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Diabetic Foot Complications

Current Challenges and Future Prospects
Part II

Edited by
José Luis Lázaro-Martínez and Luigi Uccioli

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Contents

About the Editors

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Prof. Luigi Uccioli has extensive research experience in late diabetic complications such as neuropathy and diabetic foot. Several of his scientific contributions have addressed the influence of autonomic neuropathy on the skin microcirculatory network of diabetic patients and its role in the pathogenesis of foot ulcerations. He has been chairman of the Study Group on Diabetic Foot for the Italian Society of Diabetes and Treasurer of the Diabetic Foot Study Group for the European Association for the Studies on Diabetes (EASD), as well as chairman of the regional section of the Italian Society of Diabetes (SID). He has been a member of the working group that developed the International Guidelines on Diabetic Foot (IWGDF) since 1999. He has been a participant of the Eurodiale network that includes leading centers for the cure of diabetic foot in Europe and has produced many reference papers in this area. On 16 September 2022, he was awarded "The DFSG LIFE TIME ACHIEVEMENT AWARD 2022" by the Diabetic Foot Study Group of European Association Studies on Diabetes (EASD). He has written more than 150 articles in peer-reviewed scientific journals and has been invited as speaker and chairman to many national and international symposia.

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Article

Evaluation of Adjuvant Antibiotic Loaded Injectable Bio-Composite Material in Diabetic Foot Osteomyelitis and Charcot Foot Reconstruction

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Abstract: The management of diabetic foot osteomyelitis (DFO) is extremely challenging with high amputation rates reported alongside a five-year mortality risk of more than fifty percent. We describe our experience in using adjuvant antibiotic-loaded bio-composite material (Cerament) in the surgical management of DFO and infected Charcot foot reconstruction. We undertook a retrospective evaluation of 53 consecutive patients (54 feet) who underwent Gentamicin or Vancomycin-loaded Cerament application during surgery. The feet were categorised into two groups: Group 1, with infected ulcer and DFO, managed with radical debridement only (n = 17), and Group 2, requiring reconstruction surgery for infected and deformed Charcot foot. Group 2 was further subdivided into 2a, with feet previously cleared of infection and undergoing a single-stage reconstruction (n = 19), and 2b, with feet having an active infection managed with a two-stage reconstruction (n = 18). The mean age was 56 years (27–83) and 59% (31/53) were males. The mean BMI was 30.2 kg/m^2 (20.8–45.5). Foot ulcers were present in 69% (37/54) feet. At a mean follow-up of 30 months (12–98), there were two patients lost to follow up and the mortality rate was 11% (n = 5). The mean duration of post-operative systemic antibiotic administration was 20 days (4–42). Thirteen out of fifteen feet (87%) in group 1 achieved complete eradication of infection. There was a 100% primary ulcer resolution, 100% limb salvage and 76% bony union rate within Group 2. However, five patients, all in group 2, required reoperations due to problems with bone union. The use of antibiotic-loaded Cerament resulted in a high proportion of patients achieving infection clearance, functional limb salvage and decrease in the duration of postoperative antibiotic therapy. Larger, preferably randomised, studies are required to further validate these observations.

Keywords: diabetic foot ulcers; diabetic foot infection; Charcot foot reconstruction; diabetic foot osteomyelitis; biocomposite; calcium sulphate

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1. Introduction

Diabetic foot ulcers (DFU) are difficult to manage and can lead to major lower limb amputation (MLEA) with a mortality rate of over 50% at 5 years [1]. As a result, the surgical management of the diabetic foot has seen a resurgence of interest, not only for infection control surgery, but also for addressing structural foot deformities, with the goal of achieving a functional limb and reducing the risk of recurrent DFU [2,3]. However, such surgical intervention presents several significant challenges, including a compromised soft tissue envelope, delayed healing due to loss of protective sensation, impaired tissue oxygenation, and the likelihood of infection recurrence. The presence of diabetic foot osteomyelitis (DFO), whether accompanied by Charcot foot deformity or not, can pose a significant challenge to both clinicians and patients. This complexity often leads to suboptimal clearance of infection, which can result in the development of multi-resistant organisms that

require prolonged and repeated antibiotic therapies [4]. As a result, effectively treating DFO requires a comprehensive and targeted approach.

The surgical debridement of an infected diabetic foot involves the removal of all visibly infected and necrotic tissues, including bone, resulting in a significant reduction in the local infection burden. If followed by targeted antibiotic therapy, complete eradication of any residual infection can be achieved. However, it is crucial to correct any coexistent significant deformity following infection eradication to prevent ulcer recurrence and maintain ambulatory status. Unfortunately, large bone resections during debridement of osteomyelitic bones and deformity corrective osteotomies may leave bone voids that can become a nidus for infection secondary to contiguous bacterial seeding from adjacent uncleared infected areas. This is particularly concerning as the penetration of systemically administered antibiotics into osseous voids has been shown to be poor and associated with suboptimal local drug concentrations. Equally, Charcot foot deformity correction has been demonstrated to significantly improve functional and quality of life outcomes [3,5]. However, addressing the bone voids that are often encountered during one-stage or two-stage approaches can have a notable impact on the success of the surgical outcome, including bone fusion and the durability of the correction achieved. Thus, effective management of these bone defects is critical for achieving optimal results in the treatment of DFO Charcot foot deformity correction.

The use of adjuvant antibiotic-loaded biodegradable vehicles to fill bone voids has the potential to address concerns regarding local antibiotic elution and bone formation stimulation [6]. Early experiences with a new antibiotic-loaded injectable biocomposite material (Cerament®, Bonesupport, Lund, Sweden) consisting of 60% Calcium Sulphate and 40% Calcium Hydroxyapatite have been encouraging in the treatment of chronic bone and joint infections [7]. However, the potential scope of this material is much wider [6], and its deployment in orthopaedic infection clearance is being increasingly explored [8,9], but its adoption in the surgical reconstruction of a previously osteomyelitic Charcot foot has not been reported, making it of great interest to those actively managing such patients. Therefore, we evaluated the effectiveness of antibiotic-loaded Cerament in eradicating infections and promoting bone healing during the surgical management of infected diabetic foot and in Charcot foot reconstructions.

2. Materials and Methods

2.1. Design

This was a retrospective service evaluation which collected information from case notes on patient demographics and co-morbidities, infection status, clinical features, investigations, surgical treatment, antibiotic treatment and the outcomes.

2.2. Inclusion Criteria

Consecutive patients under the care of the diabetes foot unit at King's College Hospital that had Cerament application during surgery, between September 2015 and June 2019, were included in this study. Those with active DFU had University of Texas Wound Classification Grade 3 ulceration with evidence of DFO. As a minimum, the presence of DFO was suspected both through clinical and radiological examination and confirmed through positive microbiological growth.

2.3. Patient Groups

Foot presentations were divided into two main groups dependent upon the procedures undertaken: Group 1—feet with infected ulcer and DFO, managed with radical debridement; and Group 2—those that had reconstruction surgery for deformed and infected Charcot foot. The latter was subdivided into Group 2a—feet that had previous surgical clearance of DFO, undergoing a single stage reconstruction, and Group 2b—presented with actively infected deformed Charcot foot, managed with a staged reconstruction procedure.

All individuals had application of antibiotic impregnated Cerament with either gentamicin (Cerament G) or vancomycin (Cerament V) during the surgical procedure.

Patients were managed by the multi-disciplinary diabetes foot team (MDFT), which included a diabetologist, orthopaedic surgeon, vascular surgeon, plastic surgeon, microbiologist and an orthotist as its core members.

2.4. Surgical Management

All surgical procedures were carried out by a senior orthopaedic surgeon from the MDFT, and in cases where feasible, pre-operative administration of antibiotics was delayed until multiple deep tissue samples were collected intraoperatively from all patients [10]. Aggressive debridement was performed in both groups, and all infected tissues were excised until the healthy bleeding margins of the green zone were reached [11].

All patients within group 1 were managed as a single stage procedure that included ulcer debridement and any additional procedures such as exostectomy.

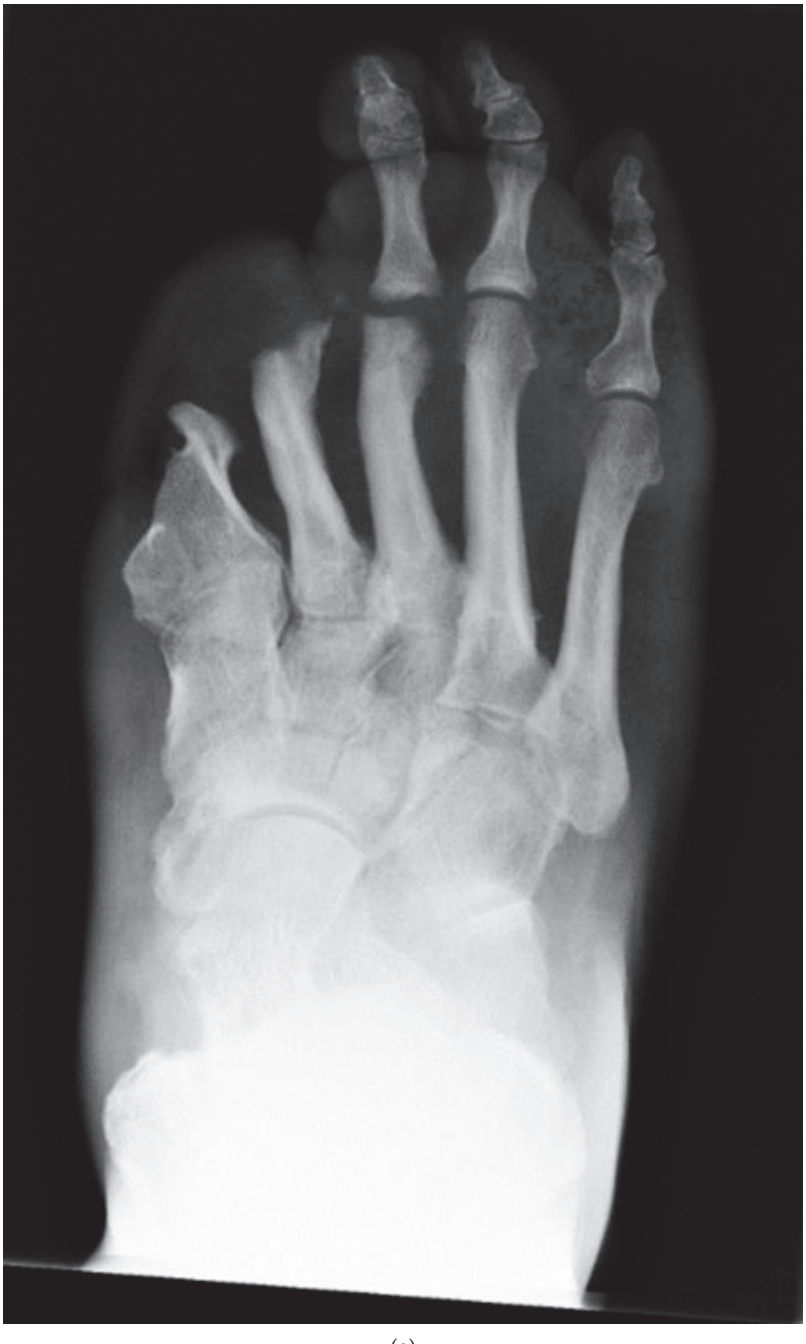
Single stage reconstruction (group 2a) was chosen among the group of patients that had a previous history of infected ulcers and DFO and shown evidence of infection clearance following previous ulcer debridement, exostectomy, and administration culture specific targeted antibiotics [12,13]. During the reconstruction procedure, the Charcot deformity correction was achieved through bone osteotomies and wedge resections and the correction was maintained with internal fixation devices. All measures were taken to achieve optimal bone opposition during fixation through compression of the bone fragments [14,15]. However, it is recognised that there are often small bone voids and gaps between the bone fragments even after compressive fixation.

Group 2b staged procedures were reserved for patients with active deep tissue infections that required a formal surgical debridement as the first stage, prior to the definitive reconstruction procedure. Our unit has described the surgical technique and protocol in a case series [16]. In the first stage, after excision of osteomyelitic bone, osteotomies are performed to improve deformity, and temporary stabilisation of the osteotomies is achieved using threaded 2.5 mm to 3.5 mm guidewires or an external fixator. To provide a high concentration of antibiotic in the surrounding tissues, a local antibiotic-eluting calcium sulphate preparation (Stimulan, Biocomposites, Keele, UK) is used to fill the bone voids. We prefer this product over Cerament at the first stage as it is less expensive and the goal is to achieve antibiotic elution only, without the need for bone healing promotion. Wounds are left open as needed and managed with negative pressure wound therapy (NWPT). Targeted intravenous antibiotics are continued along with advanced wound care and offloading of the foot in a total contact cast (TCC).

When there was clinical and serological evidence of infection eradication, usually at 6–8-week mark, the second stage of reconstruction was performed. During the second stage of reconstruction, further bone resections were performed to accomplish optimal deformity correction, and skeletal stabilisation was achieved using appropriate internal fixation techniques [16].

2.5. Cerament Instillation

Prior to the wound closure, in group 1, 2a and 2b during the second stage, Cerament V or G was applied to the bone debridement and osteotomy areas. This was performed by injecting directly into any osseous voids created or within the medullary cavities of the metatarsals following drilling and curettage, or into multiple drill holes created within the bones in the infected areas, or a combination of these [8,17] (Figure 1a,b). Care was taken to create a dry bone bed while injecting Cerament to promote interdigitation once it was set. The choice of antibiotic was based on the sensitivity results of deep tissue specimen cultures (group 1 and group 2a) or previous intraoperative bone sampling cultures in group 2b (precedence given when available). If no clear pathogen was identified or previous microbiological results were inconsistent, a discussion with microbiology was undertaken to decide on the best antibiotic option.



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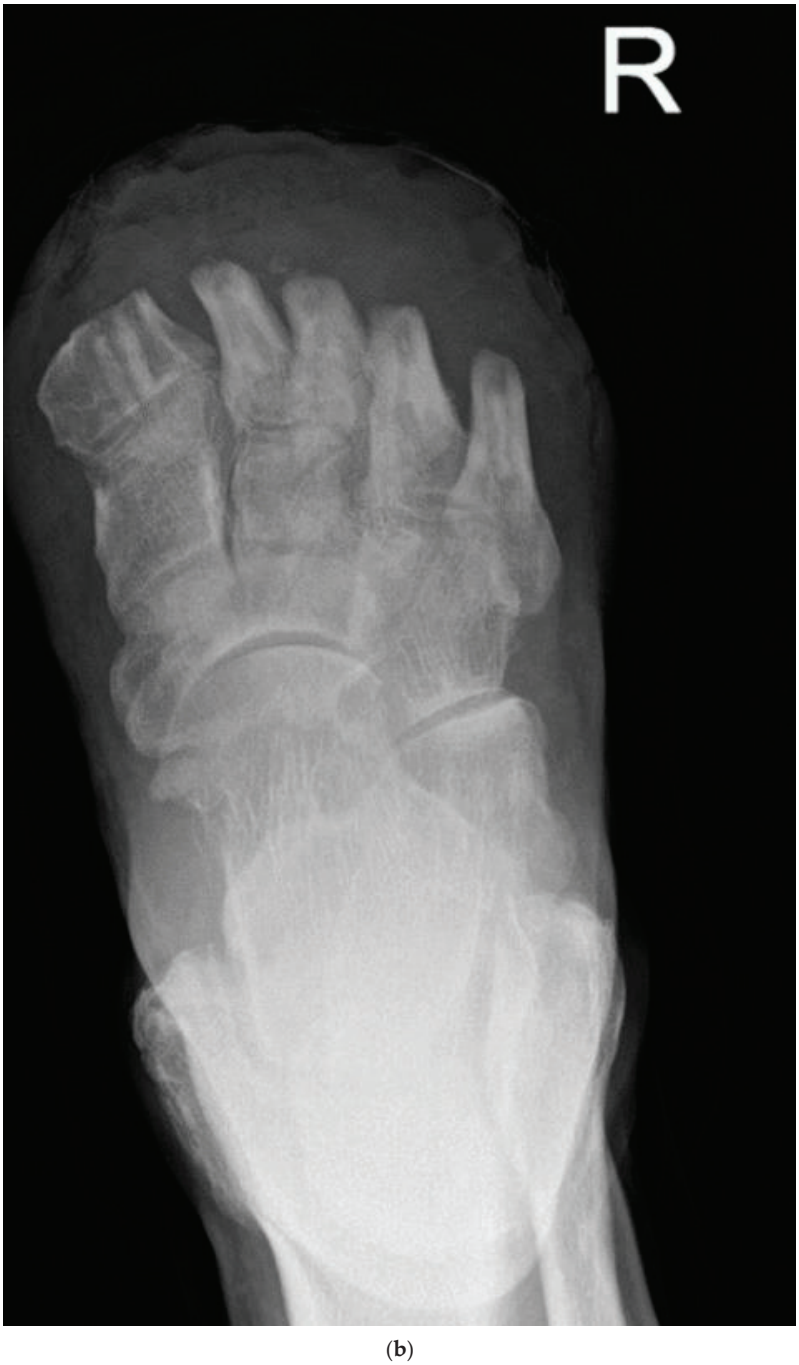
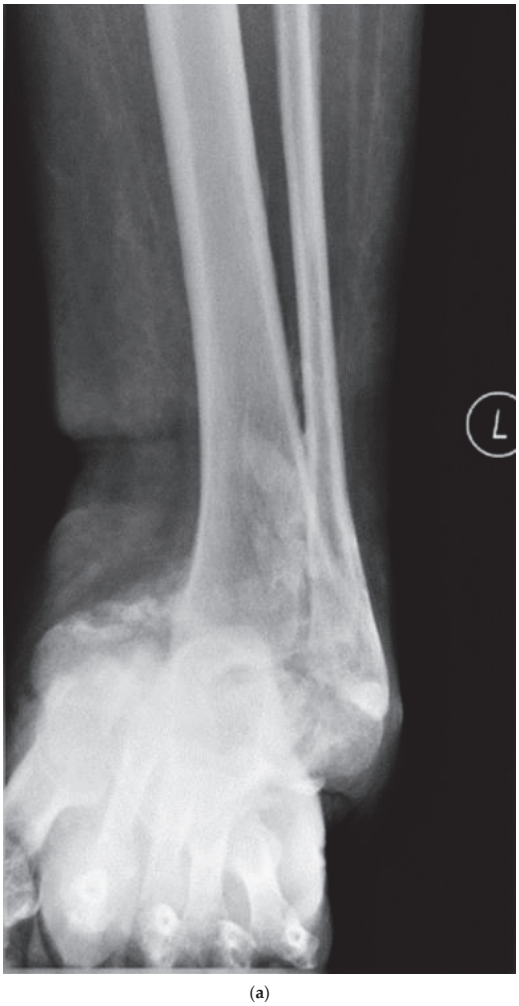


(**b**)

Figure 1. (**a**,**b**): Dorsoplantar radiographs of the foot taken before surgery (**a**) demonstrating osteomyelitic changes in the forefoot, and following trans-metatarsal amputation demonstrating Cerament application in the metatarsal stumps.

The surgical wounds were primarily closed, and the ulcers were left open when closure was not possible. The open ulcers were managed with NWPT in a bivalved TCC initially, followed by a closed TCC when the NWPT was no longer required. The intravenous antibiotics were continued until there was clinical and serological evidence of infection clearance. Patients were initially followed up, following discharge, at two weekly intervals for regular change in a TCC, and at 6 weeks, 3 months, 6 months, 12 months and annually thereafter, with radiographs for the assessment of bony union. The bone healing was considered satisfactory when consolidation of three or more cortices or bone bridging across the fusion site of more than 50% was noted on plain radiographs taken in two orthogonal views. Computerised tomography scans were obtained if their roentgenograms showed signs of delayed or non-union or motion noted clinically at the fusion site (Figure 2a–e). Patients maintained non-weight bearing in a TCC until there was evidence of bone healing and then transitioned into bespoke surgical footwear. None of the patients in Group 2b were advised to wear commercial footwear with or without bespoke insoles.



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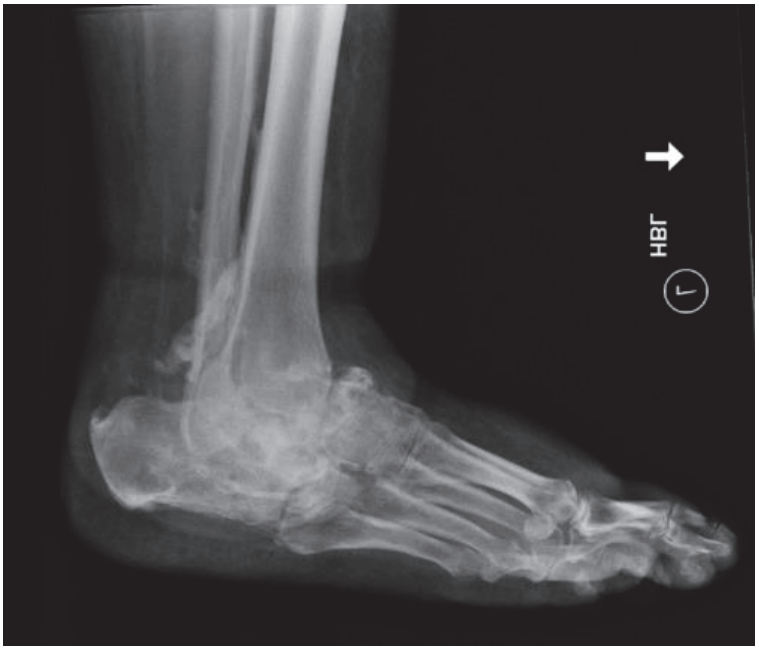

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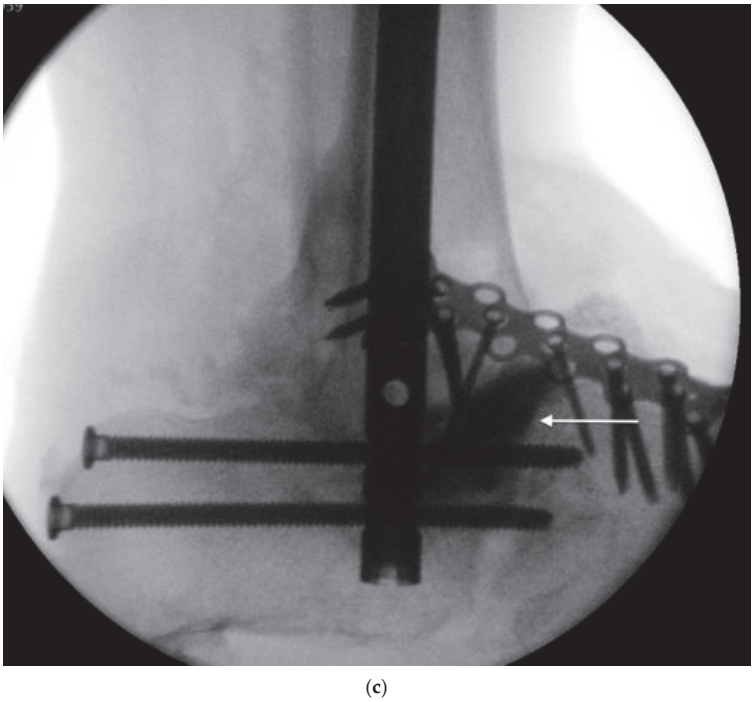

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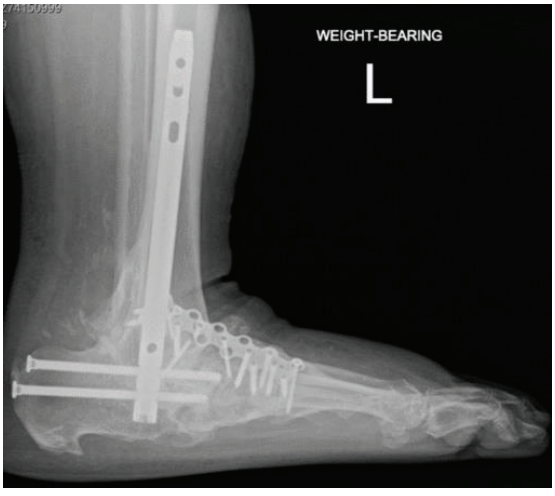

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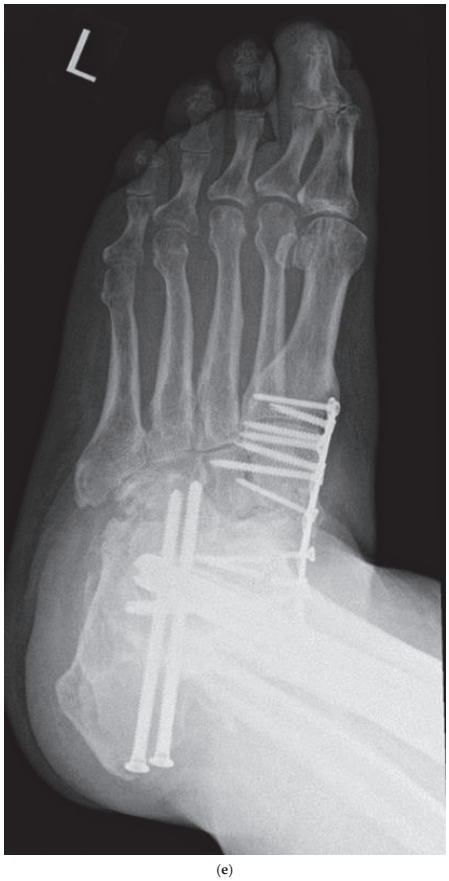

(**e**)

Figure 2. (**a**–**e**): Pre-operative weight bearing AP (**a**) and lateral (**b**) radiographs showing Charcot hindfoot and midfoot. Intra-operative fluoroscopy image (**c**) showing application of Cerament (arrow)

following fixation. Foot and ankle lateral radiograph (**d**) and foot oblique radiograph taken at 6 months following reconstruction demonstrating bony union.

2.6. Outcomes

Primary outcomes evaluated were the proportion of participants who achieved infection eradication and primary bone union. Secondary outcomes included ulcer resolution and recurrence, ambulatory status, limb salvage, mortality rate, and the need for orthopaedic re-intervention in the same area.

2.7. Governance and Approval

In discussion with the hospital trust Research & Innovation Department, and using the NHS Health Research Authority's online tool, the project was deemed to be a service evaluation. Ethical approval was not required as patients' management was not affected in any way and treatment had already been provided. All patients attending the King's Diabetic Foot Service are consented to participate in research and audit projects, which include service evaluation.

2.8. Statistical Analysis

Statistical analysis was performed using IBM SPSS statistics version 24. Categorical variables were analysed using Fisher's exact test and continuous variables were analysed using an independent samples *t*-test. Differences between the three groups for the variables described in the tables were determined using a one-way ANOVA. Statistical significance was set at $p < 0.05$.

3. Results

We identified 53 consecutive patients who underwent a surgical procedure using Cerament in a total of 54 feet. One patient underwent staged bilateral procedures. The mean age was 56 years (27–83) and 59% (31/53) were males. The mean BMI was 30.2 kg/m^2 (20.8–45.5) including five patients with a BMI $\geq$ 40. All patients presented with peripheral neuropathy; in 72% (38/53) due to type 2 diabetes, 25% (13/53) due to type 1 diabetes and 2 patients (4%) due to Charcot Marie Tooth disease. In terms of diabetic complications, 53% (27/51) had retinopathy and 41% (21/51) chronic kidney disease stage 3 or higher including 4 on renal dialysis. Additionally, 14% (7/51) had a previous revascularisation procedure for peripheral arterial disease. Chronic DFU were present in 70% (37/53) patients. Prior to a review in our clinic, 47% (25/53) were recommended a major limb amputation at their local units. Patients were followed up in the multidisciplinary foot service for a mean duration of 30 months (range 12–98), with two patients lost to follow up, one from each group.

3.1. Procedures

Group 1 consisted of 17 feet (31% of total cohort). These included six patients for minor amputations (four transmetatarsal and two fifth ray amputations), six forefoot ulcer and bone debridement, four os calcis ulcer and bone debridement, and one hindfoot ulcer debridement, along with Achilles' tendon lengthening and partial talectomy. Group 2 consistent of 37 feet of which 19 had a single stage reconstruction (group 2a, 35% of total cohort) whilst 18 required a staged reconstruction (group 2b, 33% of total cohort). There were no major differences in patient demographics between the three groups, as shown in Table 1.

Table 1. Pre-operative Patient Details. BMI = body mass index, ASA = American Society of Anaesthesiology score, eGFR = estimated glomerular filtration rate, NS = non-significant.

	Group 1	Group 2a	Group 2b	One-Way ANOVA
Number of patients (*n*=)	17	19	17	NS
Number of feet operated upon (*n*=)	17	19	18	NS
Number of patients lost to follow up	0	1	0	NS
Number of Males (*n*=)	12	11	8	NS
Mean age (years)	55.7	55.7	55.8	NS
Mean BMI (kg/m^2)	28.9	33.3	31.7	NS
Number of pre–operative ulcers (*n*=)	16	8	13	NS
Mean ASA	2.4	2.5	2.6	NS
Retinopathy	9	9	9	NS
Nephropathy (eGFR <30 mL/min)	4	7	6	NS
Renal Dialysis	0	2	2	NS
Preceding revascularisation (*n*=)	0	3	4	NS
Pre-operative Mobility:				
Independent	8	3	1	$p < 0.05$
Stick	3	6	4	NS
Wheelchair	6	10	12	NS

NS = non significant.

3.2. Microbiology

The intra-operative bone and deep tissue specimens were analysed (Figure 3). *Staphylococci* sp. were the most common organism (30%). Gram-negative organisms were identified in 34% and a polymicrobial infection was seen in 25% of isolates with a combination of gram positive, negative and anaerobes. There was no growth in 15 samples (20%). Post-operative systemic antibiotics were administered for a mean 20 days (range 4–42).

3.3. Cerament Use

Cerament V was used in 39% (21/54) feet while 65% (35/54) feet had Cerament G instilled during surgery to cover the most significant isolates; the difference was statistically significant, $p = 0.037$. In two patients, both Cerament V and G were used.

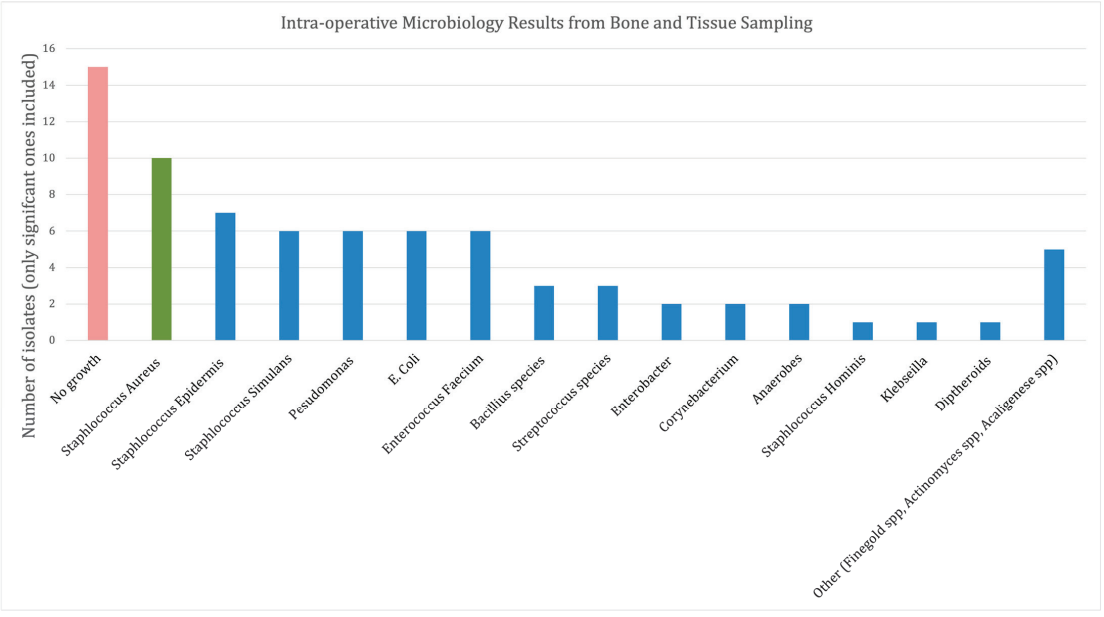

Figure 3. Intra-operative microbiology results from bone and tissue sampling.

4. Primary Outcome Measures

Two patients from group 1 were not included in the analysis as they died within 12 months from their surgery. An overview of post-operative results is provided in Table 2.

Table 2. Post-operative Results. NA = not applicable, NS = non-significant.

	Group 1A	Group 2a	Group 2b	One-Way ANOVA
Primary Bone Union	NA	13	15	NS
Non-Unions	NA	6	3	NS
Post-operative Deep Infection	None	None	1	NS
Non healing ulcer	2	None	None	NS
New Ulcer Formation (not at index site)	None	2	3	NS
Mortality within each group	2	1	3	NS
Mean post-operative Haemoglobin	109	98	103	NS
Post-operative ambulatory status:				NS
Independent in orthotic shoes	10	13	13	NS
Independent in a bivalve cast	1	3	3	NS
Partial weight bearing	3	1	1	NS
Non weight bearing/wheelchair	1	1	1	NS

4.1. Infection Resolution and Ulcer Healing

In group 1, 13/15 (87%) feet achieved complete eradication of infection and ulcer resolution following the first surgical procedure. Two feet within this group had persisting ulcers and both patients elected to continue managing their ulcers non-operatively.

Within Group 2, all the primary ulcers resolved. There were no cases of ulcer persistence or reoccurrence at the index region. Five patients (two in group 2a and three in group 2b) within the reconstruction group developed de novo ulcers in other areas of the foot.

Three of these patients had wounds attributed to metalwork prominences and underwent partial metalwork removal that resulted in wound healing. The remaining two patients developed forefoot ulcers that were treated with minor amputations.

4.2. Primary Bone Union

This was relevant for Group 2 only, and the overall bone fusion rate was 76%. In group 2a the primary bone union rate was 68% (n = 13/19), whereas it was 83% (n = 15/18) within group 2b. Details of patients not achieving primary union are shown in Table 3.

Table 3. Details of Non-Unions.

Case	Single/Two Stage	Reconstruction Location	Smoking Status	Clinical Stability	Further Procedures
1	Single	Midfoot	Ex-Smoker	Stable	Removal of metalwork—bolt to prevent further ulceration
2	Single	Mid and hindfoot	No	Unstable	Exostectomy
3	Single	Midfoot	Ex-smoker	Stable	None
4	Single	Mid and hindfoot	No	Stable	None
5	Single	Hindfoot	Yes	Unstable	Removal of broken nail and revision hindfoot fusion
6	Single	Midfoot	No	Stable	None
7	Two stage	Mid and hindfoot	No	Unstable	Revision of hindfoot nail and ulcer debridement
8	Two stage	Mid and hindfoot	Yes	Stable	None
9	Two stage	Midfoot	Yes	Infected non union	Removal of metalwork and repeat stage 1 procedure due to deep infection

4.3. Post-Operative Infection Recurrence

One patient from group 2b developed a deep infection nine months following a staged reconstruction. This required removal of all internal metalwork and further radical debridement followed by reconstruction.

5. Secondary Outcome Measures

5.1. Metalwork Infection

There was one case of infected metalwork and bone non-union in group 2b, requiring metal work removal and further debridement.

5.2. Ulcer Recurrence

There were no cases of ulcer reoccurrence at the index site in any patient groups at the end of the follow-up.

5.3. Post-Operative Ambulation

Thirty-six patients (36/51, 71%) achieved full weight bearing with orthotic shoes. A total of twelve patients (24%) required custom made ankle foot orthosis, seven of which were full weight bearing and five patients were partial weight bearing. Three (6%) patients remained wheelchair bound.

5.4. Mortality

There was a total of five deaths during the follow-up period (mortality rate 11%), two of which were in group 1, and occurred within 12 months of their surgery. None of the deaths were related to the surgery or occurred within the first 3 months post-operatively.

5.5. Limb Salvage

Within this case series, we report a 100% limb salvage rate at the end of the follow-up period (mean duration of 30 months). No major amputations were undertaken in any of those who died.

5.6. Further Procedures

Of the five cases with de novo ulcers that were treated with metalwork removal (n = 3) and minor amputations (n = 2), there were a further five operations performed due to bone union issues (Table 3). One patient had a delayed union at the ankle following a hindfoot nail fixation (group 2a) and was successfully treated through dynamisation of the nail. Three patients had aseptic non-unions (two hindfoot and one midfoot), of which two had broken hindfoot nails, requiring removal and revision hindfoot fusions, and one midfoot non-union required an exostectomy. One patient had an infected non-union (group 2b) that was treated with removal of metal work and further debridement.

6. Discussion

Prevention of infection recurrence following the surgical management of DFO remains a challenge. Complex diabetic foot infection clearance procedures often require aggressive bone resections and osteotomies, and the management of resultant dead space is considered critical. Local antibiotics delivery into these bone voids is a developing area in the management of diabetic foot infections, offering evident benefits. While non-biodegradable products such as polymethylmethacrylate (PMMA) has been utilised in the past, more recently, the easy availability of calcium sulphate derived products (natural or composite), which are biodegradable, serve as the quintessential platform, providing greater apparent safety and excellent drug release kinetics when impregnated with a range of antibiotics [6,18]. Therefore, filling of the dead space with local antibiotic eluting synthetic biodegradable bone substitutes, may help achieve infection eradication. It has been identified that this method delivers high concentrations of antibiotics locally delivered, often in the order of 10–100 times the minimum inhibitory concentration (MIC) [19,20] and potentially above the mean antimicrobial eradication concentration, without systemic toxicity. Some diabetic foot infection clearance procedures, such as Charcot deformity corrections, benefit from additional bone healing stimulation that can promote healing of osteotomies. Bone grafts that are typically used for such a purpose carry a high risk of contracting infection from bacterial seeding from adjacent previously infected areas and are generally not recommended in the presence of previous infections [6,18]. Injectable calcium hydroxy apatite material can lead to osseoconduction and promote bone healing. Cerament is an injectable and completely resorbable Calcium sulphate and hydroxyapatite biocomposite that can have added Gentamycin or Vancomycin, thus providing both local antibiotic elution and bone stimulation. Cerament impregnated with antibiotics has been shown to be effective in chronic osteomyelitis [7], but its potential in the surgical treatment of complex diabetic foot osteomyelitis has only recently been investigated [8,9,21,22]. However, the use of Cerament in Charcot reconstruction for its additional positive effect on bone healing has yet to be explored.

An important highlight Is that our report represents the first series describing the use of Cerament in diabetic foot Charcot reconstruction surgery. The rate of bone fusion in our current study was 76%, limb salvage was 100% and independent ambulation was 90%. Comparison with previous published literature is difficult, as a large proportion of our cohort (43%) had simultaneous Charcot midfoot and hindfoot reconstruction, a significantly greater undertaking than the previous series which reported a fusion rate of 90% with 100% limb salvage involved *hindfoot only* [12]. However, simultaneous mid and hindfoot arthrodesis has been shown to have a 12 times higher rate of non-union and metal work breakage, compared to isolated hindfoot or midfoot [23]. Five out of eight patients (63%) in the non-union group developed a stable pseudoarthrosis with a deformity free plantigrade foot and no further treatment was required. Cerament also contain hydroxyapatite which is

recognised for its ability to support bone formation and act as an osteoconductive scaffold. According to Nilsson et al., [24] in vitro biomechanical tests indicate that Cerament G has compression strength comparable to cancellous bone and promotes bone growth. However, despite the use of Cerament, our study's group experienced a 24% non-union rate, raising the possibility that the healing response to use of biocomposite bone substitute may be suboptimal in infected Charcot foot bones compared to those that are healthy and normal. We believe our results provide a foundation for further research exploring the efficacy of Cerament as an osteoconductive scaffold in the surgical reconstruction of Charcot foot.

Achieving durable infection eradication typically requires surgical debridement of infected, non-viable bone and soft tissues. However, current guidelines recommend [25,26], and, indeed, specialist centres provide, concurrent systemic antibiotic therapy to ensure the eradication of any remaining infection. The optimal duration for such therapy is uncertain [4,26], and patients are frequently offered extended antibiotic regimens. The mean duration of systemic antibiotic administration in our study was 20 days (range 4–42), which is considerably lower than other reported studies [7,17]. Earlier series from McNally et al. [7] and Drampalos et al. [17] received post-operative systemic antibiotic administration for between 6 to 12 weeks after the indexed procedure. Similarly, in two studies where a ring fixator was used to correct Charcot deformity and achieve stability, the postoperative systemic antibiotic therapy was for 8 and 11 weeks, respectively [27,28]. Two recent studies using Cerament in DFO have reported using systemic antibiotics between 4 and 6 weeks, indicating a possible trend that clinicians are now becoming more confident limiting systemic antibiotic duration with antibiotic impregnated Cerament instillation. Our findings taken together with the previously published reports, make a stand for antibiotic stewardship and further support the results from two recent randomised controlled trials on diabetic foot infections [29,30], challenging the notion prevalent within DFO care that long duration of systemic antibiotics are ostensibly required, if durable infection clearance is to be achieved.

Wound ooze and inflammatory reactions with calcium sulphate-based void fillers has been well described in the literature [31]. McNally reported a wound leak of 6% in his series of 100 patients [7]. We have seen a decreasing trend in both wound ooze and 'Cerament burns' (skin erythema from leakage of Cerament) amongst our patients, which we attribute to the learning curve associated with its use. We meticulously place the Cerament within the osteotomy site prior to the application rigid compression or intraosseously via drill holes, limiting the amount of Cerament leakage within the soft tissue envelope.

The use of Cerament was distinctly different within our groups. In Group 1, the main aim was infection eradication and wound closure at the time of surgery. In Group 2a, infection control had already been achieved through previous surgery and the patients were subsequently subjected to deformity correction procedure, during which Cerament was used to eradicate any residual infection and promote bone healing to achieve bone union. Similarly, Cerament was used among group 2b patients during the second stage of the procedure, with the aim of achieving eradication of any residual infection and promoting bone fusion. Cerament was not used during the first stage of two stage as it is more expensive and there was no requirement for bone fusion during this state.

The antibiotic admixed with Cerament did not unequivocally match the intra-operative microbiological isolates. The decision on the best suited antibiotic for intra-operative instillation was based on preceding microbiological data, which have shown to have only fair to moderate concordance with surgical bone specimens [32]. Furthermore, polymicrobial growths, commonly prevalent in DFO, can often limit determination of the most important isolate to target. This underscores the importance of rigorous perioperative planning, including detailed discussions with microbiology colleagues to determine the appropriate choice of antibiotic to admix with Cerament. In addition, we continued systemic antibiotics targeted against isolates from surgical specimens, until inflammatory markers were deemed controlled but not normalised.

A surprising and noteworthy finding was that the mortality rate among our cohort was lower than expected. All our patients were referred to us after a period of uncontrolled infection and DFO development and typically such severe presentations are linked to high mortality rates. For instance, the United Kingdom National Diabetic Foot audit recorded a 14% mortality rate after 12 months among individuals with severe ulceration at presentation [33,34]. In a recent study, we reported a mortality rate of up to 45% after 18 months among a cohort that experienced a diabetic foot attack requiring urgent surgical debridement [35]. In contrast, the patients in our current study were younger, did not have advanced peripheral artery disease (PAD), and had chronic low-level infections instead of severe infections compared to the other groups. However, our evaluation was not designed to investigate the impact on mortality and, along with the small sample size, these factors could have contributed to the lower mortality rate. Another possible explanation could be that ensuring complete infection resolution and maintaining mobility through deformity correction may have contributed to extended survival by reducing chronic inflammation.

The strength of our study includes the reporting of outcomes from a diabetic foot centre with a well-established pre-and post-operative protocols in the surgical management of diabetic foot infections. We have analysed the clinical outcomes, including infection eradication and complications, functional outcome, including ambulatory status and radiological outcomes on this group of complex presentations. Limitations include the retrospective nature of our evaluation, the relatively low numbers of patients within each group and that we did not have a comparator group. Nonetheless, at present, this represents the largest reported series on the use of Cerament in diabetic foot reconstructive surgery.

In conclusion, we report on our experience utilising Cerament in DFO surgery and also, for the first time, in diabetic foot Charcot reconstructive surgery. The use of Cerament resulted in high proportion of functional limb salvage and infection clearance and decrease in the duration of post-operative antibiotic therapy. In addition to improvement in functional status, we observed apparent improved survival of the individual with diabetic foot disease. Further studies, ideally larger controlled cohorts, carefully exploring these observations with Cerament instillation in complex diabetic foot orthopaedic interventions are required.

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Informed Consent Statement: Not applicable as the study was a retrospective evaluation of clinical outcomes.

Data Availability Statement: No data is provided due to privacy restrictions.

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Take Home Message:

- High proportion of patients achieving infection clearance and limb salvage in the surgical management of DFO with the use of antibiotic loaded Cerament;
- Cerament facilitates complex orthopaedic reconstruction in previously infected Charcot feet;

- Cerament usage allows shorter duration of systemic antibiotics following diabetic foot osteomyelitis surgery, even when the internal fixation metal work is used.

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Risk Factors for Surgical Site Infections in Elective Orthopedic Foot and Ankle Surgery: The Role of Diabetes Mellitus

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Abstract: Surgical site infection (SSI) after elective orthopedic foot and ankle surgery is uncommon and may be higher in selected patient groups. Our main aim was to investigate the risk factors for SSI in elective orthopedic foot surgery and the microbiological results of SSI in diabetic and non-diabetic patients, in a tertiary foot center between 2014 and 2022. Overall, 6138 elective surgeries were performed with an SSI risk of 1.88%. The main independent associations with SSI in a multivariate logistic regression analysis were an ASA score of 3–4 points, odds ratio (OR) 1.87 (95% confidence interval (CI) 1.20–2.90), internal, OR 2.33 (95% CI 1.56–3.49), and external material, OR 3.08 (95% CI 1.56–6.07), and more than two previous surgeries, OR 2.86 (95% CI 1.93–4.22). Diabetes mellitus showed an increased risk in the univariate analysis, OR 3.94 (95% CI 2.59–5.99), and in the group comparisons (three-fold risk). In the subgroup of diabetic foot patients, a pre-existing diabetic foot ulcer increased the risk for SSI, OR 2.99 (95% CI 1.21–7.41), compared to non-ulcered diabetic patients. In general, gram-positive cocci were the predominant pathogens in SSI. In contrast, polymicrobial infections with gram-negative bacilli were more common in contaminated foot surgeries. In the latter group, the perioperative antibiotic prophylaxis by second-generation cephalosporins did not cover 31% of future SSI pathogens. Additionally, selected groups of patients revealed differences in the microbiology of the SSI. Prospective studies are required to determine the importance of these findings for optimal perioperative antibiotic prophylactic measures.

Keywords: diabetic patients; surgical site infection; elective orthopedic surgery

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1. Introduction

Surgical site infection (SSI) is uncommon in adult orthopedic surgery, ranging from the lowest rate of 1% for primary hip and knee arthroplasties to the highest rate of 20–50% for Gustilo grade III open fractures [1] or amputation stumps [2,3], and up to 20% for elective orthopedic oncologic surgery in the pelvic area [4,5]. We recognize that some special groups can have a higher risk, such as diabetic or immunosuppressed patients [2,6]. These elements, such as non-glycemic control in the preoperative time of surgery or host immunosuppression, could be added as important risk factors for SSI [1,7]. Indeed, diabetes mellitus is described as an independent risk factor for SSI, or community-acquired infections, in the entire orthopedic field [8]. The pathogenesis of SSI is believed to be acquired during surgery. This is supported by the success of SSI prevention measures directed towards activities in the operating theatre [9]. However, there are currently no data on the actual proportion of SSIs in the operating theatre versus postoperative care, and host factors are also important. Malnutrition, diabetes mellitus, anticoagulation, smoking and vasculopathy, steroid therapy, or use of tumor necrosis factor-alfa inhibitors are known to affect wound healing, and some of them have been related with higher SSI risk [6,10,11].

Among many measures, perioperative antimicrobial prophylaxis helps to reduce orthopedic SSI risks to 1–3%, compared to 4–8% without antibiotics [2]. Prophylactic antibiotic agents are taken for granted for most orthopedic interventions. However, in elective foot and ankle surgery in adult patients, there are no universal recommendations [12]. The American College of Foot and Ankle Surgeons recommends routine use of antibiotic prophylaxis in selected high-risk patients with certain conditions, such as diabetes, other immunosuppressive states, and surgeries involving bone, hardware, and prosthetic joints [13]. In contrast, for a clean, uncomplicated, and elective soft tissue surgery of the foot and ankle in otherwise healthy patients, the perioperative antibiotic prophylaxis is not routinely warranted [14]. Regarding the choice of the agents, a narrow-spectrum covering *Staphylococcus aureus* should be used for patients without a past history of resistant infection. Experts suggest cefazolin (or cefuroxime) as the agent of choice and clindamycin or vancomycin for patients with a beta lactam allergy [13]. Other authors suggested almost the same, with first or second cephalosporins and also glycopeptides only if multi-resistant skin colonization is documented [6].

It is noteworthy that diabetic foot patients show significantly more infections occurring in people with diabetes than in those without it. It may be explained by the effects of hyperglycemia, obesity, and/or the effects of neuropathy and impaired tissue perfusion on injury and wound healing [15]. Thus, peripheral neuropathy, Charcot neuroarthropathy [16,17], current or past smoking, and increased length of surgery were significantly associated with SSI [18].

The microbiological characteristics of orthopedic foot surgery SSIs are well described, but not specifically for the subgroup of patients with diabetic foot surgery. Usually, the most common isolated pathogens are coagulase-negative *Staphylococcus* and *S. aureus*, and similar studies have observed proliferation in difficult-to-treat bacterial isolates of methicillin-resistant *S. aureus* and *S. epidermidis*, and vancomycin-resistant *S. aureus* and *Enterococcus* in clean elective surgery [7,14,19,20]. The question regarding a better gram-negative perioperative antibiotic prophylaxis in diabetic foot surgeries is not resolved. In addition to the skin pathogens concerned, there are more complicated patients with an open wound that could be colonized with different microorganisms, including gram-negative bacilli [21]. There is no solid data to support a change in routine antibiotic prophylaxis, but future investigation may reveal the need to change prophylaxis in this group of patients.

In this study, we establish risk factors for elective clean orthopedic foot surgeries and a group of elective clean-contaminated foot surgeries. Furthermore, we evaluate the microbiology of SSI with an emphasis on the role of diabetes mellitus in the incidence of SSI and related pathogens. Of note, we do not analyze diabetic foot infections in diabetic foot syndromes that are a different distinct entity, for which a much broader literature is already published. We only investigate the epidemiology of SSI after elective foot surgery for non-infectious indications, stratified by the presence and absence of concomitant diabetes mellitus.

2. Materials and Methods

We retrospectively analyzed the surgical episodes of elective foot and ankle surgeries in our foot center; an orthopedic referral center at the Balgrist University Hospital in Zurich, Switzerland, from January 2014 to September 2022. We used data mining from the hospital's own medical databases and verified the SSI by opening individual electronic files. We included all patients older than 18 years of age with elective foot and ankle surgeries and excluded emergency surgeries understood as any injury that requires immediate medical care [19], such as open or displaced fractures or surgeries performed for community-acquired or nosocomial infections (gangrene, severe soft tissue infection), and severe ischemia. We divided the cohort into two groups of diabetic and non-diabetic patients. We studied the key variables related to possible risk factors for SSI, including duration of the surgery, type of elective surgery, more than two previous surgeries, surgery with foreign material (osteosynthesis, external material), the ASA score, and the presence of a

chronic wound in diabetics. The local ethics committee approved our retrospective study (BASEC 2022-01755). Patients who did not provide their general informed consent upon hospitalisation for surgery were not analyzed.

2.1. Definitions

Elective orthopedic surgery: a type of procedure that is pre-planned and is not performed in an emergency [13].

Surgical site infection (SSI): the infection that occurs after surgery in the part of the body where the surgery took place. The SSI can sometimes be superficial involving the skin. Other SSIs are more serious and can involve tissues under the skin, organs, or implanted material. These infections occur up to 30 days after surgery (or up to 1 year after surgery with implants) [22].

ASA score: The physical status classification systems of the American Society of Anesthesiologists (ASA) were developed to offer clinicians a simple categorization of a patient physiologically. It is a simple tool used to assess the risk of death and complication after a surgical procedure. The categories are as follows. ASA I: normal health; ASA II: mild systemic disease; ASA III: severe systemic disease; ASA IV: severe systemic disease that is a constant threat to life; ASA V: moribund, not expected to survive without operation [23].

Clean elective surgery: the non-emergency surgery performed on intact skin, not infected [13,24].

Clean-contaminated elective surgery: the non-emergency surgery performed in a chronic open wound [13,24].

2.2. Statistical Analysis

Categorical variables are summarized as counts and percentages, while continuous variables are summarized with median and interquartile range (IQR). Comparisons between groups were performed by chi-square or Fisher exact tests for categorical data. Continuous data were analyzed using the Wilcoxon rank-sum test or the Kruskal–Walli's test. To adjust for the large case-mix, we performed univariate and multivariate analyses using logistic regression models. We used Stata software (version 16.0, Stata Corporation, College Station, TX, USA) and p-values ≤ 0.05 (two-tailed) were significant.

3. Results

We analyzed 6318 elective foot surgery episodes from 4272 patients. The patients received a prophylaxis with cephalosporin: cefuroxime 1.5 or 3 g 30 min before surgery, and, in case of allergy, 600 mg of clindamycin was administered. The prevalence of diabetes was 8.6% and obesity (IMC > 30 mg/kg^2) was 16%; 32% of the episodes were the second episode or next episodes. Diabetic and non-diabetic foot surgery patients differed significantly in the patient's demographics (Table 1). For instance, the male sex was more frequent among diabetic patients (54% vs. 42%, $p < 0.01$.) The overall surgery with hardware (internal and external fixation) was present in 1858 (30%), representing a little less in diabetic patients (n = 290, 53% vs. n = 1568, 27%. $p < 0.01$). The number of soft tissue surgeries performed only without any bone intervention was 602 (10%) and other procedures (arthroscopies) were present in 99 (1%) of the episodes.

Table 1. Clinical characteristics in elective orthopedic foot and ankle surgery.

	Overall, N = 6318	**Diabetic Patients N = 540**	**Non-Diabetic Patients N = 5778**
Sex—male	2730 (43)	292 (54)	2438 (42)
Age, range	51 (36–62)	61 (52–69)	50 (34–61)
Body mass index (kg/m^2)	26.9 (23.3–31.3)	31.8 (27.4–35.0)	26.4 (23.1–30.6)
Days of hospitalization	3 (3–5)	5 (3–14)	3 (3–5)

Table 1. *Cont.*

		Overall, N = 6318	Diabetic Patients N = 540	Non-Diabetic Patients N = 5778
Time of surgery (hours)		1.1 (0.6–1.7)	1.1 (0.5–1.9)	1.1 (0.7–1.7)
≥2 surgeries		2046 (32)	242 (45)	1804 (31)
Bone resection		5304 (84)	446 (83)	4858 (84)
	Toe surgery [1]	1482 (23)	1402 (24)	80 (15)
	Other bone surgeries (excluding toes) [2]	3743 (59)	312 (58)	3431 (59)
	Charcot neuroarthropathy	79 (2)	54 (10)	25 (0.4)
Cavus–cavovarus foot		313 (5)	28 (5)	285 (5)
Soft tissue surgery [3]		602 (10)	539 (9)	63 (12)
Other procedures [4]		99 (1)	3 (1)	96 (2)
Foreign material				
	Osteosynthesis	1695 (27)	191 (35)	1504 (26)
	External fixator	163 (3)	99 (18)	64 (1)
ASA score				
	ASA 1	1667 (26)	7 (1)	1660 (29)
	ASA 2	3581 (57)	245 (45)	3336 (58)
	ASA 3	978 (15)	266 (49)	712 (12)
	ASA 4	40 (1)	21 (4)	19 (0)
	Unidentified ASA	52 (1)	1(0)	51 (1)
Surgical site infections		***119 (1.88)***	***31 (5.74)***	***88 (1.52)***

Footnote: Data are shown in numbers (%) or median (range). [1] Deformities of the toe (bunion removal, hammertoes, and hallux deformities). [2] Pseudoarthrosis, arthrosis, luxation fracture, osteotomy, implantation of arthrodesis or other foreign bodies through the bone (70%); degenerative foot problems and other foot deformities not included in the separated group, and correction of exostosis (30%). [3] Tendinopathies or other tendon problems, plantar fasciitis, soft tissue tumors, and other surgeries related with the soft tissue debridement. [4] Foot arthroscopies.

3.1. Surgical Site Infections

In general, we observed the occurrence of SSI in 119 episodes (119/6318; 1.88%), which was substantially increased in the diabetic group (1.52% in non-diabetic patients versus 5.74% in diabetic patients, $p < 0.01$). Clean elective surgery showed proportions of SSIs, ranging from the lowest 0% to 0.3% in elective arthroscopies, toe surgeries, and foot deformities, to the highest 10% with material surgery or contaminated elective surgery, with an approximately 14% SSI rate among patients with pre-existing foot ulcers (Table 2).

3.2. Risk Factors for Surgical Site Infections

We performed a multivariate logistic regression to identify important variables associated with SSI. We identified the ASA score of 3 or 4 with an odds ratio (OR) of 1.87 (95% confidence interval (CI) 1.20–2.90), more than two previous surgeries with an OR of 2.86 (95% CI of 1.93–4.22), and internal or external osteosynthesis material with an OR of 2.33 (95% CI of 1.56–3.49) and OR 3.08 (1.56–6.07) as the most important variables associated with SSI, respectively. The complete logistic regression analysis is showed in Table 3.

Table 2. SSI risk according to the type of surgery.

Type of Foot and Ankle Surgery	Surgical Site Infection Risk
Clean Elective Surgery	
Toe surgery	4/1482 (0.3%)
Bone surgery (forefoot, midfoot, hindfoot)	94/3743 (2.5%)
Charcot neuroarthropathy	5/79 (6%)
Foot deformity surgery	1/313 (0.3%)
Bone surgery with osteosynthesis material	52/1695 (3%)
Bone surgery with external fixation	16/163 (10%)
Only soft tissue surgery	15/602 (2.5%)
Other including arthroscopies	0%
Clean-contaminated Elective Surgery	
Surgical zone with diabetic foot wound	9/65 (14%)

Table 3. Risk factors for surgical site infections in the entire cohort.

	SSI (n = 119)	Univariate (OR, 95% CI)	Multivariate (OR, 95% CI)	*p*-Value
Male sex	67 (56)	1.71 (1.19–2.47)	1.61 (1.10–2.35)	0.02
Age (years)	59 (49–71)	1.04 (1.02–1.05)	1.03 (1.02–1.04)	<0.01
Diabetes mellitus	31 (26)	3.94 (2.59–5.99)	1.42 (0.86–2.35)	0.17
ASA Score 3 or 4	51 (43)	4.03 (2. 78–5.83)	1.87 (1.20–2.90)	0.01
≥2 surgeries	76 (64)	3.79 (2.60–5.54)	2.86 (1.93–4.22)	<0.01
Internal material	52 (44)	2.74 (1.85–4.04)	2.33 (1.56–3.49)	<0.01
External material	16 (13)	9.41 (5.24–16.90)	3.08 (1.56–6.07)	<0.01
Duration of surgery (hours)	0.88 (0.43–1.85)	0.83 (0.65–1.06)	0.83 (0.67–1.04)	0.11

Footnote: Data are shown in numbers (%) or median (range).

Risk Factors for Surgical Site Infections in the Subgroup of Diabetics

As diabetes is considered a significant risk for SSI, we performed stratified and separate analyses for foot surgeries among the diabetic patient population only (Table 4). The only significant risk associations in this subgroup of patients were a pre-existing foot ulcer (in the last three months) with an OR of 2.99 (95% CI 1.21–7.41), internal and external material with OR 4.31 (95% CI 1.53–12.11) and OR 3.86 (95% CI 1.32–11.25), and more than two previous surgeries with OR 2.73 (1.19–6.22), respectively.

Table 4. Risk factors for surgical site infections among diabetic patients.

	SSI (n = 31)	Univariate (OR, 95% CI)	Multivariate (OR, 95% CI)	*p*-Value
Male sex	19 (61)	1.37 (0.65–2.88)	1.06 (0.48–2.36)	0.89
Age (years)	64 (52–74)	1.03 (1.00–1.07)	1.04 (1.00–1.08)	0.04
Wound	9 (29)	3.31 (1.45–7.54)	2.99 (1.21–7.41)	0.02
Internal material	14 (45)	3.22 (1.21–8.53)	4.31 (1.53–12.11)	0.01
External material	11 (35)	5.08 (1.83–14.16)	3.86 (1.32–11.25)	0.01

Table 4. *Cont.*

	SSI (n = 31)	Univariate (OR, 95% CI)	Multivariate (OR, 95% CI)	*p*-Value
≥2 surgeries	22 (71)	3.21 (1.45–7.11)	2.73 (1.19–6.22)	0.02
ASA Score 3 or 4	22 (71)	2.20 (0.99–4.87)	1.55 (0.65–3.67)	0.32
Duration of surgery (hours)	1.17 (0.53–1.97)	1.02 (0.71–1.46)	0.97 (0.68–1.38)	0.86

Footnote: Data are shown as numbers (%) or median (range).

3.3. Microbiological Findings in SSI

We collected the microbiological findings in 50% of the 119 SSI episodes with 85 microbiological isolates. Among those, gram-positive cocci (GPC) were the most important group (71%). In the GPC group, the coagulase-negative *Staphylococcus* represented one of the most common (33%). This was followed by *S. aureus* (28%), while gram-negative bacilli represented a minor group (21%). "Others" group represented anaerobes and some gram-positive bacilli (Figure 1). The polymicrobial characteristic (more than 1 microorganism) of each episode represented 22/119 (18%) of the general cohort, 17/68 (25%) of implant-related material, and was higher, especially with 6/9 (67%) for patients with foot ulcers before SSI diagnosis. Regarding the microbiological isolates, it should be noted that *Pseudomonas aeruginosa* represented 9/85 (11%) of the pathogens resistant to the antibiotic prophylactic agents of index surgery and 4/13 (31%) in the diabetic foot ulcer subgroup.

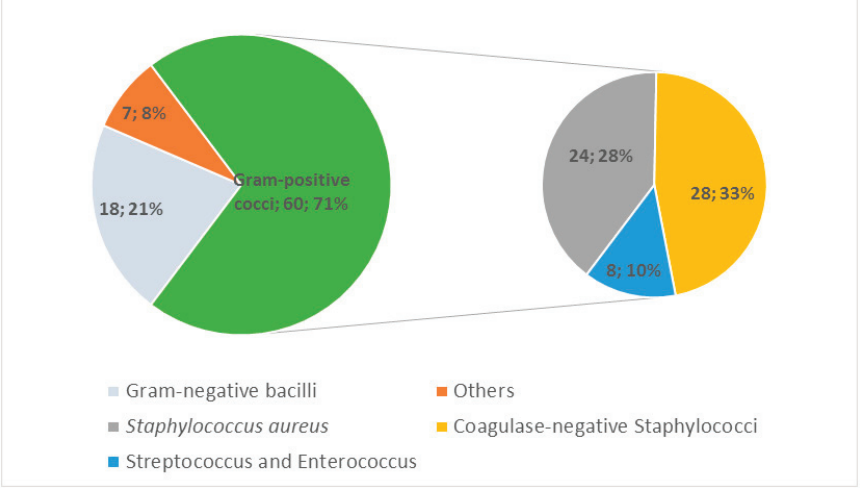

Figure 1. Microbiology of surgical site infections (pathogenic groups). Data are shown as numbers n; %.

4. Discussion

We found that the incidence of SSI in the elective orthopedic foot surgery population was 1.88%; among patients with concomitant diabetes it was 5.74%, slightly lower than that of other comparative cohorts, where the incidence was 9.5% [18]. Although surgical site infections are considered to be a burden on the health system, SSI in elective orthopedic foot and ankle surgery is still low compared to other surgical procedures, such as digestive tract surgery and colon surgery (18–25%) [3,25], and reveals some different characteristics from other orthopedic procedures. In orthopedics, the highest reported rates of SSIs are the emergency surgeries or contaminated/dirty surgeries, the Gustilo grade III open fractures or amputation stumps (20–50%), and orthopedic oncologic pelvic surgery (up to 20%) [2,5]. Skin injury with the loss of skin barrier protection, the contaminated/dirty surgeries and

the local microbial contamination with more gram-negative bacilli and anaerobes is one of the risk factors that explain the highest risk of postoperative infection in these groups. The infection risk was dominated by the extent of tissue damage according to the Gustilo grade [26,27].

Risk factors for SSIs were an ASA score of 3 and 4 points, more than two previous surgeries, or implants (internal and external material). In multivariate analysis, we could not prove diabetes as an independent risk factor, and our results were similar to those of other cohorts [18], but in the sub-analysis of diabetic patients, a foot ulcer before surgery was equally a risk of later SSI, and more gram-negative bacteria were present. Regarding these diabetic patients, Armstrong et al. [24] defined four risk groups to predict complications after elective foot surgery, which were elective and prophylactic surgeries for Class I and Class II, curative diabetic foot surgery performed in patients with an open wound for Class III, and emergency surgery for Class IV. Their results showed that the prevalence of complications after surgery in these groups was higher for class III and class IV compared to class I or II. Indeed, the proportion of ulceration/re-ulceration, postoperative infection, and amputation were 0–2.2–0% for class I, 2.2–6.7–2.2% for class II, 11–20–6.7% for class III, and 24.4–100–48.9% for class IV. However, the microbiological findings of postoperative infection were not described.

Most importantly, perioperative antibiotic prophylaxis for non-emergency foot and ankle surgeries is not universally recommended. Experts advocate general prophylaxis with second-generation cephalosporin in diabetic or otherwise immune-suppressed patients and for surgery involving bone or hardware [13]. In our results, at least 11% of all final SSI pathogens were resistant to previous prophylaxis, especially in diabetic foot patients with a previous wound with an average of 31% resistant pathogens. The selection of the best prophylaxis has been extensively discussed [28,29], especially regarding coagulase-negative staphylococci (e.g., *S. epidermidis*) that are very often resistant to cephalosporins [19,20]. These gram-positive microorganisms predominate in hardware infections and less in open chronic wound infections or foot osteomyelitis. The polymicrobial characteristics of the chronic wound in diabetics and the different skin colonization through the wound leads to the discussion of changing preoperative prophylaxis in selected situations for better coverage of gram-negative bacilli (fermenters and non-fermenters) in the specific orthopedic surgery group, which is the subject of ongoing trials [30]. A microbiologically better prophylaxis proposed for these selected groups with contaminated elective surgeries could be the addition of gram-negative antibiotic spectrum (gentamicin or 4th generation cephalosporins) to the gram-positive prophylaxis used (vancomycin, teicoplanin, or daptomycin). However, this remains speculative. In the clinical field, a prospective controlled study is urgently needed to confirm our findings and eventually propose some changes in foot and ankle surgery prophylaxis.

The main limitations of this research are the single-center study and the nature of a retrospective cohort study. Confounding factors cannot generally be ruled out, because there is heterogeneity, different sample sizes, and different characteristics between the diabetic and non-diabetic groups.

Despite the limitations, we have some important conclusions to highlight that make us consider a different prophylaxis for the specified groups during orthopedic surgery. The higher polymicrobial characteristic of SSIs, including gram-negative bacteria in diabetic foot ulcers, should be observed in detail to determine whether there is a need to change antibiotic prophylaxis when an elective surgery is performed in these specific groups.

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Microcirculation Improvement in Diabetic Foot Patients after Treatment with Sucrose Octasulfate-Impregnated Dressings

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Abstract: To assess the patients' microcirculation evolution during the treatment with a sucrose octasulfate-impregnated dressing, fifty patients with neuroischaemic DFU treated with TLC-NOSF dressing were included in a prospective study between November 2020 and February 2022. $TcpO_2$ values were measured on the dorsalis pedis or tibial posterior arteries' angiosome according to the ulcer location. $TcpO_2$ values were assessed at day 0 and every 4 weeks during 20 weeks of the follow-up or until the wound healed. A cut-off point of $tcpO_2 < 30$ mmHg was defined for patients with impaired microcirculation. The $TcpO_2$ values showed an increase between day 0 and the end of the study, 33.04 ± 12.27 mmHg and 40.89 ± 13.06 mmHg, respectively, $p < 0.001$. Patients with impaired microcirculation showed an increase in the $tcpO_2$ values from day 0 to the end of the study ($p = 0.023$). Furthermore, we observed a significant increase in the $TcpO_2$ values in the forefoot DFU ($p = 0.002$) and in the rearfoot DFU ($p = 0.071$), with no difference between the ulcer locations ($p = 0.694$). The local treatment with TLC-NOSF dressing improved the microcirculation in patients with neuroischaemic DFU, regardless of microcirculation status at the baseline, and in the forefoot, regardless of the location.

Keywords: diabetic foot; neuroischaemic diabetic foot ulcer; microcirculation; sucrose octasulfate dressing; transcutaneous oxygen pressure

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1. Introduction

Neuroischaemic diabetic foot ulcers (DFUs) have become the commonest ulcer type among patients with diabetes [1]. Foot tissues can become ischemic because of macrovascular disease (atherosclerosis); however, when we are evaluating the genesis of diabetic foot complications, not only macrovascular complications, but also the presence of microvascular complications seem to be important predictors for the development and the prognosis of the DFU [2]. The relationship between DFU and peripheral arterial disease (PAD) has been well recognized. Nevertheless, the role of impaired microcirculation is yet to be fully understood [3].

It has been demonstrated that microangiopathy may play a significant role in the pathogenesis of tissue breakdown, and it may become an important factor in the poor healing of wounds [3]. It is essential for skin nutrition, fluid homeostasis, thermoregulation, the provision of defense, and the repair of cells and cytokines following injury and infection. Consequently, impaired microcirculation will impact these essential processes [4]. Microangiopathy comprises detrimental changes in the nerve microvasculature's structure and function, which in turn cause reduced endoneurial perfusion and hypoxia. Consequently, reducing the supply of oxygen to nerves and tissues causes a disturbance in the metabolism of cells, which significantly impedes the viability of tissues [3].

Several technological methods have enabled us to evaluate the microcirculation status, such as laser Doppler flowmetry, capillaroscopy, hyperspectral imaging, or transcutaneous oxygen pressure ($tcpO_2$) techniques, among others [5]. Of them, the $tcpO_2$ method is a non-invasive method that evaluates skin microcirculation and reflects tissue perfusion and oxygen delivery [6]. Additionally, $tcpO_2$ is considered to better evaluate the microvascular function and its role in predicting foot ulcer healing and lower limb amputations [7].

Previous studies have documented the usefulness of different local therapies in microcirculation restoration and their potential effect on its improvement [8–14]. Of these, a recent pilot study has reported that sucrose octasulfate dressings (TLC-NOSF (Technology Lipido-Colloïd-Nano-OligoSaccharide Factor)) result in an increase in the skin oxygen pressure [15]. The power of this study remains poor due to the small sample size and the pilot study design. The development of these therapies that improve diabetic foot-impaired microcirculation could change the microvascular status of diabetic patients in the long-term follow-up period. Therefore, we aimed to assess the patients' microcirculation improvement during a treatment with a sucrose octasulfate-impregnated dressing.

