Review

DOI: 10.5582/ddt.2023.01086

Which biomarkers predict hard-to-heal diabetic foot ulcers? A scoping review

Qi Qin[1], Daijiro Haba[1,2], Gojiro Nakagami[1,2,*]

[1] Department of Gerontological Nursing/Wound Care Management, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan;
[2] Global Nursing Research Center, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan.

SUMMARY Diabetic foot ulcers (DFUs) often develop into hard-to-heal wounds due to complex factors. Several biomarkers capable of identifying those at risk of delayed wound healing have been reported. Controlling or targeting these biomarkers could prevent the progression of DFUs into hard-to-heal wounds. This scoping review aimed to identify the key biomarkers that can predict hard-to-heal DFUs. Studies that reported biomarkers related to hard-to-heal DFUs, from 1980 to 2023, were mapped. Studies were collected from the following databases: MEDLINE, CINAHL, EMBASE, and ICHUSHI (Japana Centra Revuo Medicina), search terms included "diabetic," "ulcer," "non-healing," and "biomarker." A total of 808 articles were mapped, and 14 (10 human and 4 animal studies) were included in this review. The ulcer characteristics in the clinical studies varied. Most studies focused on either infected wounds or neuropathic wounds, and patients with ischemia were usually excluded. Among the reported biomarkers for the prediction of hard-to-heal DFUs, the pro-inflammatory cytokine CXCL-6 in wound fluid from non-infected and non-ischemic wounds had the highest prediction accuracy (area under the curve: 0.965; sensitivity: 87.27%; specificity: 95.56%). CXCL-6 levels could be a useful predictive biomarker for hard-to-heal DFUs. However, CXCL6, a chemoattractant for neutrophilic granulocytes, elicits its chemotactic effects by combining with the chemokine receptors CXCR1 and CXCR2, and is involved in several diseases. Therefore, it's difficult to use CXCL6 as a prevention or treatment target. Targetable specific biomarkers for hard-to-heal DFUs need to be determined.

Keywords Delayed wound healing, prediction, prevention, treatment target

1. Introduction

Diabetic foot ulcer (DFU) is one of the major complications of diabetes mellitus, and one of the main causes of hard-to-heal wounds (*1*). A large-scale study conducted by the U.S. Wound Registry reported that within a 1-year follow-up period, around 33% of the DFUs failed to heal and developed into hard-to-heal wounds (*2*). Although the definition of a "hard-to-heal wound" varies, it can be broadly described as one that fails to heal with standard therapy in an orderly and timely manner (*3*). Hard-to-heal DFUs require sophisticated therapies that account for a large proportion of medical resources, with an average cost of $10,472 per episode (*4*). Despite the use of advanced treatments, this cohort experiences a greater risk of lower extremity amputations and mortality (*5,6*). Therefore, predicting the outcome early and replacing standard therapy with advanced therapies to return hard-to-heal wounds to a healing trajectory could be a useful approach to improving efficiency in wound care and minimizing the enormous burden on medical resources.

Wound healing is a dynamic and complex biological process that can be divided into four partly overlapping phases: hemostasis, inflammation, proliferation, and remodeling. These phases involve multiple functional cells, as well as cytokines, growth factors, and enzymes (*7,8*). Diabetes causes impaired wound healing by affecting one or more biological mechanisms that are triggered by hyperglycemia, micro- and macro-circulatory dysfunction, and tissue hypoxia (*9*). Therefore, to predict wound outcomes, two types of approaches have been adopted in previous studies, clinical and molecular biomarker assessments, which involve the macroscopic changes in the wound and the microscopic changes underneath during the wound healing process. Clinical assessment usually includes data such as the patients' basic characteristics, assessment of inflammatory signs, the efficiency of blood supply, and wound status. However, the prediction

rate is unsatisfactory, as a recent study has reported a prediction model using bedside assessment data with a 0.77 area under the curve (AUC) (*10*). In contrast, studies using molecular biomarkers such as C-X-C motif chemokine ligand 6 (CXCL6) exhibited a higher accuracy in predicting wound healing (*11*). However, patients with severe ischemia were not included in that study, so whether its results can be applied to people with angiopathy remains unknown. Several studies have extensively investigated dysregulated biomarkers related to wound healing, such as serpin family B member 3 (SERPINB3), miR-155, CXCL5, *etc.*, to elucidate the mechanism involved in delayed wound healing (*12-14*). The sensitivity and specificity of these biomarkers are unknown, and it is uncertain whether they can serve as predictive indicators for hard-to-heal DFUs in patients with any type of difficult-to-heal wounds.

We aimed to map biomarkers related to wound healing and identify the specific biomarkers that can be used to predict the progression of hard-to-heal DFUs, and answer the following research questions: (1) Which specific population among patients with DFUs was studied? (2) What is the definition of hard-to-heal DFUs in most studies? Or, how do most studies define a DFU exhibiting delayed healing? (3) What type of specimen was used to detect the biomarkers? (4) Which methods have been used to detect the biomarkers related to hard-to-heal DFUs? (5) Which analytical techniques were used to detect the biomarkers? (6) Which biomarkers were found to attribute to delayed wound healing in DFUs? (7) Can the detected biomarkers be used for the prediction of hard-to-heal wounds and what level of accuracy is provided by them?

2. Methods

2.1. Protocol

This scoping review was conducted by following the steps outlined in the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA)-ScR extension for scoping reviews (*15*).

