Journal of Diabetes Research

The Unattended Borderline of Diabetic Neuropathy

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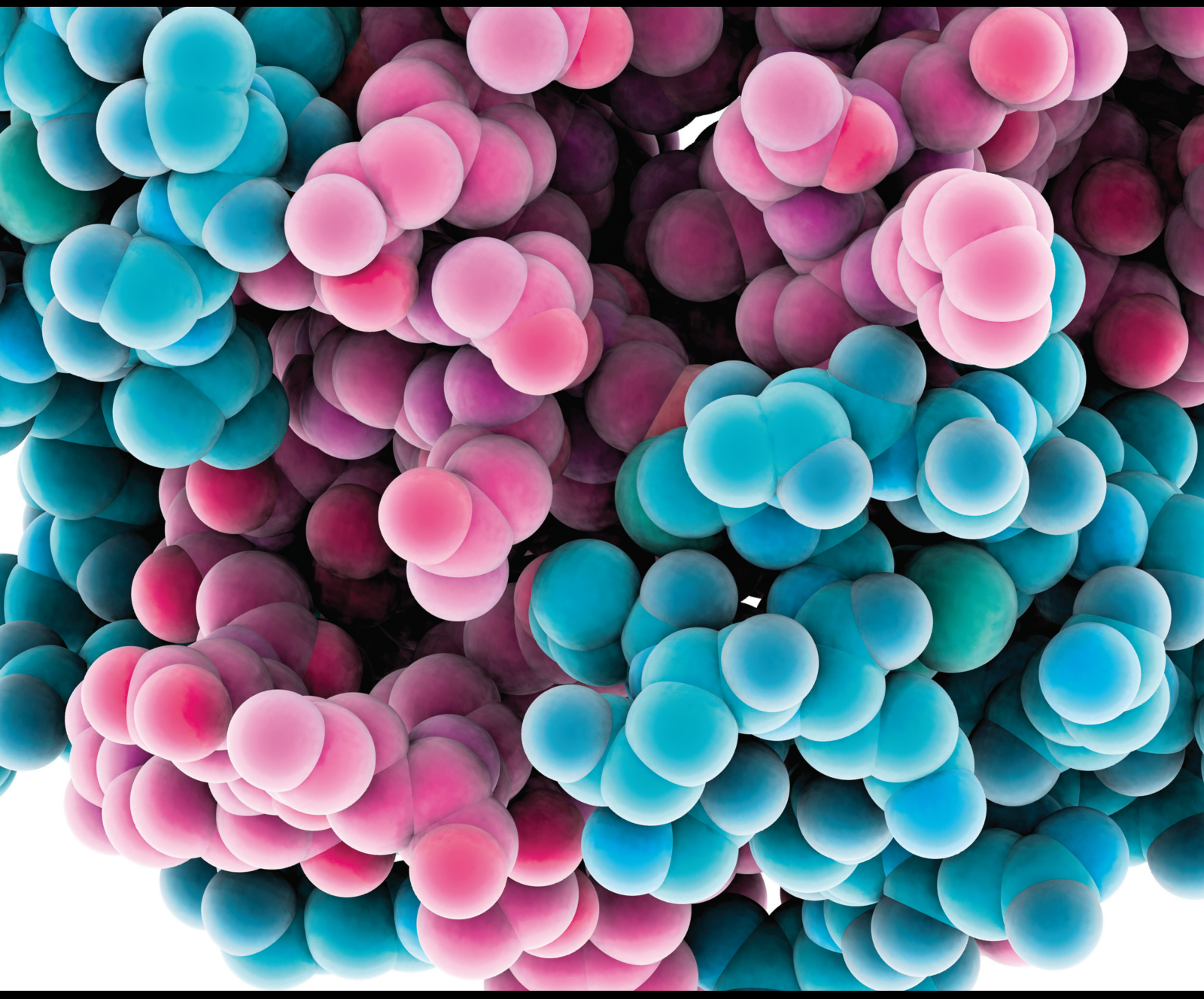

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Research Article

Skin Advanced Glycation End Products among Subjects with Type 2 Diabetes Mellitus with or without Distal Sensorimotor Polyneuropathy

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Aim of the Study. To examine the correlation between skin AGEs and parameters of distal sensorimotor polyneuropathy (DSPN) in type 2 diabetes mellitus (T2DM). *Materials and Methods*. We included 132 subjects (88 men) with a mean age of 64.57 years and median T2DM duration of 14.5 years. Skin AGEs were measured with AGE reader mu connect (Diagnoptics) on the dominant arm. The device enables single and automated triplicate measurements: both of these were performed. DSPN was diagnosed through the neuropathy disability score (NDS). Small nerve fibre function was assessed by temperature and pinprick sensation on the foot. Bilateral measurement of the vibration perception threshold (VPT) on the hallux was carried out by using a neurothesiometer (Horwell Scientific Laboratory Supplies). *Results*. Single and triplicate AGE measurements were positively correlated with each other (Pearson's correlation coefficient $r = 0.991$, 95%CI = 0.987-0.994, $p < 0.001$). AGEs were higher among subjects with vs. those without DSPN ($p < 0.001$). Furthermore, they were higher among subjects with reduced vs. normal temperature sensation ($p < 0.001$), among subjects with reduced vs. normal pinprick sensation ($p = 0.002$), among those with abnormal vs. normal monofilament examination ($p < 0.001$), and among those with abnormal vs. normal VPT ($p < 0.001$). AGEs were correlated with NDS, VPT, and monofilament score. *Conclusions*. In T2DM, skin AGEs are increased in the presence of DSPN. This holds true both for large and for small nerve function impairment. Moreover, AGEs are correlated with DSPN severity.

1. Introduction

Diabetic neuropathy remains a major chronic complication of diabetes mellitus [1]. It may, in turn, lead to further complications, notably diabetic foot, neuropathic pain, and autonomic failure [2]. Its commonest manifestation is chronic distal sensorimotor polyneuropathy (DSPN) [3]. Chronic hyperglycaemia represents a major underlying pathogenic mechanism [4]. Excess serum glucose activates several biochemical pathways and, among others, leads to the formation of advanced glycation end products (AGEs) [5, 6]. The latter promote inflammation and impair normal electrical activity in neurones [6]. AGEs may be measured by the enzyme-linked immunosorbent assay (ELISA), high-performance liquid chromatography (HPLC), mass spectrography, and tissue biopsy or through evaluation of skin autofluorescence [5, 6]. In recent years, their measurement in the skin has attracted considerable interest, because it is noninvasive and accurate [5–7]. It has been discussed that skin AGEs measurement might serve as a risk marker of atherosclerotic disease [7, 8] or of microvascular complications of diabetes [6, 9].

However, the association of AGEs with DSPN is still not demonstrated enough. Therefore, the aim of this study was to examine the correlation between skin AGEs and parameters of DSPN in subjects with type 2 diabetes mellitus (T2DM).

2. Materials and Methods

This study included 132 subjects (88 men, 44 women) with a mean age of 64.57 ± 8.21 years and median T2DM duration of 14.5 years (range 7.00-20.00) who were attending the Diabetes Centre of the Second Department of Internal Medicine at Democritus University of Thrace, Greece. These were randomly chosen and offered an examination. The study was approved by the institutional ethics committee, and all patients gave their informed consent.

Inclusion criteria were age above 18 years and T2DM. Exclusion criteria were as follows: age ≥ 85 years, inability to undertake the examination, severe illness, severe infection, hypoglycaemia, liver cirrhosis, alcohol abuse, B12 depletion, other causes of neuropathy, heart failure, dermatologic disease at the measuring site, tattoos, exposure to skincare creams or any other substance that may have fluorescent properties, self-tanning agents in the past 10 days, Fitzpatrick skin type > V, and chronic kidney disease (estimated glomerular filtration rate < 60 mg/dl) [6, 7].

Skin AGEs were measured with AGE reader mu connect (Diagnoptics, NL) on the dominant arm, according to the manufacturer's instructions [4]. Subjects were asked to place their dominant forearm on the device, the elbow being aligned with the reader's edge. Measurements were taken once the dominant arm was in the correct position. The device illuminates a small portion of the skin (approximately 4 cm^2) on the volar side of the examinee's forearm [4]. It produces light on the selected area with an excitation light source of ~370 nm. Emission light and reflected excitation light emanating from the skin are measured using a glass fibre in the 300-600 nm range [4]. The device enables single and automated triplicate measurements: both of these were performed [4]. AGEs were expressed in arbitrary units, as per the manufacturer [4].

Diagnosis of DSPN was based on the neuropathy disability score (NDS), an established clinical examination score [10]. DSPN was defined as NDS ≥ 3 [10]. In a simplified approach based on its original classification [10], DSPN was defined as absent (NDS 0-2), mild (NDS 3-5), and moderate/severe (NDS 6-10).

Small nerve fibre function was evaluated by temperature and pinprick sensation on the foot [11]. These were evaluated on the dorsal foot aspect using a Tiptherm rod and a sterile single-use lancet, respectively [11].

The vibration perception threshold (VPT) on the hallux was measured bilaterally with a neurothesiometer (Horwell Scientific Laboratory Supplies) [12]. Abnormality was defined as VPT > 25 V, using the lower of the two measurements [12]. Then, patients were classified into those with normal (<16 V), mildly impaired (16-25 V), and severely impaired VPT (>25 V) [13, 14].