2. Materials and Methods

2.1. Subjects

A prospective study was conducted between November 2020 and February 2022 on 50 patients with DM who had non-infected neuroischaemic DFU in a specialized diabetic foot unit.

The inclusion criteria were: having confirmed type 1 or 2 DM, being aged >18 years old, presenting with non-infected neuroischemic DFU of grade IC or IIC, as defined by the University of Texas Diabetic Wound Classification System, having their glycemic control confirmed by an HbA1c (hemoglobin A1c) of $\leq$10% (85.8 mmol/mol) in the previous 3 months, and having a wound area surface size of between 1 to 30 cm^2 at the moment of inclusion.

The exclusion criteria were: having a critical limb ischemia [16], end-stage renal disease or dialysis, the presence of edema from a vascular, renal, or cardiac disease, and patients with chronic obstructive pulmonary disease, which could alter oxygen saturation at a systemic level, patients who have suffered from a stroke in the last 3 months, those with acute Charcot foot, and those who had undergone surgical revascularization in the past 3 months before inclusion in the study.

This study was approved by the ethics committee of our teaching hospital in May 2020 (Code: 20/386-O_P). Before inclusion in this study, all of the patients provided their written informed consent according to the principles of the Declaration of Helsinki [17].

2.2. Clinical Assessment

The baseline patient assessment was carried out before the inclusion in the study, including diabetes type and duration, associated comorbidities, the HbA1c (%) values from the last blood test, and foot-related complications.

Neuropathy was diagnosed using a Biotensiometer and Semmes–Weinstein 5.07/10 g monofilament (Novalab Iberica, Madrid, Spain) [18]. Peripheral arterial disease (PAD) was diagnosed based on the distal pedal pulse palpation, ankle–brachial index (ABI), toe-brachial index (TBI), and $tcpO_2$ [16].

The neuroischemic patients were defined as having an ankle–brachial index ABI of $\leq$0.9 and an ankle systolic blood pressure (ASBP) of $\geq$70 mmHg or a toe systolic blood pressure (TSBP) of at least 50 mmHg. In the patients with ABI > 0.9, we considered PAD when the toe-brachial pressure index (TBI) was <0.7 [15].

The patients were classified depending on the microcirculatory status at the baseline based on whether they had normal or impaired microcirculation. A cut-off point of $tcpO_2$ < 30 mmHg was set for the patients with impaired microcirculation. For the patients with normal microcirculation, a cut-off point of >30 mmHg was set [19].

2.3. Wound Management and Follow-Up

All of the patients were dressed with a sucrose octasulfate dressing (UrgoStart Contact, 10 × 10 cm, Laboratories Urgo Medical, Paris, France). The patients came twice weekly to the outpatient clinic for dressing care until they were healed. Additionally, the patients received a high standard of care (SoC) with offloading, following the International Working Group of the Diabetic Foot (IWGDF) offloading Guidelines [20]. In addition, when it was necessary, sharp debridement was performed to remove the non-viable skin, including the peri-wound skin. Wound area surface, photographs, and Wollina scores were assessed during each study visit, monthly, and at the end of the study.

Microcirculation was measured using a $tcpO_2$ TCM400 measuring device (Radiometer) following the angiosome concept according to the ulcer location [21]. The electrode was placed on the dorsalis pedis for the DFU located on the forefoot or in the posterior tibial artery for the DFU located on the midfoot or rearfoot. The values were recorded in mmHg after a calibrating them for a time of 10 min. The patients were supine during the examination, and they were asked not to move or speak. The $TcpO_2$ values were assessed at the baseline (day 0) and every 4 weeks during 20 weeks of follow-up or until the wound healed.

The study's main outcome was to evaluate the patients' microcirculation improvement during the treatment with a sucrose octasulfate-impregnated dressing in patients with neuroischaemic diabetic foot ulcers.

2.4. Statistical Analysis

All of the statistical analyses were performed using the software package SPSS version 25.0 (IBM Corp. Released in 2017. IBM SPSS Statistics for Macintosh, Version 25.0. Armonk, NY, USA: IBM Corp.).

The assumption of normality of all of the continuous variables was verified using the Shapiro–Wilk test. The normally distributed variables (Shapiro–Wilk test with $p \geq 0.05$) are reported as mean and standard deviations.

The categorical variables are reported as a frequency and percentage, while the continuous variables are reported as the mean ± standard deviation (SD; parametric distribution) or the median and interquartile range (IQR; non-parametric distribution).

The student t-test for paired samples was used to explore the differences in the $TcpO_2$ values within the treatment with the sucrose octasulfate dressing because of the normal distribution of the variables. p-values < 0.05 were considered to be statistically significant, with confidence intervals of 95%.

3. Results

A total of 50 patients with non-infected neuroischaemic diabetic foot ulcers were included in the present study and followed for 20 weeks or until they were healed. The demographic characteristics, DM, and related foot complications at the baseline are shown in Table 1.

Table 1. Demographics characteristics of patients at baseline.

Variables	Patients (n = 50)
Male, n (%)	45 (90%)
Female, n (%)	5 (10%)
Mean age ± SD (years)	62.60 ± 8.94
Type 1 diabetes, n (%)	4 (8%)
Type 2 diabetes, n (%)	46 (92%)
Glycated hemoglobin (%)	7.81 ± 1.47
Duration of diabetes ± SD (years)	20.04 ± 11.43
Risk factors	
Retinopathy, n (%)	18 (36%)
Nephropathy, n (%)	9 (18%)
Cardiopathy, n (%)	22 (44%)

Table 1. *Cont.*

Variables	Patients (n = 50)
Hypertension, *n* (%)	39 (78%)
Hypercholesterolemia, *n* (%)	28 (56%)
Tobacco use, *n* (%)	7 (14%)
Previous ulceration, *n* (%)	45 (90%)
Previous amputation, *n* (%)	40 (80%)
Vascular assessment	
History of revascularization, *n* (%)	16 (32%)
Bypass surgery, *n* (%)	3 (18.75%)
Endovascular surgery, *n* (%)	13 (81.25%)
Presence of dorsalis pedis pulse, *n* (%)	19 (38%)
Presence of posterior tibial pulse, *n* (%)	13 (26%)
Ankle brachial pressure index, mean ± SD	1.08 ± 0.36
Toe brachial pressure index, mean ± SD	0.69 ± 0.28
$TcpO_2$ Day 0 (mmHg) ± SD	33.04 ± 12.27
Systemic antiplatelet treatments	35 (70%)

BMI, body mass index; $TcpO_2$, transcutaneous oxygen pressure; SD, standard deviation.

At the baseline, 20 (40%) patients had impaired microcirculation ($tcpO_2$ < 30 mmHg), with mean $tcpO_2$ values of 20.20 ± 5.38 mmHg at the moment of inclusion. Additionally, the forefoot was the most frequent location; 40 (80%) and 10 (20%) of the DFUs were in the rearfoot. The wound characteristics are shown in Table 2.

Table 2. Wound characteristics of patients at baseline.

Wound Characteristics	Patients (n = 50)
Wound duration (weeks), median (IQR)	2.50 (2–8)
Wound area (cm^2), median (IQR)	1.55 (1.20–2.35)
Pollina score, mean ± SD	4.60 ± 1.80
University of Texas Diabetic Wound Grade Classification	
IC: Ischemic, not infected, superficial wound, *n* (%)	44 (88%)
IIC: Ischemic not infected wound penetrating to tendon or capsule, *n* (%)	6 (12%)

IQR, interquartile range; SD, standard deviation.

The $TcpO_2$ values after TLC-NOSF dressing application showed an increase between day 0 and the end of the study in the whole population (33.04 ± 12.27 and 40.89 ± 13.06 mmHg, respectively) (p < 0.001). Additionally, when they were analyzed separately, both of the groups showed a local improvement in tissue oxygenation. The patients with impaired microcirculation showed an increase in the $tcpO_2$ values from day 0 (20.20 ± 5.38 mmHg) to the end of the study (31.28 ± 13.74 mmHg) (p = 0.023) (Table 3). The patients with normal microcirculation also increased from 41.60 ± 6.80 mmHg at the point of inclusion to 46.73 ± 8.53 mmHg (p = 0.007).

From the 20 patients who had impaired microcirculation, at the end of the study, 13 (65%) patients achieved a normal microcirculation value (p < 0.001). Out of the whole study population, 13 (26%) patients did not achieve wound healing after 20 weeks of follow-up.

Furthermore, were observed a significant increase in the $tcpO_2$ values in the forefoot DFU between day 0 (32.85 ± 12.76 mmHg) and until the wound closed (41.34 ± 12.02 mmHg) (p = 0.002) and in the rearfoot DFU between day 0 (33.80 ± 10.66 mmHg) and until the wound closed (39.25 ± 17.21 mmHg) (p = 0.071), with no difference between the ulcer locations (p = 0.694) (Table 4).

Table 3. Differences in $tcpO_2$ values in the feet of patients after sucrose octasulfate dressing application depending on microcirculation impairment (whole population and impaired microcirculation).

Visit	All Patients (n = 50)	p Value	Impairment Microcirculation Patients (n = 20)	p Value
Day 0	33.04 ± 12.27	-	20.20 ± 5.38	-
Week 4	33.87 ± 12.58	<0.001 *	26.53 ± 10.21	0.002 *
Week 8	30.60 ± 11.83	0.402	24.67 ± 10.02	0.390
Week 12	44.30 ± 11.79	0.046 *	41.50 ± 7.77	<0.001 *
Week 16	44.85 ± 5.89	<0.001 *	44.50 ± 12.02	<0.001 *
Week 20	49.50 ± 2.12	<0.001 *	51.00	-
Wound closure	40.89 ± 13.06	<0.001 *	31.28 ± 13.74	0.023 *

* Differences were assumed significant at $p < 0.05$ for a confident interval of 95%.

Table 4. Differences in $tcpO_2$ values in the feet of patients after sucrose octasulfate dressing application depending on DFU location (forefoot and rearfoot).

Visit	Forefoot Location (n = 40)	p Value	Rearfoot Location (n = 10)	p Value
Day 0	32.85 ± 12.76	-	33.80 ± 10.66	-
Week 4	34.69 ± 13.57	<0.001 *	30.85 ± 7.9	0.914
Week 8	29.29 ± 10.82	0.277	34.33 ± 14.8	0.007 *
Week 12	44.16 ± 10.34	0.287	44.50 ± 15.45	0.523
Week 16	47.6 ± 4.15	0.854	38.00 ± 2.82	<0.001 *
Week 20	48.00	-	51.00	-
Wound closure	41.34 ± 12.02	0.002 *	39.25 ± 17.21	0.071

* Differences were assumed significant at $p < 0.05$ for a confident interval of 95%.

4. Discussion

The results of the present study confirm an improvement in the microcirculatory status derived from the sucrose octasulfate-impregnated dressings in the neuroischaemic DFU treatment. The transcutaneous oxygen pressure showed an increase in the local oxygenation during the wound healing. Additionally, this enhancement happened regardless of the vascular status at the baseline or the forefoot location. These findings indicate an added value to sucrose octasulfate dressings to support the first line of treatment in neuroischemic patients recommended by the IWGDF [22].

Several studies have explored potential therapies that could improve microcirculation due to the growing prevalence of PAD and compounds by the diffused nature of the vascular affection. Local therapies, such as hyperoxygenated fatty acids [8,9] and topical Vitamin E acetate [23], or physical procedures, such as whole body vibration [24], have recently documented their usefulness as a primary and secondary preventive tools in non-ulcerated diabetic patients.

On the same vein, some local and systemic treatments for DFU, such as low-intensity laser irradiation [10,25], injections of adipose-derived stromal vascular fraction cells [11], a systemic and local natural extract from the bark of the French maritime pine [12], the use of some heparins (e.g., dalteparin) [13], other systemic treatments with antiplatelets properties [26], or the use of local skin flaps [27] have been reported to produce a local increase in skin microcirculation.

Despite this, some of these are invasive processes or systemic treatments that need additional local wound care, thus increasing the direct costs. Finally, they used different methods to evaluate skin oxygenation. Consequently, it is difficult to compare these findings with the results of our study.

A previous pilot study [15] performed on a total of 11 patients with the same neuroischemic characteristics showed a local improvement in the $tcpO_2$ values after using the sucrose octasulfate dressing between day 0 (29.45 ± 7.38 mmHg) and until the wound

closed (46.54 ± 11.45 mmHg) ($p < 0.016$). Following the same trend derived from the current research, we observed an improvement from 33.04–12.27 mmHg at day 0 and 40.89–13.06 mmHg until the wound closed ($p < 0.001$). Thus, we could confirm the beneficial effect of this local procedure on the microcirculation, leading to an increase in tissue oxygenation.

Following this trend, another two therapies (systemic hyperbaric oxygen and autologous combined leucocyte, platelet, and fibrin) have reported promising results in the enhancement of the microcirculatory status. These therapies are recommended by the wound healing interventions guidelines (IWGDF), and they must be considered in non-healing ischaemic diabetic foot ulcers as adjunctive treatments [22].

Amir N. Wadee et al. [25] demonstrated a local improvement of the $tcpO_2$ values by the treatment of chronic DFU with systemic hyperbaric oxygen. They found a significant increase between the baseline (20.26 ± 5.26 mmHg) and the three post-measures in the second (29.15 ± 5.78 mmHg), fourth (39.48 ± 8.43 mmHg), and sixth weeks (50.15 ± 11.13 mmHg) of the treatment ($p = 0.000$).

Moreover, Dubsky et al. [28] demonstrated an improvement of the $tcpO_2$ values from 20.8 ± 9.6 to 41.9 ± 18.3 mmHg ($p = 0.005$) after 12 weeks of treatment with autologous cell therapy (ACT) in patients with diabetes and no-option chronic limb-threatening ischemia and foot ulcers, which are above those of the standard treatment ($p = 0.034$). However, this did not change significantly in the ACT group from 12 to 24 weeks.

Both of them reported similar results to ours in a similar population and with a similar measurement methodology of skin oxygenation and follow-up. Nevertheless, it is a fact that both of them are more expensive therapies with poor accessibility for this kind of population.

The growing interest in microcirculation studies in recent times is obvious and is reflected in the number of publications that try to demonstrate which therapies promote an effect on it.

The results of our study confirm the previously reported effect of sucrose octasulfate-impregnated dressings [15] on the microcirculation of neuroischemic patients. Microcirculation improvement could be related with mechanisms that sould be studied in further research.

Finally, our results should be interpreted with caution due to a major limitation: there is no control group for a comparison to be made. Therefore, further research should confirm the present results in a controlled and randomized clinical trial, in which the variables that could influence on peripheral oxygenation such as HbA1c or others must be evaluated. The main strength of the present study is that it is the first prospective study that demonstrates an improvement in the microcirculatory status in patients with impaired diabetic microcirculation at inclusion derived from the use of a dressing recommended by the IWGDF Guidelines in neuroischemic patients. These findings could help patients during and after the treatment to improve their microcirculatory status, even in the presence of satisfactory or delayed blood flows. The authors look forward to providing subsequent data from the 1 year follow-up prospective study after the patients have healed to analyze if a sustained improvement of the microcirculation could potentially have a positive effect on the recurrence rate of these lesions.

5. Conclusions

The local treatment with TLC-NOSF dressing improved microcirculation in patients with DFU regardless of their vascular status at the baseline or the forefoot location.

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Re-Epithelialization of Neuropathic Diabetic Foot Wounds with the Use of Cryopreserved Allografts of Human Epidermal Keratinocyte Cultures (Epifast)

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Abstract: The application of tissue-engineering technology to wound healing has become an option for the treatment of diabetic foot ulcers (DFU). A comparative, prospective study was conducted to assess the efficacy of a cryopreserved allograft of human epidermal keratinocytes (Epifast) to enhance wound healing in granulating DFU. Eighty patients were assigned to receive Epifast (n = 40) or Standard Care (SC) treatment (n = 40). The Epifast group displayed a shorter duration of the epithelialization phase (3.5 ± 4 vs. 6.4 ± 3.6 weeks, $p < 0.05$) and upon the entire wound healing process than the SC group (10 ± 5.7 vs. 14.5 ± 8.9 weeks, $p < 0.05$), reaching wound closure at 16 and 30 weeks, respectively. The Kaplan–Meier analysis revealed that Epifast group patients were 50% more likely than the SC to heal wounds faster (Cox-hazards ratio of 0.5, 95% CI = 0.3–0.8, $p < 0.0001$; Likelihood Ratio of 7.8. $p < 0.05$). Patients in the control group displayed a slower healing as the Saint Elian (SEWSS) severity grade increased (group differences of 0.6, 3.8, and 4.3 weeks for grades I, II, and III, respectively). DFW treated with Epifast displayed a shorter time to complete re-epithelialization than wounds treated with standard care.

Keywords: diabetic foot; living skin equivalents; classification; wound healing; keratinocytes

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1. Introduction

Foot ulcerations are among the most complex and heterogeneous complications in diabetic patients. The application of tissue-engineering technology to wound healing has become an option for the treatment of chronic wounds [1–6]. Skin equivalents are constructed from cultured keratinocytes that form an epidermal layer without dermal components. Epifast promotes cellular migration and produces growth factors that stimulate cell proliferation from ulcerated skin to achieve re-epithelialization [7].

Many factors play a role in the wound healing process, such as foot pressure, ischaemia, growth factors and cytokines and infection [ref]. Based on our experience with a previously published grading system [8], one important factor for wound progress is the wound healing phase. Clinical trials usually assess the efficacy of living skin equivalents in terms of percent of wound healing and frequency of amputation. We propose that their wound healing efficacy is useful during the granulation phase. In this study, we hypothesize that Epifast enhances re-epithelialization and reduces wound healing time in patients with diabetic foot wounds. Thus, we assessed the efficacy of an allograft of human epidermal keratinocytes (Epifast) to shorten the time to achieve 100% of wound closure and the epithelialization healing time. (A period started when granulation tissue is completed and re-epithelialization from the wound border begins).

2. Materials and Methods

2.1. Patients

A randomised, comparative, prospective study was undertaken to evaluate the effect of Epifast in DFUs. Consecutive type 2 diabetes patients from the foot clinic at our center with diabetic foot wounds were screened for eligibility for the study but included by random allocation when granulation tissue was in progress. Patients with type 2 diabetes who were over 18 years of age and had wounds at or distal to the malleoli with different degrees of neuropathy, anatomic, and tissue affection were considered for inclusion. An ulcer was defined as a full skin thickness wound below the ankle in a diabetic patient, irrespective of their etiology or duration. Only patients with non-infected, granulating wounds at or distal to the malleoli with loss of protective sensation and at least one pedal pulse detected by Doppler were included. Wound size was evaluated by measuring the maximum length and the maximum width. (When >1 ulcer was present only the largest ulcer was included).

Exclusion criteria included the following: (a) arterial disease (diagnosed by the absence of both foot pulses on the affected extremity, an ankle/brachial index below 0.5, or toe pressure < 30 mm Hg and a toe/brachial index < 0.30); (b) osteomyelitis and/or total gangrene of the foot or forefoot; (c) severe cardiovascular and renal failure; (d) severe neurological problems; (e) and other situations that would make the patient a poor candidate for the study (e.g., confined to a bed or lacking family assistance). All patients provided written informed consent. The study was reviewed and approved by the Human Subjects Ethics Committee.

2.2. Protocol

Once the granulating wound healing phase was determined by clinical inspection; all eligible subjects were randomly assigned to receive either Epifast (Group 1) or continued under standard management (control Group 2). Patients were blinded with respect to treatment and were informed only that a high-tech dressing could be randomly selected for their treatment. Both groups received comprehensive care and evaluation. All patients were treated according to an outpatient ambulatory model that included appropriate surgical debridement, aggressive parenteral/intramuscular broad spectrum antimicrobial administration, appropriate off loading, and strict glycemic control. Patients in both treatment groups were seen every third day or once a week. All patients were instructed to use a wheelchair or crutches to reduce load bearing on the affected foot and to rest as much as possible, although most patients have difficulty complying with these instructions. Compliance was assessed by directly questioning the patient and their caretaker, as well as by inspecting the dressings.

Patients randomized to the study group received the keratinocyte skin culture application (Epifast®; Bioskinco, Mexico City, México) [7]. Epifast is free from HIV-1, HIV-2, HBV, CMV, and toxic substances. The Epifast allografts were placed over a sterile tissue mesh between two protective plastic nets (7.00 × 8.00 cm, total surface of 56 cm^2). The allografts were then applied as a dressing over the wounds and changed every 7 days for one month. Patients were advised about malodor not related to infection.

Subjects under standard care treatment received hydrofiber, alginate, or Vaseline dressings. Alginate or hydrofiber dressings were used when the wound was exudative. Vaseline dressings were used during the non-exudative granulation phase until complete wound healing occurred.

2.3. Primary Objective and Measurements

The primary endpoint was the duration in weeks for the epithelialization phase to achieve 100% closure of the wound, which was measured from the advance of new skin from the baseline wound border until complete healing. Epithelialization was defined as the proliferation of epithelial cells providing cover for the new granulation tissue to restore an intact epidermal barrier. Granulation tissue is pink/red, moist tissue composed

of new blood vessels, connective tissue, fibroblasts, and inflammatory cells that fills an open wound during healing. Epifast was applied while the granulation phase was in progress. Changes in the surrounding skin and granulation tissue were assessed by direct clinical observation. The presence of healthy tissue surrounding the ulcers was considered a clinical sign that tissue toxicity was absent or minimal. Outcomes were assessed over a mean follow-up period of 52 weeks (365 days). The follow-up period was part of the normal treatment duration according to healing success or failure and a secondary follow-up with a minimum of 6 months for delayed wound healing. Once ulcer healing was achieved, patients were released from the study.

Baseline demographic measurements were performed at the first visit. The diagnosis of diabetes was made prior to enrollment. Doppler studies were performed, and toe or ankle/brachial indices were calculated. Pulse palpation, ABI (Hand-held Doppler–Huntleigh, Getinge AB; 8 MHz Doppler probe) and TBI were performed sequentially to assess the vascular status in every patient included in the study. Toe pressure was measured using a 2.5 cm wide × 12 cm long digital cuff on the proximal aspect of the hallux to calculate the TBI. A PPG unit Hadeco Smartdop 30EX was used when no toe artery was found using a Doppler and ischemia severity was graded as normal (0), mild (1), moderate (2) and severe (3). The degree of neuropathy was assessed using a 128-Hz vibration from a tuning fork at the hallux or on the basis of a decrease in or absence of Semmes–Weinstein (5.7/10-g) monofilament sensation at 2 of 3 points (first toe and fifth and first metatarsal head). Neuropathy for severity score purposes was sub-grouped as (0) none, (1) mild (diminished protective sensation to vibration or monofilament), (2) moderate (absence of sensation to vibration or monofilament), and (3) severe when diabetic neuro-osteoarthropathy (DNOA) was found. Infection was assessed and scored according to the Infectious Disease Society of América as part of the Saint Elian System [8]. Infection was excluded and scored as (0) without signs or symptoms. Mild infection affected skin superficially and was scored as 1 and diagnosed as erythema between 0.5 mm and 2 cm, induration, tenderness, warmth, and purulent discharge. Moderate infection (scored as 2) was identified by erythema more than 2 cm, muscle, tendon, bone, or joint infection. Osteomyelitis was diagnosed by a positive bone-to-probe test, by X-ray film, or biopsy. Severe infection (scored as 3) was identified by systemic inflammatory response or severe metabolic disturbances (hyperglycemia or hypoglycemia) that required patient hospitalization or was difficult to control. Neuropathy, infection and vascular assessments and wound characteristics were included as part of the Saint Elian Wound Score System for the Diabetic Foot [8,9]. According to this score, the 10 wound variable categories measured at patient presentation were as follows: (1) primary location, (2) topographic aspects, (3) number of affected zones, (4) ischemia, (5) infection, (6) edema, (7) neuropathy, (8) depth, (9) area, and (10) wound healing phase. All categories were subcategorized with an ascending score from mild (1) to severe (3 points).

The maximum score was 30 points. A score of 10 points or fewer was graded as I (mild, likely successful wound healing). A moderate score of 11–20 points was graded as II (partial foot threatening; outcome related to "state of the art" therapies used and associated with a good patient response). A score of 21–30 points was graded as III (limb- and life-threatening; outcome unrelated to "state of the art" therapies due to a poor patient response).

Consecutive measurements were recorded on different dates with the following variables in the left column: date, therapy, the ten wound variable categories, score, cumulative difference, and grade. Data were registered according to the date for each item as many times as needed.

Wound images and wound measurements were recorded in a database for systematic data collection.

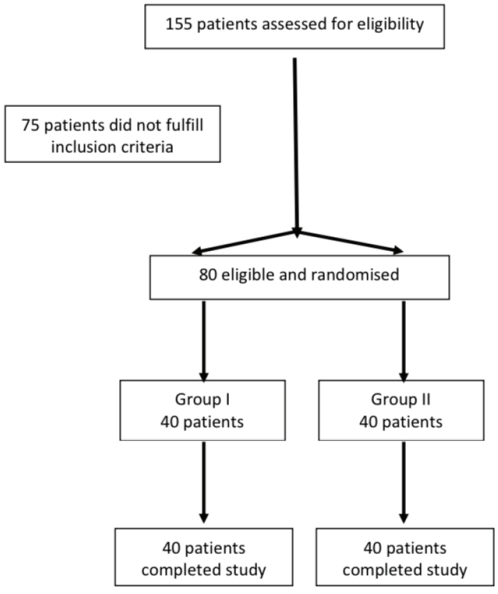
2.4. Statistical Analysis

Significance was considered at $p < 0.05$. Values for chi-squared with Yate's correction or Fisher's exact test with 2 × 2 tables and variance ratios for natural and treatment analysis

of variance were calculated. Kaplan–Meier probability, log-rank test, and Cox Hazard Ratio analyses were performed for wound healing time and were measured against the study groups. Simple regression was performed for variables such as wound size and time assuming a dependent relationship of wound size during the time period of examination. The p value for slopes differences was determined by Student's *t*-test (two-tailed). The sample size was required to have 80% power to detect differences for a one-sided test hypothesis: H_o: $P_1 = P_2$ versus H_a: $P_1 < P_2$ or $P_1 > P_2$. A 5% α (0.05) significance level, an 80% (0.80) power test (1-β), a 90% (0.90) success proportion in arm 1 (P_1), an 80% (0.80) success proportion in arm 2 (P_2), and a 1:1 group ratio were set for calculations. A total sample size for arm 1 and 2 of 68 patients was required, with 34 patients in each group. Forty patients per group were included, assuming a 20% drop-out rate. Patients assessed for eligibility were submitted to block randomization with an allocation ratio of 1:1. Two members of staff scored the Saint Elian System and assessed the epithelialization phase and healing progress independently. Kappa agreement index was performed using 2 × 2 tables to detect differences between the two observers. A kappa value (K $\frac{1}{4}$ Po−Pe/1−Pe; Po $\frac{1}{4}$ observed agreement, Pe $\frac{1}{4}$ expected agreement by random) between 0.61 and 1.0 was representative of substantial to excellent agreement.

3. Results

Of 155 patients initially assessed, 75 were excluded because of the patient's decision, the refusal to complete the protocol or the failure to achieve the granulation healing phase (Figure 1). A total of 80 patients were included and were randomized to receive either Epifast (n = 40, Group 1) or standard care (n = 40, Group 2).



Group I: Patients treated with EPIFAST
Group II: Patients treated with Standard of Care

Figure 1. Flowchart showing patient randomisation.

There were no differences in demographics and clinical characteristics between the groups (Table 1). The 10 categories and subcategories for wound severity (Saint Elian classification) displayed similar proportions among the groups. The wound duration (lapse from the initial wound to patient presentation) was 7.2 (range 3–15) weeks. The treatment duration was 9.0 weeks (range 1–30). Healing times according to the initial assessment of the San Elian severity grades were 6.0 (range 1–35), 10.4 (range 1–122) and 26.8 (range 1–64) weeks for grades I, II and III, respectively ($p < 0.05$).

Table 1. Baseline demographic and clinical characteristics.

Characteristic	Group 1 Epifast n = 40 (%)	Group 2 Standard Care n =40 (%)	*p* Value
Age yr means ± SD +	65 ± 11.8	63.1 ± 11.3	0.42
Sex *			
Male	22 (52.5)	19 (47.5)	0.20
Female	18 (45)	21 (52.5)	
Diabetes duration in years means ± SD +	18.8 ± 11.3	18.7 ± 9.7	0.70
HbA1c means ± SD, % [mmol/mol] **	8.9 ± 1.9 [74]	8.2 ± 2.3 [66]	0.64
Obesity	26 (65)	30 (75)	0.54
Smoking *	6 (28)	9 (30.3)	0.33
Palpable peripheral pulses *	40 (100)	40 (100)	
Wound history in weeks means ± SD +	4.7 ± 6.1	6 ± 7.6	0.49
Saint Elian Score means ± SD+	19 ± 1.6	16 ± 2.0	0.81
Saint Elian Severity Grades *			
I (good prognosis for wound healing)	6 (15)	5 (12.5)	0.60
II (partially foot-threatening)	30 (75)	33 (82.5)	
III (limb- and life-threatening)	4 (10)	2 (5)	

+ Kruskal–Wallis. * Chi-squared ** IFCC standardization system (NGSP = [0.09148 × IFCC] + 2.152).

Ulcer size in cm^2 was similar between the two groups (11.1 range (2.5–32.5) vs. 12.2 (range 3–28) cm^2, $p > 0.05$, respectively, Table 1). The Saint Elian severity grades and score sums initially performed displayed no differences in distribution between groups for grades I through III.

Although total wound healing was 10% better for Group I (95 vs. 85%, respectively; Fisher's exact test), this difference ($p = 0.09$) was not significant (Table 2). Three patients in Group 1 and six patients in Group 2 did not heal. Patients included in Group 1 (Table 2) healed faster than those in Group 2 (10 ± 5.7 vs. 14.5 ± 8.9 weeks, $p < 0.05$; respectively), with a shortened duration of epithelialization phase (3.5 ± 4 vs. 6.4 ± 3.6 weeks, $p < 0.05$).

Table 2. Average duration of epithelialization, wound healing and severity grades for study groups.

Outcomes	Group 1 Epifast n = 40 (%)	Group 2 Standard Care n = 40 (%)	*p* Value
Complete wound healing *	38 (95)	34 (85)	0.09
Time to healing [weeks mean ± SD] **	10 ± 5.7	14.5 ± 8.9	0.003
Epithelialization time [wks mean ± SD] **	3.5±4	6.4 ± 3.6	0.001
Wound healing by severity grades (SEC) [wks mean ± SD] ***			
Grade I	1.7 ± 0.4	2.3 ± 3.0	0.003
Grade II	2.3 ± 2.0	6.1 ± 3.0	
Grade III	12.2 ± 3.3	16.5 ± 3.4	

(SEC) = Saint Elian Classification * Fisher's exact test ** Kruskal–Wallis *** ANOVA.

The Kaplan–Meier survival probability analysis revealed significant differences in healing times between Group 1 and Group 2 (Figure 2).

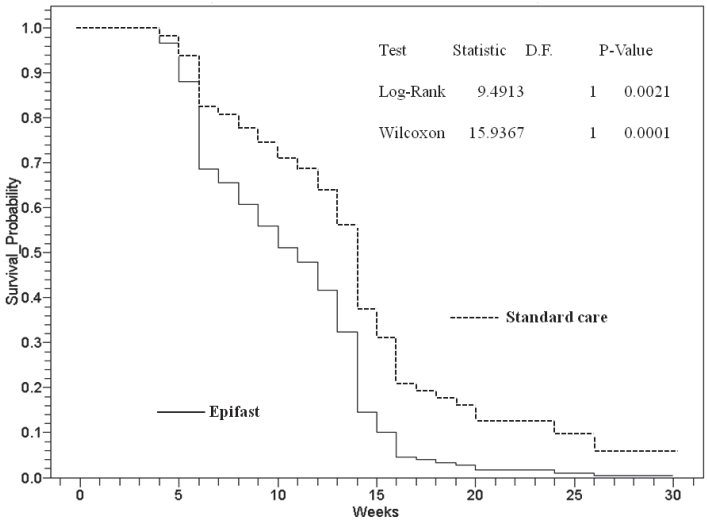


Figure 2. Kaplan–Meier survival probability analysis of wound healing failure by weeks for study groups with a mean follow-up period of 365 days. Survivors were non-healing patients.

Cox proportional hazards showed that Group 1 had a 50% faster healing time than the standard care group (Hazard ratio 0.5, 95% CI = 0.3–0.8; $p < 0.01$), with a likelihood ratio of 7.8 for delayed healing in Group 2 ($p < 0.05$). The Epifast group healed faster at 16 weeks versus the SC group that reached 100% wound closure by 30 weeks ($y = -1.6429x + 14.571$; $r = 0.94464$ and $y = -2.5x + 12.5$; $r = 0.89928$, respectively).

At 52 weeks of follow-up, one patient from Group 1 developed re-ulceration at a different location. No cases of re-ulceration occurred in Group 2. Excessive granulation tissue was observed in eight patients in Group 1 and 2 patients in Group 2 ($p < 0.05$). The excessive granulation tissue was removed surgically as many times as necessary (mean, 4.2 ± 3.2 weeks). After complete re-epithelialization, no further excessive granulating activity was observed during the follow-up period.

In Group 1, one patient had major amputation secondary to severe acute renal failure (ARF), remaining chronically wounded after the end of the study follow up (two patients) and reinfection (one patient). Patients in Group 2 failed to heal due to reinfection (four patients), and death secondary to ARF (one patient), and major amputation (one patient).

4. Discussion

This is the first study to show improved wound healing with application of Epifast to the wound in patients with non-healing DFUs compared to standard care. Overall patients in the former group had improved wound epithelialization and significant faster time to healing.

Our study reports an average healing time of 67 days for Group 1 vs. 97 days for the control. In Group 2, the completeness of wound closure (95% for Epifast vs. 85% for standard care) was similar but slightly superior to a previous study of Dermagraft which reported a median percent wound closure of 91% compared to 78% for the control group [6].

In the present study, we showed that patients treated with Epifast healed faster than the standard care group due to shortened re-epithelialization time of diabetic foot ulcers. The healing time was reduced compared to the control group. The similar proportions achieved for the higher wound healing success rates in both study groups indicates that patients included in our study were randomly assigned during the advanced granulation phase, that was not included within the results and discussion of the Dermagraft study. During this phase, infection, ischemia, or other aggravating factors were not present due to

good treatment response achieved prior to randomization with strong progress towards wound healing.

Patients included in the present study by random assignment were a selected sample with a high probability of healing and then randomized to active or control groups. Even those patients with severity grades of III showed good progress toward healing at the time of randomization. As we reported previously, this response is achieved in only 30% of grade III patients [8].

We also showed that Epifast enhanced epithelialization to varying extents according to wound severity. Differentiating wound severity (mild, moderate and severe) was important for analyzing the time to healing and was used as a confounder to control bias in group comparisons. Epifast shortened the wound healing time compared with the control group as wound severity increased. The severity of wounds was graded using the Saint Elian Scoring System [8,9] avoiding limitations and erroneous interpretations when documenting diabetic foot ulcers characteristics [10,11]. Conversely, the SEWSS internal and external validation reports an inter-observer analysis with high concordance between two observers [8,9]. In the present study, the observers showed a high Kappa agreement index of 0.88 and 0.92 to score categorization and grade of the Saint Elian system and for the assessment of healing progress during re-epithelialization, respectively.

Different biological treatments have been investigated to facilitate wound healing in patients with diabetic foot ulcers, such as Leucopatch [12], stem cell therapy [13,14], and Dermagraft [6] with varying results. Recent advances in stem cell therapy using mesenchymal stem cells and pluripotent stem cells is promising [15,16], but has not been translated into better wound healing and diabetic foot outcomes [17]. The differences in the biological characteristics of human skin equivalents compared to natural skin are due to the autologous nature of human skin equivalents, as well as their structure and composition of a mixture of biological and non-biological substances that are categorized as epidermal, dermal, and full-thickness skin substitutes. Epifast, which belongs to the first category, is a cryopreserved allograft of neonatal human epidermal foreskin cultured keratinocytes that constitute an epidermal layer without dermal components [7]. The use of keratinocytes stimulate migration of native keratinocytes from the wound edge [18]. A cell-based therapy is a highly promising approach for DFU treatment for ischemic DFU including mesenchymal stem cells that can be effective to provide an adjuvant therapy for limb salvage. The superiority of some specific cell types for DFU treatment is controversial. Undoubtedly, cell therapy is a potent tool for the treatment of DFU. However, further high-quality clinical research to determine the most effective cell type for DFU treatment must be conducted.

4.1. Side Effects

Our results indicate an incidence of benign hyper-granulation tissue. This condition is likely a side effect related to growth factor activity that enhances the formation of granulation tissue. Growth factors and extracellular matrix proteins are present in the frozen cultured sheets of human epidermal keratinocytes used during wound healing [18]. Although cancer risk has been reported in patients treated with recombinant human platelet-derived growth factor [19], hyper-granulation tissue in the present study was completely benign.

4.2. Study limitations

This trial was a single blind study that compared standard care instead of placebo, in contrast to a double-blind trial design. Although sample size calculations were made, the lack of significant results for rates of total wound healing (p equal to 0.09) can be explained by type II error (the null hypothesis is not rejected when it is in fact false) and must be addressed by increasing the sample size in future studies. For Kaplan–Meier analysis, a minimum sample size of 30 patients is required; therefore, this test assumption was

satisfied. However, a multicenter study with a bigger sample size could help to strength these preliminary results.

5. Conclusions

In conclusion, we have demonstrated significantly faster healing in patients with diabetic foot ulcers that were treated with human epidermal keratinocytes (Epifast) than standard care due to better re-epithelialization of the ulcers (Figure 3). Our results might be extended to those patients who achieve a granulation phase and are prone to complete re-epithelialization and therefore could facilitate faster wound healing.

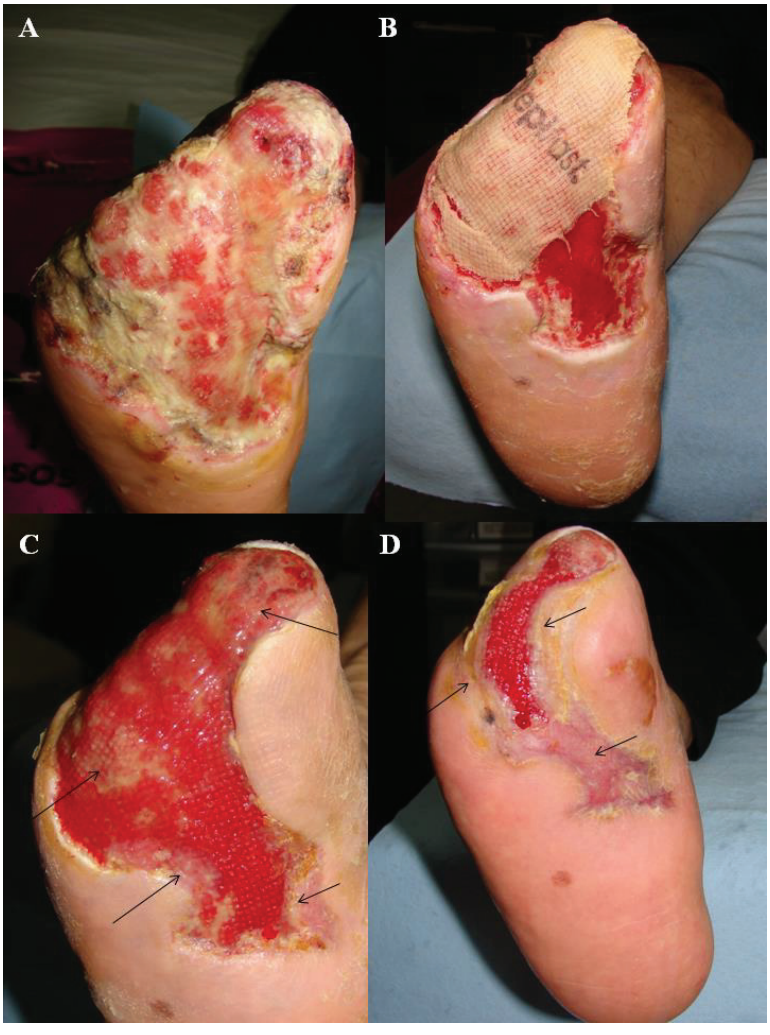


Figure 3. (**A**) 100 cm^2 complex, difficult to heal diabetic foot wound in the granulation phase after random assignment to the Epifast group. (**B**) A granulating wound covered by Epifast after 3 weeks, enhanced by growth factors present in the Epifast. (**C**) Arrows indicate newly formed skin 8 weeks after treatment. (**D**) Spread of epithelialization after 16 weeks. Total epithelialization was achieved 2 weeks later.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki, and approved by the Institutional Ethics Committee of our Center. Code number 0008/2015A.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Data supporting reported results can be found within the archives of the clinical charts in our center.

Conflicts of Interest: The authors declare no conflict of interest.

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Validation of the Ischaemia Severity Scale (ISS) Based on Non-Invasive Vascular Assessments (SEWSS) for Predicting Outcomes of Diabetic Foot Attack

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Abstract: Assessment of ischaemia severity includes a variety of measures, such as pedal pulse palpation, the ankle/brachial index (ABI), and the toe/brachial index (TBI), but there is a lack of consensus regarding which ischaemia scale is the most effective for determining outcome prognosis. The purpose of this study is to validate the application of the ischaemia severity scale (ISS) in the effective prediction of wound healing, amputations, and mortality for diabetic foot wounds (DFW). This prospective study included 235 consecutive patients graded according to the Saint Elian Wound Score System (SEWSS). The ISS is part of this system, with patients being scored as non-ischaemic (0) or having mild (1), moderate (2), or severe (3) ischaemia. Age, diabetes duration in years, and ulcer size were found to be associated with a longer mean ischaemia of increasing severity. A trend of reduction in the pulse palpation rates (70.4%, 50%, 8.5% to 0%; $p < 0.01$), ABI (1.1 ± 0.1, 0.86 ± 0.3, 0.68 ± 0.2, 0.47 ± 0.2, $p < 0.01$), TBI average values (0.90 ± 0.35, 0.62 ± 0.52, 0.50 ± 0.33, 0.10 ± 0.42, $p < 0.01$), wound healing success (88.7%, 57.7%, 40.7%, 12.9%; $p < 0.01$), and delay in weeks (Kaplan–Meier: log-rank 44.2, $p < 0.01$) was observed with increasing values of the ISS (0, 1, 2, and 3). The odds ratio for adverse outcomes increased for each additional level of ischaemia severity. Thus, we demonstrate that the ISS is useful in effectively predicting adverse outcomes for DFW.

Keywords: wound healing; amputations; ischaemia; diabetic foot; ankle/brachial index; toe/brachial index

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1. Introduction

Diabetic foot syndrome is defined as an acute or chronic attack characterised by one or more foot wounds that can differ in aetiology, complexity, and severity grade factors, including extent, depth, anatomic zones and aspect locations, infection, ischaemia, oedema, and neuropathy, and it is associated with increased amputations and death risk in persons with diabetes [1]. Foot wounds are among the most complex and heterogeneous complications in diabetic patients. A foot wound is defined as a breakdown in the protective function of the skin below the ankle in a diabetic patient, irrespective of its aetiology or duration. In our previous study [2], we demonstrated that the outcomes for ischaemia are a relevant part of comprehensive severity wound scoring for variables that positively or negatively affect wound healing progress. Ischaemia is included among the prognosis factors scored and graded homogeneously, from mild to severe, as part of this classification system. Ischaemia has the worst prognosis of the ten severity factors assessed for wound healing progress, amputations, and death in diabetic foot patients. Patients with ischaemia of varying severity have increased odds ratios for major amputations and wound healing failure compared with non-ischaemic patients [2]. The assessment of ischaemia in a clinical setting includes questioning and clinical examination in combination with a variety of

measures, such as pedal pulse palpation, the ankle/brachial index (ABI), the toe/brachial index (TBI), and waveform analysis, but there is no consensus regarding which assessment method is the most effective for diagnosis [3,4]. The ABI is a very useful clinical index for assessing arterial blood supply to the foot, but there are limitations when used in consideration of people with diabetes [4]. Medial calcification in diabetes, known as Mönckeberg's sclerosis, causes the incompressibility of the foot arteries, which might affect the accuracy of the ankle/brachial index [5,6]. Autonomic neuropathy and chronic renal insufficiency are highly associated with Mönckeberg's sclerosis [7]. Interval ABI and TBI results are used to monitor the efficacy of revascularisation procedures in the lower extremities and to predict wound healing and future cardiovascular-related morbidity and mortality [8]. There is limited research on the reliability of these non-invasive vascular tests in patients with varying stages of diabetic foot wounds. We hypothesised that a comprehensive diagnostic approach with the addition of several tests, including a register of the Doppler waveform analysis, would improve the accuracy of prognoses, so we used this approach for the assessment of the ischaemia severity level as part of the system for classifying diabetic foot wound severity [2]. In this study, we validate the application of the severity grade scores for ischaemia that are included in wound severity classification for predicting the wound healing rate, major amputations, deaths, and treatment abandonment in patients with diabetic foot wounds.

2. Materials and Methods

Consecutive patients with type 2 diabetes and diabetic foot wounds who arrived for care at our centre were initially included in the study.

2.1. Primary Objective and Measurements

The primary endpoint was the rates of wound healing, major amputations, deaths, and patient treatment abandonment for each level of the ischaemia severity scale (ISS) in patients with diabetic foot wounds. All patients provided written informed consent, and the Human Subjects Ethics Committee reviewed and approved the study.

The inclusion criteria were patients with type 2 diabetes who were over 18 years of age and had wounds at or distal to the malleoli with different degrees of infection, oedema, ischaemia, neuropathy, anatomic factors, and tissue affection. The extent and depth of tissue death was included as part of each wound assessment. Wound size was measured as the maximum length by the maximum width. Wounds secondary to gangrene debridement or after the surgical removal of infected tissue were included. Exclusion criteria included a diagnosis of severe cardiovascular or renal failure or severe neurological problems that would make the patient a poor candidate for the study (e.g., being bedridden). Patients with no family assistance were also excluded from the study.

2.2. Demographic, Clinical, and Wound Characteristics

Baseline demographic measurements were performed at the first visit. The diagnosis of diabetes was made prior to enrolment. The severity and type of diabetic foot attack was immediately diagnosed at presentation. Oedema, neuropathy, vascular, and infection assessments and wound characteristics were included and assessed according to the Saint Elian Wound Score System for diabetic foot attack. This system uses the following ten wound variables categories, which are measured at patient presentation: (1) primary location, (2) topographic aspects, (3) number of affected zones, (4) ischaemia, (5) infection, (6) oedema, (7) neuropathy, (8) depth, (9) area, and (10) wound healing phase. Each factor was subcategorised using an ascending severity score from mild (1 point) to severe (3 points). The score sum was a maximum of 30 points. A score sum of 10 points or fewer was graded as I (mild; successful wound healing likely). A moderate score of 11 to 20 points was graded as II (partial foot threatening; outcome related to "state-of-the-art" therapies used and associated with a good patient biological response), and 21 to 30 points was

graded as III (limb- and life-threatening; outcome unrelated to "state-of-the-art" therapies due to poor biological patient response).

2.3. Non-Invasive Vascular Assessment to Categorise Severity Grades of Ischaemia (ISS)

Ischaemia levels of mild (1 point), moderate (2), and severe (3) were categorised following non-invasive vascular assessment. We chose the test with a reputed higher accuracy when there were any discrepancies between the results of one or more methods used to scale ischaemia severity. The evidence-based predictive values escalated from pedal pulse palpations to ABI, TBI, and waveform pulse analysis. Doppler waveform analysis with graphic report was performed only in patients with ischaemia. Patients were diagnosed, clinically or according to the ABI, TBI, and waveform analysis results, for subcategorisation as ischaemic (scaled 1 to 3) or non-ischaemic (scaled as zero) patients.

2.3.1. Pulse Palpation

Two different members of staff determined ischaemia severity according to the scale using the dorsalis pedis and tibialis posterior arteries of the foot: palpation of a bound strong arterial pulse (0, non-ischaemic), palpable but slightly diminished (1, mild), thready and scarcely palpable (2, moderate), and non-palpable pulses (3, severe).

2.3.2. Ankle/Brachial and Toe/Brachial Index

All participants were required to lay supine for a minimum of ten minutes prior to any assessment, and systolic pressure was measured. The tibialis posterior and dorsalis pedis artery pressures were assessed and used for the ABI calculation (Hand-held Doppler–Huntleigh, Getinge AB; 8 MHz Doppler probe). A regular pulse was found, and the sphygmomanometer was pumped up slowly to a maximum of 200 mmHg to occlude digital blood flow. The systolic pressure was obtained by deflating the cuff. Toe pressure was measured using a 2.5 cm wide $\times$ 9 cm long digital cuff on the proximal aspect of the hallux to calculate the TBI. A PPG unit Hadeco Smartdop 30EX was used when no toe artery was found using a Doppler. The toe/brachial index and ABI were determined by dividing the higher systolic pressure of the toe or ankle with the maximum blood pressure of the arms. The ABI and TBI were separately calculated for each leg, and the measurement of the wounded limb of the two values was taken as the result for the study patient. Ischaemia was defined as an ABI < 0.9 and TBI < 0.75. The following range categories of the ABI for the ISS were used: 0.9 to 1.2 (0, normal), 0.7 to 0.89 (1, mild), 0.5 to 0.69 (2, moderate), and <0.50 (3, severe). The following TBI levels for the ISS were used: >0.75 (0, normal), 0.60 to 0.74 (1, mild), 0.30 to 0.59 (2, moderate), and <0.30 (3, severe).

2.3.3. Index Agreement

Two members of staff independently conducted each non-invasive test for the assessment of the ISS. The kappa agreement index was calculated using 2 $\times$ 2 tables to detect differences between the two observers. A kappa value (K $\frac{1}{4}$ Po-Pe/1-Pe; Po $\frac{1}{4}$ observed agreement, Pe $\frac{1}{4}$ expected agreement by random) between 0.61 and 1.0 indicates substantial to excellent agreement.

2.4. Standard of Care Treatment and Therapeutic Intervention

All patients were treated using an outpatient ambulatory model, which included appropriate surgical debridement, aggressive parenteral/intramuscular broad-spectrum antimicrobial administration, appropriate off-loading, and strict glycaemic control. Angioplasty and bypass were performed in patients with ischaemia who accepted the procedure. All patients were initially followed on a daily basis and, depending on the condition of the wound, were seen every third day or once weekly. Cardiovascular disease, nephropathy, retinopathy, and neurological problems were assessed. The follow-up period was part of the normal treatment duration according to the healing success or failure plus a secondary follow-up within a minimum of 6 months for delayed wound healing. The patient was

deemed to have completed the study once total ulcer healing was achieved. Early and direct deaths were those that occurred as a consequence of the diabetic foot wounds during therapy or within 30 days after. Delayed mortality occurred after this early period as a consequence of conditions other than an acute diabetic foot problem. Consecutive score measurements were recorded at different dates. Daily wound images and wound measurements were recorded in a database for systematic data collection.

Significance was considered at $p < 0.05$. Values of chi-square with Yate's correction or the Fisher's exact test with 2×2 tables or Mantel–Haenszel χ^2 for linear trends, and variance ratios for natural and treatment analysis of variance, were calculated. Kaplan–Meier probability, log-rank test, and Cox hazard ratio analyses were performed for wound healing time and measured against the study's ischaemia severity scores. The study population included every patient who was consecutively assessed by their random presentation. Because the entire population was included, it was not necessary to calculate the sample size.

3. Results

3.1. Patients

A total of 235 patients with type 2 diabetes and diabetic foot wounds who consecutively presented for care at our centre were initially assessed and included in the study. Each patient was invited to participate in the study at presentation and informed that the assessment was part of their diagnosis and treatment and was being performed for research purposes. Outcomes were assessed during a mean follow-up period of 52 weeks, which was extended to 156 weeks for assessing delayed mortality. The treatment period duration was 9.3 ± 9.2 weeks (range 1 to 30 weeks).

3.2. Demographic, Clinical, and Wound Characteristics

According to the ischaemia severity scale, there were 159 non-ischaemic patients (67.6%), 24 (10.2%) patients with mild ischaemia, 25 (10.6%) patients with moderate ischaemia, and 27 (11.4%) patients with severe ischaemia. There were no differences due to gender, smoking, HbA1c, or wound history in weeks. Age, diabetes duration in years, ulcer size, and severity wound score average exhibited an increasing trend for increasing grades of ischaemia severity (Table 1). Ischaemia occurred at a very low rate in patients at SEWSS grade I with a mild severity score (8.3%) and successful wound healing, and no patient with this grade was observed when the ISS was moderate and severe. However, at Saint Elian severity wound grade II (partially foot-threatening), there were no differences in patient ratings for any ISS. A significant ascending trend of ISS patient ratings of severity wound grade III (limb- and life-threatening) was observed (Table 1). The ten categories of Saint Elian classification.

The severity factor scores of the Saint Elian classification exhibited similar proportions ($p > 0.05$) to the Ischaemia Severity Score (from zero to three), aside from in the number of affected zones and the area size grades ($p < 0.05$). The percentages of patients with small wound sizes diminished as the severity of ischaemia increased (83.6%, 70.8%, 64.5%, and 44.4% from 0 to 3, respectively), and this was also the case for medium (10.1%, 12.5%, 16%, and 37%, respectively) and large (6.3%, 16.7%, 20%, and 18.5%, respectively) wound sizes ($p < 0.01$). There was a descending trend in ischaemia rates for one (76.4%, 10.8%, 6.8%, and 6.1%, respectively), two (56.3%, 9.4%, 18.8%, and 15.6%, respectively), and three affected foot zones (43.5%, 8.7%, 13%, and 34.8%, respectively). Non-ischaemic patients exhibited a decreasing trend in ischaemia rates from one to two and three affected foot zones (71.1%, 22.6%, and 6.3%, respectively). Conversely, patients with increasing ischaemia severity exhibited an increasing number of affected zones.

Table 1. Baseline demographic, clinical, and wound characteristics.

	Ischaemia Severity Scale				
	0	**1**	**2**	**3**	
	No	**Mild**	**Moderate**	**Severe**	
Characteristic	***n* = 159**	***n* = 24**	***n* = 25**	***n* = 27**	***p* Value**
Age (y) +	61.3 ± 11.1	71.9 ± 9.5	69 ± 10.5	71.3 ± 7.3	<0.01
Gender **					
Male	68 (42.8)	14 (58.3)	13 (52)	14 (51.9)	0.21
Female	91 (57.2)	10 (41.7)	12 (48)	13 (48.1)	
Diabetes duration in years +	17.7 ± 8.9	22.6 ± 10.9	19.8 ± 11.5	24.1 ± 12.7	0.03
HbA1c +	8.6 ± 2.3	7.4 ± 2.3	8.0 ± 1.9	7.8 ± 2.1	0.19
Smoking **	52 (33)	6 (25)	6 (24)	7 (26)	0.29
Wound history (weeks) +	5.8 ± 7.5	8.6 ± 9.8	6.6 ± 8.8	7.5 ± 5.8	0.41
Wound size cm^2	11.3 ± 36.4	13.9 ± 22.3	19.8 ± 36.5	19.1 ± 18.9	<0.01
Saint Elian score means ± SD +	14.6 ± 3.7	15.6 ± 4.0	17.8 ± 3.6	19.3 ± 3.2	<0.01
Saint Elian wound severity grades **					
I (good prognosis for wound healing)	23 (14.5)	2 (8.3)	0	0	0.03
II (partially foot-threatening)	124 (78)	18 (75)	18 (72)	18 (66.7)	0.16
III (limb- and life-threatening)	12 (7.5)	4 (16.7)	7 (28)	9 (33.3)	<0.01

Values are the mean ± SD or actual (percent); ** Mantel–Haenszel chi square for trends or + Kruskal–Wallis.

Non-Invasive Vascular Tests to Categorise Severity Grades for Ischaemia

A pulse palpation test was performed, and the ABI calculated for 100% of the study patients (30% resulted with Mönckeberg's sclerosis). The TBI was calculated in 88.1% of the population because no pulse was found or because there was a previous amputation or a wound involving the first ray. Doppler waveform analysis with graphic reporting was performed in 81 patients (34.4%). All patients were submitted to angioplasty or bypass (15.5%), including Doppler waveform analysis, as part of the surgical protocol to assess the arterial flow starting at the femoral common level. The pulse palpation rates of the foot arteries revealed a decreasing trend when progressing from non-ischaemic patients to a mild, moderate, and severe grade of ischaemia. There were no pulse palpations on the dorsalis pedis and tibialis posterior arteries when ischaemia was categorised as severe. The systolic ankle pressures were 124 ± 44.4, 125 ± 58.7, and 95 ± 61.8 and toe pressures were 91.5 ± 62.3, 81.3 ± 77.4, and 26.5 ± 48.2 for mild, moderate, and severe grades, respectively ($p < 0.01$). A reducing trend in the average ankle and toe/brachial index values with increasing ischaemia severity grades was confirmed. In total, 81 of the 235 (34.4%) patients who were previously diagnosed as patients with ischaemia could be differentiated based on their ISS levels when including pulse palpation and the ABI and TBI values. Pulse waveform analysis confirmed the absence of ischaemia with a normal triphasic wave in five of these patients (3.1%) and different grades for ischaemia in 76 of these patients (96.9%). No normal triphasic waveforms were recorded for any ISS score for ischaemia severity. A biphasic waveform was prevalent for mild ischaemia. A monophasic waveform was prevalent at a moderate score (76%). No pulse wave was recorded for severe ischaemia in 81.4% of the patients. The test results to categorise ischaemia revealed significant differences in non-ischaemic patients compared to patients with ascending grades of ischaemia severity (Table 2).

Table 2. Non-invasive vascular assessment to categorise ischaemia according to grade of severity.

	Ischaemia Severity Scale				
	0	1	2	3	
	No	Mild	Moderate	Severe	
Non-Invasive Vascular Tests	***n* = 159**	***n* = 24**	***n* = 25**	***n* = 27**	***p* Value**
Pulses palpation of foot arteries					
Dorsalis pedis	112 (70.4)	12 (50)	2 (8.5)	0	<0.01 *
Tibialis posterior	103 (64.7)	10 (41.6)	1 (4)	0	<0.01 *
Ankle/brachial index	1.1 ± 0.1	0.86 ± 0.3	0.68 ± 0.2	0.47 ± 0.2	0.01 +
Toe/brachial index	0.90 ± 0.35	0.62 ± 0.52	0.50 ± 0.33	0.10 ± 0.42	<0.01 +

Values are the mean ± SD or actual (percent); * Mantel–Haenszel chi-square for trends or + Kruskal–Wallis.

3.3. Kappa Agreement Index

The observers showed high agreement, 0.91 and 0.94, for the categorisation of normal pulse palpation, wherein non-ischaemic patients scored 0, and the absence of pulse palpation (scored as 3 points) in severe ischaemic patients, respectively. The agreement scores descended to 0.7 and 0.68 for mild and moderate ischaemia, respectively, based on scoring by pulse. The ABI, TBI, and waveform pulse recordings showed a kappa agreement index above 0.78 for all levels of the ischaemia severity scale.

Outcomes for Primary Endpoints

Wound healing success and failure were validated according to the subcategories for ischaemia. The Kaplan–Meier survival probability analysis for wound healing failure by weeks demonstrated that the delay in the wound healing time differed according to the graded ischaemia severity (Figure 1).

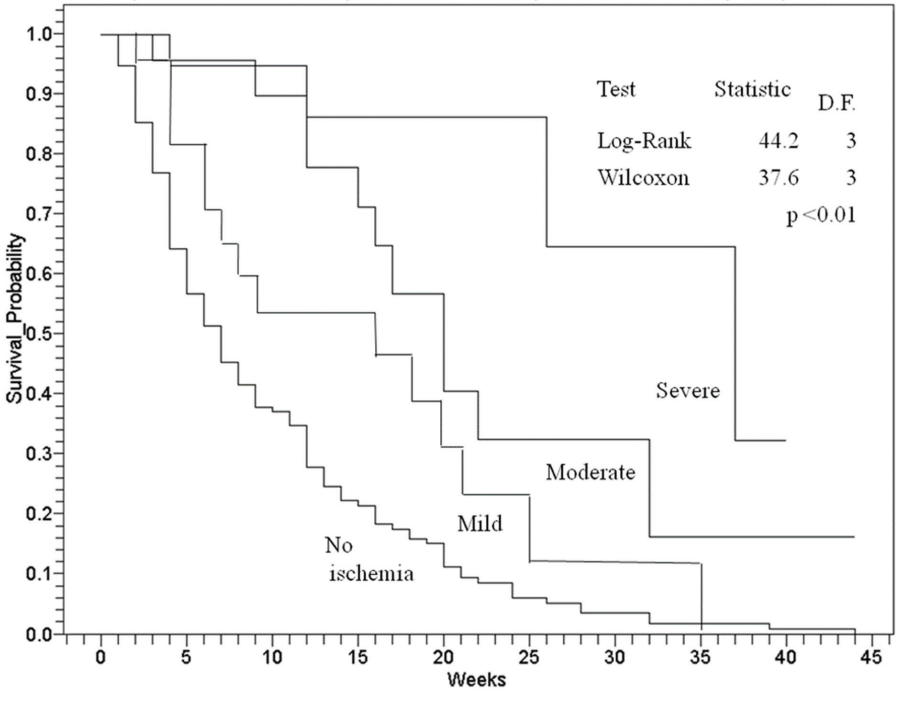

Figure 1. Kaplan–Meier survival probability analysis for wound healing failure by weeks according to the severity of ischaemia.

The Cox proportional hazard scores demonstrate that the survival probability for wound healing success was significantly higher for mild ischaemia (hazard ratio: 9.5; 95% CI 2.1–41.2, $p < 0.01$) and non-ischaemic patients (hazard ratio: 13.8; 95% CI 3.4 to 56.1, $p < 0.01$) than for severe ischaemia (likelihood ratio of 52; $p < 0.05$). The hazard ratio was not significantly different when comparing moderate and severe ischaemia ($p < 0.05$). Wound healing rates decreased significantly according to the severity of ischaemia, and the levels of amputations and patient abandonment of treatment increased (Table 3).

Table 3. Outcome rates and odds ratio for ischaemia severity scale in diabetic foot patients.

	Ischaemia Severity Scale			
Outcomes	**0 No** ***n* = 159**	**1 Mild** ***n* = 24**	**2 Moderate** ***n* = 25**	**3 Severe** ***n* = 27**
Wound healing *	134 (88.7)	15 (57.7) [2.7, 1.0–7.5]	11 (40.7) [9.5, 3.8–23.5]	4 (12.9) [74.4, 16.5–335]
Major amputation *	7 (4.4)	3 (12.5) [3.4, 0.8–14.4]	4 (16) [3.9, 1.0–14.8]	19 (70.3) [51.9, 17–59.2]
Abandonment of treatment *	28 (17.6)	7 (29.2) ** [2.0, 0.7–5.3] ***	9 (36) [2.7, 1.1–6.5]	13 (48.1) [4.6, 1.9–10.9]
Early deaths **	3 (1.9)	0	2 (8) [4.2, 0.6–26.9]	1 (3.7) [1.9, 0.1–19.7]

Wound healing: percentages are for success and odds ratio for failure. Values are actual (percent) and odds ratio [OR, 95% C.I.]. * Significant values for Mantel–Haenszel chi-squared for trend, ** non-significant values. All odds ratio values are significant for logistic regression, except *** non-significant value.

The probabilities of healing failure, major amputations, delayed death, and treatment abandonment were determined using odds ratio calculations of the ischaemia severity grades against non-ischaemic patients (Table 3) with diabetic foot wounds (likelihood ratios of 79.7, 60.5, 25, and 15.1, respectively). The odds ratio for delayed mortality as a consequence of kidney or cardiac failure or stroke increased significantly as the ischaemia severity grade increased, but the Cox hazard ratios were not significantly different. Early mortality as a direct consequence of diabetic foot was not significant and found to be independent of the ischaemia severity scale.

4. Discussion

The results of this study confirm that there are differences between scales according to ischaemia severity grades. The range of score subcategories from non-ischaemic patients, to mild, moderate, and severe ischaemia (0–3), was predetermined by a review of published values for pedal pulses, the ABI, TBI, and waveform analysis. A variance or failure of concordance of the scores of each test used to categorise the ISS was expected as an alternative to the null hypothesis. However, the characterisation of ischaemia severity levels was validated by the hard data obtained from the non-invasive tests in our study. The variability in the published reports in terms of the reliability of these tests is controversial, particularly with regard to their predictive value when used as a single test. Most of these studies were designed to evaluate sensitivity and specificity to detect the percentage of arterial stenosis using angiography as the "gold standard" [8]. Reliability in measuring perfusion distal to the ankle is required to adequately assess and treat patients with diabetic foot, where the concern is to determine ischaemia severity as a predictor of wound healing and amputation outcomes. Angiography is an invasive test that is not currently used for diagnosis, but it is fundamental in planning the surgical approach when necessary. Angiograms are not safe, and possible complications include allergic reactions to the contrast dye, damage to blood vessels, blood clots, bleeding, and kidney damage, particularly for patients with diabetes and whose kidney functions are already impaired. Knowledge of the length, level, and number of stenoses provided by angiography in the diabetic foot is needed to plan and perform the corrective surgical procedure after diagnosis, and this level of arterial obstruction was achieved using vascular non-invasive tests. Therefore, we performed

predictive analysis using discrete choice models based on logistic regression (odds and likelihood ratios) and the survival probability of Kaplan–Meier analysis for non-healing patients. The reliability may be improved by adding the values of different non-invasive tests to increase the accuracy of clinical judgements. The palpation of pedal pulses is a subjective measurement, and the palpation of pulses becomes increasingly difficult as ischaemia becomes more severe. There is also large inter-rater variability among inexperienced clinicians when determining the palpation of pedal pulses [3,9–11], but pedal pulse palpation is the only way to assess the arterial perfusion of the feet in many primary care settings. Our study provides data to support the application of a pulse palpation assessment of the ischaemia severity scale in selecting patients for referral to a vascular or diabetic foot unit or deciding to continue their care at the same level. The ABI is a reliable measure of peripheral arterial disease in patients without diabetes, with excellent sensitivity and specificity [12]. However, the ABI has limited applicability in patients with long-standing diabetes because of the likelihood of falsely elevated readings [3,7]. Neither the Society for Vascular Technology [13] nor a consensus paper [14] explain how the limits of the ABI range were derived. The ranges for the ABI appear to be derived from original data in Yao [13], Cornwall [14], and Sumner [15]. Numerous methods of calculating the ABI have been described [16–20] based on variances in the numerator in the ABI equation: (a) the current method uses the high ankle pressure (HAP) of the two ankle systolic arterial pressures as the numerator in the ABI equation [18]; (b) a second method uses the low ankle pressure (LAP) of the two ankle systolic arterial pressures as the numerator [17,18]; (c) a third calculation uses the average of the two ankle systolic pressures as the numerator in the ABI equation [18]; (d) a few studies have used the tibialis posterior artery systolic pressure to calculate the ABI [20]. We used the HAP to calculate the ABI based on the dorsalis pedal or tibialis posterior arteries. A comparison of the four assessment methods is underway to determine which ABI modalities are superior in predicting outcomes. The limitations to using the ABI are that the ABI is age- and blood pressure-dependent, it encompasses the arterial flow of the anterior and tibialis posterior arteries, and it does not identify any occlusion or calcification of vessels distal to this site [21]. Calculating the toe brachial index (TBI) solves these problems [21], but it is not feasible to register the digital pulses in some patients if there is a previous amputation or a wound at the first toe (11.9% in the present study). Using a Doppler probe, toe pulses were not detected in 28 patients (15%), and the use of the PPG unit Hadeco Smartdop was a useful alternative [22]. Toe pressure and the TBI may be used as an adjunct to a standard peripheral arterial assessment performed by general practitioners, podiatrists, vascular surgeons, and nurses to obtain quantitative baseline measures or to confirm the diagnosis and severity of ischaemia in diabetic foot patients. The pulse palpation, the ankle brachial pressure index, and the toe brachial pressure index were useful for assessing the severity of ischaemia. Our results validate the ISS with non-invasive assessments, which must be available for health care professionals in primary settings. Our study shows that toe pressure, as a non-costly test, is easy to determine for use in grading ischaemia severity, increasing its potential when combined with pulse palpation, plethysmography, and pulse wave registration. If the primary care setting lacks these sets of tests, then pulse palpation provides a reasonable alternative to the ISS scale. In one classification [23], the scale for ischaemia was validated according to TcPo2, the determination of which is expensive and frequently unavailable at diabetic foot centres. We were unable to measure transcutaneous oxygen pressures (TcPO2), as the diagnostic kit for this was not available for our patients. We were advised of its variability and would suggest it has value as part of a scoring system rather than in isolation. As part of the WiFi classification, TcpO2 fails to be superior over toe pressure measurements for haemodynamic monitoring during endovascular revascularisation [24]. Future research must clarify the impacts of these systems in health care for the prevention of amputations due to diabetic foot attack secondary to ischaemia.

The results of the present study clearly differentiate non-ischaemic from ischaemic patients and their severity grades. Non-ischaemic patients were found to be younger

and have shorter diabetes duration and the smallest wound sizes in cm^2. Patients with ischaemia revealed ascending severity scores for age, diabetes duration, and wound size, which negatively affected the prognoses for wound healing, amputations, and deaths. The Kaplan–Meier analysis and odds ratio results confirm differences in wound healing failure according to ischaemia severity grades, which may be explained as a consequence of the "poor" biological response of these patients, caused by the ageing process and body damage secondary to a longer duration of diabetes. The study population included every patient who was consecutively assessed by their random presentation. The entire population, and not a sample, was submitted to Kaplan–Meier analysis to avoid violations of the test assumptions. Ischaemia severity correlated with the Saint Elian severity grades with a high impact on outcomes, which were positive for grade I and negative for grade III. The odds ratio for direct early mortality secondary to a diabetic foot wound was not significant, but there is an increase in the OR for delayed mortality according to the ISS in the follow-up. Our report is in accordance with the current evidence for the use of the ABI in the identification of patients at high risk of future cardiovascular and cerebrovascular mortality [24,25]. A systematic review of 11 published studies on 44,590 subjects was performed by Heald et al. [26], who reported that an ABI < 0.9 is associated with an increase in all causes of mortality, cardiovascular and cerebrovascular mortality, coronary heart disease, and fatal and non-fatal stroke. In our study, we confirmed that the odds ratio increases for delayed mortality (cardiovascular, renal, or stroke) accordingly with the ischaemia severity grades in persons with diabetes and diabetic foot wounds.

