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Interleukin-8/Matrix Metalloproteinase-9 Axis Impairs Wound Healing in Type 2 Diabetes through Neutrophil Extracellular Traps-Fibroblast Crosstalk

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Keywords: interleukin-8 | matrix metalloproteinase-9 | neutrophil extracellular traps | type 2 diabetes mellitus | wound healing

ABSTRACT

Neutrophils interact with and activate fibroblasts through the release of neutrophil extracellular traps (NETs). We investigated the role of NETs-fibroblast crosstalk in the cutaneous wound healing of type 2 diabetes (T2D). Neutrophils/NETs, serum, and primary human skin fibroblasts (HSFs) were obtained from individuals with T2D and age/sex-matched controls. NET-stimulation studies were performed on neutrophils/HSFs, with and without specific inhibitors, while HSF healing capacity was assessed using a scratch wound healing assay. T2D HSFs display a profibrotic phenotype, showing increased CCN2/CTGF, α-smooth muscle actin, and collagen release, albeit with impaired healing capacity, elevated type I collagen C-terminal telopeptide, and collagen degradation associated with increased (~3.5-fold) matrix metalloproteinase-9 (MMP-9) in T2D neutrophils/NETs. IL-8 induced the expression of MMP-9 in neutrophils/NETs. Moreover, T2D neutrophils/NETs exhibited increased IL-8 content, which acted in an autocrine/paracrine fashion to further augment its production by neutrophils/HSFs. The findings were validated in normoglycemic individuals during a hyperglycemic clamp with concomitant lipid infusion and further corroborated immunohistochemically in diabetic plantar ulcer biopsies. This novel, vicious circle of NETs/interleukin-8/MMP-9/HSFs was hindered by IL-8 or MMP-9 blockade via specific inhibitors or by dismantling the NET-scaffold with DNase I, suggesting candidate therapeutic targets in wound healing impairment of T2D.

Abbreviations: CCN2, cellular communication network factor 2; COL1, collagen type I; CTGF, connective tissue growth factor; CTX1, C-terminal cross-linked telopeptide of type I collagen; DFU, diabetic foot ulcer; DPP4i, dipeptidyl peptidase-4 inhibitors; FFA, free fatty acid; GLP1RA, glucagon-like peptide-1 receptor agonists; HCG, hyperglycemic clamp; HI, healthy individuals; HSFs, human skin fibroblasts; MMP-9, matrix metalloproteinase-9; NE, neutrophil elastase; NETs, neutrophil extracellular traps; SGLT2i, sodium/glucose co-transporter-2 inhibitors; T2D, type 2 diabetes mellitus; αSMA, alpha smooth muscle actin.

Dimitrios Tsilingiris, Anastasia-Maria Natsi, and Efstratios Gavriilidis contributed equally to this work and share first authorship.

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1 | Introduction

Diabetic foot ulcers (DFUs) constitute one of the most frequent and serious complications of type 2 diabetes mellitus (T2D).

Wound healing is a complex and dynamic function where immune and stromal cells migrate, proliferate, and interact, leading to extracellular matrix deposition and tissue remodeling [1, 2]. Its classical conception involves four partly overlapping phases: hemostasis phase, where platelets orchestrate the first step of tissue repair; inflammatory phase, where immune cells infiltrate the affected tissue and interact with stromal cells; proliferative phase, where fibroblasts dispose the extracellular matrix (e.g., collagen) and the remodeling phase where the collagen is remodeled and organized mainly by matrix metalloproteinases (MMPs) [1, 2].

Neutrophils are the first immune cells to migrate during the inflammatory phase of wound healing. To date, several studies implicate neutrophils in the impaired wound healing of T2D via the release of neutrophil extracellular traps (NETs) [3–5]; however, the exact mechanisms involved in this process are not clearly understood. It has been demonstrated that neutrophils via NETosis are able to interact with stromal cells, such as fibroblasts, leading them to acquire disease-specific properties [6, 7]. Fibroblasts, in turn, as indicated by a recent study by our group [7], during their crosstalk with neutrophils, are capable of releasing neutrophil chemoattractants, such as IL-8, sustaining an amplification loop. Notably, the "protein quality" of NETs can vary in different disease settings, as neutrophils, in response to environmental stimuli, can undergo transcriptional reprogramming, a procedure called neutrophil plasticity [8].

MMPs are zinc endopeptidases responsible for the degradation of extracellular matrix components. They participate in all wound healing phases, but more importantly in remodeling [9]. Several reports have shown that MMPs, such as MMP-9, are found in abundance in DFUs [10, 11]. Additionally, available evidence suggests that MMPs can decorate NET structures and induce tissue damage [12, 13]. Nevertheless, to date, the putative role of MMPs as components of NETs in T2D remains unexplored.

In this study, we aimed to investigate the role of the crosstalk between neutrophils/NETs and human skin fibroblasts (HSFs) in wound healing impairment of T2D, focusing on MMP-9 and IL-8 as components of this interaction.

2 | Results

2.1 | HI HSFs Treated with T2D NETs Acquire a Profibrotic but Dysfunctional Phenotype Similar to T2D HSFs

We initially examined whether HSFs from unaffected skin areas exhibit an impaired fibrotic potential in T2D compared with controls. We found that T2D HSFs exhibit augmented alpha-smooth muscle actin (aSMA) staining, consistent with an activated myofibroblast-like functional status (Figure 1A,C,E), together with increased cellular communication network 2 (CCN2) at protein (Figure 1A–F) and mRNA (Figure 1G) levels, as well as increased collagen production (Figure 1H), compared with HSFs from healthy individuals (HI HSFs).

Since HSFs' migration during cutaneous healing occurs across the produced type I collagen matrix [14], we examined whether the increased fibrotic readiness in T2D is reflected in an augmented migration of HSFs during an in vitro scratch wound healing assay. Interestingly, HSFs from the T2D project a defective migratory capacity compared with HI HSFs (Figure 1I).

Recent studies of our group highlighted that the crosstalk between neutrophil/NETs axis and fibroblasts drives immunofibrosis in COVID-19 and Crohn's disease [6, 7], while previous studies provided evidence that neutrophils/NETs are also implicated in wound healing impairment of T2D [3, 15, 16]. Furthermore, neutrophils are the primary population of immune cells initially migrating into the site of cutaneous injury, where they come at physical proximity with HSFs [1, 2]. Consequently, we investigated the effects of neutrophils and NETosis on the functional state of HSFs. For this purpose, we in vitro stimulated HI HSFs with T2D NETs to simulate the local inflammatory environment at the site of cutaneous injury. Prior to stimulation and inhibition studies, isolated NETs were quantified performing an MPO/DNA complex ELISA (Figure S1A).

Incubation of HI HSFs with NETs from T2D, but not from HI, induces a profibrotic phenotype similar to T2D primary HSFs, characterized by upregulation of CCN2 at protein (Figure 2A,B) and mRNA (Figure 2C) level, along with increased collagen release (Figure 2D) and positive aSMA staining (Figure S1B–D). However, they appear functionally impaired as assessed by a scratch wound healing assay (Figure 2E).

Collectively, similarly to the already affected primary diabetes HSFs, HI HSFs stimulated by T2D NETs exert a profibrotic phenotype characterized by increased collagen release, although they appear functionally impaired. This highlights neutrophil-fibroblast interaction through NETs as a key component in the defective wound healing of T2D.

2.2 | T2D NETs Induce Increased Collagenase Activity in Control HSFs through Augmented MMP-9 Production

Neutrophils are a known source of MMP-9 [17, 18], which is known to possess collagenase activity [19]. Accordingly, we found that NETs from T2D compared with HI demonstrate higher MMP-9 concentrations (approximately 3.5-fold) (Figure 3A,B). On the other hand, in serum, free MMP-9 levels were comparable between diabetes and HI (Figure S2A).

Moreover, treatment of HI HSFs with T2D NETs led to enhanced immunostaining for type I collagen cross-linked c-telopeptide (CTX1), a product of type I collagen enzymatic breakdown [20], indicating that the produced collagen undergoes excessive catabolism into nonfunctional fragments during neutrophil-HSF crosstalk in T2D. This effect was reversed after pretreatment of T2D NETs with either DNase I, an agent that dismantles the DNA scaffold of NETs, or a specific inhibitor of MMP-9 (MMP-9-IN-1),