Finally, 10 g Semmes Weinstein monofilaments were used on 10 foot sites [14, 15] bilaterally. The monofilament score was the number of correct answers [14]. Abnormality was defined as monofilament score < 8, using the lower of the two measurements [14, 15].

2.1. Statistical Analysis. Analysis was carried out using the IBM Statistical Package for the Social Sciences (SPSS), version 19.0 (IBM Corp., Armonk, NY, USA). The normality of quantitative variables was tested by the Kolmogorov-Smirnov test. Normally distributed quantitative variables were expressed as mean ± standard deviation (SD), while qualitative variables were expressed as absolute and relative (%) frequencies. The association of AGEs with patients' demographic and clinical characteristics was assessed using Student's t-test and analysis of variance (ANOVA); post hoc comparisons were performed using Tukey's test. Correlations were assessed by Pearson's r and intraclass (ICC, two-way mixed effects model with average measures) correlation coefficients.

Receiver operating characteristic (ROC) analysis was used to evaluate the diagnostic significance of single AGEs measurement and triplicate AGEs measurement for large fibre impairment (impaired tuning fork perception or ankle reflexes), small fibre impairment (impaired temperature or pinprick sensation), and overall DSPN. The area under the ROC curve (AUC), sensitivity, specificity, and positive and negative predictive values were calculated, while Cohen's kappa was used to assess agreement. The optimal cut-off values were derived according to the Youden index [16]. All tests were two-tailed. Statistical significance was defined at 5% ($p < 0.05$).

3. Results

Single AGEs measurement was positively correlated with their triplicate measurement (Pearson's correlation coefficient $r = 0.991$, 95%confidence interval (CI) = 0.987-0.994, $p < 0.001$; intraclass correlation coefficient (ICC) = 0.995, 95 %CI = 0.994-0.997, $p < 0.001$).

In single measurement, AGEs were higher among subjects with vs. those without DSPN (3.31 ± 0.73 vs. 2.55 ± 0.56, $p < 0.001$). Furthermore, they were higher among subjects with reduced vs. normal temperature sensation ($p < 0.001$), among subjects with reduced vs. normal pinprick sensation ($p = 0.002$), among those with abnormal vs. normal VPT ($p < 0.001$), and among those with abnormal vs. normal monofilament examination ($p < 0.001$) (Table 1). Identical significant differences were observed in triplicate measurement (data not shown).

AGEs (single measurement) showed positive correlations with age ($r = 0.343$, $p < 0.001$), T2DM duration ($r = 0.275$, $p = 0.001$), NDS ($r = 0.551$, $p < 0.001$), VPT right foot ($r = 0.475$, $p < 0.001$), VPT left foot ($r = 0.422$, $p < 0.001$), monofilament score right foot ($r = -0.462$, $p < 0.001$), and monofilament score left foot ($r = -0.484$, $p < 0.001$). Similarly, AGEs (triple measurement) showed positive correlations with age ($r = 0.361$, $p < 0.001$), T2DM duration ($r = 0.283$, $p = 0.001$), NDS ($r = 0.555$, $p < 0.001$), VPT right foot

Table 1: AGEs (single measurement) and parameters of DSPN.

	AGEs (single measurement)		
Parameter	With DSPN	Without DSPN	p value
	3.31 ± 0.73	2.55 ± 0.56	<0.001
	Reduced temperature sensation	Normal temperature sensation	p value
	3.18 ± 0.72	2.51 ± 0.56	<0.001
	Reduced pinprick sensation	Normal pinprick sensation	p value
	3.86 ± 0.32	2.84 ± 0.72	0.002
	Abnormal VPT	Normal VPT	p value
	3.45 ± 0.69	2.74 ± 0.68	<0.001
	Abnormal monofilament	Normal monofilament	p value
	3.19 ± 0.69	2.62 ± 0.67	<0.001

AGEs: advanced glycation end products; DSPN: distal sensorimotor polyneuropathy; VPT: vibration perception threshold.

Table 2: AGEs in relation to the severity of DSPN.

	AGEs (single)	p value	AGEs (triplicate)	p value
NDS		<0.001		<0.001
No DSPN ($n = 76$)	2.55 ± 0.55		2.55 ± 0.54	
Mild DSPN ($n = 43$)	3.27 ± 0.74		3.25 ± 0.71	
Moderate/severe DSPN ($n = 13$)	3.50 ± 0.69		3.47 ± 0.71	
Multiple comparisons				
No DSPN vs. mild DSPN	—	<0.001	—	<0.001
No DSPN vs. moderate/severe DSPN	—	<0.001	—	<0.001
Mild DSPN vs. moderate/severe DSPN	—	0.500	—	0.484
VPT		<0.001		<0.001
Normal ($n = 62$)	2.58 ± 0.58		2.56 ± 0.56	
Mildly impaired ($n = 54$)	3.02 ± 0.77		3.00 ± 0.74	
Severely impaired ($n = 16$)	3.58 ± 0.56		3.59 ± 0.58	
Multiple comparisons				
Normal vs. mildly impaired	—	0.001	—	0.001
Normal vs. severely impaired	—	<0.001	—	<0.001
Mildly impaired vs. severely impaired	—	0.010	—	0.005

AGEs: advanced glycation end products; DSPN: distal sensorimotor polyneuropathy; NDS: neuropathy disability score; VPT: vibration perception threshold.

($r = 0.482$, $p < 0.001$), VPT left foot ($r = 0.422$, $p < 0.001$), monofilament score right foot ($r = -0.472$, $p < 0.001$), and monofilament score left foot ($r = -0.482$, $p < 0.001$).

AGEs in relation to the severity of DSPN (evaluated by NDS and VPT) are shown in Table 2. AGEs (both single and triplicate measurements) were significantly higher among subjects with severe impairments.

Table 3 summarises ROC analysis for the evaluation of the diagnostic significance of AGEs (single measurement) for large fibre impairment, small fibre impairment, and overall DSPN. The optimal cut-offs were ≥3.15 for large fibre impairment, ≥2.75 for small fibre impairment, and ≥2.95 for overall DSPN. The corresponding AUC values (and 95% confidence intervals) were 0.766 (0.671-0.861), 0.765 (0.685-0.846), and 0.790 (0.712-0.869) (on each occasion, $p < 0.001$). With these cut-offs, AGEs (single measurement) yielded moderately high agreement (>71%), sensitivity (>61%), specificity (>69%), and negative predictive value (NPV) (>68%), as well as low-moderate positive predictive value (PPV) (>51%). The highest sensitivity and PPV were seen for small fibre impairment, while the highest specificity and NPV were seen for large fibre impairment. ROC analysis was the same for triplicate AGE measurements (data not shown). ROC curves for both single and triplicate AGE measurements are shown in Figures 1–3.

4. Discussion

In T2DM, the present study has shown that skin AGEs are increased in the presence of DSPN. This holds true both for large and for small nerve fibre function impairment.

Table 3: ROC analysis for the evaluation of the diagnostic significance of AGEs (single measurement) for large fibre impairment, small fibre impairment, and overall DSPN.

	Large fibre	*p* value	Small fibre	*p* value	Overall DSPN	*p* value
AUC (95% CI)	0.766 (0.671-0.861)	<0.001	0.765 (0.685-0.846)	<0.001	0.790 (0.712-0.869)	<0.001
Cut-off	≥3.15		≥2.75		≥2.95	
Sensitivity (%)	61.8 (43.6-77.8)		74.0 (62.4-83.6)		69.0 (55.5-80.5)	
Specificity (%)	79.6 (70.3-87.1)		69.5 (56.1-80.8)		77.0 (65.8-86.0)	
PPV (%)	51.2 (39.6-62.7)		75.0 (66.6-81.9)		70.2 (60.0-78.7)	
NPV (%)	85.7 (79.5-90.3)		68.3 (58.6-76.7)		76.0 (67.9-82.6)	
Overall agreement (%)	75.0		72.0		73.5	
Cohen's kappa	0.388	<0.001	0.434	<0.001	0.461	<0.001
OR (95% CI)	6.30 (2.70-14.72)	<0.001	6.47 (3.02-13.87)	<0.001	7.45 (3.43-16.20)	<0.001

AUC: area under the curve; CI: confidence interval; DSPN: distal sensorimotor polyneuropathy; NPV: negative predictive value; OR: odds ratio; PPV: positive predictive value.

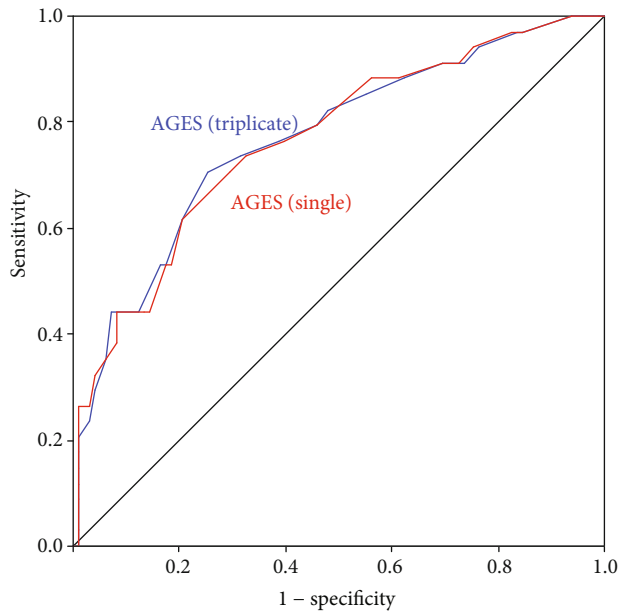

Figure 1: ROC analysis for the evaluation of the diagnostic significance of AGEs (single measurement) and AGEs (triplicate measurement) for large fibre impairment.