5. Conclusions

Our results may assist health care professionals in wound care within a diabetic foot clinic or at primary care facilities through using the ISS to select the most appropriate treatments based on the probability of healing in patients with wounds along with monitoring. The Saint Elian score [1,2,27–29] for severity wound grades provides a platform for prevention, diagnosis, and prognosis. Aggravating factors, such as oedema, infection, neuropathy (Charcot) [30], and ischaemia, must be independently assessed and treated in therapeutic decisions. These treatments may involve revascularisation, amputation, or conservative management. The ischaemia severity scale provides the clinician with a better understanding of healing potential and whether there is an opportunity for the vascular team to improve the flow to the extremities using revascularisation techniques, such as angioplasty or bypass surgery. The ISS is useful for predicting adverse outcomes of DFW.

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Data Availability Statement: Data supporting the reported results can be found within the archives of the clinical charts in our Centre.

Conflicts of Interest: The authors declare no conflict of interest.

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Mortality Risk Associated with Diabetic Foot Complications in People with or without History of Diabetic Foot Hospitalizations

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Abstract: The aim of this study was to evaluate the risk of death after hospitalizations for diabetic foot (DF) complications, comparing two different cohorts of people with or without a prior history of DF hospitalizations across the years 2011 to 2018 in Tuscany, Italy. The DF complications were categorized by administrative source datasets such as: amputations (both major and minor), gangrene, ulcers, infections, Charcot and revascularizations. A further aim was to present the trend over time of the first ever incidents of diabetic foot hospitalizations in Tuscany. The eight-year-mortality rate was higher in the cohort with prior hospitalizations (n = 6633; 59%) compared with the cohort with first incident DF hospitalizations (n = 5028; 44%). Amputations (especially major ones) and ulcers had the worst effect on survival in people without basal history of DF hospitalizations and respectively in those with a history of prior DF hospitalizations. In both cohorts, revascularization procedures, when compared to ulcers, were associated with a significantly reduced risk of mortality. The prevalence rate of minor amputations showed a slightly rising trend over time. This result agrees with the national trend. Conversely, the progressive increase over time of revascularizations, associated with the fractional decrease in the rate of gangrene, suggests a trend for more proactive behavior by DF care teams in Tuscany.

Keywords: diabetic foot complications; mortality risk; hospitalizations; first ever incident diabetic foot hospital admission; amputations; diabetic foot ulcers

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1. Introduction

Diabetic foot (DF) is a leading cause of disability; it represents huge costs for healthcare systems and early mortality in people with diabetes [1–10]. A point which remains to be more extensively addressed is evaluating the different impact on death risk after a first ever incident of diabetic foot hospital admission for DF complications compared to the risk for people who experienced a previous DF hospitalization: all this would help to better understand the natural history of DF, its costs and, consequently, the resources to be allocated for care or prevention of DF and its complications. A recent paper, for instance, has shown that lower limb amputations are among the most expensive complications of diabetes and that their cost is significantly high, particularly after a first ever incident of diabetic foot hospitalization [11]. In addition, while among all DF complications amputations and diabetic foot ulcers seem to be associated with a higher risk of mortality [12,13], evidence has also been accumulated over time that revascularization procedures, which have an important role in the strategy of anatomical and functional rescue of lower limbs,

protect from premature death [14,15]. To better elucidate these issues, we have carried out a retrospective observational study using administrative data sources regarding death incidence related to hospitalizations for DF in the region of Tuscany, in central Italy, over the years 2011 to 2018. The first aim of the present study was to evaluate the trend of first incident hospitalizations due to each DF complication across the entire period of eight years in this population. A further goal was grading the risk of death associated with each single foot lesion, as diagnosed from hospital discharges in two different cohorts: in people with prevailing DF lesions at basal as testified by the history of prior DF hospitalizations, and in those who were hospitalized for diabetic foot complications for the first time.

2. Materials and Methods

2.1. Study Design and Data Source

The population under study consisted of all identified people with diabetes residing in Tuscany, a region of central Italy, as of 1 January 2011, retrospectively followed up until 31 December 2018. The diagnosis of diabetes was based on a validated algorithm by utilizing administrative databases at the Regional Health Agency of Tuscany, in Florence, Italy, as previously detailed [16]. Such regional dataset has been validated and shown to cover more than 80% of all diabetic patients living in Tuscany [17]. This initial population was divided into two cohorts: the first including all individuals who had no previous hospitalizations for DF complications as of 1 January 2011, or at entry into the study. The second cohort included all individuals with a history of previous DF hospitalizations at baseline.

2.2. Definition and Classification of DF Complications

DF hospitalizations were recorded according to any of the following ICD-9 CM codes: ulcers: 440.23, 707.14, 707.15; Charcot neuro-arthropathy: 713.0, 713.5, 713.8; infections: 6811, 6819, 6826, 6827, 6829, 730.07, 730.17, 730.27, 99.21; gangrene: 440.20, 440.21, 440.22, 440.23, 440.29, 443.9, 785.4, 440.0, 440.24; major and minor lower extremity amputations: 84.10–84.19; revascularizations (surgical: 39.25, 39.29; endoluminal: 39.50, 39.90). In both cohorts, the presence of co-morbidities was diagnosed according to the Charlson index. This index is an integrated indicator referring co-morbidities as from all previously hospital discharges [18] and scored as 0, 1 or 2, thus reflecting the increase in their complexity and severity.

2.3. Outcomes and Statistical Procedures

The incidence of death (all-cause mortality) occurring within the period 1 January 2011 to 31 December 2018 was retrieved in both cohorts from the database of the regional registry office. Time to event was considered as the interval from the first ever incident of diabetic foot hospitalizations or from 1 January 2011 to death or to end of study, and survival rates were determined through Kaplan-Meier curves.

After testing for proportionality of risks, the Cox proportional hazards model has been used to assess the hazard ratios (HRs) of all-cause death after any incidental first-ever DF complication in a model where foot ulcers were the reference group and after adjusting for Charlson index, sex, age and antidiabetic therapy. In this cohort, the incidence rates of DF complications across the entire period 2011–2018 was calculated by trend test after chi-square. Among those with prevailing DF hospitalizations at basal, death HRs were calculated by means of Cox proportional hazards models, and the time to event was considered as the interval from 1 January 2011 to death or to end of study, after adjusting for the same covariates and with foot ulcer as the reference group.

All data were anonymized and based on administrative datasets, preventing any disclosure of patients' identity as well as of any other sensitive information. Because of such formal protection, no informed consent or any approval by an Ethics Committee was required, according to current national and regional rules.

All analyses were performed using SAS ver. 9.3, SAS Institute Inc., Cary, NC, USA.

3. Results

The main characteristics of the two cohorts under study are reported in Table 1. Both cohorts contained about the same number of hospitalizations (11,246 vs. 11,529), and age was on average more advanced in those without prior DF hospitalizations (74 ± 10 yr vs. 71 ± 11 yr; $p < 0.05$). Males were more represented in both cohorts, even if with a preponderance significantly lower among those without previous hospitalizations at basal (61.6% vs. 66.1%; $p < 0.05$). Most first ever DF hospitalizations were due to revascularization procedures or to gangrene, with lower rates for amputations and Charcot. The incidence rate of ulcers was similar to that of infections: 22.3; 95% CI 21–23.8 per 1000 p-y vs. 20.4; 95% CI 21–23.8 per 1000 p-y. Comorbidities were more severe in those with prior DF hospitalization at baseline, with the percentage of Charlson index ≥2 approximately twice as high: 75.8% vs. 41.7%; $p = 0.0001$. Therapy with insulin was about twice as prevalent in the cohort with prior DF hospitalizations at baseline as compared with the cohort with first incident hospitalizations. In the cohort without prior hospitalizations for diabetic foot, there were 5028 deaths with a 56% survival rate at the end of follow-up. Instead, in the cohort with prior hospitalizations, there were 6633 deaths with a survival rate at follow-up of 41%; $p < 0.05$. The rate of deaths was higher after any first incident hospitalized complication, especially after both major or minor amputations and gangrene. However, revascularizations and ulcers had approximately the same mortality incidence rate in the two cohorts (0.40; 95% CI 0.37–0.42 per 1000 p-y vs. 0.39; 95% CI 0.37–0.42 per 1000 p-y for revascularizations and 0.67; 95% CI 0.60–0.74 per 1000 p-y vs. 0.72; 95% CI 0.67–0.77 per 1000 p-y for ulcers). The prevalence rates for any diabetic foot complications, evaluated by trend test after chi-square across the total eight-year period, showed no significant trend, except for the curve of gangrene, which had a negative slope (Figure 1; Table 2). The rates of minor amputations and revascularizations increased over the entire period; trend test: $p < 0.0001$ for both. Survival analysis estimated by Kaplan-Meier curves showed that in the cohort without prior hospitalizations, major amputations had the worst survival rate over time, while in the cohort with prevalent diabetic foot complications at baseline, ulcers were associated with the poorest prognosis (Figure 2). Both major and minor amputations showed a significantly higher risk of death only in the cohort without previous hospitalizations. This was verified after calculating adjusted HRs of death through Cox regression models, considering ulcers as the reference group (Figure 3). Ulcers had a worse prognosis compared to gangrene, infections and Charcot in the cohort with prevalent diabetic foot at baseline. Revascularizations had a protective effect against mortality by about 30–40% in both cohorts.

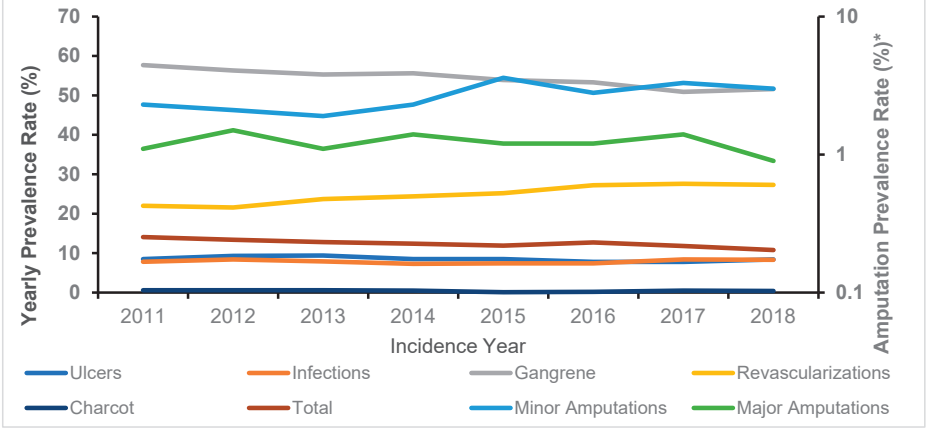

Figure 1. Prevalence rates of hospitalizations for diabetic foot complications across the years 2011 to 2018 in Tuscany (* Logarithmic scale).

Table 1. Descriptive analysis for variables of both cohorts under study, with or without a prior history of hospitalizations for diabetic foot complications.

Diabetic Foot Lesions	Major Amputations	Minor Amputations	Revascularizations	Gangrene	Infections	Charcot	Ulcer	Total
without Prior Hospitalizations for Diabetic Foot								
No. (%)	143 (1.2)	306 (2.6)	2854 (24.7)	6282 (54.5)	908 (7.9)	51 (0.4)	985 (8.5)	**11,529**
Incidence rate of first hospitalization per 1000 p-y (95%CI)	3.0 (2.5–3.5)	6.5 (5.8–7.3)	77.3 (74.5–80.2)	293.2 (286–300.6)	20.4 (19.1–21.7)	1.1 (0.8–1.4)	22.3 (21.0–23.8)	**4.6 (4.5–4.7)**
Mean Age yr (SD)	74 (12)	68 (13)	70 (10)	73 (10)	66 (13)	62 (13)	72 (12)	**71 (11)**
Male Sex No. (%)	73 (51)	199 (65)	1875 (66)	3865 (54.4)	548 (60.3)	30 (58.8)	518 (52.6)	**61.6**
Charlson index								
0 No. (%)	46 (31.2)	145 (47.4)	1149 (40.3)	2364 (37.6)	435 (47.9)	22 (43.1)	413 (41.9)	**39.7**
1 No. (%)	22 (15.4)	54 (17.6)	520 (18.2)	1182 (18.8)	165 (18.2)	10 (19.6)	188 (19.1)	**18.6**
2+ No. (%)	75 (52.4)	107 (35.0)	1185 (41.5)	2736 (43.6)	308 (33.9)	19 (37.3)	384 (39.0)	**41.7**
Therapy (%)								
Insulin	14.0	13.4	12.0	11.9	10.6	15.7	13.1	**12.0**
Oral	42.7	41.5	41.5	44.4	36.6	29.4	44.2	**42.9**
Insulin/oral	10.5	13.1	11.8	11.8	13.0	25.5	15.3	**12.3**
None	32.9	32.0	34.6	31.9	39.9	31.4	27.4	**32.8**
No. of deaths; Incidence rate of death per 1000 p-y (95%CI)	96; 1.63 (1.33–1.99)	122; 0.58 (0.48–0.69)	939; 0.40 (0.37–0.42)	3087; 0.76 (0.73–0.79)	310; 0.40 (0.36–0.45)	12; 0.20 (0.12–0.36)	462; 0.67 (0.60–0.74)	**5028;** 0.61 (0.59–0.63)
with Prior Hospitalizations for Diabetic Foot								
No. (%)	39 (0.3)	86 (0.8)	1561 (13.9)	7049 (62.7)	1273 (11.3)	113 (1.0)	1125 (10.0)	**11,246**
Mean Age yr (SD)	71 (13)	69 (14)	74 (9)	75 (10)	69 (13)	67 (14)	73 (11)	**74 (10)**
Male Sex (%)	31 (72)	54 (60)	1099 (71)	4822 (64.8)	739 (58.1)	63 (55.8)	629 (55.9)	66.1
Charlson index (%)								
0 No. (%)	11 (25.6)	23 (25.6)	70 (4.5)	440 (6.2)	185 (14.5)	14 (12.4)	44 (3.9)	**7.0**
1 No. (%)	11 (25.6)	23 (25.6)	284 (18.3)	1122 (15.9)	287 (22.6)	21 (18.6)	183 (16.3)	**17.2**
2+ No. (%)	21 (48.8)	44 (48.8)	1199 (77.2)	5487 (77.9)	801 (62.9)	78 (69.0)	898 (79.8)	**75.8**
Therapy (%)								
Insulin	16.3	21.1	14.2	21.4	25.6	30.1	34.7	**22.3**
Oral	37.2	35.6	42.6	37.8	31.8	23.0	27.7	**36.6**
Insulin/oral	9.3	13.3	9.9	16.3	17.9	18.6	22.8	**16.2**
None	37.2	30.0	33.3	24.5	24.7	28.3	14.8	**24.9**
No. of deaths; Incidence rate of death per 1000 p-y (95%CI)	22; 0.54 (0.35–0.81)	39; 0.30 (0.22–0.40)	818; 0.39 (0.37–0.42)	4320; 0.56 (0.55–0.58)	633; 0.35 (0.32–0.38)	52; 0.30 (0.23–0.40)	749; 0.72 (0.67–0.77)	**6633; 0.51 (0.50–0.53)**

Table 2. Prevalence rates of hospitalizations for diabetic foot complications across the years 2011 to 2018 in Tuscany. Hospitalization rate for diabetic foot complications = −038 × incidence rate + 14.2.

Year	Ulcers	Infections	Gangrene	Charcot	Revascularizations	Major Amputations	Minor Amputations	Total
	No. (%)	No. (%)	No. (%)	No. (%)	No. (%)	No. (%)	No. (%)	No. (%)
2011	138 (8.5)	127 (7.8)	940 (57.7)	9 (0.6)	358 (22.0)	18 (1.1)	38 (2.3)	1628 (14.1)
2012	144 (9.3)	130 (8.4)	871 (56.3)	10 (0.6)	335 (2.6)	24 (1.5)	33 (2.1)	1547 (13.4)
2013	139 (9.4)	117 (7.9)	814 (55.3)	9 (0.6)	348 (23.7)	16 (1.1)	28 (1.9)	1471 (12.8)
2014	122 (8.5)	104 (7.3)	795 (5.6)	7 (0.5)	349 (24.4)	20 (1.4)	33 (2.3)	1430 (12.4)
2015	117 (8.5)	102 (7.4)	743 (53.9)	1 (0.1)	348 (25.2)	17 (1.2)	50 (3.6)	1378 (11.9)
2016	114 (7.8)	109 (7.4)	780 (53.3)	3 (0.2)	399 (27.2)	18 (1.2)	41 (2.8)	1464 (12.7)
2017	106 (7.8)	115 (8.4)	695 (50.9)	7 (0.5)	377 (27.6)	19 (1.4)	45 (3.3)	1364 (11.8)
2018	105 (8.4)	104 (8.3)	644 (51.6)	5 (0.4)	340 (27.3)	11 (0.9)	38 (3.0)	1247 (10.8)
P for trend	NS	NS	0.0001	NS	<0.0001	NS	0.007	0.0001

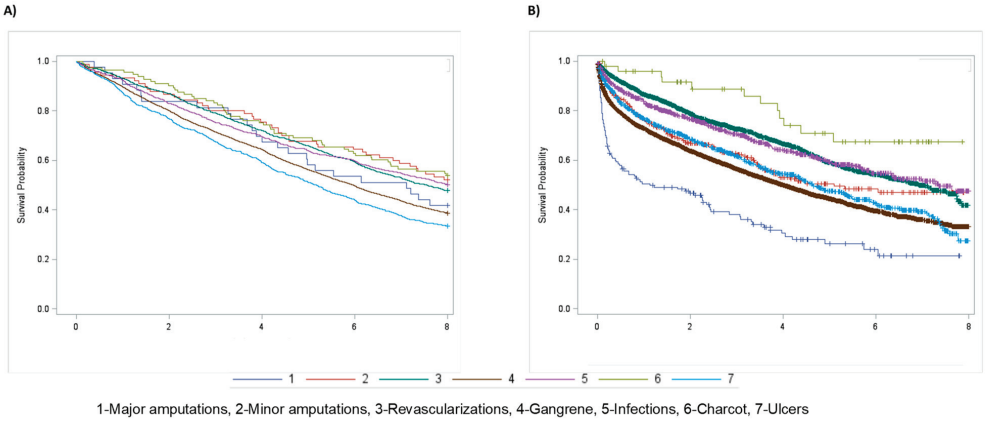

Figure 2. Survival probability by Kaplan Meier analysis for diabetic foot complications in people with previous hospitalizations for diabetic foot (**A**) and after first incident hospitalization for diabetic foot (**B**).

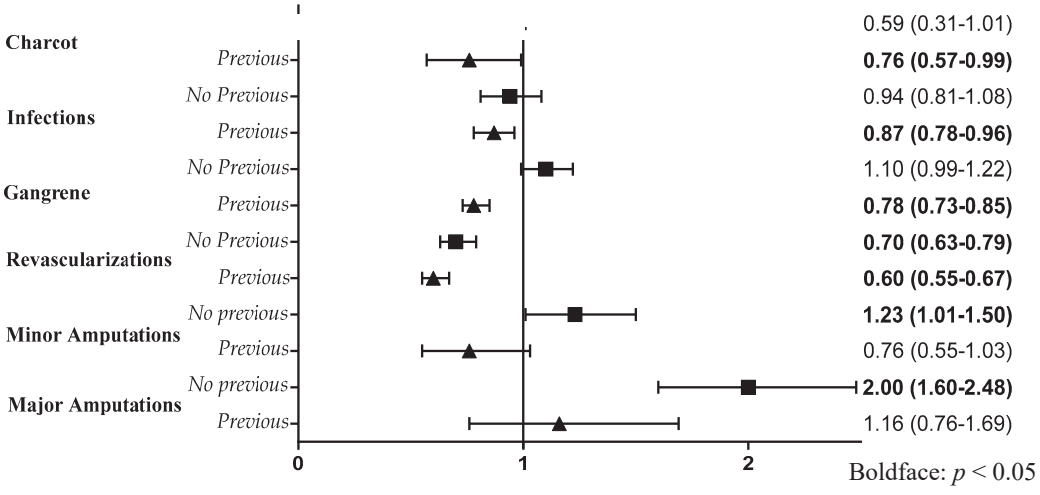

Figure 3. Adjusted Hazard Ratios (HR) of diabetic foot complications in both cohorts with (▲) and without (■) previous hospitalizations for diabetic foot. Ulcers are considered here as the reference group.

4. Discussion

Diabetic foot is associated with a significant increase in the risk of premature death [3–12,19]. It has, indeed, been rightly said that the reduction in life expectancy of patients with diabetic foot can be compared to that of those affected with cancer [3]. The main purpose of the present study was to better define the role played by different DF complications in increasing the risk of mortality, comparing two different cohorts of people: those with or without prior hospitalizations for DF complications. The patients were followed retrospectively for eight years (2011–2018) in Tuscany, an Italian region that has about 3.5 million people. A further matter in question was to verify what role the procedures of revascularization played in eventually modifying the risk of death: a point that has not always been addressed by most prior studies. The yearly prevalence rate of first ever incidents of diabetic foot hospitalizations in Tuscany was substantially stable over time. The prevalence rate of major amputations was very low and remained unchanged over time. This result reflects, at least in part, the trend towards a

continuous slight decline of major amputations in Italy in last decade [20]. The prevalence rate of minor amputations was slightly rising over time, and this is again in agreement with a similar national trend [20], while the progressive increase over time of revascularizations, associated with the fractional decrease in the rate of gangrene, suggests more proactive measures by diabetic foot care teams in our region, particularly targeted at procedures for the rescue of lower limbs in patients with more advanced vascular ischemic diseases. Both cohorts under study, with or without prior DF hospitalizations, were significantly different in several respects: the cohort with prior hospitalizations was younger, contained more males, had more comorbidities and was more frequently treated with insulin, while the adjusted risks of death for each complication appeared substantially more impacting after a first hospitalization more closely related to ischemic vascular complications such as amputations and gangrene. Regardless of DF complications, total mortality rates at 8 years were higher in the cohort with prior hospitalizations (59%) as compared with the mortality of the cohort counting first incident hospitalizations for DF (44%); $p < 0.05$. This agrees with the range of death incidence rates reported by previous epidemiological studies referring to cohorts with or without a history of DF [5,6,13,21,22]. It is, however, difficult to compare mortality rates across different countries, since most studies were designed to compare the risk of mortality between people with diabetic foot lesions and those without diabetic foot, and, additionally, many studies did not distinguish between first and recurrent hospitalizations. It is noteworthy, however, that the greatest risk of death was represented by ulcers in those with prior hospitalizations and by major amputations in patients experiencing a first ever hospitalization for DF. In this respect, a recent epidemiological study regarding the incidence of hospitalizations and of overall mortality in Piedmont, a region of northwestern Italy, clearly demonstrated that mortality risk was significantly higher after the new incident hospitalizations for both minor and major amputations of lower limbs, considered as the most selective expression of vascular DF [23]. The higher prevalence of women (39% vs. 34%) among first incident DF hospitalizations compared with those with DF at basal is in line with what was previously reported [24]. This study, moreover, shows that in the cohort with prior hospitalizations at basal, in the presence of the competing risk of premature death after amputations, diabetic foot ulcers appear as the lesions with the worst prognostic effect regarding survival, in agreement with what is widely reported by the literature [2,6,7]. A recent study has moreover demonstrated that ulcers are associated with a lower amputation-free survival rate [13], and, consequently, ulcers, especially ischemic ulcers, could significantly predict amputations in both cohorts, mediating by this way their final effect on death risk, even if the design of this study is not able to clarify this aspect. In addition, in those with prior hospitalizations for DF complications, it is interesting to note that even a classical ischemic lesion such as gangrene has a lesser mortality risk when compared to ulcers seemingly associated with both ischemic and non-ischemic pathogenesis [25]. Revascularizations, on the contrary, are characterized by a significant reduction in the overall risk of mortality compared to ulcers in both cohorts, further highlighting the importance of revascularization procedures, not only to save the functional integrity of the lower limbs but also to improve life expectancy in these patients. In this respect, interestingly, the positive effect of revascularization is evident not only when it represents a first event but also among those with a history of previous hospitalizations for DF.

4.1. Limitations and Strengths of the Study

As with all retrospective cohort studies based on administrative data, the main limitation of this study is that the lack of clinical data prevents a more thorough evaluation of the eventual interrelationships between death risk and severity of foot lesions. A further limitation is having considered only hospitalizations (both ordinary and day-hospital discharges), excluding other care settings, even if hospitalizations could reasonably include all more complicated clinical situations. The strength of our study may be found in the vast sample of the population involved and in the solid methods used to identify diabetes, as well as in the homogeneity in treatment of patients, as expected from a single regional public health system with free access to all resident citizens.

4.2. Conclusions

The prevalence rates for diabetic foot complications generally showed no significant trend, except for gangrene, which had a negative trend. Rates of minor amputations and revascularizations have instead increased throughout the entire period. The progressive increase over time for revascularizations, associated with the fractional decrease in the rate of gangrene, suggests a trend for more proactive measures by DF care teams, particularly targeted at procedures for the rescue of lower limbs in these patients. According to this study, moreover, the adjusted risk of mortality after hospitalizations for DF complications was completely different in cohorts with or without a prior history of hospitalized DF complications. Those without prior history showed an overall lesser percentage rate of deaths compared those with a prior history of hospitalizations due to complications. Amputations (especially major) had the worst effect on survival in people without a history of DF hospitalizations at basal. In those with a history of prior DF hospitalizations, ulcers predicted the worst prognosis. In both cohorts, revascularization procedures significantly reduced the risk of mortality. These peculiarities should be taken fully into consideration when evaluating data from epidemiological studies about the death risk associated with DF complications and could be useful for health care providers and policy makers.

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Nutritional Status Assessed with Objective Data Assessment Correlates with a High-Risk Foot in Patients with Type 2 Diabetes

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Abstract: Malnutrition and diabetes are likely to co-occur. There are few reports on the association between nutritional status and foot risk in patients with type 2 diabetes (T2D). Therefore, we aimed to investigate this relationship in this cross-sectional study. We investigated the relationships between objective data assessment (ODA), especially Controlling Nutritional Status (CONUT) score and foot risk, evaluated by the International Working Group on the Diabetic Foot (IWGDF), in consecutive patients with T2D. Patients were divided into groups 0 to 3 by IWGDF, and groups 1 to 3 were defined as high-risk groups. Among 469 patients, 42.6% (n = 200) of them had high-risk foot. Patients with high-risk foot were significantly older (71.2 ± 11.3 vs. 64.2 ± 13.4 years, $p < 0.001$) and had a longer duration of diabetes (18.0 ± 12.0 vs. 11.5 ± 10.0 years, $p < 0.001$) than those in the low-risk group. In the high-risk group, serum albumin level, total lymphocyte count, hemoglobin, and CONUT score were significantly worse, especially in older patients (≥75 years). Multivariate logistic regression analysis showed that there was a positive correlation between CONUT score and high-risk foot in older patients (OR, 1.37; 95% CI, 1.05–1.86; p = 0.021). Our results indicated that nutritional status, assessed by ODA, correlated with high-risk foot, especially in older patients with T2D.

Keywords: clinical practice; diabetes; foot risk; nutritional status

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1. Introduction

Diabetic foot ulcers and gangrene are known to be caused by diabetic peripheral neuropathy (DPN) and peripheral artery disease (PAD), which affects 25% of patients with diabetes [1,2]. The infection causes or worsens foot ulcers due to complications of DPN and PAD [3]. Moreover, amputation of lower limbs due to diabetic foot ulcer and gangrene reduces patients' quality of life and results in a physical and mental burden on them and their families, as well as a huge financial burden on society [4]. Therefore, the importance of foot screening and foot care in patients with diabetes is widely recognized.

Malnutrition is influenced by several factors, and the nutritional status of patients with diabetes worsens due to diabetic complications and comorbidities [5]. Malnutrition worsens underlying diseases and leads to unfavorable prognosis in older patients with diabetes [6]. Malnourished patients with diabetes have been shown to be twice as likely to have foot injuries compared with nourished patients [6]. Maintaining and improving nutritional status is important in the treatment of foot ulcers and gangrene [7]; however, there are few reports on the relationship between nutritional status and the risk of diabetic foot in patients with diabetes. Therefore, we performed a cross-sectional study of patients with

type 2 diabetes to investigate the relationship between nutritional status, assessed using an objective data assessment (ODA), and diabetic foot risk, proposed by the International Working Group on the Diabetic Foot (IWGDF) [8].

2. Materials and Methods

2.1. Study Participants and Data Collection

We performed this study in accordance with the Declaration of Helsinki and obtained informed consent from all patients. This study was approved by the Ethics Committee of Otsu City Hospital (No. 213). We included patients with type 2 diabetes >20 years of age who were the outpatients of Otsu City Hospital (Otsu, Japan) and whose legs were examined and tested. Patients were assessed for smoking status using a self-administered questionnaire.

Blood samples were gathered in the morning after an overnight fast to measure hemoglobin (g/dL), total lymphocyte count (count/mL), hemoglobin A1c (%), creatinine (μmol/L), total cholesterol (mmol/L), cholinesterase (U/L), and serum albumin (g/dL). Complete blood counts and examinations were performed using a Beckman Coulter LH 780 instrument and Bio Majesty JCA-BM 6050 (JEOL, Tokyo, Japan). The Controlling Nutritional Status (CONUT) score was calculated using the data of serum albumin levels, total cholesterol levels, and total lymphocyte counts [9]; albumin levels $\geq$3.5, <3.5 and >3.0, <2.99 and $\geq$2.5, and <2.5 g/dL were scored as 0, 2, 4, and 6 points, respectively; total lymphocyte count of $\geq$1600, 1599–1200, 1199–800, and <800/mm^3 were scored as 0, 1, 2, and 3 points, respectively; and total cholesterol levels $\geq$180, 140–179, 100–139, and <100 mg/dL were scored as 0, 1, 2, and 3 points, respectively. The CONUT score was defined as the sum of scores, ranging from 0 to 12, with higher scores indicating a worse nutritional status.

Patients with acute inflammatory or infectious diseases, hematological diseases, malignancy, severe organ damage, including nephrotic syndrome or liver cirrhosis, or blood diseases were excluded from our study.

Type 2 diabetes was diagnosed as previously reported [10], and diabetic foot risk was categorized into groups using the IWGDF classification, as follows [8]: (0) (no loss of protective sensation (LOPS) and no peripheral artery disease (PAD)), (1) (LOPS or PAD), (2) (LOPS + PAD or LOPS + foot deformity or PAD + foot deformity), and (3) (LOPS or PAD and one or more of the following: history of a foot ulcer, a lower-extremity amputation (minor or major) and end-stage renal disease). We defined groups 1–3 as the high-risk group according to a previous report [11]. Examination of the lower limbs was performed by a certified nurse for diabetes nursing, a diabetologist, or a certified diabetes educator. DPN was diagnosed using the diagnostic criteria for diabetic neuropathy proposed by the Diagnostic Neuropathy Study Group [12]. Two or more abnormalities of three examination items were used to diagnose DPN: neuropathic symptoms such as neuropathic pain, paresthesia and numbness, decreased or absent ankle reflex (bilateral), and decreased distal sensation assessed by C128 Hz tuning fork without evident non-diabetic peripheral neuropathy. Diabetic retinopathy was diagnosed by an ophthalmologist as previously reported [13], and diabetic nephropathy was defined as nephropathy with urine microalbuminuria >30 mg/gCre [14]. PAD was diagnosed if at least one of the following was confirmed: ankle brachial pressure index (ABI) < 0.9 or absence of two or more pedal pulses on palpation. Foot deformity and musculoskeletal abnormalities were examined to detect hallux valgus deformity, hammer/claw toe deformity, and hallux limitus (limited motion at the metatarsophalangeal joint). Stratified analysis was performed between older ($\geq$75 years) and younger patients. We divided the patients according to statin use because statin usage decreases total cholesterol levels, which leads to increased CONUT scores.

2.2. Statistical Analysis

For statistical analysis, JMP v.9.0 (SAS Institute Inc., Cary, NC, USA) was used, and statistical significance was set at $p < 0.05$. A chi-square test, unpaired Student's *t*-test or analysis of variance, or post hoc Tukey–Kramer test was used for comparison analyses

between the groups. The data were analyzed by cross-tabulation, Pearson χ2 test, or Fisher's exact test. Multivariate logistic regression analysis was used to adjust for factors associated with nutritional status and high-risk foot. We selected covariates for multivariate analysis, including sex, BMI, age, duration of diabetes, current smoking status, creatinine level, HbA1c level, and hypertension.

3. Results

In this study, a total of 553 patients were included. Among them, 68 patients were excluded because of malignancy or blood diseases (n = 27), foot ulcers (n = 17), severe tissue damage (n = 12), liver cirrhosis (n = 4), acute inflammatory or infectious disease (n = 3), nephrotic syndrome (n = 3), and acute massive hemorrhage (n = 2). The clinical characteristics of study participants according to the IWGDF criteria are described in Table 1. Patients in group 1 and group 2 assessed using the IWGDF criteria were significantly older and had a longer duration of diabetes than those in group 0. Total cholesterol was significantly worse in group 2, and cholinesterase was significantly worse in group 1. Serum albumin level, hemoglobin, and CONUT scores were significantly worse in group 1 and 2.

Table 1. Clinical characteristics of the participants.

	Group 0	Group 1	Group 2	Group 3	p
n	269	150	38	12	
Age (years)	64.2 ± 13.4	70.6 ± 11.1 *	73.6 ± 11.0 *	68.8 ± 12.4	<0.001
Male (%)	62.5	54.4	51.2	75.0	0.077
Duration of type 2 diabetes (year)	11.5 ± 10.0	16.6 ±11.4 *	22.7 ± 12.0 *,†	17.5 ± 8.9	<0.001
BMI (kg/m^2)	25.0 ± 4.4	23.9 ± 4.8	24.4 ± 3.6	25.0 ± 5.0	0.225
Hemoglobin A1c (%)	7.2 ± 1.1	7.4 ± 1.2	7.7 ± 0.9	7.5 ± 1.0	0.142
Creatine (μmol/L)	69.7 ± 32.7	90.8 ± 86.9 *	91.9 ± 34.2 *	89.3 ± 36.4	0.002
Current smoking (%)	7.8	8.0	12.7	16.7	0.901
Statin use (%)	35.3	42.0	47.4	50.0	0.128
Hypertension (%)	54.6	70.7	81.6	83.3	0.001
Retinopathy (%)	22.3	40.0	42.1	41.7	<0.001
Nephropathy (%)	35.7	58.7	73.9	75.0	<0.001
Total cholesterol (mmol/L)	4.7 ± 0.9	4.5 ± 0.8	4.1 ± 0.5 *,†	4.1 ± 1.0	<0.001
Cholinesterase (U/L)	337.7 ± 95.6	309.4 ± 91.5 *	314.8 ± 92.9	311.2 ± 85.5	0.032
Serum albumin (g/dL)	4.2 ± 0.4	4.0 ± 0.4 *	3.9 ± 0.4 *	3.9 ± 0.6	<0.001
Hemoglobin (g/dL)	13.8 ± 1.6	12.8 ± 2.0 *	12.8 ± 1.8 *	13.8 ± 1.5	<0.001
Lymphocyte (count/mL)	2037 ± 857	1848 ± 714	1851 ± 848	1877 ± 589	0.134
CONUT	1 (1–3)	2 (1–5) *	3 (1–9) *	2 (1–4)	0.001

Continuous variables are presented as means ± 1 SD. Skewed variables are presented as medians (interquartile range). Categorical variables are presented as numbers (percentage). Group 0, no LOPS and no PAD; Group 1, LOPS or PAD; Group 2, LOPS + PAD or LOPS + foot deformity or PAD + foot deformity; and Group 3, LOPS or PAD and one or more of the following: history of a foot ulcer, a lower-extremity amputation (minor or major), or end-stage renal disease. LOPS, loss of protective sensation; PAD, peripheral artery disease; BMI, body mass index. * $p < 0.05$ vs. Group 0; and † $p < 0.05$ vs. Group 1.

In Table 2, the clinical characteristics of study participants were compared according to low- or high-risk IWGDF criteria. Patients with high-risk foot, assessed using the IWGDF criteria, were significantly older and had a longer duration of diabetes than those in the low-risk group. Total lymphocyte count, hemoglobin, cholinesterase, serum albumin level, and CONUT scores were significantly worse in the high-risk foot group (Table 2).

Table 2. Comparisons of variables between low and high foot risk category in all patients.

	Low Foot Risk	High Foot Risk	*p*
n	269	200	
Age (years)	64.2 ± 13.4	71.2 ± 11.3	<0.001
Male (%)	62.5	55.0	0.147
Duration of type 2 diabetes (year)	11.5 ± 10.0	18.0 ±12.0	<0.001
BMI (kg/m^2)	25.0 ± 4.4	24.1 ± 4.7	0.051
Hemoglobin A1c (%)	7.2 ± 1.1	7.4 ± 1.2	0.059
Creatine (μmol/L)	70.7 ± 35.3	88.4 ± 79.7	<0.001
Current smoking (%)	8.2	8.5	0.776
Statin use (%)	38.7	43.5	0.093
Hypertension (%)	57.2	73.5	<0.001
Retinopathy (%)	22.5	40.5	<0.001
Nephropathy (%)	37.9	62.0	<0.001
Total cholesterol (mmol/L)	4.7 ± 0.9	4.5 ± 0.8	0.005
Cholinesterase (U/L)	337.7 ± 95.6	313.1 ± 94.2	0.007
Serum albumin (g/dL)	4.2 ± 0.4	4.0 ± 0.4	<0.001
Hemoglobin (g/dL)	13.8 ± 1.6	12.9 ± 1.9	<0.001
Lymphocyte (count/mL)	2037 ± 857	1858 ± 724	0.017
CONUT	1 (1–3)	2 (1–4)	0.001

Continuous variables are presented as means ± 1 SD. Skewed variables are presented as medians (interquartile range). Categorical variables are presented as numbers (percentage). BMI, body mass index; CONUT, controlling nutritional status.

A comparative analysis indicated that older patients (≥75 years) had worse nutritional status, as assessed by several ODAs, whereas no significant difference was found in glycemic status and the proportion of statin use. The proportions of hypertension and microangiopathy were higher in older patients (Table 3).

Table 3. Comparisons of variables between patients <75 and ≥75.

	<75 Years	≥75 Years	*p*
n	339	130	
Age (years)	61.1 ± 10.8	80.8 ± 4.3	<0.001
Male (%)	61.7	53.1	0.197
Duration of type 2 diabetes (year)	12.3 ± 10.5	20.4 ±11.8	<0.001
BMI (kg/m^2)	25.2 ± 4.8	23.3 ± 3.8	<0.001
Hemoglobin A1c (%)	7.3 ± 1.2	7.4 ± 1.0	0.694
Creatine (μmol/L)	70.7 ± 53.3	88.3 ± 44.1	0.022
Current smoking (%)	9.1	6.2	0.191
Statin use (%)	38.6	47.7	0.101
Hypertension (%)	63.1	71.5	0.027
Retinopathy (%)	27.4	38.5	0.011
Nephropathy (%)	43.1	64.6	<0.001
Total cholesterol (mmol/L)	4.6 ± 0.9	4.5 ± 0.8	0.019
Cholinesterase (U/L)	339.3 ± 99.8	297.9 ± 82.8	<0.001
Serum albumin (g/dL)	4.1 ± 0.4	3.9 ± 0.5	<0.001
Hemoglobin (g/dL)	13.7 ± 1.6	12.6 ± 1.8	<0.001
Lymphocyte (count/mL)	2047 ± 833	1647 ± 584	<0.001
CONUT	1 (1–3)	2 (1–4)	<0.001

Continuous variables are presented as means ± 1 SD. Skewed variables are presented as medians (interquartile range). Categorical variables are presented as numbers (percentage). BMI, body mass index; CONUT, controlling nutritional status.

A stratified analysis between older (≥75 years) and younger groups showed that serum albumin was significantly low in group 3 and hemoglobin was significantly low in group 1 in the older group (Table 4a). In the group younger than 75 years of age, serum albumin levels in group 1 and 2 were low and hemoglobin was low in group 1 at significant

levels (Table 4b). In all age groups, there were no significant differences in BMI and HbA1c with or without foot risk. The proportions of hypertension and nephropathy had significant differences in each group, and the disease duration was significantly longer in group 2 in both the older and younger groups (Table 4a,b).

Table 4. (**a**) Comparisons of variables in patients ≥75 years. (**b**) Comparisons of variables in patients <75 years.

(a)					
	Group 0	**Group 1**	**Group 2**	**Group 3**	***p***
n	45	64	22	4	
Age (years)	79.6 ± 4.2	80.8 ± 4.3	83.8 ± 4.2 *	78.8 ± 3.8	0.021
Male (%)	57.8	51.6	45.5	75.0	0.510
Duration of type 2 diabetes (year)	16.0 ± 11.1	21.5 ±11.1	28.1 ± 13.3 *	17.8 ± 11.0	0.002
BMI (kg/m^2)	22.8 ± 3.5	23.1 ± 3.6	23.9 ± 3.8	23.9 ± 2.9	0.576
Hemoglobin A1c (%)	7.3 ± 0.9	7.4 ± 0.9	7.9 ± 1.1	7.4 ± 1.5	0.058
Creatine (μmol/L)	70.6 ± 26.4	91.9 ± 55.1	93.6 ± 35.6	94.3 ± 56.8	0.153
Current smoking (%)	6.7	4.7	4.5	25.0	0.883
Statin use (%)	44.4	39.1	40.9	50.0	0.842
Hypertension (%)	60.0	65.6	90.9	75.0	0.048
Retinopathy (%)	17.8	42.2	45.5	50.0	0.003
Nephropathy (%)	48.9	57.8	81.8	75.0	0.012
Total cholesterol (mmol/L)	4.8 ± 1.0	4.4 ± 0.8	4.3 ± 0.6	4.4 ± 1.4	0.149
Cholinesterase (U/L)	305.5 ± 85.8	290.1 ± 84.4	307.6 ± 84.0	281.0 ± 92.3	0.779
Serum albumin (g/dL)	4.1 ± 0.6	3.9 ± 0.4	3.8 ± 0.3	3.5 ± 0.7 *	0.005
Hemoglobin (g/dL)	13.1 ± 1.6	11.9 ± 1.9 *	12.2 ± 1.8	13.1 ± 1.9	0.009
Lymphocyte (count/mL)	1692 ± 762	1613 ± 593	1702 ± 712	1873 ± 417	0.871
CONUT	2 (0–3)	2 (1–5)	3 (1–9)	5 (1–11)	0.049
(b)					
	Group 0	**Group 1**	**Group 2**	**Group 3**	***p***
n	224	86	16	8	
Age (years)	59.5 ± 11.5	64.1 ± 9.7 *	64.3 ± 7.0	62.6 ± 11.3	0.004
Male (%)	63.4	58.1	50.0	75.0	0.289
Duration of type 2 diabetes (year)	10.5 ± 9.5	12.9 ±10.4	17.6 ± 12.1 *	15.3 ± 9.7	0.007
BMI (kg/m^2)	25.4 ± 4.5	24.5 ± 5.5	24.6 ± 3.9	25.6 ± 5.5	0.496
Hemoglobin A1c (%)	7.2 ± 1.2	7.4 ± 1.3	7.4 ± 0.7	7.5 ± 0.8	0.788
Creatine (μmol/L)	73.3 ± 38.1	76.3 ± 32.3 *	88.7 ± 41.5 *	81.8 ± 31.6	<0.001
Current smoking (%)	8.5	10.5	12.5	12.5	0.511
Statin use (%)	37.5	44.2	56.3	50.0	0.438
Hypertension (%)	56.7	74.4	68.8	87.5	0.029
Retinopathy (%)	23.2	38.3	37.5	37.5	0.075
Nephropathy (%)	35.7	59.3	56.3	75.0	<0.001
Total cholesterol (mmol/L)	4.7 ± 0.9	4.8 ± 0.9	4.2 ± 0.6	4.0 ± 0.9	0.011
Cholinesterase (U/L)	344.9 ± 96.2	323.7 ± 102.4	323.0 ± 109.6	331.1 ± 77.6	0.368
Serum albumin (g/dL)	4.2 ± 0.3	4.0 ± 0.5 *	3.9 ± 0.5 *	4.1 ± 0.3	0.002
Hemoglobin (g/dL)	13.9 ± 1.6	13.3 ± 1.9 *	13.4 ± 1.7	14.1 ± 1.2	0.038
Lymphocyte (count/mL)	2106 ± 860	2010± 758	1979 ± 980	1890 ± 650	0.729
CONUT	1 (1–3)	1 (1–3)	3 (1–7)	2 (1–3)	0.062

Continuous variables are presented as means ± 1 SD. Skewed variables are presented as medians (interquartile range). Categorical variables are presented as numbers (percentage). Group 0, no LOPS and no PAD; Group 1, LOPS or PAD; Group 2, LOPS + PAD or LOPS + foot deformity or PAD + foot deformity; and Group 3, LOPS or PAD and one or more of the following: history of a foot ulcer, a lower-extremity amputation (minor or major), or end-stage renal disease. LOPS, loss of protective sensation; PAD, peripheral artery disease; BMI, body mass index; CONUT, controlling nutritional status. *, $p < 0.05$ vs. Group 0.

Multivariate logistic regression analyses showed that the CONUT score was associated with a high-risk foot in the older group, after adjusting for several factors. This relationship was not observed in the younger group of patients (Table 5). Moreover, multivariate logistic regression analyses showed a correlation between CONUT score and high-risk foot in the

older group, regardless of statin use (Table S1a). This relationship was not observed in the younger group (Table S1b).

Table 5. Multivariate-adjusted ORs (95% CI) for high-risk diabetic foot assessed with IWGDF.

	<75 Years		≥75 Years	
	OR (95%CI)	*p*	OR (95%CI)	*p*
Age	1.05 (1.02–1.08)	0.002	1.02 (0.93–1.12)	0.667
Male	1.82 (1.04–3.23)	0.037	2.68 (1.05–7.19)	0.038
Duration of type 2 diabetes	1.02 (0.99–1.04)	0.157	1.06 (1.01–1.10)	0.007
BMI	0.99 (0.93–1.05)	0.701	1.10 (0.96–1.25)	0.174
Hemoglobin A1c	1.32 (1.05–1.65)	0.022	1.53 (0.97–2.53)	0.157
Creatine	1.02 (1.01–1.03)	<0.001	1.01 (0.99–1.02)	0.319
Hypertension	1.52 (0.83–2.79)	0.223	1.78 (0.67–4.81)	0.241
Current smoking	1.53 (0.66–3.47)	0.301	2.24 (0.44–13.5)	0.332
CONUT	0.94 (0.87–1.01)	0.107	1.37 (1.05–1.86)	0.021

OR, odds ratio; CI, confidence interval; IWGDF, International Working Group on the Diabetic Foot; BMI, body mass index; CONUT, controlling nutritional status.

4. Discussion

This study revealed that patients with type 2 diabetes and with high-risk foot were older, had a longer duration of diabetes, had poor glycemic control, and had a worse renal function. In addition, their nutritional status, as assessed by ODAs, was significantly worse, especially in older patients.

Diabetes is often associated with malnutrition, especially in older patients, and the association has been previously reported [15–18]. Malnutrition in patients with diabetes and high-risk foot is known to be associated with inflammation-related atherosclerosis, leading to amputation of the lower extremities in addition to known risk factors [19]. Therefore, timely nutritional assessment is needed for patients with diabetes and high-risk foot. Although Mini Nutritional Assessment (MNA) and subjective global assessment (SGA) are well-known nutritional assessment screening tools [20,21], they are not always easy to perform routinely in clinical practice. SGA is a well-established tool for nutritional assessment [20]; however, it is subjective and requires an evaluator with some training and specialized knowledge for accurate assessment. MNA is an excellent nutritional assessment tool for older individuals [21], but it is relatively time-consuming because of many questions.

On the other hand, ODA is useful for nutritional evaluation in daily medical care because it is relatively easy to obtain and cost-effective. Serum albumin level and BMI are well-known markers of malnutrition, and the relationship between malnutrition and total mortality has been reported in older people [22,23]. Moreover, a serum albumin level of <3.5 g/dl has been shown to correlate with decreased visceral protein [24] and is reported to be an independent risk factor of all-cause mortality [25]. However, physicians should be cautious in evaluating nutritional status with serum albumin levels because of the effect of age and various conditions, including inflammation and liver or kidney diseases [26,27]. BMI is an important index in patients with diabetes; however, a previous report indicated that >30% of patients with diabetes diagnosed with malnutrition had a BMI $\geq$ 30 kg/m^2 [28]. Therefore, it may be difficult to evaluate the nutritional status of patients with diabetes by BMI alone. In this study, BMI was lower in older patients; however, no significant differences were found between the high-risk and low-risk foot groups at all ages.

CONUT is a complex ODA, calculated using total lymphocyte count, total cholesterol level, and serum albumin level [9]. CONUT evaluates nutritional status from various perspectives using three types of objective biomarkers: protein metabolism, immune function, and lipid metabolism [9]. A positive relationship between CONUT score and SGA was also reported previously [29]. In addition, previous studies showed that the CONUT score is a useful marker for mortality [30,31], healing of foot ulcers [32,33], and subclinical

atherosclerosis [34]. In the present study, multivariate logistic regression analysis indicated that the CONUT score was significantly associated with a high-risk foot in the older group, with or without statin use. Serum albumin levels were low in the high-risk foot group in all age groups, but CONUT was significantly poor in the high-risk foot group only in the older group in this study. Since the limitation of nutritional assessment with serum albumin level alone has been pointed out [26,27], it might indicate severe malnutrition in the high-risk foot group in older patients.

All ODAs were poor, and the microvascular complications of diabetes were advanced in the older group with the high-risk foot; therefore, these patients might be at high risk of foot ulcer development and might need much time to heal once foot ulcers occur. It is important to be proactive with foot risk evaluation and pay attention to nutritional status assessed with ODA in clinical practice, especially in older patients with a high-risk foot. Monitoring nutritional status in older patients with type 2 diabetes might be helpful to prevent future foot ulcers.

This study had several limitations. First, because of the study's cross-sectional design, causal relationships could not be mentioned. Second, there is no information about the subjective nutritional indicators and sarcopenia assessed by skeletal muscle mass with body composition tests. Third, we categorized patients as low-risk and high-risk to perform multivariate analysis in this study. However, grouping patients with risk foot 1, 2, and 3 might lead to biased results, due to the heterogeneous characteristics and small sample size. Finally, this study was performed at a single institution, and all participants were Japanese. Therefore, whether our findings can be applied to other populations is uncertain.

5. Conclusions

In conclusion, it was shown that nutritional status assessed with ODA was significantly worse in patients with type 2 diabetes and high-risk foot in the older population.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/jcm11051314/s1, Table S1. (a). Multivariate-adjusted ORs (95% CI) for high-risk diabetic foot assessed with IWGDF in patients taking statin. (b). Multivariate-adjusted ORs (95% CI) for high-risk diabetic foot assessed with IWGDF in patients not taking statin.

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Evaluation of the Use of Antibiofilmogram Technology in the Clinical Evolution of Foot Ulcers Infected by *Staphylococcus aureus* in Persons Living with Diabetes: A Pilot Study

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Abstract: Infected diabetic foot ulcers (DFUs) represent a serious threat to public health because of their frequency and the severity of their consequences. DFUs are frequently infected by bacteria in biofilms, obstructing antibiotic action. Antibiofilmogram was developed to assess the impact of antibiotics to inhibit biofilm formation. This pilot study aimed to determine the benefits of this technology in predicting antibiotic activity on the outcome of 28 patients with Grade 2 DFUs that were infected by a monomicrobial *Staphylococcus aureus*. Patients with diabetes were followed during the antibiotic treatment (day 14) and the follow-up period of the study (day 45). The contribution of Antibiofilmogram was compared between patients with non-concordant results (n = 13) between antibiogram and Antibiofilmogram versus concordant results (n = 15). The clinical improvement of wounds (80.0% vs. 38.5%, p = 0.0245) and the absence of exudates (0% vs. 33.3%, p = 0.0282) were observed in concordant vs. discordant groups. This pilot study provides promising results for the interest of Antibiofilmogram in the prescription of antibiotics to prevent biofilm formation in infected DFUs.

Keywords: Antibiofilmogram; antibiotics; biofilm; diabetic foot infections; *Staphylococcus aureus*; wound healing

1. Introduction

Foot ulceration is one of the most frequently recognized complications in patients living with diabetes, as an ultimate result of a triopathy associating sensory, autonomic, and motor neuropathies, immunopathy, and lower limb arteriopathy [1]. Infection of these ulcers is a frequent (40–80%) and costly complication, increasing diabetes-related hospital admissions, mortality, and morbidity [1]. The management of this complication is a challenging problem, and wound-healing outcomes are often poor [2].

Diabetic foot ulcers (DFUs) are often infected with commensal and pathogenic microorganisms, especially containing *Staphylococcus* spp. [3,4]. For clinicians, the difficulties in distinguishing between infection and colonization of DFUs frequently leads to non-adapted antimicrobial treatment with overly broad-spectrum or excessively prolonged treatment [1]. This increases the risk of non-traumatic lower limb amputations [1] and the emergence of multidrug-resistant organisms [1,3–6]. Moreover, among these chronic wounds, 60–80% of microorganisms are organized in biofilm, increasing the difficulty of treating these lesions because sessile bacteria have a higher tolerance towards antibiotics, and bacterial biofilms play a crucial role in delayed wound healing [7–9]. It is also known that some antibiotics have an inductive effect around therapeutic doses on biofilm behavior, with consequences for the duration of remission and/or recurrence of the wound infections [10].

To date, clinicians have no available routine information or tools to investigate the role of antibiotics in wound healing, or to predict wound evolution. Recently, a diagnostic tool derived from the BioFilm ring test (BioFilm Control, St Beauzire, France) was elaborated to investigate the capacity to study the early biofilm formation of bacteria, and it has been used to assess the impact of antibiotics to inhibit the installation of this early biofilm formation [11]. Antibiofilmogram provides complementary information for the traditional antibiogram in order to decipher the efficiency of antibiotics against biofilm formation. Here, we conducted a pilot study to test the benefit of Antibiofilmogram use for the clinicians, providing information on the efficiency of antibiotics against biofilm formation regarding the risk of failure of an antibiotic regimen on the evolution of DFUs infected by *S. aureus.*

2. Materials and Methods

2.1. Study Design

This prospective, multicenter, observational pilot study was approved by the South Mediterranean III Ethics Committee (clinicaltrials.gov (accessed on 30 January 2020) #NCT02378493). From 16 December 2015 to 14 July 2019, we enrolled persons with diabetes who were admitted to three French diabetic foot clinics (Nîmes, Nantes, and Lyon) with a suspected new episode of diabetic foot infection (DFI) (Grade 2, according to the PEDIS (Perfusion Extent, Depth, Infection, Sensation) classification of the International Working Group of Diabetic Foot (IWGDF) consensus conference [1]), without antibiotic treatment in the past 14 days and with monomicrobial culture of *S. aureus*. The presence and severity of the infections was assessed by a trained diabetologist or infectious disease specialist. Demographic, comorbidities and clinical data were collected in this study. Arteriopathy was clinically assessed by the presence or absence of suggestive symptoms, such as intermittent claudication or leg pain at rest, and signs such as cold legs or feet, pale or bluish color of the skin, and foot pulses. In addition, according to local usual practices, a Doppler ultrasound examination, ankle-brachial pressure index (ABPI), and transcutaneous oxygen pressure ($TcPO_2$) were implemented. Neuropathy was assessed by the presence or absence of paresthesia or cramps, as well as dry skin or hyperkeratosis of the foot, Charcot foot, and other foot deformities and protective sensation (using the 10 g Semmes–Weinstein monofilament testing, as recommended by the IWGDF). After wound debridement, samples of the bacterial cultures were obtained by scraping and swabbing at the wound base, or by tissue biopsies [1]. Antibiotics were prescribed for 14 days following the local protocol of each hospital and the IWGDF recommendations [1]. Each center has followed its own protocol to manage the wounds. All patients had general measures, including a prescription for offloading devices, dressings changed by nurses, the controlling of blood glucose, and an anti-tetanus vaccination if needed. Patients were followed-up on days 14 and 45. Wound evolution was assessed via surface area and depth and the presence of inflammatory signs and exudates. An outcome was considered 'unfavorable' in patients seeking an early review and for worsening/stagnating wounds or 'favorable' for completely or partially re-epithelialized wounds. The definition of healing was based on the criterion of reaching at

least a 40% reduction of the initial ulcer area at the end of the study (day 45), as previously proposed by Edmonds et al. [12] and the French High Authority of Health [13].

2.2. Bacteriological Study

The isolates were identified using the Vitek® MS system (bioMérieux, Marcy L'Etoile, France). Antimicrobial susceptibility testing was performed by a disk diffusion test (Bio-Rad, Marnes-la-Coquette, France) or broth microdilution procedures (UMIC) (Biocentric, Bandol, France), according to EUCAST recommendations (https://www.eucast.org/clinical_breakpoints (accessed on 10 September 2021)). Vancomycin and teicoplanin MICs were determined using the broth microdilution procedures (UMIC) (Biocentric, Bandol, France).

Multilocus sequence typing (MLST) was performed on *S. aureus* on inclusion, day 14, and day 45 [14]. Seven housekeeping genes (*arc*, *aroE*, *glpF*, *gmk*, *pta*, *tpi* and *yqi*) were sequenced to determine the allelic profile. The strains were assigned to an ST using the MLST database [15].

2.3. Antibiofilmogram

The Biofilm ring test was used to study antibiotic action on biofilm formation and to determine an Antibiofilmogram (BioFilm Control), as previously described [10]. Briefly, experiments were performed with the bacterial isolate using the brain heart infusion medium. The 96-well microtiter plates containing bacteria, magnetic beads and antibiotics (20 µL of antibiotic solutions) were incubated at 37 °C for 4 h before visual reading. At this time, the plates were placed onto a magnetic block, read after magnetic attraction (1 min), and analyzed using a microplate scanner with the BioFilm Control software (BFC Elements 3.0), which generated a biofilm formation index (BFI). Using a second algorithm (Algo CMIb), the biofilm minimal inhibitory concentration (bMIC) was assessed for 13 antibiotics (cloxacillin, ceftazidime, amoxicillin/clavulanic acid, teicoplanin, vancomycin, fosfomycin, ofloxacin, rifampicin, cotrimoxazole, gentamicin, clindamycin, erythromycin and fusidic acid). The bMICs were determined based on the BFIs using an algorithm developed and validated in-house. Four wells without antibiotics, filled with the bacterial suspension and magnetic beads, were used as the positive control (there was an absence of spots, due to beads immobilization in biofilm). Assays were performed in triplicate. The interpretations were performed by comparing the bMICs obtained with the EUCAST breakpoints (V 1.1 April 2020; www.eucast.org/clinical_breakpoints (accessed on 10 September 2021)). The oxacillin breakpoint was used as a proxy amoxicillin/clavulanic acid and ceftazidime breakpoint. The final result was communicated to the clinician at the end of the study and was a susceptibility classification of the particular strain (sensitive, intermediate, resistant) toward the selected antibiotics.

2.4. Statistical Analysis

The primary outcome was the role of antibiogram/Antibiofilmogram concordance (in terms of *S. aureus* strains and prescribed antibiotics) on the presence/absence of *S. aureus* strains on day 14 (at the end of antibiotic treatment). The secondary outcome was the role of antibiogram/Antibiofilmogram concordance in wound improvement and healing. Patients were classified to the concordant group if all the antibiotics prescribed were active against *S. aureus* and efficient against the biofilm formation, or the discordant group if one or two antibiotics were inactive against the *S. aureus* strain or inefficient against biofilm formation.

This study was exploratory; however, the inclusion of 32 patients would allow us to demonstrate a relative risk (RR: the probability of absence of *S. aureus* at the end of antibiotic therapy in case of concordance/the probability of absence of *S. aureus* at the end of antibiotic therapy in case of discordance) equal to 2 for a concordance rate between 43% (e.g., teicoplanin), and 71% (e.g., vancomycin); and an RR of 3 for a concordance rate of 88% (e.g., erythromycin/fusidic acid), with a power of 80% and a bilateral alpha risk of 5%—taking into account the consecutive inclusion of patients and considering a 15%

rate of patients with non-exploitable data. These data, used for sample size calculation, came from preliminary data using the BFC software and were obtained with an antibiotic panel chosen by the investigators from Nîmes University Hospital. The concordance rate by antibiotic was very variable and depended on the antibiotics tested: 5% to 95% (unpublished laboratory data), but mostly higher than 40%. We expected a relatively large concordance effect on the absence of *S. aureus* at the end of antibiotic therapy, but this is not yet quantified.

The normality of the quantitative variables' distribution was determined using the Shapiro–Wilk normality test with a threshold of 0.01 and coefficients of kurtosis and skewness. Statistical results were to be presented as the means ± standard deviations (SD) for quantitative variables following a Gaussian distribution, and means and 95% back-transformed confidence intervals for Gaussian variables after transformation. Medians and interquartile (IQ) ranges were used for the other variables. For the qualitative variables, the numbers and the associated percentages were to be presented. A univariate analysis was determined concerning patient characteristics at inclusion and the rate of absence of *S. aureus* at D14 between groups. Qualitative variable comparisons were carried out using a chi-square test or Fisher's exact test. Quantitative variable analyses between the two groups were performed using a Kruskal–Wallis test. DFU healing at the end of the study was compared between the two groups via the chi-square test or Fisher's exact test.

The potential role of Antibiofilmogram and the pre-defined cofactors in predicting wound evolution was studied between groups. The scores established from this matrixial analysis were compared between two groups and certified by Soladis (Lyon, France). A comparison of wound evolution on day 14 and day 45 between groups was performed using a chi-square or Fisher's exact test for qualitative variables, and a Student's test or Wilcoxon rank sum test was used for quantitative variables. The individual trajectories of clinical course wound area and depth during the study were represented graphically. The statistical analysis was to be conducted under the SAS (SAS institute, Cary, NC, USA) version 9, or R 2.9.2 (R development Core Team 2009, R foundation for Statistical Computing, Vienna, Austria). Differences were considered statistically significant when the degree of significance (*p*-value) of the test was ≤ 0.05.

3. Results

3.1. Studied Population

Thirty-five patients were screened, with seven excluded due to the problem of bacterial identification at inclusion ($n = 2$), bacterial conservation ($n = 2$), antibiotic therapy in the last 2 weeks ($n = 2$) and non-formation of biofilm with Antibiofilmogram ($n = 1$) (Figure 1). Finally, 28 patients were definitively included: $n = 18$ at Nîmes, $n = 7$ at Lyon and $n = 3$ at Nantes.

Most of the included patients were male (22, 78.6%) with a mean age of 61.2 years (±11.92) and type 2 diabetes (26, 92.9%) (Table 1). The median Charlson score was 3 (±2). The median wound surface area was 119 mm^2 (±197.65), and the median depth was 3 mm (±9), with exudates in 6 wounds (24%).

Seventeen patients (60.7%) received bitherapy (Table 1). The main antibiotics administered were β-lactams ($n = 19$, 67.9%), notably amoxicillin/clavulanic acid ($n = 15$, 53.6%), followed by clindamycin ($n = 9$, 32.1%), ofloxacin ($n = 6$, 21.4%), and rifampicin and cotrimoxazole ($n = 5$, 17.9%).

The bacteriological analysis identified 28 *S. aureus* at inclusion. Seven (25%) patients had *S. aureus* infection on day 14 and 9 (32.1%) on day 45. A total of 44 isolates were analyzed by Antibiofilmogram.

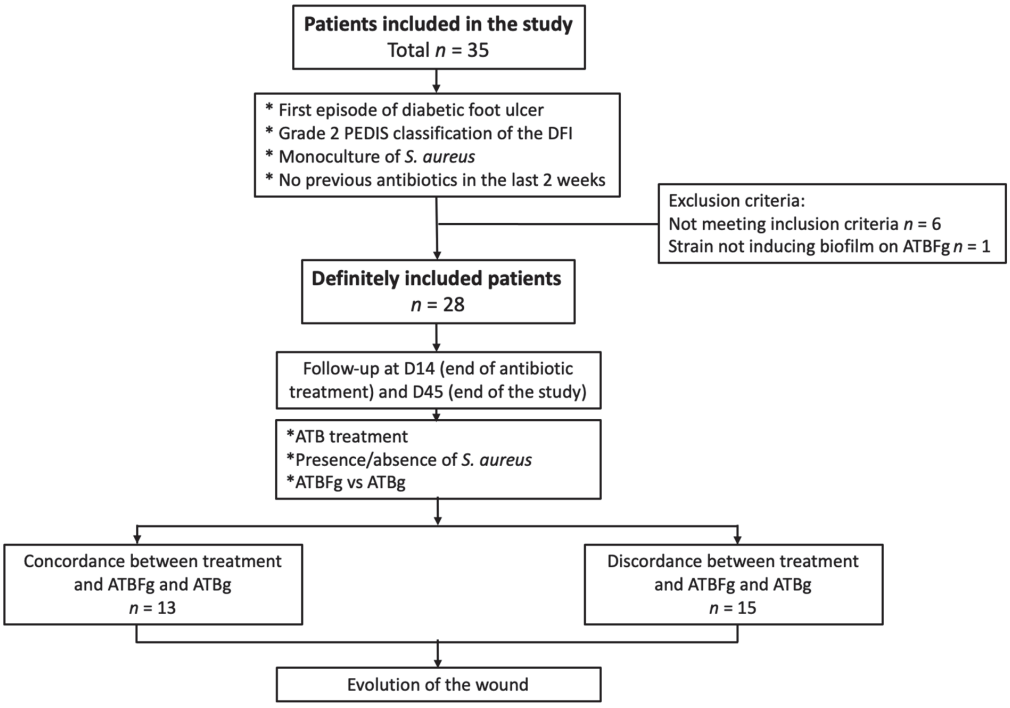

Figure 1. Flowchart of the study. ATBFg, Antibiofilmogram; ATBg, antibiogram.

Table 1. Demographic and clinical characteristics of the study population at inclusion.

Characteristics	Concordant Group (*n* = 15)	Disconcordant Group (*n* = 13)	Total (*n* = 28)	*p*-Value Concordant vs. Disconcordant
Age (years, SD [a])	60.1 (±13.1)	62.4 (±10.9)	61.2 (±11.9)	0.6273
Male/Female (*n*,%)	11 (73.3)/4 (26.7)	11 (84.6)/2(15.4)	22 (78.6)/6 (21.4)	0.4865
BMI [b] (kg/m^2, SD)	29.93 (±5.18)	33.67 (±7.94)	31.66 (±6.75)	0.1462
Comorbidities				
Charlson index (median, IQ [c])	2 (3)	4 (1.5)	3 (2)	0.4591
McCabe Score	1	1	1	>0.99
Arteriopathy (*n*,%)	14 (93.3)	12 (92.3)	26 (92.3)	>0.99
Neuropathy (*n*,%)	13 (86.7)	12 (92.3)	25 (89.3)	>0.99
Diabetes duration median (years, IQ)	15 (±10)	16 (±10)	15.5 (±10.7)	0.473
HbA1c mean (%, SD)	8.60 (±2.18)	7.93 (±1.4)	8.29 (±1.86)	0.3536
Type 1/Type 2 diabetes mellitus (*n*,%/*n*,%)	1 (6.7)/14 (93.3)	1 (7.7)/12 (92.3)	2 (7.1)/26 (92.9)	0.9201
Characteristics of the wounds				
Initial wound depth median (mm, IQ)	3 (±4.25)	7 (±14)	3 (±9)	0.2621
Initial wound surface area median (mm^2, IQ)	117.8 (±168.8)	120.1 (±265.75)	119 (±197.65)	0.9632
Exsudative wound (*n*,%)	2 (13.3)	4 (30.8)	6 (24)	0.3720

Table 1. *Cont.*

Characteristics	Concordant Group (*n* = 15)	Disconcordant Group (*n* = 13)	Total (*n* = 28)	*p*-Value Concordant vs. Disconcordant
Monotherapy/Bitherapy (*n*,%)	9 (60.0)/6 (40.0)	2 (15.4)/11 (84.6)	11 (39.3)/17 (60.7)	**0.0238**
Treatment duration (day, SD)	13 ± 5	14 ± 3.5	13 ± 3.8	**0.0393**
β-lactams (*n*,%)	10 (66.7)	9 (69.2)	19 (67.9)	0.7051
Macrolides and related (*n*,%)	7 (46.7)	3 (23.1)	10 (35.7)	0.1145
Cotrimoxazole (*n*,%)	0 (0)	5 (38.5)	5 (17.9)	**0.0131**
Glycopeptides (*n*,%)	0 (0)	2 (15.4)	2 (7.1)	0.2063
Fluoroquinolones (*n*,%)	0 (0)	6 (46.2)	6 (21.4)	**0.0046**
Rifampicin (*n*,%)	0 (0)	5 (38.5)	5 (17.9)	**0.0131**

[a] SD, standard deviation; [b] BMI, body mass index; [c] IQ, interquartile; *p*-value was calculated using the Student test for demographic data, the Wilcoxon test for the Charlson score, the diabetes duration, the characteristics of the wounds and the treatment duration, and the Fisher exact test for the other variables. In bold, significant results ($p < 0.05$).

Thirteen patients showed discordant results and 15 concordant between the antibiogram and Antibiofilmogram (Table 2). Groups were demographically similar; however, monotherapy was more common and of a shorter duration in the concordant group (60.0% monotherapy vs. 15.4%, $p = 0.0238$; and 13 ± 5 days vs. 14 ± 3.5, $p = 0.0393$, respectively) (Table 1). Cotrimoxazole, ofloxacin and rifampicin were exclusively used in the discordant group (0% vs. 38.5%; 0 vs. 46.2%; 0 vs. 38.5%). Using Antibiofilmogram, clindamycin (9 strains/9) and rifampicin (3/3) were always efficient against biofilm formation, whereas ofloxacin (6/6), cotrimoxazole (5/5), and vancomycin (1/1) never were (Table 2).

Table 2. Results of antibiogram and Antibiofilmogram of *S. aureus* strains isolated from DFI against the final antibiotics prescribed.

Classification Group	Patients	Antibiotics Prescription [a]	Result of Antibiogram [b]	Results of Antibiofilmogram
Concordant	C01P004	CLN	S	S
	C01P009	CLN	S	S
	C01P012	AMC	S	S
	C03P001	AMC	S	S
	C03P003	CLN	S	S
	C03P004	CLN	S	S
	C03P006	CLN	S	S
	C03P008	AMC and CLN	R/S	R/S
	C03P009	CLN	S	S
	C03P010	AMC and CLN	R/S	R/S
	C04P002	AMC	R	R
	C04P003	AMC	S	S
	C04P004	AMC	S	S
	C04P005	AMC	S	S
	C06P001	AMC	S	S
Discordant	C01P001	OFX + RIF	S/S	R/S
	C01P002	AMC + OFX	S/S	S/R
	C01P005	AMC + OFX	S/S	S/R
	C01P008	AMC + SXT	S/S	R/R
	C01P010	OFX + CLN	S/S	R/S
	C01P011	SXT + OFX	S/S	R/R
	C01P013	SXT + RIF	S/S	R/S
	C03P002	OFX + SXT	S/S	R/R
	C03P007	CLN + VAN	S/S	S/R
	C04P001	SXT	S	R
	C04P007	AMC	S	R
	C04P008	AMC + RIF	S/S	R/S
	C06P003	AMC	S	R

[a] AMC, amoxicillin/clavulanic acid; CLN, clindamycin; OFX, ofloxacin; RIF, rifampicin; SXT, cotrimoxazole; VAN, vancomycin; [b] S, susceptible; R, resistant.