2.2. Eligibility criteria

Population: People with DFUs, or diabetic animal models. Concept: Hard-to-heal wounds or wounds exhibiting delayed healing. Studies comparing delayed healing with non-diabetic wounds were excluded. Context: Delayed wound healing-related biomarkers. Original studies that reported molecular biomarkers related to hard-to-heal DFUs regardless of design, including clinical or animal experimental studies, were included in this scoping review. Both experimental and quasi-experimental study designs, including randomized controlled trials, non-randomized controlled trials, and before-and-after studies were considered. However, review papers were excluded. Since the definition of hard-to-heal wounds varied between studies, and many studies described hard-to-heal wounds as impaired wound healing, delayed wound healing, non-healing wounds, or poorly healed wounds; hence, we included all studies related to wound healing. The following studies were excluded: (1) Studies that addressed biomarkers that promote wound healing or are related to rapid healing. However, if the study reported the determination of biomarkers related to delayed wound healing and further experimentally confirmed their inhibitory effect on wound healing, they were included. (2) Studies that did not include people with diabetes or diabetic animal models, or those that only included comparisons with non-diabetic wounds. (3) Studies that did not describe the criteria for defining "hard-to-heal" wounds. (4) Studies that did not describe the biomarker sampling methods. (5) Studies not published in English or Japanese.

2.3. Information sources

The following electronic databases were searched and data from the inception of the database until the date on which the searches were performed (Oct 20, 2023) were included in the search: MEDLINE (PubMed interface), Cumulative Index to Nursing and Allied Health Literature (CINAHL), EMBASE, and ICHUSHI (Japan Medical Abstracts Society). These databases were searched using the following keywords: "diabetic foot" OR "diabetes mellitus" AND "wounds and injuries" AND "foot ulcer" OR "diabetic ulcer" OR "diabetic foot ulcer" OR "diabetic wound") AND "delayed wound healing" OR "non-healing" OR "impaired wound healing" OR "hard to heal" OR "poor wound healing" AND "biomarkers" OR "RNA" OR "proteins" OR "DNA".

2.4. Selection of sources of evidence

All the articles from the different databases were uploaded to a reference management software. Duplicate articles were removed. Two reviewers screened the titles and abstracts and excluded irrelevant studies, separately, based on the inclusion criteria. After screening the titles and abstracts, the full text of the articles was assessed and considered for review by two independent reviewers; the articles that matched the exclusion criteria were rejected and the reasons are shown in the flow chart. Next, the included studies were quantitatively synthesized. The whole process was conducted by two reviewers to screen the studies independently, and any lack of consensus was discussed with a third reviewer.

2.5. Data charting process

Data from eligible studies were charted by two

independent reviewers, using a data collection form developed by the authors.

2.6. Data items

We charted the following variables: study population and sample size, study design, patient/animal demographic data, wound characteristics (wound size, wound age, severity, and evaluation method), criteria of hard-to-heal DFUs, specimen-related information (sample type, collection method, collection timing, sample process, and sample storage conditions), targeted biomarkers (up-regulation/down-regulation and type of biomarker) and prediction accuracy.

The results were separated into two tables based on the subjects: human subject studies and animal subject studies, as shown in Table 1. Tables were produced to allow the comparison of the different collection techniques based on the key characteristics of the extracted data. One reviewer summarized it while the other reviewer double-checked the contents. Any lack of consensus was discussed with a third reviewer.

3. Results

3.1. Search flow (Figure 1)

The scoping review process is visually summarized in the flow diagram. Initially, a total of 808 records were identified from various databases, including CINAHL, EMBASE, MEDLINE, and ICHUSHI. After eliminating 86 duplicate records, the remaining 722 records underwent initial screening. Among these, 672 were excluded as they did not meet the specified inclusion criteria. In the subsequent screening phase, 50 reports were selected for retrieval. However, 21 of these reports could not be obtained due to various reasons, such as limited access to full-text articles or disparities in terms of population, concept, or context. During the eligibility assessment phase, 29 reports were thoroughly evaluated, resulting in the exclusion of 15 reports primarily due to their lack of alignment with the desired population and research concept. Ultimately, 14 studies were considered suitable for inclusion in the review, comprising 10 clinical studies and 4 animal studies.

3.2. Clinical studies (Table 1)

3.2.1. Which specific population among patients with DFUs was studied?

Among the nine clinical studies, six focused on diabetic neuropathic wounds, and one focused on infected wounds. Most studies focused on patients with diabetes aged between 18-90 years, presenting with neuropathic ulcers graded under the Texas Grading System, ranging from grades 2 to 3 (*11,13,16-19*). The majority of the patients were admitted to the inpatient department, and some studies had specific exclusion criteria for infection, such as systemic infection, being under microbial treatments, or other immunological disorders that might affect inflammatory markers (*11,16,19,20*). One study focused on patients with ischemic diabetic ulcers that required transluminal angioplasty and foot surgery (*21*), while MacDonald *et al.* focused on only infected diabetic foot ulcers (*22*).

3.2.2. What is the definition of hard-to-heal DFUs in most studies?

The definition of hard-to-heal or non-healing DFUs varied across studies. Common definitions included ulcers persisting or increasing in size, development of new ulcers, requirement of amputations, or patient death. Most studies defined non-healing as ulcers that did not heal or enlarged within a specific timeframe, commonly between three and six months, with six months being the most frequent benchmark (*11,13,16,17*). Some studies considered a wound size reduction of over 50% within four weeks as a healing ulcer (*18,19*), while others used a three-month period (*19,21*), and one study defined non-healing based on a one-year timeframe (*23*).