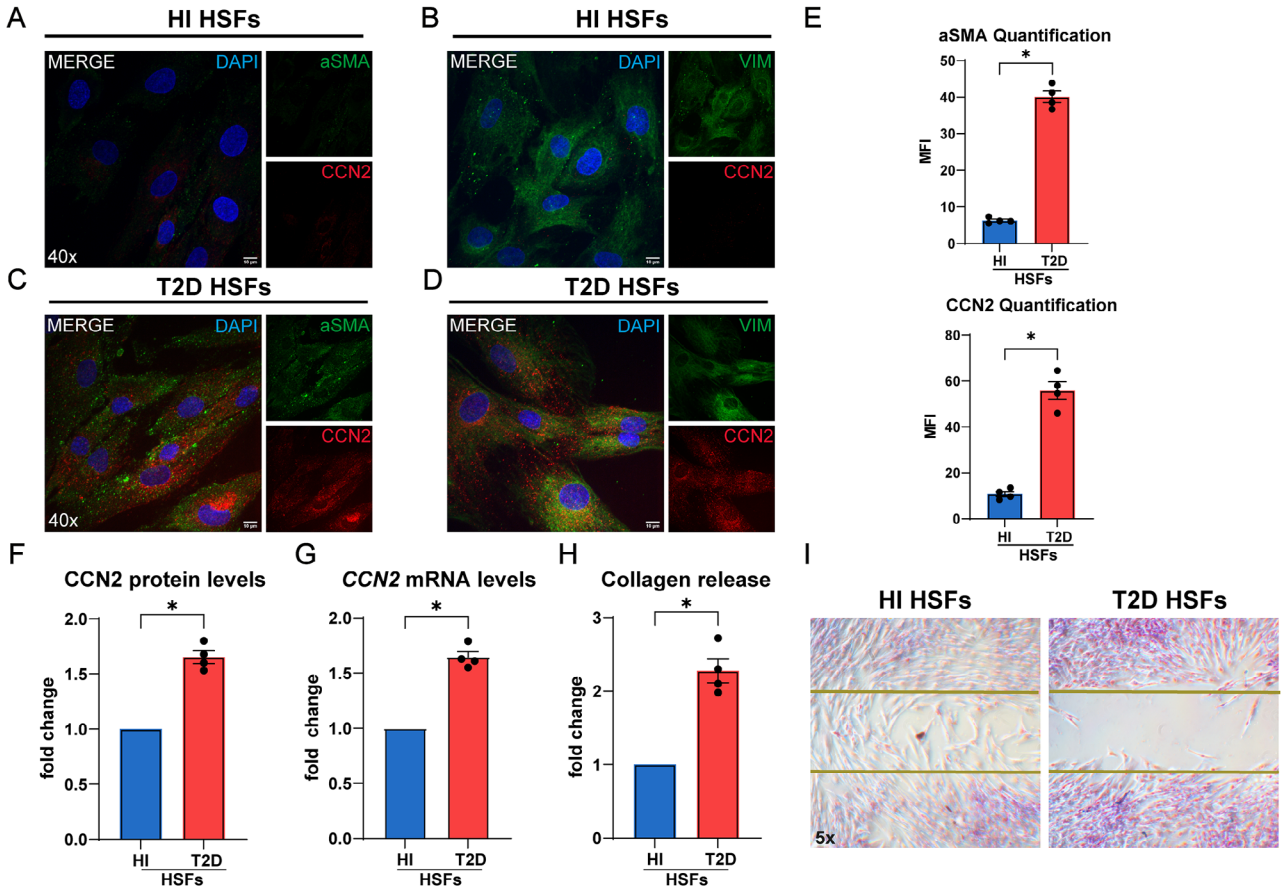

FIGURE 1 | T2D fibroblasts are characterized by a profibrotic but functionally impaired phenotype. (A–D) Immunostainings in HSFs isolated by HI (A), (B), and T2D patients (C), (D). (A), (C) blue: DAPI, green: aSMA, red: CCN2; (B), (D) blue: DAPI, green: Vimentin, red: CCN2. (E) Corresponding mean fluorescence intensity (MFI) quantification of aSMA and CCN2. CCN2 protein (F) and mRNA (G) levels were assessed by cytoblot and RT-qPCR, respectively, in HI and T2D HSFs. (H) Sircol collagen release assay. (I) Wound healing assay. (A–D), (I) One representative example out of four independent experiments is presented. (A–D) Confocal microscopy. Magnification: 40×, scale bar 10 μm. (I) Optical microscopy. Magnification: 5×. (E–G) Mann–Whitney *U*-test was applied, $n = 4$, $^*p < 0.05$. aSMA, alpha smooth muscle actin; CCN2, cellular communication network factor 2; HI, healthy individuals; HSFs, human skin fibroblasts; T2D, type 2 diabetes mellitus.

corroborating the major role of MMP-9/NETs in the degradation of collagen (Figure 3C). Additionally, HI HSFs stimulated with T2D NETs preincubated with either DNase I or MMP-9-IN-1 presented intact wound healing capacity (Figure 3D) together with low immunoreactivity of CTX1 (Figure S2D), indicating the functional role of MMP-9-bearing NETs. These findings were in conjunction with increased collagenase activity in cell lysates of HSFs treated with T2D NETs, a phenomenon also reversed by preincubation of NETs with DNase I or MMP-9-IN-1 (Figure 3E).

Next, to investigate whether the increased MMP-9 concentrations may be associated with glycemic perturbations, we examined the relationship of MMP-9 in NETs with glycated hemoglobin (HbA1c), an index reflecting the average glycemia of the preceding 2–3 months. NET-bound MMP-9 concentrations were significantly higher in patients with T2D with poor than those with good glycemic control (28.2 vs. 14.9 ng/mL, $p < 0.05$ for HbA1c ≥7% and <7% respectively, Figure 3F), showed also a positive correlation with HbA1c ($r = 0.42$, $p < 0.05$, Figure 3G). In contrast, no such associations were noted regarding serum MMP-9 (Figure S2B,C).

Taken together, these findings suggest that neutrophil MMP-9 is increased alongside hyperglycemia T2D, and its release through NETs induces phenotypic and functional alterations in HSFs associated with the increased collagen degradation and defective wound healing capacity.

2.3 | Sustained IL-8 Production through Neutrophil-HSF Interaction Feedbacks IL-8/MMP-9-Bearing NETs

Since IL-8 is a powerful neutrophil chemoattractant, and neutrophils have been shown to be persistently present in chronic DFUs [21], we proceeded to investigate its role in neutrophil-HSF crosstalk in T2D.

Firstly, we found that T2D neutrophils exhibit higher IL-8 expression than HI neutrophils at mRNA (Figure 4A) and protein (approximately 2.5-fold) levels (Figure 4B,C). Furthermore, exposure of HI HSFs to NETs from T2D but not HI, strongly induced IL-8 at mRNA (Figure 4D) and protein level (Figure 4E), an effect that is prevented upon the neutralization of IL-8 on

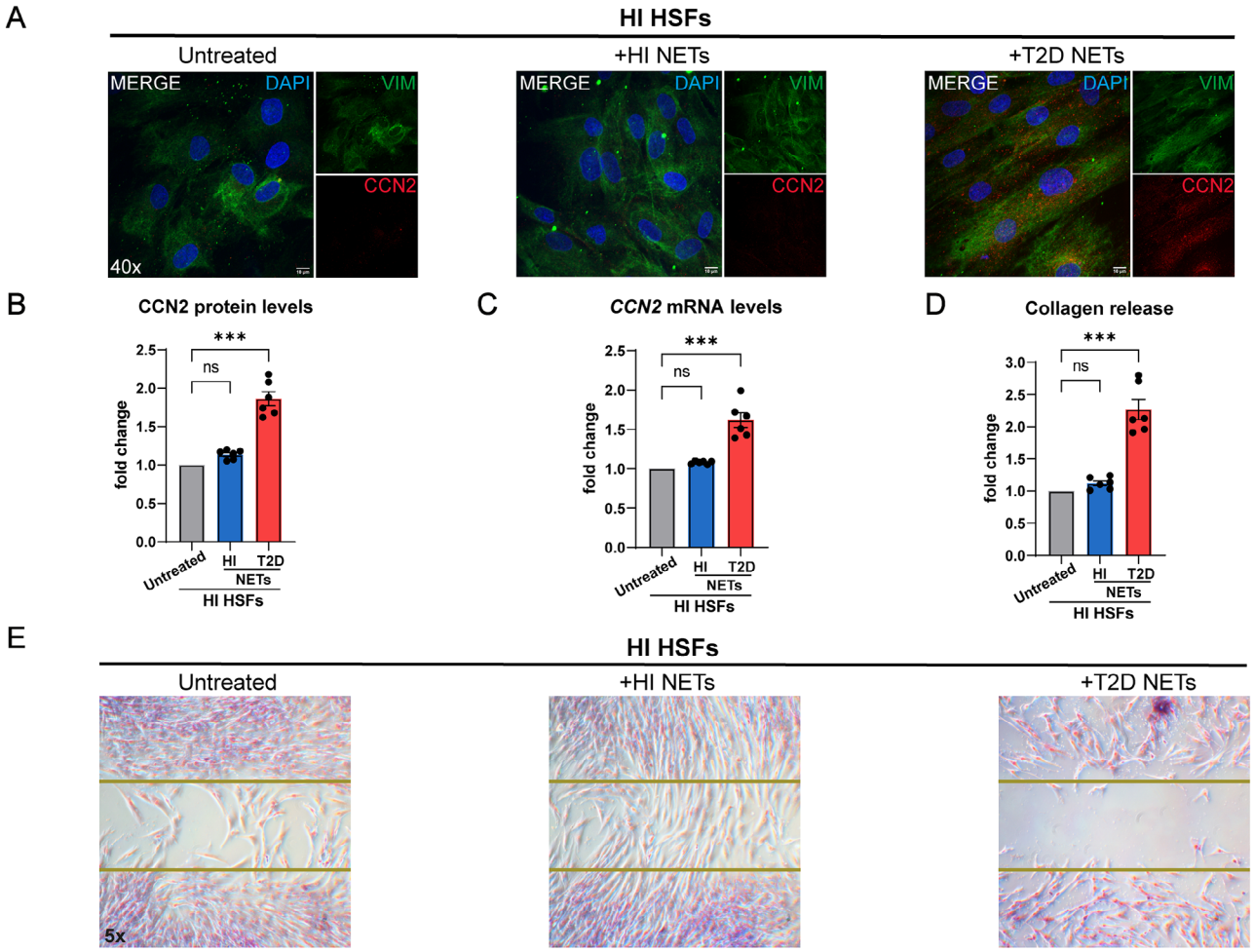

FIGURE 2 | Healthy fibroblasts, during their crosstalk with T2D NETs, acquire a functionally defective phenotype, similar to primary T2D fibroblasts. (A–E) Stimulations of HI HSFs with either HI or T2D NETs. Assessment of CCN2 by (A) immunostaining (blue: DAPI, green: Vimentin, red: CCN2), (B) Cytoblot and (C) RT-qPCR. (D) Sircol collagen release assay. (E) Wound healing assay. (A), (E) One representative example out of four independent experiments is presented. (A) Confocal microscopy. Magnification: 40×, scale bar 10 μm. (E) Optical microscopy. Magnification: 5×. (B–D) Kruskal–Wallis followed by Dunn's test for multiple comparisons, was applied, $n = 4$, $^{***}p < 0.001$, ns, not significant. CCN2, cellular communication network factor 2; HI, healthy individuals; HSFs, human skin fibroblasts; T2D, type 2 diabetes mellitus.