3.2. Presence of S. aureus during the Follow-Up of the Patients

No significant differences were observed for *S. aureus* presence at the follow-up between the discordant (n = 3, 23.1% at day 14 and n = 4, 30.8% at day 45) and concordant groups (n = 4, 26.7% at day 14 and n = 5, 33.3% at day 45) (p = 0.574) (Table S1). The seven *S. aureus* isolated at inclusion and day 14 belonged to the same ST, suggesting that the strains were identical. On day 45, seven *S. aureus* always belonged to the same ST with two (C03P008 and C04P004) present in the three samples (inclusion, day 14 and day 45). In two cases (C04P005 and C06P003) a new ST was detected.

3.3. Antibiofilmogram and Evolution of the DFU

On day 14, fewer wounds were exudative in the concordant group (0% vs. 30.8%, p = 0.0282). Moreover, these patients showed clinical improvement (80.0% vs. 38.5%, p = 0.0245) and reduced wound depth (2 mm ±1.25 vs. 3 ±14.0), but these results were not significant (p = 0.0516). A non-significant greater diversification of species was isolated in the concordant group (1.79 vs. 1.58) (Table 3).

Table 3. Evolution of the DFU infected by *S. aureus* at the end of treatment (day 14) and at the end of the follow-up (day 45).

Characteristics	Concordant Group (n = 15)	Discordant Group (n = 13)	Total (n = 28)	p-Value Concordant vs. Discordant
End of treatment (Day 14)				
Wound depth median (mm, IQ [a])	2 ± 1.25	3 ± 14	2 ± 4.5	0.0516
Wound surface area (mm^2, IQ)	43.55 ± 72.12	75.8 ± 220.15	43.55 ± 135.8	0.4556
Exsudative wound (n, %)	0 (0)	4 (30.8)	4 (14.3)	**0.0282**
Inflammatory signs (n, %)	8 (53.3)	11 (84.6)	19 (67.9)	0.0823
Number of species (mean, SD [b])	1.79 ± 1.05	1.58 ± 0.51	1.69 ± 0.84	0.55
Gram-Negative Bacilli (n, %)	5 (33.3)	6 (46.2)	11 (39.3)	0.6922
Gram-Positive Cocci (n, %)	8 (53.3)	7 (53.8)	15 (53.6)	>0.999
Anaerobes (n, %)	0 (0)	0 (0)	0 (0)	>0.999
Clinical improvement (n, %)	12 (80.0)	5 (38.5)	17 (60.7)	**0.0245**
Wound healing (n, %)	8 (53.3)	4 (30.8)	12 (42.9)	0.2219
End of follow-up (Day 45)				
Wound depth median (mm, IQ)	3 ± 2	3 ± 18	3 ± 2	0.5482
Wound surface area (mm^2, IQ)	22 ± 70.2	42.4 ± 185.3	29.85 ± 157.62	0.0595
Exsudative wound (n, %)	2 (13.3)	3 (20.0)	5 (17.9)	0.3217
Inflammatory signs (n, %)	7 (46.7)	9 (69.2)	16 (57.1)	0.4404
Number of species (mean, SD)	1.87 ± 1.13	1.69 ± 0.75	1.79 ± 0.96	0.6395
Gram-Negative Bacilli (n, %)	5 (33.3)	3 (20.0)	8 (28.6)	0.686
Gram-Positive Cocci (n, %)	10 (66.7)	7 (53.8)	17 (60.7)	0.7
Anaerobes (n, %)	0 (0)	3 (20.0)	3 (10.7)	0.0873
Clinical improvement (n, %)	12 (80)	8 (61.5)	21 (75.0)	0.5295
Wound healing (n, %)	11 (73.3)	8 (61.5)	19 (67.9)	0.4953

[a] IQ, interquartile range; [b] SD, standard deviation; p-value was calculated using the Wilcoxon test for wound characteristics and the Fisher exact test for the other variables. In bold, significant results ($p < 0.05$).

The representation of each individual evolution of the wounds showed that most patients in the concordant group had improved DFUs on day 14, in contrast to the discordant group (Figure 2). A clear amelioration of the wound surface and depth was noted in the concordant group (n = 12 with full or partial wound healing and n = 3 with stabilization/worsening) on day 45. The evolution in the discordant group was diverse: improvement (n = 8) and stabilization or aggravation (n = 5).

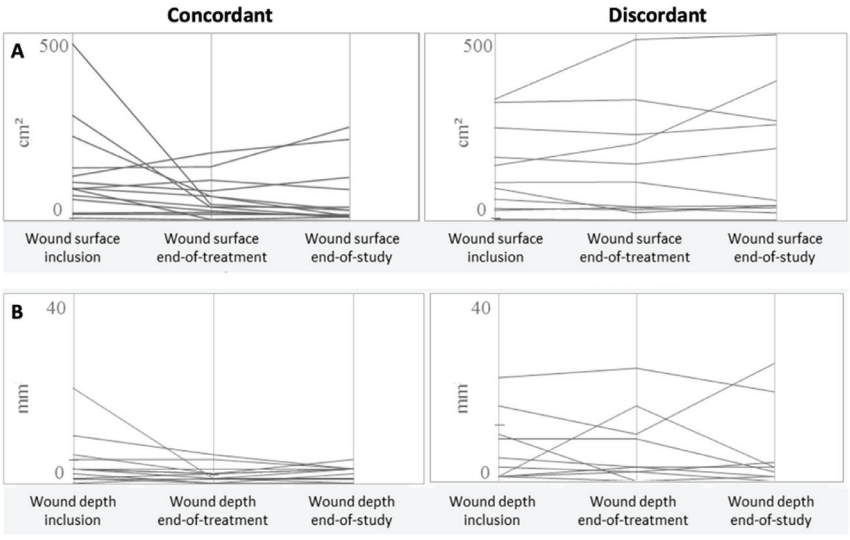

Figure 2. Individual evolution of the wound surface area (**A**) and wound depth (**B**) measurements at inclusion, at the end of treatment (day 14) and at the end of the follow-up (day 45) of patients with DFU infected by *S. aureus* and belonging to discordant and concordant groups based on the results of the Antibiofilmogram.

Finally, at the end of the antibiotic treatment (day 14), 17 patients had favorable wound evolution (with 12 patients experiencing healing) and 11 unfavorable. An antibiogram/Antibiofilmogram concordance was noted in the patients with a favorable evolution, with a relative risk of 3.1 (95% CI:1–9.2) (Figure 3).

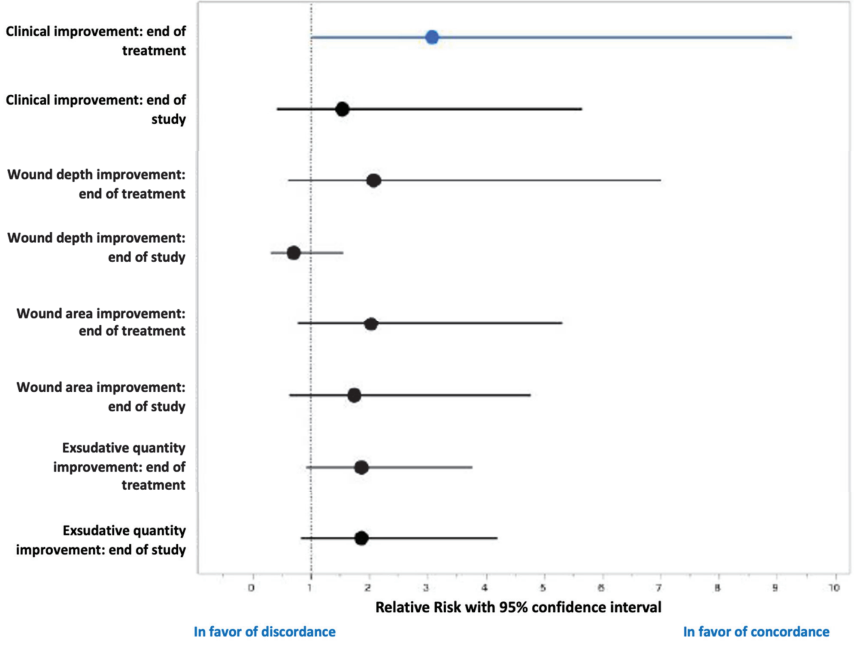

Figure 3. Forest plot (relative risk and 95% confidence interval) presenting the effect of the antibiogram/Antibiofilmogram concordance on the wound evolution.

4. Discussion

Many factors influence the healing of DFUs. Among them, the polymicrobial biofilm represents one of the causes of delayed healing [7]. This non-healing of DFU appears to arise from a bacterial biofilm at the wound bed [7,16,17] and the organization of microorganisms in functionally equivalent pathogroups [7,18]. These sessile bacteria are difficult to treat, and few antibiotics are effective [19,20]. Standard antibiograms have limited ability to determine antibiotic effectiveness at the site of infection and on sessile bacteria. Recently, the Antibiofilmogram, based on the use of the BioFilm ring test [11,21], was adapted to evaluate the ability of antibiotics to inhibit biofilm growth [11]. Our first pilot multicenter re-study of the Antibiofilmogram contribution to guide clinicians in the treatment of DFU infected with *S. aureus* demonstrated promise for the clinical evolution of these wounds. The concordance between an antibiogram and Antibiofilmogram (meaning that the antibiotic would be effective against both planktonic and biofilm-form) was associated with the clinical improvement of the wound, fewer exudates at the end of antibiotic treatment (day 14), and a decreased wound area at the end of the follow-up (day 45).

Among the concordant group (concordance between antibiogram and Antibiofilmogram), a large majority of patients had a clinical improvement of their wound (n = 12/15, 80%) (Table 3). When we focused our attention on the three remaining patients, we noted that two had a worsening evolution of their wounds on day 14 (C01P004) and on day 45 (C04P004) (Figure 2), due to the presence of *P. aeruginosa* and *P. aeruginosa* + *K. oxytoca*, respectively. As these two patients received clindamycin alone (always efficient on biofilm installation) and amoxicillin/clavulanic acid alone (efficient on the biofilm installation of C04P004), we concluded that the two regimens were not adapted to treat the Gram-negative bacilli, even if recently Orazi et al. showed that *P. aeruginosa*-secreted products could increase the antibiotic activity to kill *S. aureus* in biofilm [22]. In the third case (C03P004), the administration of clindamycin alone seemed to be adapted, while *S. aureus* was not detected on day 14 and day 45, whereas the wound was stabilized but not improved. Only *Corynebacterium striatum*, a commensal bacterium of the skin, was detected in the follow-up of the patient. We could not exclude that other parameters, such as offloading, were not correctly applied, and that they influenced the wound evolution. A comparison between the two groups also showed a more important diversity in the number of bacterial species isolated from DFU in the concordant group, compared to the discordant group (1.79 vs. 1.58, respectively) during the follow-up of the wounds. One hypothesis should be that debridement is associated with a remodeling of cutaneous microbiota and that numerous species can colonize the wounds, explaining this greater bacterial diversification that could protect the ulcer to pathogenic bacteria and prevent infections.

Interestingly, only 7 patients (25%) presented persistent DFU colonization on day 14 and day 45 by a related ST strain. This constatation is in accordance with a recent observation performed in our hospital, where 25% of our panel (n = 48) harbored a related *S. aureus* isolate during a period of four weeks, with a median persistence of 12 weeks, and only one patient (2.1% of our panel) presented a successive *S. aureus* belonging to a same clonal lineage over time for an extended period exceeding 30 weeks [23]. This suggested that long-term persistence of *S. aureus* in DFI has a weaker implantation rate compared to other chronic conditions [24]. The debridement and antibiotic therapy could explain this low rate, yet the debridement appears to be sometimes insufficient, and many factors influence healing in persons with diabetes (e.g., offloading, antibiotic uptake, and glycemic balance). The MLST results also confirmed the important diversity of the *S. aureus* clones, as previously noted [25–27]. No clone was associated with the worsening evolution or an *S. aureus* persistence in the wound.

The main study limitation is the small size of our population (n = 28). The inclusion criteria were restrictive with only Grade 2—whereas, our specialized clinics followed mainly Grade 3 and 4 DFI and a monoinfection to *S. aureus*—and these wounds were preferentially polymicrobial [3,6,7]. Some antibiotics were more effective against biofilm infections and usable alone (β-lactams or related macrolides), or in combination (with

rifampicin), but always with the need of the Antibiofilmogram. Larger prospective studies should confirm the value of Antibiofilmogram.

In conclusion, although not all of our outcomes showed significant differences—due to the small-sized cohort—our findings may suggest that an antibiotic strategy, which also incorporates information regarding antibiotic action against biofilms, may be a promising approach to improving wound healing outcomes for patients with DFUs.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/jcm10245928/s1, Table S1: Comparison of MLST results of the *S. aureus* strains isolated from DFI at inclusion, at the end of the treatment, and at the end of the study.

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Psychometric Validation of the Cardiff Wound Impact Schedule Questionnaire in a Spanish Population with Diabetic Foot Ulcer

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Abstract: Diabetic foot ulcers (DFU) negatively affect the quality of life (QoL) of people with diabetes. The Cardiff Wound Impact Schedule (CWIS) questionnaire has been designed to measure the QoL of people with chronic foot wounds. However, no studies have been specifically designed to validate this instrument in a Spanish population. In this prospective study, a total of 141 subjects with DFU were recruited. DFU was determined by performing physical examinations. Medical records were exhaustively reviewed to collect clinical variables. The CWIS was transculturally adapted by a group of experts and a group of patients with DFU. The SF-36 and EQ-5D generic instruments were used as reference tools. The questionnaires were administered at 7 days and 4, 12, and 26 weeks after the baseline assessment by personal interview with each of the study subjects. The psychometric properties of the instrument were assessed using statistical methods. The content validity had an average of 3.63 (90.7% of the maximum score of 4). The internal consistency of the CWIS subscales had a standardized Cronbach's alpha range from 0.715 to 0.797. The reproducibility was moderate with an intraclass correlation coefficient (ICC) range from 0.606 to 0.868. Significant correlations between CWIS domains and SF-36 and EQ-5D subscales were observed, demonstrating a good criterion validity of the CWIS questionnaire ($p < 0.001$). However, the construct validity of the CWIS was not validated with a comparative fit index (CFI) of 0.69, a root mean square error of approximation (RMSEA) of 0.09, and a standardized root mean square residual (SRMR) of 0.10. The sensitivity to changes over time was optimal in the three domains (i.e., social life, well-being, and physical symptoms) ($p < 0.001$). In conclusion, the Spanish version of the CWIS shows acceptable psychometric properties to assess the QoL of subjects with DFU, except for its construct validity.

Keywords: diabetic foot ulcer; type 2 diabetes; quality of life; psychometric validation; reliability; validity

1. Introduction

Diabetic foot ulcer (DFU) is an important complication of diabetes, with an incidence rate of 1–4% and a lifetime risk of 15–25% [1,2]. This condition is defined as an ulceration of the foot associated with diabetic neuropathy which show any grade of ischemia and infection [3]. Moreover, this diabetic complication is often associated with other serious complications such as osteomyelitis and lower limb amputation [2]. The five-year mortality rate among patients with DFU is around 40%, with a 2.5 times higher risk of mortality in comparison with patients without DFU [4]. The pathogenesis of DFU involves multiple factors, such as peripheral neuropathy, artery disease, traumas and foot deformities, as well as abnormal joints [3]. Additionally, a recent meta-analysis about the global epidemiology of DFU demonstrated that subjects with DFU were older, had lower body mass index, longer diabetes duration, and showed higher frequency of hypertension, diabetic retinopathy, and smoking habit in comparison with those without DFU [5]. Additionally, DFU negatively impacts the health-related quality of life (HRQoL) of the affected patients, especially those with unhealed ulcers [6–11].

HRQoL is a patient-reported outcome (PRO) that takes into account the presence of biological or physiological dysfunction, symptoms and functional impairment [12]. Moreover, quality of life (QoL) is a multidimensional, subjective, and dynamic measure of the physical, psychological, and social aspects of daily life [12]. Patients with DFU report pain, and limited daily and social activities that worsen their QoL [2]. Moreover, a poorer QoL is associated with several clinical factors, such as pain, fatigue, wound infection, restricted mobility, and social isolation [13]. Furthermore, QoL can be negatively influenced by other factors such as the frequency of attending clinic visits and hospitalizations, and the presence of disturbed daily life activities [13]. The importance of measuring a PRO can be focused on providing valuable information about the effectiveness of a treatment or intervention care [4]. In addition, it can help us to understand how DFUs impact on patients' QoL, with the aim of helping to improve their health care [4].

The Cardiff Wound Impact Schedule (CWIS) questionnaire was designed and validated to assess the impact of chronic wounds on the QoL of patients [14,15]. Although this is not a specific questionnaire for DFU, it is able to discriminate between healed and unhealed ulcers [14–16]. In addition, the CWIS showed sensitivity to healed wounds in a randomized clinical trial when different types of dressings were evaluated for DFU [16]. Furthermore, its domains were also strongly correlated with SF-36 subscales as the gold standard [8,15]. This suggests that CWIS is a valid disease-specific measure of QoL in subjects with DFU [11,15,17,18]. The CWIS, originally developed in English in the UK, has been validated in other countries, such as China, Sri Lanka, Canada, Sweden, and Portugal [15,17–21], while it has only been translated and culturally adapted in German, French, and US English [22]. In Spain, there is no available Spanish questionnaire to specifically assess the QoL of patients with chronic wounds [14]. Thus, the aim of the study was to translate the CWIS into Spanish and prospectively assess its validity and reliability in a group of patients with DFU.

2. Materials and Methods

2.1. Design and Settings

Participants were patients with DFU treated by an expert in chronic wounds at the Department of Endocrinology, University Hospital Arnau de Vilanova between June 2013 and January 2015. A description of the study participants has been provided in a previous publication [23]. The inclusion criteria were a diagnosis of diabetes mellitus (type 1 or type 2 diabetes) with a DFU; a first ulcer, or a new-onset ulcer with $\leq$3 months duration; over 18 years old; and the presence of one or more ulcers located below the malleoli. The exclusion criteria were having psychological or cognitive deterioration, having a terminal illness, or having been hospitalized. The ethics committee from the University Hospital Arnau de Vilanova approved the study. Written informed consent form was obtained from all participants.

2.2. Clinical and Sociodemographic Variables

A detailed description of clinical variables has been described in our previous publication [24]. These data were collected through individual interviews with all participants. Furthermore, a careful review of their clinical records was performed. Hypertension and dyslipidemia were determined if participants were specifically being treated with drugs for these two conditions. Diabetic foot disease and Charcot neuroarthropathy were diagnosed by performing a podiatric examination, as detailed in a previously published study [25]. After physical examination, a previous lower-limb amputations (minor or major), foot abnormalities, the presence of Charcot foot disease and an assessment of the local ulcer features was determined [25–27]. The diagnosis of DFU was established following the standard recommendations of the International Working Group on the Diabetic Foot (IWGDF) [28]. Peripheral arterial disease was appraised using the ankle-brachial index (ABI) and was categorized as normal (from 0.91 to 1.30), moderate ischemia (from 0.41 to 0.90), severe (from 0 to 0.40), and non-compressible because of the detection of calcification (more than 1.30) [29]. Moreover, the pedal or posterior tibial pulse was analyzed in those study participants with an ABI value over 1.30. The determination of peripheral arterial disease was defined by the presence of non-palpable pulses. Following the IWGDF consensus, the type of ulcer was classified as neuropathic, ischemic and neuroischemic [28]. The presence of two symptoms or greater of inflammation (i.e., redness, induration, warmth, and tenderness/pain), or purulent secretions was determined to diagnose an infection of ulcer. Moreover, signs of systemic inflammation (i.e., leukocytosis, fever and C reactive protein) were also evaluated and the grade of the infection was appropriately classified [30].

2.3. Instruments

The questionnaires (CWIS, SF-36 and EQ-5D) were administered at 7 days and 4, 12, and 26 weeks after the baseline assessment by individual interviews with each of the participants.

2.3.1. Cardiff Wound Impact Schedule (CWIS)

CWIS was designed and validated to specifically assess the QoL of subjects with chronic wounds (leg ulcers and DFU) [15]. This questionnaire contains 47 items divided into four scales: demographic and clinical characteristics (3 items), global HRQoL (1 item), satisfaction with HRQoL (1 item) and impact of the wound on lifestyle. This last scale includes 3 domains: social life (14 items in total, 7 related to stress and 7 to experience), well-being (7 items), and physical symptoms and everyday living (24 items in total, 12 related to stress and 12 to experience). All three domains are scored on a 5-point scale, from "not at all" to "always". The final score ranges from 0 (poorer QoL) to 100 points (higher QoL).

2.3.2. 36-Item Short-Form Health Survey (SF-36)

The SF-36 questionnaire is a generic tool that evaluated the health status of the subject [14]. This contains 36 items that are grouped to eight subscales: physical role, physical functioning, general health, bodily pain, social functioning, vitality, emotional role, and mental health. These eight subscales are incorporated into physical and mental health summary scores. Each subscale ranged from 0 (poorer health status) to 100 points (better health status) and is normalized using US norms. This questionnaire is commonly used to validate other tools related to QoL.

2.3.3. EuroQoL 5D Health Utility Index (EQ-5D)

This is a generic questionnaire designed used to assess HRQoL in different diseases, as well as in the general populations of several countries [9]. This instrument includes 5 dimensions: self-care, mobility, pain/discomfort, usual activities, and anxiety/depression. Each item is divided into three categories: no problems, some problems, and extreme problems. This questionnaire shows a visual analogue scale (VAS) to rate the current health status of the study participants on a scale scored from 0 (poorer health status) to 100 points

(higher health status). An index value (EQ-5D index value) is calculated by combining the five dimensions using UK weights for health status defined by each combination.

2.4. Transcultural Adaptation of the CWIS Questionnaire

Two fluent translators in both languages translated the original English version independently to Spanish. The two translated versions were later compared by a group of experts and by a group of patients. Both groups discussed the differences between both versions and reached an agreement. A third translator back-translated the proposed Spanish version for the research group to compare this back-translation with the original English version and correct it if required. The content validity of the final Spanish version was assessed by seven experts using a Likert scale that ranged from 1 (of little relevance) to 4 points (very relevant). They evaluated the relevance of each item to assess the impact of DFU on the QoL of the patients (available at https://www.irblleida.org/media/upload/arxius/VARIS/Questionari_CWIS.pdf) (accessed on 2 September 2021).

2.5. Sample Size

The sample size was based on Cronbach's alpha, a measure of internal consistency. Accepting an alpha risk of 0.05 and a beta risk of 0.2 in a two-sided test and without dropouts, 124 patients were needed to detect Cronbach's alpha coefficients of 0.3 and higher as statistically significant. Anticipating a maximum dropout rate of 15%, the required minimum sample size was 143.

2.6. Statistical Analysis

Descriptive statistics, including mean and standard deviation for quantitative variables and absolute and relative frequencies for qualitative variables, were used.

The experts' assessment for content validity was 3.63 on average (90% of the maximum score of 4). Reliability was measured by internal consistency and reproducibility. Internal consistency was measured using the α-Cronbach coefficient [31], where coefficients of 0.70 or higher were considered adequate in accordance with the study protocol. In addition, reproducibility or test-retest reliability was determined using the CWIS results at baseline and one day 7 visit after the first treatment for patients with no healed diabetic ulcers, assuming no changes for them. It was quantified using the intraclass correlation coefficient (ICC), defined by a single rater, two-way, mixed-effects model for quantitative variables.

Validity assessment was based on criterion and construct validity. In this study, the criterion validity was only determined in terms of concurrent validity with the domains and summary measures of the SF-36 and EQ-5D questionnaires by estimating Pearson's correlation coefficients with the scores of the CWIS subscales. Criterion validity was considered for values over 0.30 (signifying moderate to large correlations). Construct validity was assessed using a confirmatory factor analysis of the three CWIS subscales for impact of the wound on lifestyle. The comparative fit index (CFI), the root mean square error of approximation (RMSEA), and the standardized root mean square residual (SRMR) were estimated. Values of 0.95 or higher, 0.06 or lower, and 0.08 or lower, respectively, are indicative of a good fit to the subscales of the original CWIS (i.e., the three subscales related to the impact of the wound on lifestyle).

The sensitivity to change over time was graphically assessed through the smoothed trends from baseline (visit 1) until visit 5 (visits corresponding to the questionnaire assessments at 7 days and at 4, 12, and 26 weeks or wound cure from baseline assessment) and depending on the healing state of the ulcer at the last available visit. Changes from their inclusion until the last available treatment visit between healed and non-healed patients were compared using the Mann–Whitney test. The R software [32] was used for statistical analysis, with a significance level of 0.05.

3. Results

The characteristics of the 141 participants recruited in the study are shown in Table 1. A high frequency of patients with type 2 diabetes (95.0%), hypertension (82.3%), dyslipidemia (61.7%), and neuropathy (92.9%) was observed in this sample. In addition, the study group showed a low educational level (40.4% had not even completed primary school). Macrovascular complications were observed in a high proportion of patients (89.4%). Neuropathic ulcer was the most prevalent etiology within the study group (61.7%).

Table 1. Baseline clinical and sociodemographic characteristics of the study group.

Characteristics	Study Group (n = 141)
Age (years)	68.3 (13.3)
Sex (men)	95 (67.4)
Ethnicity (Caucasian)	140 (99.3)
Educational level	
Not even primary	57 (40.4)
Completed primary	47 (33.3)
Secondary high school	28 (19.9)
Graduate or higher	9 (6.4)
Employed	24 (17.0)
Smoking	
Never	63 (44.6)
Current or former	78 (55.4)
Type 2 diabetes	134 (95.0)
BMI, kg/m^2	29.0 (4.9)
HbA1c, %	7.5 (1.6)
Hypertension	116 (82.3)
Dyslipidemia	87 (61.7)
Microvascular complications	
Retinopathy	96 (68.1)
Nephropathy	51 (36.2)
Neuropathy	131 (92.9)
Cardiovascular disease	126 (89.4)
Diabetes therapy	
OAD	41 (29.1)
OAD + insulin	57 (40.4)
Insulin	36 (25.5)
Diet	7 (5.0)
Antiplatelet agents	94 (66.7)
Dialysis	8 (5.7)
Type of ulcer	
Neuropathic	87 (61.7)
Ischemic	9 (6.4)
Neuroischemic	45 (31.9)
Infection of ulcer	83 (58.9)
Type of previous amputation	
Minor	41 (29.1)
Major	2 (1.4)
Presence of Charcot foot disease	9 (6.4)

Data are shown as mean (SD) for continuous variables or n (%) for categorical variables. BMI, body mass index; HbA1c, glycated hemoglobin.; OAD, oral antidiabetic drugs.

The content validity had an average score of 3.63 (90.7% of the maximum score of 4). Internal consistency of the CWIS domains was acceptable, with a standardized Cronbach's alpha of 0.715 for social life (items adding experience and stress), 0.729 for well-being, and 0.797 for physical symptoms and everyday living (items adding experience and stress) domains (Table 2). Internal consistency of the CWIS domains were not improved or only marginally improved by the deletion of items. In terms of reproducibility, the CWIS well-being domain showed moderate reproducibility (ICC = 0.63), while the other domains

(i.e., social life, physical symptoms, HRQoL, and satisfaction with HRQoL) showed good reproducibility (ICC from 0.80 to 0.88).

Table 2. Inter-item internal consistency and reproducibility of the Cardiff Wound Impact Schedule (CWIS) domains.

CWIS Domains	Number of Items [1]	Range of Correlations [2]	Average Inter-Item Correlation	Cronbach's Alpha	Reproducibility ICC (95%CI)
Social life [3]	14	−0.086–0.533	0.264	0.715	0.80 (0.72–0.85)
Well-being	7	0.019–0.565	0.278	0.729 [5]	0.63 (0.51–0.73)
Physical symptoms and everyday living [4]	24	0.040–0.767	0.247	0.797 [6]	0.84 (0.79–0.88)
Self-reported HRQoL	1	-	-	-	0.87 (0.82–0.91)
Satisfaction with HRQoL	1	-		-	0.88 (0.83–0.91)

[1] Number of items per domain. [2] Inter-item Pearson's correlations. [3,4] Experience and stress item correlations are summated. [5] Improved from 0.729 to 0.743 if the third item for this subscale is deleted. [6] Improved from 0.797 to 0.800 if the eight item for this subscale is deleted. HRQoL, health-related quality of life; ICC, intraclass correlation coefficient; CI, confidence interval.

The analysis of the concurrent criterion validity is shown in Table 3. Significant Pearson's correlations were found between the impact of the wound on lifestyle domains and self-reported quality of life assessed by CWIS and the domains and summary scores of the SF-36 and EQ-5D. Thus, the CWIS social life domain was largely correlated with the SF-36 social functioning domain and moderately correlated with the SF-36 domains of role physical and overall physical component ($r \geq 0.4$, $p < 0.001$), as well as with the SF-36 domains of physical functioning, vitality, bodily pain, emotional role, and mental component summary ($r > 0.30$, $p < 0.001$). The CWIS well-being assessment was moderately correlated with all EQ-5D and SF-36 domains except for SF-36 general health assessment. The CWIS symptoms assessment was largely correlated with the SF-36 domains of physical functioning, role and component summary, bodily pain, and social functioning ($r \geq 0.50$, $p < 0.001$) and moderately correlated with the EQ-5D, for both the VAS and index, and with the SF-36 vitality and emotional role domains. The CWIS domains of HRQoL and satisfaction with HRQoL were largely correlated with mental health and mental component summary ($r \geq 0.50$, $p < 0.001$) and moderately correlated with EQ-5D index and VAS and with SF-36 bodily pain, vitality, social functioning, and emotional role ($r < 0.30$, $p < 0.001$).

Table 3. Linear regression between domains of the Cardiff Wound Impact Schedule (CWIS) and the SF-36 and EQ-5D overall and subscale scores.

Domains	CWIS Domains				
	Social Life	Well-Being	Physical Symptoms and Everyday Living	Global HRQoL	Satisfaction with HRQoL
SF-36 Subscales					
Physical functioning	0.399 **	0.334 **	0.610 **	0.247 *	0.199 *
Role physical	0.443 **	0.356 **	0.528 **	0.228 *	0.251 *
Bodily pain	0.341 **	0.298 **	0.544 **	0.431 **	0.399 **
General health	0.212 *	0.240 *	0.250 *	0.348 **	0.390 **
Vitality	0.397 **	0.366 **	0.436 **	0.467 **	0.418 **
Social functioning	0.523 **	0.365 **	0.530 **	0.424 **	0.447 **
Role emotional	0.316 **	0.308 **	0.345 **	0.303 **	0.367 **
Mental health	0.284 *	0.330 **	0.281 *	0.511 **	0.524 **
Overall physical component [1]	0.406 **	0.321 **	0.619 **	0.253 *	0.220 *
Overall mental component [2]	0.327 **	0.336 **	0.268 *	0.493 **	0.535 **

Table 3. *Cont.*

Domains	CWIS Domains				
	Social Life	Well-Being	Physical Symptoms and Everyday Living	Global HRQoL	Satisfaction with HRQoL
EQ-5D subscales					
VAS	0.221 *	0.347 **	0.307 **	0.463 **	0.487 **
EQ-5D index value	0.261 *	0.315 **	0.419 **	0.396 **	0.365 **

[1,2] calculated according to the SF-36 subscales involved physical and mental roles. HRQoL, health-related quality of life; VAS, visual analog scale. * $p < 0.05$; ** $p < 0.001$.

On the other hand, the confirmatory factor analysis assessing construct validity of the CWIS showed that their structural definition of domains was not validated (Figure 1). The CFI was only 0.69, the RMSEA was 0.09, and the SRMR was 0.10, indicating that the CWIS structure for subscales lacked construct validity. Exploratory factor analysis with three factors showed that items 3 (family overprotective) and 6 (not going out for fear of bumping wound) from the social life domain loaded more in the well-being domain, while items 3 (healing confidence) and 5 (wound unpleasant look) from the well-being domain loaded more in the social life domain. Among the items of the symptoms domain, items 5 (wound suppuration) and 8 (wound unpleasant smell) loaded more in the well-being domain, while items 10 (adapted footwear), 11 (amount of treatments) and 12 (economic cost) loaded more in the social life domain.

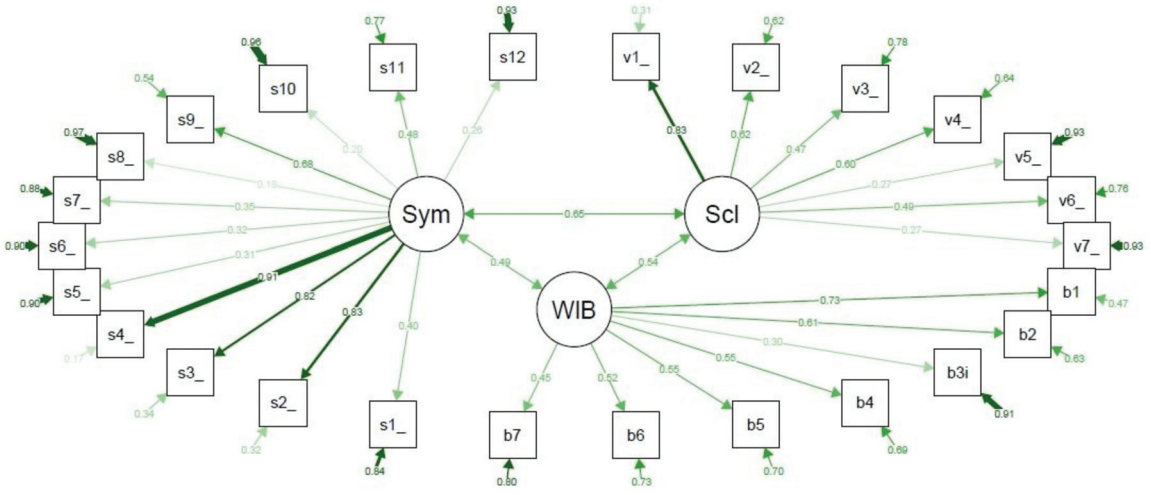

Figure 1. Confirmatory factor analysis of the Cardiff Wound Impact Schedule (CWIS) domains. Sym, Physical symptoms and everyday living; Scl, Social life; WIB, Well-being. v1–v7 are the items of the Social life domain. b1–b7 are the items of the Well-being domain. s1–s12 are the items of the Physical symptoms and everyday living domain. Each arrow between the questionnaire items and the subscale that they are measuring shows the standardized pattern coefficients for this relationship, where values closer to 1.0 (wider and darker) are indicative of better fit, and the circled arrow represented in each questionnaire item shows the residuals. The arrows connecting the subscales show the pairwise correlation between them. Comparative fit index (CFI) = 0.69; root mean square error of approximation (RMSEA) = 0.09; standardized root mean square residual (SRMR) = 0.10.

We analyzed whether the Spanish version of the CWIS had a high sensitivity to detect changes between healed and unhealed ulcers (Table 4). The three domains of the CWIS for impact of the wound on lifestyle (i.e., social life, well-being and physical symptoms)

showed a high sensitivity to change according to the healed group ($p < 0.001$). The HRQoL and satisfaction with HRQoL domains did not show significant changes ($p = 0.903$ and $p = 0.085$, respectively).

Table 4. Descriptive analysis of the sensitivity to change assessment of the Cardiff Wound Impact Schedule (CWIS) domains according to healing status.

Domains	Unhealed (n = 34)	Healed (n = 107)	p-Value
Change in Social life from baseline	2.7 (0.0–8.9)	12.5 (3.6–19.6)	<0.001
Change in Well-being from baseline	0.0 (−2.7–7.2)	35.7 (21.4–46.4)	<0.001
Change in Physical symptoms and everyday living	0.0 (0.0–10.4)	10.4 (4.2–16.7)	<0.001
Global HRQoL	0.0 (−1.0–1.0)	0.0 (−1.0–1.0)	0.903
Change in Satisfaction with HRQoL	0.0 (−1.0–0.0)	0.0 (−1.0–1.0)	0.085

Data are median (95% confidence interval). HRQoL, health-related quality of life.

4. Discussion

The psychometric validation of the Spanish version of the CWIS was acceptable in a sample of patients with DFU. Our results suggest that the internal consistency of the CWIS domains was acceptable, while the reproducibility was excellent in physical symptoms, global HRQoL, and satisfaction with HRQoL domains. The CWIS domains were strongly correlated with SF-36 and EQ-5D subscales demonstrating an excellent criterion validity of the instrument, although well-being was not correlated with these two generic questionnaires. However, the CWIS structure in Spanish was not validated, showing a poor construct validity. On the other hand, this instrument showed a high sensitivity to detect changes between healed and unhealed ulcers.

The results of this study suggest that the Spanish version of the CWIS showed good internal consistency. Nevertheless, other versions of the questionnaire have reported a higher internal consistency (i.e., higher Cronbach α coefficient than our Spanish version), including the Sri Lankan (Cronbach $\alpha = 0.89$), Swedish (Cronbach $\alpha = 0.92$), and Chinese (Cronbach $\alpha = 0.93$) version, as well as the original English form (Cronbach $\alpha = 0.96$) [15,18–20]. However, in our study, the well-being domain did not show a high Cronbach α coefficient. This was similar to the Sri Lankan and Swedish versions [19,20], whereas the Chinese and English versions found a high internal consistency for all the CWIS domains [15,18].

In the present study, the test-retest stability of the instrument showed a moderate reproducibility in the well-being domain. Moreover, reproducibility was good for social life, physical symptoms, global HRQoL, and satisfaction with HRQoL. This was discordant with the Sri Lankan questionnaire, which found a poor reproducibility for the well-being domain and acceptable results for the other CWIS domains [19]. However, our results were similar to the Swedish questionnaire as they showed an excellent stability for the physical domain, and an acceptable reproducibility for well-being and social life domains [20]. The original English CWIS also found a high level of reproducibility as well as the Canadian CWIS questionnaire [15,17].

Criterion validity of the CWIS was excellent with stronger correlations with EQ-5D and SF-36 subscales. This is aligned with the validation studies performed in the other countries. They found similar correlations between CWIS domains and SF-36 subscales, which has been extensively used to assess HRQoL [15,17–20]. This indicates that the Spanish CWIS is a valid disease-specific measure of QoL in patients with DFU, although the well-being domain was not well correlated with the EQ-5D and SF-36 subscales. This could be due to the fact that well-being is a measure that should be assessed with a specific questionnaire designed and validated for a specific purpose/disease area. Furthermore, generic instruments are designed to study health status or HRQoL and not for specific components of QoL like well-being [33].

The original English CWIS was designed and validated with a high construct validity [15]. This is in contrast with our results which showed a poorer construct validity due to the cultural differences between the countries and populations. In our study involv-

ing Spanish subjects, the lifestyle variables related with well-being, social life, and ulcer symptoms were differentially grouped. For this reason, some changes in these items of the questionnaire might improve the construct validity. In the Chinese version, authors had to delete one item due to the cultural setting of China [18]. However, a confirmatory factor analysis to determine the construct validity of the subsequently translated version of the questionnaire was not performed [17,19,20].

Our results showed a high sensitivity to detect changes between healed and unhealed ulcers in the social life, well-being, and physical domains. This is similar to the other translated versions of the CWIS, except for the original English questionnaire which did not find differences between both groups in any domain [15,17–20].

This study has some limitations. This sample of patients showed other comorbidities that could influence the results of the QoL. Moreover, reproducibility of the CWIS was performed at seven days from baseline, whereas patients with DFU were treated at baseline to ensure the ulcers were cared for correctly because these patients have a high-risk of complications and mortality. However, this study has several strengths. At this moment, this is the first study to assess the reliability and validity of a Spanish version of the CWIS questionnaire. Despite this being an instrument designed to assess HRQoL in patients with chronic wounds, this study reports good psychometric properties. Furthermore, the CWIS correlated with the SF-36 and EQ-5D measures, which confers more quality and precision in the validation process. Another strength is the prospective design of the study that can assess changes in QoL and ulcer status over time.

5. Conclusions

The Spanish version of the CWIS questionnaire showed an acceptable validity in some respects, such as reproducibility, criterion validity, sensitivity to ulcer changes over time, and reliability to assess the QoL of patients with DFU. However, its construct validity was poor, indicating cultural differences between populations from different countries. Therefore, we strongly feel that further studies in other Spanish settings are warranted.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Ethics Committee of University Hospital Arnau de Vilanova from Lleida.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data presented in this study are available on request from the corresponding author. The data are not publicly available due to privacy reasons.

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The Association between Foot and Ulcer Microcirculation Measured with Laser Speckle Contrast Imaging and Healing of Diabetic Foot Ulcers

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Abstract: Diagnosis of peripheral artery disease in people with diabetes and a foot ulcer using current non-invasive blood pressure measurements is challenging. Laser speckle contrast imaging (LSCI) is a promising non-invasive technique to measure cutaneous microcirculation. This study investigated the association between microcirculation (measured with both LSCI and non-invasive blood pressure measurement) and healing of diabetic foot ulcers 12 and 26 weeks after measurement. We included sixty-one patients with a diabetic foot ulcer in this prospective, single-center, observational cohort-study. LSCI scans of the foot, ulcer, and ulcer edge were conducted, during baseline and post-occlusion hyperemia. Non-invasive blood pressure measurement included arm, foot, and toe pressures and associated indices. Healing was defined as complete re-epithelialization and scored at 12 and 26 weeks. We found no significant difference between patients with healed or non-healed foot ulcers for both types of measurements (p = 0.135–0.989). ROC curves demonstrated moderate sensitivity (range of 0.636–0.971) and specificity (range of 0.464–0.889), for LSCI and non-invasive blood pressure measurements. Therefore, no association between diabetic foot ulcer healing and LSCI-measured microcirculation or non-invasive blood pressure measurements was found. The healing tendency of diabetic foot ulcers is difficult to predict based on single measurements using current blood pressure measurements or LSCI.

Keywords: laser speckle contrast imaging; diabetes mellitus; diabetes complications; foot ulcer; microcirculation; peripheral artery disease; wound healing

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1. Introduction

Diabetes Mellitus is a metabolic disease and its patient population is growing worldwide, with a prevalence of 9.3% of the adults between 20 and 79 years old. A total of 463 million people are living with diabetes [1]. One of the major complications of diabetes is diabetic foot disease. Mortality, high morbidity, costs, and a reduced quality of life are all associated with diabetic foot disease [2–5]. Peripheral neuropathy and peripheral artery disease (PAD) are both major causes for diabetic foot ulceration, and PAD also contributes to poor healing outcomes [6,7]. Recognizing the levels of ischemia of the lower limb arteries is therefore essential in the treatment of foot ulcers in people with diabetes. However, this has been identified by various researchers and clinicians as one of the key challenges in diabetic foot disease [7,8].

Multiple methods are used to identify diabetic foot ulcers that are suspicious to poor healing as a result of PAD. Diagnostic arteriography and non-invasive blood pressure measurements are recommended in guidelines of the International Working Group on the Diabetic Foot (IWGDF) for the treatment of diabetic foot ulcers [9]. Indications for vascular consultation include ankle blood pressure < 50 mmHg, toe pressure < 30 mmHg, and ankle/brachial index (ABI) < 0.5 [6,10]. However, these non-invasive blood pressure measurements have various disadvantages. For example, it has been shown that ABI underestimates the prevalence of PAD in people with diabetes due to the arterial circular calcification of the media and the consequent non-compressibility [11,12]. Toe pressure does not reflect the vascular situation at the ulcer location, and does not measure microcirculatory status. Microcirculation can be estimated with transcutaneous oxygen pressure measurements (TcpO2), where a value above 25 mmHg has been demonstrated to predict ulcer healing potential; however, the presence of oedema can affect the accuracy of these measurements, and as this measurement is confined to the dorsal side of the foot it frequently does not reflect vascular status at the ulcer location [6,10,11,13]. In a recent systematic review on the performance of prognostic markers in the prediction of ulcer healing or amputation among foot ulcers in diabetes, it was concluded that wound healing was associated with a better perfused foot (skin perfusion pressure $\geq$ 40 mmHg, toe pressure $\geq$ 30 mmHg, or TcpO2 $\geq$ 25 mmHg) [8]. However, in most studies included in this systematic review, likelihood ratios of these tests in accurately predicting healing were small. While this is partly the result of coexisting factors (e.g., infection, comorbidities) also impacting on ulcer healing, it also suggests that other measurements overcoming the disadvantages of these non-invasive blood pressure measurements may result in better predictive values.

Novel optical imaging techniques such as laser speckle contrast imaging (LSCI) are available to complement the currently used non-invasive blood pressure measurements in people with diabetic foot disease [14]. LSCI is a non-invasive optical imaging technique able to measure blood flow in the skin [15,16]. In general, the reproducibility of LSCI is high, and it has low inter-subject variability [17–20]. LSCI is an interesting technique to measure blood flow in diabetic foot disease, because it has a widely validated track record of non-invasive in vivo blood flow measurements compared with other established methods of large-area microcirculation [14]. Furthermore, LSCI can provide non-invasive real-time feedback on changes in perfusion, and is able to monitor the microcirculation in the outer layer of the skin. Such microcirculation measurements give an indication of the perfusion directly in and around the ulcer, which overcomes the disadvantage of measurements such as ABI, toe pressure, and TcpO2.

In a previous study among patients with a diabetic foot ulcer, we demonstrated that LSCI is a stable technique with a high inter- and intra-user reliability [21]. We concluded that LSCI can be used in the clinical setting complementing non-invasive blood pressure measurements. However, for assessing its clinical benefit, insight in its prognostic accuracy compared with non-invasive blood pressure measurements is required. Therefore, the aim of this study is to investigate and compare the prognostic values of LSCI and non-invasive blood pressure measurements in relation to healing of diabetic foot ulcers.

2. Materials and Methods

The clinical dataset used for this study was obtained as part of a larger study [21]. This study was approved by a registered medical ethics committee and registered in the Dutch trial register (NTR5116). A total of 33 patients with a diabetic foot ulcer participated and both the non-invasive blood pressure measurements and LSCI measurements of each ulcerated foot was available. This dataset was supplemented with people with a diabetic foot ulcer who were eligible for regular treatment, as LSCI was implemented in daily practice following completion of the above-mentioned study. All patients with a diabetic foot ulcer who were presenting at the outpatient clinic at ZGT Hospital, located in Almelo, the Netherlands, were scanned with LSCI. If the patient fulfilled the inclusion criteria and

gave permission to use the data for scientific purposes, the patient data were included in this study. All examinations used in the current study were part of regular treatment and therefore the second cohort of this study was exempt from medical ethical review according to the Medical Research Involving Human Subjects Act in the Netherlands. These two cohorts together form the participants of this current single center, observational cohort study. All study actions were in line with the principles of the Declaration of Helsinki.

Inclusion criteria were a confirmed diagnosis of type 1 or type 2 diabetes mellitus, and one foot ulcer (defined as break of the skin of the foot that involves at least the epidermis and part of the dermis [22]). Exclusion criteria were having multiple foot ulcers, an amputation of the forefoot or an amputation at a more proximal location of the foot (e.g., midfoot or hindfoot), moderate or severe foot infection (IWGDF grade 3 or 4; [23]), being incapacitated or undergoing cancer treatment. All patients were treated in accordance with the local protocol, which is based on the Dutch guidelines [24] and the IWGDF guidelines [25]. Treatment consisted of offloading, ulcer debridement and wound dressings, antibiotic treatment in case of mild infection, and blood pressure measurements to assess PAD, and surgical revascularization when required. Regular blood pressure measurements included both the non-invasive blood pressure measurements (i.e., arm pressure, ankle pressure, ABI, toe pressure, and TcpO2). Regular microcirculatory measurements included LSCI scans in and around the ulcer location.

Measurements were performed after ulcer debridement, and consisted of first doing LSCI scans, followed by non-invasive blood pressure measurements. LSCI scans were performed of the ulcer foot with a PeriCam PSI NR (Perimed AB, Stockholm, Sweden). Either the plantar or the dorsal side of the foot was scanned, depending on the ulcer location, with the ulcer location to be included in the scan. Perfusion was expressed in perfusion units (PU). During the scan, the patient lay supine on the examination table barefoot. After 5 min, for the patient to get used to the room temperature (kept between an ambient 21–22 degrees), the LSCI scans were acquired. During the scans, three different time periods of interest (TOI) were measured: baseline, biological zero, and post-occlusion hyperemia measurements. The baseline was a measurement in the first stage of the scan when the measured perfusion was stable on visual inspection for 30 s. Subsequently, a cuff around the ankle was inflated to stop blood flow to the foot. During this time the perfusion dropped to the biological zero value of the patient. When the perfusion did not further decrease for 30 s, the biological zero was measured. After this measurement, the ankle cuff was released. The maximum measured blood flow after release of pressure was used as the post-occlusion hyperemia value. During each TOI, different regions of interest (ROI) of the foot were measured (i.e., foot, ulcer, and ulcer edge). As described in detail in our previous paper [21], each ROI was manually selected in the scans in order to measure the mean perfusion of different areas of the foot and ulcer. The ROIs were positioned at the beginning of each TOI and repositioned during the scan to correct for possible movement of the foot during the scan [21]. Non-invasive blood pressure measurements consisted of measuring arm pressure, ankle pressure, toe pressure, and TcpO2, with a PeriFlux 6000 (Perimed AB, Stockholm, Sweden), all according to the manufacturer's instructions.

Each patient was classified as non-ischemic, ischemic, or critical-ischemic based on non-invasive blood pressure measurements, following IWGDF criteria [6,26]. Patients were classified as critical-ischemic when ABI $\leq$ 0.39, or ankle pressure < 50 mmHg, or toe pressure or TcpO2 < 30 mmHg. Patients not classified as critical-ischemic but had an ABI between 0.4–0.79, or an ankle pressure between 50–100 mmHg, or a toe pressure or TcpO2 between 30–59 mmHg, were classified as ischemic. Patients were classified as non-ischemic with ABI $\geq$ 0.8 and an ankle pressure > 100 mmHg and a toe pressure and TcpO2 $\geq$ 60 mmHg [6,10,26].

Clinical background and different parameters (Table 1) of the patient were obtained at baseline. The level of neuropathy was measured with a 10 g Semmes–Weinstein monofilament [6], HbA1c was measured with blood tests, and other parameters such as smoking were collected or measured during anamnesis. Follow-up for outcomes was until ulcer

healing or for a maximum 26 weeks. Healing of the foot ulcer was defined as complete re-epithelialization of the ulcer without revascularization or major amputation [22] and was scored by an experienced clinician at 12 and 26 weeks during the outpatient clinic visits. Patients who died, who underwent revascularization, or major amputation were excluded.

Table 1. Patient characteristics at baseline, and separated between healed and non-healed patients at 12 and 26 weeks.

Variable		Baseline	12 Weeks			26 Weeks		
		Mean ± SD N (%)	Healed Mean ± SD	Non-Healed Mean ± SD	*p*-Value	Healed Mean ± SD	Non-Healed Mean ± SD	*p*-Value
Patient Characteristics		53 (100%)	23 (43.4%)	30 (56.6%)		36 (67.9%)	17 (32.1%)	
Age (Years)		66.7 ± 12.8	68.9 ± 13.1	65.1 ± 12.7	0.300	67.3 ± 11.9	65.7 ± 15.1	0.679
Gender	Male	42 (79.2%)	18 (78.3%)	24 (80%)	0.877	30 (83.3%)	12 (70.6%)	0.286
	Female	11 (20.8%)	5 (21.7%)	6 (20%)		6 (16.7%)	5 (29.4%)	
Height (cm)		179.4 ± 9.6	179.1 ± 11.1	179.7 ± 8.4	0.858	179.7 ± 10.7	178.7 ± 6.6	0.744
Weight (kg)		96.0 ± 19.9	98.0 ± 21.4	94.4 ± 18.8	0.546	96.9 ± 20.1	93.9 ± 20.0	0.660
BMI		29.7 ± 5.5	30.4 ± 5.5	29.2 ± 5.6	0.475	29.9 ± 5.2	29.4 ± 6.4	0.808
HbA1c (mmol/mol)		63.6 ± 21.0	59.5 ± 14.4	67.6 ± 25.6	0.221	63.0 ± 15.9	65.7 ± 33.4	0.729
Smoking	Yes	11 (52.4%)	3 (42.9%)	8 (57.1%)	0.590	6 (46.2%)	5 (62.5%)	0.436
	No	4 (19.0%)	1 (14.3%)	3 (21.4%)		2 (16.4%)	2 (25.0%)	
	Stopped	6 (28.6%)	3 (42.9%)	3 (21.4%)		5 (38.5%)	1 (12.5%)	
	Unknown	32	16	16		23	9	
Diabetes Type	1	3 (5.7%)	1 (4.3%)	2 (6.7%)	0.717	2 (5.6%)	1 (5.9%)	0.962
	2	50 (94.3%)	22 (95.7%)	28 (90.0%)		34 (94.4%)	16 (88.2%)	
Diabetes Duration	≤10 years	20 (43.5%)	8 (38.1%)	12 (48.0%)	0.394	12 (36.4%)	8 (61.5%)	0.180
	>10 years	26 (56.5%)	13 (61.9%)	13 (52.0%)		21 (63.6%)	5 (38.5%)	
	Unknown	7	2	5		3	4	
Dialysis	Yes	3 (5.7%)	1 (4.3%)	2 (6.7%)	0.266	2 (5.6%)	1 (5.9%)	0.998
	No	47 (88.7%)	22 (95.7%)	25 (83.3%)		32 (88.9%)	15 (88.2%)	
	In the past	3 (5.7%)	0 (0.0%)	3 (10.0%)		2 (5.6%)	1 (5.9%)	
Infections	Yes	8 (15.1%)	4 (17.4%)	4 (13.3%)	0.683	5 (13.9%)	3 (17.6%)	0.721
	No	45 (84.9%)	19 (82.6%)	26 (86.7%)		31 (86.1%)	14 (82.4%)	
Neuropathy	Yes	48 (96.0%)	22 (100.0%)	26 (92.9%)	0.201	35 (100.0%)	13 (86.7%)	0.027 *
	No	2 (4.0%)	0 (0.0%)	2 (7.1%)		0 (0.0%)	2 (13.3%)	
	Unknown	3	1	2		1	2	
UT-classification					0.776			0.704
	0A	4 (7.5%)	2 (8.7%)	2 (6.7%)		3 (8.3%)	1 (5.9%)	
	1A	30 (56.6%)	15 (65.2%)	15 (50.0%)		22 (62.9%)	8 (47.1%)	
	1B	1 (1.9%)	0 (0.0%)	1 (3.3%)		0 (0.0%)	1 (5.9%)	
	1C	1 (1.9%)	0 (0.0%)	1 (3.3%)		1 (2.8%)	0 (0.0%)	
	2A	5 (9.4%)	1 (4.3%)	4 (13.3%)		3 (8.3%)	2 (11.8%)	
	2B	5 (9.4%)	2 (8.7%)	3 (10.0%)		3 (8.3%)	2 (11.8%)	
	3A	3 (5.7%)	1 (4.3%)	2 (6.7%)		2 (5.6%)	1 (5.9%)	
	3B	3 (5.7%)	2 (8.7%)	1 (3.3%)		2 (5.6%)	1 (5.9%)	
	3C	1 (1.9%)	0 (0.0%)	1 (3.3%)		0 (0.0%)	1 (5.9%)	
History of Ulcers	Yes	31 (58.5%)	15 (65.2%)	16 (53.3%)	0.384	20 (55.6%)	11 (64.7%)	0.528
	No	22 (41.5%)	8 (34.8%)	14 (46.7%)		16 (44.4%)	6 (35.3%)	
Minor Amputation	Yes	8 (15.1%)	2 (8.7%)	6 (20.0%)	0.255	5 (13.9%)	3 (17.6%)	0.721
	No	45 (84.9%)	21 (91.3%)	24 (80.0%)		31 (86.1%)	14 (82.4%)	
Vascular Status	Non-ischemic	7 (13.2%)	1 (4.3%)	6 (20.0%)	0.925	4 (11.1%)	3 (17.6%)	0.275
	Ischemic	28 (52.8%)	16 (69.6%)	12 (40.0%)		23 (63.9%)	5 (29.4%)	
	Critical-ischemic	18 (34.0%)	6 (26.1%)	12 (40.0%)		9 (25.0%)	9 (52.9%)	

* $p < 0.05$; note: UT-classification is the University of Texas Diabetic Wound Classification [27].

Statistical Analysis

To investigate differences between the healed and non-healed participants at 12 and 26 weeks, t-tests were conducted for all numerical variables and a Chi^2 test for all categorical variables. Statistical relevance was considered with a *p*-value less than 0.05. ROC curves were created and the sensitivity and specificity of different parameters were calculated. The thresholds to calculate positive and negative likelihood ratio (LLR+ and LLR–) were chosen based on the highest combination of both sensitivity and specificity. A LLR– between 0.5–1 or LLR+ between 1–5 indicates no small change, while a LLR– between 0.1–0.5 or LLR+ between 5–10 were considered as moderate. LLR– below 0.1 or LLR+ above 10 were considered as large effect [8].

3. Results

A total of 61 patients were included. One patient died during follow up, two patients underwent major amputation, and five patients underwent revascularization. Of the 53

patients included for analysis, 23 (43.4%) healed within 12 weeks, 36 (67.9%) in 26 weeks, while 17 patients (32.1%) did not heal in 26 weeks or received revascularization treatment (Figure 1, Table 1).

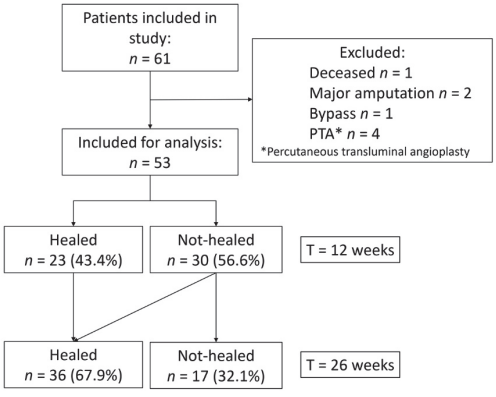

Figure 1. Schematic overview of patient population and clinical outcomes at 12 and 26 weeks.

Patients were on average 67 years, predominantly male, and with an average BMI of 29.7 (Table 1). Average healing percentages for the non-ischemic, ischemic, and critical-ischemic groups were 4.3%, 69.6%, and 26.1% at 12 weeks and 11.1%, 63.9%, and 25.0% at 26 weeks. There were no significant differences between healed and non-healed patients at 12 weeks (p = 0.925) and also no significant differences between these groups at 26 weeks (p = 0.275; Table 1). Furthermore, for the majority of other patient characteristics, no significant difference was found between healers and non-healers (p-values ranging from 0.027–0.949; Table 1).

There were no significant differences in any of the perfusion measurements between the healed and non-healed group, neither for LSCI at the foot, ulcer, or ulcer edge, nor for any of the non-invasive blood pressure measurements (p-values ranging from 0.136–0.983; Table 2). There were also no significant differences when we compare the 95% confidence intervals of both the LSCI perfusion measurements and non-invasive blood pressure measurements (Figure 2).

Table 2. Mean values of laser speckle contrast imaging (in perfusion units (PU)) and non-invasive blood pressure measurements (mmHg) at baseline and between patients with healed versus non-healed foot ulcers.

Variable	Baseline	12 Weeks			26 Weeks		
	Mean ± SD N (%)	Healed Mean ± SD	Non-Healed Mean ± SD	p-Value	Healed Mean ± SD	Non-Healed Mean ± SD	p-Value
Laser Speckle Contrast Imaging (PU)							
Foot							
Baseline	50.3 ± 14.6	49.3 ± 15.1	51.1 ± 14.5	0.654	49.4 ± 13.9	52.3 ± 16.3	0.508
Biological zero	12.8 ± 7.7	12.7 ± 7.3	12.8 ± 8.1	0.959	12.5 ± 6.5	13.5 ± 10	0.637
Post occlusion peak	77.3 ± 26.6	76.7 ± 24.4	77.8 ± 28.6	0.889	77.4 ± 23.2	77.2 ± 33.6	0.983
Ulcer							
Baseline	104.8 ± 34.6	108.8 ± 33	101.8 ± 36.1	0.467	109.1 ± 35.7	95.8 ± 31.2	0.197
Biological zero	25.2 ± 15.3	26.4 ± 17.9	24.3 ± 13.2	0.631	25 ± 16.3	25.7 ± 13.4	0.884
Post occlusion peak	104.0 ± 33.4	107.8 ± 32.6	101.1 ± 34.3	0.473	108.2 ± 35.2	95.2 ± 28.2	0.190
Ulcer Edge							
Baseline	92.2 ± 30.7	96.3 ± 33.4	89.1 ± 28.6	0.402	94.2 ± 33.8	88.1 ± 23	0.509
Biological zero	20.1 ± 10.7	20.5 ± 10.8	19.8 ± 10.9	0.840	19.4 ± 10	21.7 ± 12.3	0.465
Post occlusion peak	102.0 ± 32.9	108.1 ± 33.9	97.3 ± 31.9	0.239	104.8 ± 35.3	96 ± 27.4	0.373
Non-invasive Blood Pressure Measurements (mmHg)							
Ankle	121.9 ± 41.0	121.3 ± 46.3	122.3 ± 37.2	0.931	126.9 ± 41.4	110.4 ± 39	0.183
Toe	88.7 ± 45.3	97.7 ± 45.1	81.8 ± 45.1	0.220	95.4 ± 45.2	75.2 ± 43.8	0.136
ABI	0.90 ± 0.31	0.88 ± 0.3	0.92 ± 0.32	0.698	0.94 ± 0.32	0.82 ± 0.3	0.188
TBI	0.68 ± 0.37	0.73 ± 0.32	0.64 ± 0.41	0.410	0.73 ± 0.38	0.57 ± 0.34	0.151
TcpO_2	47.9 ± 17.5	44.8 ± 15.1	50.3 ± 19.1	0.262	46.8 ± 14.1	50.1 ± 23.6	0.526

Note: PU = perfusion units; ABI = ankle/brachial index; TBI = toe/brachial index; and TcpO_2 = transcutaneous oxygen pressure.

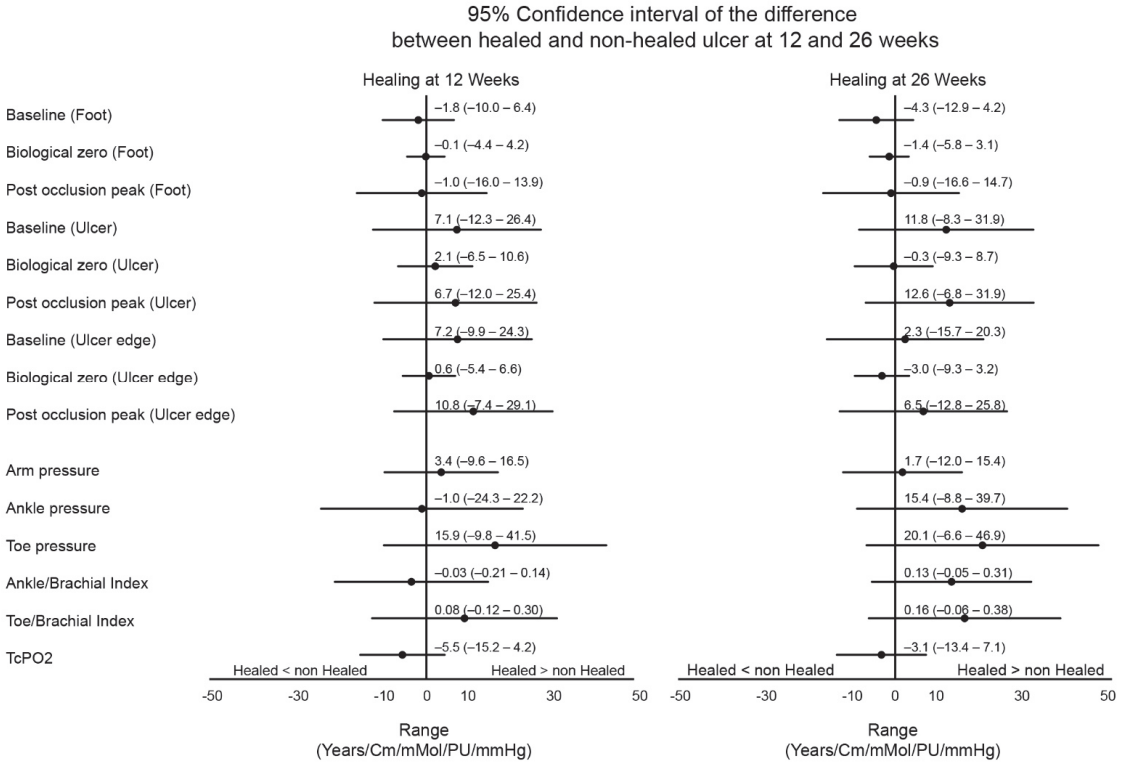

Figure 2. Difference in range of parameters between healed and non-healed patients at 12 and 26 weeks.

The ROC curves for LSCI scans and non-invasive blood pressure measurements demonstrated poor to moderate sensitivity and specificity (Figure 3).

Both LLR+ and LLR– showed a small to no effect (LLR+ 1.06–4.72; LLR– 0.36–0.89; Figure 3, Table 3). The largest effect for prognosis of healing at 12 weeks was found for LSCI at the ulcer during baseline or post-occlusive peak (LLR–: 0.36). The largest effect for prognosis of healing at 26 weeks was found for LSCI at the ulcer edge during baseline or post-occlusive peak (LLR+: 4.72 and 2.60) and for ankle and toe pressure (LLR–: 0.40).

With no significant differences found between patients who healed and those who did not heal, we repeated all tests for the group of participants classified as ischemic only (n = 28). We chose to do so, because advanced blood pressure assessment is most important in this group from a clinical perspective, as these are patients for whom diagnosis and prognosis are in a grey area. However, these post hoc analyses did not result in different findings (results not shown); again, no differences were seen in blood pressure measurements between patients who healed and patients who did not heal.

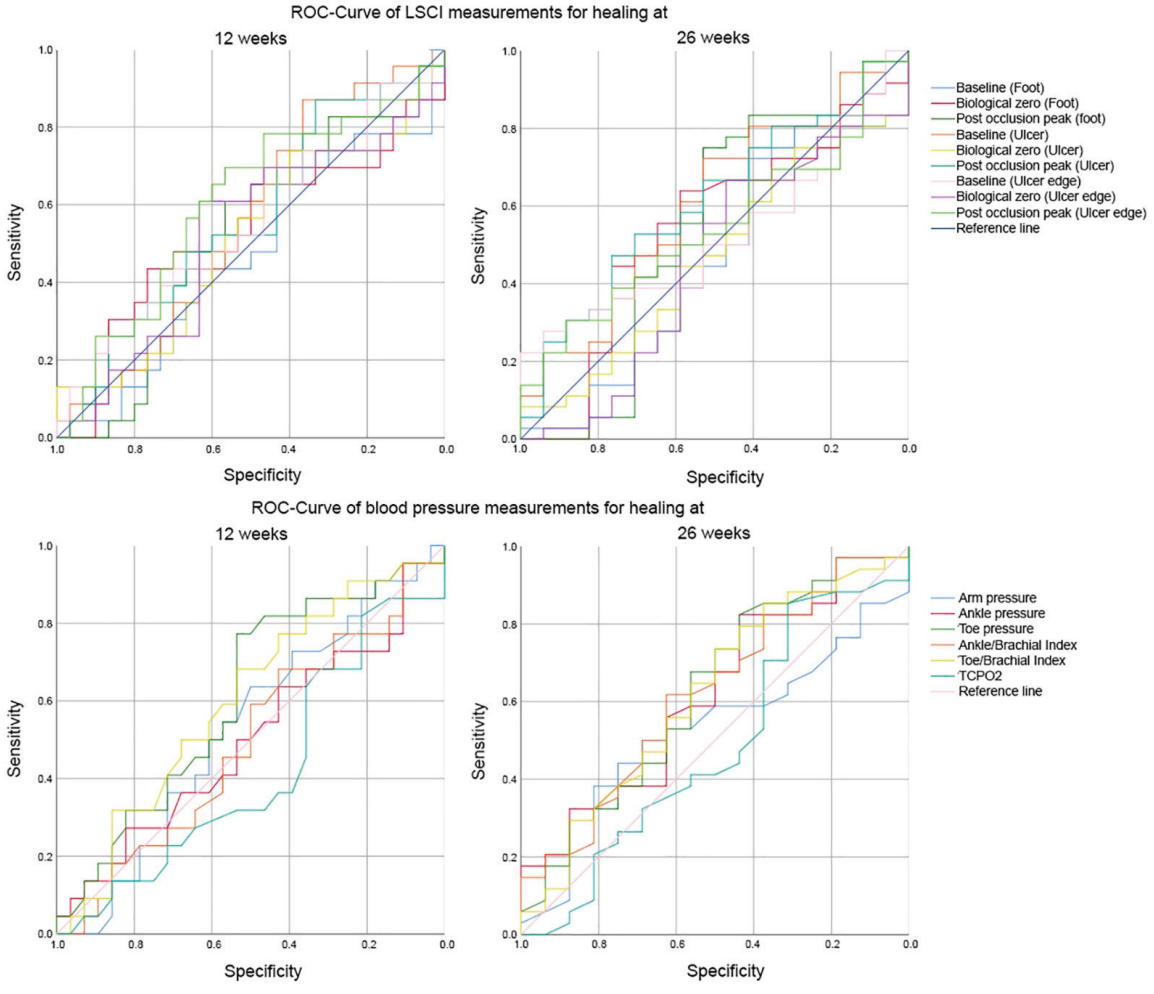

Figure 3. ROC curves showing sensitivity and specificity for laser speckle contrast imaging measurements and non-invasive blood pressure measurements as prognostic tests for ulcer healing at 12 or 26 weeks.

Table 3. Threshold, sensitivity, and specificity for non-invasive blood pressure measurements and laser speckle contrast imaging measurements, for healing after 12 weeks and 26 weeks.