3.2.3. What type of specimen was used to detect the biomarkers?

Most studies utilized wound exudates, with some used serum (*18,21*), plasma (*17,23*), skin biopsies (*13,16*), or wound tissue samples (*19,22,23*). Wound exudates were mostly collected using the swabbing technique (*11,17,19,20*). These specimens were typically collected at the initial clinic visit or specific post-wounding time points in longitudinal studies.

3.2.4. Which methods and analytical techniques have been used to detect the biomarkers related to hard-to-heal DFUs?

The predominant method used for biomarker detection was Enzyme Linked Immunosorbent Assay (ELISA), since the studies primarily conducted protein analysis. Besides ELISA, studies employed techniques including proteomics analysis, protein arrays, multiplex immunoassay, real-time RT-PCR, and 16S rRNA genomic sequencing.

3.2.5. Which biomarkers were found to attribute to delayed wound healing in DFUs?

Several biomarkers were identified across the studies. These included downregulation of CXCL6 (*11*), ENA-78 (CXCL5) (*17*), SERPINB3, and upregulation of neutrophil elastase (*13,16*), citrullinates histone H3

Table 1. Clinical studies that reported potential biomarkers for predicting hard-to-heal DFUs

	Author, year, country	Wound characteristics	Definition of hard-to-heal	Specimen-related information	Analysis methods	Biomarkers	Accuracy of prediction
Protein	Wang *et al*., 2019, USA (*11*)	Diabetic neuropathic wounds Texas Grading System (grade 2–3) Exclusion: Ischemia, infection, and immunological disorder	Non-healing: Ulcer persisted or increased in size, new ulcers appeared, amputations were required, or the patient died.	Wound exudates (swab)	ELISA	CXCL6 ↓	AUC: 0.965 Cutoff value: 846.90 ng/mL Sensitivity: 87.27% Specificity: 95.56%
Protein	Li *et al*., 2019, China (*17*)	Diabetic neuropathic wounds Texas Grading System (grade 2–3)	Nonhealing: The ulcer persisted or was even enlarged, development of new ulcers, amputations, or death	Wound exudates (swab) and plasma	Protein array; candidate markers were then analyzed using ELISA	ENA-78↓	AUC: 0.705 (95% CI 0.608–0.801, $P < 0\,001$) Cutoff value: 1792.00 ng/mL Sensitivity:45.90% Specificity: 89.58%
Protein	Li *et al*., 2013, China (*18*)	Diabetic neuropathic wounds Texas Grading System (grade 1–3) Wound duration: 14 to 90 days Size: > 0.5 cm^2 Exclusion: Arteriopathy of the lower limbs	Poor healers: A decrease in wound area < 82% in 4 weeks	Serum samples at the first clinic visit and the end of 4-week treatment	ELISA for MMP-9, MMP-2, TIMP-1 and TIMP-2	MMP-9/TIMP-1 ratio↑	AUC:0.658 (Sensitivity: 63.6%; specificity: 58.6%)
Protein	Fadini *et al*., 2014, Italy (*13*)	Diabetic neuropathic wounds Exclusion: Ischemia, systematic infection	Non-healing: Ulcer persisted or was even enlarged in 6 months	Skin biopsy	Proteomics analysis, ELISA, Real-time RT-PCR	SerpinB3↓	AUC: 0.665 Sensitivity: 75%; Specificity: 62.5% Cutoff value: 1.13 ng/mL/ total protein μg/μl
Protein	Fadini *et al*., 2016, Italy (*16*)	Diabetic neuropathic wounds Exclusion: Ischemia, systematic infection	Non-healing: Ulcer persisted or was even enlarged in 6 months	Serum samples	ELISA (elastase, NGAL, lactoferrin, PR-3)	Neutrophil elastase↑	AUC: 0.815 (95% CI 0.686–0.944) (ulcer infection)
Protein	Yang *et al*., 2020, China (*23*)	Diabetic wounds Exclusion: Traumatic amputation, Buerger's disease, vasculitis, acute arterial occlusion	Non-healing: Did not heal in one year with multidisciplinary management of DFU	Peripheral blood plasma and wound tissues	ELISA (NET-related markers, elastase level)	CitH3↑, Neutrophil elastase↑	AUC: 0.84 [95% CI 0.76–0.90]
Protein	Loffle *et al*., 2011, Germany (*20*)	Diabetic ulcer located below the ankle Not receiving any antimicrobial treatments in 3 months	Healing: within no soft-tissue infection group: Healed within 6-month follow-up period	Wound fluid (swab)	Wound fluid lactate concentration	Lactate concentration↑	NA
Protein	Vieceli Dalla Sega *et al*., 2022, Italy (*21*)	Ischemic ulcer that requires percutaneous transluminal angioplasty and foot surgery	Optimal healing: healed at 3 months; Others: new limb revascularization, new lesions or recurrence	Peripheral blood serum	Multiplex immunoassay: sCD40L, IFN)-γ2, IFN-γ, 1RA, IL-2, IL-4, IL-5, IL-6, IL-10, IL-13, IL-18, TNF-α, Angiopoietin-2, Endoglin, Endothelin-1, sE-Selectin, Thrombomodulin, s-RAGE, sICAM-1, P-Selectin, sVCAM-1, and PAI-1; ELISA: vWF	Non-healing: sICAM-1 ↑, Endothelin-1↑ Revascularization: PAI-1↑, Endothelin-1↑ New lesions or recurrences: IL-10↑, IL1RA↑,sCD40L↑, Thrombomodulin↓	Decision tree accuracy for lower risk of new lesion: 0.812 (95% CI = 0.6192–0.937) (sCD40L < 18 pg/mL and thrombomodulin levels ≥ 2 pg/mL)