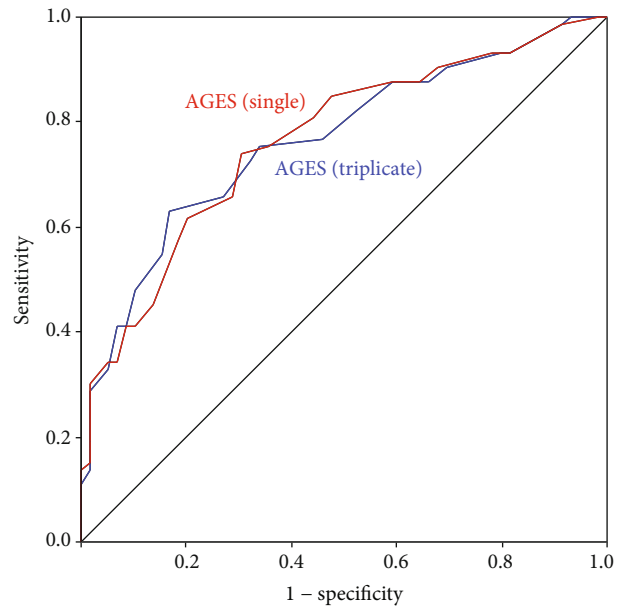

Figure 2: ROC analysis for the evaluation of the diagnostic significance of AGEs (single measurement) and AGEs (triplicate measurement) for small fibre impairment.

Moreover, a positive correlation between skin AGEs and DSPN severity was noted.

Our findings are in line with previous reports [17–22]. Indeed, in a multicentre study including 497 participants with diabetes mellitus (including both diabetes types), a significant increase in skin AGEs was seen in the presence of DSPN (defined using the Toronto Clinical Neuropathy Score, the Neuropathy Symptom Score, and the NDS) [17]. Skin AGEs were elevated among participants with NDS ≥ 3, compared with those exhibiting a lower NDS score [17]. Further evidence pointed to an increased accumulation of skin AGEs in T2DM Japanese subjects with DSPN (diagnosed by the presence of ≥1 neuropathic symptoms, abnormal vibration perception, and absence of ankle/knee reflexes), as compared with those without [18, 19]. When DSPN was defined as history of diabetic foot ulceration, a positive correlation was seen with the higher tertiles of skin AGEs, even following adjustment for several confounding factors including macrovascular disease [20].

A more recent work including 820 T2DM Chinese participants used nerve conduction study for DSPN diagnosis [21]. Higher skin AGEs were linked with a five-fold increased risk of DSPN (odds ratio = 5.15; 95%CI = 1.48 -4.53, $p < 0.01$), and a cut-off value > 2.57 predicted a three-fold increased risk of DSPN [21]. Taken together, our and previous reports reveal an association between elevated skin AGEs and both DSPN presence and severity. Of note, the latter has been diagnosed by various modalities.

Importantly, the present study appears to be the only one to have looked separately at large and small fibre impairment. Indeed, skin AGEs were increased in both small and large nerve fibre impairments. As regards the

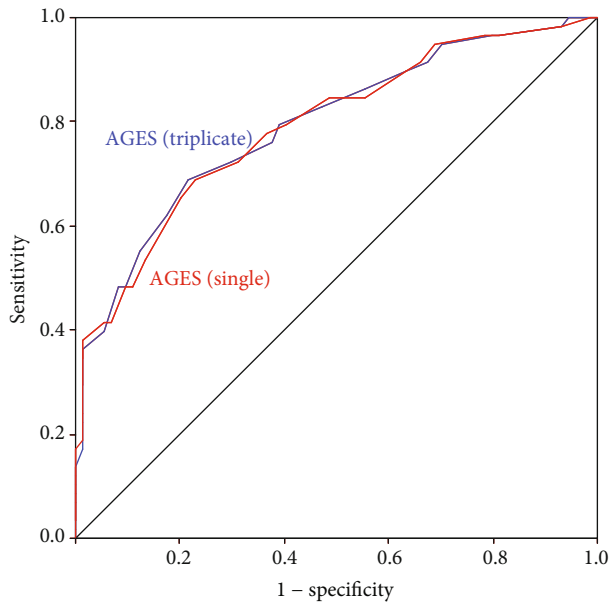

Figure 3: ROC analysis for the evaluation of the diagnostic significance of AGEs (single measurement) and AGEs (triplicate measurement) for overall DSPN.

former, reduced temperature and pinprick sensation (estimates of small nerve fibres [14, 15]) were seen in participants with higher skin AGE levels. The same was seen for parameters assessing the latter (abnormal VPT and monofilament). So far, a positive association between skin AGEs and increased VPT, even before reaching the VPT cut-off indicating high risk of foot ulceration, has been reported [23, 24]. Similarly, nerve conduction velocity and amplitude of the median, sural, and peroneal nerve were negatively correlated with skin AGEs among diabetic participants (both diabetes types) with or without DSPN [25].

As regards small nerve fibres, our findings are in line with the previously reported inverse relationship between skin AGEs and electrochemical skin conductance [26]. However, we assessed small fibre impairment differently from the previous study.

Skin AGEs (either single or triple measurement) in our study showed positive correlations with age, T2DM duration, NDS, VPT, and monofilament score in each lower extremity. Again, these results are in line with previous reported associations of AGEs with age, smoking, renal function, macroangiopathy, and microvascular complications [19, 27]. Other associations have previously included body mass index, HbA_{1c}, high-density lipoprotein cholesterol, and albumin-to-creatinine ratio [28]. Interestingly, in a study registering the most ancient available previous HbA_{1c} before admission to the study [20], skin autofluorescence was independently related to this and not the most recent HbA_{1c}, potentially demonstrating glucose memory as one of the main variables for skin AGEs accumulation.

Triplicate measurements were highly correlated with single measurements. All results and associations observed with single measurements were also observed with triplicate measurements with almost identical numbers. This finding is novel and suggests that triplicate AGEs measurements are not required to study the association with DSPN. This holds true, although triplicate measurements are also simple and quick to perform.

Interestingly, with appropriate cut-offs identified by ROC analysis, AGEs measurement (both single and triplicate) yielded moderately high sensitivity, specificity, and NPV, while its PPV was low-moderate. This held true for large fibre impairment, small fibre impairment, and overall DSPN. The highest sensitivity and PPV were seen for small fibre impairment, while the highest specificity and NPV were seen for large fibre impairment. Based on these observations, the contribution of AGEs measurement to DSPN diagnosis is rather moderate. Arguably, they may be slightly more useful for the diagnosis of small fibre impairment and for the exclusion of large fibre impairment, but caution and further experience are needed.

The strength of the study is the inclusion of several DSPN parameters. A limitation is the smaller number of female than male participants. A second limitation is the clinical diagnosis of DSPN. Indeed, we did not use more sophisticated modalities, such as nerve conduction velocity, intraepidermal nerve fibre density via skin biopsy, or corneal confocal microscopy, which may enable earlier diagnosis of subclinical DSPN [1, 2, 29]. Moreover, subjects were included in a tertiary care centre, and so results may not be directly applicable to primary care and/or the general T2DM population. Furthermore, our study offers no prospective data on DSPN development, but this was beyond its scope. Finally, extending our observations to other microvascular complications (microalbuminuria, diabetic retinopathy) would be interesting, but this was beyond the scope of this work focusing on neuropathy.

The implications of the present study may be outlined as follows. Skin AGEs are higher in the presence of DSPN and are associated with its severity. Whether they represent an aetiological factor or whether they are merely a manifestation of nerve damage cannot be answered by the present study. Indeed, longitudinal data looking at the risk of developing DSPN in relation to AGEs would be required to clarify this issue. We also need more information as to how skin AGEs might, perhaps, be used as a screening tool of DSPN in everyday practice. For this purpose, simplicity and rapidity of the examination would be useful advantages, but the cost and limited availability of the device are important disadvantages.

5. Conclusions

In T2DM, skin AGEs are increased in the presence of DSPN. This holds true both for large and for small nerve function impairment, as well as for loss of protective sensation. Moreover, AGEs are correlated with DSPN severity. These results add to our insights into the role of AGES in DSPN and suggest that their further study including prospective data is justified.

Data Availability

Data is available upon reasonable request.

Conflicts of Interest

NP has been an advisory board member of Astra-Zeneca, Boehringer Ingelheim, MSD, Novo Nordisk, Pfizer, Takeda, and TrigoCare International; has participated in sponsored studies by Astra-Zeneca, Eli-Lilly, GSK, MSD, Novo Nordisk, Novartis, and Sanofi-Aventis; has received honoraria as a speaker for Astra-Zeneca, Boehringer Ingelheim, Eli-Lilly, Elpen, MSD, Mylan, Novo Nordisk, Pfizer, Sanofi-Aventis, and Vianex; and attended conferences sponsored by TrigoCare International, Eli-Lilly, Galenica, Novo Nordisk, Pfizer, and Sanofi-Aventis. The other authors report no conflicts of interest.