12 Weeks	Threshold	AUC	Sensitivity	Specificity	LLR+	LLR–
Laser speckle contrast imaging (PU)						
Foot						
Baseline	43.5 PU	0.467	0.696	0.400	1.16	0.76
Biological zero	14.3 PU	0.528	0.435	0.767	1.86	0.74
Post occlusion peak	73.5 PU	0.517	0.609	0.567	1.40	0.69
Ulcer						
Baseline	84.3 PU	0.558	0.870	0.367	1.37	0.36 **
Biological zero	15.7 PU	0.510	0.739	0.400	1.23	0.65
Post occlusion peak	89.4 PU	0.561	0.870	0.333	1.30	0.39 **
Ulcer edge						
Baseline	103.0 PU	0.552	0.435	0.700	1.45	0.81
Biological zero	19.9 PU	0.519	0.609	0.633	1.66	0.62
Post occlusion peak	96.6 PU	0.603	0.696	0.567	1.61	0.54
Non-invasive blood pressure measurements (mmHg)						
Arm pressure	130.5 mmHg	0.528	0.636	0.500	1.27	0.73
Ankle pressure	153.0 mmHg	0.500	0.273	0.821	1.53	0.89
Toe pressure	77.5 mmHg	0.608	0.773	0.536	1.66	0.42 **
Ankle brachial index	0.83	0.494	0.682	0.429	1.19	0.74
Toe brachial index	0.57	0.599	0.682	0.536	1.47	0.59
TcpO_2	30.5 mmHg	0.416	0.818	0.214	1.04	0.85
26 weeks	**Threshold**		**Sensitivity**	**Specificity**	**LLR+**	**LLR–**
Laser speckle contrast imaging (PU)						
Foot						
Baseline	41.9 PU	0.454	0.722	0.412	1.23	0.67
Biological zero	10.9 PU	0.540	0.639	0.588	1.55	0.61
Post occlusion peak	62.3 PU	0.541	0.750	0.529	1.59	0.47 *
Ulcer						
Baseline	92.3 PU	0.606	0.722	0.529	1.53	0.52
Biological zero	12.7 PU	0.469	0.750	0.294	1.06	0.85
Post occlusion peak	109.5 PU	0.609	0.472	0.765	2.01 *	0.69
Ulcer edge						
Baseline	118.5 PU	0.525	0.278	0.941	4.72 *	0.77
Biological zero	14.0 PU	0.455	0.667	0.471	1.26	0.71
Post occlusion peak	123.0 PU	0.547	0.306	0.882	2.60 *	0.79
Non-invasive blood pressure measurements (mmHg)						
Ankle pressure	96.0 mmHg	0.619	0.824	0.438	1.46	0.40 *
Toe pressure	54.0 mmHg	0.626	0.824	0.438	1.46	0.40 *
Ankle brachial index	0.89	0.619	0.618	0.625	1.65	0.61
Toe brachial index	0.51	0.618	0.735	0.500	1.47	0.53
TcpO_2	30.5 mmHg	0.484	0.853	0.313	1.24	0.47 *

Note: AUC = area under the curve; LLR+ = positive likelihood ratio; LLR– = negative likelihood ratio; TcpO_2= transcutaneous oxygen pressure measurements; * small effect, ** moderate effect.

4. Discussion

The aim of this study was to investigate the association between foot and ulcer (micro) circulation (measured with both LSCI and non-invasive blood pressure measurements) and healing of diabetic foot ulcers at 12 and 26 weeks. We found no significant differences in any of the measurements between the group of healed and non-healed patients, neither at 12 nor 26 weeks. Positive and negative likelihood ratios showed no or only small effects. In our cohort, both LSCI and non-invasive blood pressure measurements were not useful as a standalone prognostic test for diabetic foot ulcer healing. This result is not in line with the outcomes of a recent systematic review by Forsythe et al. [8] in which it was concluded that some non-invasive blood pressure measurements may have prognostic value. However,

the majority of studies included in this review showed similar likelihood ratios as found in the current study [8]. This implies that prognostic quality of non-invasive blood pressure measurements on its own are not always a valuable predictor for healing of diabetic foot ulcers.

While our study had a different approach in calculating the cut-off values for different non-invasive bedside blood pressure measurement tests, we can still use the results of the studies included in Forsythe et al. [8] to put the effect of the found likelihood ratios in perspective. First, in our study, the cut-off values for the different blood pressure measurements were based on the optimal combination of both sensitivity and specificity for ulcer healing. This is a different approach compared with other studies in which they used fixed cut-off values and in which they calculated corresponding likelihood ratios based on those values. We chose this approach, because no cut-off values for LSCI measurements are available yet. Therefore, it was necessary to find the cut-off values with the highest prognostic power. To compare LSCI with the non-invasive blood pressure measurements, we used the same technique and calculations with this bedside test as well. Despite this difference in approach, the likelihood ratios were comparable. For example, we found a LLR+ for healing after 12 and 26 weeks based on the ankle pressure of 1.53 and 1.46 with a cut-off values of >153mmHg and >96.0 mmHg. Other studies found an LLR+ of 1.08 (>50 mmHg) [28], 1.46 ($\geq$50 mmHg) [29], 2.52 ($\geq$80 mmHg) [29], 3.24 ($\geq$70 mmHg) [30], and 6.40 ($\geq$100 mmHg) [31]. Although the studies with a higher threshold (>70 mmHg) showed a higher LLR+, this effect was still small (LLR+: 2.52–3.24) to moderate (LLR+ 6.40), while other studies observed no change in effect based on ankle pressure measurements (LLR+: 1.08–1.46).

Similar findings are seen when comparing the found LLR+ and LLR– for toe pressure and TcpO2. Our study found LLR+ and LLR– for toe pressure and TcpO2 of 1.46, 0.40, and 1.24, 0.47, respectively. Other studies reported similar LLR+ and LLR– for toe pressure measurements. For example 1.12, 0.88 [28]; 1.28, 0.33 [29]; 2.47, 0.21 [30]; 2.88, 0.64 [32]; 4.30, 0.25 [29]; and 5.00, 0.88 [32]. Although the LLR are not exactly identical, the results are similar in effect and range from no effect (LLR+: 1–2; LLR–: 0.5–1), to a small effect (LLR+: 2–5; LLR–: 0.2–0.5), comparable to our findings of no effect (LLR+: 1.46) and a small effect (LLR–: 0.40).

When we compared our LLR+ for TcpO2 with other LLR+ values, some studies did find larger effects: LLR+ of 10.03 [32] and 5.14 [33] were found for TcpO2 thresholds $\geq$30 mmHg, indicating a moderate to large prognostic effect. However, those findings were not unanimous as other studies found lower LLR+ (1.21 and 2.73) [34,35]. This is an indication that the prognostic power of different tests are influenced by the specific patient populations and other factors such as environment and time period.

As this is the first study to investigate LLR+ and LLR– for LSCI in relation to diabetic foot ulcer healing, there are no findings for direct comparison. However, in light of the above-mentioned studies, our findings are within the expected range. Despite the advantages of measuring at and around the exact ulcer location, and including both baseline perfusion values and stress-test values, LSCI did not result in improved prognostic likelihood ratios for ulcer healing in this cohort, compared with regular non-invasive blood pressure measurements.

The following limitations of this study should be considered. First, the combination of more than one prognostic test may provide more useful information on the probability of healing than a single test or test used in isolation [8]. However, in the current study, we analyzed the different blood pressure tests individually instead of combining them. While it is interesting to do so in a follow-up study, with the low likelihood ratios found, the benefits of combining may be small.

Second, the exclusion of patients that underwent a major amputation can be considered as another limitation. A major amputation could have been considered as endpoint, too, in addition to wound healing. This could be useful for clinicians in order to identify the patients with a higher probability of healing without revascularization to pursue a

conservative approach. Furthermore, it could be of importance to identify patients with an unacceptable high risk of a major amputation. For those patients, adequate revascularization should be a priority [8]. Therefore, amputation incidence can help in assessing the impact of disease. However, it is not necessarily a good measure of the quality of care and amputation incidence is partially based on the clinical choice of the attending physician [36]. Therefore, we decided to focus on the healing of the diabetic foot as a biological endpoint.

Third, the follow-up period of six months and the use of two measurement moments (at 12 and 26 weeks) dichotomizes healing, rather than using the more detailed time to healing in days or weeks. This dichotomization results in diverse groups, where both short healing times (<4 weeks) and long healing times (>20 weeks) could end up in the same group. In further research it might be better to use time to heal (in weeks) as an endpoint for that study. This provides a better understanding and more useful parameter to obtain meaningful insights into the patients' healing tendency, since the time needed to heal a chronic diabetic foot ulcers usually varies a lot. We decided to use a different approach in this study for several reasons. First, we wanted to compare our results with previous research [8]. Second, dichotomization is recommended for the assessment of data in diabetic foot research (e.g., [37]). Third, diagnostic values cannot be calculated for a continuous outcome measure.

A fourth limitation of this study is that drug use and specific additional treatment of the patient (for example offloading or wound dressings) were not taken into account in this study. Where the first might influence microcirculation measurements, the latter might influence healing outcomes. However, because all patients were treated in the same center and by the same clinicians, clinical decisions were considered similar, and therefore not accounted for in analyses. Furthermore, while some drugs might affect microcirculation, no previous study on prognosis has found an effect of such drugs on likelihood ratios for prognosis [8].

Finally, it is questionable whether we can compare our results with outcomes of previous studies. Although we see comparable results, it is likely that the included populations differ. Whereas in the past, the majority of patients with diabetic foot ulcers had been treated in hospitals, currently only the more complex cases visit hospitals or specialized care centers for diabetic foot ulcers. For future research it would be interesting to compare and validate our findings with more recent studies.

5. Conclusions

No association between healing of diabetic foot ulcers and microcirculation measured with LSCI or non-invasive blood pressure measurements was found. We can conclude that both types of measurements were not useful as a standalone prognostic instrument for diabetic foot ulcer healing.

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Institutional Review Board Statement: All study actions were conducted according to the guidelines of the Declaration of Helsinki. The first cohort of this study was approved by a registered medical ethics committee METC TWENTE and registered in the Dutch trial register (NTR5116, 25-03-2015). Ethical review and approval were waived for the second cohort of this study because all examinations used in this cohort were part of regular treatment. Therefore, the second cohort of this study was exempt from medical ethical review according to the Medical Research Involving Human Subjects Act in the Netherlands.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data used to support the findings of this study are included within the article. Additional source data can be requested from the corresponding authors (O.A.M. and W.S.).

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Autologous Peripheral Blood Mononuclear Cells for Limb Salvage in Diabetic Foot Patients with No-Option Critical Limb Ischemia

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Abstract: Peripheral blood mononuclear cells (PBMNCs) are reported to prevent major amputation and healing in no-option critical limb ischemia (NO-CLI). The aim of this study is to evaluate PBMNC treatment in comparison to standard treatment in NO-CLI patients with diabetic foot ulcers (DFUs). The study included 76 NO-CLI patients admitted to our centers because of CLI with DFUs. All patients were treated with the same standard care (control group), but 38 patients were also treated with autologous PBMNC implants. Major amputations, overall mortality, and number of healed patients were evaluated as the primary endpoint. Only 4 out 38 amputations (10.5%) were observed in the PBMNC group, while 15 out of 38 amputations (39.5%) were recorded in the control group ($p = 0.0037$). The Kaplan–Meier curves and the log-rank test results showed a significantly lower amputation rate in the PBMNCs group vs. the control group ($p = 0.000$). At two years follow-up, nearly 80% of the PBMNCs group was still alive vs. only 20% of the control group ($p = 0.000$). In the PBMNC group, 33 patients healed (86.6%) while only one patient healed in the control group ($p = 0.000$). PBMNCs showed a positive clinical outcome at two years follow-up in patients with DFUs and NO-CLI, significantly reducing the amputation rate and improving survival and wound healing. According to our study results, intramuscular and peri-lesional injection of autologous PBMNCs could prevent amputations in NO-CLI diabetic patients.

Keywords: peripheral blood mononuclear cells; PBMNCs; cell therapy; critical limb ischemia; no-option critical limb ischemia; NO-CLI; diabetic foot; major amputation; amputation-free survival; AFS; wound healing

1. Introduction

Critical limb ischemia (CLI) has a high incidence in patients with diabetes and is related with high morbidity and mortality rates [1]. Limb salvage is associated with percutaneous or surgical revascularization, in comparison to the medical treatment in patients with peripheral arterial disease (PAD) and diabetic foot ulcers (DFUs) [2]. However, up

to 25% of diabetic patients are not eligible for revascularization as a result of the inability to overcome vessel obstruction and/or for critical general conditions [3,4]. Of the one million annual amputations worldwide, 75% are performed on patients with type 2 diabetes (T2DM) [5]. No-option critical limb ischemia (NO-CLI) remains a strong unmet clinical need: at 1 year follow-up, NO-CLI diabetic patients showed, respectively, lower rates of limb salvage (13.8% vs. 73.4%, $p < 0.0001$), higher rates of amputation (30% vs. 4.5%, $p = 0.0001$), and higher mortality rates (50% vs. 8.9%, $p < 0.0001$) in comparison to revascularizable CLI patients [4]. Autologous cell therapy, and the use of autologous PBMNCs in particular, has arisen as a possible strategy to treat NO-CLI patients as well as diabetic foot patients [6–9]. Recently, Rigato et al. [10], in a recent meta-analysis of NO-CLI patients, showed that autologous cell therapy had the potential to modify the natural history of intractable CLI. In separate cell type analyses, PBMNCs, but not other cell types, were associated with a significant decrease in amputation and increase in amputation-free survival [10]. Accordingly, Liew et al., in a meta-analysis of 16 randomized trials, showed that PBMNCs lowered the risk of major amputation and significantly increased ulcer healing [11]. The primary mechanism of action of PBMNCs is the induction of therapeutic angiogenesis with collateral vessel formation [12] through the paracrine activities of growth factors, cytokines, and messenger molecules, as well as through exosomes [13]. Moreover, PBMNCs, monocytes/macrophages, and lymphocyte/Treg populations play a key role in tissue regeneration in persistent trophic lesions through inflammatory macrophage M1 polarization to the M2 regenerative phenotype [14,15]. CD14+ monocytes have also been proven to be efficient in patients with diabetes as opposed to the decreased angiogenic activity of CD34+ stem cells [16]. Recent technology improvements have led to the development of less invasive, operator independent, and user-friendly point of care devices based on peripheral blood selective filtration to produce fresh autologous immobilized peripheral blood mononuclear cells, with evidence in term of adequate potency in therapeutic angiogenesis in vitro and in vivo [17]. Promising results were obtained by immobilized PBMNCs produced by point of care selective filtration in different clinical trials [18,19], including in diabetic patients. The aim of this study is to evaluate PBMNC implants in comparison to standard care treatment in NO-CLI patients with DFUs.

2. Materials and Methods

This study is a retrospective cohort study approved by the local ethics committee. A cohort of 76 NO-CLI patients with DFUs that were not eligible for revascularization in the first instance according to ESVS ESC 2017 criteria [20], or after multiple revascularization failures, were enrolled and treated with standard medical therapy from January 2014 to February 2019. Data were collected in the hospital's local database and analyzed retrospectively.

Patients in both groups received the same standard therapy: surgical debridement, local dressings, antiplatelet drugs, pain relief therapy and antibiotics in case of infection signs, and offloading of the affected foot, in accordance with international guidance [21]. Since October 2016, PBMNC filtration technology has been available in our center, and 38 patients were treated with standard care and in addition with autologous PBMNCs.

The inclusion criteria of both cohort groups were: (a) ulcers with inadequate perfusion, as indicated by a transcutaneous oxygen pressure value ($TcpO_2$) < 30 mmHg; (b) ulcers with grade I or II or III stage C as defined by the Texas University Classification System [21]; (c) evidence of no run-off pedal vessels, failure after several percutaneous interventions (where re-intervention was no longer possible), or failure after infra-genicular bypass grafting; (d) possibility to save foot support. Exclusion criteria were: (a) lesion site above the tibial–tarsal joint; (b) moderate or severe infection according to the WIFi classification system (The Society of Vascular Surgery—Wound Ischemia and Foot Infection Classification System) [22]; (c) NYHA class IV; (d) anemia (Hb < 8 g/dL); (e) coagulation disorder/thrombocytopenia (PLT < 50,000/μL); or (f) active cancer/leukemia or lymphoma hematological disease.

Both the standard care control group (38 patients) and the PBMNC group (38 patients) received the same diagnostic–therapeutic multidisciplinary approach: diabetes control was maximized by the diabetologist; comprehensive foot assessment was carried out by the nurse, together with the diabetologist, including determination of vibration perception threshold, 10 g monofilament test, and $TcpO_2$ measurement; the standard of care includes dressings, off-loading and systemic therapy according to the IWGDF guidelines [23], antibiotic therapy prescribed by infectious disease specialists, and vascular assessment and revascularization procedures performed by cardiologists, vascular surgeons, or interventional radiologists.

Informed consent for participation in the study during the clinical trial was obtained from all subjects.

The concentration of autologous PBMNCs was produced according to the instructions for use by MonoCells–Pall Celeris (Athena) filtration-based point of care device for the rapid preparation of TNC concentrate from 120 mL of anticoagulated blood, for use in human cell therapy applications (now available as Hematrate Blood Filtration System–Cook Regentec). This system is the first point of care device conceived to concentrate an MNC-enriched population of TNCs with high angiogenic potential from PB without apheresis by means of a filtration system. The cell product obtained has been extensively characterized in terms of composition, recovery, and FACS cell population analysis [17]. Briefly, TNCs were enriched 2.97-fold and MNCs were enriched 4.2-fold (average dose implanted = 1.06 ± 0.28 ($\times 10^8$); the CD34+ progenitor cell subpopulation was enriched by 5.6% ± 4.2% versus peripheral blood with a mean CD34+ cell count of 1.37×10^6. The efficiency of the CD34+ hematopoietic stem cell enrichment of this selective filtration system is comparable with the CD34+ concentration obtained by the use of a point of care device for bone marrow cells (BMAC 2) [17,24]. All procedures were performed in an operating room with anesthesiologic support (propofol and/or peripheral block). After appropriate surgical debridement of the wound bed, multiple perilesional and intramuscular injections of 10 mL PBMNC cell suspensions (0.2–0.3 mL in boluses) were injected along the relevant axis below the knee, at intervals of 1–2 cm and to a mean depth of 1.5–2 cm, using a 21 G needle. This procedure was repeated three times for each patient at intervals of 30–45 days from each other. Foot-sparing surgery in patients treated with PBMNCs was performed at the same time as the final cell implant, and only when the $TcpO_2$ value was above 30 mmHg (excluding all patients without foot perfusion improvement). Major amputation was defined as above the ankle amputation. Healing was defined as complete coverage by epithelial regeneration.

Amputations, risk of death, and healed patients were evaluated as primary outcomes. $TcPO_2$ and healing time were evaluated as secondary outcomes. After the first treatment, patients were regularly followed up for two years, with evaluations at 1, 3, 6, 12, 18, and 24 months. See Figure 1 for flow diagram.

Statistical Analysis

A baseline assessment was carried out to estimate any differences among the standard care control group and the PBMNC group. Due to the small sample size, the evaluation was performed through non-parametric tests (Mann–Whitney U test for independent samples for continuous variables, and Cochrane chi-square test for discrete variables). For patient features and baseline demographics, Bonferroni correction for multiple comparisons was applied and a p value equal to 0.003 was considered as the threshold for statistical significance.

A multivariate survival analysis was performed using the Kaplan–Meier survival analysis model by statistical epidemiological software SPSS, version 25. The study size was designed to show a 90% power to identify a proportion of avoided amputations of 70% or greater. Results were considered statistically significant when measures had an estimated error under the 5% threshold: for p values <0.05, the null hypothesis was then rejected.

The estimate of relative risk (RR), absolute risk reduction (ARR), relative risk reduction (RRR), and number needed to treat (NNT) was then achieved, with a 95% confidence interval, 5% alpha error, and 20% beta error.

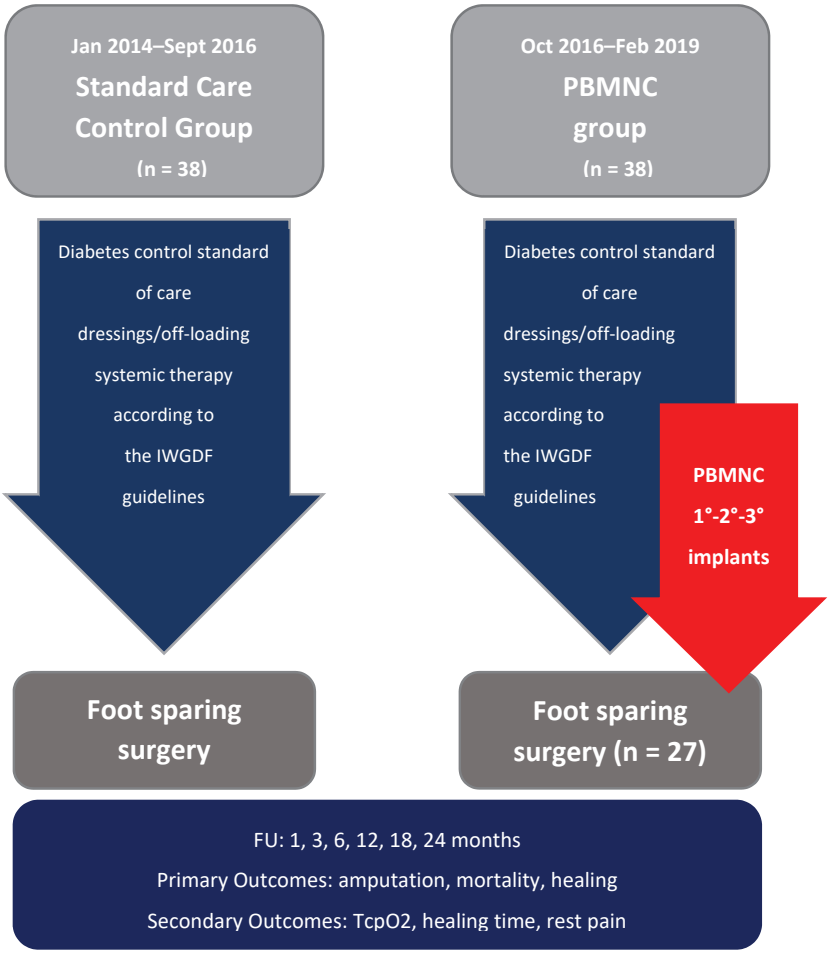

Figure 1. Study flow diagram.

3. Results

3.1. Patient Features and Baseline Demographics

The study group was composed of 76 patients: 38 patients in the standard care control group and 38 patients in the PBMNC group.

Baseline demographic, clinical, and ulcer characteristics of both groups are reported in Table 1. No significant difference in age, gender, diabetic status (type, duration of disease, and glycated hemoglobin), site of lesion, or number of comorbidities between the two groups was recorded. The prevalence rate of retinopathy was higher in the standard therapy group ($X^2_C = 10.077$, $p = 0.002$).

Table 1. Patient features and baseline demographics.

	PBMNC Group	Control Group	Statistical Test	*p* Value
Age	77.00 ± 6.72	77.58 ± 10.73	U = 664.500	$p = 0.55$
Gender	26M (68.4%) 12F (31.6%)	26M (68.4%)12F (31.6%)	$X^2_C = 0.000$	$p = 1.000$
Type of diabetes	Type 1 = 3 (7.9%) Type 2 = 35 (92.1%)	Type 1 = 1 (2.6%) Type 2 = 37 (97.4%)	$X^2_C = 1.056$	$p = 0.304$
Duration of diabetes	16.45 ± 8.96	18.63 ± 8.60	U = 621.000	$p = 0.291$
Site of lesion	Forefoot (78.9%); hindfoot (21.1%)	Forefoot (73.7%); hindfoot (26.3%)	$X^2_C = 0.291$	$p = 0.589$
HbA1c %	7.48 ± 0.69 (58 mmol/L)	7.62 ± 0.77 (60 mmol/L)	U = 622.000	$p = 0.389$
Rheumatologic disease	12 (31.6%)	9 (23.7%)	$X^2_C = 0.592$	$p = 0.442$
Cardiopathy	23 (60.5%)	27 (71.1%)	$X^2_C = 0.935$	$p = 0.333$
Stroke/TIA	8 (21.1%)	17 (44.7%)	$X^2_C = 4.828$	$p = 0.028$
Retinopathy	8 (21.1%)	21 (55.3%)	$X^2_C = 10.077$	$p = 0.002$ *
Neuropathy	26 (68.4%)	31 (81.6%)	$X^2_C = 1.754$	$p = 0.185$
Wound extension (Texas University Classification)	2C = 9 (23.7%) 3C = 29 (76.3%)	2C = 5 (13.2%) 3C = 33 (86.8%)	$X^2_C = 1.401$	$p = 0.237$
WIFi	W1I3Fi0 = 10 (26.3%) W3I3Fi0 = 28 (73.7%)	W1I3Fi0 = 4 (10.5%) W3I3Fi0 = 34 (89.5%)	$X^2_C = 3.152$	$p = 0.076$
$TcpO_2$	11.59 ± 5.2	14.05 ± 5	U = 581.500	$p = 0.196$
Renal failure	21 (55.3%)	19 (50.0%)	$X^2_C = 0.211$	$p = 0.646$
Angioplasty Failure	30 (78.9%)	21 (55.3%)	$X^2_C = 4.828$	$p = 0.028$ *
Not feasible	8 (21.1%)	15 (40.5%)	$X^2_C = 3.348$	$p = 0.067$
Bypass occlusion	5 (13.2%)	4 (10.8%)	$X^2_C = 0.098$	$p = 0.754$
Tibial/pedal absence	23 (67.6%)	29 (76.3%)	$X^2_C = 0.67$	$p = 0.412$
Calcification	24 (75.0%)	34 (89.5%)	$X^2_C = 2.56$	$p = 0.109$

Legend: * $p < 0.003$ (p value with Bonferroni correction). HbA1c % = glycated hemoglobin; TIA = transient ischemic attack.

3.2. Clinical Outcome

The 38 patients treated with PBMNCs showed a significant improvement in all primary outcomes. Kaplan–Meier survival analysis was performed to evaluate amputation-free survival after 1, 3, 6, 12, 18, and 24 months follow-up, comparing the PBMNC and the standard therapy group.

The Kaplan–Meier curves and the log-rank test results showed a significantly lower amputation rate in the PBMNC group ($p = 0.000$; Figure 2) at each point of follow-up. Only 4 out 38 (10.5%) amputations were observed in the PBMNC group, while 15 out of 38 amputations (39.5%) were recorded in the standard care control group ($p = 0.0037$).

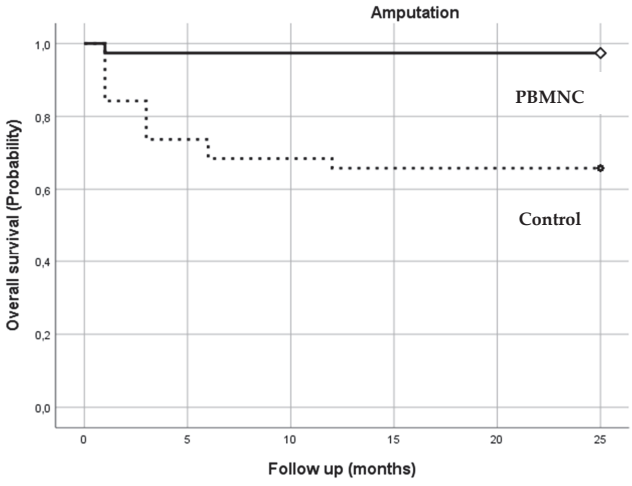

Figure 2. Amputation-free survival: the number of patients alive without amputation in both groups during the follow-up period (1–24 months).

	Number at Risk					
Months	**1**	**3**	**6**	**12**	**18**	**24**
PBMNC group	36	34	34	34	34	34
Control group	32	26	24	23	23	23

Furthermore, mortality risk was significantly lower in the PBMNC group ($p = 0.000$). As illustrated in Figure 3, at the end of the two-year follow up period, nearly 80% of the PBMNC group was still alive ($n = 30$), compared with only 20% of standard therapy group ($n = 8$).

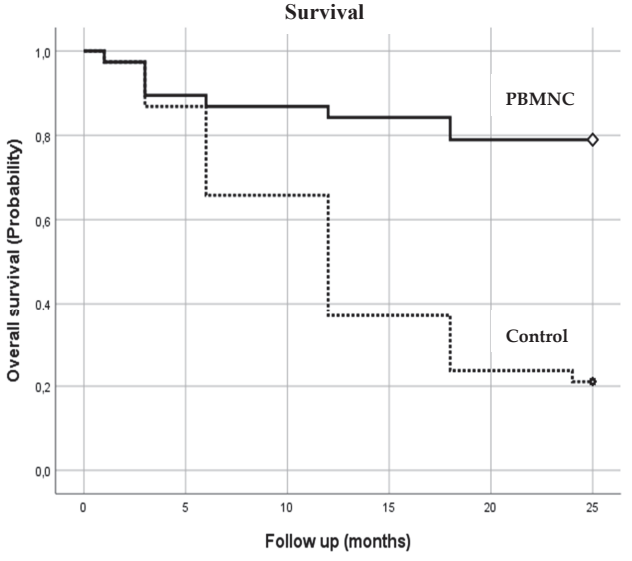

Figure 3. Overall survival: the number of patients alive in both groups during the follow-up period (1–24 months).

	Number at Risk					
Months	**1**	**3**	**6**	**12**	**18**	**24**
PBMNC group	37	34	33	32	30	30
Control group	37	33	27	16	11	8

As illustrated in Figure 4, almost all the healing events occurred in the PBMNC group ($p = 0.000$). Healing at the two-year follow up occurred in 86.8% ($n = 33$) of the PBMNC group, compared to 2.6% ($n = 1$) of the standard therapy group. Most of the healing events (31 out of 33) in the PBMNC group took place within 6 months of treatment.

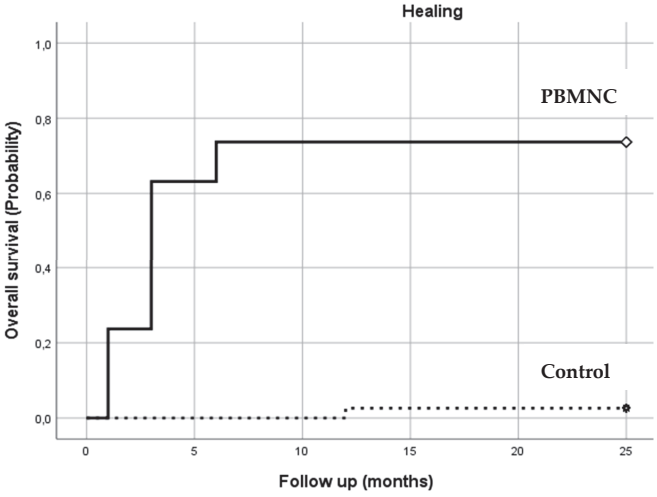

Figure 4. Wound healing: the number of patients healed in both groups during the follow-up period (1–24 months).

	Number at Risk					
Months	**1**	**3**	**6**	**12**	**18**	**24**
PBMNC group	9	25	31	32	33	33
Control group	0	0	0	1	1	1

Moreover, PBMNC-treated patients showed a decreased risk (RR = 0.11, 95% CI = 0.02–0.52) of amputation compared with the control group, with an absolute risk reduction (ARR) of 0.29 (ARR = 0.29, 95% CI = 0.12–0.46) and a relative risk reduction of 0.85 (RRR = 0.85, 95% CI = 0.36–0.96). The number needed to treat in order to prevent one amputation was 3.45 (NNT = 3.45, 95% CI = 2.19–8.15). Mortality risk within 24 months was higher in the control group (RR = 0.07, 95% CI = 0.02–0.21). Absolute risk reduction was 0.58 (ARR = 0.58, 95% CI = 0.40–0.76) and relative risk reduction was 0.73 (RRR = 0.73, 95% CI = 0.50–0.86), compared with the controls. The number needed to treat to avoid one death was 1.73 (NNT = 1.73, 95% CI = 1.31–2.53).

For the secondary endpoint, $TcpO_2$ in the PBMNC group increased by 24 mmHg (median = 24; IQR = 11.5–31.0) at the end of the cell therapy treatment from baseline data (under 25 mmHg is characteristic of CLI). No changes in $TcpO_2$ value were observed in the control group during the follow-up period (14 ± 5 mmHg). Healing time was 71.66 ± 42.24 days in the PBMNC group, while only one patient healed in the standard therapy group. No minor or major side effects were observed in the PBMNC group.

4. Discussion

Diabetic patients usually suffer from long-segment vascular obstruction, and the predominantly distal vessel disease make these patients poor candidates for revascularization, resulting in continued disease progression, amputation, and death [25,26]. No-option CLI remains a significant unmet medical need, and innovative approaches, such as cell therapy, to induce vascular regeneration and achieve limb salvage are urgently needed. Both mobilized and immobilized PBMNCs have shown promising preliminary results in diabetic patients [6–11,18,19]. Our aim was to evaluate PBMNC implant in addition to standard care in NO-CLI patients with DFUs. Despite the limited number of observed patients (n = 76) and the scarce sample size (n = 38) of the PBMNC patient group, a significant decrease in amputations was observed (4 amputations out of 38 patients) compared to the standard care control group (15 out of 38). Moreover, we observed a low number of deaths (n = 8 patients) in the PBMNC group, compared to 30 deaths in the control group. A reduced mortality risk (93% reduction) within two years was recorded for the PBMNC group compared with the standard therapy group.

Furthermore, the additional autologous cell therapy treatment showed a positive impact on healing outcome. Indeed, only one patient treated with traditional therapy healed. The effectiveness of PBMNCs is also highlighted by the assessment of the number needed to treat (NNT) to prevent one additional negative outcome (in our study, death, or amputation). In our study, less than two PBMNC-treated patients should be achieved to avoid one death within 2 years (NNT = 1.73, 95% CI = 1.31–2.53) and 3.45 patients should be treated with PBMNCs to prevent one amputation (NNT = 3.45, 95% CI = 2.19–8.15). The wound healing potency of PBMNCs was previously reported in a meta-analysis, including 16 RCTs and involving 774 CLI patients, where this cell therapy not only significantly lowered the risk of major amputation, but also significantly increased ulcer healing [11].

Death after major amputation in diabetic patients has been well described by Jones et al., who showed that 3 years after below the knee amputation (BKA), 33.3% of patients were dead, and after above the knee amputation (AKA), 71.4% of patients died ($p < 0.001$). At 5 years after BKA, 63.3% of patients were dead, and after AKA, 85.7% of patients were dead ($p = 0.05$) [27]. Persiani et al. [19] reported a 9.4% rate of major amputations in 18 no-option patients with diabetes treated with PBMNCs (produced by the same point of care device used in our study), which is comparable to the 10% amputation rate we observed. The same result was also previously reported in 2009 by Moriya [6], who observed a major amputation rate of 10.5% and a mortality rate of 21.5% at 2-year follow-up in the first published trial on immobilized PBMNC implants in NO-CLI patients. Regarding the standard care control group, our result is similar to a study on 574 NO-CLI patients (of which 70% were diabetic), which reported a 23% major amputation rate and a 31.6% death rate, primarily from cardiovascular disease, after 2 years [28]. Instead, in our study, only 10.5% of patients were amputated and 21.05% died in the PBMNC-treated population at the end of the two-year follow-up. In a previous study on diabetic NO-CLI patients, a 11.1% major amputation rate in the autologous cell therapy group compared with a 50% rate in the control group was observed at 6 months, with no difference between bone marrow cells (BMMNCs) and peripheral blood cells (PBMNCs) [9]. We observed a healing rate of 81.6% and 84.3% at 6 and 12 months compared to a rate of 2.6% in the standard therapy group. Moreover, most patients (31 out of 33) healed after PBMNC treatment within 6 months. The wound healing rate in our study is higher than the rate reported by Dubsky et al. corresponding to 63% and 82% in 31 diabetic NO-CLI patients treated with autologous cell therapy (20 patients treated with BMMNCs produced by a BMAC SmartPrep point of care device and 11 patients treated by G-CSF-mobilized PBMNCs produced by apheresis), respectively) [29]. Interestingly, in the same study, the authors reported a comparable improvement of CLI major amputation with autologous cell therapy compared with repeated PTA, and more effective healing of foot ulcers in the cell therapy group [30].

Recently, Meloni et al. reported a 30% amputation rate and 50% mortality rate for NO-CLI diabetic patients at 1 year follow-up in a retrospective cohort study [4]. Few diseases connote a higher mortality rate: among 22 different types of malignancy, only six have a 5-year mortality rate higher than that of CLI [29]. This tremendously high mortality rate demonstrates the need to identify new therapeutic strategies to reduce major amputation in this fragile population. The 21% mortality rate we observed at two years follow-up in the PBMNC group is a remarkable result compared to the 80% mortality rate of our standard therapy group at two years.

In addition to the positive clinical outcome on amputation mortality and wound healing, the possibility to perform foot-sparing surgery was significantly higher in the PBMNC group (71.05%) compared to the standard therapy group (7.9%), in which the data are similar to a study about this type of surgery in no-option CLI (13%) [31]. $TcpO_2$ in the PBMNC group increased by 24 mmHg at the end of cell therapy treatment, while no increase was detected in the control group. A significant increase of $TcpO_2$ after PBMNC implants in diabetic patients was previously observed in two clinical trials [9,19]. It was not possible to compare the time to healing between groups because only one patient showed ulcer healing in the control group at the end of the follow-up period.

Pain control is a challenging issue in no-option CLI patients, and it is often only partially controlled by paracetamol and opioids, despite their common side effects such as constipation and drowsiness. In the treated group, pain relief was achieved following the first PBMNC implant, as evaluated by the NRS scale, but data regarding rest pain for the control group were not recorded. Rest pain evaluation with the NRS scale [25] in the PBMNC group showed a mean baseline value of 8.46 +/− 2.01, which decreased to 4.58 +/− 8.39 after the first implant, and ultimately to 2.15 +/− 5.77, allowing the discontinuation of painkillers. Although a direct comparison between the two groups is not possible, rest pain reduction immediately after the first cell implant has also been observed in previous PBMNC clinical trials utilizing PBMNCs generated by a point of care selective filtration system [18,19]. This effect could be partially explained by the fact that macrophages, when polarized in the M2 anti-inflammatory activation state, release powerful natural opioid substances [32]. Interestingly, it has been shown that in streptozotocin-induced diabetic rats, the implantation of peripheral blood mononuclear cell fractions is associated with an improvement in motor nerve conduction velocity (MNCV) due to arteriogenic effects in the sciatic nerve, and that VEGF may contribute to this effect [33]. A reduction in rest pain in CLI patients after PBMNC treatment has been previously reported in clinical trials, as well as in meta-analyses [6–11,27].

The frailty of no-option CLI patients and the delicate management of the diabetic foot require that PBMNC therapy, as for the standard therapy, is performed by a multidisciplinary team, which could include care relating to every single feature of the diabetic CLI patient (including the optimization of glycemic control, the reduction of cardiovascular risk factors, the early diagnosis and therapy of infection, pain control, foot surgery, and the early mobilization and rehabilitation of the patient).

Autologous PBMNCs cell therapy could represent an innovative therapeutic strategy to treat these critical patients. PBMNCs offer several advantages over other autologous cellular therapies produced from bone marrow aspirate (such as BM-MNC, or cellular concentrate produced from adipose tissue, such as the stromal vascular fraction (SVF) or micro fragmented adipose tissue), in addition to the obvious non-invasiveness of blood collection. Firstly, PBMNC implants can be repeated easily; a recent randomized controlled trial showed that CLI patients who received four repeated BM-MNC injections versus one single implant show a better pain-free walking distance, suggesting the frequency of implant is superior to cell quantity [34]. Accordingly, Kang et al. confirmed that increasing the injection frequency enhances the survival of the injected bone marrow derived mesenchymal stem cells in a CLI animal model [35]. Secondly, PBMNCs can be easily produced by a point of care selective filtration system intra-operatively and are ready to use in less than 15 min. Thirdly, diabetes heavily impairs bone marrow cell

populations, as well adipose tissue cells, both in terms of angiogenic and regenerative ability [36,37]. In the recent MOBILE randomized double-blind study on 152 no-option CLI patients at Rutherford stage 4 or 5 treated with BM-MNC or placebo, the 2-year post-hoc analysis showed that while BM-MNCs did provide a significant benefit for patients without diabetes at Rutherford stage 4, it did not provide any benefit for patients with diabetes and/or those at Rutherford stage 5, suggesting a negative impact of diabetes on cell therapy with BM-MNC for CLI [38]. Recently, the SCELTA trial suggested the "non-inferiority" of non-mobilized PBMNCs compared to BM-MNCs [8]. Given the current absence of evidence of the superiority of bone marrow versus peripheral blood cells, the advantage of peripheral blood as a cell source is the avoidance of bone marrow harvesting disadvantages such local pain, hematomas, and anemia, as well as a longer surgical procedure [10]. Dong et al. showed that there are no differences in amputation-free survival in patients treated with purified CD34+ or PBMNCs in a randomized trial [39]. Diabetes impairs the angiogenic capacity of human adipose-derived stem cells, mainly by the reduction of the CD271 + subpopulation [40]. So far, there are few studies on the use of adipose tissue cell concentrates for CLI in diabetic patients, and adipose tissue concentrates have not been included in meta-analyses [41,42]. A recent study observed a dysfunction in mesenchymal stem cells from the adipose tissue of diabetic patients, probably due to oxidative stress and autophagy, suggesting a limit to their therapeutic use [43]. On the contrary, adipose tissue concentrate has been shown to be safe and efficient to treat chronic venous ulcers [44].

Although this study is exposed to several potential biases as a result of its nonrandomization and the relatively small sample size, the intramuscular and peri-lesional injection of autologous PBMNCs showed very encouraging results without any adverse effects on all primary end points evaluated (amputation, death, and wound healing) in the two-year follow-up period.

5. Conclusions

In the last few years, a huge number of papers studying the mechanism of action of PBMNCs have been published, both on their characteristic angiogenic potency and on their regenerative and immunomodulatory capacity through the polarization of macrophages [12,43,44]. The new concept of the immune-centric revolution shifts the focus from stem cells to immune cells, particularly monocytes/macrophages and lymphocyte-based cell therapy, in regenerative medicine [44]. In our study, autologous PBMNCs, produced easily in the operating room by a dedicated selective filtration point of care device, seem to be a very promising therapy, with the potential to modify the natural history of intractable CLI and diabetic foot in terms of major amputation and overall survival rates. PBMNC therapy opens a new frontier in the management of these critical patients.

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Informed Consent Statement: Informed consent was obtained from all subjects involved in this study.

Data Availability Statement: The data presented in this study are available on request from the corresponding author. The data are not publicly available due to privacy reason.

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Review

Cardiovascular/Stroke Risk Stratification in Diabetic Foot Infection Patients Using Deep Learning-Based Artificial Intelligence: An Investigative Study

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Abstract: A diabetic foot infection (DFI) is among the most serious, incurable, and costly to treat conditions. The presence of a DFI renders machine learning (ML) systems extremely nonlinear, posing difficulties in CVD/stroke risk stratification. In addition, there is a limited number of well-explained ML paradigms due to comorbidity, sample size limits, and weak scientific and clinical validation

methodologies. Deep neural networks (DNN) are potent machines for learning that generalize nonlinear situations. The objective of this article is to propose a novel investigation of deep learning (DL) solutions for predicting CVD/stroke risk in DFI patients. The Preferred Reporting Items for Systematic reviews and Meta-Analyses (PRISMA) search strategy was used for the selection of 207 studies. We hypothesize that a DFI is responsible for increased morbidity and mortality due to the worsening of atherosclerotic disease and affecting coronary artery disease (CAD). Since surrogate biomarkers for CAD, such as carotid artery disease, can be used for monitoring CVD, we can thus use a DL-based model, namely, Long Short-Term Memory (LSTM) and Recurrent Neural Networks (RNN) for CVD/stroke risk prediction in DFI patients, which combines covariates such as office and laboratory-based biomarkers, carotid ultrasound image phenotype (CUSIP) lesions, along with the DFI severity. We confirmed the viability of CVD/stroke risk stratification in the DFI patients. Strong designs were found in the research of the DL architectures for CVD/stroke risk stratification. Finally, we analyzed the AI bias and proposed strategies for the early diagnosis of CVD/stroke in DFI patients. Since DFI patients have an aggressive atherosclerotic disease, leading to prominent CVD/stroke risk, we, therefore, conclude that the DL paradigm is very effective for predicting the risk of CVD/stroke in DFI patients.

Keywords: diabetics; diabetic's foot infection; cardiovascular/stroke risk stratification; deep learning; AI bias

1. Introduction

Foot ulcers are the leading cause of morbidity and amputation in people with diabetes. These complications also contribute to significant healthcare expenditure, as indicated by the fact that 20 to 40% of healthcare resources are spent on diabetic feet associated with diabetes [1,2]. As per the World Health Organization (WHO), diabetic foot syndrome (DFS) is described as "ulceration of the foot (distally from the ankle and including the ankle) linked with neuropathy and various grades of ischemia and infection" [3]. It is a severe long-term complication of diabetes mellitus (DM) that can lead to disability, amputations, cardiovascular diseases, and a lower quality of life [4,5].

In the United States, approximately 73,000 lower-extremity amputations are carried out each year due to diabetes [6]. Foot ulceration is the primary and sole factor that causes 80% of these complications [7,8]. The existence of foot ulceration is believed to be a significant risk factor for morbidity, death, and disability. This notion is confirmed by the fact that the diabetic condition is responsible for approximately 80% of nontraumatic amputations and that 85% of these amputations are preceded by foot ulceration [9]. It is thought that 15% of diabetics will get an ulcer on one of their lower limbs at some point during their disease [10]. A connection between a diabetic foot infection (DFI) and cardiovascular disease (CVD) has been discovered by several investigations [11–13]. DFI is an indicator of diabetes, and when active and uncontrolled, raises the risk of CVD [14–16].

The greatest risk factors for coronary heart disease (CHD) and diabetes include obesity, high blood pressure, and high blood cholesterol [17,18]. The diabetic foot ulcer (DFU) disease also causes inflammatory reactions, which can contribute to the development of atherosclerosis, promoting coronary artery disease (CAD), and the worsening of CVD [19–23]. Multiple studies relate more advanced stages of a DFI to more severe forms of atherosclerotic cardiovascular disease (ASCVD) [15,23–25]. As a result, a DFI contributes to the development of CVD. It is essential to understand the connection between a DFI and CVD to reduce the risk of heart attacks, cardiovascular events (CVE), and stroke [9,26].

The development of calcifications and hemorrhagic formation characteristics, as seen in a DFI, increases the risk of CVD [27,28]. Foot wound imaging is an essential procedure in examining a DFI [29]. It is essential to use foot imaging to monitor changes in a DFI to provide an accurate assessment of the prevalence of diabetics [30]. It is suggested that coronary imaging be performed to determine the risk of developing CVD [23]. In addition,

imaging of the coronary arteries is necessary to identify plaque in CAD [31,32]. Intravenous ultrasonography (IVUS) and optical coherence tomography (OCT) are two examples of effective imaging technologies that can be used to diagnose coronary plaque [33–35]. Since surrogate markers are well established for CAD, such as carotid artery imaging and its quantification, thus, there is a need for (i) accurate and computerized carotid plaque load assessment, (ii) effective detection of atherosclerotic disease in DFI patients and (iii) CVD risk stratification. All three aspects are essential to prevent DFI-driven CVD from becoming severe. Hence, there is a need for the automated and early assessment of a diabetic foot infection (DFI) and CVD severity in patients to avoid morbidity and mortality.

Artificial intelligence (AI) has fundamentally altered the dynamics of the healthcare sector [36]. Machine Learning (ML) and Deep Learning (DL) algorithms have been implemented in a variety of medical applications [37,38]. AI-based technologies are data-driven, which means they make decisions based on information in databases, and have been used to diagnose diabetes [39,40], liver [41], thyroid [42], and skin cancer [43], just to name a few. Regarding CVD, the results show that there are nonlinear connections between the input predictors and the cardiovascular outcomes [44,45]. In contrast to the statistical risk estimation techniques currently in use [44,46], ML-based algorithms may use intricate quasi-relationships among several risk predictors (or attributes) that are input simultaneously.

DL algorithms extract characteristics directly from the input data to generate predictions. Some examples include the characterization of carotid wall tissue, the segmentation of pictures, and the stratification of CVD risk [47,48]. It has also been established that DL algorithms with convolution neural networks (CNNs) extract features, which can then be used to train and test an ML classifier to obtain a final classification [49,50]. Recently, images of the DFI foot wound have been utilized to predict the severity of the disease. It has been demonstrated that algorithms based on ML and DL can accurately predict a DFI [29,30]. Because of this, it is conceivable for AI-based solutions to allow the analysis of image-based diabetic foot inputs [51]. This is made possible by eliminating the demand for human intervention. Several applications of carotid ultrasonography that use AI-based algorithms have shown a lot of promise [52–54]. Thus, it means that these AI-based methods could be used to evaluate a patient's risk and treat both DFI and CVD disorders concurrently.

The usage of alternative imaging for the visualization of CAD helps in the categorization of DFI patients into appropriate CVD risk categories [55–57]. This is because CAD is easier to see with surrogate imaging. Thus, to gain a more in-depth insight into the pathophysiology of diabetes, diabetes foot ulcer, and cardiovascular disease, this study focuses on the use of low-cost carotid artery and diabetic foot ultrasound imaging. Using techniques such as ML and DL, it is possible to identify patients who are at significant risk of developing CVD complications [58]. To best analyze the above study, we have adopted the search strategy and the distributions.

2. Search Strategy Using PRISMA Model

The Preferred Reporting Items for Systematic reviews and Meta-Analyses (PRISMA) model (Figure 1) is used as the basis for the search method. PubMed, IEEE, and Google Scholar are three databases that are used to search for and screen relevant papers. These databases are searched with keywords such as "diabetic foot ulcer", "diabetic foot disease", "diabetic foot infection", "diabetes", "CVD", "diabetic foot ulcer and CVD", "diabetic foot ulcer and coronary artery disease", "diabetic foot imaging", "diabetes and carotid imaging", "artificial intelligence", "artificial intelligence and CVD", "machine learning and CVD", "deep learning and CVD", "classifiers and CVD/stroke risk stratification", and "atherosclerotic plaque tissue classification". There was a total of 324 papers located on PubMed, and there were 548 articles initially selected from Google Scholar and IEEE. To narrow the list down to just 872 articles, sophisticated criteria such as time and relevancy were utilized. After considering whether or not to include them in this evaluation, a total of 140 articles were narrowed down to the articles that made the final list. The following are the three criteria that were used to exclude studies: (i) studies that did not relate in

any way to our study objective, (ii) papers that did not contain useful information, and (iii) studies that contained insufficient data in the studies. Following the elimination of 422, 103, and 140 investigations (respectively denoted with the letters E1, E2, and E3), a final pool of 207 studies was chosen for the final analysis out of a total of 450 studies. Figure 2 depicts the comprehensive screening procedure for the selection of the research paper.

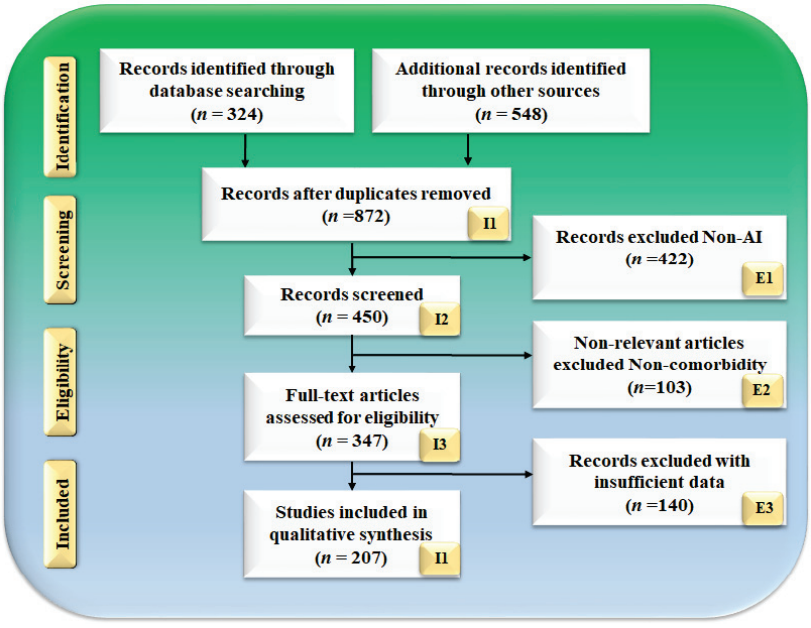

Figure 1. PRISMA model for selection of studies.

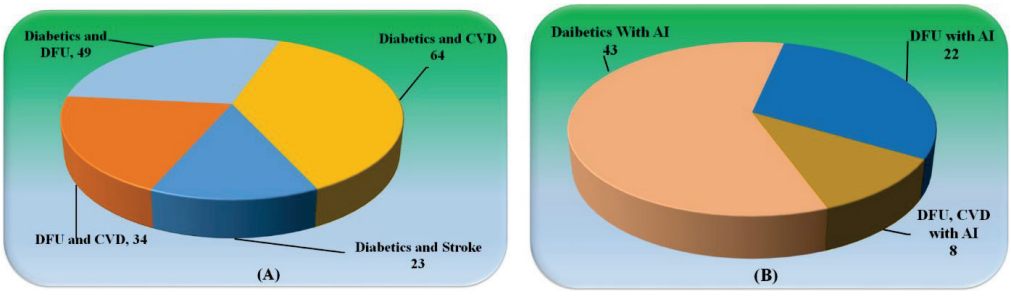

Figure 2. (**A**) Studies related to Diabetics with CVD, Stroke, and DFU. (**B**) Studies explaining the role of AI in Diabetics with DFU and CVD.

Statistical Distribution

Figure 2a shows the studies related to (i) diabetes and DFU, (ii) diabetes and CVD, (iii) DFU and CVD, and diabetes and stroke. A number of the articles explain the role of diabetics leading to the development of CVD in a patient. Figure 2b shows the distribution of studies of AI with (i) Diabetics, (ii) DFU, and (iii) DFU and CVD. Each study had an examination utilizing a feasibility analysis, which was followed by a cross-check using scientific validation to guarantee that it came as close as possible to meeting our goals.

3. Pathobiological Mechanisms of Diabetes, CVD, and Diabetic Foot

Figure 3 shows the biological link between diabetes mellitus and CVD. The survival rate of diabetic patients is lower than that of nondiabetic patients [59]. In the context of CVD, many studies showed that diabetes patients had 2–4 folds increased morbidity and mortality rates than patients without diabetes mellitus (DM) [60]. In addition, DM patients suffering from a foot infection have increased morbidity and mortality rates due to CVD about twice as much compared to patients with DM without a foot disease. A paper published by Pinto et al. [61] demonstrated an increased risk of CVD morbidity and mortality in DM patients who experienced amputation due to a foot infection compared to DM patients without a foot disease. Furthermore, in this study, authors also mentioned that patients suffering from a DFI have higher levels of serum cholesterol, serum triglycerides, and microalbuminuria or proteinuria, which are considered CV risk factors, compared with DM patients without a foot infection [62–64]. Another recent five-year follow-up study showed an increased risk of cerebrovascular events in DM patients with a foot disease compared to DM patients without a foot disease [25]. The published works [62–64] demonstrate that patients with a DFI are more prone to increased mortality and morbidity due to CVD than diabetic patients without a foot disease. We, thus, hypothesize that longstanding nonhealing ulcers in diabetes patients result in the activation of cytokine production, which further damages the heart (stage A of Figure 3). Interestingly, supporting our hypothesis, Jeffocate et al. [65], in their recent article, specified that patients with a DFI are more prone to developing an inflammatory cascade of increased levels of proinflammatory cytokines such as interleukin-1beta (IL-1β) and tumor necrosis factor-alpha (TNF- α), compared with diabetic patients without foot diseases. Additionally, Weigelt et al. [66] also showed that a DFI is responsible for the increase in circulation of acute phase cytokines such as interleukin 6 (IL6) and C-reactive protein (CRP). The above evidence demonstrated that immune activation in chronic nonhealing wounds is the key source of developing CV risk factors in patients with DM (stage A of Figure 3). These increased proinflammatory cytokines due to immune activation can trigger intracellular and extracellular reactive oxygen species (ROS). Furthermore, (stage C of Figure 3) results in damage to endothelial cells and causes the opening of inter endothelial junctions in a blood vessel [67]. Thus, this damage in the endothelium layer results in the penetration of native low-density lipoprotein (LDL) particles inside the tunica intimal layer, and this process is known as transcytosis [68]. Oxidative stress due to increased levels of ROS results in the formation of oxidized LDL (OxLDL), formed by the peroxidation of phospholipid molecules on the surface of LDL particles (Stage D of Figure 3). This process is known as lipid peroxidation [69]. Due to the presence of cellular and humoral innate immunity, OxLDL is taken by the macrophage, and this triggers the accumulation of many OxLDL inside the macrophage, resulting in the development of foam cells (stage E of Figure 3) [70,71]. Excess accumulation of foam cells increases the intake of more cholesterol, causing apoptosis and necrosis and progressing to the formation of the necrotic core (stage F of Figure 3) [72,73]. These attract the aggregation and adhesion of platelets, resulting in the development of atherosclerotic plaque (stage G and H of Figure 3) [74].

The endogenous and exogenous metabolic disruptions concerning glucose metabolism and their respective molecular repercussions contribute to an elevated risk of cardiovascular disease in patients with diabetes. The revelation of the cardiovascular outcome trial (CVOT) data and the discovery of certain unexpected advantages of major adverse cardiovascular events (MACE) in these trials highlight that higher levels might have both direct and indirect impacts. The metabolic balance is severely thrown off by normal glucose levels, which exacerbates risk factors for cardiovascular disease.

In addition to these endogenous sources of abnormality, the process of glucose metabolism, and exposure to external substances, such as those found in advanced glycation end products (AGEs), may be amplified by factors in nutrition as well as in the environment, leading to the activation of proatherogenic processes. Although a plethora of research has exposed the deleterious effects of glucose on extra and intracellular character-

istics, their long-term unfavorable effects, such as on glycation and epigenetic variables and metabolic memory [75,76], have also been suggested to play crucial roles in CVD in diabetes mellitus. Moreover, diabetes mellitus on the disturbance of lipid/lipoprotein metabolic activities, in addition to their unique and independent effects, also interrelate with all these glucose-driven processes. This is because the glycation of lipids and lipoproteins could alter those species' function and, through receptor for advanced glycation endproducts (RAGE)-dependent mechanisms, may mediate and exacerbate cellular perturbation [76,77]. As a result, diabetes mellitus is associated with an increased risk of immediate and long-term effects triggered by glucose.

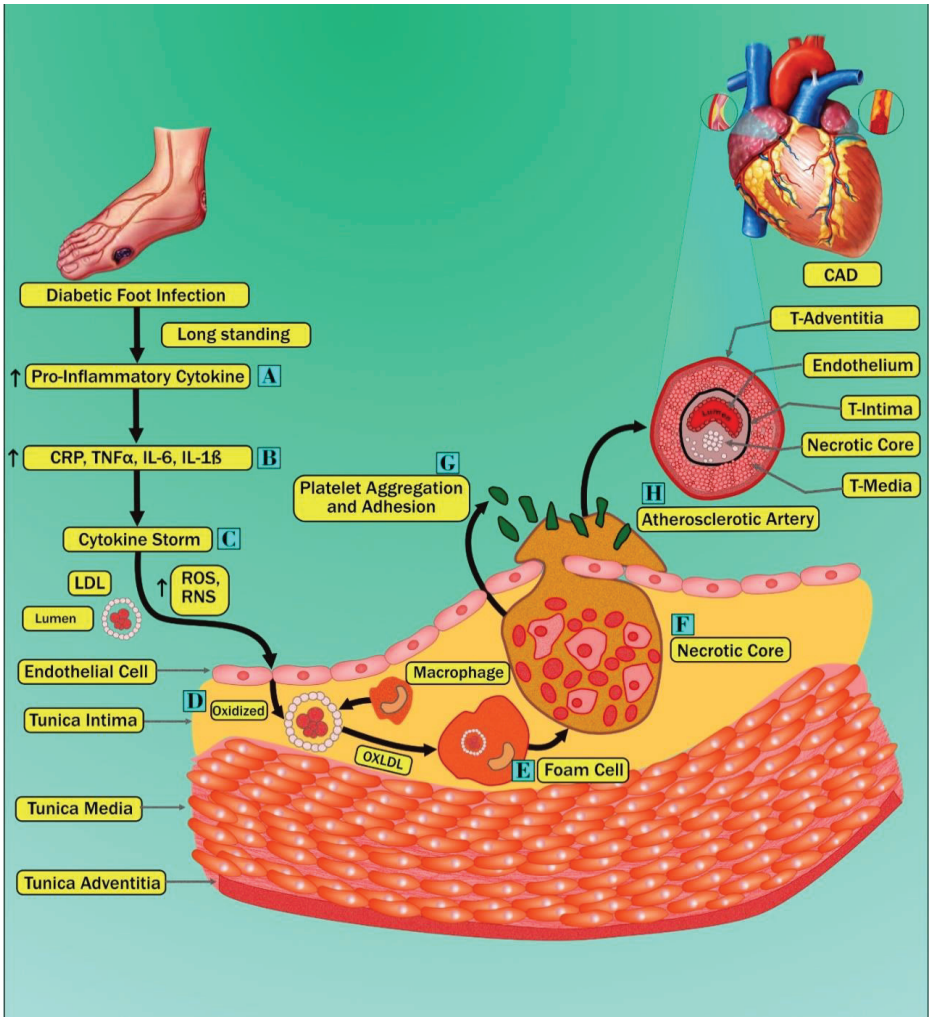

Figure 3. Pathobiological mechanisms of diabetes mellitus, cardiovascular disease, and diabetic foot are shown by different stages marked as A–H.

As altered gene expression patterns and signaling pathways combine with immune cells, blood vessel cells malfunction, increasing the risk of vascular and cardiovascular disease in patients with certain metabolic abnormalities [26].

Vascular Complications in Diabetes Mellitus

Vascular abnormalities in diabetes are caused by a state of chronic hyperglycemia [78]. These difficulties can develop in large blood arteries, characterized by diabetic macroangiopathy, and in small blood vessels, characterized by diabetic microangiopathy [78]. Such vascular irregularities are due to the irrevocable glycation of proteins that occurs nonenzymatically, as well as changes in the cellular redox potential. Elevation in oxidative stress and the condition of inflammation lead to the development of endothelial dysfunction and a state of increased hypercoagulability.

The resolution of inflammation is hampered in diabetic patients, which correlates to the increased levels of TNF-, IL-6, and other proinflammatory cytokines in these patients, as well as to the development and progression of nephropathy and atherosclerosis, and other complications of diabetes [79]. Recent research has demonstrated that proresolving lipid mediators, such as lipoxins, resolvins, and protectins, play a significant role in the resolution of inflammation [22]. These mediators work by suppressing polymorphonuclear and monocyte recruitment and protecting cells from damage, transforming the cytokine environment from proinflammatory to proresolving (Figure 4). As a result, these proresolution lipid mediators have significant therapeutic potential in diabetic renal and cardiovascular disorders [21,80]. The inefficient metabolites of magnification lipid mediators in muscle and adipose tissue contribute to the persistence of chronic inflammation in obesity [81]. This suggests that these lipids could be used to treat insulin resistance, diabetes, and the problems that come with these conditions [82]. Table 1 represents various studies that link DFI and CVD relations.

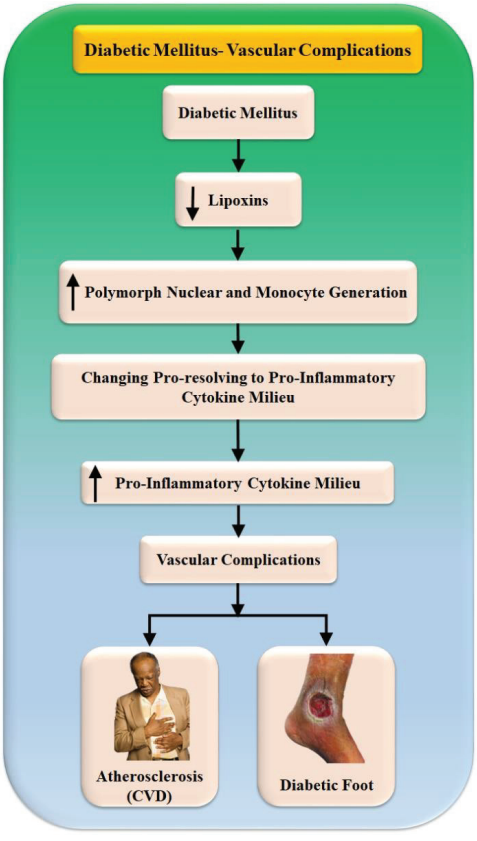

Figure 4. Vascular complications due to diabetes mellitus.

Table 1. Relationship between the diabetic foot, diabetic syndrome, and cardiovascular disease.

SN	Citations	Relationship	ME	PS	OUTCOME	TRE
1	Feleke et al. [28] (2007)	DFI and CVD	LBBM, OBBM	2818	DFI Infections led to morbidity, with the combined effect of CVD leading to mortality. Following diabetic foot ulcers came TB, skin and subcutaneous infections, and pneumonia.	NR
2	Brownrigg et al. [14] (2012)	DFI with CVD risk of mortality	LBBM	3619	DFI patients have a higher risk of all-cause mortality than other diabetics. CVD contributes to this risk.	NR
3	Matheus et al. [83] (2013)	Diabetes and CVD	LBBM	NR	Diabetes prevention is the most effective way to lower CVD risk. Traditional, changeable heart disease risk factors are still essential for diabetes people.	NR
4	Tuttolomondo et al. [16] (2015)	DFS as a Cardiovascular Marker	LBBM	NR	In addition to peripheral sensory neuropathy, deformity, and trauma, other risk factors, including calluses, edema, and peripheral vascular disease, have been identified as etiological contributors to the formation of diabetic foot ulcers.	NR
5	Domingueti et al. [13] (2015)	Diabetes and CVD	LBBM	NR	Vascular problems in type 1 and type 2 diabetes are closely linked to endothelial dysfunction, hypercoagulability, inflammation, and the poor resolution of inflammation.	NR
6	Al-Rubeaan et al. [27] (2015)	DFI and CVD	LBBM	NR	Neuropathy and PVD are major risk factors for diabetic foot problems. Diabetic retinopathy is a major independent risk factor for diabetic foot issues. CVD risk factors are common among diabetics, and primary and secondary prevention strategies are essential to reduce morbidity and expense from this chronic condition.	NR
7	Bertoluci et al. [11] (2017)	Diabetes and CVD	LBBM	NR	CVD risk is increased 2- to 4-fold in people with type 2 diabetes, however, due to the disease's extreme variability, the two conditions cannot be regarded as risk equivalents. To tailor care to each patient, risk assessment is essential.	NR
8	Dietrich et al. [15] (2017)	DFI as a Predictor of CVD and Mortality	LBBM	NR	DFS is linked to CVD and death. DFI's connection with renal failure and retinopathy indicates the evolution of micro- and macrovasculopathy, neuropathy, chronic inflammation, and lipotoxicity.	NR
9	Mishra et al. [24] (2017)	DFI and CVD	LBBM	NR	Patients diagnosed with DFI have an increased risk of death from any cause compared to other diabetics. The risk is increased by cardiovascular disease.	NR
SN	Citations	Relationship	ME	PS	OUTCOME	TRE
10	Petrie et al. [84] (2018)	Diabetes and vascular complication	LBBM	NR	Diabetes and hypertension increase the possibility of CVD. Oxidative stress, inflammation, and fibrosis, which cause microvascular and macrovascular problems of diabetes, also cause vascular modification.	NR
11	Serhiyenko et al. [85] (2018)	Cardiac autonomic neuropathy in diabetes	LBBM	NR	CAN is a frequent, undiagnosed consequence of DM that increases CV morbidity and mortality. As cardiac denervation could be prevented and partially reversed in early disease stages, DM patients should be screened for it.	Yes
12	Shariful et al. [12] (2020)	Diabetes and CVD	LBBM	1262	Diabetes increased CVD risk at an early age. To reduce future CVD risks, diabetics must reduce cigarette usage and improve BP control.	NR

Table 1. *Cont.*

SN	Citations	Relationship	ME	PS	OUTCOME	TRE
13	Balasubramanian et al. [20] (2021)	DFI and Microcirculation	LBBM	NR	Microcirculation plays a crucial function in tissue injury and inflammation homeostasis and resistance. Furthermore, the latest evidence supports the disruption of microcirculation as the weak link in the sequence of events that leads to DFI.	NR
14	Karhu et al. [86] (2022)	Diabetes and CVD	LBBM	2535	Intermittent hypoxia is worse in people with preexisting CVD, and diabetes and CVD accelerate IH deterioration. Intermittent hypoxia is a pathophysiological hallmark of sleep anemia that increases the risk for severe health consequences. Patients with diabetes or CVD should receive additional attention for sleep anemia screening and follow-up monitoring.	NR
15	Schuett et al. [87] (2022)	Diabetes and CVD	LBBM	NR	Diabetes and hypertension trigger CVD. Oxidative stress, inflammation, and fibrosis promote microvascular and macrovascular diabetic complications.	NR
16	Qiu et al. [57] (2022)	DFI and CVD	LBBM	423	The development of a diabetic foot ulcer was associated with a considerably greater death risk from all causes as well as from cardiovascular disease compared to that of a control group of those who had diabetes mellitus but did not have DFI.	NR

SN: serial number, RELATION Diabetic Foot and CVD, ME: method of evaluation, PS: patient size, OE: outcome, TRE: Treatment, NR: not reported, CVD: Cardiovascular disease, DFI: Diabetic Foot Ulcer, DFS: Diabetic Foot Syndrome, DM: Diabetic Mellitus, CAN: Cardio Autonomic Neuropathy, LB: Lab-base, OB-Office base, TB: Tuberculosis, PAD: Peripheral Arterial Disease.

4. ML/DL-Based CVD/Stroke Risk Assessment in Diabetics Foot Ulcer Patients

There is evidence that ML/DL is being used in every industry, including medical imaging [47,88,89]. Deep neural networks (DNNs), a subset of DL, are designed to function like the human brain and have been shown to have several applications [36,90–92]. DL makes automatic feature extraction, classification, and segmentation possible via the power of convolution, max-pooling, and various channel maps such as spatial and temporal attention [93–96]. Multiple publications have detailed the use of AI in the diagnosis and prognosis of CVD [97–99] and the forecasting of lesions due to a DFI [51,100–104]. Furthermore, DL has played a crucial role in DFI identification during the presence of comorbidities, including diabetes [105], Parkinson's disease (PD) [106–110], rheumatoid arthritis [111], and pneumonia [91,112]. In addition to CVD and diabetes,, the presence of such comorbidities in patients profoundly impacts the nonlinear dynamics [113]. As a result, the importance of DL is growing in identifying moderate and high-risk patients with CVD/stroke risk [114–116]. Considering this, for superior CVD/stroke risk, an improved set of biomarkers for DFI severity is needed.

Section 4.1 explains the ML/DL-based architecture for evaluating the risk of CVD/stroke in DFI patients. CUSIP quantification using DL which includes the design of wall segmentation using UNet, UNet+, UNet++, and UNet3P, one of the most advanced paradigms, will be discussed in Section 4.2. Furthermore, DL for DFI lesion segmentation and quantification is discussed in Section 4.3. Section 4.4 discussed the challenges in imaging modalities models for CVD risk stratification in DFI patients.

4.1. ML/DL-Based Architecture for Evaluating the Risk of CVD/Stroke in DFI Patients

ML techniques were developed for superior segmentation and classification [97,99,114,117,118]. Despite that, it lacked automated feature extraction. In contrast, ML/DL is a powerful framework because it can create automated features by utilizing the underlying knowledge base. It also provides an improved training paradigm in which the nonlinearity between variables and the gold standard can be dynamically adjusted.

These two aspects combine to make ML/DL a powerful framework [97,99,114,117,118]. Separating data into training and testing sets is a fundamental tenet of AI algorithms. Our team has already experimented with several DL use cases [119–121]. As a result, we arrange our data so that the classes are balanced or if augmentation is needed. Data preparation and the selection of an appropriate cross-validation strategy are two of the most crucial factors to think about before dividing a dataset.