Table 1. Clinical studies that reported potential biomarkers for predicting hard-to-heal DFUs (continued)

	Author, year, country	Wound characteristics	Definition of hard-to-heal	Specimen-related information	Analysis methods	Biomarkers	Accuracy of prediction
Microbiome	MacDonald *et al.*, 2019, USA (22)	Infected diabetic foot ulcer	Persistent infections at week 12	Wound tissue debridement	16S rRNA genomic seq (microbial species) and qPCR (bacterial abundance)	Higher abundance of Bacteroidales and Streptococcaceae; Low level of Actinomycetales	NA
Microbiome	Min *et al.*, 2020, USA (*19*)	Vascularized plantar neuropathic DFU Duration; > 4 weeks Size: > 0.5 cm^2 Exclusion: Receiving antibiotics over 2 weeks	Non-healing wounds: < 50% closed by week 4	Plantar skin swab, ulcer debridement of the wound edge before wound cleansing.	16S rRNA next generation sequencing	Gram-positive anaerobic cocci ↑	NA

(citH3) (*23*), higher level of soluble intercellular adhesion molecule-1 (sICAM-1) and endothelin-1 (ET-1) (*21*), Gram-positive anaerobic cocci (*19*), and a higher abundance of Bacteroidales and Streptococcaceae and a lower level of Actinomycetales in non-healing wounds (*22*). In Vieceli Dalla Sega *et al*. study, they further mentioned that plasminogen activator inhibitor-1 (PAI-1) and ET-1 levels were associated with the need for revascularization within 12 months from the previous treatment. The levels of interleukin-10 (IL-10), IL1RA, and CD40L, were linked with an increased risk of developing new lesions or recurrences after DFU healing. Conversely, thrombomodulin levels were inversely associated with this risk (*21*).

3.2.6. Can the detected biomarkers be used for the prediction of hard-to-heal wounds and what level of accuracy is provided by them?

Several studies provided accuracy metrics for the biomarkers. For instance, CXCL6 exhibited a high level of predictive accuracy with an AUC of 0.965, indicating high sensitivity (87.27%) and specificity (95.56%) at a cutoff value of 846.90 ng/mL (*11*). ENA-78 had an AUC of 0.705 with a sensitivity of 45.90% and a specificity of 89.58% (*17*). SERPINB3 showed an AUC of 0.665 with a sensitivity of 75% and a specificity of 62.5% (*13*). Neutrophil elastase had an AUC of 0.815 (*16*), while CitH3 displayed an AUC of 0.84 (*23*). One study developed a decision tree for the endpoint of recurrences and new lesions based on sCD40L and thrombomodulin levels, which showed an accuracy of 0.812 (95% CI = 0.6192-0.937) for the outcome; however, the delayed wound healing prediction model was not shown (*21*). These metrics suggest that these biomarkers have potential predictive value for hard-to-heal DFUs.

3.3. Animal studies (Table 2)

3.3.1. Which specific population among animal models was studied?

The studies utilized various mouse models, including fibroblast growth factor-7 (FGF-7)-null diabetic mice (*24*), *p66Shc*-KO STZ-induced diabetic mice (*25*), *Flii*$^{+/-}$ mice (*26*), and *db/db* thrombospondin-2 (TSP2) KO mice (*27*).

3.3.2. What are the main findings?

Key findings included the significant delay in wound contraction and healing due to the absence of FGF-7 in diabetic mice (*24*), accelerated healing in *p66Shc* knockout diabetic mice (*25*), increased inflammation due to elevated Flii levels (*26*), and accelerated re-epithelialization in TSP2-deficient mice (*27*).

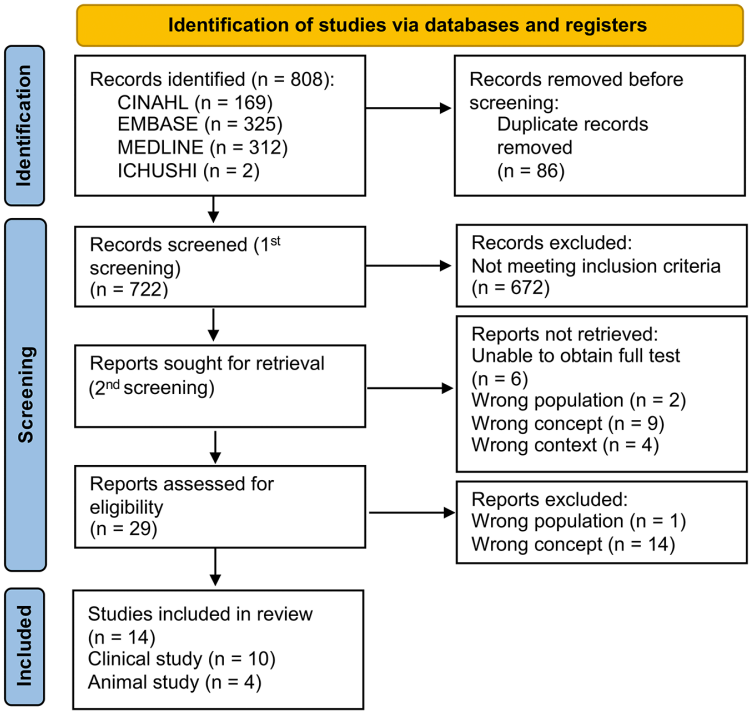

Figure 1. Scoping review flow diagram.

