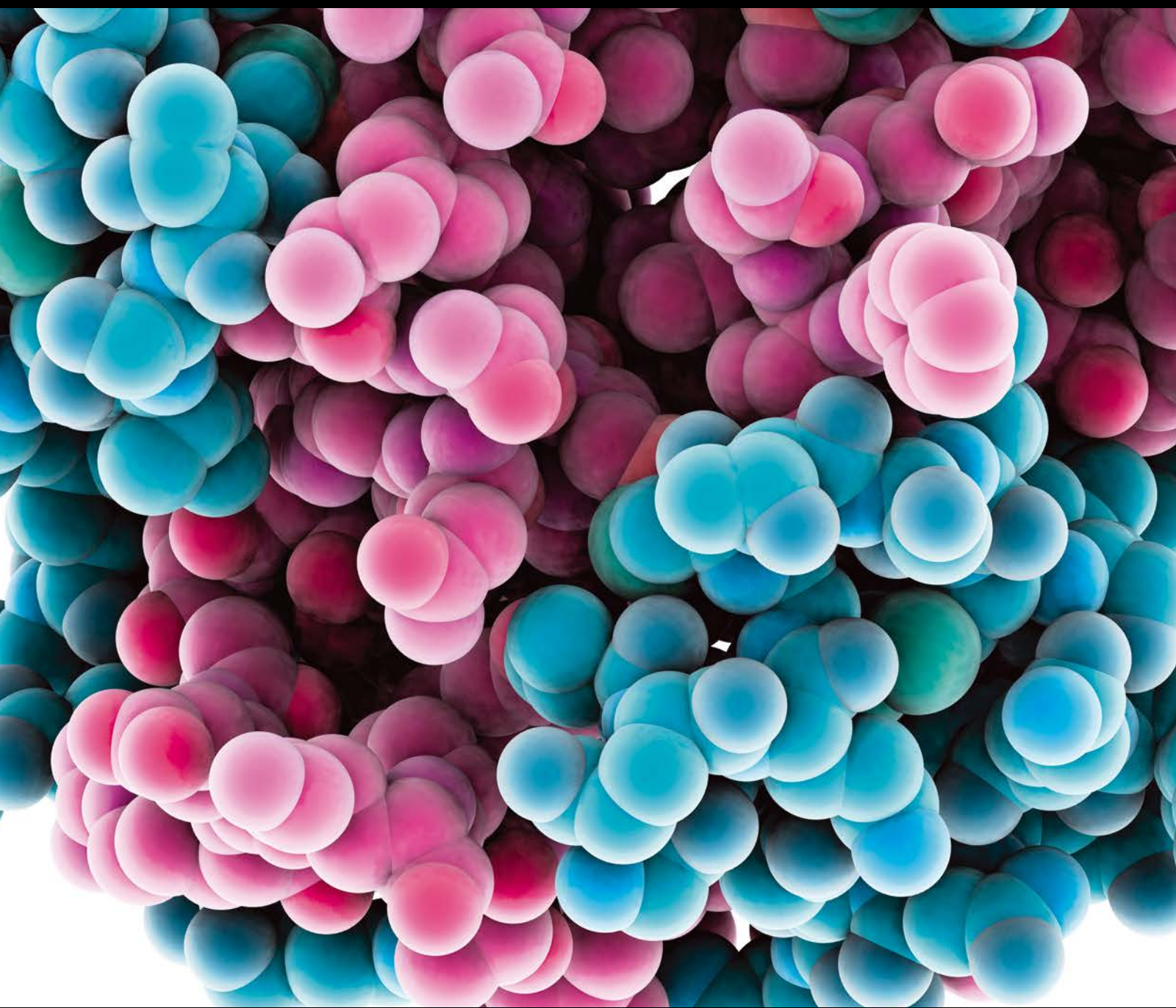
Journal of Diabetes Research

Diabetic Foot: Current Status and Future Prospects

Guest Editors: Didac Mauricio, Edward Jude, Alberto Piaggesi, and Robert Frykberg



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Editorial

Diabetic Foot: Current Status and Future Prospects

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Diabetic foot disease is a debilitating complication of diabetes mellitus, ultimately affecting up to 50% of patients with both type 1 and 2 diabetes. Currently, this complication is still leading to significant loss of quality and years of life of the affected patient [1, 2]. Furthermore, it represents at least 12–15% of the overall cost associated with diabetes and up to 40% in developing countries [2, 3]. Additionally, current available treatments for diabetic foot disease are usually not as effective as they should be [4]. This is mainly explained by the insufficient knowledge of its underlying mechanisms and treatment tools because of the insufficient interest and research resources allocated to the study of this complication worldwide.

All, except one, of the papers included in this special issue are dealing with clinical and epidemiological aspects of diabetic foot disease with an observational design. One paper is devoted to the investigation of a novel therapeutic approach in an experimental animal model. The content of this issue mirrors the current clinical and research background of this diabetic complication; that is, there is ample room for improvement in all aspects of its prevention and treatment.

In one of the papers, using a retrospective study design, N. K. Chammas et al. assessed mortality and its causes in a large sample of patients from a single foot clinic in the UK and confirmed that death occurred 5 years earlier in patients with a diabetic foot ulcer. As for the general diabetic population, cardiovascular mortality was the main cause of death in the cohort, and the same was observed in patients with neuropathic foot ulcers where ischemic heart disease was the main cause of death. As found in previous studies, these results confirm the close link between diabetic microangiopathic disease and atherosclerotic cardiovascular disease. As for retinopathy and diabetic kidney disease, the results point again to neuropathic foot disease as a cardiovascular risk condition.

In a very large cohort of patients from Japan, a retrospective study by M. Tomita et al. aimed at assessing the incidence of diabetic foot ulcers according to the presence and severity of microvascular disease, that is, retinopathy and albuminuria. During a long follow-up period, after adjusting for other potential major contributing factors, the authors showed that the presence of concomitant retinopathy and albuminuria greatly increased the risk of developing diabetic foot disease. Thus, these findings should be taken into account in terms of preventative measures in patients with diabetic microangiopathic complications.

In the field of epidemiology, C. I. Bondor et al. performed a post hoc analysis using the data from a previous study of quality of life in a cross-sectional survey done in Romania in 2012. Although the study was based on self-reported

data, they showed a very high frequency of foot ulcers and amputations in this large cohort of more than 21,000 diabetic patients. These results confirm the great burden created by diabetic foot disease and highlight the importance of epidemiological studies to raise the awareness of this important health care problem around the globe.

M. T. V. Quilici et al. performed a cross-sectional study in patients admitted to a Brazilian hospital because of diabetic foot infections. The findings of the study showed a high rate of amputations in this study cohort in which most patients had neuroischemic feet. Additionally, the study underlines the importance of early referral of patients with diabetic foot infections.

An interesting paper by M. May et al. showed the data on amputations from a large insurance company delivering health care to some 2.9 million people from 2 regions in Germany before and after the implementation of 6 networks for specialized care of the diabetic foot. They found that there was a decrease of overall amputations. However, the amputation rates were still high and indicate the need for further improvement in specialized care of the diabetic foot.

Finally, in the field of experimental research, X. Zhang et al. conducted an experimental study using Tuo-Li-Xiao-Du-San, a traditional Chinese medicine formula, to treat wounds in a rat model of diabetes. They were able to show that this treatment was able to enhance the healing of ulcers through several mechanisms: reduction of inflammatory cell mediators and increase in angiogenesis and collagen deposition.

To conclude, although the interest in research and management of diabetic foot disease has increased in recent years, it is still the Cinderella among diabetes complications in terms of research efforts and in resource allocation and outcomes. Additionally, the complexity of its management needs to be addressed on a multidisciplinary approach that should gather all expertise necessary for the optimal management of each aspect of this complication [2, 5]. The future should bring much more high-quality research to the area of diabetic foot disease, from basic research to properly conducted clinical trials.

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Research Article

Increased Mortality in Diabetic Foot Ulcer Patients: The Significance of Ulcer Type

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Diabetic foot ulcer (DFU) patients have a greater than twofold increase in mortality compared with nonulcerated diabetic patients. We investigated (a) cause of death in DFU patients, (b) age at death, and (c) relationship between cause of death and ulcer type. This was an eleven-year retrospective study on DFU patients who attended King's College Hospital Foot Clinic and subsequently died. A control group of nonulcerated diabetic patients was matched for age and type of diabetes mellitus. The cause of death was identified from death certificates (DC) and postmortem (PM) examinations. There were 243 DFU patient deaths during this period. Ischaemic heart disease (IHD) was the major cause of death in 62.5% on PM compared to 45.7% on DC. Mean age at death from IHD on PM was 5 years lower in DFU patients compared to controls (68.2 ± 8.7 years versus 73.1 ± 8.0 years, $P = 0.015$). IHD as a cause of death at PM was significantly linked to neuropathic foot ulcers (OR 3.064, 95% CI 1.003–9.366, and $P = 0.049$). *Conclusions.* IHD is the major cause of premature mortality in DFU patients with the neuropathic foot ulcer patients being at a greater risk.

1. Introduction

There is strong epidemiological evidence of excess mortality in association with the diabetic foot syndrome. There is a greater than twofold increase of mortality in diabetic foot ulcer (DFU) patients compared to nonulcerated diabetic patients, regardless of age, type and duration of diabetes, treatment of diabetes, glycated haemoglobin concentration, history of lower extremity amputation, and cumulative pack years of cigarette use [1]. Diabetic patients with leg and foot ulcers have a lower 5-year survival (43%) than nondiabetic ulcerated subjects (56%) and general population controls (68%) [2].

Reported mortality rates for diabetic foot ulcer (DFU) patients range from ≈10% on a median follow-up of 16 months [3] to 24% after 5 years [4]. A study from a Liverpool foot clinic indicated a 5-year mortality rate as high as 44% in patients presenting with new DFUs [5]. A large community based Norwegian study over a 10-year follow-up period reported an increased mortality of 49% in diabetic patients with a history of DFU compared with 35.2% of diabetic patients without a history of foot ulcers and 10.5% of nondiabetic individuals [6]. We previously reported preliminary results on our patients but only had information on 112 of 243 death certificates and 41 out of 80 postmortem examinations that were carried out [7]. The present paper has information on the full number of 243 death certificates and 80 postmortem examinations. The aim of this study was to establish the precise cause(s) of death in DFU patients and to examine the relationship between cause of death and ulcer type.

2. Materials and Methods

To delineate causes of death in DFU patients, we conducted an 11-year retrospective (death search) audit of all deceased subjects who attended the Diabetic Foot Clinic at King's College Hospital and whose records were available in this institution. Occurrence of death was confirmed from hospital medical notes and general practitioners' records. Information

Table 1: Characteristics of diabetic foot ulcer patients and controls.

Characteristic	DFU (n = 243)	Controls (n = 121)	P
Mean age in years	71.2 ± 11.1	72.8 ± 10.1	0.090
Sex			
Males	147 (60.5%)	54 (44.6%)	0.005
Females	96 (39.5%)	67 (55.4%)	
Type of diabetes			
Type 1	28 (11.5%)	12 (10 %)	
Type 2	169 (69.5%)	109 (90 %)	0.3
Unknown	46 (19%)	0	
Place of death			
Hospital	187 (77%)	103 (85%)	0.07
Home	56 (23%)	18 (14.9%)	

provided for each patient included full name, address, date of birth, date of death, age at death, and place of death.

Causes of death were established for deaths occurring between April 1989 and January 2000 from the following:

(a) death certificates obtained from the central register for England and Wales held in Southport through the Family Record Centre in London. In cases where death certificates could not be retrieved from the central register, individual registry offices of various boroughs of London were approached for those certificates;

(b) postmortem examination results as stated in hospital records and coroners' reports on death certificates.

Study subjects included type 1 and type 2 diabetes patients as per WHO classification (1997) (Table 1). DFU patients were stratified into two categories according to type of ulcer. Ischaemia was diagnosed by absence of foot pulses [8]. Neuropathy was determined by the presence of a stocking distribution of sensory loss to light touch (cotton wool). Patients with signs of neuropathy and palpable pulses were said to have neuropathic ulcers and patients with absent foot pulses with or without neuropathy were deemed to have ischaemic ulcers.

We sought a control group of diabetic patients who attended the diabetic clinic at King's College Hospital and died over a 10-year period from March 1990 to March 2000. These patients had diabetes mellitus but no history of foot ulceration. They were stratified to match the DFU group in age and type of diabetes. In the UK, the major immediate cause of death is classified as 1a, 1b, or 1c on the death certificate. Death from ischaemic heart disease (IHD) was defined as death due to coronary artery disease or fatal myocardial infarction from occlusive coronary thrombosis or secondary fatal dysrhythmias.

2.1. Statistical Analysis. Mean age at death was calculated, including its standard deviation, and significance was tested at 95% confidence interval. A P value of <0.05 was considered statistically significant. The significance of the risk of death from IHD in diabetic patients with neuropathic ulcers compared to those with ischaemic ulcers was also assessed using the Chi-squared and Fischer exact tests (software used was GB STAT v6.5). The odds ratio was also determined with confidence intervals calculated at 95% level of certainty. Also, levels of significance of causes of death between DFU patients and control patients and between neuropathic and ischaemic ulcer patients on both death certification and postmortem results were calculated and expressed as P values in Tables 2, 3, and 4.

3. Results

We identified 268 patients who no longer attended the diabetic foot clinic between April 1989 and January 2000. There were 243 confirmed deaths in DFU patients who attended the clinic during the above period. The remaining 25 subjects with DFU whose deaths could not be confirmed or information on cause of death was unobtainable were excluded from the study. Overall, loss to follow-up during this period was 9.3%. The DFU group consisted of 147 (60.5%) male patients and 96 (39.5%) female patients with an age range of 30 to 95 years. Of these, 187 had ischaemic ulcers and 56 had neuropathic ulcers as defined in Materials and Methods. Death certificates confirmed that 187 DFU (77%) subjects died in hospital.

A control group of 121 deceased diabetic patients without foot ulceration was identified from the general diabetic clinic at King's College Hospital. These were patients who attended the clinic between March 1990 and March 2000. Of the 139 patients who no longer attended the diabetic clinic, 121 patients had confirmed deaths and causes of deaths were obtained as for the ulcerated group. In 12.9%, the cause of death could not be obtained. The control group comprised 54 (44.6%) male patients and 67 (55.4%) female patients with an age range of 44 to 92 years. The majority of the control patients, 103 (85%), died in hospital, which was comparable to the DFU group. Data regarding the presence of neuropathy or ischaemia was not available for the control group. The characteristics of both DFU and control group patients are summarised in Table 1. There was no significant difference apart from the proportion of male patients which was significantly higher in the DFU group compared with the controls.

In addition to death certification, 80 (33%) DFU subjects and 30 (25%) control patients had postmortem examination.

3.1. Subjects Lost to Follow-Up in That the Cause of Death Could Not Be Obtained. There were 25 DFU patients and 18 control patients whose cause of death could not be acquired. These patients may have moved from one region to another within the UK or possibly emigrated abroad. There are no reliable procedures for confirmation of death in this group, as deaths have not been notified to the Central Register in England and Wales. It was not possible to obtain the death certificate (or postmortem results) for these subjects as there was no trace at the Family Records Centre or the local registry office. We therefore excluded these subjects from our study.

The DFU group consisted of 12 (48%) male patients and 13 (52%) female patients. The mean age was 73.35 ± 9.32

Table 2: Causes of death in diabetic foot ulcer patients and control group on death certification and postmortem examination.

Cause of death	DFU pts on DC (n = 243)	Control pts on DC (n = 121)	P	DFU pts on PM (n = 80)	Control pts on PM (n = 30)	P
Ischaemic heart disease	111 (45.7%)	55 (45.5%)	0.968	50 (62.5%)	21 (70%)	0.465
(i) CAD/atherosclerosis	89	45	0.976	47	16	0.609
(ii) MI/coronary thrombosis	47	32	0.123	27	11	0.775
(iii) Cardiac arrest	5	1	0.401	0	0	0.629
Other cardiac causes	13 (5.3%)	7 (5.8%)	0.864	7 (8.8%)	3 (10%)	0.839
Bronchopneumonia	39 (16 %)	21 (17.4%)	0.752	3 (3.8%)	4 (13.3%)	0.085
Cancer	20 (8.2%)	19 (15.7%)	0.033	2 (2.5%)	1 (3.3%)	0.812
Cerebrovascular accidents	11 (4.5%)	6 (5%)	0.854	1 (1.3%)	0	0.932
Septicaemia	10 (4.1%)	6 (5%)	0.712	0	0	0.629
Renal failure	10 (4.1%)	3 (2.5%)	0.433	0	0	0.629
Pulmonary thromboembolic disease	8 (3.3%)	1 (0.8%)	0.187	7 (7.5%)	1 (3.3%)	0.349
Gastrointestinal bleeding	5 (2.1%)	0	0.245	2 (0.8%)	0	0.671
Chronic obstructive pulmonary disease	3 (1.2%)	1 (0.83%)	0.727	1 (1.25%)	0	0.932
Ruptured aortic aneurysm	2 (0.8%)	1 (0.83%)	0.997	2 (2.5%)	0	0.671
Other causes	11 (4.4%)	1 (0.83%)	0.098	5 (6.25%)	0	0.318

DC: death certification, PM: postmortem, CAD: coronary artery disease, and MI: myocardial infarction.

Table 3: Association of ischaemic heart disease with ulcer type on postmortem examination.

Ulcer type	Death from IHD	Death from other causes	Total	P
Neuropathic	19 (79.2%)	5 (20.8%)	24	0.049
Ischaemic	31 (55.4%)	25 (44.6%)	56	
Total	50	30	80	

years. There was no significant difference between patients whose cause of death was ascertained and those whose cause of death was not obtained for both age (P = 0.174) and proportion of males to females (P = 0.230).

The control group consisted of 9 (50%) male and 9 (50%) female patients. Mean age was 75.88 ± 10.33 years. There was no significant difference between patients whose cause of death was ascertained and those whose cause of death was not obtained for both age (P = 0.107) and proportion of males to females (P = 0.670).

3.2. Causes of Mortality in DFU Patients and Control Group on Death Certification. Overall, IHD was the major immediate cause of death (stated as cause 1a, 1b, or 1c on the death certificate) in 111 (45.7%) DFU patients (Table 2). The IHD category comprised coronary atheroma and coronary artery disease which were reported as cause of death in 89/111, myocardial infarction (MI) or coronary thrombosis and occlusion in 47/111, and cardiac arrest in 5/111 death certificates. Each one of IHD subcategories may have been reported alone or in combination with one or more of the other subcategories. The "other cardiac causes" category comprised deaths due to hypertensive heart failure, myocardial degeneration and fibrosis, and haemopericardium.

The results were similar in the control group with IHD accounting for 55 (45.5%) of all deaths. In the IHD category, coronary atheroma and coronary artery disease were stated in 45/55, MI or coronary thrombosis and occlusion in 32/55, and cardiac arrest in 1/55 on death certificates.

There was no significant difference in the other causes of death between DFU patients and controls apart from a significantly higher number of deaths in the control group from cancer (19/121) compared with the DFU group (20/243) (odds ratio 2.077, 95% confidence interval 1.062–4.060, and P = 0.032) (Table 2).

3.3. Causes of Mortality in DFU and Control Groups on Postmortem Examination. Postmortem examination results were obtained for 80 DFU patients. The results confirmed that IHD was the commonest immediate cause of death and the proportion had risen to 62.5% of all DFU deaths compared with 45.7% from death certification (Table 2). The postmortem results in 30 control group patients showed a slightly higher proportion of IHD deaths, 70.0% (21) compared to 62.5% (50) for the DFU group. This difference, however, did not reach statistical significance (P = 0.465) (Table 2). On postmortem examination, there was no significant difference in other causes of death including cancer between the DFU and control groups.

3.4. Ulcer Type and Cause of Death as Determined by Postmortem and on Death Certification. To examine the relationship between cause of death and ulcer type, patients were stratified into two categories: neuropathic and ischaemic

Table 4: Deaths and causes of death in neuropathic and ischaemic ulcer patients on death certification and postmortem examination.