The first step, "data preparation or preprocessing", works in tandem with the second step, "data partition". Step three generates offline training using training data, and step four estimates the risk of coronary artery disease or cardiovascular disease on the test data (see Figure 5). Two basic procedures make up data preparation or preprocessing: (i) normalizing the data using a typical scalar paradigm that translates the features (risk factors) between 0 and 1, and (ii) augmenting the data using a SMOTE model [95,96]. It has been seen that several algorithms use "PCA-based pooling" which is an established unstructured statistical attribute selection technique as part of the data preparation in the ML area and has been well adapted by our group [34,122].

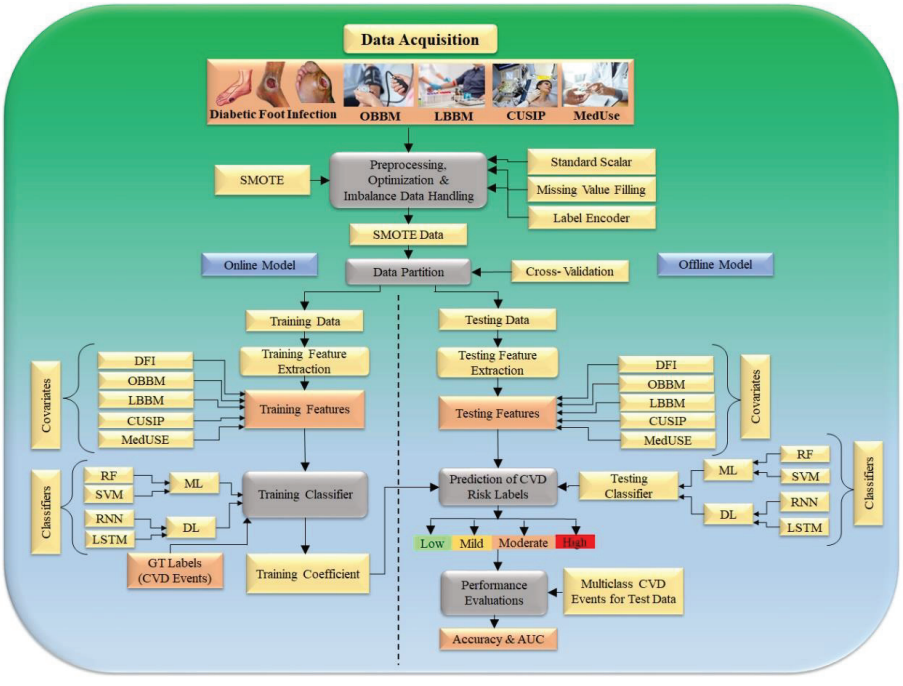

Figure 5. Hybrid model to predict the severity of CVD/Stroke in DFI framework (Courtesy of AtheroPoint™, Roseville, CA, USA permission granted).

The second step of the system is responsible for data partitioning; here, the training and testing sets are created with a K10 cross-validation methodology that uses 90% training and 10% testing data. The third step of the architecture is a model generator, where risk variables and the CAS serve as inputs to deep learning classifiers, such as recurrent neural network (RNN) and long short-term memory (LSTM), which generate the offline coefficients. Part four is a prediction paradigm, where the produced model is used to change the test datasets to predict the CAD risk. Keep in mind that the CV is a multimodal paradigm, thus, we will get the predicted CAD value for all the 10 combinations in a cyclic sequence, making sure that no two combinations overlap and that no test data are included in the training set [99,123,124].

One important thing to remember is that the learning algorithm's embedded feature optimization is a prerequisite [99,125]. The online system is enhanced with a performance component, which calculates accuracy considering the known reference values for the test dataset. The right side below also shows the performance evaluation should the cohort be used using cross-validation protocol, which consists of the computing accuracy, sensitivity, specificity, precision, recall, and *p*-value as conducted in several of our applications [34,39,122]. Table 2 represents various studies used for DFI and CVD prediction. The predictive output labels are either heart failure (cardiovascular events) or stroke (cerebrovascular events) and can be categorized into four parts, such as low, mild, moderate, and high. [126].

Table 2. Studies show the role of AI in the diagnosis, and prediction of, DM, DFI, and CVD.

SN	Citations	IC	DS	REL	PRE	ClassTy	TOC	ML/DL	ACC %	AUC	SEN	SPE	F1	MCC
1	Parthiban et al. [127] (2012)	LBBM	341	DM, CVD, and AI	CVD	SVM	NB	ML	74.23	0.73	0.79	NR	NR	NR
2	Jelinek et al. [128] (2016)	OBBM, LBBM	88	DM, CVD, and AI	CVD	SVM	RF	ML	81.00	0.89	0.91	0.89	NR	NR
3	Zarkogianni et al. [129] (2017)	OBBM, LBBM	560	DM, CVD, and AI	CVD	SVM	NB	ML	76.34	0.87	0.79	0.76	NR	NR
4	Basu et al. [130] (2018)	OBBM, LBBM	2529	DM, CVD, and AI	Death	PCA	KNN, DT	ML	84.34	0.843	0.87	NR	0.76	0.843
5	Dinh et al. [101] (2019)	OBBM, LBBM	131	DM, CVD, and AI	DM, CVD	XGBoost	RF	ML	84.10	0.81	0.78	0.73	NR	NR
6	Segar et al. [131] (2019)	OBBM, LBBM	319	DM, CVD, and AI	Heart Failure	LDA	RF	ML	76.00	0.778	0.76	NR	0.79	0.778
7	Aggarwal et al. [116] (2020)	OBBM, LBBM	526	DM, CVD, and AI	CVD	SVM	ANN	ML	86.00	0.863	NR	0.81	0.71	NR
8	Derevitskii et al. [115] (2020)	OBBM, LBBM	8139	DM, CVD, and AI	Stroke, DM	XGBoost	NB	ML	84.53	0.87	0.91	0.86	NR	NR
10	Hossain et al. [132] (2021)	OBBM, LBBM	4819	DM, CVD, and AI	CVD	SVM	RF	ML	88.16	0.80	NR	NR	0.88	NR
11	Longato et al. [103] (2021)	OBBM, LBBM	24676	DM, CVD, and AI	CVD	SVM	CNN	DL	79.81	0.76	0.84	NR	0.79	NR
SN	Citations	IC	DS	REL	PRE	ClassTy	TOC	ML/DL	ACC %	AUC	SEN	SPE	F1	MCC
13	Hyerim et al. [102] (2022)	OBBM, LBBM	10442	DM, CVD, and AI	DM, CVD	LR, DT	CNN	DL	80.88	0.86	0.81	NR	NR	NR
14	Goyal et al. [30] (2020)	OBBM, LBBM	7136	DFI and AI	Diabetic foot Infection	NR	CNN	DL	91.21	0.93	0.84	0.89	NR	NR
15	Alzubaidi et al. [51] (2020)	OBBM, LBBM	754	DFI and AI	DFI	KNN	DNN	DL	93.04	0.91	0.87	0.83	0.94	NR
16	Khandekar et al. [100] (2021)	LBBM (IR)	202	DFI and AI	Diabetic foot	6 Models	CNN	DL	92.51	0.92	NR	NR	0.81	NR
17	Isaza et al. [29] (2021)	OBBM, LBBM	146	DFI, CVD, and AI	DFI	PCA	CNN	DL	88.24	0.84	0.86	0.79	NR	NR

SN: serial number, IC: input covariates, DS: data size, REL: Relation, PRE: Prediction, ClassTy: Classifier type, OBBM: Office base biomarker, LBBM: Lab base biomarker, FE: feature extraction, TOC: Type of classifier, ACC: Percentage accuracy, SEN: Sensitivity, SPE: Specificity, MCC: Mathew coefficient correlation, AUC: Area under curve, DL: Deep learning, ML: Machine Learning, CNN: Convolution neural network, DFI: Diabetic Foot Infection, DNN: Deep neural network, RF: Random forest, SVM: Support vector machine, DT: Decision tree, LR: Logistic Regression, US: Ultrasound, NR: not reported.

4.1.1. CVD Risk Stratification Using ML-Based Classifiers

An ML-based classifier's purpose is to sort the data it receives into one of several predetermined categories or labels [133]. In the case of a task involving the prediction of CVD or stroke events, for instance, applying the input features to the trained classifier results in a prediction of either the "event" or "no-event" category. The ML-based classifier in this work assigns each patient to either the low-risk or high-risk category, depending on which risk profile they fit into. Meanwhile, we mentioned the fact that the purpose of this study was to devise an ML system that was both effective and economical; therefore, an RF classifier was included in the ML system to perform the risk stratification on the patients [134]. Various studies effectively show the ML-based plaque risk stratification using a Random Forest (RF) classifier. Jamithkar et al proposed (shown in Figure 6) an RF-based ML algorithm that, compared to other ML-based algorithms, has been shown to have a higher predictive capacity [135,136]. As a result, the RF classifier was chosen for the risk stratification of the patients [137].

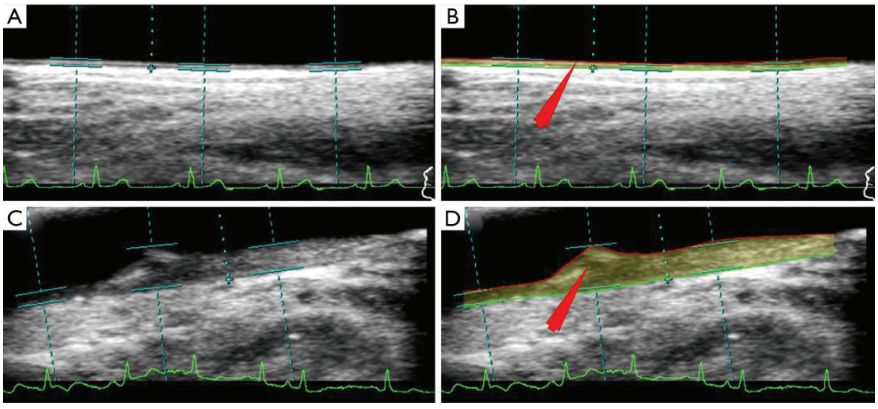

Figure 6. CVD risk stratification is based on an automated AtheroRisk-ML Integrated system. Row 1 (**A**,**B**) is low risk, and Row 2 (**C**,**D**) is High Risk [137].

4.1.2. CVD Risk Stratification Using DL Classifiers

Recurrent Neural Network (RNN) Classifier: A study by Rumelhart et al. [138] explained the concept of a subtype of neural network known as an RNN. Using RNNs to approximate nonlinear unknown dynamical systems is a robust architecture [139,140]. Two of the biggest difficulties in training an RNN are the vanishing gradients problem, which has a direct influence on the stability of the model, and (ii) the difficult optimization target [141]. Figure 7 depicts the suggested hybrid design, which consists of a single RNN unit activated with ReLU and four dense layers layered on top of it. There are 64, 32, and 8 nodes, respectively, in the ReLU-activated intermediate dense layers. There are four softmax-activated nodes in the output layer. A complete model is trained to determine a patient's atherosclerotic risk category based on their input characteristics. Training the model occurred with the help of the loss function categorical cross-entropy loss (CEL) and the optimizer Adaptive Moment Estimation (ADAM). Figure 7 provides a high-level view of an RNN's structure.

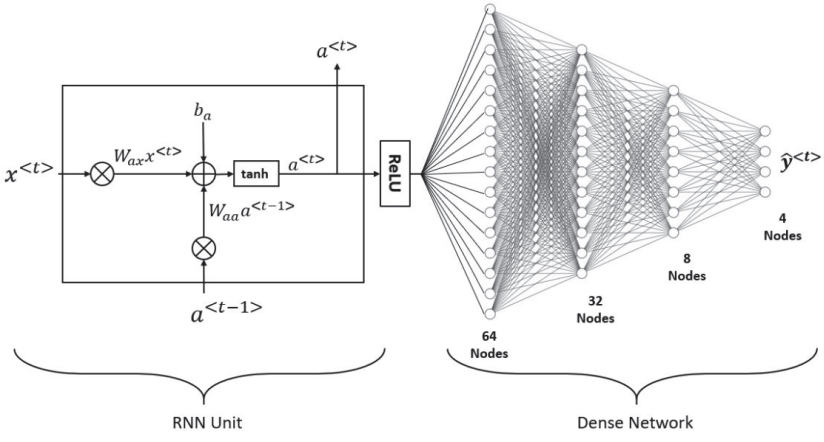

Figure 7. The overall architecture for RNN.

LSTM classifier: Long-term short memory (LSTM) is one of the types of DL algorithms that can be used to predict the likelihood of developing CVD or a stroke [96]. The issue of long-term dependency is specifically designed to create an LSTM as shown in Figure 8. They do not have to put in a lot of effort to learn how to remember things for extended

periods because it is nearly part of their routine. The structure of an RNN always takes the form of a series of modules of the neural network that are repeated. In basic RNNs, this repeating module would frequently produce the same results as a single tanh layer. One of the most important characteristics of an LSTM is its capacity to perform analysis on multiple varieties of data points, such as a single observation. This design incorporates four primary elements, namely, cells, update gates, output gates, and null gates. The design is based on a single component called a cell. The values are stored in the cell at random intervals, and the flow of information or features into and out of the cell is controlled by three gates [142–144]. The LSTM consists of four fully connected layers that are fully coupled to one another and stacked on top of one another. When it comes to creating long-term linkages in data, an LSTM performs better than other methods [145].

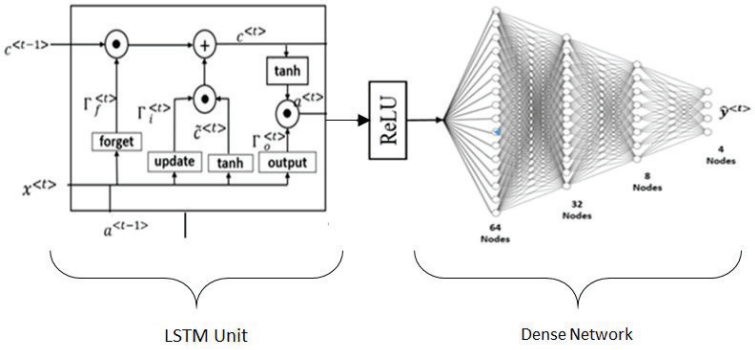

Figure 8. The basic model of LSTM architecture.

The dropout strategy is difficult to implement, which makes it difficult to prevent overfitting in LSTMs, which is a common problem with these models. Dropout is a regularization method that works by leaving out the input and recurrent links to LSTM units during the activation and weight-updating steps of training a network. The behavior of an LSTM after being subjected to a variety of random weight initializations is, as a result, quite comparable to that of a feed-forward neural network. Instead, they chose initialization with a small amount of weight [96].

4.2. CUSIP Quantification Using UNet Architectures: UNet, UNet+, UNet++, UNet3P

Since the morphology of the plaque has variability, one needs out-of-the-box techniques which use knowledge-based systems for CUSIP measurements [31]. Such knowledge-based systems evolve a training program that can undergo nonlinear adjustment, as was previously demonstrated in the context of CVD risk stratification [97,98,137,146,147]. The image-based phenotypes that are generated from carotid ultrasound scans are regarded to be CUSIP [67,148]. These phenotypes include total plaque area, average and maximum carotid intima-media thickness (cIMT), intima-media thickness variability (IMTV), geometric total plaque area (gTPA), morphological total plaque area (mTPA), and lumen diameter (LD) [149–151] (AtheroEdge™ 3.0, AtheroPoint™, Roseville, CA, USA). This CUSIP is then used to improve the ML algorithm results shown in Figure 9. The segmentation of the carotid wall is helpful in the process of identifying the presence of plaque buildup [152–154]. The GT is an important component in the design of ML-based CVD risk stratification. This GT can be a CAD indicator, such as a CT score derived from the CT imaging. CT scoring can also be estimated using a DL framework or one can use plaque tissue characterization using optical coherence tomography (OCT) [155]. The paper by Suri et al. [156] discusses the CT-based scoring system. One can also use an IVUS-based solution for detecting CAD lesions [33,157,158].

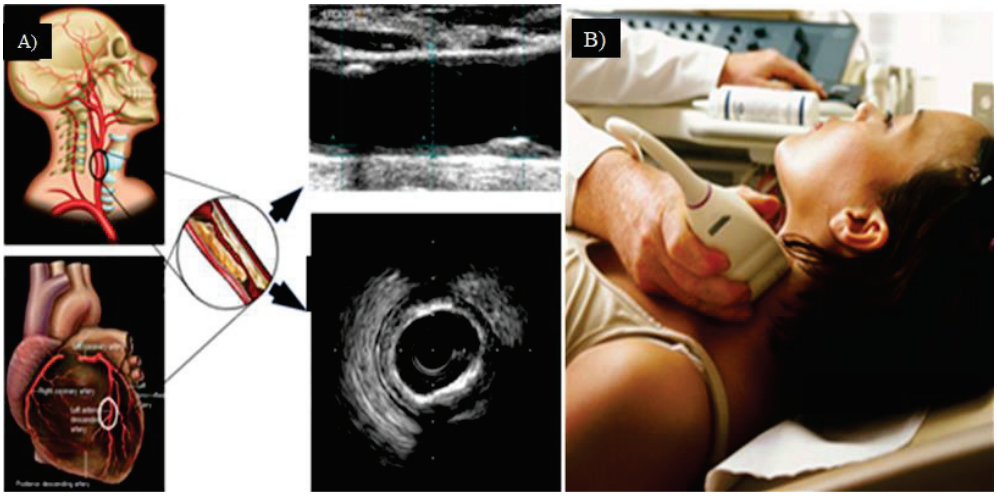

Figure 9. (**A**) CTAD is a potential surrogate marker for COAD, shown using an IVUS-based vascular cross-sectional scan. (**B**) B-mode carotid longitudinal imaging system using linear ultrasound [159].

Jain et al. [121] have proposed the UNet model for the segmentation of atherosclerotic plaque as shown in Figure 10. The model represents a four-layer DL-based UNet design consisting of four encoders and four decoders on each side of the U-shaped network. The encoder takes down samples while the decoder takes up samples. Each UNet encoder stage has a 2D-convolution, ReLU, and MaxPooling layer. Each decoder stage includes a stack of up-convolution-2D, depth-concatenation, 2D-convolution, ReLU, and MaxPooling layers. Encoder stage one receives a 224 × 224 grayscale US carotid scan. Stage one had 64 convolution filters, and each subsequent stage doubled that number. Each stage has 128, 256, and 512 filters. Each decoder stage halves the number of filters, such as 512, 256, 128, and 64, which are the bottom numerals in the illustration. The bridge network connects the encoder and decoder units. The bridge network has 3 × 1024 filters. Bridge network features can be concatenated to the last encoder stage after downsampling from the first upsampling level. Each encoder stage's spatial features are sent to the decoder through a skip connection. These functionalities are added to the decoder or bridge network layers. After the final decoder step, the plaque region and backdrop are identified using the softmax classifier layer (pink). An ADAM optimizer reduced plaque segmentation cross-entropy loss.

Deep learning has been improved by the addition of two models that operate independently of each other, a technique known as hybrid deep learning (HDL) [32,160–162]. As a result, an SDL-based UNet architecture can be used to create an HDL-based UNet, which may result in improved performance. In addition, given the arrangement of the convolution layer configuration, one can leverage the parallelization notion to increase the HDL designs' overall performance. The UNet advanced algorithms, such as UNet++ and UNet3P, are shown in Appendix A.

Jain et al. [121] show the role of UNet on two sets of carotid artery scans taken from Japanese and Hong Kong databases and in an unseen AI framework, which allows training on dataset A and testing on dataset B. The UNet model was trained on 330 Japanese DB photos and then evaluated on 300 Hong Kong DB images in the first experiment, referred to as "Unseen AI-1 (Tr: JAP, Te: HK)" [96]. Figure 11 shows the visualization of the carotid data. The UNet training model's *nine* classification parameters considered were as follows: (i) the reliability coefficient (CC); (ii) the area under the curve (AUC); (iii) the accuracy; (iv) the sensitivity; (v) the specificity; (vi) the precision; (vii) Mathew's correlation coefficient; (viii) the dice similarity coefficient (DSC); and (ix) the Jaccard index (JI). The mean values

of the *nine* classification parameters for the 300 images in the HK DB are 0.8, 0.87, 98.55, 95.41, 98.64, 67.82, 79.29, 78.38, and 65.42 [121].

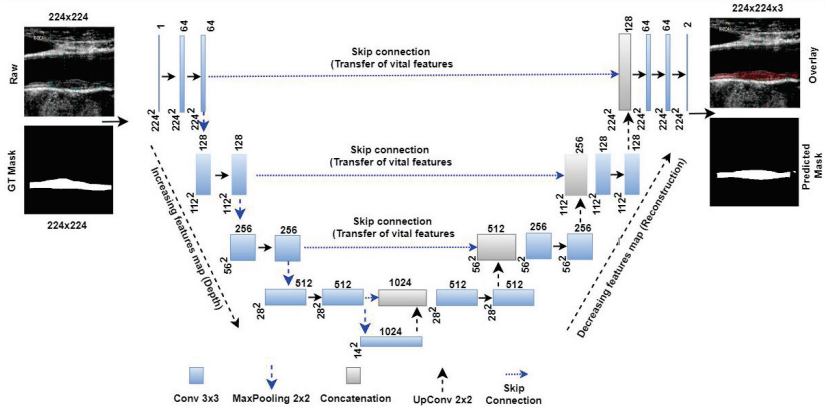

Figure 10. UNet model for segmentation of the wall of an atherosclerotic plaque [121].

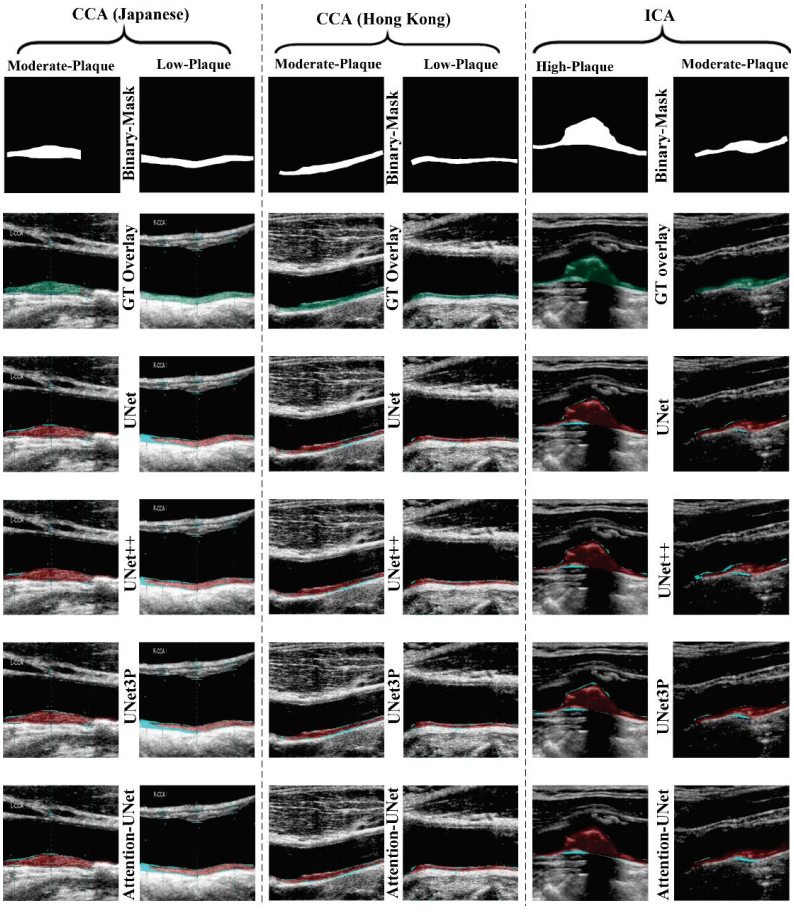

Figure 11. Visualizations of the Japanese, Hong Kong, and United Kingdom (ICA) databases were segmented using UNet, UNet++, UNet3P, and Attention-UNet models [96].

4.3. Deep Learning for Diabetic Foot Ulcer Lesion Segmentation and Its Quantification

Multiple investigations utilizing a variety of imaging techniques have demonstrated DL's effectiveness in detecting DFI lesions [163–165]. In reality, DL has been tried out for lesion detection in several different settings, including (i) the common carotid artery [111,119,166], (ii) the coronary artery [33,167,168], (iii) the brain tumor [169–171], (iv) skin cancer [43,122], and (v) CT-based pulmonary imaging [172,173]. The DFI typically has amorphous shapes and permeable boundaries. The skin around a DFI might seem different at different phases, such as redness to callus formation, blistering, granulation, sloughing, bleeding, and scaly skin [174]. The skin around a DFI is crucial because it reveals whether or not the DFI is healing, and it is also a potential extension area [175,176]. Ischemia, inflammation, aberrant pressure, maceration from exudates, and other conditions all raise the likelihood of fragile skin. Similarly, if the skin around the DFI looks healthy, the wound is healing well. The medical imaging of diabetes-related foot ulcers remains complicated [164]. For the representation, we use a smartphone-captured foot image for the modality. However, CT/MRI/Xray images can be used for the imaging modality of foot ulcers [100].

To improve the process of extracting significant features that are connected to the classification of a DFI, a novel model of a deep CNN-based architecture has been proposed by Alzubaidi et al. [51]. The Directed Acyclic Graph (DAG) principle served as the inspiration for its structure during the design process. When employing these kinds of networks, two major concerns must be addressed. For certain uses, a network that consists of a limited number of different layers and has a straightforward structure is adequate. Furthermore, DFI categorization requires a network that has a more intricate structure to retrieve more information to differentiate between typical and abnormal classes. This not only contributes to an increase in the number of details that can be learned but also to an improvement in the correctness of that learning. Figure 12 illustrates the overall process that our classification follows.

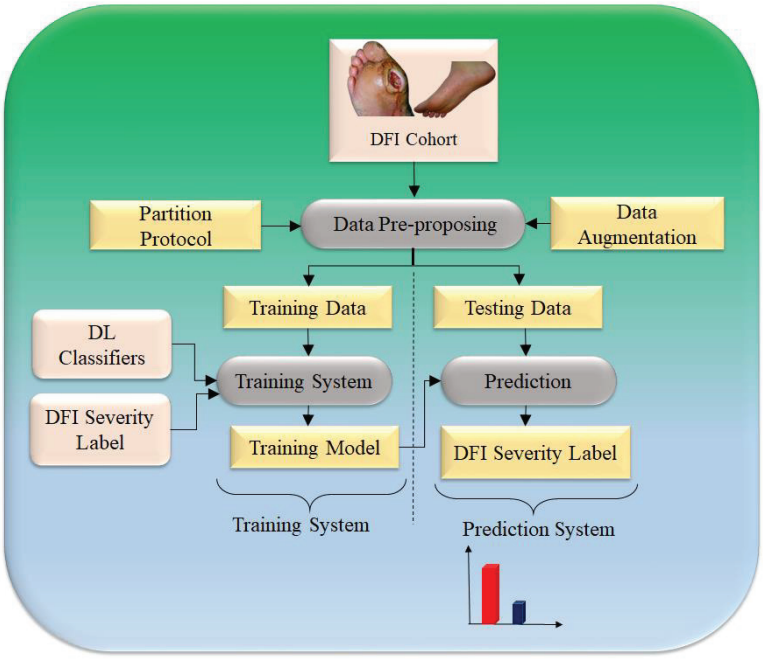

Figure 12. CNN-based model for DFI predication [51].

The color, consistency, and discharge of the surrounding skin are all analyzed, and the area is palpated for signs of warmth, swelling, and soreness. Inflammation, usually

caused by a wound infection, is indicated by the presence of redness. Black discoloration may indicate ischemia. If something is white and wet, it is because of maceration, but if it is white and dry, it is usually because of increased pressure. Understanding that skin tones affect how things look is crucial. Sometimes, skin lesions that show up red or brown on white appear black or purple. Darker skin colors may hide even mild cases of redness. The process of segmentation is designed by first extracting texture features and color variables from small patches of wound images, and then using ML algorithms to identify the patches of skin as either normal or aberrant [177–180].

Here, we focus on an image-based DFI lesion segmentation and its quantification that extracts features (covariates) during the DL paradigm. In DL, manual delineations of DFI lesions are challenging and are also vital for the design of offline DL training models. Figure 13 shows a few instances in which FCN-AlexNet and FCN-32s models can detect the small DFI and distinct surrounding skin or detect a very small part of them. Hyperparameter adjustment during training is a crucial part of DL for achieving optimal system performance. To avoid overfitting and ensure generalization, it is necessary to optimize (i) the learning rate, (ii) the number of epochs, (iii) the batch size, (iv) the normalization of batches, and (v) the addition of dropout layers. As a corollary, the ideal DL architecture necessitates the use of many biomarker sets, each with its unique collection of data, on a big data platform that guarantees a multiresolution platform for speedy implementation [94]. To guarantee faster performance, such pretrained models can benefit from transfer learning when used for DFI lesion segmentation [120,180–183].

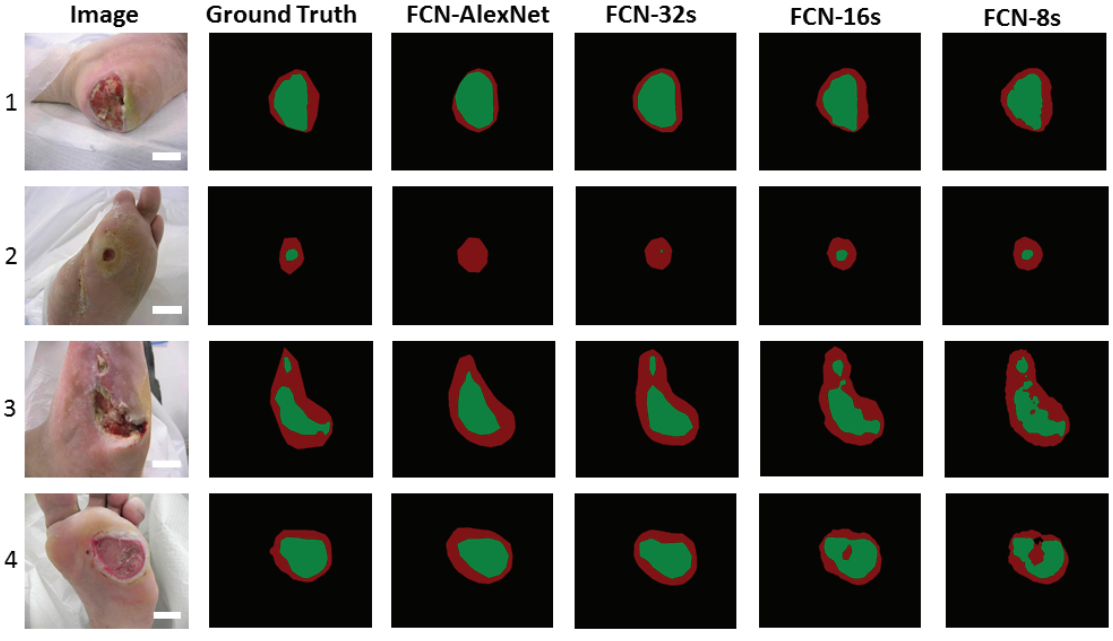

Figure 13. Four different FCN models (columns 3–6) and the gold standard (column 2) demonstrate the segmentation of the DFI area (green) from the skin (red) around it [51].

4.4. Challenges in CVD Risk Stratification on DFI Patients

Despite the availability of a wide range of diagnostic imaging techniques for the examination of diabetes-related foot problems, it is still difficult to differentiate between neuroarthropathy and osteomyelitis. The early and precise diagnosis of diabetic foot problems can assist in lowering the prevalence of infection-related comorbidities, the requirement for hospitalization, the length of hospitalization, and the prevalence of major limb amputations.

The main procedures that are used at this time for the examination of diabetes-related foot problems include traditional radiography, computerized tomography, nuclear medicine scintigraphy, magnetic resonance imaging, ultrasound, and positron emission tomography [184,185]. On the other hand, each one of these modalities cannot provide enough information by itself; therefore, a multimodal approach is required to arrive at an accurate diagnosis [186].

Therefore, we hypothesize that DL models can execute specific tasks, such as automated disease diagnosis, with more precision and efficiency than ML models, and that they serve as a second level of validation on the diagnosis. Models that have been trained using DL can be used for a broad variety of challenges, such as differential diagnosis, enhancements to image acquisition, and picture-based quantification.

The AI models have some challenges: (i) The data size must be large. If the data size is not big enough, SMOTE should be used during training to make it bigger. (ii) GT should be evaluated correctly for CVD risk, such as CAD in the training model. (iii) Optimization must be performed during the training of the CVD design. (iv) The correct CUSIP should be found by using UNET with attention channel maps. (v) All biomarkers, such as OBBM, LBBM, CUSIP, MedUSE, and DFI Severity, must be collected in the right way. (vi) DFI Severity DL system should give the risk appropriate and be validated by the Diabetologist or even surgeons dealing with foot amputations. (vii) Strong ML or DL models, such as XGBOOST, RNN, and LSTM, must be taken into account. (viii) If the ML models are not strong, one can switch to ML or DL ensemble models.

5. Discussion

5.1. Principal Findings

This is the first study to investigate the risk factors and gold standards for CVD and stroke in DFI patients based on their symptoms. The findings highlight the importance of selecting CVD and stroke risk-assessment approaches for DFI patients, especially those at high risk for CVD and stroke. Diagnosing a heart issue in a patient with a DFI is aided by surrogate carotid artery imaging. It has become clear from our research that ultrasound-based imaging techniques are the most practical for carotid atherosclerotic imaging. Furthermore, under the DFI framework, AI-based algorithms are the best option for the risk stratification of CVD/stroke.

A DFI is widely considered harmful to the brain and the heart. The review shows how a DFI worsens CVD and stroke in a progressive chain of events. We propose an approach to employing AI to aid in the diagnosis of CVD/stroke risk stratification in the DFI framework. Therefore, we can employ gold standards, such as coronary artery CT scores or coronary IVUS plaque burden, for superior AI training-based design for offline model generation, which can then be used for transforming the test patient features for CVD/stroke risk prediction. Using an AI-based model, we can effectively monitor these patients and prevent any CVD-related adverse long-term effects. Thus, for the DFI framework, ML and DL models can help provide a more precise assessment of the risk of CVD and stroke. The model could be taught so that it operates automatically and quickly. This is a game-changer for modern healthcare systems, particularly in identifying CVD and stroke risks in DFI patients. Clinicians can use the AI models' vascular and cerebrovascular data-based results to better counsel DFI patients and advise them on their CVD/stroke risk stratification.

5.2. Benchmarking

An analysis of the available data reveals that a DFI and CVD have been connected in a few studies using OBBM, LBBM, and MedUSE. In the study, AI's role in identifying combined CVD/stroke and a DFI has only been briefly mentioned. The AI model is only utilized by selecting a few articles within the DFI framework to describe the severity of CVD.

Parthiban et al. [127] explained the role of classifiers that can be helpful in the early diagnosis of the diabetic patient's susceptibility to developing heart disease. The patients can then be warned to adjust the way they live as a result. Diabetic individuals will be less

likely to develop heart disease, leading to lower mortality rates and, therefore, less overall healthcare costs. An SVMs classifier was explored that used a cross-validation protocol and showed an accuracy of 83.32%. Therefore, the use of this SVM model for the categorization of the diabetic dataset is something that may be advocated.

Jelinek et al. [128] focused on automatically identifying severe diabetic neuropathy using a brand-novel algorithm called Glioblastoma Multiforme (GBML). The study evaluated the specificity and sensitivity of the findings using GBML and compared the results against other ML methods. The patient size was 242. The uses K5 CV protocol. The GBML test for identifying acute diabetic neuropathy reached the highest degree of performance, with a sensitivity of 0.98 and a specificity of 0.89.

Zarkogianni et al. [129] carried out a study into the application of cutting-edge ML methods, the bilinear model, and ensemble learning to produce CVD risk scores for a population with type 2 diabetes. The utilization of a subsampling learning strategy resulted in the production of several primary models based on Hybrid Wavelet Neural Networks (HWNN) and self-organizing maps (SOM). The independently trained primary models' results were combined using DL and the results were then compared with one another. The models were evaluated using information taken from the medical records of 560 T2DM patients. The best discrimination performance achieved an area under the curve (AUC) of up to 71.48%.

Segar et al. [131] proposed an innovative risk prediction tool, WATCH-DM, which was tested on a well-phenotyped clinical study of patients with type 2 diabetes and cardiovascular disease or risk factors, but no history of heart failure at baseline. It identified patients who face a heart failure risk of up to 20% in the next five years. Since the data needed to calculate the WATCH-DM risk score are collected during the routine clinical care of patients with type 2 diabetes, therefore, integrating the WATCH-DM risk score into electronic health record systems or mobile health applications will provide a powerful tool for clinical practice. The advantage of WATCH-DM is that it does not require a particular cardiovascular biomarker or supplementary imaging examination. More research needs to be done to determine whether or not the WATCH-DM can be effective compared to other therapeutic options that are now accessible, such as sodium-glucose transport proteins (SGLT2i).

Aggarwal et al. [116] demonstrated diabetes mellitus (DM) causes hyperglycemia. Type 1 and type 2 diabetes are insulin-deficiency and insulin-resistance conditions. It can induce atherosclerosis, stroke, and MI. Neurodegeneration and autonomic dysfunction are also present. Autonomic balance regulates nonlinear physiological factors. The data size of 526 was produced from ECG data to evaluate 13 regressive HRV parameters and test ANN. With these inputs, an ANN design (13:7:1), at a 0.01 learning rate, achieved 86.3% classification accuracy. SVM differentiated diabetic and controlled individuals with an accuracy of 90.5%. Nonlinear HRV parameters reveal different changes owing to diabetes, so they can be combined with ML algorithms to construct a noninvasive, low-cost real-time diabetes prognosis system.

Derevitskii et al. [115] proposed that DM is among the most frequent forms of diabetes, also known as chronic diabetes. This particular form of diabetes is among the healthcare industry's most pressing concerns today. This disease is linked to several other conditions that simultaneously raise the risk of CVD and premature impairment. Patients diagnosed with type 2 diabetes have an elevated risk of various problems. In the case of patients such as these, medical doctors required methods that were more realistic for estimating the potential for future difficulties.

Karhu et al. [86] explained that the role of diabetes is extremely common in individuals who have already been diagnosed with CVD or chronic heart failure, and it is associated with a large increase in the likelihood of unfavorable outcomes. However, the persistently poor outcomes of people with diabetes mellitus highlight the importance of diabetes-specific systematic reviews and novel therapeutics aimed at specific pathophysiological requirements such as diabetic vascular and heart disease.

Schuett et al. [87] proposed that diabetes is prevalent in individuals who have already been diagnosed with CVD or chronic heart failure. It is essential to provide holistic care that focuses on lowering overall cardiovascular risk by employing various prevention methods to significantly cut the risk of cardiovascular events, progress to CHF, and mortality. However, the continually poor results of individuals with DM emphasize the importance of a diabetes-specific systematic review. Innovative therapeutics for particular pathophysiological conditions require an assessment of diabetic vascular and heart disease. To the best of our knowledge, no AI study has ever been able to provide us with information that is both clear and helpful regarding the CVD and stroke risk classification of DFI patients. The benchmarking analysis for the studies listed in Table 3 is presented below.

Table 3. Comparing the proposed review against previous reviews on joint DFI and CVD.

SN	Citations	Year	DFI[a]	DM[b]	CVD[c]	DI[d]	WI[e]	AI[f]	RSG	ClassTy[h]	ML/DL[j]	ACC %[k]	AUC[l]	SEN[m]	SPE[n]	FI[o]
1	Parthiban et al. [127]	2012	×	✓	✓	×	×	✓	×	✓	✓	✓	✓	×	×	×
2	Jelinek et al. [128]	2016	✓	✓	✓	×	×	×	×	×	×	×	×	×	×	×
3	Zarkogianni et al. [129]	2017	×	×	✓	✓	×	✓	×	✓	✓	✓	×	×	×	×
4	Segar et al. [131]	2019	✓	✓	✓	×	×	×	×	×	×	×	×	×	×	×
5	Dinh et al. [101]	2019	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	×	×	×	×
6	Aggarwal et al. [116]	2020	✓	×	×	✓	×	✓	×	✓	✓	✓	✓	×	×	×
7	Derevitskii et al. [115]	2020	✓	✓	✓	×	×	×	×	×	×	×	×	×	×	×
8	Karhu et al. [86]	2022	✓	✓	✓	×	×	×	×	×	×	×	×	×	×	×
9	Schuett et al. [87]	2022	✓	✓	✓	×	×	×	×	×	×	×	×	×	×	×
10	Hossain et al. [132]	2021	✓	✓	✓	×	✓	✓	×	✓	✓	✓	✓	×	×	×
11	Longato et al. [103]	2021	✓	✓	✓	×	×	✓	×	✓	✓	✓	✓	×	×	×
12	Hyerim et al. [102]	2021	✓	✓	✓	×	×	✓	×	✓	✓	✓	✓	×	×	×
13	Maindarkar et al. (proposed)	2022	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓

DFI[a]: Diabetic foot Infection, DM[b]: Diabetic Melliuties, CVD[c]: Cardiovascular diseases, WI[d]: Wound Imaging, CI[e]: Carotid Imaging AI[f]: Artificial Intelligence, RS[g]: Risk Stratification, ClassTY[h]: Type of Classifier, ACC[k]: Accuracy, AUC[l]: Area under curve, SEN[m]: Sensitivity, SPE[n]: Specificity.

5.3. Special Note on Casual Relationship between DFI and CVD

DFIs are vascular complications of diabetes mellitus associated with high mortality and morbidity. A few authors discovered a higher prevalence of major, previous, and new-onset cardiovascular and cerebrovascular events in diabetic patients with foot ulcers than in those without these complications [23,52,187,188]. This is consistent with diabetes' complicated interplay of factors with inflammatory metabolic diseases and their effects on the cardiovascular system, which could explain the increased morbidity and mortality levels in diabetic patients with amputations [189]. Inflammatory markers, such as IL-6 plasma levels and resisting, in diabetic participants validated the pathogenic issue of the "adipovascular" axis, which may add to the cardiovascular risk in type 2 diabetics. This "adipovascular axis" could be linked to the cause of foot ulcers in people with diabetes through microvascular and inflammatory mechanisms [2].

5.4. A Short Note on the Effect of COVID-19 on DFI Patients

COVID-19 has been shown to have affected several organs of the human body, such as the brain and heart [190]. A DFI causes more disability and death than any other diabetes condition. DFIs that do not heal despite treatment are the primary cause of hospitalization, amputation, disability, and mortality among people with diabetes [191]. People with diabetes, especially those with extensive foot ulcers, present significant issues in the face of a global pandemic such as COVID-19 [192]. To face the COVID-19 outbreak, the traditional diabetic foot treatment routine is no longer appropriate. Various studies have commented on a novel procedure for treating a patient with a DFI in the setting of the worldwide COVID-19 pandemic [188,193,194]. DFIs were classified as (i) mild (having no wound or tiny wound, no infection, and stable condition), (ii) moderate (having complex and refractory infection wound), or (iii) severe (having dry gangrene, sore in the injury, body

temperature, and sepsis symptoms) [195]. Patients with generalized diabetic foot issues can receive treatment at home with the help of telemedicine. This allows clinicians to instruct patients and encourage them to do a selfexamination of the foot, how to change wound dressings, and administer medications [192]. Patients with severe problems are referred to the hospital's outpatient clinic for treatment following a positive COVID-19 screening. Patients with a severe DFI who have been diagnosed or suspect that they have a COVID-19 infection require immediate isolation and ongoing quarantine. Patients with a low or mild DFI will be discharged to continue their care at home under telemedicine monitoring and physician supervision, while patients with a critical DFI will be admitted to the hospital following a COVID-19 screening [196]. During their hospital stay, patients with a DFI in a serious condition will receive a variety of treatments, ranging from rest and medication to debridement and local dilatation, and even amputation [197].

5.5. A Short Note on Bias in Deep Learning Systems for CVD/Stroke Risk, DFI, CUSIP Measurements

Bias was unnoticed in early computer-aided diagnosis systems [198]. Recently, the role of bias estimation in AI models has quickly emerged. Several factors are important, such as the sample size used in the training model design step of the DL algorithms, which is very important to consider. Furthermore, there is bias in AI due to several factors, including (i) a lack of clinical testing of AI techniques, (ii) scientific validation, (iii) failing to meet the gold standard, (iv) comorbidities, (v) a lack of big data configuration, (vi) failing to perceive the proper disease severity ratio, and (vii) variabilities in CVD [199]. As a consequence of this, when DFI-associated CVD symptoms (or risk variables) are investigated as inputs to an AI model, it is essential that the AI model be stable, accurate, and have a small amount of AI bias [152,156,173,200,201]. It is possible to observe that the database contains patient characteristics that are particular to a given region. Because of this, the model can produce false positive or negative results for other places, which would make the algorithm biased [185,202].

5.6. Work Flow for CVD Risk Stratification for DFI Patients

The workflow of the CVD/stroke risk stratification of DFI/DM patients can be seen in Figure 14. The pipeline consists of three major systems, labeled A, B, and C. System A consists of a DFI severity estimation given the patient's condition if the patient has a DFI. This DFI is an online system called **A-on**. System B consists of the CUSIP measurements which is also an online AI-based system, called **B-on**. The final system C is also an online system, such as a machine or deep learning system, for CVD/stroke risk stratification labeled as **C-on**. Note that all three online AI-based systems are supervised and, hence, must be executed by the trained offline systems called **A-off**, **B-off**, **and C-off**. Note that the **A-on** system accepts real camera phone images of the DFI whose DFI severity needs to be estimated using the **A-off** system. The output of the **A-on** system is the DFI severity. The **B-on** system accepts the surrogate imaging of CAD, so-called carotid imaging, along with the **B-off** trained system leading to the CUSIP measurements. Finally, the **C-on** system is triggered by taking the inputs of online laboratory-based biomarkers, such as LBBM, OBBM, CUSIP, MedUSE, and DFI-severity, and the **C-off** trained system to estimate the CVD/stroke risk stratified system.

The main feature of the model is cost-effectiveness. The imaging device used for diabetic foot infection image capturing is a smartphone. CUSIP is used for the carotid artery scan. There is no necessity for extra devices.

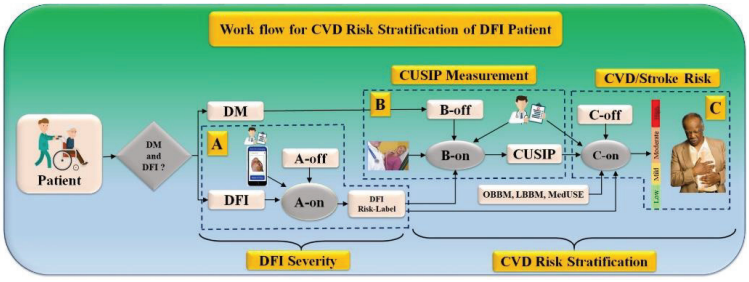

Figure 14. The overall architecture of CVD screening on DM and DFI patients. **A-on**: Online DL-based FDI severity system, **A-off**: Offline DL-based FDI severity system, **B-on**: Online DL-based Carotid wall quantification system, **B-off**: Offline DL-based Carotid wall quantification system, **C-on**: Online ML-based CVD Risk Assessment system, **C-off**: Offline ML-based CVD risk assessment system, DM: Diabetes Mellitus, DFI: Diabetic foot infection, CUISP: Carotid ultrasound image phenotype.

The main feature of the model is cost-effectiveness. The imaging device used for diabetic foot infection image capturing is a smartphone. CUSIP is used for the carotid artery scan. There is no necessity for extra devices.

5.7. Strengths, Weakness, and Extensions

The presented research article explains the various essential aspects of risk stratification for CVD and stroke patients with a DFI disease. Because of its improved nonlinear adjustment between the variables and the gold standard, DL provides better training and more accurate risk prediction. Additionally, the system gives it thorough predictors, such as OBBM, LBBM, CUSIP, MedUSE, and DFI as covariates, in addition to providing an estimation of the lesion size based on the wound scans of the diabetic foot. The role of an LSTM or RNN, an extremely effective strategy for creating the DL system for predicting the risk of CVD and stroke, was given. In conclusion, the DL system is generalized, and this generalization can be changed by including additional covariates and comorbidities, such as diabetes, rheumatoid arthritis, renal disease, coronary artery disease, etc.

While DL brings some benefits to the system, one must always ensure that the system is optimized to take advantage of these benefits. In addition, the DL system needs a solid gold standard for (a) lesion annotations and (b) CVD/stroke gold standard collection in cohorts. Both of these steps take a significant amount of time, and they also have associated costs. Last, but not least, as was said before, deep learning systems are vulnerable to artificial intelligence bias because of their overperformance in terms of accuracy and lack of interpretability.

When it comes to the design of extensions, ensemble-based methodologies allow for the creation of superior DL systems. Big data are an option that could be considered to strengthen the DL system by using a larger sample size and more data sources. If only a few participants are in the cohort, the DL system can be improved by incorporating augmentation designs. One can also integrate the conventional image-processing models with advanced DL models for superior feature extraction [5]. Furthermore, as part of the extension, one can learn about ulcers using multimodality imaging [203]. Another important component is to monitor the CVD/stroke risk with the changing DFI lesions. This can incorporate tools for image registration [204]. Last, but not least, the DL system needs to be updated with the latest round of pruning so that smaller training storage models [205] and evolutionary approaches [206] can be used.

6. Conclusions

This in-depth study brought to light the significance of CVD and stroke risk predictions for people with a DFI living in a diabetic environment. Additionally, we demonstrated how a DFI combined with hypertension can lead to strokes in both the vascular and cerebral

systems. This review focused on how a DFI may contribute to the already complex nature of CVD and stroke. Therefore, it is essential to classify DFI patients' risk of CVD and stroke. Carotid screening is a noninvasive, reduced alternative to traditional imaging that can be used to monitor people with a DFI for CVD and stroke. The low-cost B-mode ultrasonography will also help to describe the plaque tissue in patients with a DFI, which can improve the estimation of the risk of CVD and stroke. The severity of the DFI can be diagnosed and quantified using wound scan pictures of foot lesions. This information can then be used as a covariate in the DL design process.

An artificial intelligence-based model for predicting the risk of CVD and stroke in DFI patients was described using the AI framework. Because of this, we have discussed the function of an AI-based model that, based on the DFI risk profile of the patient, can reliably categorize patients diagnosed into risk groups for CVD and stroke. Finally, we explore the function that AI plays in this setting as well as the engagement of a DFI in the CVD/stroke paradigm.

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Appendix A UNet+ and UNet++, and UNet3P Architecture

The UNet+ and UNet++ designs are depicted below in Figures 10 and A1, respectively [207]. Both of these networks are enhanced variations of the UNet's architecture. In each of these architectural designs, the links between the encoder and decoder stages are handled by something called a "dense skip network (DSN)". The UpConv layer is the first in the DSN, which is then proceeded by concatenation and two levels of convolution. The output of the subsequent encoder stage is passed through the UpConv layer and into the concatenation layer, where it is merged with the output of the same encoder level. Both UNet+ and UNet++ have the same quantity of DSNs at every stage of the encoding and decoding process. It is important to note that, in the case of the UNet+ architecture, each DSN is only connected to its previous skip network output, as shown in Figure 10, whereas in the case of the UNet++ architecture, every DSN is linked to all prior DSNs in the same phase via avoiding network outputs, as shown in Figure A1. Figure 10 shows the UNet+ architecture, and Figure A1 shows the UNet++ architecture.

The UNet3P network is yet another iteration of the original UNet protocol. This model presents a novel approach to full-scale skip connection that improves upon the utility of multiscale features. High-level definition of feature maps generated from multiscale features is combined with lower-level specifics of the region of interest to use these full-scale skip connections. A lack of interconnectivity between features on

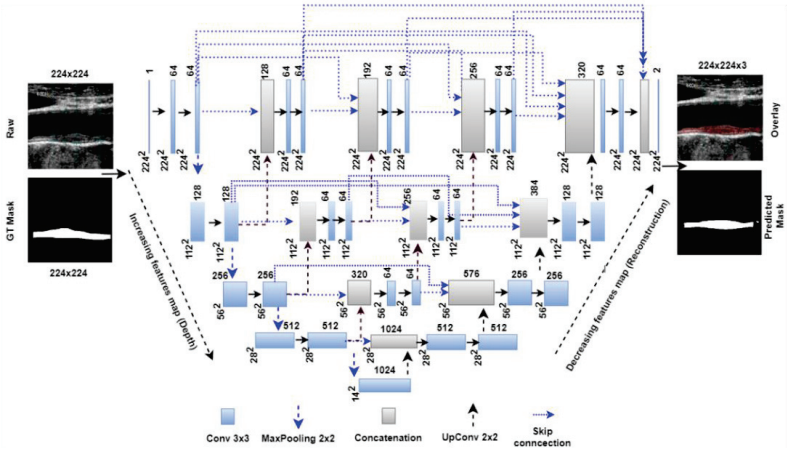

Figure A1. UNet++ Architecture.

Different scales are a weakness shared by UNet, UNet+, and UNet++. Therefore, UNet3P takes advantage of the multiscale features by incorporating lower-scale characteristics from the transmitter side with high-scale characteristics from the decoder side. In the UNet3P architecture, Decoder Stage 1 combines the characteristics map from Encoder Phase 1 (same scale), Decoder Phases 2, 3, and 4, and the bridge connection (large-scale). The characteristics map from Encoder Step 1, Encoder Stage 2, Decoder Stages 3, 4, and the bridge are combined in Decoder Stage 2 (large scale). The information from the first two stages of the encoder (at a lower scale), the third stage of the encoder (at the same scale), the fourth stage of the decoder, and the bridge are combined in the third stage of the decoder (large scale). Stage 4 of the decoder combines the information from stages 1–3 of the encoder (smaller scale), stage 4 of the encoder (same scale), and the bridge. The UNet3P architecture is depicted as a block diagram in Figure A2.

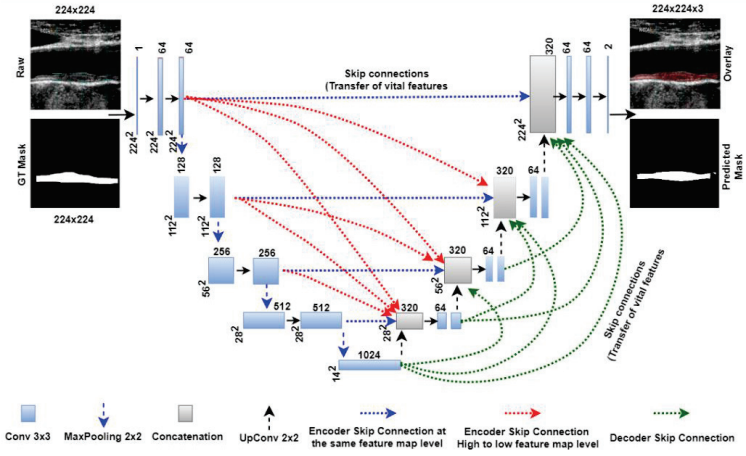

Figure A2. UNet++ Architecture.

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Review

Ultrasound-Assisted Wound (UAW) Debridement in the Treatment of Diabetic Foot Ulcer: A Systematic Review and Meta-Analysis

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Abstract: A systematic review and meta-analysis were carried out to investigate the effect of ultrasound-assisted wound (UAW) debridement in patients with diabetic foot ulcers (DFUs). All selected studies were evaluated using the Cochrane risk of bias tool to assess the risk of bias for randomized controlled trials. PubMed and Web of Science were searched in October 2021 to find randomized clinical trials (RCT) assessing the effect of UAW debridement on DFUs. RevMan v5.4. was used to analyze the data with the Mantel–Haenszel method for dichotomous outcomes. A total of 8 RCT met our inclusion criteria, with 263 participants. Concerning the healing rate comparing UAW versus the control group, a meta-analysis estimated the pooled OR at 2.22 (95% CI 0.96–5.11, $p = 0.06$), favoring UAW debridement, with low heterogeneity ($x^2 = 7.47$, df = 5, $p = 0.19$, $I^2 = 33\%$). Time to healing was similar in both groups: UAW group (14.25 ± 10.10 weeks) versus the control group (13.38 ± 1.99 weeks, $p = 0.87$). Wound area reduction was greater in the UAW debridement group (74.58% ± 19.21%) than in the control group (56.86% ± 25.09%), although no significant differences were observed between them ($p = 0.24$). UAW debridement showed higher healing rates, a greater percentage of wound area reduction, and similar healing times when compared with placebo (sham device) and standard of care in patients with DFUs, although no statistically significant differences were observed between groups.

Keywords: ultrasound assisted wound debridement; diabetic foot ulcers; diabetic foot; treatment

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1. Introduction

Among complications caused by diabetes mellitus, diabetic foot ulcer (DFU) is one of the most serious and costly [1]. Diabetic foot syndrome is defined as the presence of infection, ulceration, or destruction of foot tissues associated with peripheral arterial disease (PAD) and neuropathy [2]. Approximately 19–34% of diabetic patients will develop a DFU during their lifetime, leading to amputation of the affected limb [3,4]. Eighty-five percent of amputations in patients with diabetes will be preceded by the presence of a foot ulcer, reaching a mortality rate of seventy percent at five years after initial amputation [3,5].

Standard of care (SOC) in patients with DFU is based on infection control, use of pressure off-loading devices, PAD management, local wound care, metabolic control of diabetes, and treatment of co-morbidities [6]. Wound debridement is a fundamental part of the local treatment of ulcers and consists of removing devitalized tissue from the wound bed to obtain viable tissue to promote healing [7]. There are different types of debridement, including mechanical, sharp/surgical, autolytic, enzymatic, or biological

debridement [8]. The International Working Group on the Diabetic Foot (IWGDF) recommends sharp/surgical debridement in preference to other techniques because it is the least expensive, fastest method of wound bed preparation and is available in all geographic areas [7,9]. Sharp/surgical debridement requires specific clinical skills as there is the potential for extensive damage to the wound bed with exposure of bone, joint tissue, or ligament [7].

Currently, in developed countries, it is estimated that approximately 50% of patients with diabetes and foot ulceration have PAD, and it is estimated that 65% of DFUs have an ischemic component; therefore, an effective alternative to traditional debridement techniques is ultrasound-assisted wound (UAW) debridement, which is useful when sharp/surgical debridement is contraindicated, such as in patients with poor vascular status [8,10,11].

There are two modalities of UAW debridement—contact and non-contact—which have identical effects on wound healing. The only difference between the two modalities is how ultrasound is applied: non-contact UAW delivers ultrasound energy to the wound bed through a fine mist of sterile saline applied at a distance between 5 and 15 mm from the wound [12,13].

The effectiveness of UAW debridement is due to the cavitation and micro-streaming effects of ultrasound. Cavitation refers to the formation of oscillating gas microbubbles in a fluid medium; when it occurs, microbubbles expand, contract, and implode, allowing the removal of non-viable tissue and biofilms without damaging healthy tissue [14–16]. Likewise, micro-streaming refers to the flow of interstitial fluids caused as a result of the vibration generated by the ultrasound device; this effect alters cell membrane permeability and second messenger activity, resulting in increased protein synthesis, mast cell degranulation, and increased growth factor production, which ultimately leads to neo-angiogenesis and fibroblast stimulation at the wound site [17,18].

Several studies have shown that UAW treatment favors granulation tissue formation in the wound bed, resulting in increased healing rates and reduced healing times of hard-to-heal wounds [12,19,20]. A case series published by Lázaro-Martinez et al. on the effect of UAW debridement in neuroischaemic DFUs showed a significant bacterial load reduction, independent of bacterial species. Bacterial load reduction was associated with improved clinical wound characteristics and a significant reduction in wound size [21]. A recent open-label randomized and controlled parallel clinical trial comparing UAW debridement versus surgical debridement in patients with DFU over a 6-week treatment period demonstrated a significant improvement in cell proliferation and reduction of bacterial load, resulting in a reduction in healing time with the use of UAW debridement [22].

To build upon these previous findings, the purpose of this systematic review and meta-analysis is to assess the effect of UAW debridement on cure rates, time to healing, and wound area reduction in patients with DFUs.

2. Material and Methods

This systematic review and meta-analyses have been performed following the general guidelines and recommendations of preferred reporting items for systematic reviews and meta-analyses (PRISMA) [23].

2.1. Literature Search

The PubMed and Web of Science databases were systematically searched in October 2021. The keywords used for the search were: (((ultrasound) OR (ultrasonic)) AND (debridement)) AND (diabetic foot ulcer). To identify additional reports, the reference list of retrieved studies was cross-checked.

2.2. Article Selection

Inclusion criteria were randomized controlled clinical trials (RCTs) published in English, including humans >18 years old and assessing the effects of UAW debridement

compared to SOC and placebo in DFUs. Exclusion criteria were animal or in vitro studies, studies on the wound of different etiologies, and studies with insufficient data for analysis.

Title and abstract review were performed independently by two reviewers (S.F.-E. and F.J.Á.-A.); any discrepancies between the two reviewers were discussed with a third reviewer (J.L.L.-M.).

The articles included in the systematic review were divided into two groups, one comparing UAW debridement versus placebo and the other UAW debridement versus SOC. Placebo refers to the use of a sham device, whereas SOC is based on local wound care using moist dressings, infection control, and use of pressure off-loading devices [6].

2.3. Data Collection

A customized Microsoft Excel spreadsheet was used to extract the data from the studies. The extracted data included: author name, year of publication, study design, number of included patients, intervention evaluated and comparison, and outcome measures (healing rate, time to healing, and wound area reduction).

2.4. Assessment of Risk of Bias and Quality of Evidence

Risk of bias in each of the included studies was estimated using the Cochrane risk of bias tool [24], according to six specific domains: random-sequence generation (selection bias), allocation concealment (selection bias), blinding (performance bias and detection bias), incomplete outcome data (attrition bias), selective reporting (reporting bias), and other bias (including supposed financial support). Each domain was evaluated for low, high, or unclear risk for bias. Further, the quality of the evidence was judged to be high, moderate, low, or very low according to the grading of recommendations, assessment, development, and evaluations (GRADE) system, based on the risk of bias, inconsistency, indirectness, imprecision, and publication bias (GRADEpro/GDT, https://gdt.gradepro.org/ accessed on 15 March 2022) [25].

The assessment was conducted independently by two reviewers (S.F.-E. and F.J.Á.-A.); any discrepancies between the two reviewers were discussed with a third reviewer (J.L.L.-M.).

2.5. Statistical Analysis

Frequency and descriptive analyses were performed using SPSS (IBM Corp. Released 2017. IBM SPSS Statistics for Macintosh, Version 25.0. Armonk, NY, USA: IBM Corp.).

The Shapiro–Wilk test was used to verify the assumption of normality of all continuous variables. Student's *t*-test and Mann–Whitney U test were performed for normally and abnormally distributed quantitative variables, respectively.

The patient was the unit of analysis for all studies. When studies comparing similar interventions reported the same outcome measures, their data were combined for meta-analysis. Review Manager (RevMan, Version 5.4. The Cochrane Collaboration, London, UK, 2020) was used to analyze the data with the Mantel–Haenszel method for dichotomous outcomes and inverse variance method for continuous outcomes according to a fixed-effect or random-effects model. Estimates of the intervention's effects are expressed as the odds ratio (OR) (95% CI) for dichotomous outcomes and standardized mean difference (SMD) (95% CI) for continuous outcomes.

Heterogeneity was estimated clinically and methodologically, and when I square (I^2) exceeded 50%, a random-effects model was used [26]. The significance of any discrepancies in the estimates of the treatment effects from the different trials was assessed using the Cochrane test for heterogeneity and the I^2 statistic.

3. Results

3.1. Literature Search

A total of 155 manuscripts were identified from the literature. After screening the titles and abstracts, we identified 126 potential records. After a full-text review, a total of eight

RCTs met the selection criteria and were included in this systematic review [20,22,27–32] (Figure 1).

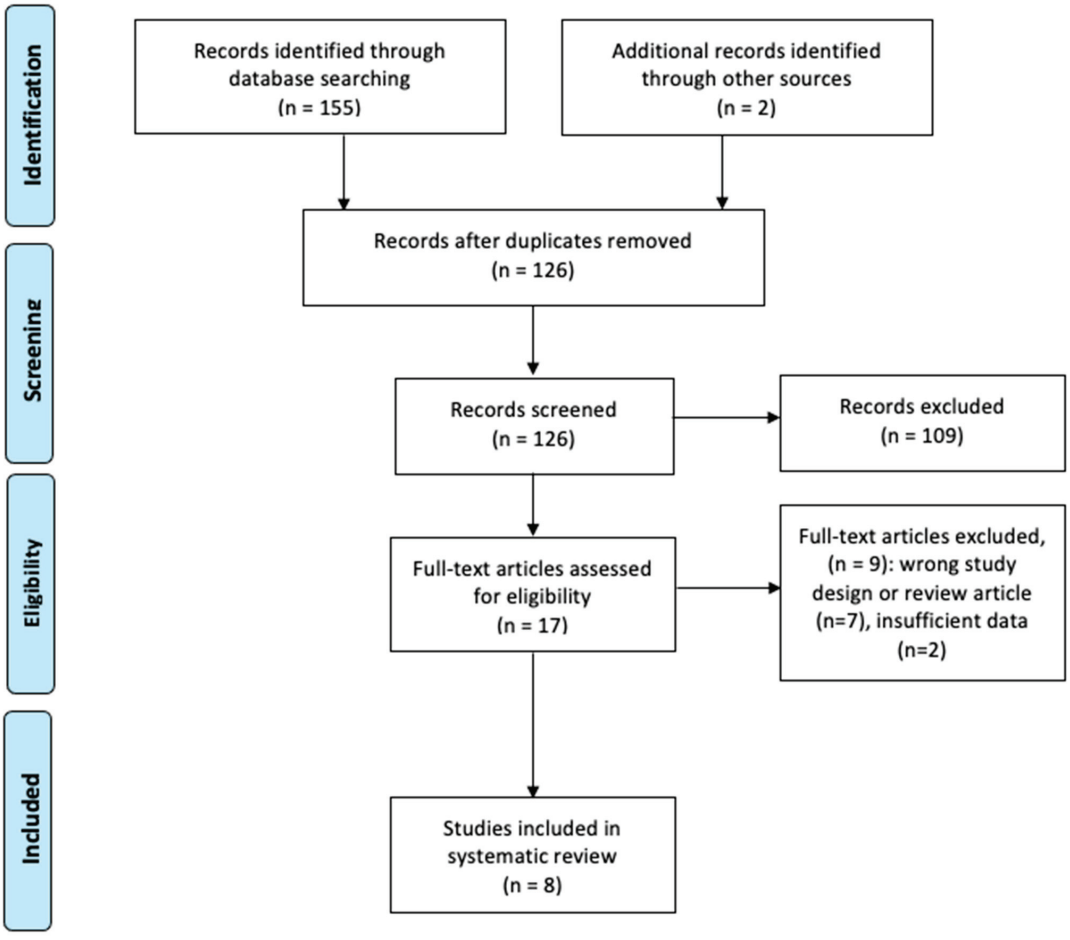

Figure 1. Flow diagram of the literature search and study selection for the systematic review.

3.2. Assessment of Risk of Bias and Quality of Evidence

The studies included in the systematic review were published between 2005 and 2020 and included 263 participants. The sample size ranged from 8 to 60 patients per study, with a mean size of 32.87 $\pm$ 21.08 patients.

According to the GRADE system, quality of the evidence was considered "very low" because of the imperfect study design, small sample size, significant heterogeneity, and potential publication bias. The results are summarized in Table 1.

Table 1. GRADE assessment for the effect of UAW on the healing of DFU.

Outcome	Number of Studies	Study Design	Certainty Assessment					Effect	Certainty
			Risk of Bias	Inconsistency	Indirectness	Imprecision	Other Considerations		
Healing Rate	6	RCT	Serious [a]	Serious [b]	Not serious [c]	Serious [d]	Publication bias strongly suspected	OR (95% CI) 2.22 (0.96, 5.11)	⊕○○○○○ Very low
Time to Healing	3	RCT	Serious [a]	Serious [b]	Not serious [c]	Serious [d]	Publication bias strongly suspected	SMD (95% CI) −1.41 (−3.43, 0.61)	⊕○○○○○○ Very low
Wound Area Reduction	3	RCT	Serious [a]	Not serious	Not serious [c]	Serious [d]	Publication bias strongly suspected	SMD (95% CI) 0.23 (−0.09, 0.55)	⊕○○○○○○ Very low

[a] The randomization method, allocation concealment, and blinding method of some included studies were not clear, and some studies did not carry out blinding method. [b] Differences were observed between the studies in relation to the time of application and frequency of the intervention as well as in the follow-up time of the patients (high heterogeneity). [c] All included studies were related to research questions and no indirect comparisons were made. [d] The sample size was small. ⊕ and ○ means very low certainly.

The risk of bias assessment of the eight RCTs included in the systematic review is summarized in Figures 2 and 3.

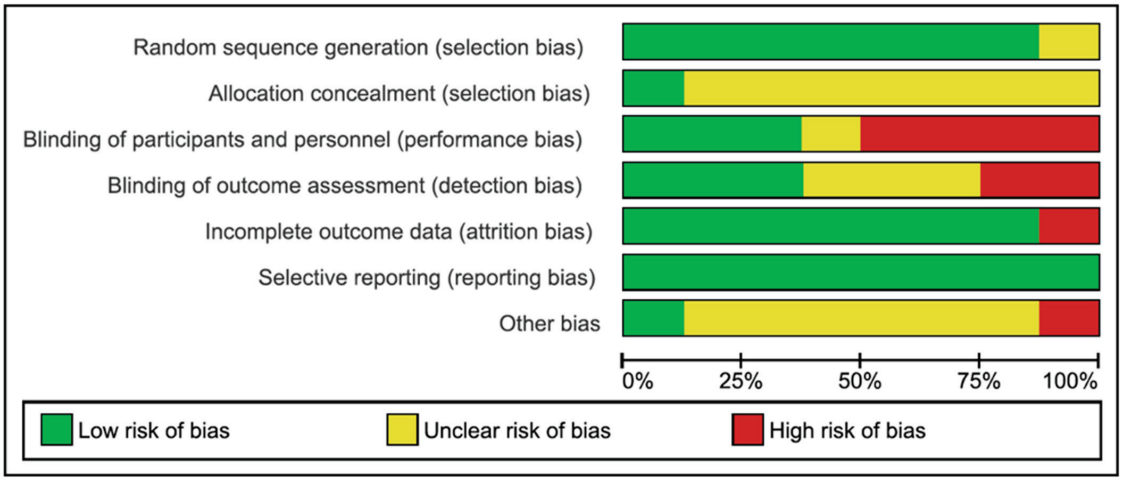

Figure 2. Risk of bias graph: Review authors' judgments about each risk of bias item presented as percentages across all included studies.