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Review Article

What Is in the Field for Genetics and Epigenetics of Diabetic Neuropathy: The Role of MicroRNAs

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Despite the high prevalence of diabetic neuropathy, its early start, and its impact on quality of life and mortality, unresolved clinical issues persist in the field regarding its screening implementation, the understanding of its mechanisms, and the search for valid biomarkers, as well as disease-modifying treatment. Genetics may address these needs by providing genetic biomarkers of susceptibility, giving insights into pathogenesis, and shedding light on how to select possible responders to treatment. After a brief summary of recent studies on the genetics of diabetic neuropathy, the current review focused mainly on microRNAs (miRNAs), including the authors' results in this field. It summarized the findings of animal and human studies that associate miRNAs with diabetic neuropathy and explored the possible pathogenetic meanings of these associations, in particular regarding miR-128a, miR-155a, and miR-499a, as well as their application for diabetic neuropathy screening. Moreover, from a genetic perspective, it examined new findings of polymorphisms of miRNA genes in diabetic neuropathy. It considered in more depth the pathogenetic implications for diabetic neuropathy of the polymorphism of MIR499A and the related changes in the downstream action of miR-499a, showing how epigenetic and genetic studies may provide insight into pathogenetic mechanisms like mitochondrial dysfunction. Finally, the concept and the data of genotype-phenotype association for polymorphism of miRNA genes were described. In conclusion, although at a very preliminary stage, the findings linking the genetics and epigenetics of miRNAs might contribute to the identification of exploratory risk biomarkers, a comprehensive definition of susceptibility to specific pathogenetic mechanisms, and the development of mechanism-based treatment of diabetic neuropathy, thus addressing the goals of genetic studies.

1. Introduction: Unresolved Clinical Issues in Diabetic Neuropathy

Both distal symmetric polyneuropathy (DPN) and cardiovascular autonomic neuropathy (CAN) are common complications of diabetes. DPN and CAN affect 30% and 20%, respectively, of the unselected population with diabetes, and their prevalence increases according to age and diabetes duration [1, 2]. Moreover, they both start early in the natural history of diabetes and are present also in prediabetes with a prevalence from 5.7% to 13% for DPN and 11% for CAN [3, 4]. Beyond the impact on the quality of life of painful forms of DPN and the clinical forms of autonomic neuropathy and the primary pathogenetic role of DPN in foot complications, both DPN and CAN have a heavy toll on survival also before the development of foot ulceration with an increased (up to 4 times) risk for mortality [5–9]. Notwithstanding, diabetic neuropathy is without doubt the least screened and diagnosed complication [10].

Moreover, not all patients needing symptomatic relief receive appropriate treatment, the latter is not universally effective [11, 12], and conclusive evidence-based efficacy of disease-modifying treatments is still lacking, or limited evidence for some of them has not yet reached the necessary requirements of the main regulatory agencies for their inclusion in guidelines. Thus, there are a number of unresolved clinical issues in the field of diabetic neuropathy, such as screening implementation, understanding of the mechanisms,

identification and qualification of valid biomarkers, and developing disease-modifying treatments.

2. How May Genetics Address Diabetic Neuropathy Needs?

Even when considering the most effective preventive strategy (i.e., intensive glycemic control in type 1 diabetes that is able to prevent up to more than 60% of new cases of CAN and DPN), it is a matter of fact that for many patients (2% yearly), current strategies for optimizing glucose control are insufficient to fully prevent or delay the development of neuropathic complications [13]. Thus, the development and progression of neuropathic complications in any single patient cannot be completely anticipated by the control of hyperglycemia or other risk factors, and, consequently, genetic factors come into play. Indeed, the wide interindividual variability observed in diabetic neuropathy, in terms of susceptibility, clinical manifestations, and disease severity, has suggested that also genetic factors may influence the natural course of development of these complications.

The search for biomarkers, i.e., a measurable indicator of a pathophysiological condition, has become a main research topic in the field of DPN with the need to identify outcome biomarkers for clinical efficacy in clinical trials (surrogate for clinical endpoint), sensitive biomarkers for the very early stages of disease (useful to discern which patients in a preclinical stage are at a higher risk of developing clinical DPN), prognostic biomarkers for the tough consequences of DPN, and biomarkers pertaining to pathogenetic mechanisms in order to identify responsive patients to therapeutic agents targeted at these mechanisms [14, 15].

Genetics may address diabetic neuropathy needs by providing exploratory genetic biomarkers of susceptibility for disease development, by giving insights into the pathogenesis of neuropathy and neuropathic pain, and by shedding light on how to select the responders to treatment.

3. Candidate Gene and GWAS Approach

Genetics has in turn its own needs and requirements. First of all, two main different approaches are possible. The candidate gene approach starts with a hypothesis based on current knowledge of what kind of gene you intend to look for. It involves a limited number of variants, selected in candidate genes, and requires lower statistical power. Regarding diabetic neuropathy, candidate genes should encode proteins involved in the known mechanisms of nerve protection or damage in diabetes.

Genome-wide association study (GWAS) involves scanning the entire genome (or codifying genes) from different people, using platforms investigating thousands of variants [single-nucleotide polymorphisms (SNPs)] and looking for consistent difference between frequencies in variants associated with a disease. Without a priori-hypothesis, newly identified genetic markers can open to new knowledge. GWASs require large sample sizes (large-scale biobanks), rigorous thresholds for statistical significance ($p < 5 \times 10^{-8}$), wide coordination between different researchers and centers (Consortia), and considerable resources and replication studies [16].

Both these approaches are aimed at identifying genes and polymorphisms associated with the disease. They will then require further investigations to understand their possible functional role in the activity of the encoded proteins. Thus, the applicability of genetic variability studies for diabetic neuropathy involves the identification of genetic variants capable of influencing the level or the function of a clinical variable of interest for the complication, leading to pathogenetic consequences. Moreover, further studies should then verify if these genetic variants are associated with particular manifestations through a genotype-phenotype study.

4. Genetics of DPN: Where We Are

While genetic research has provided wide data on genetic variants associated with the risk of diabetic nephropathy and diabetic retinopathy, relatively little has been done regarding the genetics of neuropathy [17, 18] as also documented by less than half the number of publications than those for retinopathy and less than a third of those for nephropathy.

As expected, research on the genetics of diabetic neuropathy has mainly been oriented towards its pathogenesis. These multiple and interconnected mechanisms include abnormalities in glucose or lipid metabolism, oxidative stress, inflammation, endothelial dysfunction, endoplasmic reticulum stress, impaired nerve function, gene expression, and DNA damage [19]. These pathways might be at various stages the target of proteins encoded by genes whose polymorphisms are documented as relevant in diabetic neuropathy.

Four of these genes [i.e., the ACE gene, methylenetetrahydrofolate reductase (MTHFR) gene, glutathione peroxidase-1 (GPx-1) gene, and catalase (CAT) gene] have received greater attention, and even meta-analyses are available, with their polymorphisms possibly involved in the increased renin–angiotensin system activity, hyperhomocysteinemia, and reduced defense against oxidative stress [20]. Moreover, the pentose phosphate pathway has been considered for its pathogenetic relevance in hyperglycemic state, a potential therapeutic approach, and genetic variability, which has been documented for genes of thiamine transporter [21], transketolase, and glyoxalase [22, 23]. In addition, the role of genetic factors in the development of neuropathic pain has become increasingly recognized, with documentation in patients with painful DPN of variants of genes of μ-opioid receptor [24], a purinergic receptor [25], and the sodium channel Nav1.7 [26–28], possibly responsible for changes in opioid pain modulation or in increased excitability.

Finally, four GWASs are available in diabetic neuropathy, 3 from the datasets from UK Genetics of Diabetes Audit and Research in Tayside Scotland (GoDARTS) and pertinent to the phenotype of neuropathic pain and foot ulceration [29–31] and the most recent developed in the US Action to Control Cardiovascular Risk in Diabetes (ACCORD) study [32].

Thus, there is increasing attention given to the genetics of diabetic neuropathy, and although a suitable genetic

biomarker has not yet been developed, promising findings can be seen on the horizon.

This review is focused on the genetics and epigenetics of microRNAs in diabetic neuropathy, given the research done by our group on this topic in the last few years.

5. The Case of MicroRNAs for Diabetic Neuropathy

MicroRNAs (miRs or miRNAs) are small RNA molecules (20–22 nucleotide length) that act as regulators in biological processes. At least 20–30% of all human genes are regulated by miRNAs through targeting sequences in their 3′ untranslated region. DNA regulatory regions might be involved in the control of development processes, hematopoietic cell differentiation, apoptosis, cell proliferation, and organ growth and in disease development. Increasing evidence supports the involvement of miRNAs in diabetes and its micro- and macrovascular complications [33].