Cause of death	Neuropathic on DC ($n = 56$)	Ischaemic on DC ($n = 187$)	P	Neuropathic on PM ($n = 24$)	Ischaemic on PM ($n = 56$)	P
Ischaemic heart disease	28 (50%)	83 (44.4%)	0.460	19 (79.2%)	31 (55.4%)	0.049
(i) CAD/atherosclerosis	23	57	0.141	16	24	0.055
(ii) MI/coronary thrombosis	17	30	0.019	13	13	0.009
(iii) Cardiac arrest	0	5	0.519	0	0	0.116
Other cardiac causes	1 (1.8%)	12 (6.4%)	0.188	0	7 (12.5%)	0.176
Bronchopneumonia	7 (12.5%)	32 (17.1%)	0.411	1 (4.2%)	2 (3.6%)	0.082
Cancer	8 (14.3%)	12 (6.4%)	0.067	0	2 (3.6%)	0.606
Pulmonary thromboembolic disease	1 (1.8%)	7 (3.7%)	0.482	1 (4.2%)	5 (8.9%)	0.469
Cerebrovascular accident	1 (1.8%)	10 (5.4%)	0.285	0	1 (1.8%)	0.865
Septicaemia	2 (3.6%)	8 (4.3%)	0.816	0	0	0.678
Chronic obstructive pulmonary disease	0	3 (1.6%)	0.616	0	2 (3.6%)	0.606
Renal failure	4 (7.1%)	6 (3.2%)	0.205	0	0	0.678
Gastrointestinal bleeding	1 (1.8%)	4 (2.1%)	0.870	1 (4.2%)	0	0.232
Ruptured abdominal aortic aneurysm	1 (1.8%)	1 (0.5%)	0.392	1 (4.2%)	1 (1.8%)	0.544
Other	2 (3.6%)	9 (4.8%)	0.701	1 (4.2%)	4 (7.1%)	0.619

DC: death certification, PM: postmortem, CAD: coronary artery disease, and MI: myocardial infarction.

foot ulcers. Of the 80 DFU patients who had postmortem examination, 24 were neuropathic and 56 were ischaemic DFUs. The postmortem data demonstrated that mortality from IHD was significantly higher in the neuropathic group compared to the ischaemic group (79.2% versus 55.4%, resp.) (odds ratio 3.064, 95% confidence interval 1.003–9.366, and $P = 0.049$) (Table 3). Specifically, there was a significantly increased mortality from myocardial infarction/coronary thrombosis in the neuropathic group (13/24) compared with ischaemic group (13/56) (odds ratio 3.909, 95% confidence interval 1.417–19.783, and $P = 0.009$) and also a trend to increased mortality from CAD/atherosclerosis in the neuropathic group (16/24) compared with ischaemic group (24/56) (odds ratio 2.667, 95% confidence interval 0.981–7.250, and $P = 0.055$) (Table 4). There was no other significant difference in the cause of death between patients with neuropathic and ischaemic ulcers.

Regarding patients whose death certificate was available, 56 had neuropathic and 187 had ischaemic ulcers. Death certificate data showed that mortality from myocardial infarction/coronary thrombosis was significantly increased in the neuropathic group (17/56) compared with the ischaemic group (30/187) (odds ratio 2.281, 95% confidence interval 1.434–4.551, and $P = 0.0193$) (Table 4). There was no other significant difference in the cause of death between patients with neuropathic and ischaemic ulcers.

3.5. Age at Death. We further determined the mean age at death in patients who had a postmortem examination. The mean age at death from IHD in the DFU group was five years lower than that in the control group (68.2 ± 8.7 years versus 73.1 ± 7.96 years, $P = 0.015$). In the neuropathic ulcer group, the mean age at death from IHD on postmortem was 67.9 ± 8.5 years compared with a mean age at death for the ischaemic ulcer patients of 68.5 ± 8.9 years ($P = 0.407$). The mean age at death for all causes combined (on death certificates and postmortem examinations) showed no significant difference between the DFU group and the control patients, 71.2 ± 11.1 years versus 72.8 ± 10.1 years ($P = 0.091$). The mean age at death from IHD (on death certificates and postmortems combined) in the DFU group was 69.5 ± 9.5 versus 72.6 ± 8.3 years for the control group ($P = 0.051$).

4. Discussion

This 11-year retrospective audit directly defines the precise causes of death in DFU patients. Postmortem results were examined to avoid inaccuracies as can occur with death certification [9]. The results confirmed IHD as the major cause of death in DFU patients. In particular, death from myocardial infarction was significantly higher in the neuropathic group compared with the ischaemic group both on death certification and postmortem findings. Our results concur with other studies which showed the increased mortality risk to be ascribed to cardiovascular disease in particular IHD [10, 11]. In addition, our study established that the risk of premature mortality from IHD is greater in patients who develop neuropathic ulceration. We have earlier addressed the role of IHD in the increased mortality rate associated with DFU through the application of a proportionate model of the DFU population [7]. We used the model to show that a 25% reduction in the number of neuropathic DFU patients dying earlier than nonulcerated subjects eliminated increased mortality. The link between neuropathic foot ulceration and the excess mortality from IHD demands further clarification.

Latest evidence shows that DFU has a major independent influence on lower extremity amputation and mortality risk which is quite apart from other baseline complications

[12]. Our postmortem data indicated a significantly high frequency of IHD in neuropathic ulcer patients (79.2%) compared to the ischaemic group (55.4%). This is in keeping with the study of Boyko et al. stating that, amongst patients with diabetic foot ulcers who died, 64% of these ulcers were judged to be due to neuropathy, and mortality was independent of macrovascular disease as measured by ankle-arm index [1]. More recent studies have confirmed that mortality in patients presenting with neuropathic ulcers was unexpectedly high with an average 14-year reduction in life expectancy related to neuropathy whether an ulcerated neuropathic or a Charcot foot [13].

4.1. Possible Reasons for Link between Neuropathy and Death

4.1.1. Peripheral Somatic Neuropathy. Large nerve fibre dysfunction related to diabetes, as measured by vibration perception threshold, is strongly linked with a high risk of foot ulceration [14]. It also predicts amputation and mortality even in young type 1 diabetes patients and is associated with increased cardiovascular risk [15]. Because of the established strong association between lower extremity neuropathy and diabetic foot lesions, death related to diabetic foot problems (including ulceration) has been used as an estimate of mortality associated with peripheral neuropathy [16]. In a 14-year observational study, the main microvascular complications of diabetes (peripheral sensory neuropathy and nephropathy) were associated with increased mortality in diabetic patients although abnormal vibration threshold was more strongly associated with increased mortality than other microvascular complications [17].

4.1.2. Autonomic Neuropathy. Patients with large fibre neuropathy also have evidence of small fibre neuropathy including autonomic neuropathy, which is associated with increased mortality from cardiovascular disease, particularly sudden cardiac death [18]. Peripheral autonomic neuropathy (small fibre) is associated with the development of foot ulceration in diabetic subjects. Measures of peripheral autonomic neuropathy in terms of peripheral vascular, cardiovascular, and neurophysiological measurements are worse in neuropathic ulcer patients when compared with nonulcerated patients [19]. Diabetic patients with autonomic dysfunction affecting cardiac efferent sympathetic signals have impaired sympathetically mediated dilation of coronary resistance vessels and the severity is related to the degree of sympathetic dysfunction. Impaired dilation of these vessels can lead to myocardial ischaemia and left ventricular dysfunction, even in the absence of overt atherosclerosis [20, 21]. Silent ischaemia is significantly more common in diabetic men with autonomic neuropathy than in those without as it prevents the development of angina pain. Evidence of fresh infarction may not always be found at postmortem in sudden deaths in patients with autonomic neuropathy [22].

4.1.3. Neuropathy and Calcification. Neuropathy is also closely linked to calcification of vascular smooth muscle, a process thought to be mediated by receptor activator of nuclear factor kappa B ligand (RANK-L)/osteoprotegerin signalling pathway implicated in coronary and peripheral vascular disease. Vascular calcification in diabetic neuropathy may be a significant factor in increased cardiovascular risk in neuropathic ulcerated patients independent of autonomic neuropathy and cardiac denervation [23]. Therapeutic options targeting these emerging pathways may help modulate macrovascular complications and have a beneficial effect on cardiovascular outcomes in this population of diabetes patients [24].

Neuropathy may also be a marker of associated nephropathy which is a well-established risk factor for cardiovascular death. Microalbuminuria, an independent predictor of progressive nephropathy, is associated with endothelial damage and reflects atherosclerotic disease and vascular dysfunction and has also been shown to be strongly associated with the development of diabetic foot ulcers in type 2 diabetic patients [25]. Patients with diabetic nephropathy have a high frequency of autonomic neuropathy and both factors are associated with and contribute independently to the risk of silent ischaemia [26]. Cardiac autonomic neuropathy is also an independent risk factor for cardiovascular morbidity and mortality in type 1 diabetic patients with nephropathy [27]. Moreover, survival after amputation is lower in diabetic patients with chronic kidney disease and those on dialysis and this may be related to the severity of neuropathy amongst other comorbidities in these patients [28]. Autonomic neuropathy, however, is difficult to diagnose on postmortem studies, let alone ascertain the degree of severity of autonomic dysfunction.

Some of the excess mortality has also been thought to be due to uncontrolled sepsis [12]. We have shown that foot infection with *Staphylococcus aureus*, which is a very common offender in DFUs, increases the mortality rate 2.6 times compared to those without *Staphylococcus* infection [29]. It is postulated that *Staphylococcus aureus* could increase the risk of mortality through a cytokine response, which might cause plaque rupture and subsequent death from myocardial infarction. Strong evidence exists for the importance of a vagus nerve-mediated pathway in controlling cytokine production essential for preventing pathological inflammation [30]. The activation of the efferent vagus nerve stimulates the release of acetylcholine which inhibits the release of tumor necrosis factor (TNF), high mobility group box-1 (HMGB1), and other proinflammatory cytokines from resident tissue macrophages (the cholinergic anti-inflammatory pathway) without affecting the production of anti-inflammatory cytokines. This inhibits excessive systemic inflammation and protects against endotoxaemia and ischaemia reperfusion injury [31, 32]. This process is attenuated in autonomic dysfunction resulting in decreased vagus nerve anti-inflammatory output, which might be associated with loss of tonic suppression of inflammatory processes [30]. Thus neuropathic patients may be at increased risk of more flagrant inflammation and more extensive endothelial dysfunction of their coronary arteries.

Potential confounding factors of the relationship between cause of death and ulcer type include ageing. Neuropathy is frequently associated with ageing [33]. The increased mean age of this sample increases the possibility of IHD being

a late diabetic complication. On the other hand, published data show that diabetes *per se* is associated with excess mortality, even in an area with high background death rates from cardiovascular disease [34]. More recently, studies have implicated QTc prolongation in type 2 diabetes patients with foot ulcers in the excess mortality observed in these patients [35].

4.2. Strengths and Limitations of Study. The use of our local nonulcerated diabetic population, as opposed to the general population, as a control group minimised bias in the estimates of relative mortality rates. The classification of causes of death facilitated comprehensive comparisons of all causes of mortality between the two groups with and without foot ulcers. Notwithstanding the loss to follow-up rate of 9.3% in acquiring the cause of death in DFU patients, the data collection method allowed for representation of most of the population in the South East of England referred with foot ulceration to this centre with no specific groups excluded. It was not possible to acquire death certificates in 25 of the DFU group and 18 of the controls. However, there was no significant difference between patients whose cause of death was ascertained and those whose cause of death was not obtained in both DFU and control patients for both age and proportion of males to females. This retrospective study looks primarily at the causes of death in DFU patients. It was not feasible to look at lifestyle factors or analyse the events preceding their deaths. It was also not possible to adjust for independent risk factors for IHD in the neuropathic group, for example, lipid profile and smoking habits. Other comorbid factors such as the presence of nephropathy have not been looked at. It is accepted that the deaths took place from 1989 up to 2000. Since then, newer diabetes treatments may have improved cardiovascular outcome. However, despite modern treatments, mortality of diabetic foot patients is still high [36].

Controlling for DFU patients also poses a significant problem as the general population does not provide specific controls, which in the case of DFU patients is difficult to determine. Although the DFU group had a higher proportion of males compared with the control group, the frequency of the causes of death was similar. While death certification showed a significantly higher number of deaths in the control group from cancer compared with the DFU group, this was not confirmed on postmortem studies. Data was not available for the presence of ischaemia or neuropathy in the control group. This emphasises the importance of a prospective study to account more accurately for levels of ischaemia and neuropathy and possibly the rate of change of these baseline categorisations.

5. Conclusion

IHD has long been recognised as an increased risk for individuals with diabetes. Our results suggest that ulcer type influences the incidence of IHD. This study provides strong evidence to reiterate its importance as a factor, placing neuropathic diabetic foot ulcer patients at a considerable risk of premature death. We have attempted to elucidate the mechanisms implicating IHD in the excess premature mortality in neuropathic DFU patients. The survival benefits of introducing an aggressive cardiovascular risk management programme in DFU clinics have also been proven and this can direct future implementation of national programmes [37].

Abbreviations

DFU: Diabetic foot ulcer
IHD: Ischaemic heart disease.

Competing Interests

There is no relevant conflict of interests to disclose.

Authors' Contributions

Dr. N. K. Chammas researched data, analysed data, and wrote the paper. Dr. R. L. R. Hill reviewed and analysed data and contributed to statistics and discussion. Professor M. E. Edmonds initiated and directed research project and edited the paper. Dr. N. K. Chammas is the guarantor of this work, and as such, had full access to all the data in the study and takes responsibility for the integrity of data and the accuracy of the data analysis.

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Research Article

Diabetic Microangiopathy Is an Independent Predictor of Incident Diabetic Foot Ulcer

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Aim. To determine the diabetic foot ulcer incidence and examine its association with microangiopathy complications, including diabetic retinopathy (DR) and albuminuria (Alb), in type 2 diabetes patients. *Methods.* This was a retrospective cohort study of 1,305 patients with type 2 diabetes who were assigned to the following groups: Category 1, normoalbuminuria without DR ($n = 712$); Category 2, Alb without DR ($n = 195$); Category 3, normoalbuminuria with DR ($n = 185$); and Category 4, Alb with DR ($n = 213$). Cox proportional hazard models were used to compare the risks of developing diabetic foot ulcers across the categories. *Results.* During 14,249 person-years of follow-up, 50 subjects developed diabetic foot ulcers, with incidence rates of 1.6/1,000, 1.5/1,000, 3.4/1,000, and 12.5/1,000 person-years in Categories 1, 2, 3, and 4, respectively. After adjusting for the presence of diabetic neuropathy and macroangiopathy, the hazard ratios and 95% confidence intervals (CIs) for the risk of diabetic foot ulcer development were 0.66 (95% CI, 0.18–2.36), 1.72 (95% CI, 0.67–4.42), and 3.17 (95% CI, 1.52–6.61) in Categories 2, 3, and 4, respectively, compared with Category 1. *Conclusion.* The presence of DR and Alb significantly increases the risk of diabetic foot ulcer development.

1. Introduction

Diabetic complications have become a serious issue in Japan, and the dramatic rise in the number of patients with diabetes has exacerbated this problem. However, awareness of one serious health condition, namely, diabetic foot disease, is inadequate among patients and healthcare providers [1, 2]. In 2008, the Japanese government introduced a management fee for diabetic complications to facilitate the prevention of diabetic foot ulcers. Consequently, the number of foot care outpatient clinics that specialize in preventing diabetic foot ulcers has risen along with the increase in the number of patients with diabetes in Japan. However, only a few Japanese reports are available that describe investigations into the long-term occurrence of diabetic foot ulcer and its risk factors [3]. Although diabetic neuropathy and angiopathy have been reported as classical risk factors for diabetic foot ulcer [1, 4], few studies' findings demonstrate the association between the occurrence of diabetic foot ulcer and microangiopathy complications, including albuminuria (Alb) and diabetic retinopathy (DR). Indeed, patients with advanced DR or Alb are very likely to develop diabetic neuropathy or angiopathy. Moreover, DR reduces visual acuity in adults, and the findings from previous studies have shown that a reduction in visual acuity is a risk factor for diabetic foot ulcer [4, 5]. In addition, Alb and elevated serum creatinine (sCr) levels have been reported as risk factors for diabetic foot ulcer [6, 7].

To determine the diabetic foot ulcer incidence and examine the association between diabetic foot ulcer and microangiopathy complications, namely, DR and Alb, we conducted a retrospective study of 1,305 patients with type 2 diabetes who had received in-hospital foot care education.

2. Materials and Methods

2.1. Study Design and Patients. This retrospective cohort study was conducted at Saiseikai Central Hospital. All subjects who had undergone a 2-week education program for diabetic patients between January 1999 and May 2005 were considered for inclusion in the study. During hospitalization, all subjects attended a 1-hour group lecture about foot care. The following exclusion criteria were applied: (1) the presence of an active diabetic foot ulcer at the time of inclusion, (2) an estimated glomerular filtration rate (eGFR) $< 30\,\text{mL/min/1.73}\,\text{m}^2$ or a strong suspicion of renal disease other than diabetic nephropathy, for example, glomerulonephritis, and (3) a follow-up period < 1 year. Of the 1,958 study candidates, 648 were transferred to other facilities for medical or personal reasons, or they withdrew from the study for unknown reasons within 1 year, 3 subjects had eGFRs $< 30\,\text{mL/min/1.73}\,\text{m}^2$, and 2 subjects had active diabetic foot ulcers. Consequently, 1,305 subjects were included in the cohort. Data relating to the patients' ages, sexes, body mass indexes, diabetes durations, and smoking statuses were collected from the medical records at the time of hospitalization. Past histories of cardiovascular disease, including the occurrences of myocardial infarctions, coronary interventions, and ischemic strokes, and the presence of diabetic neuropathy and angiopathy were determined from the patients' medical records.

2.2. Clinical Measurements and Assessments. Blood samples were collected in the early morning of day 2 of hospitalization following an overnight fast. The glycosylated hemoglobin (HbA1c) (%; NGSP) levels on admission were determined using high-performance liquid chromatography.

The presence of diabetic neuropathy was evaluated using the Semmes-Weinstein monofilament test and the vibratory sensation test [4]. The Semmes-Weinstein monofilament test involved assessing a foot's sensation using a 10 g monofilament at five sites on the plantar aspect of each foot, namely, the hallux, and the first, second, third, and fifth metatarsal heads [8]. The results from the vibratory sensation test were considered abnormal if the perception of vibration ceased within 10 s after a 128-Hz tuning fork was applied to the medial malleolus. If the sensations from the 10-g monofilament or the vibration sensation tests were reduced, the patient was diagnosed as having sensory neuropathy.

When the patients had histories of intermittent claudication and rest pain, peripheral arterial disease, or absent or reduced pedal pulses, ankle brachial index (ABI) measurements were performed. Every patient rested in a supine position for at least 5 min in a quiet room before the ABI was measured. If either or both ABI values were ≤0.9, the patient was diagnosed with angiopathy.

In addition, 24-hour urine samples were collected from the patients from day 2 until day 3 of hospitalization. The clinical staging of diabetic nephropathy was based mainly on the urinary albumin excretion rate (AER) and, at the advanced stage, it was based on reductions in the eGFR. Alb was defined as a urinary AER $>20\,\mu\text{g/min}$ that was determined during the 24-hour urine sample collection. The eGFR, which was used as an index of nephropathy, was obtained using the following formula [9]:

$$\text{eGFR} = 194 \times \text{sCr}^{-1.094} \times \text{age}^{-0.287} \times (\text{woman})\, 0.739. \quad (1)$$

All of the subjects underwent funduscopic examinations that were carried out by a qualified ophthalmologist during or just before hospital admission. A diagnosis of DR was made based on the Davis classification.

The subjects were categorized into four groups based on their diabetic microangiopathy complications at baseline, as follows: Category 1, normoalbuminuria without DR (n = 712); Category 2, Alb without DR (n = 195); Category 3, normoalbuminuria with DR (n = 185); and Category 4, Alb with DR (n = 213).

The subjects were treated for diabetes mellitus and, when necessary, for coexisting diseases at our outpatient clinics at intervals of between 1 and 4 months, depending on the status of each disease. The follow-up histories for incident diabetic foot ulcers were obtained from the medical records. The data used in the present study were collected by May 1, 2015.

2.3. Ethical Considerations. The study's protocol abided by the Japanese government's ethical guidelines for epidemiological research, and it was reviewed and approved by the ethics committee of Saiseikai Central Hospital (Protocol number 311). All of the procedures were undertaken in accordance with the ethical standards established by the institutional and national committees on human experimentation and in accordance with the Declaration of Helsinki.

2.4. Statistical Analyses. The patients' baseline characteristics were determined after they had been stratified into the four categories. The differences among the categories were evaluated using a one-way analysis of variance. The diabetic foot ulcer incidence was calculated in person-years. The 5-year and 10-year cumulative diabetic foot ulcer incidence rates were estimated using the Kaplan-Meier method. The log-rank test was used to examine the differences among the four patient categories in relation to the diabetic foot ulcer incidence. We constructed Cox proportional hazard models to estimate the associations between diabetic complications and the diabetic foot ulcer incidence, using the follow-up time as a dependent variable. Age-, sex-, and multivariable-adjusted hazard ratios (HRs) were calculated using Cox proportional hazards regression models. Model 1 was the age- and sex-adjusted model. The multivariable-adjusted models were adjusted for the presence of diabetic neuropathy alone (Model 2) and for the presence of diabetic neuropathy and angiopathy (Model 3). The multivariable-adjusted HRs were used to calculate the relative risks of diabetic foot ulcer occurrence in each diabetic microangiopathy complications category. A P value < 0.05 was considered to indicate statistical significance. STATA version 10 (StataCorp LP, College Station, TX, USA) was used for the statistical calculations.