4. Discussion

The primary objective of this scoping review was to map biomarkers related to wound healing and to identify specific biomarkers that can predict the progression of hard-to-heal DFUs. Our findings provide a comprehensive overview of the current state of research in this area and highlight potential avenues for future investigations.

Our scoping review has identified several biomarkers associated with delayed wound healing in DFUs. The downregulation of CXCL6 in non-healing ulcers was highlighted by Wang *et al*. (2019) with high sensitivity and specificity, making it a potential promising biomarker for predicting hard-to-heal DFUs (*11*). CXCL6, a chemokine, is recognized for its involvement in various inflammatory processes. This finding underscores the central role inflammation plays in wound healing. Although our review did not find other studies directly supporting this observation, the significance of inflammation in wound healing is a recurring theme in the literature. While it exhibits high accuracy in predicting non-healing DFUs, it has also been implicated in various other diseases, including alcoholic liver disease (*28*), inflammatory bowel diseases and gastrointestinal tumors (*29*), neuro-inflammatory disease (*30*), and other inflammatory diseases (*31*). Because it is not specific to wounds, it might lead to false positives. Even if it could be used as a predictive measure, it might be a difficult treatment target. Li *et al*. (2019) reported the downregulation of ENA-78 in non-healing DFUs (*17*). ENA-78 plays a crucial role in neutrophil recruitment, further emphasizing the importance of inflammation in wound healing. This finding aligns with the broader understanding of inflammation's significance in wound healing, as seen in the studies by Wang *et al*. (2019) and Li *et al.* (2013) (*11,18*) which observed an increased matrix metalloproteinase (MMP-9)/TIMP-1 ratio in patients with poor wound healing. This balance between MMPs and TIMPs is essential for wound remodeling as described in many previous studies (*18,32-34*). While this observation is unique to their study, it offers a potential avenue for further research into the role of MMPs and TIMPs in wound healing. Loffle *et al.* (2011) found elevated lactate concentrations in the wound fluid of patients with soft-tissue infections. Lactate, a byproduct of anaerobic metabolism, can indicate tissue hypoxia or bacterial metabolism (*20*). Both factors can hinder wound healing. Fadini *et al*. (2014) noted a downregulation of SerpinB3 in rapidly healing ulcers (*13*). SerpinB3 is involved in various cellular processes, including apoptosis and inflammation. While this study suggests its potential protective role in wound healing, further studies are needed to confirm this observation and its implications. Levels of sICAM-1 and ET-1, both molecules expressed by the endothelium, were found to be inversely related to wound healing within three months in patients with critical limb ischemia (*21*). Elevated levels of these molecules are indicative of endothelial dysfunction. Elevated sICAM-1 levels are linked to inflammation and indicate either endothelial stimulation or damage. ET-1impacts vascular smooth muscle cells, serving as a powerful vasoconstrictor.

Table 2. Biomarkers of delayed wound healing in diabetic animal models

Author, year, country	Animal type	Wound model	Definition of delayed wound healing (hard-to-heal)	Specimen-related information	Analysis method	Biomarkers	Main findings
Peng *et al.*, 2011, China (*24*)	11-12 weeks old FGF-7-null diabetic mice: FGF-7 -/- Lepr db/db; FGF-7 null mice: FGF-7 -/- Lepr +/+; Diabetic mice: FGF-7 +/+ Lepr db/db; Wide type: FGF-7 +/+ Lepr +/+;	φ10 mm full-thickness dorsal excisional wound	Wound contraction rate: Contraction/initial area of wound size; Epithelialization rate: re-epithelialization/ (Initial area of wound size – contraction); Open wound rate: Open wound/initial area of wound size	For histological analysis: Wound tissue on day 7 and day 14 For RNA analysis: Wound tissue on day 7	Immunohistochemistry for Ki67; Real time PCR (α-SMA, Col-Ia, TGF-β1, bFGF,EGF, IGF-1)	FGF-7	Lack of FGF-7 in diabetic mice significantly delayed wound contraction and wound healing, however, did not affect reepithelialization of cutaneous compared to diabetic mice
Fadini *et al.*, 2010, Italy (*25*)	p66Shc-KO STZ-induced diabetic mice with and without hind limb ischemia; non-diabetic mice with or without hind limb ischemia	Diabetes (blood glucose level > 300 mg/dl). Hind limb ischemia (2 weeks after femoral artery ligation and excision) φ 4 mm full-thickness skin wounds	Wound size Healing time Granulation area	Wound tissue at mid-closure time	Histological analyses (H&E, MT, IF (B4-isolectin))	p66Shc	p66Shc knockout accelerates healing of diabetic and ischemic wounds. p66Shc knockout rescues the impaired granulation tissue in diabetic wounds.
Ruzehaji *et al.*, 2013, Australia (*26*)	12-16 weeks old female BALB/c Flii +/– (low Flii); WT (normal) STZ-induced diabetic mice; Flii Tg/Tg (high Flii) STZ-induced diabetic mice; Non-diabetic group	Two φ 6 mm full-thickness skin wounds	Wound area on day 7	Wound tissue on day 7 post-wounding	Immunohistochemistry for Flii, TLR9, TLR4, MyD88 and NF-kB.	Flii	Increased levels of Flii in diabetic mouse wounds led to increased TLR4 and NF-KB production which led to excessive inflammation and chronicity.
Kunkemoeller *et al.*, 2019, USA (*27*)	10–12-week db/db,DKO, STZ, and wild-type (WT) mice. db/db TSP2 KO (DKO)	two φ 6 mm full-thickness skin wounds	Wound closure time	3 mm wound surrounding tissue on 7, 10, or 14-days post-wounding	IHC (TSP2 , vimentin, CD31) Quantitative RT-PCR and Western Blot (TSP2, HSP90, O-GlcNAc ,OGT, and p65)	TSP2	TSP2 deficiency in diabetic mice accelerated reepithelialization and increased granulation tissue formation, fibroblast migration, and blood vessel maturation.