NETs by using a specific anti-IL-8 antibody (Figure 4D,E). These suggest a positive feedback loop of IL-8 production through neutrophils/NETs/IL-8/fibroblasts interplay. Similarly, IL-8 is induced in HI neutrophils following exposure to T2D NETs but not HI NETs at mRNA (Figure 4F) and protein levels (Figure 4G), comparably with the stimulation of HI neutrophils with a recombinant IL-8 (mRNA level) (Figure 4F). These results are hindered after successful inhibition of NET-derived IL-8 by anti-IL-8 neutralization, stipulating a self-sustaining, autocrine neutrophil/IL-8 pathway (Figure 4F,G).

Next, in view that IL-8 is a potent inducer of MMP-9 production in neutrophils through PKC/ERK1-2 or Src-kinases pathways [22] and NETs proved to be a source of IL-8, we exposed HI neutrophils to T2D NETs or recombinant IL-8. Interestingly, both stimuli induced MMP-9 production by HI neutrophils (Figure 4H,I), in a manner similar to that observed in ex-vivo neutrophils from T2D. This effect was prevented by interleukin-8 blockade (Figure 4H,I), suggesting an additional vicious amplification loop of MMP-9 by IL-8.

Collectively, these indicate that neutrophil-derived IL-8 exerts a potent autocrine action, thereby inducing not only its own expression but also MMP-9 production. Moreover, IL-8-enriched T2D NETs further stimulate the IL-8 expression by HSFs.

2.4 | Neutrophil Alterations of T2D are Replicated in Normoglycemic Individuals After Lipid/Glucose Infusion

Elevated circulating free fatty acid (FFA) and glucose concentrations are metabolic hallmarks of T2D. Consequently, we sought to investigate whether the ascertained neutrophil deviations may also be inducible during transient elevation of FFA and glucose concentrations. We conducted hyperglycemic clamps (target glucose 200 mg/dL in the steady state of 120 min) following a 120 min infusion of lipid emulsion in lean, normoglycemic individuals ($n = 3$, Table S2, Figure S4, timepoint 240', T240').

Neutrophils exhibit increased IL-8 and MMP-9 production after lipid plus glucose infusion (T240'), similar to ex-vivo diabetic

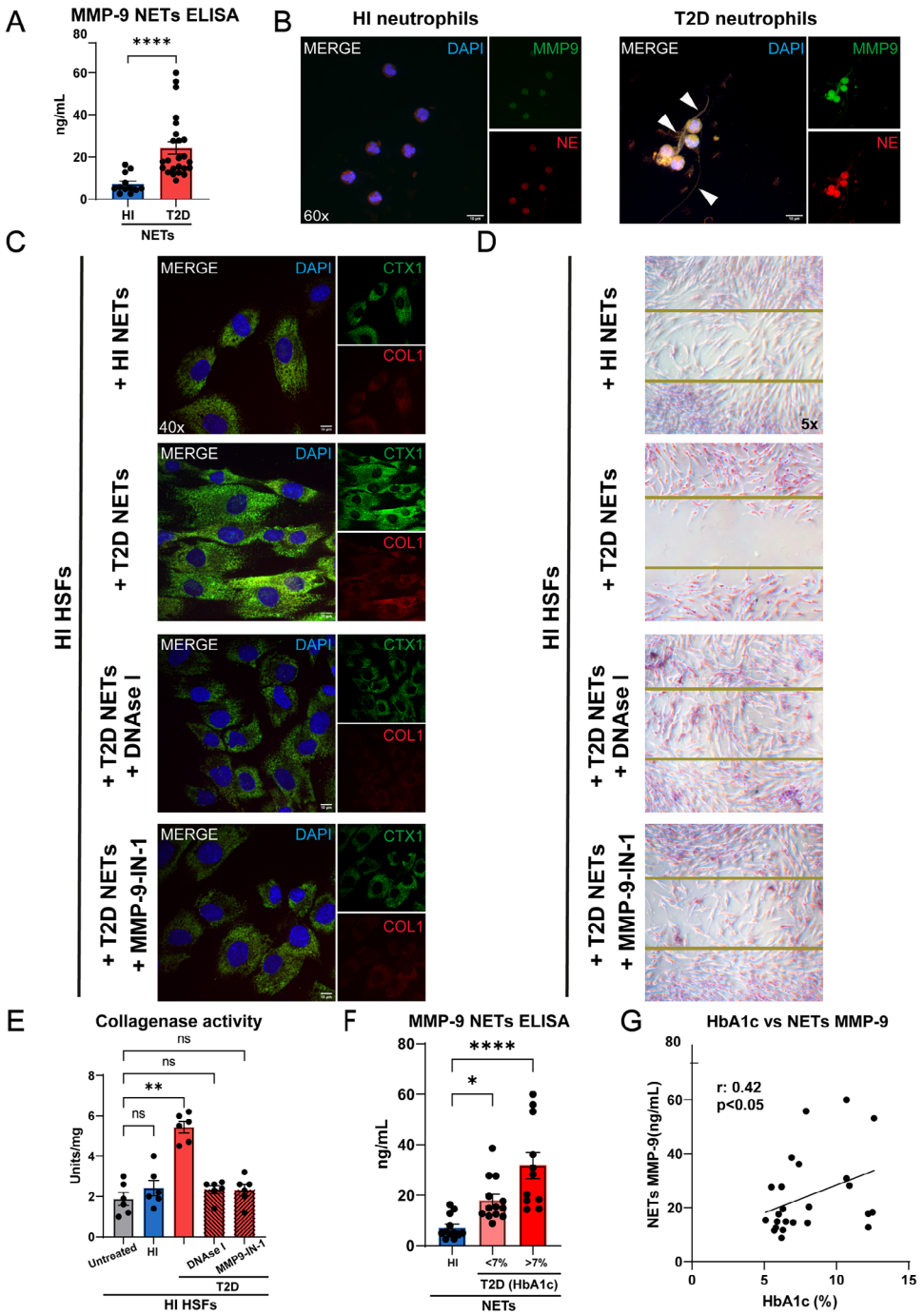

FIGURE 3 | Hyperglycemic T2D NETs overexpress MMP-9, which impairs the wound healing process. (A, B) MMP-9 concentration in HI and T2D NETs assessed by ELISA (A) and immunostaining (B) (blue: DAPI, green: MMP-9, red: NE). (C) Immunostaining (blue: DAPI, green: CTX1, red: COL1) and (D) wound healing assay of HI HSFs stimulated with HI or T2D NETs in the absence/presence of DNAse I or MMP-9-IN-1. (E) Collagenase activity assay of HI HSFs treated under the aforementioned conditions. (F) MMP-9 ELISA measured in NETs obtained from patients with good (HbA1c<7%) and poor (HbA1c>7%) glycemic control. (G) Correlation between MMP-9 concentration in NETs and HbA1c levels. (A), (E), (F) Kruskal–Wallis followed by Dunn's test for multiple comparisons was applied, $*p < 0.05$, $**p < 0.01$, $****p < 0.0001$, ns, not significant. (A), (F) HI, $n = 12$; T2D, $n = 24$; (E) $n = 6$. (B–D) One representative example out of four independent experiments is presented. (B), (C) Confocal microscopy. (B) Magnification: 60×, scale bar 10 μm. White arrowheads depict NETs. (C) Magnification: 40×, scale bar 10 μm. (D) Optical microscopy. Magnification: 5×. (G) Spearman's rank correlation coefficient was used. CTX1, C-terminal cross-linked telopeptide of type I collagen; COL1, collagen type I; HbA1c, hemoglobin A1c; HI, healthy individuals; HSFs, human skin fibroblasts; MMP-9, matrix metalloproteinase-9; MMP-9-IN-1; matrix metalloproteinase-9 inhibitor-1; NE, neutrophil elastase; T2D, type 2 diabetes mellitus.