3. Results

3.1. Baseline Clinical Characteristics of the Patients in the Four Categories. Table 1 shows the baseline characteristics of the study participants who were grouped according to their diabetic microangiopathy complications. The mean age of the study cohort was approximately 60 years, approximately 70% of the patients were men, and the mean diabetes duration was approximately 10 years. The mean HbA1c level was 9.1%. Of the 1,305 study participants, 54.6% ($n = 712$) were assigned to Category 1, 14.9% ($n = 195$) were assigned to Category 2, 14.2% ($n = 185$) were assigned to Category 3, and 16.3% ($n = 213$) were assigned to Category 4. Several baseline characteristics showed significant differences among the microangiopathy complications categories. For example, the subjects in Category 4 were older, were predominantly male, and they had longer disease durations compared with the subjects in the other categories.

3.2. Cox Regression Analysis of Albuminuria and Diabetic Retinopathy at Baseline and the Diabetic Foot Ulcer Incidence. Of the 1,305 subjects evaluated, 93 died, 28 were transferred to other facilities for medical or personal reasons, and 201 withdrew for unknown reasons during the 10-year follow-up period. The follow-up rate at 10 years was 75.3%. During the median follow-up period of 11.8 years, 50 cases were diagnosed with new-onset diabetic foot ulcers, yielding 14,291 person-years (Table 2). The annual diabetic foot ulcer incidence rates were 1.6, 1.5, 3.4, and 12.5 per 1,000 person-years in Categories 1, 2, 3, and 4, respectively.

The Kaplan-Meier curves showed that the individuals in Category 4 had a significantly higher diabetic foot ulcer incidence (log-rank test, $P < 0.001$) (Figure 1). The 10-year cumulative diabetic foot ulcer incidence increased according to the microangiopathy complications category, with 1.7%, 1.2%, 4.2%, and 12.7% of subjects developing diabetic foot ulcers in Categories 1, 2, 3, and 4, respectively (Table 2).

Associations between the presence of DR, Alb, diabetic neuropathy, and angiopathy and the diabetic foot ulcer incidence were identified using Cox regression analysis (Table 3). After adjusting for age and sex (Model 1), the presence of diabetic microangiopathy complications was positively and significantly associated with the diabetic foot ulcer incidence. In Model 3, which was additionally adjusted for the presence of diabetic neuropathy and angiopathy, the HRs and the 95% confidence intervals (CIs) for the diabetic foot ulcer incidence were 0.66 (95% CI, 0.18–2.36), 1.72 (95% CI, 0.67–4.42), and 3.17 (95% CI 1.52–6.61; $P = 0.002$) for Categories 2, 3, and 4, respectively, compared with Category 1.

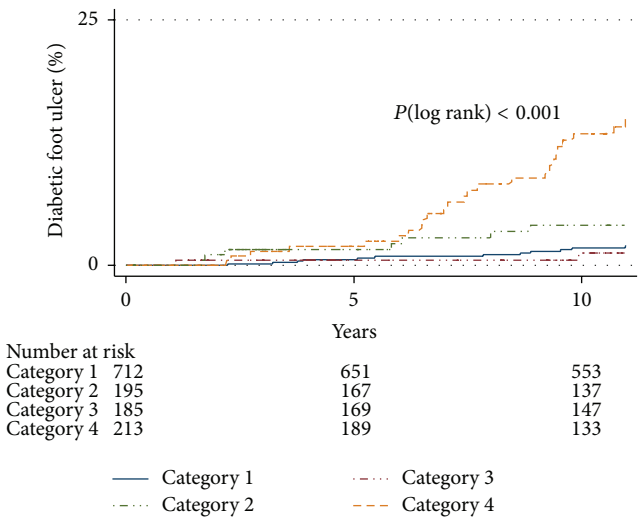

Figure 1: Unadjusted Kaplan-Meier estimates of the occurrence of diabetic foot ulcers over 10 years. The data shown are for patients with different microangiopathy complications at baseline.

4. Discussion

This study aimed to determine the diabetic foot ulcer incidence in patients with type 2 diabetes and to investigate the association between diabetic foot ulcer and microangiopathy. Following in-hospital education, the 5-year and 10-year cumulative diabetic foot ulcer incidence rates in patients with type 2 diabetes were 1.9% and 3.7%, respectively. Importantly, the 10-year cumulative diabetic foot ulcer incidence rate in Category 4, which comprised patients with both DR and Alb, was 12.7% and was up to threefold higher than the 10-year cumulative diabetic foot ulcer incidence rate in Category 1. After adjusting for the presence of diabetic neuropathy and angiopathy, the presence of diabetic microangiopathy complications was positively and significantly associated with the occurrence of diabetic foot ulcers. The findings from this study provide information about the risk of patients developing diabetic foot ulcers, and they enable more realistic risk estimations in relation to the occurrence of diabetic foot ulcers in individuals with DR and Alb.

Although the number of foot care outpatient departments has grown in response to the increase in the number of patients with diabetes in Japan, few long-term follow-up studies have investigated the diabetic foot ulcer incidence, and the results from short-term studies only are available in the literature [3]. Reports from Asian countries are particularly uncommon [10], and an association between the diabetic foot ulcer incidence and the presence of DR and/or Alb has not been proposed. Diabetic microangiopathy progresses with the duration of a patient's diabetes, and the diabetic complications observed are interrelated. The results from this study may enable type 2 diabetes patients with DR and/or Alb to understand the actual risk in relation to diabetic foot ulcer development. Moreover, the 10-year cumulative diabetic foot ulcer incidence rates in the patients in Categories 3 and 4, which included those with DR, were higher than the 10-year cumulative diabetic foot ulcer incidence rates in the patients in Categories 1 and 2, which contained patients who did not have DR, indicating that DR is a particularly important factor associated with the occurrence of diabetic foot ulcers. Indeed, Boyko et al. [6] and Leese et al. [11] found that poor vision, which was defined as a corrected visual acuity of 20/40 or lower, is a risk factor for diabetic foot ulcer. In addition, DR is a risk factor for all causes of death, cardiovascular events, and arteriosclerosis. In their study, Ohtomo et al. [12]

Table 1: Baseline characteristics of the study population according to the microangiopathy complications category.

	All patients	Category 1: NA (DR−)	Category 2: Alb (DR−)	Category 3: NA (DR+)	Category 4: Alb (DR+)	*P* values
Number of patients, *n* (number of men, *n*)	1,305 (904)	712 (472)	195 (156)	185 (112)	213 (164)	
Age (years)	59.9 ± 9.1	59.4 ± 8.9	60.1 ± 9.3	61.4 ± 8.8	60.3 ± 9.4	0.03
BMI (kg/m^2)	23.8 ± 3.9	23.4 ± 3.6	25.5 ± 4.7	22.8 ± 3.4	24.3 ± 4.1	<0.001
Diabetes mellitus duration (years)	10.3 ± 8.2	8.4 ± 7.3	8.5 ± 7.9	14.3 ± 8.0	15.1 ± 8.6	<0.001
HbA1c (%; NGSP)	9.2 ± 1.8	9.1 ± 1.8	9.2 ± 1.8	9.1 ± 1.5	9.5 ± 1.8	0.0092
Neuropathy, *n* (%)	520 (39.8)	202 (28.4)	67 (34.4)	99 (53.5)	152 (71.4)	<0.001
Angiopathy, *n* (%)	33 (2.5)	11 (1.5)	9 (4.6)	2 (1.1)	11 (5.2)	0.003
Prior cardiovascular disease, *n* (%)	136 (10.4)	49 (6.9)	29 (14.9)	25 (13.5)	33 (15.5)	<0.001
Smoker, *n*, none/past/current	707/276/322	388/153/171	86/53/56	118/30/37	115/40/58	0.011
Treated with insulin, *n* (%)	528 (40.5)	211 (29.6)	66 (33.9)	111 (60.0)	140 (65.7)	<0.001
Diabetic retinopathy, *n*, none/background/more advanced stage or prior photocoagulation	907/259/139	712/0/0	195/0/0	0/125/60	0/134/79	<0.001
eGFR (mL/min/1.73 m^2)	79.3 ± 20.1	80.8 ± 18.3	77.8 ± 22.4	78.9 ± 20.6	75.7 ± 22.5	0.02
Diabetic nephropathy, *n*, none/microalbuminuria/macroalbuminuria	897/327/81	712/0/0	0/173/22	185/0/0	0/154/59	<0.001

BMI: body mass index; HbA1: glycated hemoglobin; NGSP: National Glycohemoglobin Standardization Program; eGFR: estimated glomerular filtration rate; NR: normoalbuminuria; Alb: albuminuria; DR: diabetic retinopathy.

Data are expressed as the number of patients (%), number of patients, or the means ± standard deviations.

Table 2: Diabetic foot ulcer incidence according to the microangiopathy complications category at baseline.

Category	*n*	Number of cases, *n*	Time at risk (person-years)	Incidence (per 1,000 person-years)	5-year cumulative incidence (%)	(95% CI)	10-year cumulative incidence (%)	(95% CI)
Category 1	712	13	8,063	1.6	1.2	(0.4–3.1)	1.7	(0.9–3.1)
Category 2	195	3	2,025	1.5	1.1	(0.2–7.6)	1.2	(0.3–4.8)
Category 3	185	7	2,054	3.4	3.4	(1.1–10.4)	4.2	(2.0–8.8)
Category 4	213	27	2,148	12.5	3.9	(1.4–10.4)	12.7	(8.4–19.1)
Total	1,305	50	14,291	3.5	1.9	(1.1–3.3)	3.7	(2.7–5.0)

CI: confidence interval.

Table 3: Hazard ratios for diabetic foot ulcer in the adjusted models according to the microangiopathy complications category at baseline.

	Model 1 HR (95% CI)	*P* values	Model 2 Adjusted HR (95% CI)	*P* values	Model 3 Adjusted HR (95% CI)	*P* values
Category 1	1.00		1.00		1.00	
Category 2	0.74 (0.21–2.64)	0.64	0.73 (0.21–2.59)	0.62	0.66 (0.18–2.36)	0.52
Category 3	2.26 (0.91–5.69)	0.08	1.60 (0.63–4.07)	0.32	1.72 (0.67–4.42)	0.25
Category 4	6.84 (3.48–13.41)	<0.001	3.91 (1.91–7.95)	<0.001	3.17 (1.52–6.61)	0.002
Neuropathy	—	—	4.37 (2.03–9.43)	<0.001	3.67 (1.66–8.01)	0.001
Angiopathy	—	—	—		7.74 (3.87–15.5)	<0.001

Model 1: adjusted for age and sex; Model 2: Model 1 + adjusted for neuropathy; Model 3: Model 2 + adjusted for angiopathy.
CI: confidence interval; HR: hazard ratio.

found that approximately 20% of the patients with DR had coronary artery disease, which may reflect impaired vascular perfusion caused by arteriosclerosis. In another study, Ogawa et al. [13] reported that diabetic neuropathy was present in all of the patients with type 2 diabetes who were hospitalized for diabetic foot ulcers and that DR and nephropathy were present in 96.4% and 78.6% of the patients, respectively. Thus, peripheral arterial disease may be present in patients with early DR. The incidence of DR in Japanese patients with type 2 diabetes is 38.3/1,000 person-years, and the DR progression rate has been reported as 21.1/1,000 person-years [14]. Since subjective symptoms are often absent, even when DR is at an advanced stage, it is important to encourage patients with definitively diagnosed or suspected diabetes to visit an ophthalmology department.

The diabetic foot ulcer incidence has been investigated previously. From their study, Peters and Lavery [15] reported that during the 3-year follow-up period, foot ulceration occurred in 14.3%, 18.8%, and 55.8% of the patients with diabetic neuropathy, diabetic neuropathy combined with foot deformities or peripheral arterial disease, and previous histories of foot ulceration, respectively. Moss et al. [16] followed up with 906 patients who were diagnosed with type 2 diabetes before they were 30 years old and 984 patients who were diagnosed with type 2 diabetes after they were 30 years old for 14 years, and they found that amputations caused by diabetic foot ulcers were required in 7.2% and 9.9% of these patients, respectively, and they concluded that severe retinopathy was a risk factor for amputation. In our study cohort, the diabetic foot ulcer frequency was lower than the frequencies reported from these aforementioned studies, which could be a consequence of the in-hospital education that our patients received. In a Japanese study, Oe et al. [3] followed up with 578 patients with diabetes who visited a specialized outpatient department for 5 years, and they found diabetic foot ulcers in 6 (1.2%) patients. However, 70% of the subjects did not have diabetic neuropathy or angiopathy [3]; therefore, the patients' backgrounds differed from those in the current study, in which 40% of the patients had diabetic neuropathy.

Despite the patients in the current study attending a group lecture about foot care as part of an in-hospital education program, the patients with type 2 diabetes, DR, and Alb developed diabetic foot ulcers, and a sharp increase in the cumulative diabetic foot ulcer incidence was observed after 5 years (Figure 1). Therefore, since the complications associated with diabetes tend to progress with its duration, patients with microangiopathy may require recurring outpatient education programs.

Several limitations should be considered when interpreting the results from the present study. First, this was a hospital-based cohort study; hence, a selection bias cannot be excluded. Second, a considerable percentage of the subjects were lost to follow-up during the study period. In addition, changes in the glucose levels and changes to the therapeutic regimens were not considered during the outpatient treatment period. Furthermore, we did not identify an association between DR and visual acuity. Finally, the impacts of the foot care and education programs on the study's results are unknown. Future investigations into the occurrence of diabetic foot ulcer in Japan should account for these limitations.

5. Conclusions

The findings from this study showed that diabetic individuals with DR and Alb were at a significantly increased risk of developing diabetic foot ulcers. From the perspective of a diabetic patient's evaluation of risk, the presence of asymptomatic DR and/or Alb may not be considered serious; hence, early intervention and long-term follow-up programs are crucial to prevent diabetic foot ulcers.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Masuomi Tomita wrote the paper and analyzed the data. Mari Okisugi, Takeshi Katsuki, Yoichi Oikawa, and Yoshihito Atsumi reviewed/edited the paper. Yusuke Kabeya, Kempei Matsuoka, and Akira Shimada contributed to the discussion and reviewed/edited the paper.

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Research Article

Epidemiology of Diabetic Foot Ulcers and Amputations in Romania: Results of a Cross-Sectional Quality of Life Questionnaire Based Survey

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This is a post hoc analysis of quality of life in diabetic neuropathy patients in a cross-sectional survey performed in 2012 in Romania, using the Norfolk QOL-DN in which 21,756 patients with self-reported diabetes were enrolled. This current analysis aims to expand research on the diabetic foot and to provide an update on the number of foot ulcers found in Romania. Of the 21,174 patients included in this analysis, 14.85% reported a history of foot ulcers and 3.60% reported an amputation. The percentage of neuropathy patients with foot ulcers increased with age; the lowest percentage was observed in the 20–29-year age group (6.62%) and the highest in the 80–89-year age group (17.68%). The highest number of amputations was reported in the 70–79-year age group (largest group). Compared to patients without foot ulcers, those with foot ulcers had significantly higher scores for total DN and all its subdomains translating to worse QOL ($p < 0.001$). This analysis showed a high rate of foot ulcers and amputations in Romanian diabetic patients. It underscores the need for implementation of effective screening and educational programs.

1. Introduction

Diabetes represents a major risk factor for lower limb amputations; it has been estimated that the presence of diabetes is associated with a 20-fold higher risk of lower limb amputations as compared to people without diabetes [1]. Diabetes-related foot ulcers have been reported with an annual incidence of 2% and a lifetime risk of 25% and are considered a major cause of nontraumatic lower extremity amputations [2]. Additionally, it has been shown that these complications have a major impact on the quality of life (QOL) and psychological status of diabetic patients [3, 4] and, as a consequence, the patients' QOL has been recognized as a measure of treatment effect [5].

Due to increased healthcare resources utilization [6] and work-loss associated costs, diabetic foot ulcers and amputations represent a major burden for the healthcare systems in both developed and developing countries. According to a health economic analysis performed in the USA, the diabetic foot ulcers are associated with $9 billion to $13 billion

increase in the direct yearly costs, thus doubling the costs of diabetes care [6]. In Romania, extrapolating the results of local studies (unpublished data), we have estimated an annual direct expenditure only for lower extremity amputations in patients with diabetes, of around 2.5 mil EUR. In the context of increasing incidence and prevalence of diabetes, a decrease in the prevalence of ulcers and lower limb amputations cannot be expected without specifically designed population interventions.

Limited data on epidemiology of diabetic foot ulcers and lower limb amputations are available for Romania [7, 8]. A research study performed in 2003, including data from several diabetes clinics from Romania, reported that the prevalence of foot ulcers was 3.2% in patients with type 1 diabetes and 3.8% in patients with type 2 diabetes [7]. Recently, an analysis of the number of lower limb amputations in patients with diabetes showed an increasing trend between 2006 and 2010 [8]. This increase was attributable to a dramatic increase in the rates of amputations in persons with type 2 diabetes as compared to 2006; since then, the number of amputations in this population increased with 16.96% in 2007, 60.75% in 2008, 66.91% in 2009, and 104.64% in 2010 [8]. To the best of our knowledge, no additional data are available on the incidence or prevalence of diabetes foot ulcers for this population. However, it is known that the incidence of lower extremity amputations is a marker of the quality of diabetic foot disease management [9, 10], with high amputation possibly attributable to inadequate education of patients and late presentation or inadequate resources for the management of the diabetic foot [11].

The analysis presented here aims to expand the research on the status of the diabetic foot in Romania and to provide an up-to-date status on the frequency of foot ulcers. This is a post hoc analysis of the Quality of Life in Patients with Diabetic Neuropathy in Romania Study (QOL-DN Romania), which had the main objective to assess the prevalence of self-reported diabetic neuropathy in Romanian population and its impact on the QOL by using the Norfolk QOL-DN "fiber-specific" questionnaire, professionally translated to Romanian. It was a cross-sectional survey performed in 2012 which enrolled 21,756 patients with diabetes and showed prevalence of neuropathy of 79% in this population [12].

2. Materials and Methods

2.1. Protocol and Survey Population. This was a cross-sectional survey in which 25,000 Romanian-translated Norfolk QOL-DN questionnaires were distributed by 181 Romanian healthcare providers (153 physicians (diabetes specialists), 5 neurologists, 14 general practitioners, and 9 nurses) to their patients with diabetes between January and December 2012. The Romanian version of the Norfolk QOL-DN is a self-administered questionnaire comprised of 16 items that capture demographic and medical history data (not scored) and 35 scored items related to patients' perception of their own health signs, symptoms, and the impact of diabetic neuropathy on their daily life over the previous 4 weeks. For the analysis of the nonscored items, we included age and the responses to the following questions: "Do you have diabetes?," "Do you have neuropathy?," "Have you ever had ulcers on your feet?," and "Have you ever had any amputation?" Total QOL and subdomain (physical functioning/large-fiber neuropathy, symptoms, activities of daily living (ADLs), autonomic neuropathy, and small-fiber neuropathy) scores were calculated based on responses to the scored items, with higher scores corresponding to poorer QOL. The survey design, the survey population, and a detailed description of the Romanian version of the Norfolk QOL-DN were previously reported [12].

All patients were informed that their personal data would be analyzed as part of a survey registered with the Romanian authorities and consented for their data to be included in the analysis. The survey was approved by the National Supervisory Authority for Personal Data Processing under number 0006753.22-03-2012.

2.2. Statistical Analysis. Frequency tables, contingency tables, and graphics were used for the description of the qualitative variables. The total QOL scores and the scores for each subdomain are presented as mean ± standard error (SE) and were compared using the Mann-Whitney test. The age is presented as mean ± standard deviation. For the variables presented as percentages, we tested the significance of differences by one-way analysis of variance, the Scheffé post hoc test, and the Chi-square test.

All descriptive and inferential analyses were performed using IBM SPSS Statistics for Windows, Version 15.0 (Armonk, NY: IBM Corp.). The significance threshold was $\alpha = 0.05$.

3. Results and Discussions

3.1. Results. As previously described [12], of the 25,000 questionnaires distributed, 23,543 were returned. Of these, after removing those not valid, with missing answer or "No" as an answer to the question "Do you have diabetes?," 21,174 were included in the present analysis. Of these, 13,812 patients answered "Yes" to the question "Do you have neuropathy?" and 7362 answered "No" to the same question. A history of foot ulcers was reported by 3088 (14.66%) patients with self-reported diabetes. The frequency of both foot ulcers and amputations was significantly higher among patients who self-reported neuropathy compared to those who did not self-report neuropathy (2,694/13,812 (19.50%) versus 299/6229 (4.8%) patients with a history of foot ulcers and 638/13,812 (4.62%) versus 89/7,362 (1.21%) patients with a history of amputation, resp.). Of the patients who answered "Yes" to the question "Do you have diabetes?," 750 (3.5%) reported that they had an amputation (Figure 1). Mean age was similar in the total group that reported diabetes irrespective of the history of neuropathy and foot ulcers, the group that reported neuropathy, the one that reported foot ulcers, and the one that reported amputations: 60.87 ± 11.31 years, 61.73 ± 10.99 years, 62.12 ± 11.06 years, and 62.44 ± 10.91 years, respectively.