5.1. miRNA Expression and Diabetic Neuropathy in Animal Models. Regarding diabetic neuropathy, in the last decade, very few studies have addressed miRNA expression in diabetic neuropathy with it being almost exclusively preclinical [34, 35]. Studies in animal models of diabetes and diabetic neuropathy have documented changes in the expression of a few miRNAs in peripheral nerve structures as related to changes in key points of pathways known as or presumed to be involved in the diabetes-related pathogenesis of nerve damage, regeneration, and in pain generation [36–43] (Table 1). In some studies, miRNAs target novel possibly pathogenetic mechanisms and miRNA mimics or anti-miR is utilized to confirm the hypothesized biological effect. This is the case of miR-25, found to be reduced in the sciatic nerve of diabetic mice and shown to act as a protective factor against advanced glycation endproducts and its receptor (AGEs-RAGE), as well as against oxidative stress through a reduction in protein kinase C alpha (PKC-alpha) and nicotinamide adenine dinucleotide phosphate (NADP) [42] or miR-146, which is reduced in the sciatic nerves of diabetic mice and rats and is negatively related to inflammatory cytokines and whose mimics produce beneficial structural and functional effects [40, 41] (Table 1). The protective effect of nanoparticle–miR-146a-5p polyplexes (nano-miR-146a-5p) was explored in DPN rats. Nano-miR-146a-5p increased nerve conduction velocity and decreased nerve damage and demyelination, together with a decrease in inflammatory cytokines and an increase in myelin basic protein, implying that the protective effect on peripheral nerves was mediated through the regulation of the inflammatory response and apoptosis, leading to the suggestion of a regulation action of miR-146a-5p in the nuclear factor kappa-light-chain-enhancer of activated B cell (NF-κB) signaling pathway [44].

5.2. miRNA Expression and Diabetic Neuropathy in Humans. Very few studies have explored the association between the expression of miRNAs and diabetic neuropathy in humans. In a study hampered by an unclear definition of both type 2 diabetes and diabetic neuropathy, miR-199a-3p was found upregulated in the plasma of 60 patients with type 2 diabetes and in the lower limb skin of 30 patients with type 2 diabetes and DPN, compared to samples of 5 and 20 healthy volunteers, respectively [45]. Additionally, miR-199a-3p was shown in vitro to downregulate the serine protease inhibitor E2 (SerpinE2) that is known to upregulate the tissue plasminogen activator (tPA) provided with thrombolytic activity. Upregulation of miR-199a-3p was suggested by the authors to exert a procoagulant action in skin peripheral circulation and thus involved in DPN pathogenesis [45] (Table 1).

In a collaborative study between diabetologists and geneticists, we evaluated in 49 patients with type 2 diabetes the expression of 6 candidate miRNAs and assessed the presence of (1) DPN using validated scoring systems for neuropathic symptoms and signs and quantitative sensory testing for vibratory and thermal perception thresholds and (2) CAN by four cardiovascular reflex tests (CARTs) [46]. We defined the presence of probable DPN based on 2 abnormalities among symptoms, signs, and vibratory or thermal perception thresholds and of early CAN with at least one abnormal CART [2, 47]. Patients with DPN, compared to those without, showed a higher expression of miR-128a ($p = 0.015$) and a lower expression of both miR-155 ($p = 0.04$) and miR-499a ($p = 0.05$), whereas patients with CAN, compared to those without, displayed only a lower expression of miR-155 ($p = 0.05$) [46].

Using ROC analysis, we found fair diagnostic accuracy for DPN and diabetic neuropathy (DPN and/or CAN) with both a model including all three miRNAs and a model with miR-128a plus miR-155. For diabetic neuropathy, the area under the ROC curve (AUC) was 0.817 and 0.801 for the three-miRNAs and two-miRNA model, respectively, the sensitivity was 75.9% and 80.6%, and specificity was 76.5% and 70.6%, respectively. For DPN, AUC was 0.815 and 0.802, sensitivity was 74.1% and 80.6%, and specificity was 76.2% and 70.6% for the three-miRNAs and two-miRNA model [46]. Thus, the combination of two or three of these miRNAs had diagnostic accuracy for diabetic neuropathy identification, supporting the potential use of these miRNAs as epigenetic biomarkers for diabetic neuropathy.

5.3. Functions of miR-128a, miR-155, and miR-499a. To understand the possible meaning of these associations, one might consider that in animal studies, miR-128a downregulates insulin signaling pathways [48], impedes adipogenesis, and promotes lipolysis [49]. An overexpression of miR-128a might exert a pathogenetic role in diabetic neuropathy through these heightened adverse metabolic effects. Both insulin resistance in the dorsal root ganglion and dysregulation of adipogenesis and lipid metabolism have been observed in animal models of type 2 diabetes and DPN and proposed as additional factors in the pathogenesis of diabetic neuropathy [50, 51].

On the other hand, miR-155 is a multifunctional miRNA: in animal studies, it enhances insulin sensitivity, regulates inflammation and immunity [52], and exerts a neuroprotective effect [53], as well as being downexpressed in white blood cells of subjects with nondiabetic peripheral neuropathies [54], thus supporting a role in inflammation

Table 1: Studies exploring the association between miRNAs and diabetic neuropathy.

miRNA	Target	Expression	Observation	Author, year
Animal studies				
miRNA-29b	Neurotrophic activity (↑)	Downregulated	In diabetic rats, miRNA-29b was downregulated in dorsal root ganglia neurons and associated with apoptosis and axonal swelling	Zhang, 2014 [36]
mmu-let-7i	NF-κB neurotrophic activity (↑)	Downregulated	In DPN type 1 diabetes mice, mmu-let-71 was reduced and mmu-mir-341 increased in dorsal root ganglia neurons; let-7i miRNA mimics and mmu-miR-341 anti-miR improved structural and functional abnormalities	Cheng, 2015 [37]
mmu-miR-341	Neurotrophic activity? (↓)	Upregulated		
3pmiRNA-190a-5p	SLC17A6 (↓)	Downregulated	In DPN mice models, 3pmiRNA-190a-5p was downregulated and SLC17A6 overexpressed	Yang, 2017 [38]
miRNA-9	CALHM1 (↑)	Upregulated	In painful DPN rat model, miRNA-9 was overexpressed in spinal dorsal horn neurons and related to CALHM1	Liu, 2017 [39]
miRNA-146a	Proinflammatory genes (↓)	Downregulated	In diabetic mice, miR-146a mimics improved sciatic nerve vascular function, axonal myelination, and peripheral nerve function	Liu, 2017 [40]
miRNA-146a	NF-κB and inflammatory cytokines (↓)	Downregulated	In diabetic rats with DPN, miR-146a was reduced in sciatic nerves and negatively related to TNF-α, IL-1β, and NF-κB	Feng, 2018 [41]
miRNA-25	PKC-α and NADP (↓)	Downregulated	In diabetic mice, miRNA-25 was reduced in sciatic nerves and associated with increase in ROS. miR-25 mimics decrease NADP and PKC-a, and miRNA-25 anti-miR increases AGEs and RAGE	Zhang, 2018 [42]
miRNA-29c	PRKCI (↓)	Upregulated	In diabetic mice, miRNA-29c was increased in DRG and sciatic nerve and suppresses axonal growth by inhibiting PRKCI	Jia, 2018 [43]
miR-146a-5p	Inflammatory response and apoptosis	Downregulated	In diabetic rats with DPN, nano-miR-146a-5p had a protective effect on peripheral nerves (↑ NCV and ↓ nerve damage and demyelination) together with ↓ inflammatory cytokines and ↑ myelin basic protein	Luo, 2019 [44]
Human studies				
miRNA-199a-3p	SerpinE2 (↓)	Upregulated	In 60 patients with type 2 diabetes and DPN, miRNA-199a-3p was upregulated in plasma and skin with consequent downregulation of SerpinE2 (and tPA with procoagulant effect)	Li, 2017 [45]
miRNA-128a	Insulin signaling pathways (↓), adipogenesis (↓), and lipolysis (↑) (miRNA-128a)	Upregulated	In 49 T2DM, miRNA-128a was upregulated, while miRNA-155 and miRNA-499 were downregulated in plasma in those with DPN and miRNA-155 was downregulated in those with CAN	Ciccacci, 2020 [46]
miRNA-155	Insulin sensitivity (↑), inflammation, immunity, neuroprotection (↑) (miRNA-155)	Downregulated		
miRNA-499a	Apoptotic pathway and mitochondrial fission through CnA and Drp1 (miRNA-499)	Downregulated		

AGEs: advanced glycation endproducts; CALHM1: calcium homeostasis modulator 1; CnA: calcineurin; Drp1: dynamin-related protein 1; IL-1β: interleukin 1 beta; NADP: nicotinamide adenine dinucleotide phosphate; NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells; NCV: nerve conduction velocity; PKC-α: protein kinase C alpha; PRKCI: protein kinase C iota type; RAGE: receptor for advanced glycation endproduct; ROS: reactive oxygen species; SerpinE2: serine protease inhibitor E2; SLC17A6: gene for vesicular glutamate transporter 2; T1DM: type 1 diabetes; TNF-α: tumor necrosis factor alpha; tPA: tissue plasminogen activator.

and neural function. An association between biochemical inflammatory markers and both DPN and CAN has been shown in patients with type 2 diabetes [55–57], and chronic low-grade inflammation is considered a critical pathway in the pathogenesis of diabetic neuropathy [58]. Thus, miR-155 expression changes might contribute to the inflammatory and immune-mediated pathogenetic mechanisms of diabetic neuropathy.

