with resting immune cells. This distinct pattern suggests that S100A8/A9 promotes a microenvironment skewed toward sustained immune activation and away from quiescence, thereby perpetuating chronic inflammation in DFU [23 - 24]. Furthermore, to explore the upstream drivers of this pathway, we identified the transcription factor DUX4 as a potential regulator of S100A9. While DUX4 is best characterized in facioscapulohumeral muscular dystrophy, its emerging role in sterile inflammation and its ability to regulate pro-inflammatory gene expression in myoblasts and macrophages have been documented [25 - 26]. Notably, a prior ChIP-seq study in monocytes has directly demonstrated DUX4 binding to the promoter region of S100A9, a finding which our study corroborates by revealing a significant positive correlation between DUX4 and S100A9 expression in the DFU transcriptomic dataset. Collectively, based on these results, we propose a pathogenic model wherein persistent metabolic stress induces initial endothelial damage and pyroptosis, triggering S100A8/A9 release—a process potentially amplified by DUX4-mediated transcription. This in turn amplifies inflammation via IL-17 signaling and recruits neutrophils, ultimately establishing a feed-forward loop that impedes wound healing.

Limitations of the Study

Despite the compelling evidence, this study has several limitations. Firstly, the bioinformatic analysis is primarily based on publicly available transcriptomic datasets. The lack of detailed clinical information, such as ulcer duration, size, or infection status, precludes analysis of how these factors influence the expression of our identified hub genes. Secondly, while we identified a correlative link between DUX4 and S100A9, and between the hub genes and specific immune populations, the causal relationships remain to be established. The role of DUX4 in regulating S100A9 in endothelial cells or myeloid cells, and the precise mechanism by which S100A8/A9 amplifies pyroptosis and inflammation in DFU, require experimental validation. Future studies employing *in vitro* models (e.g., high glucose-treated endothelial cells with DUX4 overexpression/knockdown) and *in vivo* diabetic wound models are necessary to confirm these interactions. Finally, the cellular source of elevated S100A8/A9 in DFU—whether originating predominantly from infiltrating neutrophils, activated macrophages, or damaged endothelial cells—should be delineated through

spatial techniques such as immunohistochemistry on patient ulcer tissues.

5. Conclusion

In conclusion, our comprehensive multi-dataset analysis identifies S100A8 and S100A9 as critical hub genes linking vascular endothelial pyroptosis to a pro-inflammatory immune microenvironment in DFU. These genes are centrally positioned in key pathways including IL-17 signaling, show a strong correlation with innate immune activation, and are potentially regulated by the transcription factor DUX4. Their consistent and marked overexpression underscores their pivotal role in DFU pathogenesis. This work not only provides novel insights into the molecular mechanisms of DFU but also positions S100A8/A9, and their upstream regulator DUX4, as promising candidates for further mechanistic investigation and as potential diagnostic biomarkers or therapeutic targets for this severe diabetic complication.

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Conflicts of interest: The authors have no known conflicts of interest to declare.

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