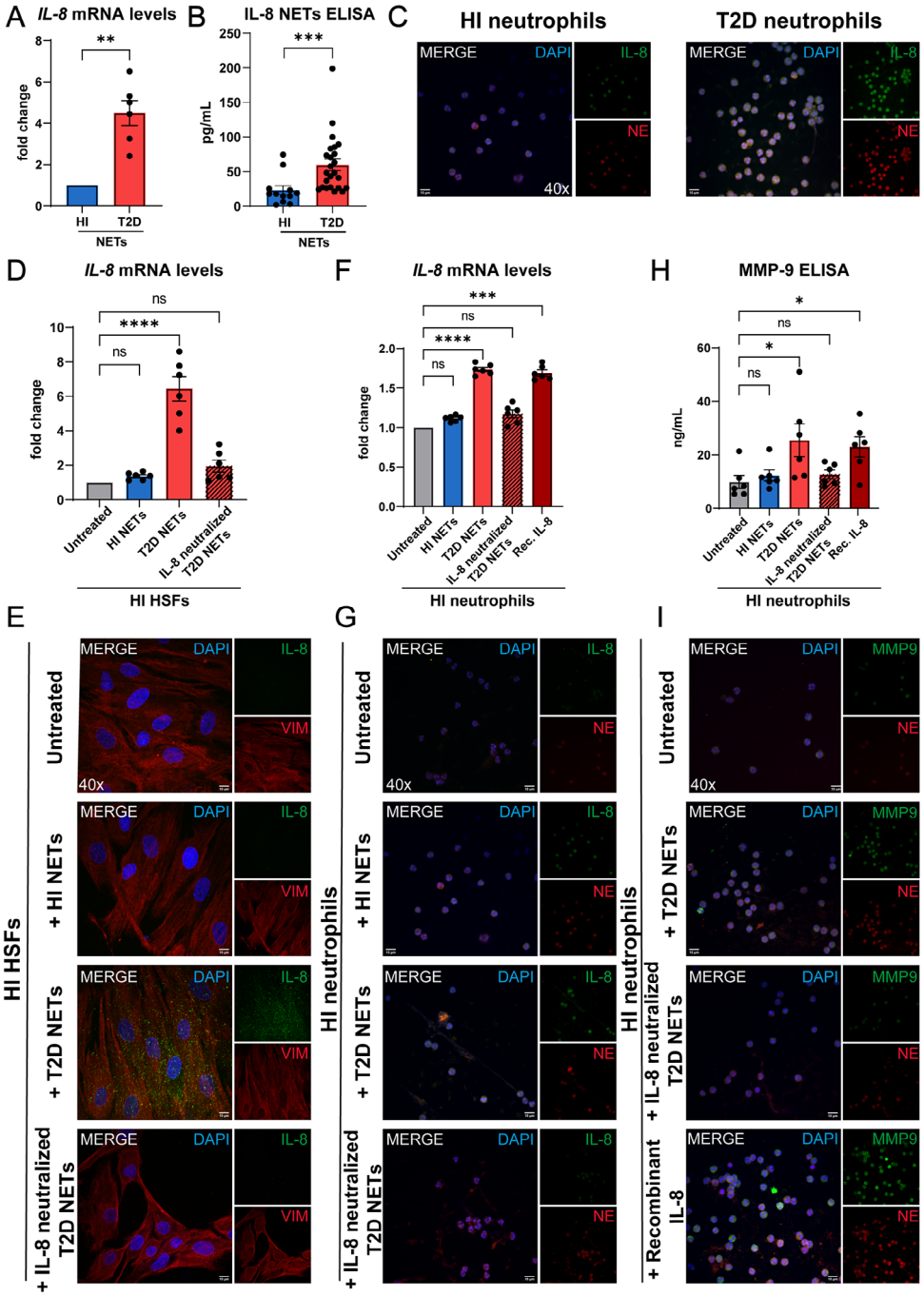

FIGURE 4 | Overproduced IL-8 by neutrophils and fibroblasts instigates autocrine and paracrine feedback loops. (A) *IL-8* mRNA levels, (B) IL-8 ELISA, and (C) IL-8 immunostaining (blue: DAPI, green: IL-8, red: NE), assessed in neutrophils/NETs obtained by HI and T2D patients. (D) *IL-8* mRNA levels and (E) IL-8 immunostaining (blue: DAPI, green: IL-8, red: Vimentin) assessed in HI HSFs stimulated with HI, T2D, or IL-8 neutralized T2D NETs. (F) *IL-8* mRNA levels and (G) IL-8 immunostaining (blue: DAPI, green: IL-8, red: NE), assessed in HI neutrophils treated with HI or T2D or IL-8 neutralized T2D NETs or recombinant IL-8. (H) MMP-9 ELISA and (I) MMP-9 immunostaining (blue: DAPI, green: MMP-9, red: NE), in HI neutrophils stimulated with HI or T2D or neutralized T2D NETs, or recombinant IL-8. (A), (B) Mann–Whitney *U*-test was applied. (A) $n = 6$, (B) HI, $n = 12$; T2D, $n = 24$. (C), (E), (G), (I) One representative example out of four independent experiments is presented. Confocal microscopy. Magnification: 40x, scale bar 10 μm. (D), (F), (H) Kruskal–Wallis followed by Dunn's test for multiple comparisons was applied, $n = 6$. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, ns, not significant. HI, healthy individuals; HSFs, human skin fibroblasts; IL-8, interleukin-8; MMP-9, matrix metalloproteinase-9; NE, neutrophil elastase; T2D, type 2 diabetes mellitus.

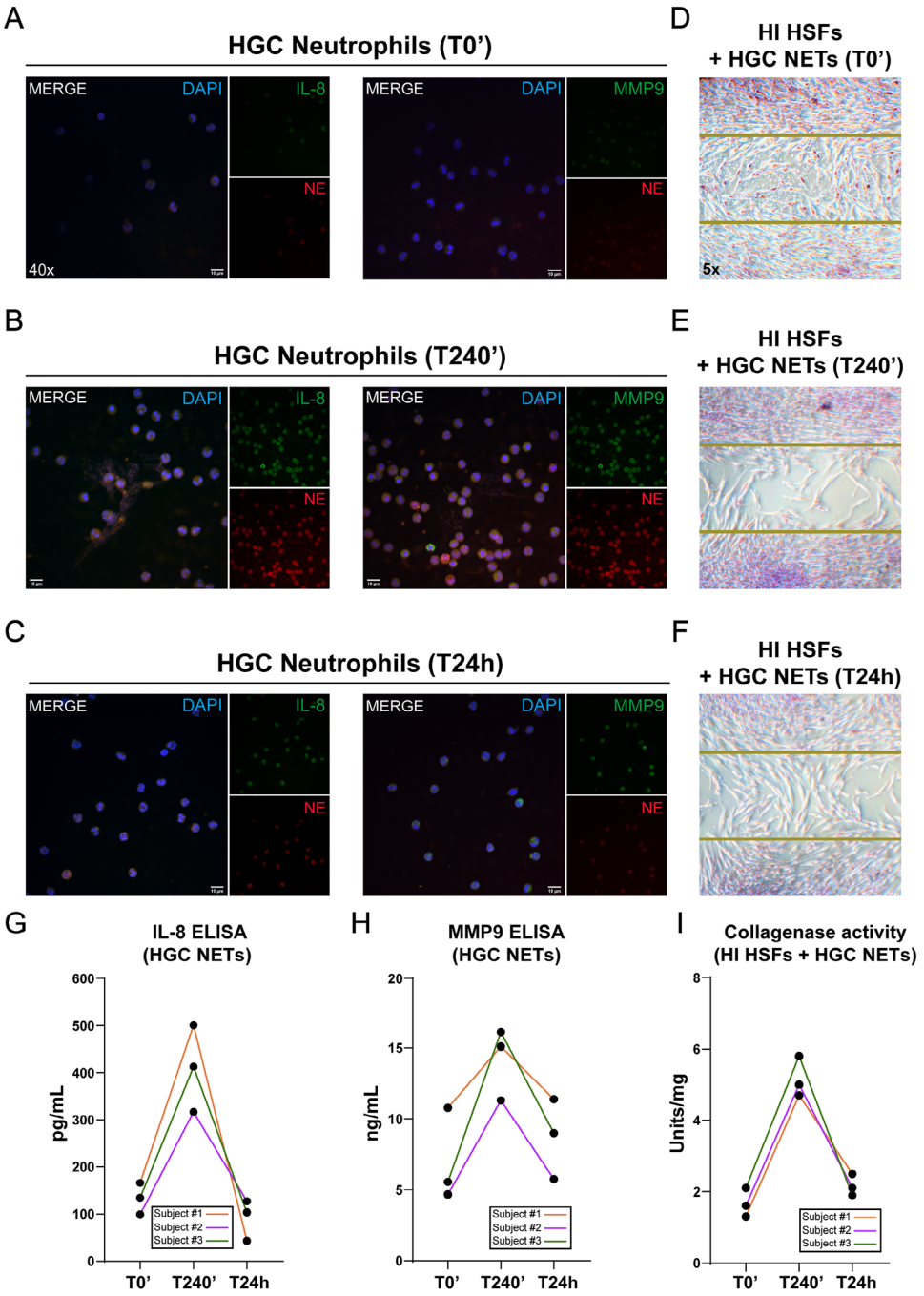

FIGURE 5 | Hyperglycemic clamps with lipid infusion induce phenotypic features in healthy neutrophils, similar to those observed in T2D. (A–C) Immunostainings depicting IL-8 (green)/NE (red) and MMP-9 (green)/NE (red) immunoreactivity in neutrophils obtained before HGC administration (timepoint 0', T0') (A), 240' minutes (timepoint 240', T240') (B), and 24 h (timepoint 24 h, T24 h) (C), after HGC infusion in healthy controls. (D–F) Wound healing assay showing the migratory capacity of HI HSFs treated with either (D) T0', (E) T240', or (F) T24 h NETs. (G) IL-8 and (H) MMP-9 ELISA measured in T0', T240', and T24h' NETs. (I) Collagenase activity of HI HSFs treated with T0, T240' or T24 h NETs. (A–F) One representative example out of three independent experiments is presented. (A–C) Confocal microscopy. Magnification: 40x, scale bar 10 μm. (D–F) Optical microscopy. Magnification: 5×. (G–I) Qualitative representation; orange line: subject #1, purple line: subject #2, green line: subject #3. HGC, hyperglycemic clamp with lipid infusion; HI, healthy individuals; HSFs, human skin fibroblasts; IL-8, interleukin-8; MMP-9, matrix metalloproteinase-9; NE, neutrophil elastase; T2D, type 2 diabetes mellitus.

neutrophils (Figures 5A–C, 5G,H). These effects are transient and subside after the restoration of the baseline metabolic state after 24 h (T24 h) (Figures 5A–C, 5G,H). Furthermore, incubation of HI HSFs with T240' NETs induces the defective wound healing capacity (Figure 5D–F) and increased collagenase activity identical to T2D (Figure 5I), an effect not observed in stimulations with T24 h NETs.