Indeed, elevated levels of ET-1 correlate with disrupted vascular tone control in diabetes (*35*). This might indicate that the variations in these biomarkers are attributable to the specific population of the study group, specifically, individuals with critical limb ischemia. In animal studies, it has been highlighted that FGF-7 is important for keratinocyte activity, and another study emphasized the role of p66Shc in inflammation and oxidative stress. Additionally, TSP2 was identified as an inhibitor of angiogenesis, which is essential for wound healing (*24-26*). While these findings are based on animal research, they present potential therapeutic targets to enhance wound healing in humans, which need to be further confirmed through clinical studies. It's noteworthy that some biomarkers, such as neutrophil elastase, were recurrent in multiple studies, suggesting a shared pathway or mechanism in delayed wound healing. The consistent theme across these studies is inflammation's role, as highlighted by markers like CXCL6 and ENA-78. Therefore, targeting inflammation could be a key strategy in promoting wound healing in DFUs.

While these biomarkers present promising prospects for predicting hard-to-heal wounds, their seamless integration into clinical practice poses challenges. Notably, some of the identified biomarkers lack specificity for wounds, as they can be elevated due to other systemic conditions. Consequently, while they may serve as predictive measures, utilizing them as treatment targets or for prevention might be intricate. For instance, biomarkers like CXCL6 and neutrophil elastase, although associated with wound healing, are also implicated in various inflammatory conditions including alcoholic liver disease (*28*), inflammatory bowel diseases and gastrointestinal tumors (*29*), neuro-inflammatory disease (*30*), and other inflammatory diseases (*31*). Thus, relying solely on these biomarkers without considering the broader clinical context may not be advisable. In the studies included in this review, the primary approach employed was the assessment of proteins, and the predominant method for collecting wound exudate was swabbing. However, it is crucial to acknowledge that the components of exudate collected *via* swabs predominantly consist of fresh wound exudate, which may differ from exudate collected over extended periods (*36,37*).

The studies incorporated into this review primarily concentrated on wounds characterized by vascular impairments or infections, with a particular emphasis on specific types of DFUs. Consequently, the biomarkers identified in these studies may have a pathological basis. However, it's important to note that this focus could potentially constrain the applicability of the findings to other types of DFUs. Furthermore, the study designs exhibited variation, encompassing both cross-sectional and longitudinal approaches; however, biomarkers were measured at a single time point in all the studies, and their cutoff levels could vary across different wound healing phases, potentially affecting the preventive strategies. Additionally, in some studies, the sample sizes were relatively small, which could potentially impact the robustness and generalizability of the findings.

Given the inherent limitations and the dynamic nature of biomedical research, future investigations can substantially benefit from the pursuit of shared biomarkers by incorporating diverse populations of individuals with various types of DFUs and utilizing a broader range of animal hard-to-heal wound models. Furthermore, continuous evaluation, involving comprehensive protein or gene expression analyses of samples that reflect the real-time conditions of the wound, such as exudate or cells collected from wound dressings, may yield deeper insights into the cellular dynamics significant to the wound healing process and advance our comprehension of it. Additionally, the use of high-resolution techniques, like single-cell RNA sequencing, represents a promising avenue for further research. This advanced methodology offers superior resolution of cellular responses and holds the potential to reveal novel biomarkers or pathways crucial to wound healing. Embracing such mechanism-driven approaches can yield more precise and actionable insights for guiding clinical interventions.

This review provides a comprehensive overview of the current state of research on biomarkers associated with hard-to-heal DFUs. However, it is important to acknowledge that our review is constrained by the existing body of literature. There might be unpublished studies or ongoing research that could provide additional insights. Additionally, the heterogeneity in study designs and populations might affect the comparability of the findings.

5. Conclusion

In conclusion, this scoping review has scoped a range of biomarkers associated with delayed wound healing in DFUs. Although these findings hold promise for potential clinical interventions, the generalizability and specificity of these biomarkers must undergo further validation. In the future, the adoption of advanced, mechanism-based research approaches has the potential to yield more precise insights, thereby paving the way for targeted interventions aimed at addressing the challenges posed by hard-to-heal DFUs.

Funding: This work was supported by the Fusion Oriented REsearch for disruptive Science and Technology (FOREST) from the Japan Science and Technology Agency (Grant No. JPMJFR205H) and JSPS KAKENHI (Grant No. 23KJ0482).