Finally, miR-499a, preferentially expressed in heart, skeletal muscle, and areas of the central autonomic network (nucleus ambiguous), has been found in animal models to be a regulator of the apoptotic pathway and mitochondrial fission in cardiomyocytes in response to ischemia or mechanical stress [59, 60] and was proposed as a marker of acute myocardial infarction and its severity [61, 62]. In fact, miR-499a targets the gene of phosphatase calcineurin A (CnA) that dephosphorylates the GTPase dynamin-related protein 1 (Drp1), the major mitochondrial fission protein. Dephosphorylated Drp1 migrates from the cytosol to the mitochondrial outer membrane and promotes mitochondrial fission and cell apoptosis [59, 60]. miR-499a overexpression might inhibit this pathway and prevent mitochondrial fission and apoptosis. Mitochondrial dynamics, i.e., fusion and fission, is also relevant in neuronal function, and mitochondrial dysfunction is believed to play a role in neurodegenerative diseases [63, 64] as well as in diabetic neuropathy. In murine models of diabetic neuropathy, Drp1 seems to mediate the hyperglycemia-driven mitochondrial damage in sensory neurons, and overactive mitochondrial fission in DRG neurons is suggested as a pathogenetic mechanism of diabetic neuropathy [65]. In addition, an imbalance in mitochondrial fusion and fission involving Drp1 was also shown as being responsible for diabetes-induced deficits in synaptic plasticity observed in the hippocampus in animal models of diabetes [66]. Thus, the role of miR-499a in neurological diabetic complications would appear to be fully supported.

6. A Genetic Perspective: Polymorphisms of *MIR146A*, *MIR128A*, and *MIR27A* Genes

Although the majority of studies are focused on miRNA expression profile investigation, recent studies have shown that also polymorphisms in miRNA genes may alter a wide spectrum of biological mechanisms and could play a role in the susceptibility to several human diseases, including diabetic neuropathy. In this regard, in our earlier studies, we used a genomic approach to investigate the relationship between miRNAs and diabetic neuropathy. The first study explored genetic polymorphisms in miRNA regions in relation to the susceptibility to type 2 diabetes [67]. This study assessed thirteen miRNAs as candidate loci—selected according to literature data and to a computational analysis—in 163 Italian subjects with type 2 diabetes and 185 healthy controls and found 6 newly described variants in addition to 9 SNPs already present in databases. In a case-control association study, two polymorphisms were found associated with type 2 diabetes susceptibility, i.e., the G allele of rs895819 in MIR27A with a protective effect (odds ratio = 0.58 and $p = 0.008$) and the G allele of rs531564 in MIR124A as a risk allele (odds ratio = 2.15, $p = 0.008$). This was the first report of genetic polymorphisms in miRNA regions as possible contributors to type 2 diabetes susceptibility [67].

Subsequently, we evaluated the possible contribution of genetic polymorphisms of miRNA genes in susceptibility to DPN and CAN [68]. Nine polymorphisms were studied in a sample of 132 patients well-defined for the diagnosis of probable DPN (based on the presence of both neuropathic symptoms and signs) and early or confirmed CAN (according to one or more abnormal CARTs, respectively). The study found an association of the rs2910164 (G>C) in MIR146A and rs11888095 (C>T) in MIR128A with DPN susceptibility. In particular, the C allele of rs2910164 in MIR146A was seen to be a protective variant (odds ratio = 0.46, $p = 0.032$), while the variant T allele of rs11888095 in MIR128A was associated with a high risk of developing DPN (odds ratio = 2.01, $p = 0.007$). The latter association was also confirmed after correction for BMI, age, disease duration, HbA1c, and gender (adjusted odds ratio = 4.89, $p = 0.002$). Moreover, the same SNP in MIR146A showed a protective effect for early CAN (adjusted odds ratio = 0.32, $p = 0.052$) and for confirmed CAN (adjusted odds ratio = 0.13, $p = 0.041$), while a polymorphism in MIR27A was associated with a higher risk of developing early CAN (adjusted odds ratio = 3.43, $p = 0.023$). An association of SNPs of MIR128A and MIR146A was also present in multiple linear regression analysis with the severity of DPN and CAN, namely, the scores for neuropathic symptoms/signs ($p = 0.026$ for MIR128A) and the score based on CARTs ($p < 0.0001$ for MIR146A) [68]. This represented the first observation of an involvement of genetic variability in miRNA genes in diabetic neuropathy susceptibility.

We described above that in animal models of diabetic neuropathy, the expression of miR-146a was found to be downregulated and inversely associated with levels of inflammatory cytokines [41] and, further, that miR-146a mimics had protective effects on peripheral nerves [40], possibly mediated by the inhibition of inflammatory response and apoptosis through the regulation of NF-κB [44]. Similarly, we presented earlier metabolic effects of miR-128a and how these might provide a meaning to the association between its expression levels and diabetic neuropathy. It is more difficult to disentangle the value of the weak link of the polymorphism of MIR27A with CAN. In addition to the evidence of its role in tumor biology, some studies have suggested that miR-27a expression was upregulated in the T cells of patients with multiple sclerosis and that in murine T cells, it impaired regulatory T cell (Treg) generation by downregulating runt-related transcription factor 1 (RUNX1) and then the forkhead box P3 (Foxp3), i.e., the master transcription factor in maintaining differentiation and suppressive function of Tregs [69]. On the other hand, the involvement of the miR-27 family in metabolic disorders and in hepatic glucose metabolism was described, with it having forkhead box O1 (FOXO1) as a downstream target [70].

The polymorphisms in these miRNA genes might affect the expression or the downstream action of the corresponding miRNAs and lead in some way to changes in metabolic and inflammatory/immune mechanisms active in the scenario of diabetic neuropathy.

6.1. Polymorphisms of miRNA Genes: MIR499A. In a more recent study, in 150 participants with type 2 diabetes, we analysed the rs3746444 SNP in the MIR499A gene to evaluate its association with susceptibility to DPN and CAN [71]. We found that the GG genotype after correction for age, sex, BMI, and HbA1c was associated with the risk of early CAN (adjusted odds ratio = 16.08, $p = 0.002$), confirmed CAN (adjusted odds ratio = 35.02, $p = 0.0005$), and DPN (adjusted odds ratio = 6.56, $p = 0.037$). In addition, MIR499A GG genotype independently contributed to early CAN together with duration and HbA1c and to DPN together with duration, HbA1c, and age. Finally, the GG genotype was associated with worse values of neuropathic deficit score, i.e., Michigan Diabetic Neuropathy Score ($p = 0.017$), vibration perception threshold ($p = 0.01$), thermal thresholds ($p = 0.01$), and CART score ($p < 0.001$): in a multiple linear regression, the GG genotype was the main variable contributing to the CART score ($p = 0.001$). Thus, the rs3746444 GG genotype might represent a marker of higher risk of DPN and CAN and of CAN severity. We described above the hypothesized actions of miR-499a and its ability to prevent mitochondrial fission as well as the imbalance in mitochondrial fusion and fission as a potential pathogenetic mechanism of diabetic neuropathy [65].

6.2. mtDNA Copies and Diabetic Neuropathy. More insight into the meaning of the association between MIR499A polymorphism and diabetic neuropathy comes from a further study by our group [72] that measured in 125 patients with type 2 diabetes the number of mitochondrial DNA (mtDNA) copies and assessed DPN, CAN, and the polymorphism of MIR499A. The study found a decrease in the number of mtDNA copies in patients with type 2 diabetes compared to healthy controls ($p = 2 \times 10^{-10}$) and further differences between patients with and without DPN ($p = 0.02$) (not between those with and without CAN). In addition, the homozygous variant genotype for the rs3746444 polymorphism of MIR499A was associated with the number of mtDNA copies, particularly in patients with type 2 diabetes with values of 22.71 ± 8.65 in patients with AA+AG genotypes and 14.43 ± 5.29 in those with the GG genotype ($p = 0.009$).

Mitochondrial biogenesis is a defense modality against hyperglycemic load and hyperglycemia-driven oxidative stress in diabetes through the increase in the mass of the mitochondrial network, with mtDNA being a marker of this process [65]. Prolonged hyperglycemia and oxidative stress induce a decrease in the mtDNA copy number [73] as also found in dorsal root ganglia from mice with chronic diabetic neuropathy [74].

The study confirmed in people with type 2 diabetes the observations obtained in animal models of diabetic neuropathy and documented for the first time that the studied polymorphism in MIR499A might affect the number of mtDNA copies, thus impairing mitochondrial biogenesis.

6.3. MIR499 Polymorphism and miR-499 Expression in DPN: Hypothesis for a Pathogenetic Role in DPN. Thus, summarizing the findings regarding the miR-499a system in diabetic neuropathy, we have evidences of (1) an association of homozygous variant genotype for the rs3746444 polymorphism of MIR499A with CAN and DPN in type 2 diabetes [71], (2) a reduction in the mtDNA copy number in type 2 diabetes, which is more pronounced in the presence of DPN [72], (3) an association in these patients between this change in the mtDNA copy number and the same polymorphism of MIR499 [72], (4) a reduced expression level of miR-499a in subjects with DPN [46], and (5) the experimental observations in rat and human cardiomyocytes that miR-499 targets the CnA gene and inhibits its expression and the CnA-mediated activation of Drp1 responsible for mitochondrial fission and apoptosis [59, 60]. These data, taken together, allow for the hypothesis that the polymorphism of MIR499A and reduced miR-499a expression may dysregulate mitochondrial biogenesis as suggested by the reduced mtDNA number and increase mitochondrial fission thus impairing mitochondrial dynamics. Mitochondrial dynamics are a pillar of mitochondrial function in neurons, able to restore homeostasis, to contrast hyperglycemia driven oxidative stress, and to maintain optimal cellular bioenergetics [64, 73]. In this way, mitochondrial dysfunction can occur, which represents a newly recognized essential mechanism in the pathogenesis of DPN [65, 73] as well as of other neurological diseases [63, 64] (Figure 1).