These results further demonstrate the importance of the lipotoxic and glycotoxic milieu as a potential instigator of the observed neu-

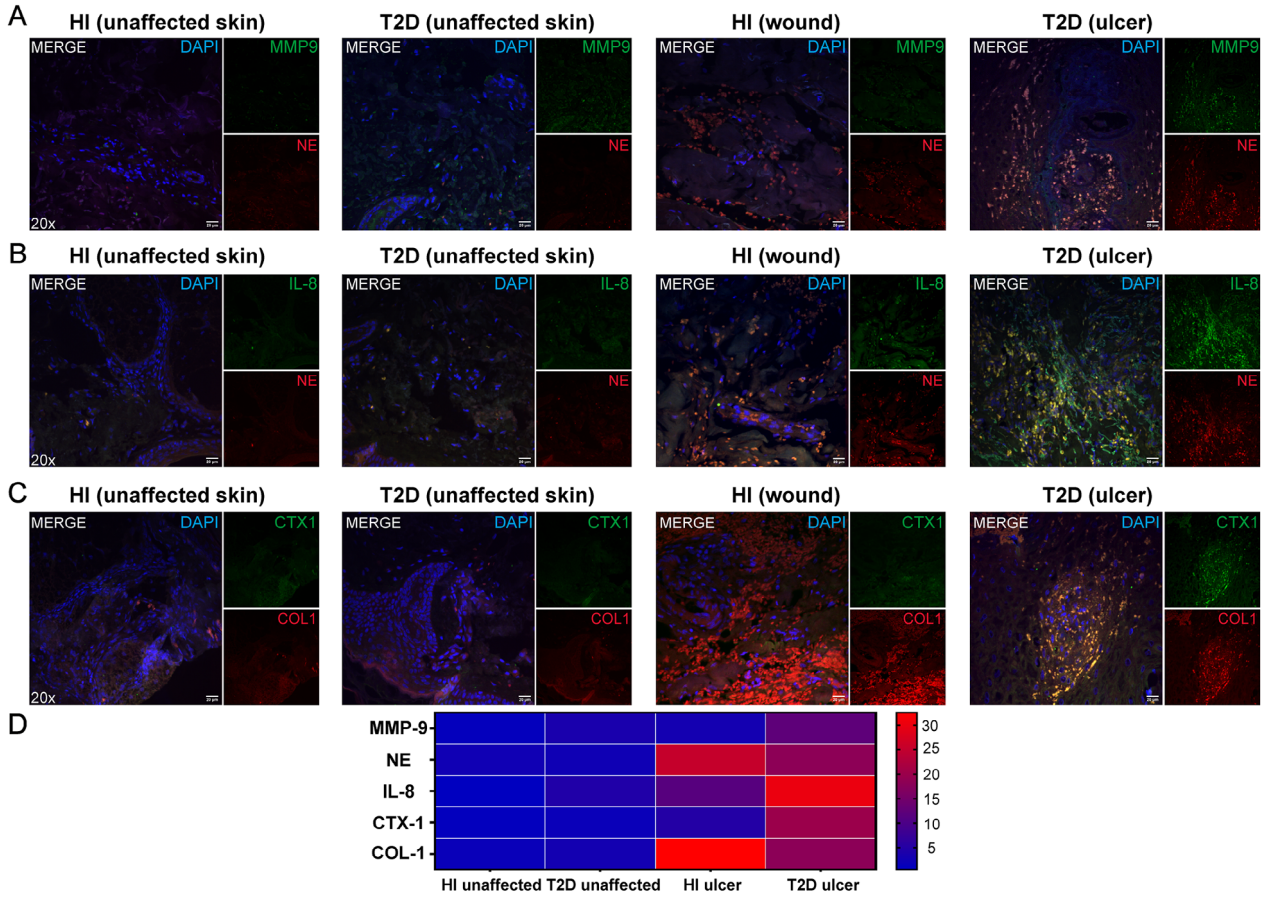

FIGURE 6 | Chronic diabetic ulcers exhibit an increased presence of MMP-9/IL-8 in neutrophils and degraded collagen. (A–C) Tissue immunostainings in unaffected skin and ulcer sections obtained from healthy and diabetic individuals. (A) Blue: DAPI, green: MMP-9, red: NE, (B) blue: DAPI, green: IL-8, red: NE, (C) blue: DAPI, green: CTX1, red: COL1. (D) MFI-based grading scale depicting MMP-9, NE, IL-8, CTX1, and COL1 immunoreactivity in the aforementioned conditions. (A–C) One representative example out of four independent experiments is presented. Confocal microscopy. Magnification: 20×, scale bar 20 μm. CTX1, C-terminal cross-linked telopeptide of type I collagen; COL1, collagen type I; HI, healthy individuals; IL-8, interleukin-8; MMP-9, matrix metalloproteinase-9; NE, neutrophil elastase; T2D, type 2 diabetes mellitus.

trophil adaptations, which in turn mandate defective cutaneous healing during neutrophil-HSF crosstalk.

2.5 | Increased MMP-9, Interleukin-8, and Degraded Collagen in Chronic Diabetic Plantar Ulcer Skin Tissue

To ascertain our in-vitro findings at the tissue level, we examined cutaneous specimens from the border of chronic neuropathic DFUs and intact forearm skin from patients with T2D, as well as plantar healing wounds and forearm skin from HI controls (n = 4 each, Table S3). All examined DFUs showed no clinical or laboratory signs of infection.

DFUs showed strong staining for MMP-9 and IL-8 in colocalization with neutrophil elastase (NE) in confocal microscopy, thus demonstrating the presence of MMP-9-bearing neutrophils (Figure 6A; Figure S3F) expressing IL-8 (Figure 6B; Figure S3G) compared with bland staining in intact skin from T2D and HI. High IL-8 levels were also observed in vimentin-marked fibroblasts of DFUs (Figure S3A–E). Furthermore, ulcer specimens exhibited increased type I collagen in conjunction with strong CTX1 immunostaining, indicating increased production of albeit-degraded collagen at the site of the chronic wound (Figure 6C; Figure S3H).

Of note, in wounds obtained from nondiabetic individuals, although neutrophils and collagen were found in abundance, immunostainings for IL-8, MMP-9, and CTX1 were faint in comparison with DFUs (Figure 6A–C; Figure S3). A grading scale, based on MFI quantifications of the abovementioned parameters, is presented in Figure 6D.

Taken together, our findings indicate that this pathogenic interplay between neutrophils and fibroblasts during cutaneous healing in T2D is characteristic of the condition, whereby it emerges as a product of elevated levels of IL-8/MMP-9 and does not apply for skin wounds in other conditions.

3 | Discussion

The presented novel results substantially expand previous data on the effects of NETosis on cutaneous healing in T2D. We introduce IL-8 and MMP-9 as core components of the crosstalk

between neutrophils and HSFs mediated by NETosis, which drives wound healing impairment in T2D. Neutrophils instigate this by interacting with HSFs, which results in the functional impairment of the latter, despite the acquisition of a pro-fibrotic phenotype, characterized by upregulation of aSMA, CCN2, and increased production of degraded collagen. The upregulated IL-8 release by diabetic neutrophils further induces not only its own expression by neutrophils and HSFs but also the production of MMP-9 by neutrophils. Subsequently, the release of MMP-9 via NETosis appears to be the culprit element behind increased collagen degradation and the induction of the overall HSF changes. The net result is the establishment of a complex autocrine and paracrine positive feedback loop of neutrophil activation-IL-8/MMP-9 release-fibroblast impairment, which sustains detrimental effects on wound closure.

The increased propensity of diabetic neutrophils toward NETosis and the deleterious effects of T2D NETs on cutaneous healing have been demonstrated in rodent models [3–5]. Accordingly, elevated myeloperoxidase-DNA complex, a surrogate of NETosis, has been shown to predict amputations in patients with T2D and DFUs [16].

Our results appoint the neutrophil-fibroblast crosstalk as the functional frame into which the detrimental action of NETs takes effect, highlighting the presence of a NETosis/IL-8/MMP-9/fibroblast axis. Increased IL-8 likely holds a cardinal position in this chain of elements since its signaling suffices to induce MMP-9. IL-8 is a potent neutrophil chemotactic factor, and higher circulating and saliva levels have been demonstrated among individuals with T2D than controls [23–25]. We demonstrated that the result of increased IL-8 expression by neutrophils and its release with NETs is the induction of its further expression by neutrophils as well as HSFs (Figure 4). This suggests that following early chemotactic signaling and initial neutrophil migration at the damaged skin area, a vicious circle of IL-8 secretion-neutrophil chemotaxis is established, which may contribute to the prolonged persistence of neutrophils in the wound area. A similar self-sustaining positive feedback loop driven by IL-8 has also been described in cases of severe COVID-19 [26].