Conflict of Interest: The authors have no conflicts of interest to disclose.

References

1. van Netten JJ, Bus SA, Apelqvist J, Lipsky BA, Hinchliffe RJ, Game F, Rayman G, Lazzarini PA, Forsythe RO, Peters EJG, Senneville É, Vas P, Monteiro-Soares M, Schaper NC, International Working Group on the Diabetic Foot. Definitions and criteria for diabetic foot disease. Diabetes Metab Res Rev. 2020; 36(Suppl 1):e3268.
2. Fife CE, Horn SD, Smout RJ, Barrett RS, Thomson B. A predictive model for diabetic foot ulcer outcome: The wound healing index. Adv Wound Care (New Rochelle). 2016; 5:279-287.
3. Atkin L, Bućko Z, Conde Montero E, Cutting K, Moffatt C, Probst A, Romanelli M, Schultz GS, Tettelbach W. Implementing TIMERS: the race against hard-to-heal wounds inflammation/infection social factors edge regeneration moisture tissue. J Wound Care. 2019; 28:S1-S50.
4. Chan B, Cadarette S, Wodchis W, Wong J, Mittmann N, Krahn M. Cost-of-illness studies in chronic ulcers: A systematic review. J Wound Care. 2017; 26:S4-S14.
5. Armstrong DG, Swerdlow MA, Armstrong AA, Conte MS, Padula W V, Bus SA. Five-year mortality and direct costs of care for people with diabetic foot complications are comparable to cancer. J Foot Ankle Res. 2020; 13:16.
6. Boulton AJM, Armstrong DG, Kirsner RS, Attinger CE, Lavery LA, Lipsky BA, Mills JL Sr, Steinberg JS. Diagnosis and management of diabetic foot complications. In: ADA Clinical Compendia. Arlington, VA, 2018; pp.1-20.
7. Stolzenburg-Veeser L, Golubnitschaja O. Mini-encyclopaedia of the wound healing - Opportunities for integrating multi-omic approaches into medical practice. J Proteomics. 2018; 18:71-84.
8. Barrientos S, Stojadinovic O, Golinko MS, Brem H, Tomic-Canic M. Growth factors and cytokines in wound healing. Wound Repair Regen. 2008; 16:585-601.
9. Baltzis D, Eleftheriadou I, Veves A. Pathogenesis and treatment of impaired wound healing in diabetes mellitus: new insights. Adv Ther. 2014; 31:817-836.
10. Bender C, Cichosz SL, Pape-Haugaard L, Hartun Jensen M, Bermark S, Laursen AC, Hejlesen O. Assessment of simple bedside wound characteristics for a prediction model for diabetic foot ulcer outcomes. J Diabetes Sci Technol. 2020; 15:1161-1167.
11. Wang X, Li J, Wang Z, Deng A. Wound exudate CXCL6: A potential biomarker for wound healing of diabetic foot ulcers. Biomark Med. 2019; 13:167-174.
12. Botusan IR, Sunkari VG, Savu O, Catrina AI, Grünler J, Lindberg S, Pereira T, Ylä-Herttuala S, Poellinger L, Brismar K, Catrina SB. Stabilization of HIF-1α is critical to improve wound healing in diabetic mice. Proc Natl Acad Sci U S A. 2008; 105:19426-19431.
13. Fadini GP, Albiero M, Millioni R, Poncina N, Rigato M, Scotton R, Boscari F, Brocco E, Arrigoni G, Villano G, Turato C, Biasiolo A, Pontisso P, Avogaro A. The molecular signature of impaired diabetic wound healing identifies serpinB3 as a healing biomarker. Diabetologia. 2014; 57:1947-1956.
14. Mori R, Tanaka K, Shimokawa I. Identification and functional analysis of inflammation-related miRNAs in skin wound repair. Dev Growth Differ. 2018; 60:306-315.
15. Tricco AC, Lillie E, Zarin W, *et al*. PRISMA extension for scoping reviews (PRISMA-ScR): Checklist and explanation. Ann Intern Med. 2018; 169:467-473.
16. Fadini GP, Menegazzo L, Rigato M, *et al.* NETosis delays diabetic wound healing in mice and humans. Diabetes. 2016; 65:1061-1071.
17. Li JY, Wang ZJ, Deng AP, Li YM. ENA-78 is a novel predictor of wound healing in patients with diabetic foot ulcers. J Diabetes Res. 2019; 2019:2695436.
18. Li Z, Guo S, Yao F, Zhang Y, Li T. Increased ratio of serum matrix metalloproteinase-9 against TIMP-1 predicts poor wound healing in diabetic foot ulcers. J Diabetes Complications. 2013; 27:380-382.
19. Min KR, Galvis A, Baquerizo Nole KL, Sinha R, Clarke J, Kirsner RS, Ajdic D. Association between baseline abundance of *Peptoniphilus*, a Gram-positive anaerobic coccus, and wound healing outcomes of DFUs. PLoS One. 2020; 15:e0227006.
20. Löffler M, Zieker D, Weinreich J, Löb S, Königsrainer I, Symons S, Bühler S, Königsrainer A, Northoff H, Beckert S. Wound fluid lactate concentration: A helpful marker for diagnosing soft-tissue infection in diabetic foot ulcers? Preliminary findings. Diabetic Med. 2011; 28:175-178.
21. Vieceli Dalla Sega F, Cimaglia P, Manfrini M, Fortini F, Marracino L, Bernucci D, Pompei G, Scala A, Trichilo M, De Carolis B, Dalla Paola L, Ferrari R, Rizzo P, Campo G. Circulating biomarkers of endothelial dysfunction and inflammation in predicting clinical outcomes in diabetic patients with critical limb ischemia. Int J Mol Sci. 2022; 23:10641.
22. MacDonald A, Brodell JD, Daiss JL, Schwarz EM, Oh I. Evidence of differential microbiomes in healing versus non-healing diabetic foot ulcers prior to and following foot salvage therapy. J Orthop Res. 2019; 37:1596-1603.
23. Yang S, Gu Z, Lu C, Zhang T, Guo X, Xue G, Zhang L. Neutrophil extracellular traps are markers of wound healing impairment in patients with diabetic foot ulcers treated in a multidisciplinary setting. Adv Wound Care (New Rochelle). 2020; 9:16-27.
24. Peng C, Chen B, Kao HK, Murphy G, Orgill DP, Guo L. Lack of FGF-7 further delays cutaneous wound healing in diabetic mice. Plast Reconstr Surg. 2011; 128:673e-684e.
25. Fadini GP, Albiero M, Menegazzo L, Boscaro E, Pagnin E, Iori E, Cosma C, Lapolla A, Pengo V, Stendardo M, Agostini C, Pelicci PG, Giorgio M, Avogaro A. The redox enzyme p66Shc contributes to diabetes and ischemia-induced delay in cutaneous wound healing. Diabetes. 2010; 59:2306-2314.
26. Ruzehaji N, Mills SJ, Melville E, Arkell R, Fitridge R, Cowin AJ. The influence of Flightless I on Toll-like-receptor-mediated inflammation in a murine model of diabetic wound healing. Biomed Res Int. 2013; 2013:389792.
27. Kunkemoeller B, Bancroft T, Xing H, Morris AH, Luciano AK, Wu J, Fernandez-Hernando C, Kyriakides TR. Elevated thrombospondin 2 contributes to delayed wound healing in diabetes. Diabetes. 2019; 68:2016-2023.
28. Jiang Y, Xi Y, Li Y, Zuo ZH, Zeng CY, Fan J, Zhang D, Tao HL, Guo YC. Ethanol promoting the upregulation of C-X-C Motif Chemokine Ligand 1 (CXCL1) and C-X-C Motif Chemokine Ligand 6 (CXCL6) in models of early alcoholic liver disease. Bioengineered. 2022; 13:4688-4701.
29. Singh UP, Singh NP, Murphy EA, Price RL, Fayad R, Nagarkatti M, Nagarkatti PS. Chemokine and cytokine