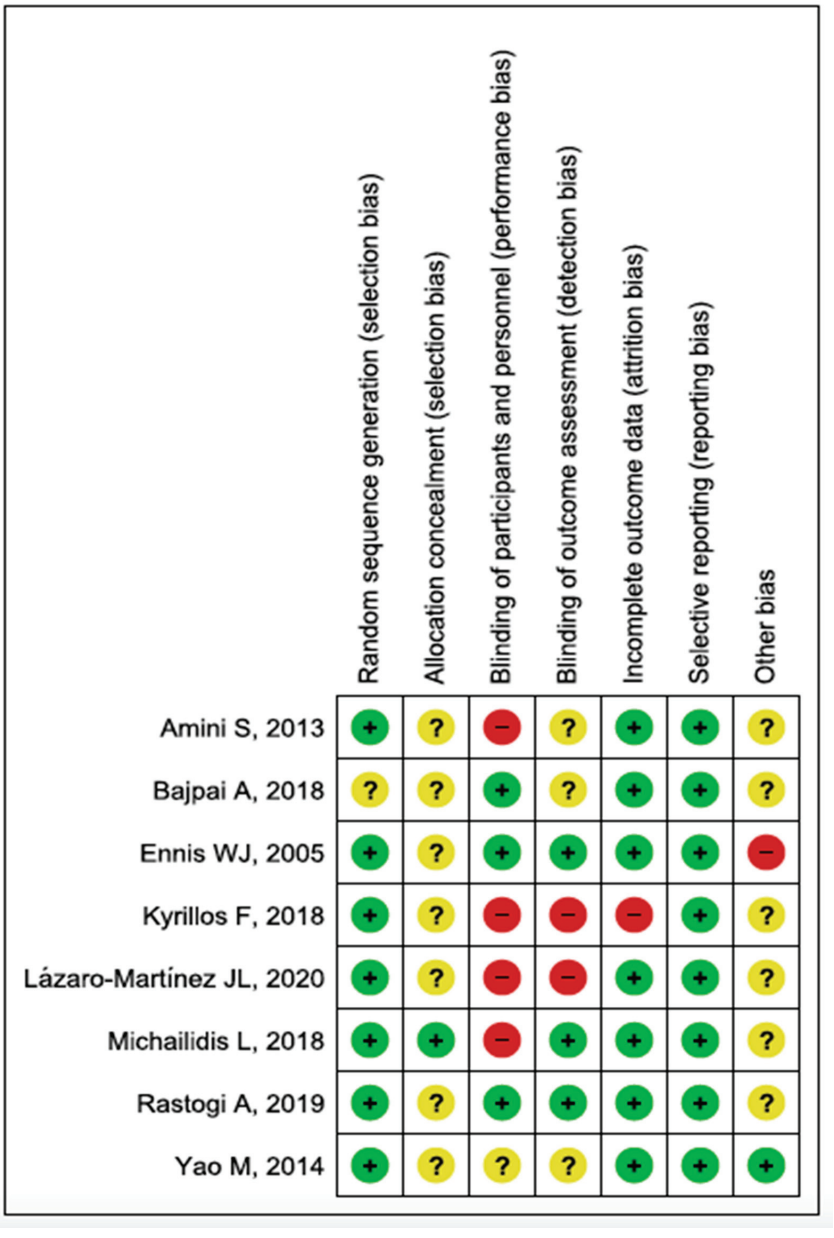

Figure 3. Risk of bias summary: Review authors' judgments about each risk of bias item for each included study. Green: Low risk of bias. Yellow: Unclear risk of bias. Red: High risk of bias.

3.3. Outcome Measures

The number of patients included in each study, type of intervention, rate, time to healing, and percentage reduction in wound area reduction are shown in Table 2. According to intervention, UAW debridement was compared with placebo (sham device) and SOC in three [27,29,32] and five trials, respectively [20,22,28,30,31]. Placebo and SOC refer to the control group.

Table 2. Characteristics of the RCTs included in the systematic review.

Author/Year	Number of Participants	Intervention	Healing Rate (%)	Time to Healing (Weeks)	Wound Area Reduction (%)
Ennis [27]/2005	Arm 1: 25 Arm 2: 28 Total: 55	Arm 1: UAW Arm 2: Placebo	Arm 1: 11 (40.7%) Arm 2: 4 (14.3%)	Arm 1: 9.12 ± 0.58 w Arm 2: 11.74 ± 0.22 w	–
Amini [20]/2013	Arm 1: 20 Arm 2: 20 Total: 40	Arm 1: UAW Arm 2: SOC	Arm 1: 12 (60%) Arm 2: 11 (55%)	Arm 1: 8.8 ± 12 w Arm 2: 11.6 ± 11.2 w	Arm 1: 87.9 ± 33.8% Arm 2: 82.4 ± 33%
Yao [28]/2014	Arm 1: 4 Arm 2: 4 Arm 3: 4 Total: 12	Arm 1: UAW 3/w Arm 2: UAW 1/w Arm 3: SOC	–	–	Arm 1: 86% Arm 2: 25% Arm 3: 39%
Bajpai [29]/2018	Arm 1: 4 Arm 2: 4 Total: 8	Arm 1: UAW Arm 2: Placebo	Arm 1: 4 (100%) Arm 2: 1 (25%)	–	–
Kyrillos [30]/2018	Arm 1: 12 Arm 2: 11 Total: 23	Arm 1: UAW Arm 2: SOC	–	–	Arm 1: 43% Arm 2: 24.4%
Michailidis [31]/2018	Arm 1: 8 Arm 2: 6 Total: 14	Arm 1: UAW Arm 2: SOC	Arm 1: 5 (62.5%) Arm 2: 5 (83.3%)	Arm 1: 29.4 ± 10.07 w Arm 2: 15.4 ± 6.1 w	–
Rastogi [32]/2019	Arm 1: 26 Arm 2: 34 Total: 60	Arm 1: UAW Arm 2: Placebo	Arm 1: 8 (23.5%) Arm 2: 3 (11.5%)	–	Arm 1: 69.4 ± 23.2% Arm 2: 59.6 ± 24.9%
Lázaro-Martínez [22]/2020	Arm 1: 27 Arm 2: 24 Total: 51	Arm 1: UAW Arm 2: SOC	Arm 1: 23 (85.1%) Arm 2: 20 (83.3%)	Arm 1: 9.7 ± 3.8 w Arm 2: 14.8 ± 12 w	Arm 1: 86.6 ± 83.8% Arm 2: 78.94 ± 68.6%

3.3.1. Healing Rate

A total of 6 studies, including 226 patients, compared the effects of UAW debridement in relation to healing rate versus control group [20,22,27,29,31,32]. A meta-analysis of this data estimated the pooled OR at 2.22 (95% CI 0.96–5.11, p = 0,06), favoring UAW debridement, with low heterogeneity (x^2 = 7.47, df = 5, p = 0.19, I^2 = 33%), although no statistically significant differences were observed between groups (Figure 4).

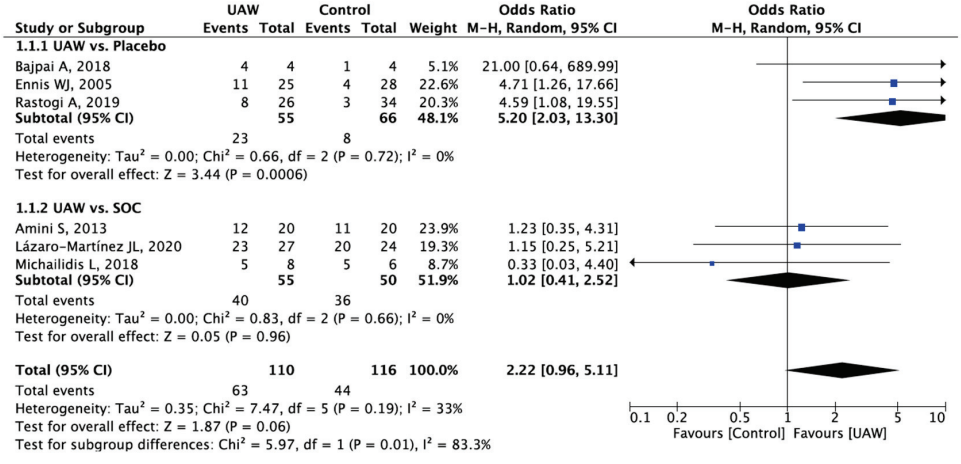

Figure 4. Forest plot of UAW debridement versus control (placebo and SOC) for complete healing rate. Bold text means overall outcomes per subgroups. Blue square: Odd Ratio for each study (measure of effect of each study).

3.3.2. Time to Healing

A total of 4 studies, including 158 patients, provided data about the healing times of DFUs and compared the effect of UAW debridement versus the control group [20,22,27,31]. Time to healing was similar in both groups, and no statistically significant differences were observed; 14.25 ± 10.10 weeks in the UAW debridement group versus 13.38 ± 1.99 weeks in the control group (p = 0.87).

3.3.3. Wound Area Reduction

A total of 5 studies, including 186 patients, compared the effects of UAW debridement in wound area reduction versus the control group [20,22,32]. Wound area reduction was greater in the UAW debridement group (74.58 ± 19.21%) than in the control group (56.86 ± 25.09%), although no significant differences were observed between them ($p = 0.24$).

4. Discussion

This systematic review with meta-analysis shows that UAW debridement in patients with DFUs is associated with higher healing rates, a greater percentage of wound area reduction than placebo and SOC, and similar healing times between UAW debridement and control groups.

In the clinical trials included in this systematic review, UAW debridement was conducted using a low-frequency ultrasound device; the frequencies used ranged from 22 to 60 kHz. There are two modalities of UAW debridement: contact and non-contact. Both are based on the effect of cavitation and micro-streaming to remove non-viable tissue from the wound bed. As the name suggests, non-contact UAW debridement generates the same effect but with a lower intensity and without direct contact with the wound surface [31].

Although the healing rates favored the UAW group with OR at 2.22 (95% CI 0.96, 5.11), no statistically significant differences were observed concerning the control group (placebo and SOC). These results could be a consequence of the small sample sizes observed in the different included studies, which ranged from 8 to 60 patients with DFUs.

The effect of UAW debridement was compared with placebo (sham device) in three studies [27,29,32]. The follow-up time of the studies ranged from 4 to 12 weeks, the frequency of debridement application varied from 1 to 3 times per week, and DFUs included were classified as Wagner 1, 2, and 3. Application time of UAW debridement was only reported in the studies published by Ennis et al. [27] and Rastogi et al. [32], and were 4 min/cm^2 and 15 min/cm^2, respectively. In all studies compared to placebo, the rate of DFU healing was higher for the UAW debridement group, with values of 23.5–100% versus 11.5–25%.

SOC effect compared to UAW debridement on DFUs was reported in five studies [20,22,28,30,31]. The follow-up time of the studies ranged from 5 to 24 weeks, the frequency of debridement application varied from 1 to 3 times per week, and DFUs included were classified according to Wagner [33] and Texas [34] classifications. Only two of five studies analyzed reported on application time of UAW debridement; in the study conducted by Lázaro-Martínez et al. [22], only neuroischaemic DFUs were included, and application time of UAW debridement was 2–3 min/cm^2, whereas the RCT published by Amini et al. [20] included neuropathic and neuroischaemic DFUs and application time of UAW debridement was 1 min/cm^2.

Regarding studies comparing UAW debridement with SOC, three studies reported on the healing rate. Amini et al. [20] and Lázaro-Martínez et al. [22] showed that the healing rate was higher with UAW debridement than with SOC; 60% and 85.1%, respectively. In contrast, Michailidis et al. [31] found a higher healing rate in the SOC group than in the UAW debridement group (83.3% versus 62.5%).

In terms of healing time, UAW debridement appears to have similar healing times to the control group. These findings could be caused by the variability of DFUs included in the RCTs, as healing time will differ depending on the wound depth and presence or absence of infection or ischemia. Another factor to consider is the variability of DFUs classification systems used in the RCTs (Wagner [33] and Texas [34] classifications).

Healing time in studies compared to placebo was only reported in the study published by Ennis et al. [27], being shorter in the UAW debridement group than the placebo group (9.12 ± 0.58 versus 11.74 ± 0.22 weeks). In relation to healing time of DFUs in studies comparing UAW debridement versus SOC, Amini et al. [20] and Lázaro-Martínez et al. [22] showed that healing times were shorter with UAW debridement (8.8 ± 12 and 9.7 ± 3.8 weeks)

than with SOC (11.6 ± 11.2 and 14.8 ± 12 weeks). Michailidis et al. [31] found that the time to healing was greater in the UAW debridement group than in the SOC group (29.4 ± 10.07 and 15.4 ± 6.1 weeks).

The results obtained in relation to healing rate and healing time in the study carried out by Michailidis et al. [31] in favor of the SOC group could be related to the small sample size and with an application time of UAW debridement, which was not precisely determined.

In addition, the reduction of wound area was greater in patients with DFUs where UAW debridement was applied. The absence of statistically significant results can be explained by the existence of the wide variation in the application time for UAW debridement and the frequency of debridement treatments, ranging from once per week to three times per week. Regarding the application time of UAW debridement, authors such as Amini et al. [20] established in their study an application time of 1 min/cm^2, whereas in the study by Bajpai et al. [29], the application time was 15 min/cm^2. The great difference in application times and frequency of UAW debridement is due to the use of ultrasound devices with different modalities (contact or non-contact ultrasound devices).

The percentage of wound area reduction in studies compared to SOC was referenced in four studies, in all of which wound area reduction was greater in the UAW debridement group than in the SOC group, with values of 43–87.9% versus 24.4–82.4%. Likewise, the percentage of wound area reduction in studies compared to placebo was referenced in one study [32]; this outcome showed a greater wound area reduction in the UAW debridement group (69.4 ± 23.2%) than the placebo group (59.6 ± 24.9%).

Regarding the level of evidence and the degree of recommendation of the included studies, all were controlled and randomized clinical trials, with a level of evidence 1b and degree of recommendation A. In 2/8 and 4/8 of the included studies, there were a high risk of bias in the blinding of results and of participants and/or professionals, respectively. There was a medium risk of bias in the allocation concealment in 7/8 studies and a low risk of bias in the random sequence in 7/8 studies. In general, in all studies, there was an unclear or low risk of bias in some of the items, mainly due to lack of information. Despite all studies being randomized clinical trials, the high risk of bias in the blinding of patients and professionals, together with the lack of information in most of the studies, limits the conclusions of this review with meta-analysis. The great difficulty in blinding patients and professionals when applying this type of instrumentalized technique should be emphasized.

To our knowledge, this study is the first systematic review with meta-analysis to assess the effect of UAW debridement on healing rates, time to healing, and wound area reduction in patients with DFUs. Therefore, it is not possible to establish comparisons with other similar previous studies.

A factor to consider regarding the literature search is the restriction of the included publications to English. The main limitation of this systematic review with meta-analysis is the small sample size of the RCTs included, which limits the generalizability of the results. Another important limitation is heterogeneity between the different RCTs, in terms of the clinical characteristics of the DFUs included (depth, infection, or ischemia), study follow-up time, time of application, and frequency of application associated with the type of ultrasound used (contact or non-contact). Finally, the lack of certain information in the studies is another limitation in evaluating some variables since it prevents the inclusion of some studies in our meta-analysis. Having this data could increase the information provided by this systematic review with meta-analysis.

Further clinical trials with low risk of bias, using control groups, with clear randomization and blinding of results could help clarify our conclusions. In addition, it is recommended to calculate the sample size of each treatment group and standardize the follow-up period of the study, the clinical characteristics of the DFUs included, and to establish a protocol about application time and frequency of UAW debridement.

5. Conclusions

Compared with placebo (sham device) and SOC, UAW debridement shows higher healing rates, a greater percentage of wound area reduction, and similar healing times in patients with DFUs, but greater quality evidence is needed to confirm these findings. UAW debridement could be an effective alternative when traditional debridement techniques are not available or are contraindicated for use. Limitations of this systematic review with meta-analysis include the small sample sizes and wide heterogeneity among RCTs in terms of clinical characteristics of DFUs, study follow-up time, application time and application frequency associated with the type of ultrasound used.

Author Contributions: Conceptualization, J.L.L.-M., F.J.Á.-A., and S.F.-E.; methodology, J.L.L.-M., F.J.Á.-A., Y.G.-Á. and E.G.-M.; software, Y.G.-Á. and M.L.-M.; validation, J.L.L.-M., F.J.Á.-A. and E.G.-M.; formal analysis, Y.G.-Á., E.G.-M. and M.L.-M.; investigation, J.L.L.-M., S.F.-E. and E.G.-M.; resources, J.L.L.-M. and Y.G.-Á.; data curation F.J.Á.-A. and M.L.-M.; writing—original draft preparation, J.L.L.-M., F.J.Á.-A., Y.G.-Á. and S.F.-E.; writing—review and editing, E.G.-M. and M.L.-M.; visualization, J.L.L.-M. and F.J.Á.-A. All authors have read and agreed to the published version of the manuscript.

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Review

The Immune-Centric Revolution in the Diabetic Foot: Monocytes and Lymphocytes Role in Wound Healing and Tissue Regeneration—A Narrative Review

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Abstract: Monocytes and lymphocytes play a key role in physiologic wound healing and might be involved in the impaired mechanisms observed in diabetes. Skin wound macrophages are represented by tissue resident macrophages and infiltrating peripheral blood recruited monocytes which play a leading role during the inflammatory phase of wound repair. The impaired transition of diabetic wound macrophages from pro-inflammatory M1 phenotypes to anti-inflammatory pro-regenerative M2 phenotypes might represent a key issue for impaired diabetic wound healing. This review will focus on the role of immune system cells in normal skin and diabetic wound repair. Furthermore, it will give an insight into therapy able to immuno-modulate wound healing processes toward to a regenerative anti-inflammatory fashion. Different approaches, such as cell therapy, exosome, and dermal substitute able to promote the M1 to M2 switch and able to positively influence healing processes in chronic wounds will be discussed.

Keywords: wound healing; diabetic foot; immune system; monocytes; lymphocytes; macrophage polarization; tissue regeneration

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1. Introduction

Diabetes is a predominant disease worldwide, with patients developing a wide variety of chronic complications, including Diabetic Foot (DF), characterized also by non-healing ulcers [1]. Current therapies for chronic non-healing diabetic wounds are still far from the optimal solution, with poor healing outcomes in many patients [1]. Nonhealing diabetic wounds produce a huge socioeconomic burden, with an estimated cost of USD 40.5 billion annually, and each amputation procedure can cost well over USD 35,000 [2]. Due to the increasing prevalence of diabetes, the total cost of diabetic ulcer care has also drastically increased in the past 20 years [3].

Extensive research tries to better highlight the diabetic wounds pathophysiology and, in particular, the role of inflammatory cell populations within the wound and how they are modified in diabetes [4].

Different cell populations such as mast cells, neutrophils, lymphocytes, monocytes, macrophages, keratinocytes, fibroblasts, and endothelial cells contribute to different stages of skin wound healing [5]. The myeloid lineage is the main supplier of inflammatory cells populations within the wound environment and plays a crucial role in the reparative phases of wounds [5,6]. It is well known that the natural wound-healing process is a four-stage progression that involves distinct and overlapping phases such as hemostasis, inflammation, proliferation, and remodeling, with different cell populations involved [7]. In contrast to acute wounds, which proceed in a well-timed fashion, chronic wounds fail to heal because they are blocked in the early inflammatory state [8].

In physiological wound healing, after the hemostasis due to platelets aggregation, injured tissues release pro-inflammatory mediators, which are essential for controlling infection, clearing necrotic debris, and the induction of the wound healing process [9]. Several cell types produce a transient connective tissue matrix, new blood vessels, and epithelial closure [7]. Newly formed tissues are remodeled.

Both innate and adaptive immune systems play an essential role in orchestrating all the phases of tissue repair and healing, as shown in both preclinical and clinical studies. The innate system, which consists of monocytes/macrophages, innate lymphocytes, basophils, natural killer (NK), granulocytes, tissue-resident mast cells, and dendritic cells, mobilizes rapidly but with low specificity. On the contrary, the adaptive system, which includes T and B lymphocytes, is activated more slowly with long-term memory and high specificity. The stimulation of innate and adaptive immune responses is activated by damage signals released from apoptotic and necrotic cells, which induce an alteration in the wound microenvironment [10,11]

Monocytes/macrophages play critical roles in host defense, tissue debridement, and cell regulatory functions [3]. Studies in monocyte/macrophage-depleted mice show that these cells are essential for normal wound healing, collagen deposition, angiogenesis, and wound closure [12,13]. The dysregulation of both monocytes/macrophages and unbalanced macrophage phenotypes may lead to impaired or reduced healing [14–17]. Impaired wound healing in diabetes has been associated with an increased number of wound monocytes/macrophages, as well as an impaired transition from pro-inflammatory into pro-healing wound [18,19]. In addition, a reduced phagocytic ability has been correlated to chronic inflammation in diabetes wounds [20,21]. Monocytes/macrophages are essential, but they do not play alone. Lymphocytes T, and in particular the subpopulation Regulatory T-cells (Treg), have been shown to promote repair and regeneration of various tissue such as skeletal and heart muscle, skin, lung, bone, and the central nervous system [22]. Recent data also suggest an unexpected key role of Treg in the angiogenesis and tissue regeneration in diabetic wound [23].

Recently, it has been recognized the influence of immune system on the regenerative therapies, according to a so-called "immune-centric revolution" or "macrophage centered approach [24–26]. For this reason, this review will give a brief insight into innovative autologous cell therapies and biomaterials able to immuno-modulate wound healing processes in a regenerative anti-inflammatory fashion. Examples of several different approaches that have been taken toward promoting anti-inflammatory (M2-like) macrophages to heal chronic wounds will be discussed.

2. Macrophage's Classification: An Overly Complex Issue

During wound healing, process macrophages assume distinct roles to guarantee proper healing [5,6]. Macrophages' phenotypes evolve along with the different stages of wound healing and can be classified roughly into the M1 class, which represents the classically activated phenotype in a pro-inflammatory state, and the alternatively activated M2 macrophages, which inhibit inflammation [27,28]. The classification of macrophages into M1 and M2 subtypes is a rather basic generalization of a more complex continuum of macrophage subtypes. Some authors describe this scenario as "the macrophage spectrum" in which cells possess varying degrees of M1- or M2-like characteristics [28]. Moreover, macrophages can go back and forth between different phenotypes depending on the cellular environment. In the attempt to classify an overly complex the dynamic macrophages populations, different classifications and nomenclatures exist based on activation, release, and surface markers. In addition, macrophage nomenclature is unclear whether the in vitro observed phenotypes are distinct or even applicable to in vivo wound healing [29]. Moreover, wound macrophages can develop a different phenotype depending on numerous factors, such as the anatomical setting of the wound, the precise area within the wound (center/edge), the environment (moist, dry), and if the wound is infected or not [30].

2.1. Macrophages' Classification Based on Activation Cues and on Cell Surface Markers

Macrophages' phenotypes change due to spatial-temporal cues during wound healing. Several different subsets of macrophages, beyond the limited over-simplification of M1 and M2, have been defined on their activation, cytokine/growth factor/chemokine release, and cell surface markers [31–33]. From the activation point of view, M1 and M2 macrophages can be activated by interferon-gamma (IFN-γ), tumor necrosis factor alfa (TNF-α), and the bacterial wall component lipopolysaccharide (LPS) in the inflammatory type M1. On the contrary, interleukins IL-4 and IL-13 induce the anti-inflammatory type M2. Depending on their activation in vitro, M2 macrophages have been further classified into different subpopulations: The M2a is activated by IL-4 or IL-13; M2b is activated by immune complexes, IL-1β, or LPS; M2c is activated by IL-10 and TGF-β; and M2d predominantly secretes IL-10 and vascular endothelial growth factor or VEGF [30].

Regarding the expression of cell surface markers, M1 macrophages express CD86 while regenerative M2 macrophages express elevated levels of the CD206 marker (mannose receptor). CD206 is a distinguishing surface marker for M2a linked to high release of arginase-1 (in mice), PDGF-BB, IGF-1, and several chemokines such as CCL17, CCL18, and CCL22 [32].

2.2. Macrophages Classification Based on Release

Regarding the cytokine/growth factor/chemokine release and in agreement with the multiple phases of wound healing, macrophages in vivo have been classified into three different sub-populations called pro-inflammatory, pro-wound healing, and pro-resolving macrophages.

Pro-inflammatory macrophages present shortly after the wound releases nitric oxide (NOS), Reactive Oxygen Species (ROS), IL-1, IL-6, TNF-α, IFN-γ, and metalloproteinases MMP-2 and MMP- 9 to digest the extracellular matrix [34].

Pro-wound healing macrophages release Platelet-Derived Growth Factor (PDGF), insulin-like growth factor 1 (IGF-1), VEGF, and Transforming Growth Factor-beta (TGF-β1) in high concentration to induce cellular proliferation, granulation tissue formation, and angiogenesis [35]. To counteract MMPs and permit ECM formation, pro-wound healing macrophages release tissue inhibitor of metalloproteinases 1 (TIMP1) [35].

Pro-resolving macrophages, in the last wound healing phase, suppress inflammation, releasing IL-10 together with arginase 1 and TGF-β1. Pro-resolving macrophages also release MMP-12 and MMP-13 to remodel and reinforce the ECM, aiming to restore tissue homeostasis and reduce fibrosis [34,36]. Pro-inflammatory and pro-resolving macrophages display some similar features as their actions overlap in the proliferation and remodeling phases. M2a macrophage sub-populations produce collagen precursors and growth factors to stimulate fibroblasts and secrete elevated levels of PDGF, which is implicated in angiogenesis [33,37,38]. M2b macrophages are characterized by CD86, CD68, and MHCII surface markers [32]. M2b macrophages reduce inflammation by releasing anti-inflammatory cytokines such as IL-10, IL-6, IL- β, and TNF, NOS, as well as several different MMPs. In vitro macrophages adopt the M2b phenotype after the neutrophil's phagocytosis [32]. M2c macrophages express CD206, MERTK, and CD163; are stimulated by glucocorticoids, IL-10, and TGF-β; and produce elevated levels of IL-10, MMP-9, IL-1β, and TGF-β and low levels of IL-12 [39]. M2c, sometimes described as deactivated macrophages, are analogous to pro-resolving macrophages. They can evolve from M1 macrophages with a "deactivated" gene profile to polarize in M2c macrophages. M2 macrophages can polarize in all a, b, and c phenotypes [32]. M2d macrophages stimulated by IL-6 and adenosine do not express either CD206 (mannose receptor) or dectin-1. They produce a high concentration of vascular endothelial growth factor (VEGF), IL-10, and TGF-β while downregulating TNF-α and IL-12 to dampen inflammation [30].

3. The M1/M2 Wound-Healing Paradigm: The Switch from KILL (M1) to HEAL (M2)

In the first phase of healing, just after hemostasis, pro-inflammatory M1 macrophages infiltrate after injury to clean the wound from bacteria, dead cells, and foreign debris [31]. When the tissue begins to repair in acute wounds, the overall macrophage population switches to the M2 phenotype, which induce anti-inflammatory and regenerative effects. It is well documented that such transition of phenotypes, defined by the term "polarization", is an essential step for wound healing [31,32].

The M2 polarization event induces the migration and proliferation of fibroblasts, keratinocytes and endothelial cells to repair the dermis, epidermis, and vasculature [37,38], and this cross-talk is impaired in diabetic wounds [39]. During this phase, both M1 and M2 are also responsible for the vascularization process, first creating new vessels trough sprouting [40], then creating anastomoses between newly formed vessels [41]. The macrophages' ability to create a functional anastomosis has been observed in in vivo time-lapse imaging, showing that a macrophage arrives at the lesion, extends filopodia or lamellipodia to physically adhere to vessels' endothelial ends, and through direct physical adhesion and mechanical traction repairs brain vasculature rupture [42]. The macrophage-mediated repair is conserved also in peripheral blood vessels [42]. This conclusion has been confirmed by the observation that macrophages secrete high concentrations of vascular endothelial growth factor (VEGF)-C to stabilize tip cell fusion and increase vascular complexity [39]. Gurevich et al., in an elegant experiment, showed, through in vivo imaging, that after tissue injury in both mice and zebrafish, macrophages could form angiogenic sprouts and drive neo-angiogenesis and consequent vessels remodeling [43]. This paper also shows that in an in vitro human co-culture model, specifically pro-inflammatory M1 macrophages are essential to initiating sprouting angiogenesis via the targeted delivery of proangiogenic cytokines and VEGF but also that a temporal phenotypic switching to M2 is a requirement to permit appropriate later vessel remodeling and regression [43].

In the final remodeling phase, macrophages release metalloproteinases (MMPs) to digest the temporary extracellular matrix, and then they start going into apoptosis [44]. In chronic wounds, pro-inflammatory macrophages persist in the M1 phenotype without transitioning to M2 anti-inflammatory phenotypes, which is believed to contribute to tissue repair impairment [36,45–47]. Therefore, controlling the phenotypic switch from M1 to M2 could represent a favorable solution for the transition from the inflammation to the proliferation stage of wound repair.

It is not clarified if the transition from M1 to M2 phenotype occurs through neodifferentiation of newly recruited monocytes from peripheral blood or/and through direct polarization of existing resident macrophages in situ to an anti-inflammatory phenotype. It has been observed that this switch can be driven by environmental changes in cytokines, miRNAs, transcription factors, exosomes [48,49], and the modulation of pro-inflammatory and anti-inflammatory receptors [50]. In the injured tissue, efficient clearance of apoptotic cells by wound macrophages (efferocytosis) is a requirement for inflammation resolution. Emerging evidence indicates that microRNA-21 (miR-21) may regulate the inflammatory response promoting efferocytosis [51]. It has been recently demonstrated that M2-derived exosomes can induce direct reprogramming of M1 into M2 with almost 100% conversion effectiveness [52]. These new reprogrammed polarized M2 macrophages produce matrix metalloproteinase (MMP) and vascular endothelial growth factor (VEGF), and their role is essential for angiogenesis re-epithelialization in the proliferative phases [52].

4. Resident Dermal Macrophages and Circulating Monocytes-Derived Macrophages

Macrophages can also be divided into two main groups according to their origin: (a) a resident tissue macrophage (RTM) population, called dermal macrophages, which are self-renewing cells derived from the embryonic yolk sack and established before birth, and (b) circulating monocytes that are recruited to injured tissue and differentiate into macrophages [34]. Skin wound macrophages originate from tissue-resident macrophages and infiltrating monocytes, with significantly higher contribution from the circulating

monocytes group [38]. Resident dermal macrophages respond to wounds through the recognition of molecules called damage-associated molecular pattern (DAMP) or, in case of infection, pathogen-associated molecular pattern (PAMP) releasing ROS, which in turn initiates a pro-inflammatory cascade [38]. Dermal macrophages also recruit neutrophils to fight the infection [38]. The main goal of resident macrophages is to retain skin integrity, homeostasis and tissue repair [53]. Characteristic surface markers of dermal macrophages are CD64+, MERTK+, and CCR2-/low. They show a high phagocytic ability but a slow turnover [54]. After remodelling, dermal macrophages self-renew and clear apoptotic cells in the resolution phase to return to tissue homeostasis [55,56]. Mapping studies have uncovered that most RTMs are principally of prenatal origin (yolk sac or fetal liver), while monocytes are constantly produced by hematopoiesis [57]. Through a combination of dynamic intravital imaging and confocal multiplex microscopy, it has been demonstrated that tissue-resident macrophages through a "cloaking" mechanism prevent neutrophil-mediated inflammatory damage, maintaining tissue homeostasis [57]. Failure of this cloaking process led to unrestricted inflammatory reactions, neutrophil swarms, and collateral tissue damage that required consequent control of neutrophil-driven inflammation by the recruitment of further circulating monocytes. RTMs represent a previously unknown immune checkpoint to prevent constant inflammatory damage.

Circulating monocytes infiltrate tissues upon initiation of the inflammatory cascade, where they can become definitive macrophages [58]. In vivo imaging showed that an initial wave of monocytes enters the wound simultaneously with neutrophils and not in a second time, as previously thought [59]. While resident macrophages initiate the local inflammatory response with short-term effects, monocyte-derived macrophages are recruited from peripheral blood for hours (about 24 h in mice) after the tissue damage [60]. The monocyte-derived macrophages' recruitment is due to signals from damaged tissue, both via DAMPs or PAMPs [61]. A common PAMPS is a lipopolysaccharide (LPS), a component of Gram-negative bacteria's outer membrane, which is recognized via binding by toll-like receptor 4, which activates NF-κB, which in turn induces the expression of pro-inflammatory genes [61]. Examples of DAMPs are extracellular DNA, RNA, and ATP released from dead cells, which attract immune cells to the injury sites [62]. Monocytes can also be recruited efficiently by chemokines and cytokines such as IL-1, IL-6, TNF-α, and MCP-1 (CCL2) [63]. In particular, MCP-1 plays a vital role in the inflammatory angiogenic response by recruiting host monocytes from the blood into the ischemic damaged tissue [64–66]. Moreover, MCP-1 promotes healing in diabetic wounds by restoring the macrophage response [66].

Resident and monocyte-derived macrophage coexist in the same wounds, and can show different states of activation. Two distinct macrophage subsets in skin wounds with distinct functions and origin have recently been demonstrated: CX3CR1hi macrophages are derived from tissue-resident macrophages and were predominantly activate in M2, while CX3CR1−/lo wound macrophages are derived from recruited monocytes and exhibit both activation phenotypes M1/M2 [67]. Migratory monocytes populate peripheral tissues in meaningful numbers and cooperate actively in tissue-protection with RTM. RTM play roles in primary prevention of inflammation and recruited monocytes in secondary resolution [57].

Circulating Monocytes and Their Role in Wound Healing

Peripheral blood monocytes are present in two categories: pro-inflammatory ("classical" monocytes, surface marker CD14+CD16− in human and Ly6C+/high in mice) and anti-inflammatory ("non-classical" monocytes, surface marker CD14low/−CD16+ in human and Ly6C−/low in mice), which are attracted to the injured tissue [61].

Olingly et al., by means of selective labelling, demonstrate that circulating non-classical monocytes are directly recruited within wounds, where they home to a perivascular niche and generate M2 wound healing macrophages [61]. Moreover it has been observed that the local delivery of a small molecule (FTY720) able to recruit non-classical monocytes supports

vascular remodeling after injury, confirming an angiogenic roles of peripheral blood monocytes [61]. Blood-derived, non-classical monocytes are major contributors to alternatively activated M2 macrophages, highlighting them as key regulators of inflammatory response and regenerative outcome.

The pro-inflammatory monocytes (CD14+CD16− in human, Ly6C+/high in mice) are derived from spleen and bone marrow, and their concentration increase in the peripheral blood after an injury; when there is no injury, they do not tightly adhere [32].The numbers of pro-inflammatory monocytes showed a short half-life (only 20 h in mice), vary depending on new cells recruitement from the bone marrow and from peripheral blood circulation, and reach a peak ~48 h after injury, while the recruited anti-inflammatory monocytes have a longer half-life (>2 days, in mice) [32]. Anti-inflammatory monocytes attach to the blood vessel wall via $\alpha_L\beta_2$ integrin (LFA-1) and L-selection (CD62L), which enables anti-inflammatory monocytes to crawl on the endothelium, even during homeostasis, so that they are ready to repair tissue and promote vascular repair when needed [32,68]. These data suggest that, in adjunct to resident tissue macrophage RTM, "resident" monocytes may also be present.

Other authors have used a different nomenclature to group human monocytes into three group: classical (CD14++CD16−), intermediate (CD14dimCD16++), and non-classical (CD14++CD16+) phenotypes. The "classically activated" CD14++CD16− monocyte phenotype represents 85% of circulating monocytes in normal healthy individuals. In comparison, the remaining 5% of the monocyte population is represented by the intermediate CD14dimCD16++ and 10% by the "non-classically activated" CD14++, CD16+ phenotype [60]. In inflammatory environments, classical monocytes differentiate into M1 macrophages, while non-classical monocytes differentiate into M2 macrophages to help in tissue repair [5,6]. Classical inflammatory monocytes are recruited to wounds in a higher amount following injury compared to non-classical monocytes, but there are evidences that inflammatory monocytes can become anti-inflammatory monocytes and differentiate into M2 macrophages [32,69]. This was also confirmed from Arnold et al. in an animal model of injured skeletal muscle, where recruited monocytes exhibiting an inflammatory profile that operates phagocytosis rapidly are able to convert into anti-inflammatory M2 macrophages, which in turn stimulates myogenesis and fiber growth [70]. Interestingly, it was recently observed that the implant of autologous peripheral blood mononuclear cells, produced by a point of care device based on selective filtration for human use, in a non-healing diabetic wound induced the polarization from M1 to M2 and the complete healing [71]. Inflammatory monocytes of the CD14++, CD16+ phenotype are strongly increased in ageing and chronic inflammatory disease [72]. Recently, a study has demonstrated that circulating CD16++ monocytes are a potential biomarker to predict the outcome of diabetic foot wound healing: In peripheral blood, the percentage of CD16++ monocytes and MMP-3 were higher in healed vs. unhealed patients [72].

5. The Secret Life of Lymphocytes

While the innate immune system is recognized to play a key role in the tissue healing process, the adaptive immune system has only recently emerged as a key player. Lymphocytes T and in particular regulatory T-cells (Treg) have been shown to promote the repair and regeneration of various tissues [22,73–76]. Treg can indirectly regulate regeneration by promoting their apoptosis neutrophils, regulating helper T-cells, and inducing macrophage polarization [77,78]. Additionally, Treg can also directly facilitate regeneration triggering resident stem cells locally [79]. Treg can improve the differentiation of stem or progenitor cells such as satellite cells to replace the damaged skeletal muscle and enhance the proliferation of neonatal cardiomyocytes for functional regeneration [80].

Tregs could regulate macrophage polarization through the suppression of IFN-γ produced by $CD4^+$ effector T cells, IFN-γ being a promoter of the formation of M1 macrophages. Another mechanism could be related to the increase in IL-10 levels in muscle [81].

In many tissues, Treg are recruited to the injured site to expedite inflammation resolution and to regulate immunity after damage [80]. Tregs promote repair in various tissue: muscle repair after cardiac injury, skin epithelial stem cell differentiation and wound healing, enhance satellite cell expansion in muscle, facilitate lung resolution, promote myelin regeneration in central nerve system, and protect kidney injury [82].

In vitro monocytes in co-culture with Treg produce diminished levels of TNF-α and IL-6 in response to LPS, while Treg alone secretes higher concentrations of IL-10, IL-4, and IL-13 [83]. Treg can also directly act on the pro-inflammatory M1 macrophages through the release of IL-10, inducing the polarization to anti-inflammatory and pro-repair M2 [84]. It has also been reported that diminished levels of Treg facilitate vascular inflammation [81]. Tregs play a highly broad variety of tissue regeneration roles such as facilitating blood flow recovery after ischemia, controlling adipose tissue inflammation, promoting muscle repair, and maintaining tissue/organ homeostasis [77]. Treg can also facilitate cutaneous wound healing, mainly by the secretion of anti-inflammatory/immunosuppressive cytokines, including IL-10, IL-35, and TGF-β [78]. Highly activated Tregs accumulate in skin early after wounding, decreasing both IFN production and proinflammatory M1 macrophage accumulation through the induced expression of the epidermal growth factor receptor (EGFR) [78]. In addition to their regeneration ability, Tregs also promote angiogenesis in ischemic tissue though apelin-mediated sprouting in diabetic patients [79,82]. In keeping, the lack of lymphocytes impairs macrophage polarization and angiogenesis in diabetic wound healing [23]. Tregs are not the only the lymphocyte population able to play a key role in wound healing and angiogenesis: It has been observed that both NK lymphocytes land CD4-T-cells modulate arteriogenesis in a murine ischemia model [85]. Accordingly, an impaired arteriogenic response has been observed in hindlimb ischemia in CD4-Knockout mice [86]. CD8+ T-cell plasticity seems to regulate vascular regeneration [87]. B cells can differentiate into antibody-producing plasma cells but can also present antigens to T cells and modulate local immune responses through the secretion of pro- and anti-inflammatory cytokines. Recent data have shown that naïve B lymphocytes injected in acute or chronic diabetic skin lesions can act as effective modulators of tissue regeneration, both in acute and chronic diabetic skin lesions, accelerating wound healing [88]. The same paper also showed that B cell treatment was associated with better-quality collagen deposition and reduced scar formation. The enhanced healing was reinforced by a higher fibroblast's proliferation, together with a diminished level of apoptosis, a regenerative modulation of cytokines, and matrix metalloproteinases. The same process was not observed by the injection of disrupted B cells or hematopoietic stem cells. All these relevant insights suggest a potential development of innovative cell therapies based on immune system cells such as monocyte/macrophages and lymphocytes to target vascular diseases associated with diabetes.

6. Monocytes/Macrophages and Lymphocytes in Diabetic Wound Healing

In a healing wound (Figure 1), just after neutrophil recruitment, a first wave of monocytes invades the tissue and differentiates into inflammatory M1 macrophages, releasing cytotoxic and proinflammatory molecule such as IL-1β, TNF-α, IL-6, and ROS, with the aim to digest damaged cell, microbes, and necrotic damaged tissue. The first wave is followed by a second wave of anti-inflammatory M2 macrophages, which, on the contrary, promote tissue remodeling, fibrosis, and wound healing through the release of TGF-β, IL-10, and other anti-inflammatory cytokines. Primarily, these M2 populations produce growth factors, anti-inflammatory mediators, although keeping the ability to clear apoptotic cells. Moreover M2s recruit endothelial stem cells and promote angiogenesis in the healing wound, allowing the development of granulation tissue and neovascularization [89].

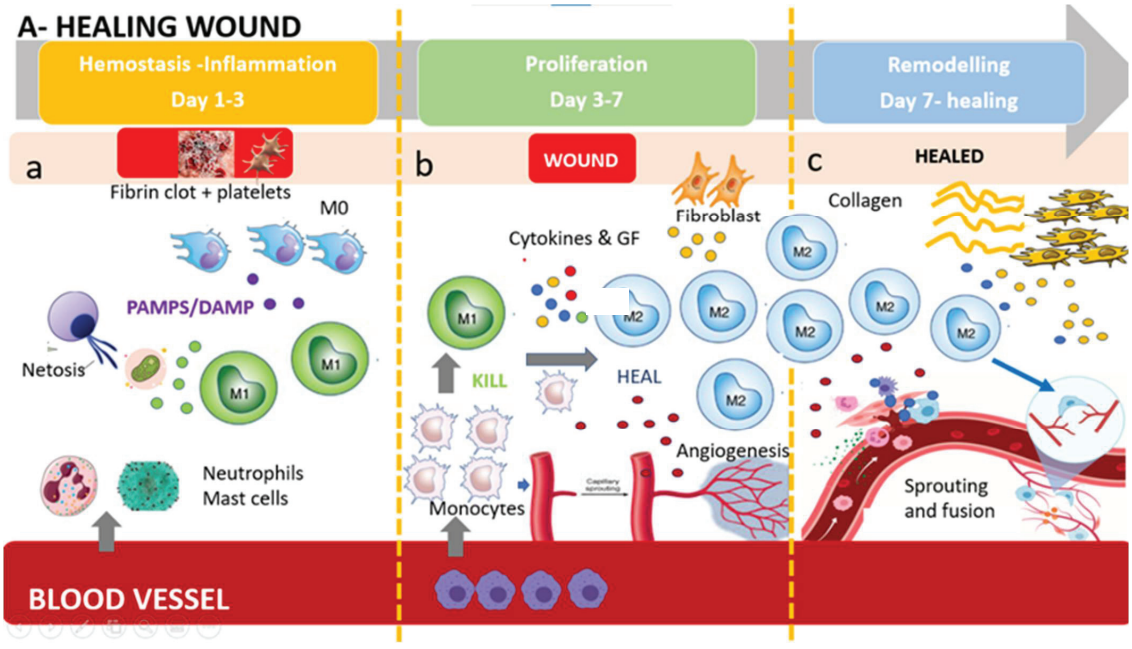

Figure 1. The healing wound: (**a**) Platelets form a fibrin clot, and chemo-attractants are released to recruit inflammatory cells (neutrophils and mast cells), releasing pro-inflammatory cytokines. NETosis (Neutrophil Extracellular Trap) helps to capture and destroy pathogens. Tissue-resident macrophages react to pathogen- and damage-associated molecular patterns (PAMPs and DAMPs). First wave of monocytes differentiates in M1(phagocytoses step). (**b**) After the resolution of inflammation, the proliferative phase starts. Angiogenesis develops via vessel sprouting. Infiltrating monocytes differentiate into M1 and M2 macrophage subsets. M1 macrophages maintain a strong inflammatory profile releasing inflammatory cytokines and ROS and eating dead bacteria and neutrophils. After M1 polarize in M2 pro-regenerative phenotype which release anti-inflammatory cytokines, growth factors, and proteases which replace the provisional ECM with collagens, induce fibroblasts proliferation, and induce new vessel formation. This process results in granular tissue and keratinocyte coverage. (**c**) Remodeling is supported by macrophages, fibroblasts, and myofibroblasts re-organizing the provisional ECM into a definitive healed tissue, principally through matrix metalloproteinases (MMPs) and their inhibitors (TIMPs), resulting in tissue with strong tensile strength and functionality. Angiogenesis is almost complete, and macrophages produce molecule bypass (fusion) between newly formed vessels, creating a functional network.

In contrast, diabetic wounds have many structural and functional differences, such as reduced angiogenesis, which produces a hypoxic wound environment and oxidative stress [20]. Diabetic foot ulcer (DFU) healing (Figure 2) does not progress through phases and is characterized by a stalled non-healing state that includes deregulated inflammation that is considered less effective to facilitate progression of healing, reduced angiogenesis, non-migratory epithelium, low response to growth factors, and fibrosis [8]. In the following paragraphs, we will describe the different behaviors of neutrophils, monocytes/macrophages, and lymphocytes in a diabetic wound.

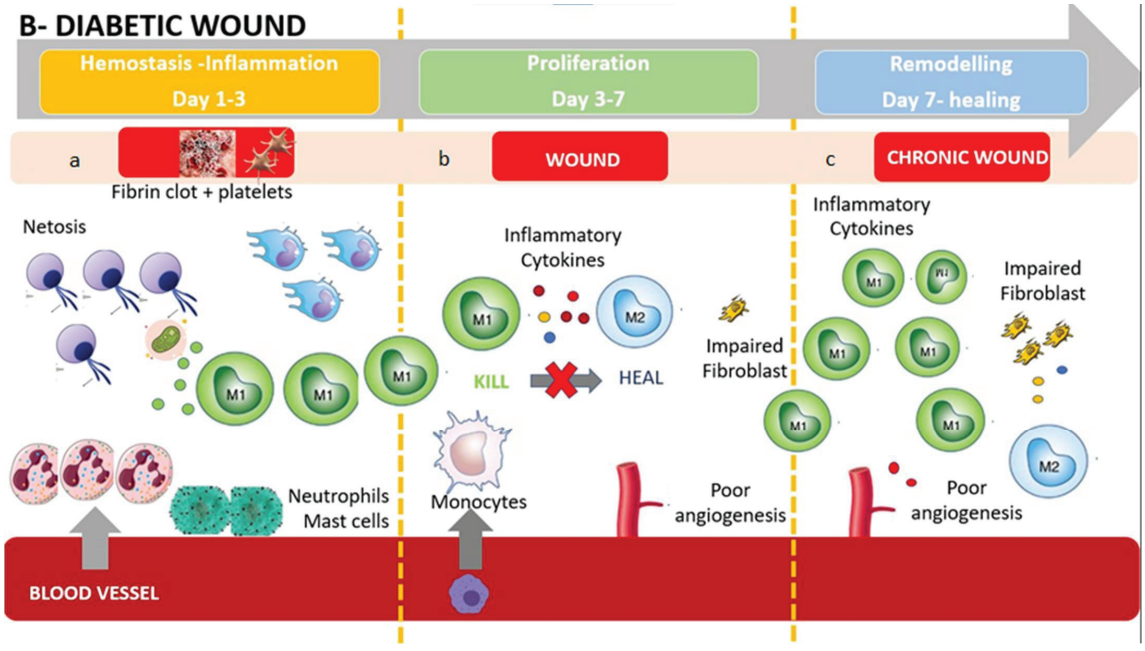

Figure 2. The diabetic wound: (**a**) Impaired wounds showed an upregulated influx of neutrophils and mast cells, leading to an intense inflammatory response, causing collateral damage, and extending the inflammatory phase to subsequent phases. The persistent higher release of inflammatory cytokines produces M1 activation with further release of inflammatory substances. (**b**) Monocyte recruitments are poor due to arterial occlusion and impaired microcirculation. Poor angiogenesis and glycated proteins result in an impaired fibroblast activity. The hypoxic environment brings oxidative stress, driving inflammatory M1 macrophage polarization and impairment of fibroblasts, resulting in poor ECM reorganization and a persistent inflammatory environment. The polarization in M2 is absent or extremely poor, causing a further accumulation of M1. (**c**) Impaired wound-resident cells remain ineffective and in an inflammatory condition. Collagen reorganization resolves poorly, resulting in weak, non-functional skin that can re-injure and potentially ulcerate, perpetually inflamed. Macrophages are still activated in the inflammatory phenotype M1. The wound does not heal.

Neutrophils in diabetic wound: A high neutrophil count within the wound and the increased neutrophil-to-lymphocyte ratio is well recognized as a characteristic of impaired diabetic wound healing [90]. As part of their antimicrobic defense, neutrophils form extracellular traps (NETs) by releasing decondensed chromatin lined with cytotoxic proteins. Unfortunately, NETs, can even cause tissue damage. Neutrophils isolated from type 1 and type 2 diabetic humans and mice were primed to produce NETs (a process termed, NETosis), and this phenomenon is responsible for delayed wound healing [91]. Accordingly, a previous study showed that a decreased ability of neutrophils to undergo NETosis led to accelerated wound closure [92,93]. This persistent neutrophil activation and induction of NETosis results in the production of further inflammation, while in the normal process, the inflammation resolution is produced by neutrophils' apoptotic body phagocytosis from infiltrating monocytes/macrophages [94]. In healing wounds, the uptake of apoptotic neutrophils resolves the inflammatory phase by limiting inflammatory cell infiltration and shifting the production of eicosanoids from pro-inflammatory to anti-inflammatory mediators [95]. In diabetic wounds, the inflammatory phase is significantly prolonged by the disruption of mechanisms which both control the influx of neutrophils as well as regulate their inflammatory processes [91]. Accordingly, it has been observed that the

transcription factor FOXM1, responsible for activation and recruitment of inflammatory cells, is downregulated in diabetic patients [96].

Monocytes-macrophages in diabetic wound: Dysregulations of the inflammatory phase seems to be associated to epigenetic polarization of innate immune cell pro-inflammatory function prior to wound infiltration, probably due to hyperglycemia [20]. The polarization of innate immune cells towards inflammatory phenotypes is correlated to the systemic inflammatory response observed in both diabetic patients and animal models [97]. Recent results in a mice diabetic model suggest that the combination of improved neutrophils numbers with reduced macrophages numbers, monocyte-derived Langerhans cells, and dendritic cells and eosinophils produces an imbalance in the immune cell composition, which may contribute to their impaired healing [98]. While in the healing wound the infiltrating monocytes differentiate into classically activated inflammatory M1 and alternatively activated M2 macrophages, in the diabetic wound, there is a strong polarization into the M1 phenotype, and the switch from M1 to M2 is heavily impaired [18–20,36,46–48]. Therefore, a lower number of M2 macrophages and a higher M1:M2 ratio will release low levels of growth factors PDGF, FGF, and VEGF and of anti-inflammatory cytokines such as IL-10, TGF-α, and TGF-β, all of which induce the proliferation phase and the effective regulation of inflammation [9]. It has been also observed that M1 macrophages release high concentrations of TNF-α in diabetic rats, and an in vitro high glucose environment facilitates M1 polarization, which are both detrimental to keratinocyte migration [99]. It has been recently shown that negative pressure wound therapy by suppressing autophagy and macrophage inflammation in a mouse model promotes wound healing [100]. Monocytes and macrophages are known to play important roles in neovascularization during wound healing [38,101]. Since macrophages are a central source of VEGF and other angiogenic molecules in wounds, the macrophage deficit may be linked to the documented decrease in wound angiogenesis that is seen in diabetic wounds [102]. A reduced VEGFR1 signaling in the diabetic wound tissue could contribute to impaired angiogenesis [103]. In addition, M2 macrophages boost wound angiogenesis both by direct (macrophage-to-endothelial cell adhesion) and by indirect mechanisms (paracrine effect) [104]. An extremely critical indicator of an effectively healing wound is an efficient controlled proliferative phase produced by an effective angiogenesis together with complete re-epithelization of the wound. The proliferative phase is characterized by granulation tissue formation, which comprises of different cell populations such as fibroblasts, as well as immune cells, together with the formation of new capillaries, which allow epithelial cell migration towards the wound surface in the process of re-epithelization. Unfortunately, in diabetic wounds, monocyte polarization towards M2 macrophages is strongly reduced, while inflammatory phenotype M1 polarization is elevated, and this causes a poor angiogenesis. Moreover, vasoreparative dysfunction has been observed in diabetic CD34+ stem cells due to impaired autocrine/paracrine function and reduced sensitivity to hypoxia, while the injection of freshly isolated circulating CD14+ monocytes into the ischemic limbs of diabetic mice improves healing and vascular growth, suggesting an important angiogenesis potency of monocytes population even in diabetic patients [105–107].

Lymphocytes in diabetic wound: A recent study underlines the key role of lymphocytes in both diabetic and non-diabetic non-healing wounds [23]. A lack of lymphocytes compromises wound healing in diabetic as well as in non-diabetic mice. Moreover, the pattern of diabetes plus a lack of lymphocytes further worsens the wound, indicating that when the innate regulatory function is missing, unbalanced M1 polarization, inadequate angiogenesis, and reduced wound healing are exacerbated [23]. Recently, it has been observed that the ischemic tissues of type-2 diabetic patients and mice have significantly more CD8+ T-cells than that of their respective normoglycemic counterparts [87]. The systemic inflammation observed in diabetic patients could limit the migration of Tregs and increase the infiltration of Th17 inflammatory population cells able to promote promote neutrophilic infiltration in the diabetic wound, explaining the prolonged inflammatory phase. Notably, the healing process of diabetic wounds may be accelerated by topical retinoic acid, in this manner

inducing T cell plasticity and the differentiation of Th17 cells towards Tregs, confirmimg the crucial role of T cells in the regulation of the inflammatory phase of diabetic wound healing [94]. It has also been observed in diabetic patients that the vascular density is negatively associated with CD4+T cells numbers after ischemic injury, while Tregs injection in the ischemic muscle increased vascular density and induced de novo sprouting angiogenesis through a paracrine effect [79]. A decrease in Natural Killer lymphocytes and high IFN-γ levels are correlated to diabetic foot complications and seem to have potential roles in predicting the infection of diabetic foot ulcers [108].

Keratinocytes and fibroblasts in diabetic wound: Chemokine Ccl2 secretion by epidermal keratinocytes is directly coordinated by Nrf2, a leading transcriptional regulator of tissue regeneration, that is activated early after cutaneous damage [39]. In diabetic wounds, Nrf2 fails to activate keratinocytes [39]. Keratinocyte-derived Ccl2 promotes macrophage EGF production, which induces keratinocyte proliferation to promote wound repair [39]. A significantly reduced skin resident cell proliferation as well as stem and progenitor cell activation was observed in diabetic foot ulcers, and it seems to be related to multiple factors such as glycation of proteins, reduced angiogenic capability, and oxidative stress, contributing to an extension of the proliferative phase [20]. Fibroblasts in impaired wounds showed ECM deposition significantly reduced abilities. DFU-derived fibroblasts were noted to produce ECMs twofold thinner than normal fibroblast, also showing a superior composition of collagen type I and fibronectin content [109].

7. The Immune-Centric Revolution: The Long and Winding Road from Stem Cells to Immune Cells Populations in Regenerative Medicine

Although stem cells have been considered promising for the treatment of degenerative diseases by 'seeding' them into damaged tissues, it has recently been observed that the regenerative capacity of stem cells is influenced and regulated by the local immune response and in particular by macrophages, which constitute a central component of the damage response and are the coordinators of tissue repair and regeneration [24]. Among the panoply of immune cells involved in the response to both acute and chronic wounds, recent discoveries have highlighted novel and often unexpected roles for certain types of immune cells in promoting a permissive local environment for effective cell replacement and restoration of tissue integrity. Some studies have shown that the control of inflammation is crucial in regenerative therapies: To be effective, regenerative therapies must block and control inflammation to allow tissue regeneration by resident stem cells [110]. Indeed, the presence of inflammation inhibits the regenerative action of tissue-resident mesenchymal stem cells (MSCs) [110]. Recent papers suggest that an innovative regenerative strategy could be to polarize macrophage from the M1 inflammatory state to the M2 anti-inflammatory state utilizing immune cells [25,32,111,112]. These reviews conclude that next-generation regenerative therapies need an immune-centric approach instead of the use of stem cells. Thus, depending on the tissue or organ targeted, regenerative strategies could be developed to stimulate macrophage polarization or to recruit subpopulations of pro-healing macrophages. Already, Mordechai in 2013 [101] and Pinto in 2014 [102] (7) have shown that the regeneration of myocardial tissue after ischemia was induced by macrophages that regulate resident stem cells and promote regeneration, suggesting that targeting macrophages could be a new strategy to improve infarct healing and repair. The regenerative and stem-cell-controlling capacity of macrophages has also recently been demonstrated in bone tissue by Gibon et al. [103], Gullard et al. [104], and Ekstrom [105]. Najar et al. [106] in 2018 clarified that mesenchymal stem cells act through a paracrine and immune-modulatory and non-differentiative mechanism and that the microenvironment and immune system regulate the activity of MSCs regardless of the tissue from which they originate. Based on the role played by several types of macrophages and lymphocytes in the wound-healing response, it is tempting to hypothesize that interventions that reduce the M1 macrophage phenotype and promote M2 may represent a new therapy to heal chronic wounds.

7.1. How to Switch to M2 Regenerative Phenotype?

Macrophages play an important role in wound healing, and the switch to anti-inflammatory M2 phenotypes is necessary for efficient healing. Questions remain regarding monocytes recruitment and macrophage differentiation, specifically whether monocytes are predetermined to differentiate in one specific phenotype, M1 or M2, or if macrophages polarize from M1 to M2 phenotypes (or vice versa) within the wound. Various approaches have been taken to immune-modulate macrophages to polarize in M2 phenotypes and/or simultaneously M1 macrophages (Figure 3). A list of methods to switch to M2, such as immune cell-based therapy, MSC, M2 exosome, and dermal substitute will be discussed.

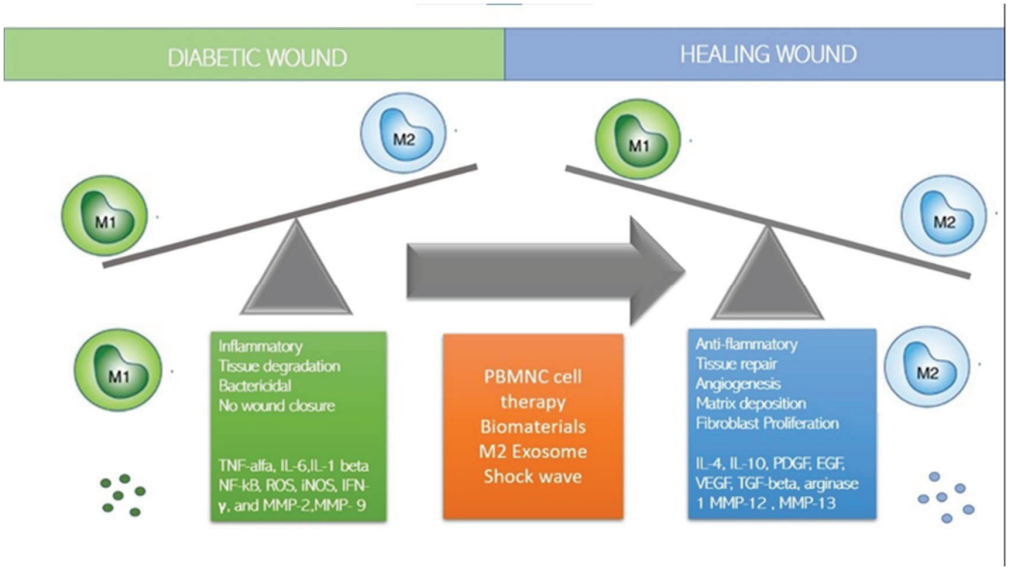

Figure 3. How to switch to M2: strategies.

7.2. Immune-Cell-Based Cell Therapy

Cell-based therapies are rapidly emerging in regenerative medicine as dynamic treatments that perform multiple therapeutic functions. Monocytes and macrophages, as innate immune cells involved in inflammation control and tissue repair, are increasing popular clinical candidates due to their angiogenic, anti-inflammatory, and regenerative ability. Table 1 shows a brief description and clinical result of clinical trials based on macrophages or peripheral blood mononuclear cells describe in this review. The treatment of chronic ulcers with blood-derived macrophages activated by hypo-osmotic shock has been used effectively in over 1000 patients in Israel [107]. Previously, Danon et al. in 1997 treated pressure ulcers in elderly patients by injecting macrophages from blood units of young, healthy donors near the wound periphery plus a portion of the cell suspension deposited on top of the wound [113]. Patients were treated with a single implant, or with a second one when delayed healing was present after 1 month later, and wound healing was compared with conventional methods (debridement, antibiotics, and wound dressings). In the macrophage-treated group, 27% healed, while only 6% healed in the control group ($p < 0.001$). Moreover, the macrophage-treated group showed a faster healing ($p < 0.02$), and no side effects were reported [113]. A second prospective controlled trial was designed to compare macrophage injections from healthy donors (66 patients) to standard care treatments (38 patients) for stage III and IV pressure ulcers in elderly patients. The results showed a significant higher percentage of completely closed wounds in the macrophages-treated group in comparison to standard care [114]. Interestingly, in the subset of diabetic patients 65.5% of wounds with

the macrophage treatment healed, while only 15.4% of healing was observed in the standard care group [114]. Magenta et al. recently published an extensive review on autologous cell therapy from different tissue sources (blood, bone marrow, and adipose tissue) to treat critical limb ischemia in diabetic patients, reporting data from basic science to clinical trials [115]. Autologous cell therapy, in particular, autologous Peripheral Blood Mononuclear Cells (PBMNC), based on monocytes/macrophages and lymphocytes represent an interesting strategy to treat non-option critical limb patients and diabetic foot patients [116–121]. Rigato et al. on a recent meta-analysis on no-option critical limb ischemia (NO CLI) patients showed that PBMNCs, but not other cell types, were associated with a significant decrease in amputation and increase in amputation-free survival [122]. The same results were observed by Liew et al. in a meta-analysis of 16 randomized trials where PBMNC lowered the risk of major amputation and increased ulcer healing significantly [123]. Three other meta-analyses on autologous cellular therapy including PBMNC on diabetic foot patients showed a benefit of wound healing and reduced amputation associated with $TcPO_2$ increase and reduced pain [124–126]. Dubsky et al. have treated 28 patients with diabetic foot disease (17 treated with bone marrow cells and 11 with PBMNC) comparing the result with a control group treated with standard care at 6 months and have reported a statistical increase in TcPO2 with no significant differences between bone marrow cells and peripheral blood cell groups, while no change in transcutaneous oxygen pressure in the control group was observed [119]. In addition, the 6 month major amputation rate was significantly lower in the cell therapy group compared with that in the control group (11.1% vs. 50%), with no difference between bone marrow cells and peripheral blood cells [119]. Interestingly, the same group reported a comparable improvement of CLI major amputation with autologous cell therapy in diabetic foot patients compared with repeated PTA and a more effective healing of foot ulcers in the cell therapy group [127]. A user-friendly point of care device based on peripheral blood selective filtration to be used for intra-operatory use in human cell therapy has been developed to produce fresh autologous PBMNC, with evidence in terms of adequate potency in therapeutic angiogenesis in vitro and in vivo [128]. Promising results have been obtained from implanting PBMNC produced by a specific device (Hematrate Blood Filtration system Cook Regentec) in different clinical trials including diabetic patients [120,121,129,130]. Persiani et al. have observed a 9.4% decrease in major amputation in 18 no-option patients with diabetes treated by PBMNC together with an increase in TCPo2 and a pain reduction at 2 years [120]. A similar result in terms of major amputation has also been previously reported on CLI non-option patients, including diabetic and Burgers patients, treated with PBMNC produced by apheresis [116]. Interestingly, it has been demonstrated by a histological examination of incisional biopsies of diabetic non-healing ulcers that autologous PBMNC implants produced by this selective filtration point of care and injected perilesionally around diabetic non-healing wounds polarize M1 macrophages in M2. Moreover, the implantation of A-PBMNC promotes relevant changes in the overall molecular setting over time [71]. The consequent cellular and biochemical adaptations favor the establishment of conditions similar to physiological ones that progressively support the regeneration of damaged tissues and finally wound healing measured as inhibition of HIF, NF-KB, and TNF-alpha, progressive polarization of M1 into M2, increase in VEGF, and newly formed capillaries [71]. As the regenerative processes occur, an increase in the vascular network formation is clearly seen [71]. These preliminary data confirm in the ability of fresh, naïve, autologous PBMNC to induce immunomodulation through macrophage polarization and that this results in complete wound healing in a diabetic ulcer. On the contrary, the delivery of macrophages polarized in vitro into M2a and M2c phenotypes and then injected into mouse wounds did not accelerate healing in wild type mice and delayed healing in diabetic mice [131]. The same study also observed a delayed re-epithelialization and persistence of neutrophils and M2 macrophages in diabetic treated wounds 15 days post-injury, suggesting that the application of ex vivo generated M2 macrophages is not beneficial and contraindicated for cell therapy of skin wounds. It seems instead that to produce a positive clinical outcome in terms of wound healing, polarization should occur

in the patients in the wounded tissue which send the right microenvironmental signals to PBMNC. The same groups showed that the implants of Matrigel supplemented with M2a and M2c macrophage subsets in a mice wound model showed an increased number of endothelial cells and tubular structures, while M1-enriched Matrigel did not, suggesting that macrophages polarized towards an M2 phenotype seem to have a higher angiogenic potential compared to other subsets [132]. Accordingly, Di Pardo et al. also observed an increase in VEGF and laminin in the diabetic wound after PBMC implant [71]. A similar results was observed for the first time by De Angelis et al. in no-option CLI patients, including a subset of diabetic patients, after PBMNC implant [121]: histological data confirmed dermal granulation tissue and an increased number of monocytes (CD68+) and newly formed micro vessels (CD31+). After the PBMNC treatment in the healed epidermis, the presence of the new vessels was observed, whereas dermal inflammation and monocyte infiltration were reduced. All these data suggest that autologous PBMNC represent a safe and effective therapy for diabetic foot non-healing wounds. Considering the low invasiveness and the repeatability, PBMNC could represent the new frontier that will replace stem cell therapy.

Table 1. Immune-Cell-based Cell Therapy—Clinical trials on diabetic patients.