The percentage of patients with neuropathy increased with age, from 39.34% in the 20–29-year age group to 76.91% in the 80–89-year age group. A similar trend was observed for the percentage of patients with foot ulcers among those with

Figure 1: Numbers of self-reported foot ulcers (a) and amputations (b) in the population included in the analysis stratified by self-reported neuropathy.

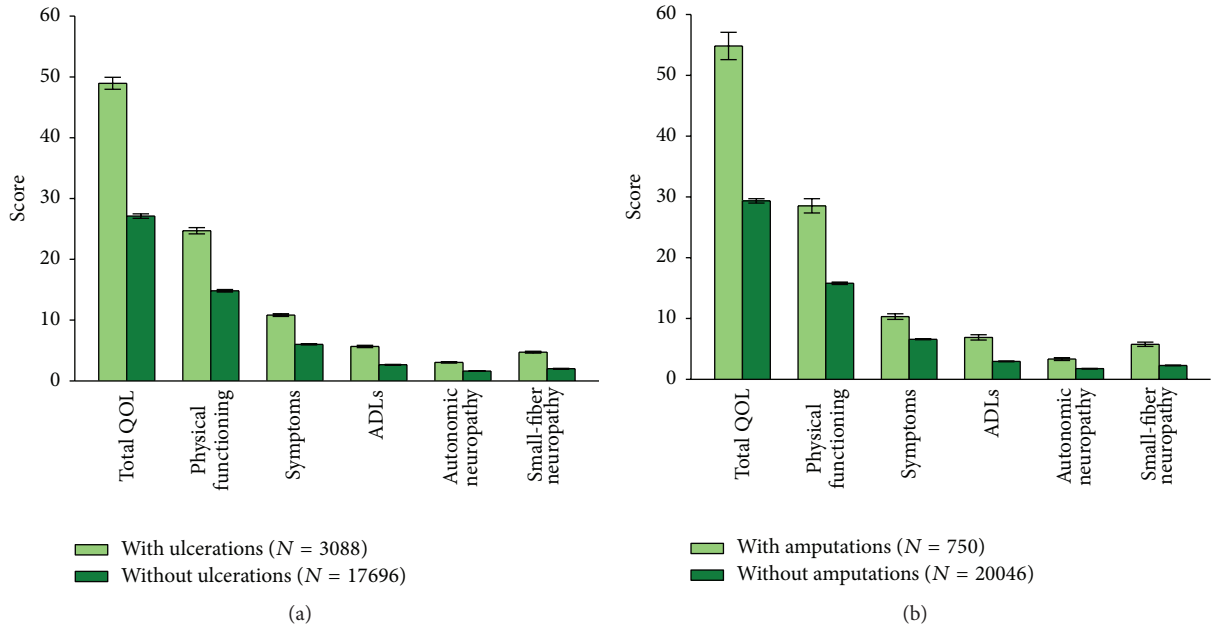

Figure 2: Norfolk QOL-DN total and subscale scores in Romanian patients with self-reported diabetes mellitus with and without foot ulcers (a) and with and without amputations (b). QOL: quality of life; ADLs: activities of daily living; N: number of patients in a given category.

neuropathy; the lowest percentage was observed in the 20–29-year age group (6.62%) and the highest in the 80–89-year age group (17.68%). The highest frequency of amputations was reported in the 70–79-year age group (15.03%) (Table 1).

In the whole analyzed group, the mean scores for total QOL, symptoms, ADLs, autonomic neuropathy, physical functioning/large-fiber neuropathy, and small-fiber neuropathy in those with and without foot ulcers and amputations are presented in Figure 2. Compared to patients without foot ulcers, those with foot ulcers had significantly higher scores for total QOL and all subdomains: 48.95 versus 27.12 for total QOL; 10.83 versus 6.01 for symptoms; 5.66 versus 2.66 for ADLs; 3.04 versus 1.62 for autonomic neuropathy; 4.72 versus 2.01 for small-fiber neuropathy; and 24.70 versus 14.82 for physical functioning/large-fiber neuropathy subdomain ($p < 0.001$). Similar differences were observed between those with amputations and those without amputations: 54.83 versus 29.36 for total QOL score; 10.31 versus 6.58 for symptoms;

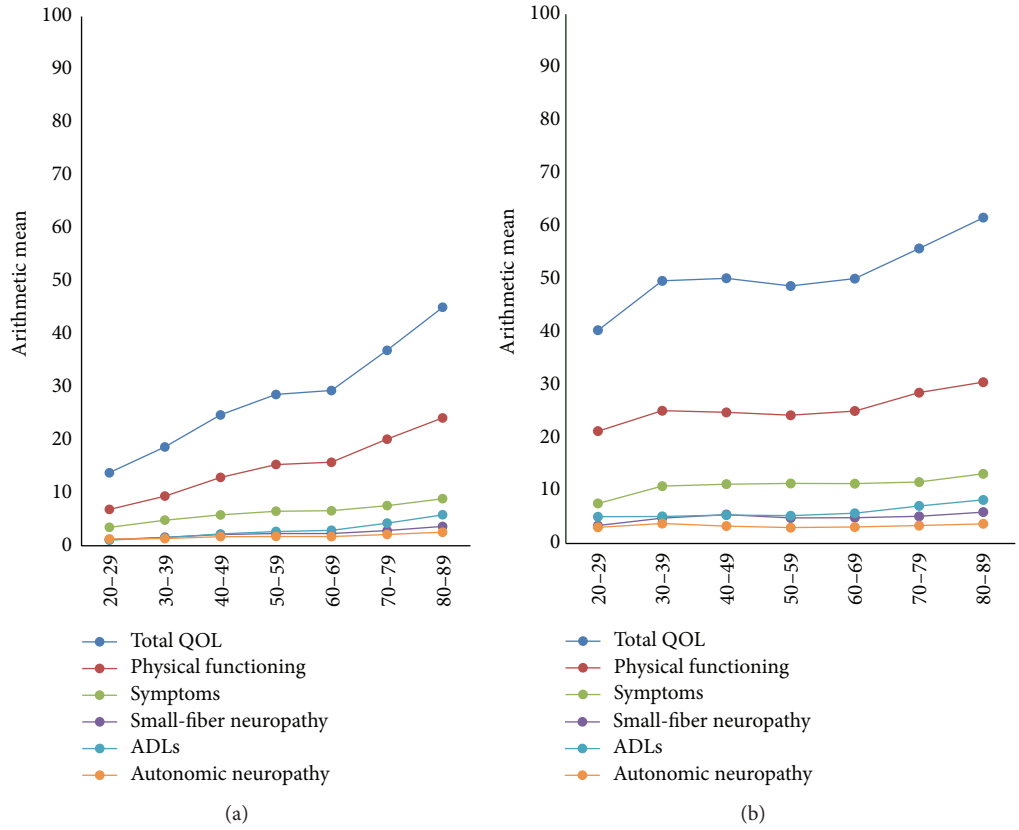

Figure 3: Norfolk QOL-DN total and subscale scores by groups of age in patients with self-reported diabetes mellitus (n = 21,174; $p < 0.001$, (a)) and diabetes, neuropathy, and foot ulcers (n = 815; only ADLs, $p < 0.05$, (b)).

Table 1: Patient distribution by age.

Age (years)		20–29	30–39	40–49	50–59	60–69	70–79	80–89
Total group (self-reported diabetes) with available data on age	n	272	555	1897	6413	7219	4000	758
Diabetes with neuropathy	n	107	310	1111	4127	4721	2817	583
	%	39.34	55.86	58.56	64.66	65.40	70.43	76.91
Diabetes, neuropathy with diabetic foot ulcers	n	18	44	212	787	886	601	134
	%	6.62	7.93	11.18	12.27	12.81	15.03	17.68
Diabetes, neuropathy with amputation	n	2	9	52	185	213	137	33
	%	0.74	1.62	2.74	2.88	2.95	33.43	4.35

n: number of patients in a given category; %: percentage.
Note: no additional data on amputation (i.e., major or minor) was collected.

6.89 versus 2.94 for ADL; 3.33 versus 1.76 for autonomic neuropathy; 5.76 versus 2.28 for small-fiber neuropathy; and 28.54 versus 15.79 for physical functioning subdomain ($p < 0.001$).

When data were compared according to the presence of neuropathy and foot ulcers, patients with neuropathy and a history of foot ulcers had the highest scores for total QOL and all subdomain scores: 51.53 for total QOL; 11.45 for symptoms; 6.00 for ADLs; 5.02 for small-fiber neuropathy; 3.21 for autonomic neuropathy; and 25.85 for physical functioning/large-fiber neuropathy subdomain. The presence of neuropathy with or without foot ulcers was associated with higher total QOL and subdomain scores as compared to the ones in patients with foot ulcers but without self-reported neuropathy. The lowest scores were reported in those with no self-reported neuropathy and no history of foot ulcers (Table 2).

Stratifying the total QOL scores on age decades (Figure 3), we observed that the QOL of a patient aged 20–29 years who reported previous foot ulcers is similar to the QOL of a patient aged 80 years with self-reported diabetes with or without neuropathy or foot ulcers.

3.2. Discussion. The analysis of this sample population of Romanian diabetic patients showed a high frequency of history of foot ulcers (14.6%). The frequency was significantly

Table 2: QOL differences in age groups shown in Norfolk QOL-DN total and subscale scores (mean ± standard error) in patients with self-reported diabetes mellitus according to the presence of self-reported neuropathy and foot ulcers.

	Self-reported neuropathy, no foot ulcers	Self-reported neuropathy, history of foot ulcers	No self-reported neuropathy, history of foot ulcers	No self-reported neuropathy, no foot ulcers
	$N = 10953$	$N = 2694$	$N = 299$	$N = 6130$
Total QOL	35.18 ± 0.24	51.53 ± 0.53	28.86 ± 1.42	12.91 ± 0.23
Physical functioning	18.96 ± 0.13	25.85 ± 0.28	15.87 ± 0.80	7.51 ± 0.14
Symptoms	7.93 ± 0.05	11.45 ± 0.12	6.08 ± 0.31	2.65 ± 0.046
Small-fiber neuropathy	2.73 ± 0.03	5.02 ± 0.08	2.14 ± 0.18	0.75 ± 0.03
ADLs	3.55 ± 0.04	6.00 ± 0.10	2.93 ± 0.26	1.09 ± 0.04
Autonomic neuropathy	2.01 ± 0.02	3.21 ± 0.06	1.83 ± 0.13	0.91 ± 0.02

QOL: quality of life; ADLs: activities of daily living.

higher among patients who self-reported neuropathy compared to those who did not self-report neuropathy (19.50% versus 4.06%) and increased with age from 6.62% in those aged 20–29 years to 17.68% in those aged 80–89 years. Our results are in line with previously reported data from the US that showed that the lifetime risk for a diabetic patient to experience a foot ulcer is 15 to 25% [13]. However, lower rates were previously reported in Europe; two cross-sectional community surveys performed in the UK showed that 5.3% to 7.4% of patients with diabetes had a history of foot ulcer [14, 15]. A study performed in Greece reported a rate of foot ulcers of 4.75% (95% confidence limits: 3.3%–6.2%) [16]; in a community in Sweden, 10% of the population included in the analysis had a history of foot ulcers and an additional 2% reported having present ulcers [17].

Although peripheral diabetic neuropathy is currently recognized as the leading risk factor for the development of the foot ulcers, it has been shown that the presence of this complication *per se* is not sufficient for the development of foot ulcers. Reiber et al. [18] showed in a clinical study that peripheral neuropathy was present in 78% of the patients who developed a foot ulcer, while foot deformities were present in 63% and peripheral vascular disease in 35% of the patients. We have not evaluated the frequency of peripheral arterial disease and of the neuropathy-associated foot deformities and therefore we cannot exclude the coexistence of these conditions in patients with self-reported neuropathy and a history of foot ulcers. Additionally, in patients without self-reported neuropathy, due to the lack of the objective evaluation of the neuropathy, we cannot claim that the foot ulcers were due to the peripheral artery disease in all cases.

In our survey, 3.50% of the patients reported a history of amputation; the frequency was significantly higher in the group with self-reported neuropathy compared to the one without (4.62% versus 1.21%). The majority of the information on the incidence and prevalence of the lower limb amputation is originating from hospital discharge data; therefore, a comparison with the reported data is difficult. In the UK and Spain, the incidence of the lower limb amputations was reported as ranging from 5.8 to 31 per 10^5 patients/year [19–21]. For Romania, the recently reported mean crude incidence of lower limb amputations in patients with diabetes is 21.3 per 10^5 [8]. Between 2006 and 2010, an increase in the lower limb amputation rates was observed for the patients with type 1 and type 2 diabetes combined, but the overall increase was due to an increase in the incidence of type 2 diabetes, while the rates in type 1 diabetes decreased [8]. This observation is in line with data from other European countries, all reporting a decrease in the incidence of the amputations in type 1 diabetes [21–23] and an increase in the amputations in type 2 diabetes [21, 22].

In our analysis, we observed an important frequency of foot ulcers and amputations in the active population aged 20–60 years. In these age groups, the frequency of foot ulcers and amputations was 6.62% and 0.74% in those aged 20–29 years and increased in parallel with age, reaching 12.27% and 2.88% in those aged 50–59 years. These results are similar to data published by Veresiu et al. [8], which showed an increase in the amputation rates for both type 1 and type 2 diabetes in persons aged 30–39 years from 2006 until 2010. The authors of this study reported a decrease in the incidence of lower limb amputations in the 20–29-year age group, while for the other age groups, the incidence increased for patients with type 2 diabetes and decreased for those with type 1 diabetes [8]. Our observations and the ones deriving from the data on the incidence of lower limb amputations are of special concern. Despite improvements in the standard of care of the diabetic foot in Romania, ulcers and amputations are being reported at young ages in the active population and are associated with higher direct and indirect costs. A possible explanation for this observation might be the variation in the delivery of preventive measures and foot care in patients at the local level; currently, physicians specialized in diabetic foot care are available only in large hospitals and large cities [8]. This may have led to limited access to specific education for an important part of the diabetic patients, with consequences on the level of health literacy, understanding and implementing preventive measures, and referral to the physician in early stages of the pathology.

To further evaluate the impact of the ulcers and amputations, we evaluated the QOL of these patients. The concomitant presence of neuropathy and ulcers or of neuropathy and amputations had a higher impact on the QOL than the presence of each of these alone. Additionally, the presence

of neuropathy alone (without a history of foot ulcers) had a higher impact on the QOL than the history of foot ulcers without self-reported neuropathy. It is accepted that patients with neuropathy and those with neuropathy and chronic foot ulcers or amputations have lower QOL compared to their diabetic fellows without neuropathy [3, 4, 24].

We acknowledge that our analysis is based on patients' self-reported information and therefore has all the limitations of such kinds of studies. The most important one is the recall bias which can influence patient's ability to correctly report previous diagnoses. At the same time, it is important to mention that the Norfolk QOL questionnaire used is a rigorously validated instrument that has the ability to discriminate between patients with and without diabetic neuropathy and between different stages of neuropathy severity [25, 26]. A recent systematic review of disease-specific measurement instruments for health-related QOL in diabetic neuropathy [27] concluded based on the evidence for test-retest reliability and known groups validity that the Norfolk QOL-DN is an instrument with the most robust psychometric properties in treatment evaluation. Whether this assumption is also valid for Norfolk QOL-DN as an instrument for epidemiological data collection remains to be investigated in future prospective studies.

4. Conclusion

This analysis showed a high frequency of foot ulcers and amputations in Romanian diabetic patients. The relative high frequency of these among the younger age groups is of special concern. This analysis offers an overview of the diabetic foot problems and underlines the need for planning and implementation of effective screening and educational programs and also the need of increasing the access of diabetic patients to healthcare providers specialized in foot care.

Disclosure

Wörwag Pharma GmbH & Co. KG, Romanian Rep. Office, was involved in all stages of the survey conduct and analysis. Wörwag Pharma GmbH & Co. KG, Romanian Rep. Office, also took charge of all costs associated with the development and the publishing of the present paper. All authors had full access to the data.

Conflict of Interests

Etta J. Vinik and Aaron I. Vinik have a patent copyright of the Norfolk QOL-DN. Eastern Virginia Medical School engaged in a licensing agreement with Wörwag; royalties were paid to Etta J. Vinik and Aaron I. Vinik. Cosmina I. Bondor reports nonfinancial support from Wörwag Pharma GmbH & Co. KG, Romanian Rep. Office, during the conduct of the study. Ioan A. Veresiu reports personal fees (speaker fees) from Wörwag Pharma GmbH & Co. KG, Romanian Rep. Office, outside the submitted work. Norina A. Gavan is an employee of Wörwag Pharma GmbH & Co. KG, Romanian Rep. Office. Bogdan Florea has nothing to disclose.

Authors' Contribution

All authors had equal contribution to the development of the present paper.

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Research Article

Risk Factors for Foot Amputation in Patients Hospitalized for Diabetic Foot Infection

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The aim of this study was to identify and quantify risk factors for amputation in diabetic patients hospitalized for foot infections. This cross-sectional study comprised 100 patients with diabetic infectious complications in the lower limbs. The variables investigated were related to diabetes, infection, and treatment compliance. Multiple Cox regression analysis was performed to identify the variables independently associated with the outcome of amputation. The most prevalent chronic complications were neuropathy and hypertension. Most patients presented with a neuroischemic foot (86%). The Morisky test showed that 72% were not compliant with diabetes treatment. Regarding patient outcome, 61% progressed to amputation, 14% to debridement, and 9% to revascularization. The results showed a 42% higher risk for progression to amputation in patients with previous use of antimicrobials. Also, the amputation risk was 26% higher for those less compliant with diabetes treatment. An increase of one point in the Wagner ulcer classification criteria corresponded to a 65% increase in the risk of amputation. Undergoing conservative, nonsurgical procedures prior to admission provided a 63% reduction in the risk of amputation. Knowledge of these factors is critical to enable multidisciplinary teams to develop treatment plans for these patients so as to prevent the need for amputation.

1. Introduction

Worldwide, the population with diabetes is currently estimated at 366 million and is expected to exceed half a billion by 2030 [1]. Foot ulcers are the principal cause of severe complications and hospitalization among patients with diabetes, substantially increasing the costs with this disease [2]. In the United States, the annual cost of foot ulcers is estimated at US$11 billion [3].

In Brazil, the population aged 30 years and over with type 2 diabetes is estimated at 6.5 million. Among these, roughly 323 000 cases of foot ulcers are reported annually, 97 000 of which require hospitalization [4].

Adding to the costs of managing infection, patients with diabetes are confronted with the risk of limb amputation, with rates 30 to 40 times higher than in individuals without the disease [2]. Studies have shown the incidence of diabetic foot to be on the order of 3% to 4%, accounting for roughly 11 million patients with this condition in 2014 [5, 6].

Peripheral neuropathy, ulceration, infection, and peripheral vascular disease are the principal factors for ulcer complications and loss of a lower limb in diabetic patients [7, 8]. Nonetheless, ambiguity remains as to which factors are most conducive to amputation outcomes and how strongly they affect these events [9]. Structured healthcare is one of the most effective approaches to reducing the indicators for diabetic foot amputation, and studies have shown that these can be reduced by as much as 75% [8].

Factors such as low socioeconomic status, smoking [10, 11], gender, renal impairment [12], ischemia, diabetic neuropathy [13], and high levels of glucose and triglycerides [14] have been reported as importantly associated with the risk of foot amputation.

Table 1: Distribution of patients with diabetes by their sociodemographic characteristics and outcomes for diabetic foot amputation.

Characteristics	Amputation		PR (95% CI)	p
	No n (prevalence %)	Yes n (prevalence %)		
Gender				0.521
Male	25 (36.8)	43 (63.2)	1	
Female	14 (43.8)	18 (56.2)	0.89 (0.62–1.27)	
Caucasian				0.281
No	11 (50.0)	11 (50.0)	1	
Yes	28 (35.9)	50 (64.1)	1.28 (0.82–2.01)	
Schooling (years)				0.709
0 to 4	25 (36.2)	44 (63.8)	1	
5 to 8	11 (44.0)	14 (56.0)	0.88 (0.59–1.30)	
>8	3 (50.0)	3 (50.0)	0.78 (0.34–1.79)	
Total income (US$/month)				0.779
<900.00	34 (39.5)	52 (60.5)	1	
>901.00	5 (35.7)	9 (64.2)	1.06 (0.69–1.63)	
Alcohol use				0.892
NO	33 (39.3)	51 (60.7)	1	
YES	6 (37.5)	10 (62.5)	1.03 (0.68–1.56)	
Smoking habits				0.828
No	32 (39.5)	49 (60.5)	1	
Yes	7 (36.8)	12 (63.2)	1.04 (0.71–1.54)	

p values <0.05 were considered statistically significant. CI: confidence interval; PR: prevalence ratio.