Diabetic neutrophils also exhibit increased MMP-9 concentration in their NETs, which is induced by autocrine IL-8 signaling. In turn, MMP-9 action results in the degradation of collagen produced by HSFs and promotes the switch of HSFs to a profibrotic, albeit functionally defective phenotype, unable to accomplish effective wound healing. The blockade of this phenomenon by MMP-9 inhibition (MMP-9-IN-1) or dismantling of the NET scaffold by DNase 1 (Figure 3C–E) indicates that not only intact MMP-9 activity but specifically its release via NETosis are of fundamental importance for the detrimental effects of neutrophil-HSFs interaction on wound healing. Elevated serum levels of MMP-9 have been demonstrated in patients with T2D compared with controls [27], as well as in patients with DM and nonhealing versus healing DFUs [28]. Likewise, increased MMP-9 in wound fluids has been shown to predict nonhealing after 12 weeks [11]. Accordingly, a number of interventions aimed at accelerating wound healing in T2D, but not specifically targeting MMP-9, have been shown to decrease its activity and expression in mouse models of diabetic wounds, and conversely, ineffective interventions are associated with an absence of such effects [29]. Excessive MMP-9 activity has also been implicated in poor healing in cutaneous wounds in conditions other than T2D, such as chronic pressure [30] and venous [31] ulcers. The novel findings of the current study substantially expand these congruent results by identifying MMP-9 and its induction by IL-8 as centerpieces of NET-mediated neutrophil-fibroblast crosstalk and subsequent wound healing impairment and offer further putative targets for therapeutic modulation. On the other hand, a modest and regulated MMP-9 action is required for extracellular matrix remodeling and wound re-epithelization [32]; these diametric effects of MMP-9 on wound healing could render strategies implementing blockade of its activity problematic. Nevertheless, local application of selective MMP-9 inhibitor (R)-ND-336 has been shown to accelerate wound healing in db/db diabetic mice [33].

An open question concerns the factors that induce the neutrophil plasticity unique to T2D, characterized by increased IL-8 and MMP-9. Our findings support that this may be influenced by the hallmark metabolic disturbances of T2D. The positive correlation of MMP-9 concentration in NETs with HbA1c and its lower values in those with optimal HbA1c levels (<7%) underpin the importance of fair glycemic control for promoting wound healing, which has been already demonstrated in observational studies [34, 35]. Furthermore, the temporary acquisition of similar plasticity in neutrophils under conditions of short-term hyperglycemia and increased FFA concentrations indicates that either these disturbances directly or the physiological responses that they elicit indirectly induce this phenotype. Importantly, treatment of HSFs with NETs obtained from healthy volunteers under these conditions induces identical phenomena to those isolated from individuals with T2D, including their functional impairment in-vitro. Clearly, a brief increase in glucose and FFA alone is insufficient to replicate the full pathophysiological context of T2D or its chronic nature [36–38]. In fact, the observed neutrophil adaptations, which resemble those seen in T2D even under these temporary conditions, further highlight the significance of these metabolic disturbances in the chronic state of diabetes.

Fibroblasts possess an impressive plasticity and modify their properties in response to the disease microenvironment [39, 40]. The plasticity of neutrophils acquired in the T2D systemic milieu induces a dysfunctional HSF state and sustains the deleterious neutrophil-HSF interactions through various mechanisms, such as NETosis. Thus, primary T2D HSFs appear intrinsically defective, presumably due to their exposure to the dysmetabolic environment of T2D and crosstalk with neutrophils.

4 | Data Limitations and Perspectives

We acknowledge the limitations of the absence of an in-vivo validation (e.g., rodent) model of wound healing as well as the lack of investigation of other MMPs, their inhibitors, and cytokines other than IL-8, which, however, do not limit the validity of the presented results.

The presented results support a pivotal role for neutrophil-HSF crosstalk mediated by NETosis in the pathogenesis of cutaneous healing impairment in T2D, and specifically for IL-8 and MMP-

9 as the culprit elements of this interaction. Considering the central role of IL-8 in inducing and sustaining the observed effects, in conjunction with the potential drawbacks that a complete inhibition of NETosis or MMP-9 could harbor, blockade of IL-8 pathway to promote wound healing either directly (e.g., receptor antagonists) [41] or indirectly (e.g. through complement inhibition) [42] arises as a particularly promising prospect. Furthermore, neutrophil-derived proteolytic enzymes, including neutrophil elastase (NE), may contribute to the pathogenesis of chronic dysmetabolic disorders such as insulin resistance [43]. The putative role of NE and other proteases on the wound healing of T2D remains to be addressed in future studies. Lastly, since our observations were made in an unselected population of individuals with T2D, the results render future research to explore the potential role of neutrophil-HSF crosstalk, IL-8, and MMP-9 in other chronic diabetic complications an attractive and much needed prospect.

5 | Material and Methods

5.1 | Participants

Patient recruitment and clinical examinations were carried out in the First Department of Internal Medicine and the Outpatient Clinic for Diabetes Mellitus and Metabolic Disorders, at the University Hospital of Alexandroupolis. Experimental procedures were conducted in the Laboratory of Molecular Hematology, integrated into the department. Biopsy samples from DFUs were obtained from the Diabetes Centre of the First Department of Propaedeutic Internal Medicine, Laiko General Hospital, Athens, Greece. The study was conducted according to the principles of the Helsinki Declaration.

A total of 36 individuals were examined (n = 24 T2D, n = 12 control group, healthy individuals-HI). The main clinical and laboratory features of the participants are presented in Table 1. Additional information can be found in the Supporting Information Material.

TABLE 1 | Main clinical and laboratory features of participants.

	T2D	Controls
n	24	12
Age (years)	68 (48–78)	64 (46–73)
Sex (female, %)	9 (37.5)	5 (41.6)
Diabetes duration (years)	12 (0, 36)	N/A
HbA1c (mmol/mol, [%])	63 (32, 148) [7.0 (5.1, 12.6)]	37 (31, 43) [5.5 (5.0, 6.1)]
Treatment (*n*, %)	6 (25.0)	0 (0.0)
No drug treatment	14 (56.0)	
Metformin	1 (4.2)	
Sulfonylurea	4 (16.7)	
DPP4i	8 (33.3)	
SGLT2i	2 (8.3)	
GLP1RA	7 (29.2)	
Basal Insulin	5 (20.8)	
Prandial Insulin		

Note Values are *n* (%) or medians (minimum, maximum).
Abbreviations: DPP4i, dipeptidyl peptidase-4 inhibitors; GLP1RA, glucagon-like peptide-1 receptor agonists; SGLT2i, sodium/glucose co-transporter-2 inhibitors; T2D, type 2 diabetes.

5.2 | Skin Biopsy from Chronic Wounds and Unaffected Skin Areas

To validate the in vitro/ex vivo findings histologically, we comparatively examined (1) skin biopsies from chronic, nonhealing (>12 weeks) neuropathic plantar ulcers from individuals with T2D (n = 4), (2) traumatic plantar wounds from matched controls without DM which healed within 1–3 weeks (n = 4), and (3) unaffected skin areas (volar aspect of underarm) from individuals with T2D as well as controls with normal glycemic indices (n = 4). Additional information can be found in the Supporting Information Material.

5.3 | Hyperglycemic Clamp with 20% Lipid Infusion

To investigate the relationship between observed neutrophil perturbations and the metabolic hallmarks of diabetes, namely hyperglycemia and increased FFA concentrations [44], we obtained neutrophils/NETs from individuals with normal weight and normoglycemia before and after transient hyperglycemia and FFA increase. Three volunteers with a body mass index <25 kg/m^2 and glycemic indices within the normal range underwent hyperglycemic clamps (HGC, target glucose 200 mg/dL) while receiving intravenous lipid 20% (Intralipid, Fresenius Kabi, Bad Homburg, Germany). HGCs were conducted according to the stage 1 protocol by the Restoring Insulin Secretion (RISE) Consortium [45].

HGC NETs were used for further stimulation studies and analyzed using IL-8/MMP-9 ELISA assays, as described below. Additional details are available in the Supporting Information Material.

5.4 | Serum Collection

Details are included in the Supporting Information Material.

5.5 | Neutrophil Isolation, Generation, and Quantification of NETs

Details are included in the Supporting Information Material.

5.6 | Isolation and Culture of Primary Human Skin Fibroblasts

In a subset of participants (T2D n = 4, controls n = 4), a 5 mm punch biopsy from the upper arm for isolation of primary HSFs was conducted, as previously described [46, 47]. Details are included in the Supporting Information Material.

5.7 | Stimulation and Inhibition Studies in Cultured Cells

Details are included in the Supporting Information Material.

5.8 | RNA Isolation, cDNA Synthesis, and Quantitative Real-Time Polymerase Chain Reaction

Details are included in the Supporting Information Material.

5.9 | In-Cell ELISA (ICE Assay, Cytoblot)

In-cell ELISA was performed in confluent monolayers of HSFs, as explained before [6, 48]. Details are included in the Supporting Information Material.

5.10 | Immunofluorescence Staining in Peripheral Blood Neutrophils and HSFs

Sample preparation and visualization of peripheral blood neutrophils and HSFs by immunofluorescence microscopy were conducted following a previously established method [49]. Details are included in the Supporting Information Material.

5.11 | Immunofluorescence Staining in Human Tissue Sections

Immunofluorescence staining was performed in formalin-fixed paraffin-embedded biopsies, as previously described [7]. Details are included in the Supporting Information Material.

5.12 | Matrix Metalloproteinase-9 ELISA

MMP-9 was measured in either NETs ex-vivo isolated from $\approx 1.5 \times 10^6$ neutrophils or serum samples using a commercial MMP-9 ELISA kit (DMP900; R&D Systems), in line with the manufacturer's protocol.