7. Genotype-Phenotype Association for Polymorphisms of miRNA Genes

A question might arise: are the observed polymorphisms of miRNA genes able to change the corresponding miRNA expression? In the previously cited study [46], in 49 patients with type 2 diabetes with and without DPN and CAN, the relationship between the expression of 6 miRNAs and the genotypic classes of the corresponding miRNA genes was assessed. We found, after adjustment for age, sex, and diabetes duration, that the rs767649 variant allele in the *MIR155* promoter region was associated with a higher expression of this miRNA compared with the wild-type allele (adjusted $p = 0.013$). The rs767649 variant allele is localized in the promoter region of the MIR155 gene and might alter the binding of transcriptional factors, which are mainly inflammatory mediators and bacterial or viral-derived toll-like receptor ligands [75] in this way increasing miR-155 expression. In two previous studies, the rs767649 variant allele was associated with a reduced susceptibility to type 1 [75] and type 2 diabetes [76].

Moreover, the rs11888095 SNP polymorphism in MIR128A was associated with higher expression level of miR-128a (adjusted $p = 0.022$). The polymorphism of MIR128A had been previously associated in a larger group of patients with type 2 diabetes with a higher risk of developing DPN [68], thus the association, here documented for the first time, between the variant allele of MIR128A and a higher risk to develop DPN might be mediated by changes in miRNA expression.

In our study, we failed to observe an association of the polymorphism in the rs3746444 polymorphism of MIR499A and the miR-499a expression. It is possible that other

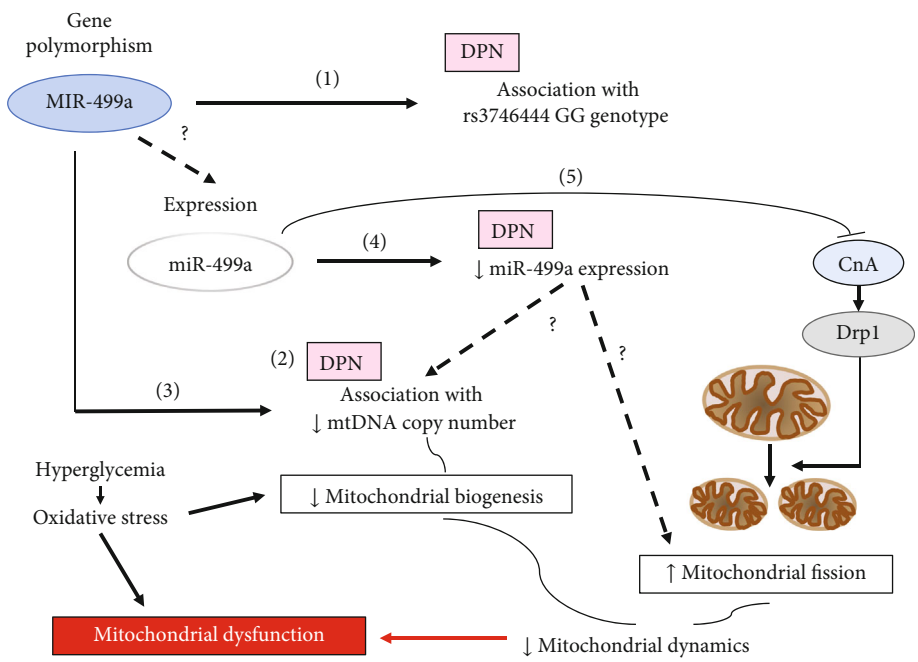

Figure 1: MIR499A polymorphism and miR-499a expression in DPN: hypothesis for a pathogenetic role in DPN. Mitochondrial dynamics are a continuous process of fusion, fission, biogenesis, and mitophagy that in neurons contrasts hyperglycemia-driven oxidative stress and maintains cellular bioenergetics [64, 73]. Mitochondrial dysfunction derives from persistent increase in metabolic load and oxidative stress in neurons in diabetes and is considered a relevant mechanism in the pathogenesis of DPN [65, 73]. Both dysregulation of the fission/fusion balance with increased fission and impaired biogenesis with reduced number of mtDNA have been found in diabetes. Recent studies have shown in people with type 2 diabetes (1) an association of the polymorphism rs3746444 of MIR499A with CAN and DPN [71], (2) a reduction in mtDNA copy number, more pronounced in the presence of DPN [72], (3) an association between this change in mtDNA copy number and the same polymorphism of MIR499A [72], (4) a reduced expression of miR-499a in subjects with DPN [46], and (5) in rat and human cardiomyocytes that miR-499a targets the gene of calcineurin (CnA), inhibits its expression and the CnA-mediated activation of dynamin-related protein (Drp) 1 responsible for mitochondrial fission and apoptosis [59, 60]. These findings allow the hypothesis that MIR499A polymorphism and changes in expression and function of miR-499a might affect both mitochondrial biogenesis and increase mitochondrial fission thus altering mitochondrial dynamics and leading to mitochondrial dysfunction and to DPN. It is not documented (dashed lines) that the studied MIR499A polymorphism affects miR-499 expression, and that in DPN, the reduced miR-499a expression is related to decreased mtDNA copy number and increased mitochondrial fission.

mechanisms contribute to changing the expression of this miRNA or that the variant allele might be involved in changing the miRNA targets rather than in regulating its expression.

Despite the small size of the studied population, this genotype-phenotype association study has shown for at least two miRNA genes that the genetic polymorphism corresponds to changes in miRNA expression with a consistency between what observed for the variant alleles and expression in their association with diabetic neuropathy. These results link the genetics and epigenetics of miRNAs and support their potential usefulness as exploratory predictive biomarkers and therapeutic targets.

8. What Does MicroRNA Genetics Put in Place for the Challenge of Diabetic Neuropathy?

The genetics of diabetic neuropathy is starting to offer results towards the achievement of biomarkers of risk and pathogenetic mechanisms, to provide support to known hyperglycemia-related mechanisms and to suggest new pathogenetic pathways and to validate therapeutic targets.

Genetic information might be integrated into a multibiomarker approach including clinical, metabolic, and imaging phenotyping to define individual susceptibility to diabetic neuropathy and its therapeutic options.

Genetic research is challenging and requires a joint force strategy to improve sample size and phenotyping. It is worthwhile proceeding, and the results in some fields might be nearer to our goals than we suspect.

In this scenario, the genetics of microRNAs is at a very preliminary stage. Notwithstanding, the study of genetics and epigenetics of miRNAs may well contribute to the identification of exploratory biomarkers of risk and pathogenetic mechanisms of diabetic neuropathy and to a comprehensive definition of susceptibility to specific pathogenetic mechanisms to favor a tailored mechanism-based treatment or prevention. The data presented here would appear to anticipate a promising development.

Conflicts of Interest

The authors do not have any conflict of interest regarding this work.

Authors' Contributions

V.S. conceived, drafted, and revised the manuscript. C.C., A.L., and P.B. provided critical input to the conception and design of the manuscript, edited, and revised the manuscript.

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Research Article

Association of Cardiovascular Autonomic Neuropathy and Distal Symmetric Polyneuropathy with All-Cause Mortality: A Retrospective Cohort Study

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Background. People with diabetic cardiovascular autonomic neuropathy (CAN) have increased cardiovascular mortality. However, the association between distal symmetric polyneuropathy (DSPN) or CAN with all-cause mortality is much less investigated. Thus, we set out to examine the effect of CAN and DSPN on all-cause mortality in a well-phenotyped cohort. *Methods*. All diabetes cases ($n = 1{,}347$) from the catchment area of a secondary diabetes care centre who had medical examination including neuropathy assessment between 1997 and 2016 were followed up for all-cause mortality in the NHS Hungary reimbursement database until 2018. We investigated the association of CAN (Ewing tests) and DSPN (Neurometer) with all-cause mortality using Cox models stratified by diabetes type. *Results*. Altogether, $n = 131/1{,}011$ persons with type 1/type 2 diabetes were included. Of the participants, 53%/43% were male, mean age was $46 \pm 12/64 \pm 10$ years, diabetes duration was $13 \pm 10/7 \pm 8$ years, 42%/29% had CAN, and 39%/37% had DSPN. During the $9 \pm 5/8 \pm 5$-year follow-up, $n = 28/494$ participants died. In fully adjusted models, participants with type 1 diabetes patients with versus without DSPN had an increased mortality (HR 2.99, 95% CI 1.4-8.63), while no association with CAN was observed. In type 2 diabetes, both DSPN and CAN independently increased mortality (HR 1.32, 95% CI: 1.07-1.64, and HR 1.44, 95% CI: 1.17-1.76). *Conclusions*. Our results are compatible with an increased risk of mortality in people with type 1 diabetes and DSPN. Furthermore, we report a similarly strong association between DSPN and CAN and all-cause mortality in type 2 diabetes mellitus.

1. Introduction

Compared to the general population, both type 1 and type 2 diabetes (T1DM, T2DM) confer a higher risk of cardiovascular complications and all-cause mortality. While guideline-directed control of conventional risk factors improves morbidity and mortality of diabetes [1], it still has an increased risk compared to the background population [2]. As cardiovascular risk factors only partly explain this risk, it is conceivable that other characteristics of diabetes (such as its complications) may also play a significant part.