This study evaluated the effect that clinical, biochemical, epidemiological, and patient-behavior-related predictors have on amputation outcomes in patients with diabetic foot. Knowledge of these factors and their influence on this outcome is critical to enable multidisciplinary teams to develop management and treatment plans for diabetic patients so as to prevent the need for foot amputation.

2. Material and Methods

This cross-sectional study comprised 100 patients with diabetic foot hospitalized at the Vascular Surgery Clinic of the Conjunto Hospitalar de Sorocaba, in Sorocaba county, São Paulo state, southeastern Brazil. Inclusion criteria were minimum age of 18 years, diagnosis of diabetes, presence of infected ulcers on a lower limb, and agreement to participate (expressed by signing a consent form). The project was approved by the Research Ethics Committee of the Universidade de Sorocaba (opinion 0028/10) and complied with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments.

The patients responded to a structured questionnaire about their sociodemographic status, knowledge of the disease, previous antibiotic use, and compliance with diabetes treatment.

Data on the clinical characteristics and health status of patients were collected from medical records. The clinical and laboratory evaluations were performed at the Laboratory for Diabetes "Conjunto Hospitalar de Sorocaba." Comorbidities had been evaluated by a group of specialists, based on consensus and guidelines [15–19]. These medical evaluations were available from the patients' records.

All foot ulcers were graded according to Wagner criteria [20]. Grade 1 ulcers are superficial, involving full skin thickness. Grade 2 ulcers are deeper, penetrating down to ligaments and joint capsule. Those of Grade 3 are deep lesions, with abscesses or osteomyelitis. Grade 4 ulcers exhibit localized gangrene. Grade 5 includes extensive gangrene, compromising more than two-thirds of foot.

Data analysis was based on debridement, revascularization, and amputation outcomes.

Compliance with outpatient treatment for diabetes was evaluated using the Morisky test [21], which consists of four simple questions. Do you ever forget to take your medication? Do you ever have problems remembering to take your medication? When you feel better, do you sometimes stop taking your medication? Sometimes, if you feel worse when you take you medication, do you stop taking it? Each negative answer is assigned one point. The higher the score, the more adherent the patient [21].

2.1. Statistical Analysis. Given the high prevalence of limb amputation, we estimated prevalence ratios and their respective confidence intervals (95% CI) for the univariate analysis of the relationships between variables and outcomes, using Shapiro-Wilk test, Student's t-test, or Mann-Whitney test. The variables with p values of less than 0.25 were selected for multivariate analysis using the Cox regression model with robust variance. The tests were performed at a significance level of 5%. All data were analyzed with Stata 11.0 statistical software (Stata Corp. LP, College Station, Texas, USA).

3. Results

Table 1 shows that age of the patients (n = 100; 32 women, 68 men) ranged from 31.9 to 89.7 years (median: 62 years), with

55% of patients older than 60. Most patients were male (68%), Caucasian (78%), poorly educated (69%), nonsmokers (81%), and alcoholics (84%) and had type 2 diabetes (99%). Of the total, 22% had been diabetic for less than five years, 24% from five to 10 years, 17% from 10 to 15 years, 16% from 15 and 20 years, and 21% for more than 20 years.

In most patients, diabetes was being monitored (79%). Most had attended annual medical appointments (73%) and, over the past year, had attended more than three appointments (67%) and tested for blood glucose levels (86%). Glucose levels at admission ranged from 4.10 to 28.7 mmol/L (mean: 12.43 ± 5.03 mmol/L).

The most frequent chronic complications were neuropathy (91%), hypertension (72%), vascular peripheral disease (63%), retinopathy (42%), dyslipidemia (41%), nephropathy (26%), coronary insufficiency (23%), and cerebrovascular insufficiency (16%). On admission, 75% of patients had Grade 4 ulcers, while 20% had Grade 3 and 5% had Grade 2 ulcers.

Less than half of the patients had undergone a prior conventional, nonsurgical procedure (debridement) (45%) or amputation (32%). For 74%, this was the first hospitalization for complications of diabetes. Most, however, had an ulcer of less than 2 cm (84%), gangrene (76%), and a neuroischemic diabetic foot (86%). Most patients showed signs of inflammation (89%) and had osteomyelitis (52%), which was also present with the high incidence of Grade 4 ulcers (75%).

Compliance with treatment was poor in 72% of patients (score 2 for 35 individuals, score 3 for 15, and score 4 for 15), while 27 were considered compliant (score 0 for 23 patients and score 1 for five).

3.1. Univariate Analysis. No statistically significant differences were observed in the prevalence of diabetic foot amputation with regard to gender, ethnicity, schooling, monthly income, alcohol consumption, or smoking.

No statistically significant differences in the prevalence of diabetic foot amputation were detected based on the occurrence of comorbidities. However, 75% of patients with two or three previous hospital admissions for chronic complications required foot amputation, whereas only 52.6% of those with one single admission experienced this outcome ($p = 0.043$; Table 2).

Table 3 shows that 78.6% of poor compliers (Morisky scores 0 or 1) had a foot amputated, whereas only 54.2% of compliant patients (scores 2–4) did so ($p = 0.012$).

Patients with a history of conservative procedures had a lower prevalence of amputation than those not subjected to this procedure ($p < 0.001$). However, previous amputation was unrelated to an amputation outcome ($p = 0.255$). Also, amputations were more frequent in patients with osteomyelitis than those lacking this condition ($p < 0.001$; Table 4).

3.2. Multivariate Analysis. To identify variables independently associated with progression to amputation, Cox multiple regressions (with robust variance) were performed on variables that showed p values lower than 0.25 on univariate analysis.

The association between ulcer grade (Wagner criteria) and treatment compliance score (Morisky test) was statistically significant ($p = 0.014$, chi-squared test). The prevalence of gangrene in patients with higher treatment compliance was 68.1%, rising to 92.8% in less compliant individuals (Morisky scores 0 or 1; Wagner Grade 4). Therefore, two models were found on multivariate analysis: one using the Morisky test (Table 5) and the other employing Wagner criteria (Table 6). Amputation outcomes proved independently associated with previous conservative procedures, previous use of antibiotics, and Morisky test scores or Wagner criteria (Tables 5 and 6).

The risk of foot amputation for patients who had received conservative treatment was 63% lower than for those with a previous amputation ($p < 0.001$; Table 5), while for individuals previously treated with antibiotics the risk of foot amputation was 42% higher than for patients not subjected to this drug therapy ($p = 0.026$).

Considering Wagner grades, the risk of foot amputation was 61% lower in individuals who had previously undergone conservative procedures than in those who had not ($p < 0.001$), Table 6. Among those previously treated with antibiotics, this risk was 36% higher than for those without antibiotic therapy ($p = 0.042$). Furthermore, for each unit increment in Wagner grade, there was a 65% increase in the risk of foot amputation in patients admitted with infectious complications in a lower limb ($p = 0.018$).

4. Discussion

In most subjects (81%), blood glucose levels ranged from 5.55 to 16.65 mmol/L. Glucose levels below 11.09 mmol/L at admission are associated with lower morbidity and mortality, and proper glycemic control is a critical factor for the infection eradication and ulcer healing. Chronic hyperglycemia is the most frequent etiological factor for complications of diabetes mellitus [22–25].

Neuropathy was reported in 91% of patients, coinciding with published data indicating a high prevalence of neuropathy in diabetic patients hospitalized for foot injuries [26]. Retinal impairment and nephropathy are the two most common microvascular complications, both of which were present in the study population (at 42% and 26%, resp.). In patients with diabetes, nephropathy is a marker for generalized vascular disease, and these patients are probably more susceptible to developing peripheral vascular disease [27]. Recent studies also suggest that the incidence of diabetic foot ulcers is more frequent in individuals with micro- and macroalbuminuria [28–30].

Patients who reported prior use of antibiotics had a 42% higher risk of major amputation than those not receiving

Table 2: Distribution of patients with diabetes by comorbidity occurrence in relation to diabetic foot amputation.

Characteristics	Amputation		PR (95% CI)	p
	No n (prevalence %)	Yes n (prevalence %)		
Number of admissions for chronic complications* (n = 96)				**0.043**
1	18 (47.4)	20 (52.6)	1	
2 or 3	10 (25.0)	30 (75.0)	1.42 (1.00–2.03)	
>3	10 (55.6)	8 (44.4)	0.84 (0.46–1.54)	
Coronary insufficiency				0.096
No	26 (33.8)	51 (66.2)	1	
Yes	13 (56.5)	10 (43.5)	0.66 (0.40–1.08)	
Hypertension				0.152
No	8 (28.6)	20 (71.4)	1	
Yes	31 (43.1)	41 (56.9)	0.80 (0.58–1.09)	
Neuropathy				0.177
No	6 (66.7)	3 (33.3)	1	
Yes	33 (36.3)	58 (63.7)	1.91 (0.75–4.90)	
Vascular peripheral disease				0.179
No	11 (30.6)	25 (69.4)	1	
Yes	28 (43.7)	36 (56.3)	0.81 (0.60–1.10)	
Cerebrovascular insufficiency				0.380
No	31 (36.9)	53 (63.1)	1	
Yes	8 (50.0)	8 (50.0)	0.79 (0.47–1.33)	
Dyslipidemia				0.679
No	24 (40.7)	35 (59.3)	1	
Yes	15 (36.6)	26 (63.4)	1.07 (0.78–1.47)	
Nephropathy				0.697
No	28 (37.8)	46 (62.2)	1	
Yes	11 (42.3)	15 (57.7)	0.93 (0.64–1.35)	
Retinopathy				0.875
No	23 (39.7)	35 (60.4)	1	
Yes	16 (38.1)	26 (61.9)	1.03 (0.75–1.41)	

p values <0.05 were considered statistically significant (indicated in bold). CI: confidence interval; PR: prevalence ratio.
*Chronic complications (coronary insufficiency, hypertension, and vascular peripheral disease).

antibiotic therapy. Similar results have been found in other studies [31, 32]. Previous prolonged use of antibiotics selects for resistant microorganisms, making treatment more difficult and increasing the risk of amputation.

The present data suggest an increased risk of amputation in patients less compliant with drug therapy. Adherence to the prescribed therapy has led to significant improvements in the health and quality of life of patients with diabetes [7, 33–37].

Compliance with medication is essential in chronic diabetes, improving control of disease progression and attenuating the severity of chronic complications. Reinforcement of guidelines on diabetes care and the importance of medication, both of which can increase treatment compliance, are facilitated when patients have more than three medical appointments per year.

In the present investigation, patients with a history of antibiotic use had an increased risk of progressing to amputation. Each unit increment in ulcer severity (measured in Wagner grades) increased the risk of amputation. Similar results were found in a Brazilian study that demonstrated a directly proportional relationship between Wagner grade and risk of limb amputation [38]. It is worth noting, however, that

Table 3: Distribution of patients with diabetes by age, time to diagnosis, and diabetic care in relation to foot amputation.

Characteristics	Amputation		PR (95% CI)	p
	No n (prevalence %)	Yes n (prevalence %)		
More than 3 appointments in the past year				**0.006**
No	7 (21.2)	26 (78.8)	1	
Yes	32 (47.8)	35 (52.2)	0.66 (0.50–0.89)	
Morisky test				**0.012**
2, 3, or 4	33 (45.8)	39 (54.2)	1	
0 or 1	6 (21.4)	22 (78.6)	1.45 (1.09–1.94)	
Age at diagnosis of diabetes				**0.030**
<40	5 (20.0)	20 (80.0)	1	
40 to 59	23 (42.6)	31 (57.4)	0.72 (0.53–0.97)	
≥60	11 (52.4)	10 (47.6)	0.60 (0.36–0.97)	
Diabetes monitoring				0.064
No	5 (23.8)	16 (76.2)	1	
Yes	34 (43.0)	45 (57.0)	0.75 (0.55–1.02)	
Glucose testing in the past year				0.073
No	3 (21.4)	11 (78.6)	1	
Yes	36 (41.9)	50 (58.1)	0.74 (0.53–1.03)	
Medical appointment in the past year				0.406
No	7 (31.8)	15 (68.2)	1	
Yes	32 (41.0)	46 (59.0)	0.86 (0.61–1.22)	
Annual medical appointment after diagnosis				0.462
No	9 (33.3)	18 (66.7)	1	
Yes	30 (41.1)	43 (58.9)	0.88 (0.64–1.23)	
Time since diagnosis (years)				0.586
<15	22 (41.5)	31 (58.5)	1	
≥15	17 (36.2)	30 (63.8)	1.09 (0.80–1.49)	

p values <0.05 were considered statistically significant (indicated in bold). CI: confidence interval; PR: prevalence ratio.

the Morisky test was originally developed for hypertension but has been used to evaluate drug treatment in patients with diabetes [39], a feature that may constitute a limitation of the present study. Another limitation is that information on previous use of antibiotics was self-reported rather than collected from medical records.

Noncompliance with pharmacological treatment of diabetes was associated with an increased risk of amputation. This risk was lower for patients who had undergone conservative treatment prior to admission.

Studies evaluating the extent of problems related to diabetic foot can provide elements for intervention policies and prevention programs—particularly in government-funded healthcare services—involving multidisciplinary teams specialized in diabetic foot care, ultimately ensuring improved treatment with more efficient use of resources.

5. Conclusion

The present findings highlight that antimicrobial therapy protocols for outpatients with diabetic foot need reviewing. Control of the disease before hospitalization can significantly reduce amputations in patients with diabetic foot.

Knowledge of these factors and their influence on amputation outcomes is critical to allow multidisciplinary teams to develop management and treatment protocols for patients with diabetes. The present findings show that limb amputation outcomes were strongly lowered by conservative treatment and compliance with diabetes drug therapy. Implemented in a preventive manner, these two measures can significantly reduce lower limb amputation in patients with diabetes.

Table 4: Distribution of patients with diabetes by disease characteristics at admission in relation to foot amputation.

Characteristics	Amputation		PR (95% CI)	p
	No n (prevalence %)	Yes n (prevalence %)		
Previous conservative procedure				**<0.001**
No	8 (14.6)	47 (85.5)	1	
Yes	31 (68.9)	14 (31.1)	0.36 (0.23–0.57)	
Osteomyelitis				**<0.001**
No	32 (66.7)	16 (33.3)	1	
Yes	7 (13.5)	45 (86.5)	2.60 (1.71–3.94)	
Wagner criteria				0.051
2 or 3	16 (64.0)	9 (36.0)	1	
4	23 (30.7)	52 (69.3)	1.93 (0.95–3.91)	
Previous amputation				0.255
No	29 (42.6)	39 (57.4)	1	
Yes	10 (31.2)	22 (68.8)	1.20 (0.88–1.64)	
Diabetic foot characteristics				0.256
Neuropathic	1 (25.0)	3 (75.0)	1	
Ischemic	7 (70.0)	3 (30.0)	0.40 (0.13–1.21)	
Neuroischemic	31 (36.1)	55 (63.9)	0.85 (0.47–1.54)	
Age at admission				0.321
<60	13 (30.9)	29 (69.1)	1	
60 to 69	10 (40.0)	15 (60.0)	0.87 (0.59–1.27)	
≥70	16 (48.5)	17 (51.5)	0.75 (0.51–1.10)	
Glucose level at admission (mmol/L) (n = 98)				0.480
<7.77	8 (47.1)	9 (52.9)	1	
≥7.77	30 (37.0)	51 (63.0)	1.19 (0.74–1.92)	
Involvement of the other lower limb				0.701
No	27 (40.3)	40 (59.7)	1	
Yes	12 (36.4)	21 (63.6)	1.07 (0.77–1.48)	

p values <0.05 were considered statistically significant (indicated in bold). CI: confidence interval; PR: prevalence ratio; PR_{adj}: adjusted prevalence ratio.

Table 5: Morisky test. Estimate of the prevalence ratio of the outcome to foot amputation in patients with diabetes using the Cox multiple regression model.

Characteristics	PR	PR_{adj} (95% CI)	p
Previous conservative procedure			**<0.001**
No	1	1	
Yes	0.36	0.37 (0.24–0.59)	
Previous use of antibiotics			**0.026**
No	1	1	
Yes	1.45	1.42 (1.04–1.92)	
Morisky test			**0.057**
2, 3, or 4 (compliance)	1	1	
0 or 1 (noncompliance)	1.45	1.26 (0.99–1.59)	

Statistically significant p values are indicated in bold. CI: confidence interval; PR: prevalence ratio; PR_{adj}: adjusted prevalence ratio.

Table 6: Wagner criteria. Estimate of the prevalence ratio of the outcome to foot amputation in patients with diabetes using the Cox multiple regression model.

Characteristics	PR	PR_{adj} (95% CI)	p
Previous conservative procedure			**<0.001**
No	1	1	
Yes	0.36	0.39 (0.25–0.61)	
Previous use of antibiotics			**0.042**
No	1	1	
Yes	1.45	1.36 (1.01–1.82)	
Wagner criteria	1.97	1.65 (1.09–2.50)	**0.018**

p values <0.05 were considered statistically significant (indicated in bold). CI: confidence interval; PR: prevalence ratio; PR_{adj}: adjusted prevalence ratio.

Conflict of Interests

There is no conflict of interests or financial support to be disclosed by the authors.

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Research Article

Decrease in (Major) Amputations in Diabetics: A Secondary Data Analysis by AOK Rheinland/Hamburg

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Aim. In two German regions with 11.1 million inhabitants, 6 networks for specialized treatment of DFS were implemented until 2008. Data provided for accounting purposes was analysed in order to determine changes in the rate of diabetics requiring amputations in the years before and after the implementation. *Method*. Data covering 2.9 million people insured by the largest insurance company between 2007 and 2013 was analysed by the use of log-linear Poisson regression adjusted for age, gender and region. *Results*. The rate of diabetics needing major amputations fell significantly by 9.5% per year ($p < 0.0001$) from 217 to 126 of 100,000 patients per year. The rate of diabetics needing amputations of any kind fell from 504 to 419 of 100,000 patients per year ($p = 0.0038$). *Discussion*. The networks integrate health care providers in an organised system of shared care. They educate members of the medical community and the general public. At the same time, a more general disease management program for people with diabetes was implemented, which may also have contributed to this decrease. At the end of the observation period, the rate of diabetics requiring amputations was still high. For this reason, further expansion of organised specialized care is urgently needed.

1. Introduction

Diabetic foot syndrome (DFS) is a lifelong consequence of diabetes mellitus which occurs in active and inactive phases. It may place the mobility of those affected under threat and consequently their independence, quality of life, and ability to work. In some cases, it may be fatal. In particular, after amputations above the ankle, mobility is impaired, as half of those affected are no longer able to walk independently [1, 2]. An important aim in the care of people with active DFS is therefore to avoid these so-called major amputations. According to the data currently available, 5–10% of people with active DFS are affected by this in standard care, while the figure in specialised care is 2–3.5% [3–5].

A substantial proportion of the spending on diabetes care in Germany is attributable to the DFS [6, 7]. Major amputations in particular, with their follow-up costs, entail significant levels of spending [8]. The development of care for people with DFS receives high priority all over the world [9].

Delivery of care to the patient is unavoidably of an interdisciplinary and interprofessional nature. It requires coordinated cooperation between all of the parties involved, which in the regions of Rhineland (9.4 million inhabitants) and Hamburg (1.7 million) joint forces into six separate regional networks since 2002. These networks integrate hospital departments, doctors and nurses working in the outpatient field, and orthopaedic shoemakers and podiatrists as healthcare service providers working in independent facilities. Coordinated treatment paths, regular quality circles, visiting physician programmes, and open benchmarking are some of the methods used in shared patient care.

Within the German health care system, baseline medical care is covered by contracts between insurance companies and organised physicians. They are called "collective contracts" because all adequately specialized physicians are free to participate. These contracts are highly regulated by federal and regional law. Disease management programs are among these collective contracts. Additionally, insurance companies can conclude contracts with groups of health care providers to offer extra services to their customers. For these contracts called "selective contracts," insurance companies are allowed to select the participating providers. AOK Rhineland/Hamburg (AOK RH) is a prime insurance company in these regions and aims to improve the care given to people with diabetes. To achieve this, in addition to a disease management program (DMP Diabetes), AOK RH together with other insurance companies supported the development of networks for the treatment of people with a diabetic foot syndrome since 2005 through selective contracts (DFS SC). After a trial period from 2005 to 2008, these contracts were concluded with network participants throughout the entire area covered by the AOK RH. From the very beginning of the contract, the aim was to produce an effect on the region as a whole. Therefore, the networks began at an early stage to make offers of further education to other facilities within the contract region. To this same aim, second-opinion procedures prior to major amputations were made available and awareness campaigns were conducted.

Publications on the incidence of amputations in Germany have so far been limited to the analysis of hospital stays with amputation events without reference to individuals. It has been argued that a change of strategy from partial amputations performed consecutively towards a unique procedure could result in reduced amputation figures in spite of an increase in the number of individuals affected and therefore a distortion of the perception of the result. The present work identifies not only the hospital stays with amputations performed, but also the number of people affected.