5.13 | IL-8 ELISA

IL-8 levels were measured in ex-vivo NETs from approximately 1.5×10^6 neutrophils, using the Human IL-8 ELISA (3560-1HP-1; MABTECH) according to the manufacturer's protocol.

5.14 | Collagen Measurement

The Sircol Collagen Assay Kit (054S1000; TEBU-BIO) was used to determine the soluble collagen types (I–V). The assay was conducted according to the manufacturer's protocol (CLRS1000; Biocolor), as previously described [6, 50]. Details are included in the Supporting Information Material.

5.15 | Collagen Degradation Assay

For the evaluation and detection of collagen breakdown by collagenases, a collagenase fluorometric assay kit (ab234624; Abcam) was used on cell lysates from HSFs after treatment with suitable agents for 24 h in low-serum (2% FBS) complete DMEM, following the manufacturer's protocol with slight modifications. Details are included in the Supporting Information Material.

5.16 | Scratch Wound Healing Assay

The wound healing-migration assay was used to evaluate the migratory ability of HSFs in-vitro, following the manufacturer's instructions and as previously conducted [6]. Details are included in the Supporting Information Material.

5.17 | Statistical Analysis

Statistical analyses were carried out in GraphPad Prism version 10 and in the Statistical Package for Social Sciences version 24.0. Values are presented as medians (25,75 interquartile range) for qualitative and n (%) for qualitative variables. Due to the nonnormality of the analyzed quantitative parameters based on the Kolmogorov–Smirnov test and the small sizes of the groups under comparison, nonparametric tests were used in the analysis. The Mann–Whitney U-test or paired Wilcoxon signed-rank and chi-squared tests were used for comparisons of quantitative and qualitative variables between groups, respectively. For comparisons involving more than two groups, the Kruskal–Wallis test, followed by Dunn's test for post hoc pairwise comparisons, was performed. Spearman's correlation coefficient was used to examine correlations between pairs of continuous variables.

Author Contributions

Conceptualization: Dimitrios Tsilingiris, Panagiotis Skendros, and Konstantinos Ritis. Methodology: Dimitrios Tsilingiris, Anastasia-Maria Natsi, and Efstratios Gavriilidis. Investigation: Dimitrios Tsilingiris, Anastasia-Maria Natsi, Efstratios Gavriilidis, Christina Antoniadou, Ioanna Eleftheriadou, Ioanna A. Anastasiou, Anastasios Tentolouris, Evangelos Papadimitriou, Panagiotis Kolovos, Victoria Tsironidou, and Alexandra Giatromanolaki. Visualization: Dimitrios Tsilingiris, Anastasia-Maria Natsi, Efstratios Gavriilidis, Evgenios Eftalitsidis, and Alexandra Giatromanolaki. Funding acquisition: Dimitrios Tsilingiris, Maria Koffa, and Panagiotis Skendros. Project administration: Panagiotis Skendros and Konstantinos Ritis. Supervision: Panagiotis Skendros and Konstantinos Ritis. Writing—original draft: Dimitrios Tsilingiris, Anastasia-Maria Natsi, Efstratios Gavriilidis, Panagiotis Skendros, and Konstantinos Ritis. Writing—review and editing: Dimitrios Tsilingiris, Anastasia-Maria Natsi, Efstratios Gavriilidis, Christina Antoniadou, Alexandra Giatromanolaki, Maria Koffa, Nikolaos Tentolouris, Panagiotis Skendros, and Konstantinos Ritis. All authors approved the final version of the manuscript. Dimitrios Tsilingiris had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Ethics Approval

The study protocol was approved by the ethics committees of the participating hospitals (approval numbers: 803/23-09-2019, 28462/04-06-2024, and 1370/03-11-2017).

Patient Consent Statement

All participants provided written informed consent prior to their inclusion.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that supports the findings of this study are available from the authors upon reasonable request.

Peer Review

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References

1. V. Falanga, "Wound Healing and Its Impairment in the Diabetic Foot," *Lancet* 366 (2005): 1736–1743, 10.1016/S0140-6736(05)67700-8.

2. D. Baltzis, I. Eleftheriadou, and A. Veves, "Pathogenesis and Treatment of Impaired Wound Healing in Diabetes Mellitus: New Insights," *Advances in Therapy* 31 (2014): 817–836, 10.1007/s12325-014-0140-x.

3. S. L. Wong, M. Demers, K. Martinod, et al., "Diabetes Primes Neutrophils to Undergo NETosis, Which Impairs Wound Healing," *Nature Medicine* 21 (2015): 815–819, 10.1038/nm.3887.

4. G. P. Fadini, L. Menegazzo, M. Rigato, et al., "NETosis Delays Diabetic Wound Healing in Mice and Humans," *Diabetes* 65 (2016): 1061–1071, 10.2337/db15-0863.

5. W. Huang, J. Jiao, J. Liu, et al., "MFG-E8 Accelerates Wound Healing in Diabetes by Regulating 'NLRP3 Inflammasome-Neutrophil Extracellular Traps' Axis," *Cell Death Discov* 6 (2020): 84, 10.1038/s41420-020-00318-7.

6. A. Chrysanthopoulou, C. Antoniadou, A.-M. Natsi, et al., "Down-Regulation of KLF2 in Lung Fibroblasts Is Linked with COVID-19 Immunofibrosis and Restored by Combined Inhibition of NETs, JAK-1/2 and IL-6 Signaling," *Clinical Immunology* 247 (2023): 109240, 10.1016/j.clim.2023.109240.

7. E. Gavriilidis, G. Divolis, A.-M. Natsi, et al., "Neutrophil-fibroblast Crosstalk Drives Immunofibrosis in Crohn's Disease through IFNα Pathway," *Frontiers in immunology* 15 (2024): 1447608, 10.3389/fimmu.2024.1447608.

8. M. Palomino-Segura, J. Sicilia, I. Ballesteros, and A. Hidalgo, "Strategies of Neutrophil Diversification," *Nature Immunology* 24 (2023): 575–584, 10.1038/s41590-023-01452-x.

9. S. M. Ayuk, H. Abrahamse, and N. N. Houreld, "The Role of Matrix Metalloproteinases in Diabetic Wound Healing in Relation to Photobiomodulation," *Journal of Diabetes Research* 2016 (2016): 2897656, 10.1155/2016/2897656.

10. K. Fu, X. Zheng, Y. Chen, et al., "Role of Matrix Metalloproteinases in Diabetic Foot Ulcers: Potential Therapeutic Targets," *Frontiers in pharmacology* 13 (2022): 1050630, 10.3389/fphar.2022.1050630.

11. Y. Liu, D. Min, T. Bolton, et al., "Increased Matrix Metalloproteinase-9 Predicts Poor Wound Healing in Diabetic Foot Ulcers," *Diabetes Care* 32 (2009): 117–119, 10.2337/dc08-0763.

12. C. Carmona-Rivera, W. Zhao, S. Yalavarthi, and M. J. Kaplan, "Neutrophil Extracellular Traps Induce Endothelial Dysfunction in Systemic Lupus Erythematosus through the Activation of Matrix Metalloproteinase-2," *Annals of the Rheumatic Diseases* 74 (2015): 1417–1424, 10.1136/annrheumdis-2013-204837.

13. A. Jo and D. W. Kim, "Neutrophil Extracellular Traps in Airway Diseases: Pathological Roles and Therapeutic Implications," *International Journal of Molecular Sciences* 24 (2023): 5034, 10.3390/ijms24055034.

14. W. Li, J. Fan, M. Chen, et al., "Mechanism of human Dermal Fibroblast Migration Driven by Type I Collagen and Platelet-derived Growth Factor-BB," *Molecular Biology of the Cell* 15 (2004): 294–309, 10.1091/mbc.e03-05-0352.

15. A. Arampatzioglou, D. Papazoglou, T. Konstantinidis, et al., "Clarithromycin Enhances the Antibacterial Activity and Wound Healing Capacity in Type 2 Diabetes Mellitus by Increasing LL-37 Load on Neutrophil Extracellular Traps," *Frontiers in immunology* 9 (2018): 2064, 10.3389/fimmu.2018.02064.

16. I. Ibrahim, Y. Nuermaimaiti, G. Maimaituxun, et al., "Neutrophil Extracellular Traps (NETs) Are Associated with Type 2 Diabetes and Diabetic Foot Ulcer Related Amputation: a Prospective Cohort Study," *Diabetes Ther* 15 (2024): 1333–1348, 10.1007/s13300-024-01579-6.

17. E. I. Deryugina, E. Zajac, A. Juncker-Jensen, T. A. Kupriyanova, L. Welter, and J. P. Quigley, "Tissue-Infiltrating Neutrophils Constitute the Major in Vivo Source of Angiogenesis-Inducing MMP-9 in the Tumor Microenvironment," *Neoplasia* 16 (2014): 771–788, 10.1016/j.neo.2014.08.013.

18. L. Huang, W. Tian, X. Chen, et al., "Peripheral Neutrophils-Derived Matrix Metallopeptidase-9 Induces Postoperative Cognitive Dysfunction in Aged Mice," *Frontiers in aging neuroscience* 14 (2022): 683295, 10.3389/fnagi.2022.683295.

19. H. F. Bigg, A. D. Rowan, M. D. Barker, and T. E. Cawston, "Activity of Matrix Metalloproteinase-9 against Native Collagen Types I and III," *Febs Journal* 274 (2007): 1246–1255, 10.1111/j.1742-4658.2007.05669.x.

20. V. Klaus, F. Tanios-Schmies, C. Reeps, et al., "Association of Matrix Metalloproteinase Levels with Collagen Degradation in the Context of Abdominal Aortic Aneurysm," *European Journal of Vascular and Endovascular Surgery* 53 (2017): 549–558, 10.1016/j.ejvs.2016.12.030.