	Description	Result	Ref.
Zuloff-Shani et al. 2004	Treatment of chronic ulcers with blood-derived macrophages activated by hypo-osmotic shock in over 1000 patients	Reduction of the healing time, reduction of risk of complications and morbidity. Improvement of the quality of life for long-suffering patients	[107]
Danon et al.	Decubital ulcers of 72 patients (average age 82), were treated by local injection of macrophages prepared from a blood unit in a closed sterile system. The remaining 127 patients (average age 79) were treated conventionally and served as controls. No exclusion criteria were applied.	In the macrophage-treated group, 27% healed, while only 6% healed in the control group ($p < 0.001$). Moreover, the macrophage-treated group showed a faster healing ($p < 0.02$)	[113]
Zuloff-Shani et al. 2010	100 consecutive elderly patients with a total of 216 stage III or IV pressure ulcers, 66 patients were assigned to the autologous macrophages group, 38 patients were assigned to the standard care treatments (38 patients.)	Percentage of completely closed wounds (wound level and patient level) were significantly better ($p < 0.001/p < 0.001$, respectively) in all patients in favor of AMS, as well as in the subset of diabetic patients ($p < 0.001/p < 0.001$).	[114]
Moriya, J et al.	Retrospective study on 42 patients with severe intermittent claudication, ischemic rest pain, or non-healing ischemic ulcers caused by peripheral arterial disease, including thromboangiitis obliterans, and who had not responded to conventional therapy that included nonsurgical and surgical revascularization (no option).	Improvement of ischemic symptoms was observed in 60% to 70% of the patients. The annual rate of major amputation was decreased significantly by treatment. The survival rate of younger responders was better than that of non-responders.	[116]
Huang, P.P et al.	150 patients with peripheral arterial disease were randomised to mobilized PBMNC 76 cases or BMMNC 74 cases implanted, follow up for 12 weeks. Primary outcomes were safety and efficacy of treatment, based on ankle-brachial index (ABI) and rest pain	Significant improvement of the ABI, skin temperature and rest pain was observed in both groups after transplantation and was better I PBMNC group. However, there was no significant difference between two groups for pain-free walking distance, transcutaneous oxygen pressure, ulcers, and rate of lower limb amputation	[117]
Liotta, F et al.	Autologous Non-Mobilized Enriched Circulating Endothelial Progenitors obtained from non-mobilized peripheral blood by immunomagnetic selection of CD14+ and CD34+ cells) or BM-MNC were injected into the gastrocnemius of the affected limb in 23 and 17 patients with no option critical limb ischemia.	After 2 yrs follow-up, both groups showed significant and progressive improvement in muscle perfusion (primary endpoint), rest pain, consumption of analgesics, pain-free walking distance, wound healing, quality of life, ankle-brachial index, toe-brachial index, and transcutaneous PO2	[118]

Table 1. *Cont.*

	Description	Result	Ref.
Dubsky, M et al.	28 patients with diabetic foot disease (17 treated by bone marrow cells and 11 by peripheral blood mononuclear cell) were included into an active group and 22 patients into a control group without cell treatment.	The transcutaneous oxygen pressure increased significantly ($p < 0.05$) compared with baseline in both active groups after 6 months, with no significant differences between bone marrow cells and peripheral blood cell groups, while no change in the control group was observed. The rate of major amputation by 6 months was significantly lower in the active cell therapy group compared with that in the control group (11.1% vs. 50%, $p = 0.0032$), with no difference between bone marrow cells and peripheral blood cells.	[119]
Persiani, F. et al.	50 diabetic patients affected by CLI underwent PBMNCs implant (32 patients underwent PBMNCs therapy associated with endovascular revascularization, 18 patients, non-option CLI)	The follow-up period was 10 months. In the PBMNC group + revascularization TcPO, pain VAS Scale improved. In PBMNCs therapy group, the mean TcPO2 improved from 16.2 ± 7.2 mmHg to 23.5 ± 8.4 mmHg ($p < 0.001$), and VAS score means decreased from 9 ± 1.1 to 4.1 ± 3.3 ($p < 0.001$). Major amputation was observed in 3 cases (9.4%), both in adjuvant therapy group and in PBMNCs therapy. (16.7%) (P $\frac{1}{4}$ 0.6) as the therapeutic choice (PBMNCs therapy group).	[120]
De Angelis, B et al.	Prospective, not randomized study based on a treated group who did not respond to conventional therapy (n = 43) when implanted with A-PBMNC cells versus a historically matched control group. Patients of both groups were suffering from CLI Fontaine scale IV with chronic ulcers	The A-PBMNC-treated group showed a statistically significant improvement of limb rescue of 95.3% versus 52.2% of the control group ($p < 0.001$) at 2 years. The A-PBMNC group also showed reduction in pain at rest, increased maximum walking distance, and healing of the wound and an overall improvement in the quality of life. Post-treatment radiological studies showed an improvement of vascularization with the formation of new collateral and by histological findings.	[121]
Dubsky et al.	31 patients with DFU and CLI treated by autologous stem cells and 30 patients treated by PTA were included in the study; 23 patients with the same inclusion criteria who could not undergo PTA or cell therapy formed the control group.	Amputation-free survival after 6 and 12 months was significantly greater in the cell therapy and PTA groups compared with controls ($p < 0.001$ and $p < 0.0029$, respectively) without significant differences between the active treatment groups. Increase in TcPO2 did not differ between cell therapy and PTA groups until 12 months but TcPO2 in the control group did not change over the follow-up period. More healed ulcers were observed up to 12 months in the cell therapy group compared with the PTA and control groups (84% vs. 57.7% vs. 44.4%; $p < 0.042$).	[127]
Scatena et al.	The study included 76 NO-CLI patients with DFUs. All patients were treated with the same standard care (control group), but 38 patients were also treated with autologous PBMNC implants.	Only 4 out 38 amputations (10.5%) were observed in the PBMNC group, while 15 out of 38 amputations (39.5%) were recorded in the control group ($p = 0.0037$). The Kaplan–Meier curves and the log-rank test results showed a significantly lower amputation rate in the PBMNCs group vs. the control group ($p = 0.000$). At two years follow-up, nearly 80% of the PBMNCs group was still alive vs. only 20% of the control group ($p = 0.000$). In the PBMNC group, 33 patients healed (86.6%) while only one patient healed in the control group ($p = 0.000$).	[129]
Di Vieste et al.	Case report of a 59-year-old patient with type 2 diabetes mellitus who had a gangrene of the right toe. After an ineffective angioplasty, it was decided to use a PBMNC therapy.	The patient underwent to amputation of the first necrotic toe and three PBMNC treatment sessions with complete surgical wound healing and limb rescue	[130]

7.3. Mesenchymal Stem Cells (MSC)

Accumulating evidence suggests that mesenchymal stem cells (MSC) promote tissue repair through the immune-modulation response and the secretion of growth factors rather than by the substitution of damaged cells [133]. MSCs release a wide range of factors, including PGE2 and interleukin-6 (IL-6), that polarize to a M2 pro-resolving profile [134]. The immuno-regulatory capacity of MSC depends on a process of "licensing" that implies the activation of MSC by the inflammatory environment. The requirement of MSC activation to induce immunoregulation is supported by data showing that the suppression of lymphocytes T proliferation induced by MSC in co-cultures is achieved only after the supplement of adequate amounts of IFN-γ and TNF-α [135]. By producing a large number of immunomodulatory molecules such as TGF-β, hepatic growth factor (HGF), nitric oxide (NO), indolamine 2,3-dioxygenase (IDO), L-10, IL-6, IL-1 receptor antagonist (IL-1Ra), hemeoxygenase-1 (HO-1), prostaglandin E2 (PGE2), and pro-angiogenic factors VEGF, angiopoietin-1, placental growth factor (PGF), HGF, basic fibroblast growth factor (bFGF), TGF-β, PDGF, and IL-6, MSCs regulate immune response and vasculogenesis, crucially contributing to the enhanced repair of injured tissues in various organ [136]. The transplantation of autologous MSCs effectively repaired corneal wounds, and macrophage depletion completely abrogated MSC-based beneficial effects, confirming that the cooperation between MSCs and macrophages was required for successful vascular regeneration [136]. MSC-injected survivals is dependent on the phenotype and function of tissue-resident macrophages [136,137]. As observed in myocardial infarction and spinal cord in murine models of injury, anti-inflammatory M2 macrophages offer a favorable environment for the engraftment of MSCs [137]. Moreover, the polarization of M1 macrophages to M2 phenotype is critical for the long-term survival of MSCs in healing tissues, suggesting that a reciprocally positive feed-back loop exists between M2 macrophages and MSCs [137]. In a similar fashion, Tregs enhance the survival and engraftment of MSCs in ischemic tissues, and Tregs may even improve the angiogenic properties of MSCs by improving VEGF production [138]. It is important to consider the special characteristics of chronic wound environments, such as low oxygen tension, and how they may influence cell functions. It has been observed that a hypoxic environment diminished macrophage plasticity in response to MSCs [139]. Moreover, in vitro studies showed that macrophages cultured in normoxic conditions with MSCs produced high levels of IL-10, however, while in hypoxic conditions (1% O_2), the release of the inflammatory cytokine was strongly reduced [139]. In vitro assays showed that MSC from diabetic patients' adipose tissue demonstrated reduced proliferative capacity and decreased VEGF paracrine release, with lower expression of the stemness gene SOX2 [140]. In keeping, the MSC from Stromal Vascular Faction (SVF) of diabetic patients did not rescue limb ischemiam and this reduced its effect and has been corelated to a significant depletion of CD271+ cells compared to non-diabetic patients [140]. Accordingly, Cianfarani et al. also showed that MSCs from diabetic mice released lower amounts of hepatocyte growth factor and insulin-like growth factor-1 and that the supernatant of diabetic ASCs manifested in a reduced capability to promote keratinocyte and fibroblast proliferation and migration, probably due to a reduce ability for macrophage polarization in M2 [141]. Moreover, the density of adipose-derived cells (ASC) was lower in the adipose tissue of diabetic rats compared with non-diabetic rats and did not promote wound healing in diabetic rats, suggesting that caution is necessary regarding the clinical use of diabetic adipose tissue for the treatment of diabetic wounds [111]. ASC from diabetic patients also exhibited a reduction in VEGF secretion and an impaired angiogenic capacity [112].

Overall, these data suggest that the therapeutic cell therapy potential from the adipose tissue of diabetic patients (SVF, MSC, Adipose derived stem cells ADSC) is dampened when compared with cells isolated from nondiabetic patients because diabetes alters MSCs' intrinsic properties and impairs their function [142].

In addition, the bone marrow of diabetic patients showed a deep remodeling, consisting of a strong reduction in micro-vessels and sensory neurons, as well as fat accumulation,

which creates an unfavorable microenvironment for resident stem cells, which in turn compromises the regenerative efficacy of bone marrow cells which could become harmful vectors of inflammation and anti-angiogenic molecules in diabetic patients [143,144]. This is an important issue that emerging autologous therapies should keep in consideration regarding diabetic non-healing wounds.

7.4. Extracellular Vesicles (EVs) and Exosome (Exo)

EV is a generic term for membrane-contained particles naturally released by cells, not containing a nucleus. EVs are traditionally divided into subtypes based on the vesicle sizes: exosomes (50–150 nm diameter), microvesicles (100–1000 nm diameter), and apoptotic bodies (50–4000 nm diameter). Exosomes are formed after the fusion of endosomes membrane with the plasma membrane, while both microvesicles and apoptotic bodies are generated by direct outward blooming from the cell surface [145]. MSC-derived extracellular vesicles (MSC-EVs) can transfer functional proteins and nucleic acids, including microRNAs (miRNAs) and messenger RNAs (mRNAs) to other cells without cell-to-cell contact. Recent studies have demonstrated that MSC-EVs reduce M1 polarization and/or promote M2 polarization in a variety of settings such as cardiovascular, pulmonary, digestive, renal, and central nervous system diseases [145]. An in vitro study revealed that MSCs derived from adipose tissue through exosome release induce M2 polarization [146]. He et al. recently showed that the early depletion of macrophages also delayed wound repair after MSC injection, confirming that MSC-mediated wound healing requires macrophages [147]. In the same paper, the authors demonstrated that MSCs from bone marrow infused systemically could translocate to reach the wound site, promote M2 polarization, and enhance wound healing [147]. The authors also observed that exosomes derived from MSCs induced macrophage polarization while the depletion of the exosomes of MSCs reduced the M2 phenotype [147]. Infusing MSCs without exosomes produced a smaller number of M2 in the wound site and delayed repair [147]. The paper also showed that miR-223, derived from the exosomes of MSCs, regulated macrophage polarization by targeting transcription factor p-knox 1 [147]. These important findings provided evidence for the first time that MSC provokes M2 polarization and could accelerate wound healing by releasing exosome-derived microRNA. Li et al. confirmed that macrophage-derived exosomes exercised anti-inflammatory effects through the inhibition of the secretion of inflammatory enzymes and cytokines and provided the healing of diabetic wound by significantly quickening angiogenesis and improving repair [148]. Another study confirmed that M2-derived exosomes (M2-Exo) induce a complete switch of M1 to M2 [52]. The subcutaneous injections of M2-Exo into the wound edge decreased the local populations of M1 and increased the M2 population and accelerated wound healing by improving angiogenesis, re-epithelialization, and collagen deposition. Accordingly, in a diabetic rat model, it has been observed that exosomes which are overexpressing transcription factor Nrf2 hasten wound healing by inducing vascularization [149]. Exosomes derived from macrophages may represent a novel therapeutic strategy in the treatment of diabetic wound damage.

7.5. Dermal Substitutes

Fully acellular dermal substitutes are used in DFU treatment because of the high safety profile and beneficial outcomes as reported in literature. Ideal scaffolds and tissue substitutes including skin matrices should be non-immunogenic, regenerative, protective, durable, and biocompatible. On the basis of the innovative macrophage-centered approach, they also should have a good capacity to induce M2 polarizations [150]. Their therapeutic outcome originates from and is dependent on their source, method of preparation, and further modification. The decellularization method and tissue source can deeply affect the wound microenvironment when the substitute is implanted. Cross-linking or the possible addition of other substances can affect the wound environment and the clinical outcome as well [151]. The chemotactic attractiveness of human fibroblasts to collagens I, II, and III has been studied for many years and is well recognized. Monocytes' adhesion to collagen

types I and III showed a noticeable effect on the secretion of different mediators, including growth factors, cytokines, and enzymes, which in turn play a key role in normative wound healing [152]. Predictably, the diverse surface morphologies and integrated active components can induce an effect on the macrophage's phenotype. Consequently, it is extremely important to study the immunomodulatory effects of dermal substitute, especially when implanted on chronic and/or diabetic wound. It has been observed that particular geometrical parameters could direct human macrophage polarization [153]. Fibrous collagen scaffolds with box-shaped pores and precise inter-fiber spacing from 100 μm down to only 40 μm facilitate primary human macrophage elongation accompanied by differentiation towards the M2 type [153]. Table 2 show commercially available dermal substitutes evaluated for their immunomodulatory and M2 polarization ability.

Yin et al. found that the pore size of a scaffold influences the phenotypes of resident macrophages, showing that a relatively larger size (~360 μm) leads to enhanced formation of blood vessels, with higher levels of VEGF+ cells and a lower level of M1 macrophages [154]. In addition to pore size, collagen-functionalizing additives may also have an effect on macrophage activation, such as Chondroitin sulfate (CS), which has been reported to play vital roles in the immune response. CS at an increasing dose range of 100–1000 μg/mL was found to significantly increase the phagocytic activity and ROS production as well as the secretion levels of NO, TNF-α, IL-6, and IL-10 by monocyte/macrophage lineage (RAW264.7) [155]. Witherel et al. studied the responses of monocyte-derived macrophages isolated from blood to four different commercially available biomaterials in vitro: OASIS® Wound Matrix, which is an extracellular matrix from porcine small intestinal mucosa; INTEGRA® Bilayer Matrix, a dermal bilayer of cross-linked bovine tendon type I collagen and chondroitin-6-sulfate plus a layer of polysiloxane; AlloMend® Acellular Dermal Matrix, a decellularized matrix composed mainly of collagen and elastin; and PriMatrix® Dermal Repair Scaffold, decellularized fetal bovine dermis rich in type I and II collagen [151]. The OASIS® and INTEGRA™ matrices downregulated the expression of M2a anti-inflammatory markers CCL22 and TIMP3, suggesting a probable inhibition of extracellular matrix secretion and fibrosis, which are crucial events for wound closure. OASIS® was also the biomaterial responsible for the greatest increase in M1 genes expression. The authors suggest that INTEGRA® inflammatory response could be related to glutaraldehyde cross-link and suggest that both OASIS® and INTEGRA™ seem to be a poor option for chronic wounds [151]. PriMatrix® as well showed a downregulation of the anti-inflammatory genes CCL22 and TIMP3 and an overexpression of both the pro- inflammatory cytokine TNF-α and CD163, associated with M2c. AlloMend® only induced an effect of the upregulation of CD163, and it was considered the biomaterial with the lowest influence on macrophage response. Agrawal et al. compare DermaMatrix®, AlloDerm®, Integra®, and DermACELL® M1/M2 polarization in an animal model [156]. Macrophage surface markers CD68 (all macrophages), CCR7 (M1 phenotype), and CD206 (M2 phenotype) were used to characterize an M1–M2 profile by an immuno-histological assay. All dermal substitutes showed a bell-shaped curve for the distribution of CD68+ macrophages, except Integra®, which showed an increasing trend of macrophages with time [156]. Moreover, DermACELL® had the highest entry of macrophages, while Integra® had the smallest [156]. AlloDerm® showed that the macrophages were mostly M1 at 7, 14, 21, and 42 days post implantation, while Integra® showed a mixed M1/M2 population of macrophages at all time-points: The trend for the M1:M2 ratio was skewed towards M2 on day 7, towards M1 on days 14 to 21, and again towards M2 on day 42 for Integra® [156]. A recent study showed that the implant of a porcine urinary bladder matrix (UBM) is associated with the modulation of wound inflammation in diabetic patients, measured as mRNA associated with M1 and M2 macrophages [157]. Recently, Montanaro et al. show investigate how the dermal substitute Nevelia®, which is a dermal substitute consisting of a three-dimensional porous matrix of type 1; purified, stabilized, bovine-origin collagen; and a layer of reinforced silicone may influence the inflammatory infiltrate and macrophages polarization [158]. The study randomly enrolled 15 diabetic patients with chronic foot ulcers, 5 treated only by standard of

care as control group, and 10 treated with Nevelia®. Biopsy was performed at baseline and after 30 days and histological, immunohistochemical, and immunofluorescence analysis was performed to evaluate the number of M1 and M2 macrophages. Dermal substitute group showed a general macrophage activation and a greater and significative polarization toward M2 subpopulation at 30 days, compared with control. The increase in M2 phenotypes population was also confirmed by confocal microscopy. Moreover, after 6 months, 6 patients (60%) of the Nevelia® completely healed, while only 1 patient (20%) healed in the control group, suggesting that this dermal substitute induce tissue reparative processes through macrophage activation and M2 reparative polarization in diabetic lesions [158]. The positive clinical outcome of this dermal substitute was previously observed by the sane authors in 41 patients with chronic diabetic wound [159]. In addition, Nevelia® dermal substitute was observed to polarize in M2 and also in an in vitro model [160].

Table 2. Dermal Substitutes tested for immunomodulatory and macrophage polarization ability.

	Primary Material Composition	Source and Other Components	Refs.
Nevelia	Porous resorbable double layer matrix 2 mm thickness made of stabilized native collagen type I and a silicone sheet 200 mm thickness mechanically reinforced with a polyester fabric. The extraction procedure and the freeze-drying process allow the structuring of the collagen into a matrix with optimal hydrophilicity, pore structure and pore size (20–125 μm)	Bovine, Native collagen Type I. No glycosaminoglycan (GAG) added to improve cell attachment and proliferation. Glutaraldehyde Cross-linking	[157–159]
Integra	Bilayer system for skin replacement made of a porous matrix of fibers of cross-linked bovine tendon collagen and glycosaminoglycan (chondroitin-6-sulfate) that is manufactured with a controlled porosity and defined degradation rate. The Integra pore size of 20 to 125 μm allows influx of cells.	Bovine Tendon Type I Collage Shark cartilage -derived chondroitin-6-sulphate (GAG). Glutaraldehyde Cross-linking	[149,150,155]
PriMatrix	Acellular dermal tissue matrix. comprising of both type I and type III collagen derived from fetal bovine dermis. This matrix is processed in a way to maintains the extracellular matrix in its native and undamaged state while removing all lipids, fats, cells, carbohydrates and non-collagenous proteins.	Fetal Bovine collagen type I and type III collagen. No cross-link	[150]
Oasis Wound Matrix	Lyophilized, decellularized porcine small intestine submucosa (SIS). Matrix is derived from a single layer of porcine small intestinal submucosa (SIS) technology. The technology provides an intact three-dimensional extracellular matrix which allows for host cell migration. The SIS is freeze-dried and sterilized with ethylne oxide gas in preparation for clinical use	Porcine small intestine submucosa (SIS). No cross-link	[150]

Table 2. *Cont.*

	Primary Material Composition	Source and Other Components	Refs.
Allomend	Decellularized donated human dermal tissue, with significant removal of cellular debris (including DNA and RNA), proteins and antigens. The process does not require the use of detergents or enzymes, thereby mitigating the possibility of harmful residuals in the tissue. The decellularization process also inactivates microorganisms through cellular disruption. USA only, not available in Europe	Human dermal tissue No cross link	[149,150,155]
DermaMatrix	Cadaveric human allograft treated with a disinfectant solution that combines detergents with acidic and antiseptic reagents. USA only, not available in Europe	Human dermal tissue No cross link	[149,150,155]
Dermacell	Decellularized regenerative human tissue matrix allograft processed using proprietary technology that removes at least 97% of donor DNA without compromising the desired biomechanical structure or biochemical properties. USA only, not available in Europe	Human dermal tissue No cross link	[149,150,154]

8. Conclusions

Both sustained increases in the number of wound macrophages together their phenotype dysregulation towards the inflammatory types, caused by intrinsic alterations in bone marrow and by a pro-inflammatory wound microenvironment, cause impaired wound healing in diabetes. Our understanding of the macrophages populations during impaired healing is still partial. Diabetic wounds with potentially devastating consequences on suffering patients remain a strong medical need. The understanding of the systemic and local immune responses is fundamental to develop innovative therapies. Moreover, it could be useful to verify how current therapy influence macrophage polarization to identify better treatment for chronic wounds in diabetic individuals. Each patient's immune system represents a dynamic history of infections, sex, age, diet, genetic characteristics, and environmental factors. Innovative therapy should be designed to manipulate the immune system to switch towards anti-inflammatory and regenerative phenotype that promotes the desired repair outcome.

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Review

Is There an Association between Sleep Disorders and Diabetic Foot? A Scoping Review

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Abstract: Diabetic foot is associated with a low quality of life since physical disabilities, mood disturbances and psychological disorders are frequent. One of the most important biological processes to ensure quality of life is sleep. Sleep disorders can impair glycemic control in patients with diabetes mellitus or even cause long-term type 2 diabetes mellitus. The aim of this study is to carry out a scoping review about the association between sleep cycle disorders and diabetic foot. PubMed, Scopus, CINAHL, PEDro, Cochrane Library, SCIELO and EMBASE databases were chosen for the search and the following terms were used: "diabetic foot","sleep*","rest-activity","mood" and"behavior". All the studies should include outcome variables about sleep and diabetic foot. Finally, 12 articles were selected, all of whichwere observational. The most frequent variables were those regarding diabetic foot ulcer aspects and diabetic neuropathy on one side, and obstructive sleep apnea, sleep duration and sleep quality on the other side. The results suggest that there is a possible association between obstructive sleep apnea and the presence or history of diabetic foot ulcers. No direct associations between sleep quality or sleep duration and diabetic foot or diabetic foot ulcer variables have been found.

Keywords: diabetic foot; diabetic foot ulcer; diabetic neuropathy; sleep; obstructive sleep apnea

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1. Introduction

In many cases, diabetes mellitus (DM) leads to several complications, of which diabetic foot (DF) is one of the most frequent [1]. It likely begins with the onset of diabetic neuropathy (DN) and peripheral arterial disease (PAD) [2].Unless this situation is prevented, it can result in diabetic foot ulcers (DFUs), which tend to become infected and show poor healing [3,4]. The risk of developing wounds is 25% higher in a patient with DM [5]. The most advanced stage of DFU often requires lower limb amputation, and this is an important source of diabetes mortality [6]. Along with the mortality and morbidity of DFUs, the economic consequences are high. In Europe, the cost of treating DFUs varies from approximately 4500 to 16,800EUR per patient [7].

DF is associated with a low quality of life, since physical disabilities and mood disturbances (among other situations) are frequent [8]. Psychological disorders, such as anxiety and depression, are also not uncommon [9]. Pain is another important factor affecting quality of life, caused by the existence of DFUs, DN and PAD symptoms [10,11], phantom limb syndrome [12] or the combination of all of these.

One of the most important biological processes to ensure quality of life is sleep, which can be altered due to sleep disorders, lifestyle, psychosocial and environmental factors, or medical conditions [13].

In fact, sleep disturbances are identified as a disruptive event that favors the appearance and chronification of pathologies. Furthermore, it has been identified that this disturbance is bidirectional, in the sense that the treatments carried out in patients with chronic pathologies have a lesser effect when they suffer from sleep disturbances [14]. Among the main effects that cause sleep disturbance are depression, fatigue, exhaustion, decreased quality of life and cardiac, systemic and metabolic alterations [14,15]. Specifically, in patients with DM, it has been observed that sleep disturbances cause alterations in glycemic control [16], a fundamental variable in the management of these patients, causing, in the long term, an increase in patients with type 2 DM [17].

Despite the fact that it has been shown that sleep disorders are directly and negatively related to the appearance, capacity for adaptation and response and possibilities of recovery from chronic diseases, to the best of our knowledge, there are no reviews published that study the association between sleep disorders and DF, either directly (due to pathophysiological reasons) or indirectly (due to psychogenic issues derived from the pathology).

The main objective of the present study is to carry out a scoping review of the literature about the association between sleep disorders and DF.

2. Materials and Methods

This review was carried out according to the guidelines and recommendations of the Preferred Reported Items for Systematic Reviews and Meta-Analysis (PRISMA) [18].

2.1. Search and Sources

PubMed (Medline), Scopus, CINAHL, PEDro, Cochrane Library, SCIELO and EMBASE databases were used. The following terms were used alongside "OR" or "AND": "diabetic foot","sleep*","rest-activity","mood","behavior".

The following database search strategy was used: ((((Sleep* [Title/Abstract]) OR Rest-Activity [Title/Abstract]) OR Mood [Title/Abstract]) OR Behavior [Title/Abstract]) AND (diabetic foot [Title/Abstract]).

2.2. Eligibility Criteria

The inclusion criteria were that studies should be observational, experimental or mixed. In these studies, the sample should consist of patients with DF. Age, sex and the type of diabetes of the sample were not considered. All documents published up to 30 July 2020 were included. The exclusion criteria were the absence of outcome variables about sleep or DF, and studies that were not conducted on humans. Documents not published in English, Spanish, German, French or Italian were also excluded.

2.3. Selection of Studies

Two independent researchers were involved in each stage of the study selection. Initially, a screening was carried out based on the title and abstract of the articles resulting from the search strategy, checking the contents of the full article if necessary. The articles were then evaluated for selection based on the previously mentioned eligibility criteria. Disagreement between the articles chosen by each reviewer was solved by the intervention of a third reviewer, who ultimately decided if a study was included or excluded.

2.4. Data Extraction and Synthesis of Results

In order to have a general approach to the studies, a Table 1 was designed to show their structural characteristics: authors, date of publication, type of study, sample size, type of diabetes, gender and age. In Table 2, the outcome variables regarding DF and sleep were presented. This table included information such as the characteristics of DFUs, DN, sleep quality, insomnia and breathing disorders, among others. These outcome variables

were extracted to analyze how often were they were studied and to determine which were the most relevant, thus facilitating the comparison of results between studies.

After the extraction and comparison of the variables related to DF and sleep, Table 3 was constructed to show the associations found in the selected studies.

3. Results

3.1. Selection of Studies

The flowdiagram (Figure 1) summarizes the study selection process, specifying the reasons for study exclusion. The main reason for exclusion was the absence of variables related to the sleep cycle or DF in the main objective of the study. There were 12 articles selected that were published since 2009 and included DF and sleep outcome variables.

All selected studies were observational, most of them cross-sectional (n = 6). The remaining studies were cohort studies (n = 2), case-control studies (n = 2), one case-report and one case series study. The sample size varied from n = 3 [19] to n = 1,656,739 [20], with the total sample size being n = 1,659,699. In 3studies [21–23], the type of diabetes in the sample was not specified. In 6studies [19,20,24–27], the sample subjects had type 2 diabetes mellitus (DM2), and in 3studies [28–30], they had both type 1diabetes mellitus (DM1)and DM2. In all of them, the population was adults(>18 years old) (Table 1).

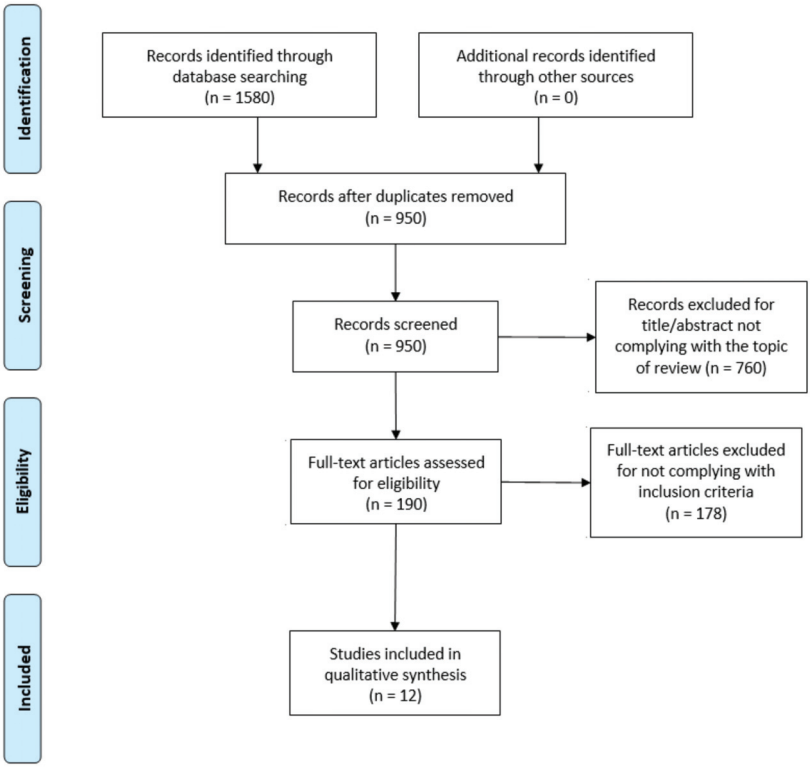

Figure 1. PRISMA flow diagram adapted with permission from The PRISMA group, 2020.

Table 1. Structural characteristics of the included studies.

Authors (Year)	Type of Study	Sample Size	Diabetes Type	Gender	Age (Mean ±SD)
Altaf et al. (2016) [24]	Observational CSS	n = 234	2	M= 48 F= 186	Range: 59,6–62,6
Andruskiene et al. (2013) [21]	Observational CSS	n = 1602	-	M= 600 F= 1002	Range: 25–64
Bener et al. (2016) [25]	Observational CSS	n = 459	2	M= 201 F= 258	48,2 ± 9,0 47,1 ± 8,3
Nair (2018) [22]	Observational CSe	n = 100	-	M= 66 F= 34	Range: 18–90
Haveleia and Gayatri (2019) [23]	Observational CSS	n = 97	-	M= 43 F= 54	54,84 ± 1,71
Maltese et al. (2018) [28]	Observational CoS Prospective	n = 94	1 (n = 28) 2 (n= 66)	M= 69% F= 31%	51,5 ± 16,2 62,7 ± 12,0
Puspita et al. (2019) [26]	Observational CSS	n = 152	2	M= 35.5% F= 64.5%	57 ± 8,61
Rutkove et al. (2009) [29]	Observational CoS Prospective	n = 82	1 2	M= - F= -	Range: 18–80
Salomé et al. (2013) [30]	Observational CSS	n = 60	1 (n = 27) 2 (n = 33)	M= 55% F= 45%	Range: 34–71
Sheahan et al. (2017) [27]	Observational C-CS	n = 77	2	M= 50 F= 27	61 ± 11
Subramanian et al. (2019) [20]	Observational C-CS Retrospective	n= 1,656,739	2	M= 902,868 F= 753,871	64.7 ± 13,3
Vas et al. (2016) [19]	Observational CR	Three cases	2	M= 3 F= 0	Range: 57, 61 - 63

Abbreviations: year (Year of publication); SD (Standard Deviation); CoS (Cohort study); C-CS (Case-control study); CSS (Cross-sectional study); CR (Case report); CSe (Case series); M (Male); F (Female).

3.2. Data Extraction and Synthesis of Results

The outcome variables analyzed in the studies were divided into DF-related and sleep-related. The most frequent variables in the first group were those regarding DFU aspects, followed by those related to DN. In the second group, the most frequent variables were those regarding obstructive sleep apnea (OSA), sleep duration and sleep quality (Table 2). All studies defined the instruments for measuring the outcome variables, except for the studies by Bener et al. [25] and Andruskiene et al. [21].

Table 3 shows a summary of the findings of the selected studies. All of them were observational, so there was no intervention to be considered and no quantitative analysis could be performed on the results. OSA showed an association with the development of DN and PAD, the severity of DFUs, the history of DFUs and healing capacity. Sleep quality was associated with the use of certain therapies for DFU healing and showed contrary associations with the presence of DFUs. Sleep duration was not associated with the presence of DFUs but was associated with the level of amputation.

Table 2. Outcome variables related to the sleep and diabetic foot.

Authors (Year)	DF Variables	DF Measurement Tools	SC Variables	SC Measurement Tools
Altaf et al. (2016) [24]	- Diabetic neuropathy - Presence of DFU - Small fiber neuropathy	- Michigan Neuropathy Screening Instrument - PARP activation - Intraepidermalnervefiber density	- Presence of OSA	- Overnight cardio-respiratory device - Apnea/Hypopnea Index
Andruskiene et al. (2013) [21]	- Diabetic foot pain	Unknown	- Problems of falling asleep - Night-time awakenings - Self-rated sleep quality - Sleep latency period - Sleepiness in daytime - Taking naps - Using of sleeping pills	- Basic Nordic Sleep Questionnaire (BNSQ)
Bener et al. (2016) [25]	- Diabetic neuropathy - Presence of DFU	- Observation	- Sleep duration - Sleep loss - Sleep disturbances	- Hours
Nair (2018) [22]	- Inflammatory symptoms - Vasodilation - Gait	- Leg swelling, foot stiffness - Skin discoloration, sensation, leg heaviness,	- Sleep quality	Unknown
Haveleia and Gayatri (2019) [23]	- Presence of DFU - Duration of DFU	Observation	- Sleep quality	- PSQI
Maltese et al. (2018) [28]	- DFU severity - DFU persistence - DFU recurrence	- SINBAD scale - Non-healing in 12-month period - Re-ulceration in a healed site	- Risk of OSA	- STOP-BANG Questionnaire
Puspita et al. (2019) [26]	- Duration of DFU - DFU assessment	- </> 6 months - Wagner scale	- Sleep quality	- PSQI
Rutkove et al. (2009) [29]	- Foot temperature - Nerve conduction studies - Quantitative sensory testing - Diabetic Neuropathy	- iButton - TSA-II NeuroSensory Analyzer - MNSI - UENS	Same as besides, but measurements were done while asleep vs. awake	- iButton - TSA-IINeuroSensory Analyzer - MNSI - UENS
Salomé et al. (2013) [30]	- Presence of DFU	- Observation	- Sleep quality	- PSQI
Sheahan et al. (2017) [27]	- Foot deformity - DFU surface area - DFU infection - DFU depth - Amputation level	- Small muscle wastage, bony prominence, prominent metatarsal heads, hammer/claw toes, limited joint mobility or Charcot deformity - Longest edge and widest edge - University of Texas scale - IWGDF classification	- Daytime sleeping - Lying down duration - Sleep duration	- Epworth Sleepiness Scale - Minutes - Minutes

Table 2. *Cont.*

Authors (Year)	DF Variables	DF Measurement Tools	SC Variables	SC Measurement Tools
Subramanian et al. (2019) [20]	- Diabetes-related foot disease	- Signs of amputation, gangrene, presence of DFU, Charcot foot, peripheral vascular disease and peripheral neuropathy	- Presence of OSA	- Previous medical diagnosis
Vas et al. (2016) [19]	- Presence of DFU - Osteomyelitis - DFU healing	- Observation - MRI - Observation	- Presence of OSA	- Previous medical diagnosis

Abbreviations: DF: diabetic foot; SC: sleep; PARP: poly ADP ribosepolymerase; OSA: obstructive sleep apnea; DFU: diabetic foot ulcer; PSQI: Pittsburgh sleep quality index; MNSI: Michigan neuropathy screening index; UENS: Utah early neuropathy scale; MRI: magnetic resonance imaging.

Table 3. Findings of selected studies that relate variables of the diabetic foot and variables of the sleep.

Sleep Variable	Diabetic Foot Variable	Findings
OSA	IENFD	- Negative correlation ($p < 0.001$) between IENFD and OSA that implies small fiber neuropathy [24]
	MNSI	- Mild OSA was associated with past history of DFU($p = 0.016$) [24]
	History of DFU	- Positive correlation with OSA presence ($p = 0.022$) [24]
	PARP	- Positive correlation ($p = 0.025$)between PARP and OSA that involves endothelial dysfunction [24]
	DF presence	- DF was significantly predictive of OSA [20]
	DFU healing	- CPAP therapy for OSA led to DFU healing in patients under treatment [19] - High risk of OSA led to poor DFU healing [28]
Sleep quality	DFU healing	- Microcurrent therapy for DFU led to a significantly better sleep quality [22]
	DFU presence	- Subjective sleep quality showed significant disparity with comprehensive sleep quality (PQSI) [23] - Poor sleep quality was significantly related to pain level ($p = 0.013$) [26] - No significant difference in sleep quality of people with diabetes with and without DFU [26] - Pain (p: 0.048) and stress (p: 0.001) were significantly related to poor sleep quality [23] - Patients with DFU had poor sleep quality (Salomé et al., 2013)
Sleep duration	Minor amputation presence	- Patients with minor amputation had lower Epworth Sleepiness Scale score (lower score = normal) than those without amputation [27]
	DFU presence	- DFU group showed no differences from DM and/or DN groups in lying down duration and sleep duration [27]

Abbreviations: OSA: obstructive sleep apnea; DFU: diabetic foot ulcer; DF: diabetic foot; CPAP: continuous positive airway therapy; DM: diabetes mellitus; IENFD: intraepidermal nerve fiber density; MNSI: Michigan neuropathy screening instrument;PARP: poly ADP ribose polymerase; PSQI: Pittsburgh sleep quality index.

4. Discussion

Obstructive sleep apnea (OSA) is a common sleep disorder in which a partial or complete obstruction of the upper airway occurs [31]. These obstructions will lead to a greater division of sleep, a decrease in oxygen saturation and a reduction in air flow [32]. OSA is an independent risk factor for cardiovascular disease [33], cognitive disorders [34]

and metabolic dysfunction [35].Intermittent hypoxia increases sympathetic activation and oxidative stress, impairing arterial function and generating inflammation [36].

It is known that untreated OSA leads to morbidity and worsening of glycemic control (insulin resistance and glucose intolerance), along with diabetic angiopathy. Research has shown that OSA is related to insensitivity of the foot and diabetic peripheral neuropathy, all of which contributes negatively to DFU healing [37,38]. In four of the studies included, DF variables were associated with OSA-related variables. Altaf et al. [24] found a positive correlation between small fiber neuropathy and OSA severity, and between the prevalence of DFUs and OSA. This suggested that OSA patients should be considered to be high risk, although the sample size did not allow regression analyses. Subramanian et al. [20] recommended anticipating the development of OSA as a risk factor in patients with DF.

Maltese et al. [28] highlighted the high prevalence of OSA among patients with DFUs, concluding that OSA severity is directly related to poor healing and re-ulceration.They used the STOP-Bang Questionnaire for OSA severity, a widely used instrument with high levels of sensitivity and specificity [39]. Therefore, the presence and severity of OSA should be considered in the treatment and prevention of DFUs. As a matter of fact, Vas et al. [19] described three cases of patients with DM2 and obesity, in which they studied how the impact of severe OSA interfered with DFU healing, despite a good local treatment. Patients under OSA treatment with continuous positive airway pressure showed significantly improved DFU healing, while patients who refused OSA treatment did not improve. Despite sample limitations, the results were promising and could represent a breakthrough in DFU treatment in patients with similar characteristics.

In another four of the studies included, sleep quality was related to DFU-related variables. Haveleia and Gayatri [23] found a significant correlation between the levels of stress and pain and subjective sleep quality in DFU patients, although they found no relationship between the severity of DFUs and sleep quality. However, it must be taken into account that this study did not include a control group in the sample. Conversely, Salomé et al. [30] did provide results that supported DFU patients having poor sleep quality, although this study did not include a control group without DFUs. Puspita et al. [26] addressed this same relationship and included a control group of people with diabetes without DFUs. As in the previous study, most of the subjects with DFUs and/or pain had poor sleep quality, although no significant differences were found.

All of these studies used the Pittsburgh sleep quality index (PSQI) to measure sleep quality, which is valid, reliable and widely used [40]. This lack of relationship between sleep quality and the presence or severity of DFUs could be explained by the high probability of DFUs not causing any pain (because of DN) [41]. Moreover, not all DFU patients suffer from sleep disorders [42].

Nairetet al. [22] analyzed the effectiveness of microcurrent therapy for the healing of chronic ulcers (including DFUs), and one of the outcome variables was sleep quality. Both sleep quality and neuropathic pain improved in most patients after receiving the therapy, however, the sleep quality measurement instrument was not specified, nor was a control group used. These results should be taken with caution and the use of microcurrent therapy should be studied in detail in future works.

Another important variable regarding sleep is its duration, which was studied by Sheahan et al. [27] in patients with DFUs under different conditions, as follows: minor amputation, major amputation, with and without off-loading elements, or peripheral neuropathy. These groups were compared to each other and did not show a significant decrease in quality or duration of sleep. This lack of correlation can be explained in a similar way as before, in that the absence of pain and psychogenic or sleep disorders may be the reason for the sleep not being impaired.

The study by Andruskiene et al. [21] found an association between diabetic foot pain and depressive states in a female population, however, no correlation was found with variables regarding the sleep. According to other authors, the depressive state can cause

sleep disturbances [43,44]. This study was the only one included in this review that took into account the consumption of sedative, antidepressant, analgesic or antitussive drugs.

In two of the studies included, DF and sleep variables were not related to each other. In the study by Bener et al. [25], sleep variables and DF variables were studied separately as risk factors for hearing loss. Rutkove et al. [29] also did not relate them. Instead, thermoregulation in the foot and DN during sleep and wakefulness were measured, and it was concluded that nocturnal thermoregulation is affected in patients with ND.

Along with our results, recent systematic reviews and meta-analyses show the association between DM on the sleep. Reutrakul et al. [45] found an association between DM1, poor sleep quality and prevalence of OSA, while another review [46] also associated the latter with DM2. Lee et al. [47] and Grandner et al. [48] found that the quality and duration of sleep influenced both glycemic metabolism in patients with DM2 and the risk of suffering from DM2. Several authors concluded that there is a high prevalence of sleep disturbances in patients with DM [35,49,50]. In the recent work by Nefs et al. [51], the reciprocal relationship between DM and sleep was approached from a behavioral science perspective, and it was stated that sleep quality should be considered with the same importance as diet and exercise in DM care.

Our results lead to several applications in clinical practice. Since the relationship between OSA and DF is the most studied, clinicians should consider OSA as a component of the multifactorial condition of DF. In addition, it may be appropriate for a diabetic foot specialist and a sleep disorder specialist to work in a multidisciplinary way with OSA and DF patients, to address prevention and treatment strategies.

Although there is a lack of evidence on the relationship between DF and the quality and duration of sleep, it is known that DFU healing is associated with poor coping and high levels of depression [52], which in turn are associated with poor quality and duration of sleep [36]. To improve the multidisciplinary treatment of patients with DF and psychological disorders or sleep disturbances, research linking these variables should be conducted in the future.

One limitation of this study is that there might be scientific literature published in a different language than those included in the inclusion criteria. In addition, a trend to link sleep disturbances with neuropathic pain has been found in the available literature. However, since neuropathic pain is not exclusive to DF, studies concerning neuropathic pain have not been the topic of this review. The same can be said for PAD, which is multifactorial and not exclusive to DM, and thereforeit has not been considered in the present work either.

5. Conclusions

In conclusion, the results suggest that there is a possible association between OSA and the presence or history of DFUs. With respect to sleep quality and duration, no direct associations with DF or DFU variables have been found. It is strongly recommended that future studies, particularly randomized controlled trials, take into account interventions for OSA, sleep quality and sleep duration. These studies should employ highly valid and reliable measurement instruments, which are widely available.

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Systematic Review

Effectiveness of Percutaneous Flexor Tenotomies for the Prevention and Management of Toe-Related Diabetic Foot Ulcers: A Systematic Review

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Abstract: There is a high prevalence of digital deformities in diabetic patients, particularly claw toe, which can result in ulceration, often located at the tip of the toe. These lesions are challenging to off-load with conventional devices and frequently lead to infection and high amputation rates. Recent guidelines recommend considering flexor tenotomies to manage these ulcerations and prevent complications. This review, which analyzed 11 studies, aimed to assess the effect of flexor tenotomies on the healing and prevention of diabetic foot ulcers (DFUs) at the toe tip. Satisfactory results were found, with a healing rate of 92% to 100% and a mean healing time of 2–4 weeks. Few mild complications were observed, and the recurrence rate was very low. Transfer lesions were the most prevalent, but simultaneous tenotomy of all toes can eliminate this risk. Flexor tenotomies are a simple, effective, and safe procedure for the treatment and management of DFUs located at the apex of the toes and should be considered part of the standard of care for diabetic feet.

Keywords: diabetic foot; diabetic foot ulcer; digital deformity; flexor tenotomy

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1. Introduction

One of the most common complications of diabetes is diabetic foot ulcers (DFUs), which have a lifetime incidence of approximately 19% to 34% [1].

Although the development of diabetic foot ulcers is multifactorial, it is most frequently associated with peripheral neuropathy and foot deformity [2]. Digital deformities such as hammer, mallet, or claw toes are commonly associated with diabetic foot ulceration, with the plantar and dorsal aspects of the toe being the most frequently affected locations [3]. Ulcers on the toes account for 43% to 55.5% of all foot ulcer cases, and while these ulcers are smaller and typically heal faster than the metatarsal head, midfoot, or rearfoot ulcers, they are often underestimated and tend to have higher rates of limb amputations compared to other foot locations [4].

This condition leads to atrophy of the intrinsic foot muscles, specifically the interossei and lumbricals. When intrinsic muscles become dysfunctional and overpowered by the extrinsic muscles (flexor digitorum longus and extensor digitorum longus), the stabilizing action is lost, which can eventually result in claw or hammer toes due to an imbalance between the intrinsic and extrinsic muscles across the metatarsophalangeal joints (MTPJs) and interphalangeal joints (IPJs) [5,6].

A claw deformity is caused by hyperextension of the MTPJ with plantar flexion of the PIPJ and DIPJ. A hammertoe is characterized by hyperextension of the MTPJ and plantar flexion of the PIFJ, but there is no contracture of the DIPJ. In contrast, a mallet toe occurs when the plantar flexion deformity is only found in the DIPJ [7,8].

In those with diabetic neuropathy, toe deformities can increase plantar pressures during midstance and toe-off, leading to the formation of calluses, minor lesions, and, ultimately, toe ulceration, particularly at the tip of the toes [9].

Off-loading and debridement are the basis of treatments to promote healing and prevent the recurrence of tip-toe ulcers [10]. Orthotic interventions such as footwear, toe silicone orthosis, or padding are standard treatments. However, conservative treatment remains unclear, has weak evidence, and often results in poor patient adherence [8,10].

Surgical interventions such as flexor tenotomies (FTs) are often considered when a toe deformity is a risk factor for developing a toe ulcer and when conservative non-operative treatment has been unsuccessful [11]. The International Working Group on the Diabetic Foot (IWGDF) recommends performing digital flexor tendon tenotomies in individuals with diabetes and abundant callus or an ulcer on the apex or distal part of a non-rigid hammer toe to prevent the first ulcer or the development of a recurrent foot ulcer [12]. The procedure consists of locating the flexor tendon by placing it under tension followed by a subsequent transversal incision in the flexor digitorium longus and brevis [11].

Two previous systematic reviews [13,14] have evaluated the effects of flexor tenotomy on the healing and prevention of diabetes-related toe ulcers. To assess the current literature, this review has been conducted due to the recent publication of new studies. Additionally, the effect of flexor tenotomies on the prognosis of further complications, such as toe deformities and transfer lesions, has not yet been evaluated.

The primary aim of this review was to assess the effectiveness of flexor tenotomies in healing and preventing diabetic foot ulcers located on the apex of the toe. The secondary objective was to evaluate the safety and efficacy of flexor tenotomies in preventing and healing diabetic foot ulcers associated with digital deformities.

2. Materials and Methods

This systematic review was carried out according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines [15] and has been registered in PROSPERO (a prospective international register of systematic reviews; identification code CRD42023396635).

2.1. Literature Search

Three electronic databases were independently searched by two reviewers (MM.C.W and M.L.M) for relevant studies on flexor tenotomies and the healing and prevention of diabetic foot ulcers located on the tip of the toe from inception up to 10 September 2022. The words “flexor tenotomy”, “healing”, “prevention”, and “diabetic foot ulcers” were used as search terms. These keywords were directly combined using the Boolean operator “AND” forming the following search strategies: flexor tenotomy AND healing AND diabetic foot ulcers, flexor tenotomy AND prevention AND diabetic foot ulcers and flexor tenotomies AND diabetic foot ulcers.

2.2. Selection Requirements

2.2.1. Inclusion Criteria

Inclusion criteria included (a) studies published in the last 12 years; (b) studies published in English or Spanish; (c) patients with digital deformities associated with diabetes that had either developed a toe ulcer or were at risk of developing a toe ulcer; and (d) studies using a prospective/retrospective case series or case–control design, cross-sectional, or cohort design and randomized clinical trials.

2.2.2. Exclusion Criteria

Exclusion criteria included (a) studies published over 12 years ago; (b) animal trials; (c) articles concerning other types of tenotomies than flexor tenotomies; and (d) articles unrelated to the treatment and prevention of diabetic foot ulcers.

2.3. Literature Screening and Data Extraction

Following the deduplication of search results, potential articles were reviewed based on title and abstract. Articles were independently screened by two authors (MM.C.W and M.L.M), and the results were compared. A third reviewer (JL.L.M) resolved any disparity between the authors.

According to the research questions, the general information of each article was arranged in a data chart, including first author, year, study design, objectives, sample, lesion characteristics, type of intervention, and follow-up.

Healing rate and healing time were included in a second table as outcomes, and complications arising from the surgical procedure and adverse effects were included in the second chart.

2.4. Quality Evaluation of Included Studies (STROBE Guidelines)

Three independent researchers analyzed the data collected from all articles. As most of the included articles were prospective and retrospective cohort studies (with only one randomized trial included), the quality evaluation was based on the standard STROBE guidelines to ensure a high-quality presentation of observational studies [16]. Raters assessed the adequacy of reported items using the STROBE guideline checklist, which provides a framework for completeness and transparency. The STROBE guidelines checklist has 22 items, including items 1 (title and abstract), 2 and 3 (introduction), 4–12 (methods), 13–17 (results), 18–21 (discussion), and 22 (funding and sponsorship). Two raters (MM.C.W and M.L.M) independently assessed each study using the STROBE guidelines, and a third rater (J.L.L.M.) was involved in achieving a consensus in case of disagreement.

2.5. Statistical Analyses

Since the included studies have great heterogeneity in research design, survey time, and outcome indicators, it would be difficult to conduct quantitative analysis, so only qualitative analyses were conducted.

3. Results

3.1. Literature Retrieval

In the first search applying the inclusion criteria, 80 articles were identified. After eliminating duplicates and reading the title and abstract, 23 articles were selected for full-text evaluation. Ultimately, 11 studies were included for analysis. Figure 1 shows the literature screening process.

3.2. Characteristics of the Included Studies

Among the included literature, ten studies were case series studies, of which seven were retrospective [17–23] and three prospective [24–26]. One randomized clinical trial was also assessed [27]. The eleven studies included 770 flexor tenotomies performed in diabetic patients.

In the study by Schmitz et al. [22], 101 tenotomies to treat digital lesions in diabetic and non-diabetic patients were evaluated; those with a curative indication in 64 diabetic feet could be evaluated in isolation, but the prophylactic group with 13 diabetic feet and 4 non-diabetic feet were analyzed together. Scheepers et al. [17] and Tamir et al. [21] also included a minority of neuropathic patients without DM in their studies but did not specify the number of tenotomies performed in diabetic patients; therefore, they could not be assessed independently.

Among the total of 770 tenotomies, 387 had a curative indication, and 388 were prophylactic; six studies included both indications, two evaluated only prophylactic tenotomies, and three evaluated only the curative indication. The study by Hedegaard Andersen et al. [23] evaluating both indications showed that in the curative tenotomy group, 14 interventions were also considered prophylactic because the patient had another finger with a preulcerative lesion (PUL) in addition to the ulcerated toe.

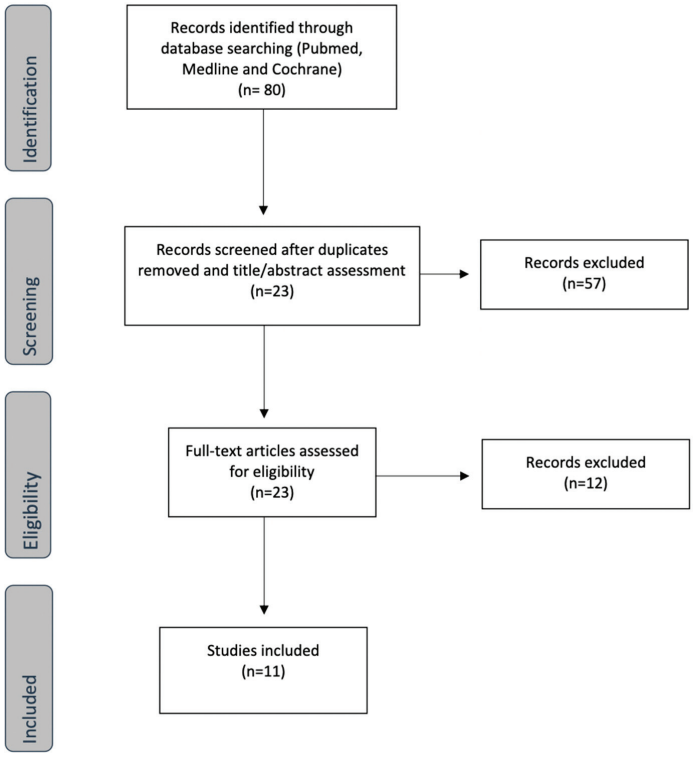

Figure 1. Flowchart of identified studies.

The studies included patients who had undergone FT to treat one or more lesions located in the apex of the toes associated with a flexible or semi-flexible digital deformity, except for the RCT by Andersen et al. [27] and the study by Smith et al. [24], in which participants with rigid digital deformities were not excluded.

Tamir et al. [21] evaluated flexor tenotomies for the treatment of DFUs in other locations than the tip and combined this technique with extensor tenotomies in selected participants in addition to performing isolated extensor tenotomies depending on the location of the lesion; these cases were not included in the outcome analysis of the present systematic review.

Another study [23] included ulcers and preulcerative lesions at locations associated with digital claw, hammer, or mallet deformities that differed from the tip of the toe, and the results for all lesion types were evaluated together.

The etiology of the lesions was neuropathic in most cases, although some articles included neuroischemic lesions [17,18,20–24]. The presence of soft tissue infection was an exclusion criterion common to all studies, but several articles included lesions with osteomyelitis (OM) [19,21,24].

Ulcer evolution times ranged from 1 to 9 months, although, for most of the studies, the average preintervention wound evolution time was around 3 months.

Regarding the surgical procedure technique, there were studies in which only the flexor digitorum longus was sectioned [17,21,22] and others in which the flexor digitorum longus and flexor digitorum brevis were approached together [17,19,20,26,27], with the incision placed proximally or distally depending on the approach. The tenotomy was performed with a scalpel [17–22,24,26]; in some cases, a percutaneous needle was used [23–25,27].

Post-surgical follow-up time ranged from 6 [19,24] to 28 months [18]; five studies followed patients for around 1 year [17,22,25–27], and three articles followed patients for approximately 2 years [19,21,23]. The research characteristics are shown in Table 1.

Table 1. General characteristics of the included studies.

First Author Year	Study Design	Objectives	Sample	Lesion Characteristics	Surgical Intervention	Follow-Up
Schepers T. 2010, [17]	retrospective	To assess the results of using flexor tenotomies to treat ulcers in flexible claw toes.	23 patients - 15 with diabetes - 5 DM + PAD 25 ulcers 17 PULs	- Wagner 0–2 (95%) - Location: the tip of the toes - The mean time of evolution = 6.8 months - Deformity: flexible claw toe	- Technique: FDL and FDB sectioned - Total *n* = 42 - Curative *n* = 42 - Prophylactic NA	11 months
Kearney TP. 2010, [18]	retrospective	To evaluate the effectiveness and safety of percutaneous tenotomy of the flexor digitorum longus for healing neuropathic ulcers in the tip of the toes.	48 patients with diabetes - 21 PAD 58 ulcers	- Location: the tip of the toes - Deformity: flexible	- Technique: FDL sectioned - Total *n* = 58 - Curative: *n* = 58 - Prophylactic NA	28 months
Van Netten JJ. 2013, [19]	retrospective	To report healing rates and healing times and to investigate the influence of preoperative treatment, time of ulcer evolution before tenotomy, and location or presence of infection on healing and healing time. They also wanted to describe the advantages of using this technique as a prophylactic intervention in diabetic patients with claw or hammertoes.	33 patients with diabetes - 31 DN - No PAD 38 ulcers	- Texas 3b majority - Location: tip of toes - Mean time of evolution = 96 days - Deformity: flexible hammer or claw toe - OM included	- Technique: FDL and FDB sectioned - Total: *n* = 47 - Curative *n* = 38 - Prophylactic *n* = 9 * 8 transfer tenotomies because they were performed on the same foot after an initial procedure	23 ± 11 months
Rasmussen A. 2013, [20]	retrospective	To examine the effectiveness of a modified flexor tenotomy technique to prevent and heal neuropathic and neuroischemic ulcers located on the tip of the toe in the presence of claw or hammertoe deformity in diabetic patients.	38 patients - 16 with 27 ulcers - 22 with 38 PULs	- Neuroischemic ulcers - Location: tip of toes - Mean time of evolution = 15 weeks - Deformity: flexible hammer or claw toe	- Technique: FDL and FDB sectioned - Total: *n* = 65 - Curative *n* = 27 - Prophylactic *n* = 38	6 months
Tamir E. 2014, [21]	retrospective	To report on the performance of percutaneous flexor and extensor tenotomies for treating neuropathic ulcers.	55 patients with diabetes * Patients with critical ischemia were excluded	- They affected mostly skin and subcutaneous cellular tissue - Location: tip, dorsum, interdigital and metatarsal head - Mean time of evolution = 33 weeks - Cellulite excluded - OM included	- Technique: FDL sectioned - Total: *n* = 103 - Curative *n* = 103 - Prophylactic NA	22 months

Table 1. *Cont.*

First Author Year	Study Design	Objectives	Sample	Lesion Characteristics	Surgical Intervention	Follow-Up
Schmitz P. 2019, [22]	retrospective	To assess whether percutaneous flexor tenotomy is an effective intervention to treat and prevent toe ulcers and whether prophylactic percutaneous tenotomy is a safe and effective way to prevent ulceration.	101 feet included 77 with DFS - 64 DFUs - 13 PULs	- 64 with DN - 1 with PAS - 18 DN + PAS - Deformity: flexible claw toe - Mean time of evolution = 124 days	- Technique: FDL sectioned - Total in DFS group: *n* = 77 - Curative *n* = 64 - Prophylactic *n* = 13 - * In both groups = curative 84 and prophylactic 17	13.4 months
Hedegaard Andersen J. 2019, [23]	retrospective	To show the outcome of percutaneous needle tenotomies and the benefit of flexor tenotomies as a treatment for claw, hammer, and mallet toes in people with diabetes.	81 patients with diabetes - >Type II - DN - 20% PAS	- Neuropathic, ischemic, and neuroischemic - Location: tip, dorsum, interdigital, and metatarsal head - Mean time of evolution = 4.5 weeks - Deformity: claw, hammer, or mallet	- Technique: Percutaneous needle - Total: *n* = 106 - Curative *n* = 36 - * (14 were considered curative + prophylactic) - Prophylactic: *n* = 70	97 weeks
Smith SE. 2020, [24]	prospective	To show the effectiveness and usefulness of percutaneous flexor tenotomies for the healing of neuropathic ulcers at the distal end of the toes performed in an outpatient setting and to show the effectiveness of percutaneous flexor tenotomies for the prevention of progression of preulcerative toe lesions to diabetic foot ulcers.	23 patients with diabetes - without PAS 11 ulcers 41 PULs	- Texas 1A majority - Location: tip of 2° and 3° toe (majority) - Mean time of evolution = 105 days - Deformity: >flexible claw toe	- Technique: FDL or FDL and FDB sectioned with needle or scalpel - Total: *n* = 76 - 51 FDL and 25 FDL + FDB - Curative *n* = 11 - Prophylactic *n* = 65	6 months
Mens MA. 2022, [25]	prospective	To evaluate the effect of percutaneous flexor tenotomy in diabetic patients on plantar pressure, toe angulation, and ulcer recurrence.	14 patients with diabetes - $\frac{1}{2}$ with PAS 19 feet 50 toes	- PUL and history of ulcer on the apex of the toes - Deformity: flexible or semi-flexible	- Technique: percutaneous needle - Total: *n* = 19 - Curative NA - Prophylactic: *n* = 19	14.4 months
López-Moral M. 2022, [26]	prospective	To evaluate the long-term clinical outcomes of patients who underwent isolated percutaneous flexor tenotomies versus multiple tenotomies to treat previous toe deformities and diabetic foot ulcers.	23 patients with diabetes - DN - without critical ischemia 31 feet	- PUL and history of ulcer on the apex of the toes - Deformity: flexible	- Technique: FDL and FDB sectioned with percutaneous needle - Total: *n* = 99 - Curative NA - Prophylactic *n* = 99 * 31 feet operated 11 with isolated tenotomies - 20 with several tenotomies	1 year

Table 1. *Cont.*

First Author Year	Study Design	Objectives	Sample	Lesion Characteristics	Surgical Intervention	Follow-Up
Andersen J. 2022, [27]	RCT	To examine the ability of tenotomies to prevent and treat hammertoe-associated ulcers in diabetic patients.	96 patients with diabetes 16 ulcers 79 PULs	- Lesions associated with flexible, semi-flexible, or rigid hammer toe deformity	- Technique: FDL sectioned - Total: n = 47 - Curative n = 8 - prophylactic n = 39 4 subgroups: PUL with SOC PUL with tenotomies + SOC DFU with SOC DFU with tenotomies + SOC	1 year

DM, diabetes mellitus; PAD, peripheral arterial disease; DN, diabetic neuropathy; PUL, preulcerative lesion; FDL, flexor digitorum longus; FDB, flexor digitorum brevis; NA, not applicable; OM, osteomyelitis; DFS, diabetic foot syndrome; DFU, diabetic foot ulcer; RCT, randomized controlled trial; SOC, the standard of care. *, additional information.

3.3. Quality of the Reporting

Items 9 (bias), 10 (study size), 19 (limitations), and 21 (generalizability) were the most poorly completed by the included studies. Table 2 shows the overall rating for the STROBE checklist.

Table 2. The overall rating for Strengthening the Reporting of Observational Studies in Epidemiology (STROBE).

Item Number–STROBE Guidelines																						
	1(a)	1(b)	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
Schepers T. 2010, [17]	No	Yes	Yes	No	Yes	Yes	No	Yes	Yes	No	No	No	No	Yes	Yes	Yes	Yes	No	Yes	No	Yes	No	No
Kearney TP. 2010, [18]	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	No	No	No	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Van Netten JJ. 2013, [19]	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	Yes	No	Yes	Yes	Yes	Yes	Yes	No	Yes	No	Yes	Yes	Yes
Rasmussen A. 2013, [20]	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	Yes	No	No
Tamir E. 2014, [21]	Yes	Yes	No	Yes	Yes	No	Yes	No	No	No	No	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	Yes	No	Yes
Schmitz P. 2019, [22]	Yes	Yes	Yes	Yes	Yes	Yes	No	Yes	No	No	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	Yes
Hedegaard Andersen J. 2019, [23]	Yes	Yes	Yes	Yes	Yes	Yes	No	Yes	Yes	No	No	No	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	Yes
Smith SE. 2020, [24]	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	Yes	No	Yes
Mens MA. 2022, [25]	No	Yes	Yes	Yes	Yes	No	Yes	Yes	No	No	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No
López-Moral M. 2022, [26]	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	No	Yes	No	Yes	Yes	Yes
Andersen J. 2022, [27]	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes

3.4. Screened Outcomes

The results obtained concerning the healing rate and healing time, complications arising from the surgical procedure, and adverse effects are shown in Table 3.

Table 3. Screened outcomes.

Researchers	Healing Rate (%)	Mean Healing Time	Adverse Events	Surgical Complications
Schepers T et al. (2010), [17]	100%	3.6 weeks	- 1 recurrence - 1 minor amputation	- Section of plantar plate

Table 3. *Cont.*

Researchers	Healing Rate (%)	Mean Healing Time	Adverse Events	Surgical Complications
Kearney TP et al. (2010), [18]	98.3%	40–52 days	- Reulceration rate in the same site 12.1% (mean time of appearance 13.9–15.2 months) - Post-surgical infection rate 5.2% not in place of incision - 1 unhealed lesion	- No complications
Van Netten JJ et al. (2013), [19]	92%	22 ± 26 days	- 3 minor amputations (of non-healing ulcers) - 7 reulcerations - 1 dorsiflexed metatarsophalangeal joint	- No complications
Rasmussen A et al. (2013), [20]	93%	21 days	- 3 recurrences - (One healed after repeating the tenotomy) - 2 transfer lesions - 2 unhealed ulcers	- 1 insufficient procedure
Tamir E et al. (2014), [21]	98%	4 weeks	- 2 unhealed ulcers - 9 transfer ulcers - 3 ruptures of the skin secondary to toe extension	- 1 mild infection - 1 patient with plantar pain
Schmitz P et al. (2019), [22]	93.8%	22 days	- Curative group: 4 infections, 1 minor amputation, 8 recurrences, and 2 transfer ulcers and 4 unhealed ulcers - Prophylactic group: 2 ulcerations	- 1 bleeding, 1 reintervention
Hedegaard Andersen J et al. (2019), [23]	94%	28 days	Curative group: - 5 recurrences - 2 unhealed Prophylactic group: - 6 progressions to active ulcer - 4 extensor tenotomies 25 transfer lesions (7 ulcers and 18 PULs) 4 amputations (3 minor and 1 major)	- 4 insufficient procedures that were repeated - Plantar pain (14%)
Smith SE et al. (2020), [24]	100%	10.2 ± 4.3 days	- Transfer lesions (15.5%) - 3 ulcers and 3 PULs	- Post-surgical infection (2.8%)
Mens MA et al. (2022), [25]	NA Recurrence 0%	NA	- No adverse events	- Without complications
López-Moral M et al. (2022), [26]	NA Recurrence 0%	NA	Insolated tenotomies: - 8 transfer lesions in 9 weeks (72.7%) - 11 adjacent HK increased + claw toes in 5 and a half weeks (100%) - 9 minor lesions in 6 and a half weeks (81%) Multiple tenotomies: - 16 floating toes (80%)	- Without complications
Andersen J et al. (2022), [27]	100% Recurrence 0%	Days (7–26)	- Curative group: no adverse effects - Prophylactic group: 5 transfer lesions, 2 PULs, and 3 ulcers	- Curative group: 2 with pain and 2 with hematomas - Prophylactic group: 21 with pain 7 with hematomas, and 1 patient with a feeling of loss of balance

%, percentage; PUL, preulcerative lesion; NA, not applicable; HK, hyperkeratosis.

3.4.1. Healing Rates and Mean Healing Times

Data on healing rates and healing times were satisfactory for all studies, with healing rates ranging from 92% to 100% and healing times around 2–4 weeks, except for the article by Kearney et al. [18], which showed a mean healing time of 5–7 weeks. The shortest healing time was observed in the cohort of Smith et al. [24], considering that most wounds were superficial and free of infection. Studies agree that lesions with infection and deeper tissue penetration had longer healing times [19,21].

3.4.2. Ulceration and Recurrence Rates

The articles that evaluated tenotomies with prophylactic indication reflected rates of progression to active ulcer and recurrence rates of 0%, except for Hedegaard Andersen et al. [23] and Schmitz et al. [22], who showed in their studies that preulcerous lesions treated with TF progressed to ulceration, but in a very low percentage.

In the study by Schmitz et al. [22], this event was observed in two patients, but they did not specify the location or whether the patient was diabetic; assessing two simultaneous populations is a limitation in this respect. The follow-up period in the study by Hedegaard Andersen et al. [23] was longer than in other studies. Additionally, in other studies, the intervention of each toe was assessed as one procedure, whereas in this case, one procedure could include one to ten toes; if the ulceration rate per toe and per procedure is calculated, the ulceration rate is 3%.

3.4.3. Complications Arising from the Surgical Procedure

Regarding complications, six articles [18,21–24,27] reported on post-surgical events such as pain and hematoma associated with the operation or infection, which were not considered serious.

Therefore, the studies agree that tenotomies are simple and safe procedures that effectively unload the apex of the toes by reducing digital deformity. Mens et al. [24] used objective biomechanical and musculoskeletal tests to demonstrate this off-loading effect; their findings show a large off-loading effect with a >50% reduction in pressure on the tip toe in line with the hypothesized causal mechanism of this minimally invasive surgery in the prevention of toe ulcers.

3.4.4. Adverse Events

Complications observed during follow-up were mostly transfer injuries and reulcerations. Several articles [19,22,27] treated transfer injuries in another episode of intervention using flexor tenotomies and showed satisfactory results, and in some cases, additional osteotomies were necessary [20].

The studies [18–23] had a total of 14 lesions that did not heal during follow-up, and in two studies, reinterventions had to be performed due to insufficiency of the initial procedure. A total of nine amputations were also found in the studies, three of which were associated with the ulcer treated with tenotomy; these lesions had osteomyelitis. Kearney et al. [18] associated the non-healing case with the presence of a pre-existing hallux amputation; in the article by Van Netten et al. [19], the ulcers that did not heal had an infection and penetrated the bone, but most of the ulcers with these characteristics did heal, almost half of them without complications.

4. Discussion

The evaluated literature presents favorable and satisfactory data regarding the effectiveness, efficacy, and safety of flexor tenotomies in treating and preventing DFUs located on the tip of the toes, which is consistent with the results obtained in previous reviews. This review quantitatively analyses outcomes, using healing rate and mean healing time to determine the effectiveness of flexor tenotomies; this reflects a strength of the study.

In addition, this review reported on the most prevalent complications resulting from flexor tenotomies, which is the main strength of the present study because these effects

have not been evaluated before in the literature. Transfer injuries were the most common adverse effect observed. It should be noted that after flexor tenotomy, the adjacent toe (due to structural and functional changes) may develop a transfer injury due to increased pressure, which can be considered serious because it may result in ulceration, infection, and subsequent amputation.

Regarding these complications, Lopez-Moral et al. conducted a study evaluating the long-term clinical outcomes of patients who underwent isolated versus multiple flexor tenotomies [26]. They found a higher rate of reulceration due to transfer injuries in the isolated tenotomy group, a higher prevalence of hyperkeratosis and deformities in adjacent toes, and higher peak barefoot pressure and pressure/time integral in toes without tenotomy in the isolated tenotomy group. These results support the idea that patients with a history of ulceration or incipient callus on the tip of the toes should undergo percutaneous flexor tenotomies on all toes to reduce long-term complications. Consistent with these findings, Hedegaard Andersen J et al. observed that the risk of transfer injury was eliminated in patients who underwent TF of all toes simultaneously [23].

In terms of limitations, most of the articles evaluated do not include a significant sample of patients with neuroischemic ulcers. In the study performed by Scheppers T et al., which included a patient with PAD, it was found that this condition was not associated with complications or delayed healing, likely due to the minimally invasive nature of the procedure [17]. The authors also reported that osteomyelitis did not affect healing but that patients took longer to heal. This finding is consistent with existing data and general principles regarding diabetes-related foot ulcers and the delay in postoperative healing caused by osteomyelitis.

Furthermore, the studies evaluated are mainly retrospective and lack high-quality evidence for analysis. There is only one RCT in the literature that compares tenotomies with SOC, highlighting the need for more of this type of study. Future research should include quantitative data analysis to enable meta-analysis, but this requires more RCTs comparing two interventions.

Regarding digital deformities, it is true that the articles define them differently, and in most cases, a complete evaluation of them is not performed, which may lead to erroneous indications for these techniques or associated complications. Moreover, there is no consensus regarding the technique and the influence of sectioning one or both flexors. Scheppers T et al. reported iatrogenesis with the section of the plantar plate resulting in a hyperextended toe that required amputation [17]. Van Netten et al. observed a patient in whom both flexors were severed, resulting in dorsiflexion of the AMTF that developed ulceration [19].

To avoid these complications, an assessment of dynamic deformities during gait should be included as a pre-surgical evaluation. Additionally, to maximize the probability of successful surgical outcomes, each patient's biomechanics should be assessed in a loading situation, and the etiology of the toe deformity should be analyzed [7,8].

The systematic use of pressure-relieving therapy with therapeutic footwear, close follow-up, correct antibiotic prophylaxis, and control of comorbidities (multifactorial approach) are essential for successful therapy [26,27], and studies that apply these principles have shown better results. Rasmussen et al. did not follow up with patients monthly after healing, as recommended by the IWGDF guidelines; therefore, the finding of reulceration events over a longer time than that identified other studies could be related to this [20].

Several studies report the use of plantar orthoses and appropriate footwear after surgery, with some studies highlighting their benefits [20,25,26]. However, other articles indicate that patients could do without custom-made or special footwear after surgery [17].

5. Conclusions

Flexor tenotomies are an effective treatment for neuropathic UPDs located at the distal end of the toes, showing a high healing rate with a short healing time. They are also an excellent prophylactic procedure, demonstrating low rates of ulceration and recurrence

and being effective in preventing UPD in the presence of digital deformity or preulcerative signs, provided their indication is correct. Therefore, these techniques should be included in the day-to-day standard of care for diabetic feet.

The presence of mild ischemia or osteomyelitis should not be considered a contraindication for the practice of these procedures. However, in these cases, there are longer healing times and a higher risk of complications during follow-up. Transfer injuries are the most prevalent secondary complication; performing a tenotomy of all toes simultaneously eliminates this risk and other complications. Therefore, it is advisable to perform multiple tenotomies rather than isolated ones. Further RCTs are required to support these conclusions with more evidence, and future research needs to include ischemia and infection data.

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