2. Materials and Methods

We analysed accounting data for the years 2007 to 2013 of the AOK RH in accordance with Sections 295, 300, and 301 of Social Security Code Book Five. This data contains information on the diagnoses according to the International Classification of Disease (ICD-10 GM), drug prescriptions according to the Anatomical Therapeutic Chemical- (ATC-) code, and surgical procedures according to the German Procedure Classification (OPS). The diagnosis of diabetes was considered to have been confirmed if indicated by more than one statement independently. These were similar to other investigations from the German healthcare system [10–12]:

(i) A 3-digit diagnosis (ICD E10* to E14*) in at least 3 of 4 consecutive quarters at the level "certain" according to the ICD 10 GM.

(ii) At least two prescriptions of antidiabetic agents (ATC A10) within 12 months.

(iii) A prescription of antidiabetic agents and a diabetes diagnosis or a glucose or HbA1c measurement within 12 months.

Major amputations were considered to be those performed at the level of the ankle or above (OPS 5-864, 5-869.0), whereas minor amputations were amputations below the ankle (OPS 5-865), which is also analogous to earlier investigations [13, 14].

Absolute frequencies were normalised to 100,000 diabetics. Adjusted amputation frequencies were presented by means of regression analysis.

The figures were compiled separately for each of the 27 regions in Rhineland and Hamburg and presented together. The breakdown corresponds to the administrative structures of AOK RH and takes into account regional specificities.

2.1. Statistics: Poisson Regression. For each of the 27 regions in Rhineland and Hamburg, the number of diabetics and their gender and age distribution, as well as the amputations themselves were determined for each year during the period investigated. This took into account possible changes in demographic developments resulting from changes in age and gender distribution or the number of insured individuals.

Using a log-linear Poisson regression [15], the annual frequency of amputations within each region was modelled with adjustments according to age and gender. We additionally applied two different offset variables: in Model 1, the offset was the absolute number of diabetics in the year under consideration within each region; in Model 2, it was the number of diabetics in the year 2007 held constant over all years. The second modelling procedure was added in order to eliminate the possible effect of any change in coding behaviour over the years.

The Poisson regression was performed using SAS version 9.2 of PROC GENMOD.

3. Results

3.1. General. Among approximately 2.9 million individuals insured by AOK RH in 2007, the diabetes prevalence was 8.2%. This figure rose in 2013 to 9.9%, with levels being 10.9% in Hamburg and 9.8% in Rhineland, respectively (Figure 1). The proportion of women was 37.2% overall and the average patient age was 69.3 (±13.8). The total number of hospital stays with amputations carried out on 6,958 diabetics in the period from 2007 to 2013 was 11,436 (3,607 with major and 7,829 with minor amputations).

3.2. Structured Care. In 2013, over 10,000 people with diabetes and DFS received structured care in networks in accordance to contracts provided by the AOK RH. In addition to the increasing numbers of participants in structured care, the proportion of diabetics cared for in the Disease Management Program (DMP) also rose to around 65% and therefore amounted to over 180,000 individuals in 2013 in absolute terms (Figure 2). Of all policyholders with diabetes who underwent amputations, 777 (11.2%) were cared for

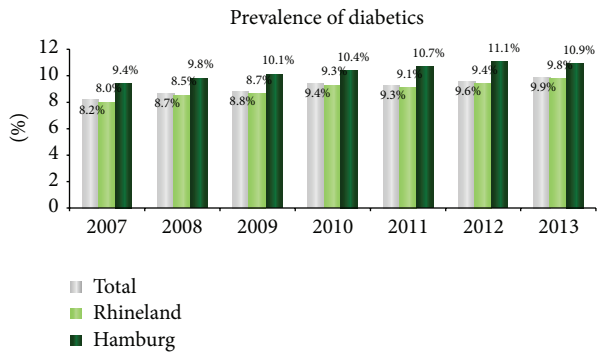

Figure 1: Prevalence of diabetics covered by AOK RH, 2007–2013.

Table 1: Overview of selective contract (SC) participants and amputation frequency.

People with diabetes and amputation from 2007 to 2013	6,958
SC participants with amputation (major or minor)	777
SC participants with major amputation	174
SC participants with minor amputation	613
Proportion of SC participants undergoing major amputations compared to the total number of amputations	22.4%

in networks (SC), with 22.4% of these undergoing major amputations (Table 1).

3.3. Amputations. It was shown that over the course of seven years up to 2013, there was a significant reduction of 41.7% in the number of patients undergoing a major amputation ($p < 0.0001$). The proportion of people with minor amputations fell by 2.1% ($p = 0.6624$) (Table 2, Figures 3(a) and 3(b)). The proportion of those who required any form of amputation fell by 17.0% ($p < 0.0001$) (results normalised in each case to 100,000 diabetics). In total, 1,537 (22.1%) diabetics underwent multiple amputations over the years and can therefore be assigned to more than one year.

The results of the Poisson regression did not provide any indications of over- or underdispersion (deviance/df = 1.35 (Model 1) and 1.10 (Model 2)).

When adjusted for age and gender distribution for each region, a significant reduction in the number of individuals undergoing major amputations of 9.5% ($p < 0.0001$) is found across all of the years. If it is assumed that the number of diabetics remains constant (Model 2), an annual decline of 8.50% ($p = 0.0002$) is recorded. The number of people affected by amputations, regardless of whether these were major or minor, fell annually by 3.7% ($p = 0.0038$).

4. Discussion

The incidence of major amputations varies worldwide between 56 and 6,000 for every 100,000 people with diabetes [16]. This variability is caused not only by differences in health care, but also by uncertainties regarding the diagnosis of the diabetic disease and whether all of the amputation events performed are recorded [17]. The incidence also varies within countries. For example, at 151 Primary Care Trusts (PCTs) in England between 2007 and 2010, the figures varied from 64 to 525 per 100,000 [18]. In Ipswich (UK), the introduction of specialised care which completely replaced the previous form of care observed a reduction in major amputations in the years 1995 to 2005 from 364 to 67 per 100,000 people with diabetes [19]. In the study presented here, the number of those affected fell from 217 to 126 per 100,000 diabetics. The number of hospital stays with an event decreased from 263 to 146 per 100,000 diabetics. This need for improvement which still exists in the international comparison might be attributable to specific aspects of the German healthcare system. Generally speaking, all hospitals with their own surgical departments can charge fees for major amputations. The complete replacement of the existing form delivering care by an alternative form is not possible here.

The specialised care is provided as an additional offer to the standard care. Indeed, only a minority of the amputations examined here were performed on patients who received care in the networks of the selective contract. The fact that the care would only be partially taken over by the networks was already foreseeable when the intervention was planned; therefore, the introduction was accompanied by a number of measures such as advanced training courses, offers of second opinions, and awareness campaigns in order to achieve a broad effect.

Two previous population-based studies of the care for people with DFS in Leverkusen, a city within the area studied here, showed a decrease in the number of amputations over the entire period under investigation (1990–2005) [20] which had not yet been seen in the years 1990–1998 [21]. In this survey, the reduction was attributed to a change in the type of care, which also formed part of the development of the regional networks.

Previous evaluations of amputation incidence from accounting data [22–24] were case and not individual related. Therefore, it was not possible to state how many people with diabetes underwent amputations and the extent to which the development in the absolute surgical figures affected the number of patients involved. This is illustrated by the study presented here, which covers the insured from two major regions and uses the figures of the largest health insurance company in these regions.

Furthermore, the analysis of the number of patients affected shows for the first time that in Germany there has been a significant decrease in the number of people with diabetes who require amputations. The number of people affected by minor amputations is falling only by a lesser extent, which is partly attributable to the fact that minor amputations are being carried out instead of major amputations.

The limitations of the study relate in particular to the selection of the patients affected due to their membership of AOK RH and the development in the incidence of the diabetes diagnoses documented. However, the selection bias remained constant over the observed period; since there were no mergers of AOK RH with other health insurance

Table 2: Absolute number of hospital stays with amputation events and diabetics, proportion of diabetics with amputation compared to total number of diabetics.

Year	Number of the insured	Number of insured diabetics	Number of hospital stays with amputations			Number of diabetics with amputation			Number of diabetics with amputation/100,000 diabetics		
			Major	Minor	Amputation (major + minor)	Major	Minor	Amputation*	Major	Minor	Amputation*
2007	2,908,300	237,164	619	975	1,594	514	761	1,196	217	321	504
2008	2,857,963	247,690	511	1,083	1,594	431	844	1,190	174	341	480
2009	2,850,008	251,623	559	1,120	1,679	456	869	1,253	181	345	498
2010	2,863,114	269,432	566	1,130	1,696	456	861	1,243	169	320	461
2011	2,881,479	267,708	515	1,120	1,635	429	884	1,245	160	330	465
2012	2,867,117	274,092	421	1,198	1,619	365	897	1,201	133	327	438
2013	2,817,703	278,647	416	1,163	1,579	352	875	1,167	126	314	419

*Patients were counted only once in the corresponding year irrespective of the type of amputation (major and/or minor).

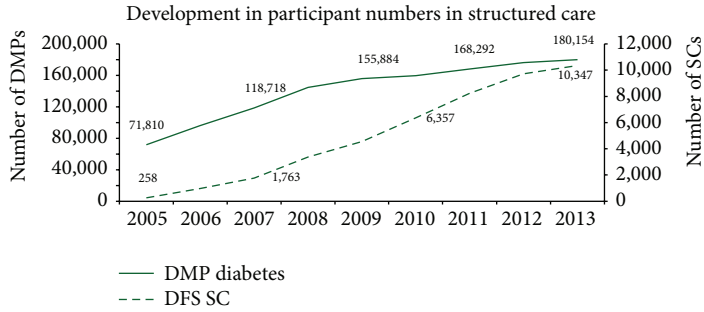

Figure 2: Development in participant numbers in the DMP and DFS selective contract (SC).

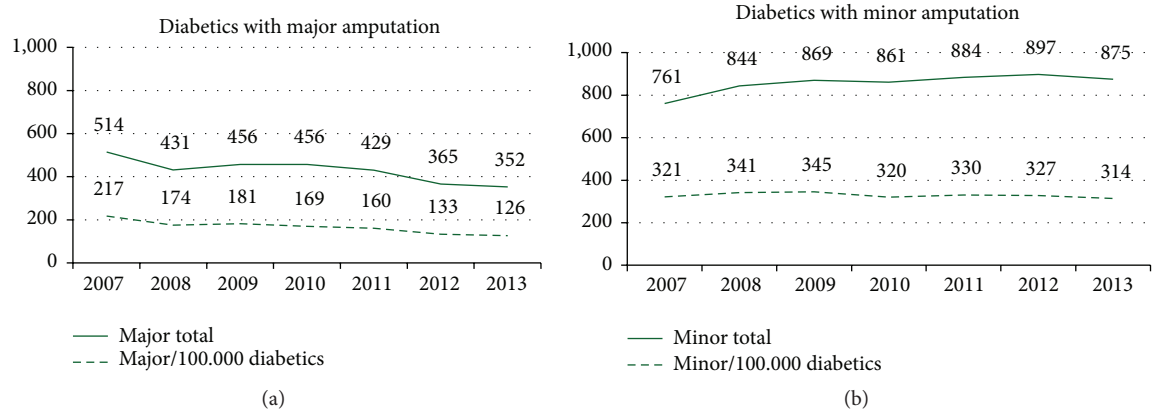

Figure 3: Number of diabetics with major or minor amputation, 2007–2013.

companies, no other trends became apparent among the insured and the number of insured individuals remained more or less the same. The increase in the number of diagnosed diabetes cases might have been attributable in part to a change in coding behaviour. For this reason, a second modelling procedure was carried out which assumed that the prevalence of diabetes remained unchanged. In this evaluation, there was also a very clear and significant reduction in the incidence of insured individuals undergoing amputations in particular major amputations.

5. Summary Assessment

The figures presented, which are based on routine data, confirm a very significant improvement in the care of people with DFS. The number of people affected who underwent major amputations with their serious consequences fell dramatically during the seven years after the introduction of organised specialised care. Both the structured treatment program DMP Diabetes and the specialized "DFS" contract are used by a large proportion of affected people in Rhineland and in Hamburg. However, the majority of those who underwent an amputation event did not use specialized care offered by the contract. The increasing number of patients in this contract alongside with the efforts to induce advances in the nonspecialized standard care might explain that improvement and should be investigated in further studies. For this reason, the expansion of the care of people with diabetic foot syndrome in structured foot networks is indispensable in the future.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

The Four-Herb Chinese Medicine Formula Tuo-Li-Xiao-Du-San Accelerates Cutaneous Wound Healing in Streptozotocin-Induced Diabetic Rats through Reducing Inflammation and Increasing Angiogenesis

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Impaired wound healing in diabetic patients is a serious complication that often leads to amputation or even death with limited effective treatments. Tuo-Li-Xiao-Du-San (TLXDS), a traditional Chinese medicine formula for refractory wounds, has been prescribed for nearly 400 years in China and shows good efficacy in promoting healing. In this study, we explored the effect of TLXDS on healing of diabetic wounds and investigated underlying mechanisms. Four weeks after intravenous injection of streptozotocin, two full-thickness excisional wounds were created with a 10 mm diameter sterile biopsy punch on the back of rats. The ethanol extract of TLXDS was given once daily by oral gavage. Wound area, histological change, inflammation, angiogenesis, and collagen synthesis were evaluated. TLXDS treatment significantly accelerated healing of diabetic rats and improved the healing quality. These effects were associated with reduced neutrophil infiltration and macrophage accumulation, enhanced angiogenesis, and increased collagen deposition. This study shows that TLXDS improves diabetes-impaired wound healing.

1. Introduction

Nonhealing wound is a hallmark of diabetes and the leading cause of nontraumatic lower extremity amputation. The lifetime risk of a person with diabetes developing a chronic foot ulcer could be as high as 25% [1]. However, the treatments for it are limited and the cost is high [2]. Therefore, developing effective and economical therapies for correcting impaired healing of diabetic wounds is an urgent clinical demand. Diabetes impairs wound healing through magnifying the inflammatory response, inhibiting angiogenesis, and decreasing extracellular matrix (ECM) deposition [3]. The ideal treatment relies on correcting the multiple deficits simultaneously through highly integrated therapeutic approaches. Traditional Chinese medicine (TCM) is characterized by the use of herbal formulas that are usually grouped by two or more medicinal herbs, which can effectively produce synergetic effects to be greater than the sum of the individual effects and reduce side effects, providing novel therapeutic strategies for diabetic ulcer. Studies showed that combining TCM with conventional treatments in diabetic wound management received better clinical outcome [4].

Tuo-Li-Xiao-Du-San (TLXDS) is a refined Chinese medicine formula consisting of four herbs: Danggui (*Radix Angelica sinensis*), Huangqi (*Radix Astragali*), Baizhi (*Angelica dahurica*), and Zaojiaoci (*thorns of Gleditsia sinensis*), in the ratio of 5 : 5 : 4 : 4 (15 g for the former two and 12 g for the latter two). It is derived from "orthodox manual of surgery" ("Wai Ke Zheng Zong" in Chinese) formulated by a famous TCM physician Shigong Chen in 1617 AD and has been used for the treatment of various refractory wounds, including pressure ulcer, venous leg ulcer, abscesses, and carbuncle. In Chinese medicine theory, TLXDS includes the therapeutic method of "TUO" represented by *Radix Angelica sinensis* and *Radix Astragali* and the therapeutic method

of "TOU" represented by *Angelica dahurica* and thorns of *Gleditsia sinensis*. Therapeutic method of "TUO" means raising "Qi" (vital energy) and nourishing "Blood" (body circulation), while therapeutic method of "TOU" refers to cleansing wound environment and eliminating toxins. For the pharmacological action of each single herb in TLXDS, *Radix Astragali* is used as "Qi" invigorator [5] and *Angelica sinensis* is prescribed as blood circulation activator [6]; *Angelica dahurica* is classified as a sweat-inducing drug able to counter harmful external influences on the skin, such as cold, heat, dampness, and dryness [7]; the thorns of *Gleditsia sinensis* have been used for the treatment of inflammatory diseases including swelling, suppuration, carbuncle, and skin diseases [8]. Hundreds of years of practice has proven the wound healing effect of TLXDS on various refractory wounds [9], and the pharmacological actions of herbs in TLXDS suggest it might be a potential remedy for diabetic wound. However, the effect of TLXDS on healing of diabetic wounds has not been explored before. The purpose of this study is to evaluate the efficacy of TLXDS on diabetic wound by using an excisional cutaneous wound model of streptozotocin-induced diabetic rats and also to clarify its active mechanism by immunohistochemical, qRT-PCR, and western blot analyses.

2. Materials and Methods

2.1. Preparation of Tuo-Li-Xiao-Du-San Ethanol Extract. Tuo-Li-Xiao-Du-San is comprised of four herbs, *Astragalus membranaceus*, *Angelica sinensis*, *Angelica dahurica*, and thorns of *Gleditsia sinensis*, in the ratio of 5 : 5 : 4 : 4 (15 g for the former two and 12 g for the latter two). The herbs were obtained from and authenticated by TASLY Pharmaceutical Group Co. Ltd. (Tianjin, China). The 70% ethanol extract of mixture of four herbs was prepared by the department of Pharmaceutical Sciences, Tianjin University of Traditional Chinese Medicine (Tianjin, China) using standardized procedure [10, 11]. Briefly, the crude herbs were powered and then extracted by 70% ethanol for three times. Gather the extracts and filter to remove the solid fragment. The solvents were removed by freeze-drying. The condensate was stored at −20°C. The extracts are freshly prepared by dissolving in sterile water to an appropriate concentration as herb solution for the later experiment.

2.2. Animals. Sprague-Dawley male rats ($n = 120$, 10 weeks old, SPF) weighing 290 ± 10 g were purchased from Beijing HFK Bioscience Co., Ltd. (Beijing, China). Rats were housed under pathogen-free conditions at the Chinese Academy of medical Sciences & Peking Union Medical College Institute of Biomedical Engineering Animal SPF facility (Tianjin, China). Rats were maintained at controlled temperature (22–25°C) and relative humidity (50%–60%) on a 12 h light-dark cycle and fed a commercial pellet diet with food and water available *ad libitum*. This study was approved by Experimental Animal Ethical Committee of Tianjin Medical University, and all procedures with animals complied with rules of the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health as well as the guidelines of the Animal Welfare Act.

2.3. Induction of Diabetes. After overnight fasting, diabetes was induced by a single intravenous injection of STZ (Sigma-Aldrich, St. Louis, MO, USA) at a dose of 50 mg/kg body weight in 0.1 mol/L citrate-phosphate buffer, pH 4.5. Control rats were injected with citrate buffer alone. Blood glucose concentration was monitored using an Accu-Chek Aviva glucometer (Roche Diagnostics GmbH, Germany) from tail vein blood. Animals with random blood glucose levels ≥16.7 mmol/L for three consecutive tests were considered diabetic.

2.4. Wound Model and TLXDS Treatment. Wound-healing model of rats was induced as described before [12]. Four weeks after STZ injection, control and diabetic rats were anesthetized by intraperitoneal injection of sodium pentobarbital (30 mg/kg body weight). The dorsal hair of rats was shaved and two 10 mm diameter full thickness wounds were created with a sterile biopsy punch. Diabetic rats were randomly allotted to diabetic treated with TLXDS (DM + TLXDS group, $n = 36$) and diabetic without drug treatment (DM group, $n = 36$), while nondiabetic rats were placed in the normal control group (NC group, $n = 10$). Rats of these three groups were randomly allocated to four experimental end points (i.e., days 5, 8, 11, and 14, 7–10 animals for each end point). Rats in TLXDS group received TLXDS ethanol extract 1.1 mL/0.2 kg body weight once daily by oral gavage starting from day 0 to each end point, and rats in NC and DM group received 1.1 mL/0.2 kg body weight water once daily by oral gavage. The dose of each herb used in rats was 1.5 g/kg body weight for *Angelica sinensis* and *Astragalus membranaceus*, 1.2 g/kg body weight for *Angelica dahurica* and *Gleditsia sinensis* thorns, which was calculated according to the dose used in patients (0.25 g/kg and 0.2 g/kg, resp.). At each experimental end point, the animals were killed simultaneously by euthanasia.

2.5. Macroscopic Analysis. The ulcers were photographed every other day with a digital camera. The percentage of completely closed ulcers was calculated with the NIH Image J analyzer by tracing the wound margin and calculating pixel area. Wound closure was calculated as Percentage Closed = [(Area on Day 0 − Open Area on Final Day)/Area on Day 0] × 100.