21. C. Wetzler, H. Kämpfer, B. Stallmeyer, J. Pfeilschifter, and S. Frank, "Large and Sustained Induction of Chemokines during Impaired Wound Healing in the Genetically Diabetic Mouse: Prolonged Persistence of Neutrophils and Macrophages during the Late Phase of Repair," *Journal of Investigative Dermatology* 115 (2000): 245–253, 10.1046/j.1523-1747.2000.00029.x.

22. S. Chakrabarti and K. D. Patel, "Regulation of Matrix Metalloproteinase-9 Release from IL-8-Stimulated Human Neutrophils," *J Leukoc Biol* 78 (2005): 279–288, 10.1189/jlb.1004612.

23. F. A. Cimini, I. Barchetta, A. Porzia, et al., "Circulating IL-8 Levels Are Increased in Patients with Type 2 Diabetes and Associated with Worse Inflammatory and Cardiometabolic Profile," *Acta Diabetologica* 54 (2017): 961–967, 10.1007/s00592-017-1039-1.

24. P. Borilova Linhartova, D. Kavrikova, M. Tomandlova, et al., "Differences in Interleukin-8 Plasma Levels between Diabetic Patients and Healthy Individuals Independently on Their Periodontal Status," *International Journal of Molecular Sciences* 19 (2018): 3214, 10.3390/ijms19103214.

25. M. Shirzaiy, Z. Dalirsani, P. Peymankar, and M. Taherizadeh, "Relationship between Salivary Levels of Interleukin-8 and HbA1c in Patients with Type 2 Diabetes," *Endocrinol Diabetes Metab* 6 (2023): e455, 10.1002/edm2.455.

26. R. Kaiser, A. Leunig, K. Pekayvaz, et al., "Self-sustaining IL-8 Loops Drive a Prothrombotic Neutrophil Phenotype in Severe COVID-19," *JCI Insight* 6 (2021), https://insight.jci.org/articles/view/150862, 10.1172/jci.insight.150862. Available at. Accessed November 29, 2021.

27. G. Derosa, A. D'Angelo, C. Tinelli, et al., "Evaluation of Metalloproteinase 2 and 9 Levels and Their Inhibitors in Diabetic and Healthy Subjects," *Diabetes & Metabolism* 33 (2007): 129–134, 10.1016/j.diabet.2006.11.008.

28. T. Dinh, F. Tecilazich, A. Kafanas, et al., "Mechanisms Involved in the Development and Healing of Diabetic Foot Ulceration," *Diabetes* 61 (2012): 2937–2947, 10.2337/db12-0227.

29. J. I. Jones, T. T. Nguyen, Z. Peng, and M. Chang, "Targeting MMP-9 in Diabetic Foot Ulcers," *Pharmaceuticals (Basel)* 12 (2019): 79, 10.3390/ph12020079.

30. A. N. Moor, D. J. Vachon, and L. J. Gould, "Proteolytic Activity in Wound Fluids and Tissues Derived from Chronic Venous Leg Ulcers," *Wound Repair and Regeneration* 17 (2009): 832–839, 10.1111/j.1524-475X.2009.00547.x.

31. D. R. Yager, L. Y. Zhang, H. X. Liang, R. F. Diegelmann, and I. K. Cohen, "Wound Fluids from human Pressure Ulcers Contain Elevated Matrix Metalloproteinase Levels and Activity Compared to Surgical Wound Fluids," *Journal of Investigative Dermatology* 107 (1996): 743–748, 10.1111/1523-1747.ep12365637.

32. A. D. Widgerow, "Chronic Wound Fluid–Thinking outside the Box," *Wound Repair and Regeneration* 19 (2011): 287–291, 10.1111/j.1524-475X.2011.00683.x.

33. T. T. Nguyen, D. Ding, W. R. Wolter, et al., "Validation of Matrix Metalloproteinase-9 (MMP-9) as a Novel Target for Treatment of Diabetic Foot Ulcers in Humans and Discovery of a Potent and Selective Small-Molecule MMP-9 Inhibitor That Accelerates Healing," *Journal of Medicinal Chemistry* 61 (2018): 8825–8837, 10.1021/acs.jmedchem.8b01005.

34. J. Xiang, S. Wang, Y. He, L. Xu, S. Zhang, and Z. Tang, "Reasonable Glycemic Control Would Help Wound Healing during the Treatment of Diabetic Foot Ulcers," *Diabetes Ther* 10 (2019): 95–105, 10.1007/s13300-018-0536-8.

35. K. L. Lane, M. S. Abusamaan, B. F. Voss, et al., "Glycemic Control and Diabetic Foot Ulcer Outcomes: a Systematic Review and Meta-Analysis of Observational Studies," *Journal of Diabetes and Its Complications* 34 (2020): 107638, 10.1016/j.jdiacomp.2020.107638.

36. A. Merovci, D. Tripathy, X. Chen, et al., "Effect of Mild Physiologic Hyperglycemia on Insulin Secretion, Insulin Clearance, and Insulin Sensitivity in Healthy Glucose-Tolerant Subjects," *Diabetes* 70 (2021): 204–213, 10.2337/db20-0039.

37. O. Rolandsson, A. Tornevi, P. Steneberg, et al., "Acute Hyperglycemia Induced by Hyperglycemic Clamp Affects Plasma Amyloid-β in Type 2 Diabetes," *Journal of Alzheimer's Disease* 99 (2024): 1033–1046, 10.3233/JAD-230628.

38. J. Szendroedi, T. Yoshimura, E. Phielix, et al., "Role of Diacylglycerol Activation of PKCθ in Lipid-Induced Muscle Insulin Resistance in Humans," *PNAS* 111 (2014): 9597–9602, 10.1073/pnas.1409229111.

39. S. Sinha, H. D. Sparks, E. Labit, et al., "Fibroblast Inflammatory Priming Determines Regenerative versus Fibrotic Skin Repair in Reindeer," *Cell* 185 (2022): 4717–4736.e25, 10.1016/j.cell.2022.11.004.

40. A. Salminen, "The Plasticity of Fibroblasts: a Forgotten Player in the Aging Process," *Ageing Research Reviews* 89 (2023): 101995, 10.1016/j.arr.2023.101995.

41. A. Nishimoto-Kakiuchi, I. Sato, K. Nakano, et al., "A Long-Acting Anti–IL-8 Antibody Improves Inflammation and Fibrosis in Endometriosis," *Science Translational Medicine* 15 (2023): eabq5858, 10.1126/scitranslmed.abq5858.

42. A. Vecchiarelli, C. Retini, A. Casadevall, C. Monari, D. Pietrella, and T. R. Kozel, "Involvement of C3a and C5a in Interleukin-8 Secretion by human Polymorphonuclear Cells in Response to Capsular Material of Cryptococcus Neoformans," *Infection and Immunity* 66 (1998): 4324–4330, 10.1128/IAI.66.9.4324-4330.1998.

43. S. Talukdar, D. Y. Oh, G. Bandyopadhyay, et al., "Neutrophils Mediate Insulin Resistance in Mice Fed a High-fat Diet through Secreted Elastase," *Nature Medicine* 18 (2012): 1407–1412, 10.1038/nm.2885.

44. S. Spiller, M. Blüher, and R. Hoffmann, "Plasma Levels of Free Fatty Acids Correlate with Type 2 Diabetes Mellitus," *Diabetes, Obesity & Metabolism* 20 (2018): 2661–2669, 10.1111/dom.13449.

45. K. J. Mather, A. H. Tjaden, A. Hoehn, et al., "Precision and Accuracy of Hyperglycemic Clamps in a Multicenter Study," *American Journal of Physiology. Endocrinology and Metabolism* 320 (2021): E797–E807, 10.1152/ajpendo.00598.2020.

46. E. Frangou, A. Chrysanthopoulou, A. Mitsios, et al., "REDD1/Autophagy Pathway Promotes Thromboinflammation and Fibrosis in Human Systemic Lupus Erythematosus (SLE) through NETs Decorated with Tissue Factor (TF) and Interleukin-17A (IL-17A)," *Annals of the Rheumatic Diseases* 78 (2019): 238–248, 10.1136/annrheumdis-2018-213181.

47. L. I. Huschtscha, C. E. Napier, J. R. Noble, et al., "Enhanced Isolation of Fibroblasts from Human Skin Explants," *Biotechniques* 53 (2012): 239–244, 10.2144/0000113939.

48. A. Chrysanthopoulou, E. Gkaliagkousi, A. Lazaridis, et al., "Angiotensin II Triggers Release of Neutrophil Extracellular Traps, Linking Thromboinflammation with Essential Hypertension," *JCI Insight* 6 (2021): e148668, 10.1172/jci.insight.148668.

49. I. Angelidou, A. Chrysanthopoulou, A. Mitsios, et al., "REDD1/Autophagy Pathway Is Associated with Neutrophil-Driven IL-1β Inflammatory Response in Active Ulcerative Colitis," *The Journal of Immunology* 200 (2018): 3950–3961, 10.4049/jimmunol.1701643.

50. A. Chrysanthopoulou, I. Mitroulis, K. Kambas, et al., "Tissue Factor-Thrombin Signaling Enhances the Fibrotic Activity of Myofibroblasts in Systemic Sclerosis through Up-Regulation of Endothelin Receptor A," *Arthritis and Rheumatism* 63 (2011): 3586–3597, 10.1002/art.30586.

Supporting Information

Additional supporting information can be found online in the Supporting Information section.