levels in inflammatory bowel disease patients. Cytokine. 2016; 77:44-49.
30. Lepennetier G, Hracsko Z, Unger M, Van Griensven M, Grummel V, Krumbholz M, Berthele A, Hemmer B, Kowarik MC. Cytokine and immune cell profiling in the cerebrospinal fluid of patients with neuro-inflammatory diseases. J Neuroinflammation. 2019; 16:219.
31. Russo RC, Garcia CC, Teixeira MM, Amaral FA. The CXCL8/IL-8 chemokine family and its receptors in inflammatory diseases. Expert Rev Clin Immunol. 2014; 10:593-619.
32. Muller M, Trocme C, Lardy B, Morel F, Halimi S, Benhamou PY. Matrix metalloproteinases and diabetic foot ulcers: The ratio of MMP-1 to TIMP-1 is a predictor of wound healing. Diabetic Med. 2008; 25:419-426.
33. Sonamuthu J, Cai Y, Liu H, Kasim MSM, Vasanthakumar VR, Pandi B, Wang HX, Yao JM. MMP-9 responsive dipeptide-tempted natural protein hydrogel-based wound dressings for accelerated healing action of infected diabetic wound. Int J Biol Macromol. 2020; 153:1058-1069.
34. Luanraksa S, Jindatanmanusan P, Boonsiri T, Nimmanon T, Chaovanalikit T, Arnutti P. An MMP/TIMP ratio scoring system as a potential predictive marker of diabetic foot ulcer healing. J Wound Care. 2018; 27:849-855.
35. Mather KJ, Mirzamohammadi B, Lteif A, Steinberg HO, Baron AD. Endothelin contributes to basal vascular tone and endothelial dysfunction in human obesity and type 2 diabetes. Diabetes. 2002; 51:3517-3523.
36. Fivenson DP, Faria DT, Nickoloff BJ, Poverini PJ, Kunkel S, Burdick M, Strieter RM. Chemokine and inflammatory cytokine changes during chronic wound healing. Wound Repair Regen. 1997; 5:310-322.
37. Schmohl M, Beckert S, Joos TO, Königsrainer A, Schneiderhan-Marra N, Löffler MW. Superficial wound swabbing: A novel method of sampling and processing wound fluid for subsequent immunoassay analysis in diabetic foot ulcerations. Diabetes Care. 2012; 35:2113-2120.

Received October 24, 2023; Revised December 11, 2023; Accepted December 13, 2023.

*Address correspondence to:
Gojiro Nakagami, Department of Gerontological Nursing/ Wound Care Management, Graduate School of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-Ku, Tokyo 113-0033, Japan.
E-mail: gojiron@g.ecc.u-tokyo.ac.jp

Released online in J-STAGE as advance publication December 22, 2023.