2.6. Immunohistochemistry. The wounds, together with unwounded skin margins, were excised, fixed with 10% formaldehyde, and embedded with paraffin. The sections (4 μm thick) were then deparaffinized and rehydrated. Antigen retrieval was performed at 95°C by microwave in 0.01 mol/L sodium citrate buffer (pH 6.0). Endogenous peroxidase activity was quenched by exposing to 3% H_2O_2. After blocking with 5% BSA in PBS, the sections were incubated with anti-CD68 (1 : 100, Thermo Fisher Scientific), anti-MPO (1 : 100, Thermo Fisher Scientific), anti-CD31 (1 : 200, Santa Cruz Biotechnology), anti-desmin (1 : 100, Thermo Fisher Scientific), and anti-collagen I (1 : 100, Thermo Fisher Scientific), respectively, followed by incubating with the corresponding HRP-conjugated secondary antibodies. The antigen-antibody complex was visualized with a Diaminobenzidine (DAB) kit. For evaluation of staining, the

overview of the positive-signal density was scored semiquantitatively as 1 (absent), 2 (low), 3 (medium), 4 (strong), and 5 (very strong). The median of scores from three observers, who were blinded to the treatment, was used for comparisons.

2.7. Collagen Estimation (Hydroxyproline Content). Wound tissues were analyzed for hydroxyproline content, which is basic constituent of collagen. The collagen composed of amino acid (hydroxyproline) is the major component of extracellular tissue, which gives strength and support. Breakdown of collagen liberates free hydroxyproline and its peptides. Measurement of hydroxyproline hence can be used as a biochemical marker for tissue collagen and an index for collagen turnover. Hydroxyproline content was analyzed using a hydroxyproline assay kit (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions. Dilute 10 μL of the 1 mg/mL hydroxyproline standard solution with 90 μL of water to prepare a 0.1 mg/mL standard solution. Add 0, 2, 4, 6, 8, and 10 μL of the 0.1 mg/mL hydroxyproline standard solution into a 96-well plate, generating 0 (blank), 0.2, 0.4, 0.6, 0.8, and 1.0 μg/well standards. Homogenize 10 mg tissue in 100 μL of water and transfer it to a 2.0 mL polypropylene tube. Add 100 μL of concentrated hydrochloric acid (HCl, ~12 M), cap tightly and hydrolyze at 120°C for 3 hours. Transfer 10–50 μL of supernatant to a 96 well plate. Place plates in a 60°C oven to dry samples. Add 100 μL of the Chloramine T/Oxidation Buffer Mixture to each sample and standard well. Incubate at room temperature for 5 minutes. Add 100 μL of the Diluted DMAB Reagent to each sample and standard well and incubate for 90 minutes at 60°C. Measure the absorbance at 560 nm (A560). The concentration of the sample was calculated as

$$\text{Concentration of the sample} = \frac{\text{OD of the sample}}{\text{OD of standard}} \times \text{Concentration of standard.} \quad (1)$$

2.8. Real-Time Quantitative PCR. Total RNA from rat ulcer was extracted using Trizol (Invitrogen, Grand Island, NY). RNA purity and integrity were assessed by spectrophotometric analysis. A total of 3 μg of RNA was reverse-transcribed using a RevertAid kit (Thermo Fisher Scientific, Waltham, MA). Reverse transcription polymerase chain reaction (RT-PCR) was performed using the CFX96 real-time PCR system (Bio-Rad, USA) with the SYBR Green PCR Kit (Takara, Otsu, Japan) for rat VEGF-A, PDGF-BB, IL-1β, and TNF-α. Primer sequences are as follows: for VEGF-A 5′ TCA AAC CTC ACC AAA GCC 3′ and 5′ GGT GAG AGG TCT AGT TCC 3′; for PDGF-BB 5′ CGC CTG CTG CAC AGA GAC 3′ and 5′ CCG CGA GAT CTG GAA CAC 3′; for IL-1β 5′ AGA AGA AGA TGG AAA AGC 3′ and 5′ CGA CCA TTG CTG TTT CCT 3′; for TNF-α 5′ TCC CAG GTT CTC TTC AAG G 3′ and 5′ GTA CAT GGG CTC ATA CCA G 3′; for GAPDH 5′ TAC CCA CGG CAA GTT CAA CG 3′ and 5′ CAC CAG CAT CAC CCC ATT TG 3′. GAPDH was defined as the reference gene. Data were analyzed with $2^{-\Delta\Delta CT}$ method.

2.9. Western Blot Analysis. Protein contents of VEGF-A, PDGF-BB, IL-1β, and TNF-α in ulcer tissue homogenates were evaluated by western blot. In brief, the protein concentrations of homogenates were determined using a BCA protein assay (Pierce Biotechnology, Rockford, IL, USA). Equivalent amount of protein samples (30 μg) was separated on an SDS polyacrylamide gel and transferred onto a polyvinylidene difluoride membrane (Millipore). After blocking in TBS containing 5% nonfat milk for 2 h at room temperature, the membranes were incubated overnight with 1 : 1,000 diluted anti-VEGF-A (Abcam), 1 : 2000 diluted anti-PDGF-BB (Abcam), 1 : 200 diluted anti-IL-1β (Santa Cruz Biotechnology), 1 : 500 diluted anti-TNF-α (Abcam), and 1 : 8000 diluted anti-β-actin (Tianjin Sungene Biotech Co., Ltd.), respectively. Binding of the primary antibody was detected using a HRP-conjugated secondary antibody (Tianjin Sungene Biotech Co., Ltd.). Positive bands were visualized using an ECL kit (Bio-Rad) and then captured on X-ray film. Housekeeping protein β-actin was used as a loading control. The density of each band was quantified using Quantity One software (Bio-Rad Laboratory) and normalized to their respective control.

2.10. Statistical Analysis. Data are presented as means ± SEM. Statistical analysis were performed using SPSS 16.0 software. One-way analysis of variance (ANOVA) test was used to determine statistical significance. $P < 0.05$ was considered to indicate a statistically significant difference.

3. Results

3.1. Diabetes Induction and Blood Glucose Level. During the 4-week diabetes induction period, 90% (72/80) of the STZ-injected rats became consistently hyperglycemic and were included in this study. During the treatment period, blood glucose levels of rats in DM group and DM + TLXDS group were significantly higher than those of rats in NC group ($P < 0.05$), and no significant difference was observed between DM group and DM + TLXDS group ($P > 0.05$), indicating that TLXDS had no effects on blood glucose (Figure 1).

3.2. Administration of TLXDS Accelerated Wound Healing. As shown in Figure 2, by the end of observation (14 days after wounding), nondiabetic wounds completely healed, while most of the diabetic wounds remained open with a low average closure rate of 61.6%. TLXDS began to significantly improve diabetic wound closure 4 days after wounding (24% versus 14%, $P < 0.05$), and by the end of observation, TLXDS increased the healing rate of diabetic wound by 25.2% (86.8% versus 61.6%, $P < 0.01$).

3.3. Administration of TLXDS Reduced Inflammatory Cells Infiltration and Stimulated Inflammation Resolution. Uncontrolled inflammation is a major characteristic of diabetic wounds. We assessed histological changes by HE staining on day 5 (Figure 3(a)) and populations of neutrophil and macrophage at the wound site by determining the constitutively expressed molecular markers MPO (for neutrophils) and CD68 (for macrophages) on day 14

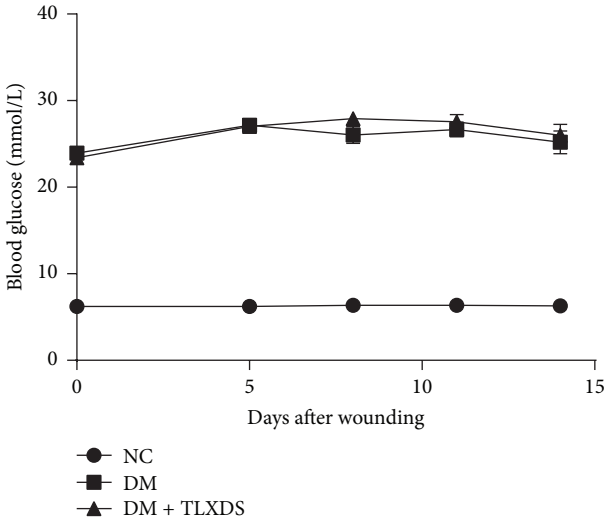

Figure 1: Blood glucose levels monitored during the treatment period. During the study period, the level of blood glucose was significantly higher in both DM and TLXDS-treated rats compared with the NC rats ($P < 0.05$). No significant difference between DM and TLXDS-treated groups was seen ($P > 0.05$). On day 0, $n = 40$ for NC, $n = 36$ for DM and DM + TLXDS; on day 5, $n = 39$ for NC, $n = 34$ for DM, and $n = 35$ for DM + TLXDS; on day 8, $n = 29$ for NC, $n = 23$ for DM, and $n = 26$ for DM + TLXDS; on day 11, $n = 19$ for NC, $n = 15$ for DM, and $n = 16$ for DM + TLXDS; on day 14, $n = 9$ for NC, $n = 7$ for DM, and $n = 8$ for DM + TLXDS.

(Figures 3(b), 3(c), and 3(d)). The infiltration of inflammatory cells and concomitant tissue necrosis in diabetic wounds was much stronger compared with nondiabetic wounds on day 5 (Figure 3(a)). Moreover, the inflammation resolution of untreated diabetic rats was significantly delayed, which was characterized by increased and prolonged neutrophils and macrophages influx on day 14 (Figure 3(b)). Administration of TLXDS led to a marked decline in inflammatory cells infiltration and tissue necrosis on days 5 and an accelerated resolution of neutrophils and macrophages on day 14 ($P < 0.05$).

3.4. Administration of TLXDS Augmented Neovascularization and Increased Granulation Tissue Deposition. Neovascularization is an essential event in the development of granulation tissue. We evaluated the growth and maturation of blood vessels by immunostaining of endothelial cell marker CD31 and pericyte marker desmin, respectively (Figure 4). The vessel density of untreated diabetic wounds was significantly decreased compared with nondiabetic wounds ($P < 0.05$), and the pericyte coverage of new vessels was discrete and incomplete. TLXDS treatment significantly enhanced neovascularization of diabetic wounds, demonstrated by increased CD31 staining. Besides, the pericyte recruitment was restored by TLXDS administration, suggesting that new vessels in TLXDS treated wounds were more mature, stable, and functional.

Meanwhile, Masson's trichrome staining and immunohistochemistry of type I collagen showed that the deposition of ECM in diabetic wounds on day 14 after wounding was significantly impaired compared with nondiabetic wounds, which was restored by the administration of TLXDS (Figure 5). Consistently, the hydroxyproline content in diabetic wounds was decreased significantly compared with nondiabetic wounds on day 14 (5.42 ± 0.29 versus 12.03 ± 0.24 μg/mg tissue, $P < 0.05$). Administration of TLXDS increased hydroxyproline content in diabetic ulcers on day 14 (7.38 ± 0.22 versus 5.42 ± 0.29 μg/mg tissue, $P < 0.05$).

3.5. Administration of TLXDS Increased Expression of Angiogenic Factors and Reduced Inflammatory Cytokine Expression. Impaired angiogenic factors production accounts for the compromised neovascularization of diabetic wounds. Therefore, we performed real-time PCR and western blotting to investigate whether TLXDS increased neovascularization through increasing angiogenic factors expression. As expected, VEGF-A and PDGF-BB levels of diabetic wounds failed to rise on day 5 after wounding to initiate the angiogenic response as compared with nondiabetic wounds (Figures 6(a), 6(c), 6(d), 6(g), and 6(h)). TLXDS increased VEGF-A and PDGF-BB expression on day 5 after wounding when active angiogenesis was undergoing ($P < 0.05$).

TLXDS stimulated inflammation resolution in diabetic wounds. Therefore, we examined the effect of TLXDS on inflammatory cytokines expression on both gene and protein levels. On day 11 after wounding, expression of inflammatory cytokines, such as IL-1β and TNF-α, was significantly higher in diabetic wounds than in nondiabetic wounds ($P < 0.05$), and TLXDS markedly reduced these inflammatory cytokine expression (Figures 6(b), 6(e), 6(f), 6(i), and 6(j)).

4. Discussion

Impaired wound healing is a serious complication in diabetes, leading to prolonged hospitalization and even amputation. However, there are limited effective and safe treatments. In this study, we demonstrated that a traditional Chinese medicine formula for refractory ulcers, named Tuo-Li-Xiao-Du-San (TLXDS), significantly improved wound healing of STZ-induced diabetic rats through reducing inflammation, increasing angiogenesis and collagen deposition.

Wounds go through three sequential and coordinate phases, inflammation, tissue formation, and tissue remodeling, to restore morphological and functional integrity [13]. Diabetes impairs wound healing through magnifying the inflammatory response, inhibiting angiogenesis, and decreasing ECM deposition [3]. Inflammation is the first and indispensable response after acute skin injury, usually subsiding in less than 5 days after wounding [14]. However, metabolic defects, such as hyperglycemia and oxidative stress, induce excessive proinflammatory cytokines (IL-1β, TNF-α, etc.) production [15, 16], sustaining a prolonged influx of neutrophils and macrophages [14, 17, 18]. As shown in the present study (Figure 3), diabetic wounds were still stuck in large amount inflammatory cells infiltration and apparent tissue necrosis on day 5 after wounding, while nondiabetic wounds already moved forward to the proliferative stage of

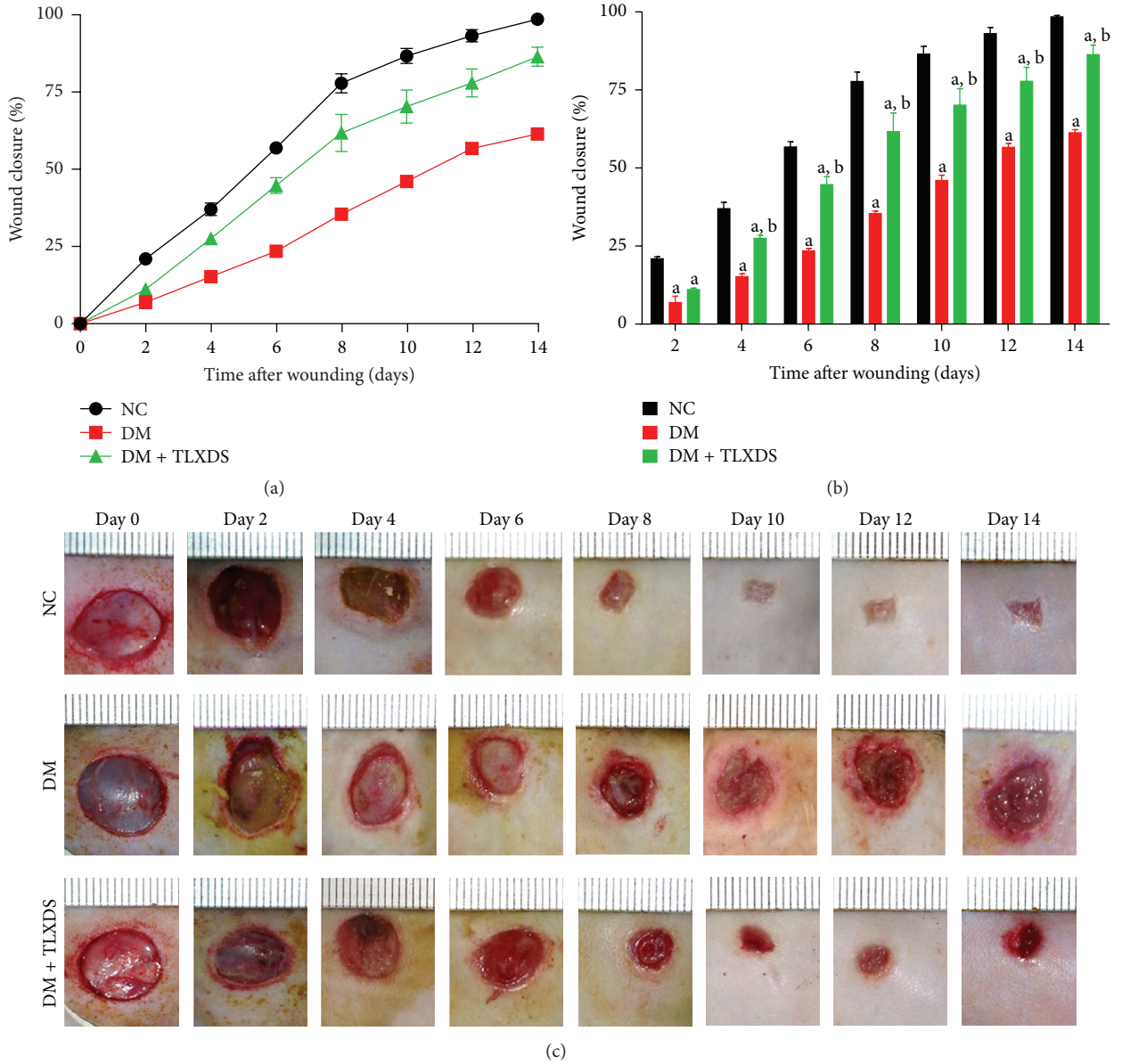

Figure 2: TLXDS treatment accelerated wound healing in diabetic rats. Percentage of wound closure (mean ± SEM) of 10 mm punch biopsies was monitored every other day until day 14 (a, b). Healing of diabetic wounds was significantly delayed compared with nondiabetic wounds at all observation time points ($P < 0.05$). TLXDS began to significantly improve wound closure on day 4 ($P < 0.05$). At the end of observation (14 days), 86.8% of the wounding area healed in DM + TLXDS group, while the closure rate in DM group was only 61.6%. (c) Typical photographs of wound healing for each group. $^{a}P < 0.05$, compared with NC group, and $^{b}P < 0.05$, compared with DM group. $n = 7$ for each group at each monitored time point.

healing. Moreover, by 14 days after wounding, neutrophils and macrophages were still abundant in diabetic wounds. Persisting inflammatory cells create a protease (neutrophil elastase, MMPs, and gelatinase) rich hostile microenvironment [19], resulting in extracellular matrix and growth factors degradation [20, 21]. Therefore, diabetic wounds are entrapped in a self-sustaining cycle of chronic inflammation and never get mature enough to move forward to the next stage of healing. The present study showed that administration of TLXDS significantly accelerated inflammation resolution by decreasing the expression of inflammatory cytokines, such as IL-1β and TNF-α, and thereby reducing the neutrophils and macrophages abundance in diabetic wounds. Reducing inflammation by TLXDS treatment might provide a favorable microenvironment for other repair cells to play their roles in healing the wounds.

In response to hypoxia after injury, endothelial cells and pericytes are recruited by angiogenic factors from existing vessels and proliferate to form new and functional blood vessels which provide the essential oxygen and blood supply for regenerating new tissues, a process known as neovascularization [22, 23]. Inadequate neovascularization

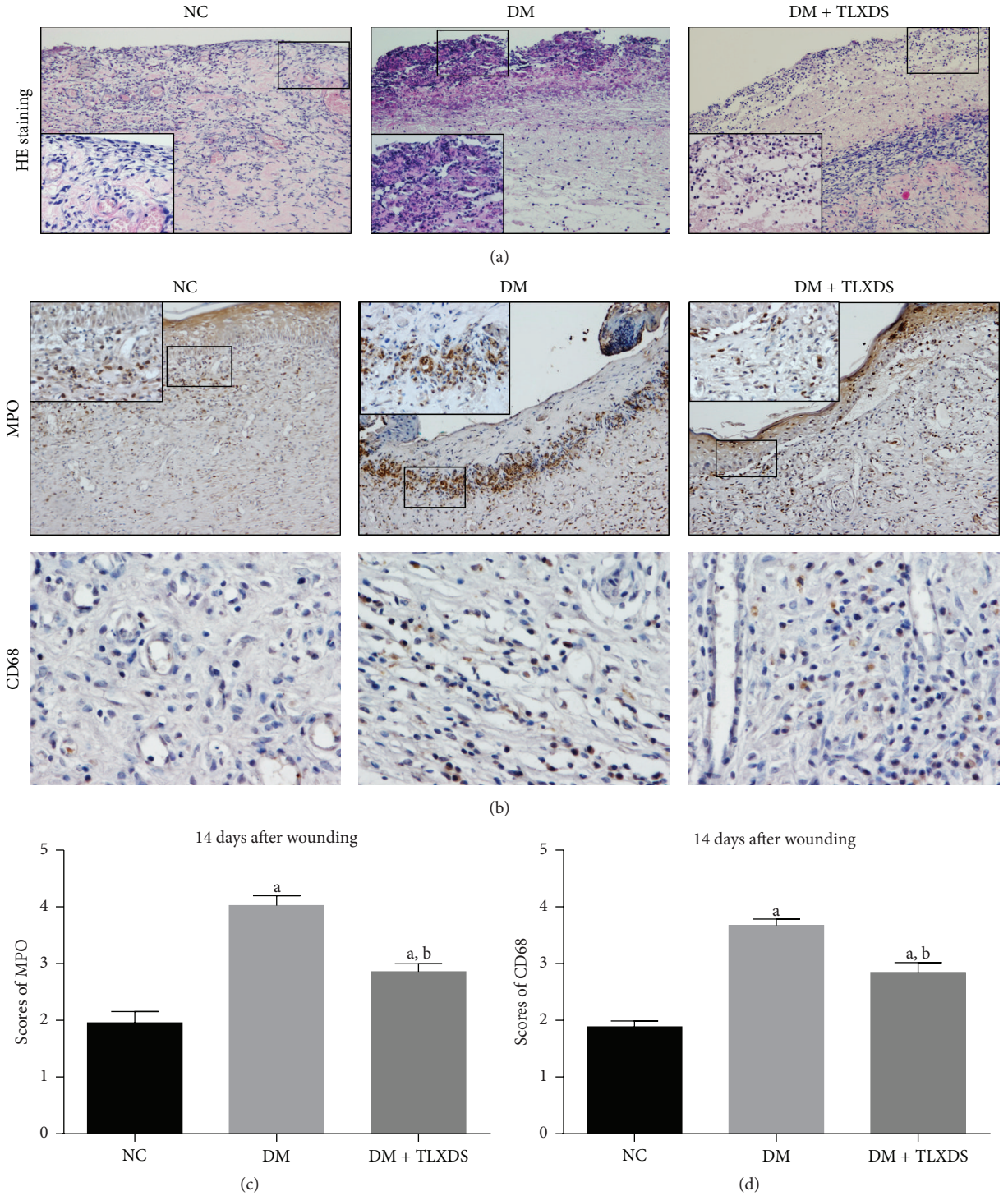

Figure 3: Effects of TLXDS treatment on inflammatory cells infiltration and inflammation resolution assessed by hematoxylin and eosin- (H&E-) stained histology on day 5 (a) and immunohistochemistry of MPO and CD68 on day 14 (b). Original magnification ×100 and insert magnification ×400. (c) and (d) Scores of MPO and CD68 staining. $n = 7$ for each group. Data are represented as means ± SEM. $^{a}P < 0.05$, compared with NC group, and $^{b}P < 0.05$, compared with DM group.

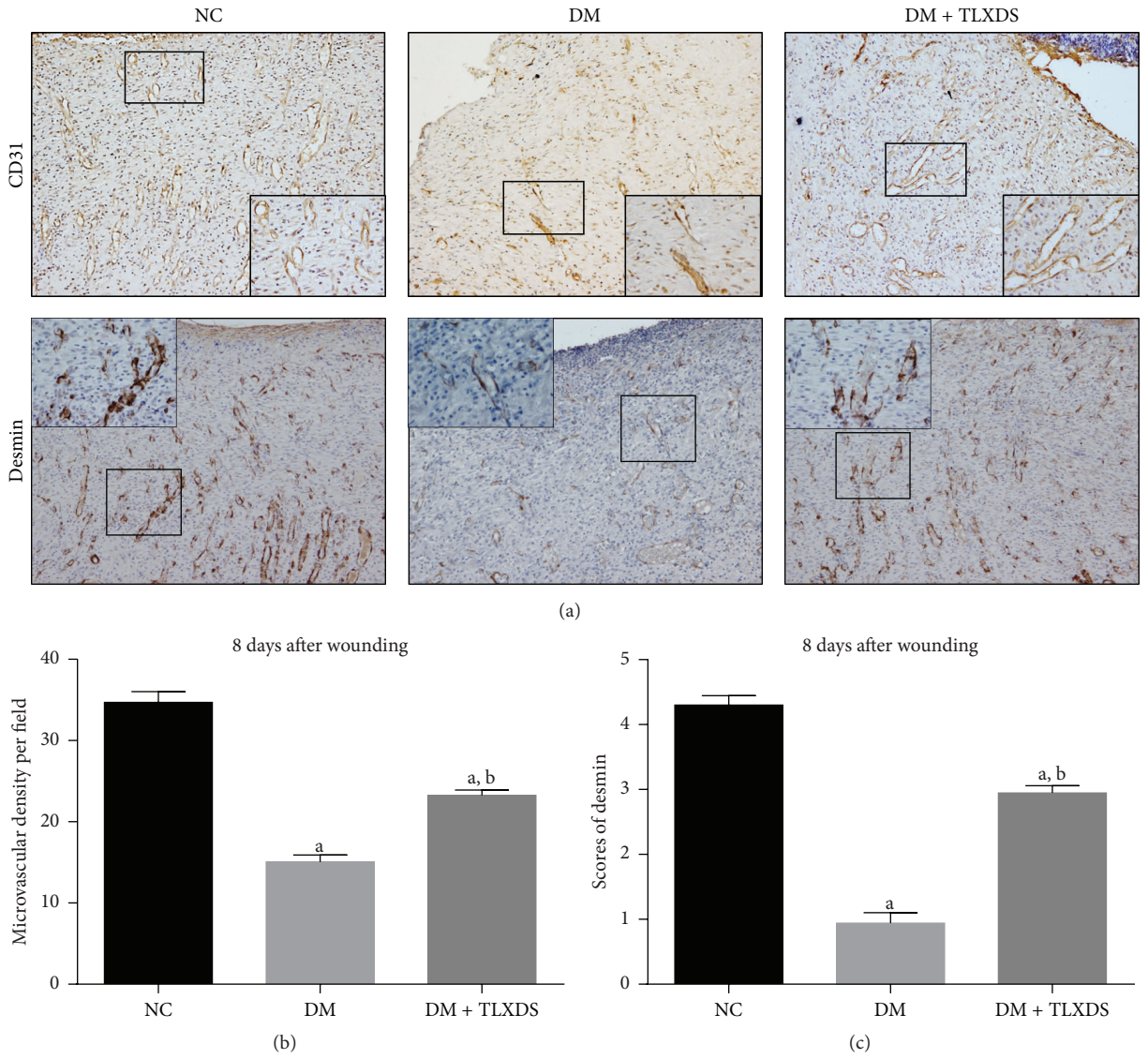

Figure 4: Effects of TLXDS treatment on angiogenesis assessed by the endothelial cell marker CD31 and pericyte marker desmin immunohistochemistry, respectively. (a) Representative CD31 and desmin-staining sections of NC group, DM group, and DM + TLXDS group on day 8 after wounding, respectively. Original magnification ×100 and inset magnification ×400. (b) Five "hot spots" in each specimen in which the CD31 antibody signal was the most intense were chosen and captured. The number of blood vessels was then counted by two investigators who were blinded to the treatment of the rats using the "manual tagging" feature in Image Pro-Plus software package. (c) Graphic visualization of scores of desmin staining on day 8. $n = 7$ for each group. Data are presented as means ± SEM. $^{a}P < 0.05$, compared with NC group; $^{b}P < 0.05$, compared with DM group.

is a cardinal feature of nonhealing diabetic wounds. The mechanisms underlying this impairment are studied a lot and now researchers widely accept that inadequate production of angiogenic growth factors, such as VEGF and PDGF, is a fundamental cause [24–26]. In this study, we observed that TLXDS restores diabetes-impaired neovascularization by increasing recruitment of both endothelial cells and pericytes. The mechanisms of TLXDS's effects in boosting angiogenesis of diabetic wounds might be related, at least in part, to the correction of reduced production of angiogenic growth factors, such as VEGF-A and PDGF-BB, which is demonstrated in this study.

Furthermore, Masson's trichrome staining and collagen type I immunohistochemistry demonstrated that TLXDS treatment increased collagen production of diabetic wounds significantly, which is important for functional recovery and healing quality [27]. This effect is probably secondary to the combined action of reduced inflammation and increased angiogenesis, since the degradation of ECM was decreased and the synthesis was fueled.

Compound formulae, usually called "FuFang" in Chinese, are combinations of TCM prescribed for treating various diseases in China. The therapeutic potencies of herbs are found additive, or even synergistic, when used

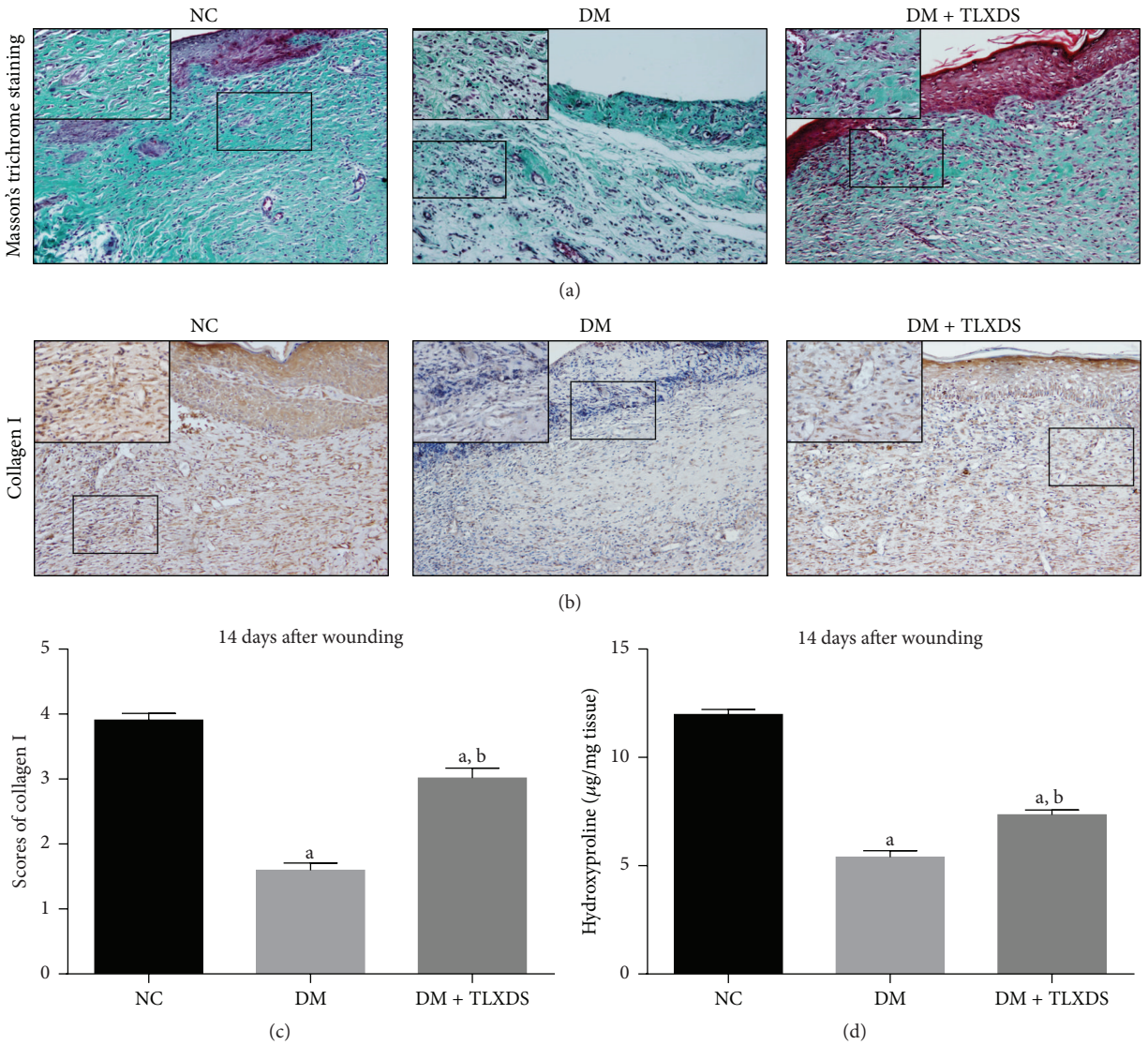

Figure 5: Effect of TLXDS treatment on extracellular matrix deposition evaluated by Masson's trichrome staining and collagen type I immunohistochemistry. Representative Masson's trichrome staining (a) and collagen type I staining sections (b) of NC, DM, and DM + TLXDS groups on day 14 after wounding. (c) Graphic visualization of scores of collagen type I staining on day 14. (d) Hydroxyproline content in the granulation tissue of each group on day 14. $n = 7$ for each group. Data are presented as means ± SEM. $^{a}P < 0.05$, compared with NC group, and $^{b}P < 0.05$, compared with DM group.

as combination. This is a great advantage for compound formulae in TCM. In Chinese medicine theory, TLXDS includes the therapeutic method of "TUO" which means raising "Qi" (vital energy) and nourishing "Blood" (body circulation) and the therapeutic method "TOU" which means cleansing wound environment and eliminating toxins. Recent researches show constituents of "TOU method," *Angelica dahurica* and thorns of *Gleditsia sinensis*, exhibit antibacterial and anti-inflammatory activities [28, 29]; the constituents of "TUO method," *Astragalus membranaceus* and *Angelica sinensis*, promote angiogenesis and the expression of angiogenic growth factors [30, 31]. Oftentimes, hundreds or even thousands of years of clinical practice has optimized formulae in TCM. The present study showed that TLXDS improves diabetic wounding healing as indicated in the ancient Chinese medicine theory and modern researching literature. There is great possibility that combining "TOU method" and "TUO method" can produce complementary and synergistic effects. It is of great interest in the future for us to reveal whether pleiotropic effects on diabetic wound healing of TLXDS in diabetic rats are attributed to the synergistic action between TUO method and TOU method. Furthermore, to clarify the active ingredients in TLXDS is of great practical significance as well.

It is noticeable that ulcer healing in diabetic ulcer and diabetic foot is complicated with many chronic problems, including long-term uncontrolled hyperglycemia, peripheral vascular disease, neuropathies, excessive pressure to the wound sites, and infection secondary to compromised immunity [2]. One pathogenic abnormality can lead to

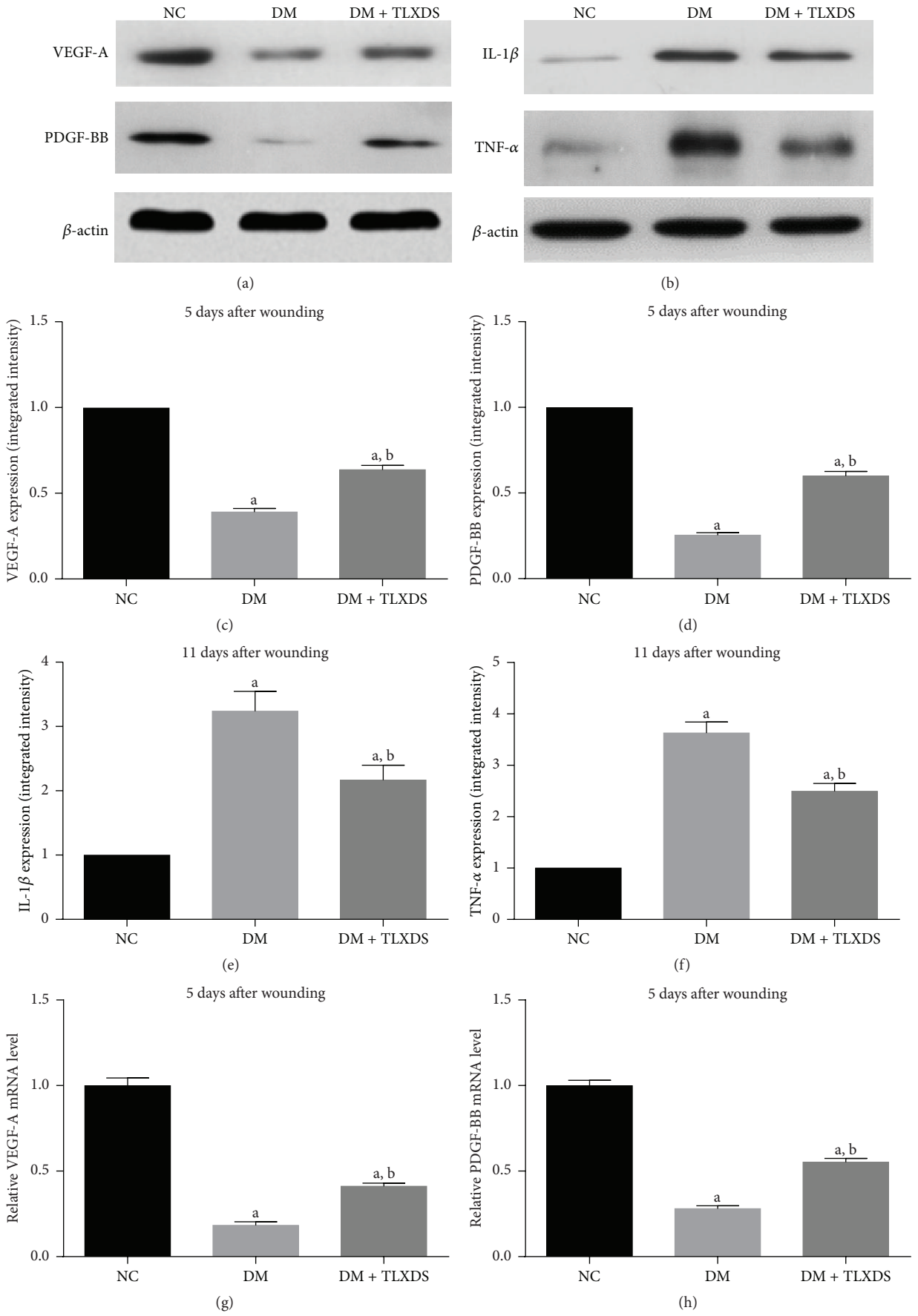

Figure 6: Continued.

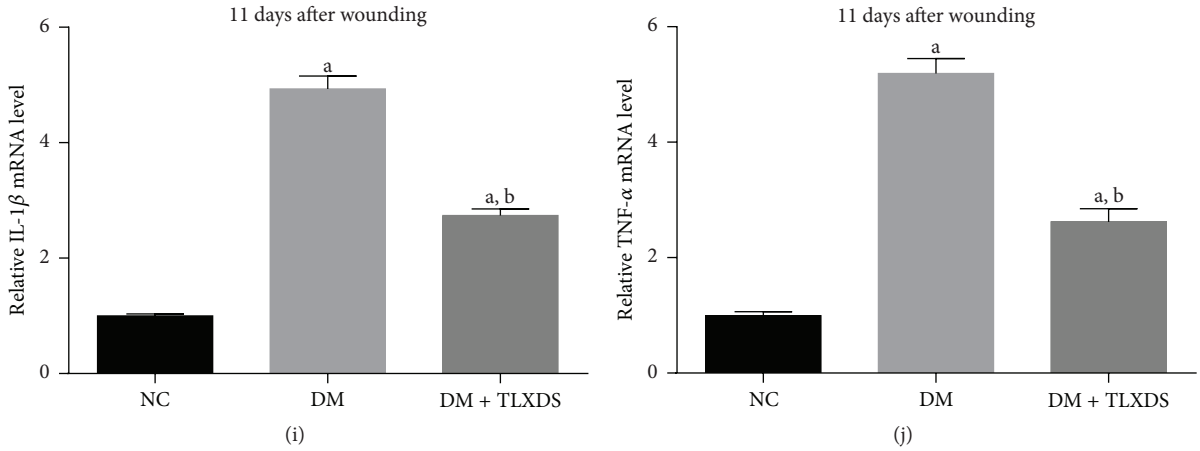

Figure 6: Effect of TLXDS treatment on the vascular endothelial growth factor-A (VEGF-A), platelet-derived growth factor BB (PDGF-BB), interleukin-1β (IL-1-β), and tumor necrosis factor-α (TNF-α) expressions assessed by western blot and real-time PCR analysis. Representative immunoblots of VEGF and PDGF-BB on day 5 after wounding ((a), $n = 9$ for each group), IL-1-β and TNF-α on day 11 after wounding ((b), $n = 7$ for each group). Quantifications of the bands (c–f). Quantification of VEGF-A, PDGF-BB, IL-1-β, and TNF-α mRNA expression (g–j). Data are presented as means ± SEM. $^{a}P < 0.05$, compared with NC group, and $^{b}P < 0.05$, compared with DM group.

another, developing vicious cycles of pathogenicity in the diabetic chronic ulcers. Considering the heterogeneity and complexity of human diabetic foot, no single animal model is capable of fully recapitulating each clinical scenario [32]. In the present study, we employed a widely used STZ-induced diabetic animal wound model [33, 34] to simulate the hyperglycemic state of diabetics and its impairment on wound healing. The results showed that TLXDS treatment is able to correct the abnormal healing process induced by hyperglycemia through reducing inflammation, increasing angiogenesis, and collagen deposition. Whether TLXDS could improve other abnormalities leading to diabetic foot, such as vascular disease and neuropathies, will be a constructive investigative direction in our further research work by using appropriate animal or cell models. And whether these findings can be extrapolated to the situation encountered in diabetic foot in patients still needs further investigation in clinical trials.

In summary, this study showed that TLXDS, a traditional Chinese medicine formula for refractory ulcers, has a positive effect on diabetes-impaired wounds. The improved wound healing is associated with reduced inflammation, increased angiogenesis, and collagen deposition after TLXDS treatment. The oral administration of traditional Chinese herbal medicine could provide an alternative and effective approach for diabetic foot ulcer therapy.

Conflict of Interests

All authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Xiao-na Zhang, Ze-jun Ma, Cong-qing Pan, and Li-ming Chen designed the study; Xiao-na Zhang, Ze-jun Ma, Ying Wang, Yu-zhu Li, Bei Sun, and Xin Guo performed the research; Xiao-na Zhang and Ze-jun Ma wrote the paper. Xiao-na Zhang and Ze-jun Ma contributed equally to this study.

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