Cardiovascular autonomic neuropathy (CAN) and distal symmetric polyneuropathy (DSPN) are early complications of diabetes that may be already present in prediabetes [3, 4]. The development of neuropathy is a complex process that involves not only hyperglycaemia but also other metabolic factors (e.g., oxidative stress and polyol pathway) [5].

While DSPN is a disabling diabetic complication that mostly affects quality of life via the development of lower limb ulcers, amputations, and frequent falling [6], autonomic nerve dysfunction is associated with an increased risk of cardiovascular mortality [7]. As CAN and DSPN share several pathophysiological mechanisms, it is likely that DSPN could also be associated with an increased cardiovascular mortality. Furthermore, DSPN through its association with falls and infectious complications could also be related to all-cause mortality. Therefore, we aimed to assess the relationship of CAN and DSPN with all-cause mortality in a retrospective cohort of a well-phenotyped diabetes population that attended a secondary care centre.

2. Methods

2.1. Setting and Participants. This is a retrospective cohort study including all adult diabetes patients who had a detailed neuropathy examination at the neuropathy laboratory of the 1st Department of Medicine, Semmelweis University in 1997-2016. The institute serves as a secondary referral centre for a suburban area of Budapest, Hungary, with ~100 thousand inhabitants. In addition to an assessment of CAN and DSPN, demographic data, anthropometrics, lifestyles, type and duration of diabetes, current and previous illnesses, and contemporary medications were collected on a standardized data entry form. Using the National Health Service (NHS) Hungary identification number, all participants were followed up for all-cause mortality in the NHS Masterfile until December 2018. This research was approved by the local ethical committee under the number of SE TUKEB 36/2017.

To reduce referral bias, we excluded data of people living outside the catchment area of our institution. We further limited the study population to diabetes patients and used the first assessment as the baseline leading to $n = 1{,}347$ eligible patients. We excluded 7% of T1DM and 15% of T2DM patients due to missing covariates or neuropathy assessments. The mortality follow-up was almost complete (100% for T1DM and 99% for T2DM). The final analytical sample included $n = 131$ (93% of those eligible) T1DM and $n = 1{,}011$ (84% of those eligible) T2DM patients (Figure 1).

2.2. Definition of DSPN and CAN. Subclinical and clinical DSPN (referred as DSPN) and CAN were diagnosed in line with the Toronto Diabetic Neuropathy Expert Group recommendation [6] and were performed using standardized protocols [8]. DSPN was evaluated by Neurometer CPT (Neurotron Inc., Baltimore, USA) [3, 8]. CAN was assessed with gold standard cardiovascular reflex tests using Ewing's battery [6]. See detailed methods in the online appendix (Supplementary material).

2.3. Covariables. From the collected information, we derived *sex* and *age* and used the zip code to investigate eligibility. *Height* and *weight* were measured. We defined current smoking as consumptions >1 cigarette/day. Weekly alcohol consumption (beer, wine spirits) was recorded and recalculated as high level of consumption (>14 units/week).

Available medical records were screened for the following medical conditions: type and duration of diabetes, hypertension, myocardial infarction, heart failure, peripheral vascular disease, cerebrovascular accident, dementia, chronic obstructive pulmonary disease, connective tissue diseases, peptic ulcer, liver disease, hemiplegia, chronic kidney disease, and malignancies. This information was supplemented with direct information from the participant.

To estimate comorbidity burden, a simplified Charlson score was calculated by summing the weighted comorbidities. As our study was limited to diabetes patients, information on diabetes, its duration, and neuropathy was not included in the Charlson score [9].

At the time of assessment, a list of *concomitant medications* (trade names) for the last week was requested and coded by the Anatomical Therapeutic Chemical (ATC) classification system.

2.4. Outcome. Hungary has a single payer health insurance system that covers most social and health care-related activities. For the current report, all participants were flagged with their NHS ID in the NHS Masterfile, and their last known status was recorded as dead or alive. Follow-up started at the time of neuropathy assessment and was censored at death or inactivation (due to expatriation) or end of follow-up (December 2018) whichever came first.

2.5. Statistical Analysis. Based on literature data, the magnitude of the association of DSPN and CAN with all-cause mortality could be within a wide range (hazard ratio [HR] 1.7 to 2.8) in type 1 diabetes [10, 11]. Given the observed mortality in the neuropathy-free population (~15%) and the prevalence of neuropathy (~35%) in our sample of type 1 diabetes, the power was between 68% and 99%. For type 2 diabetes, the reported adjusted hazard ratios were 1.33 to 1.55 that gave excellent power (100%) based on similar calculations to detect differences with similar magnitude to the literature [12, 13].

Given the large age and mortality risk differences between T1DM and T2DM, all statistical analyses were stratified by type of diabetes. Baseline comparisons between participants dead or alive at follow-up were done using 2-sample t-tests and χ^2 tests.

We fitted hierarchical Cox proportional hazards models to estimate hazard ratios (HRs) with 95% confidence intervals (CI) for the effects of CAN and DSPN on all-cause mortality. The baseline model (Model 1) was adjusted for age, sex, anthropometric factors (height, BMI), lifestyles (current smoking and alcohol consumption), and diabetes duration. Then, in Model 2, we further adjusted for the presence of hypertension, antihypertensive treatment, and systolic and diastolic blood pressure. The final model (Model 3) was further adjusted for comorbidities (simplified Charlson comorbidity index) and medications (lipid lowering, antianginal, antiarrhythmic, platelet aggregation inhibitors, and anticoagulant medications). First, we run separate models for CAN and DSPN, and finally, we entered both types of neuropathy in a mutual model.

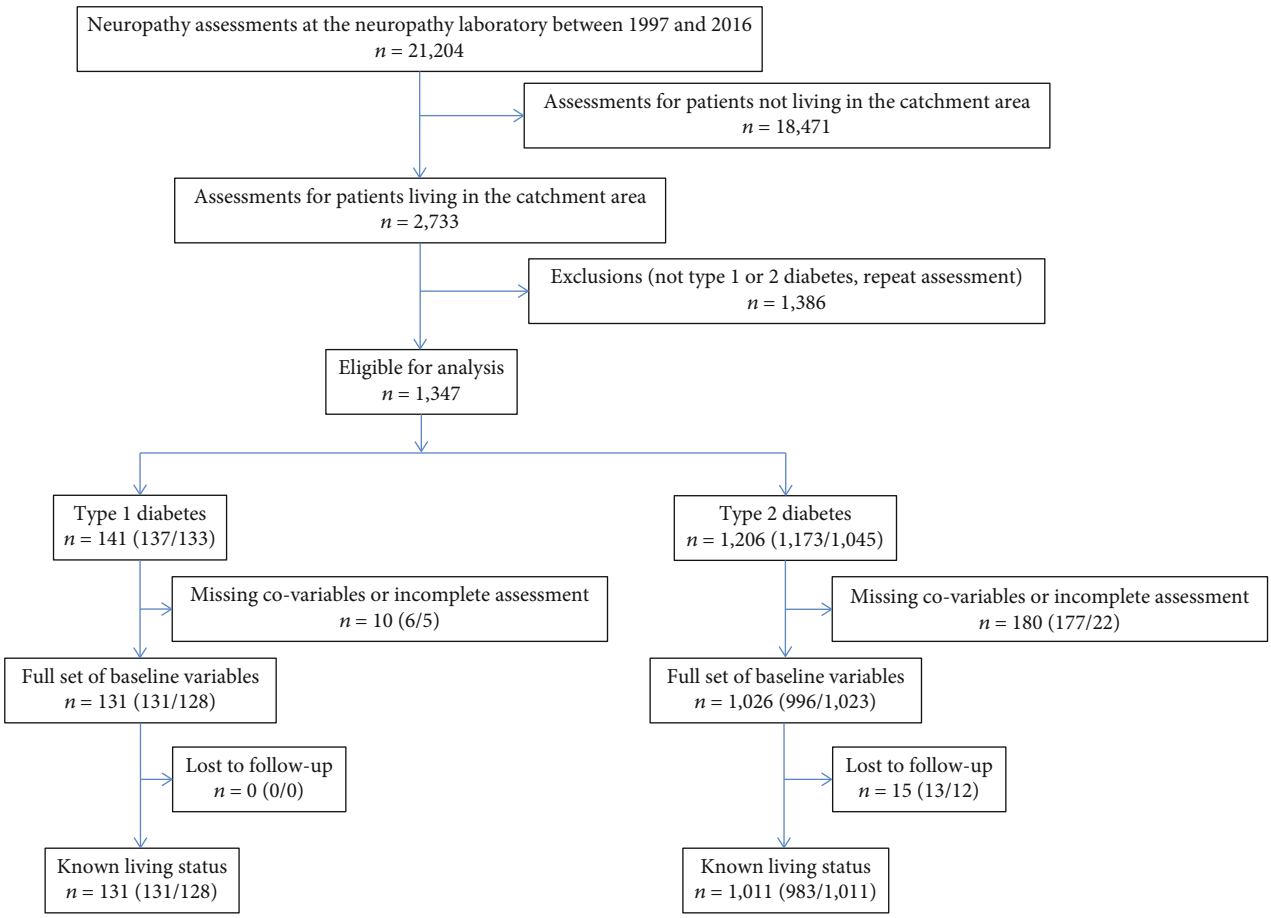

Figure 1: Flow chart of the selection of participants for the current study. Numbers are given for people with any neuropathy. Numbers in brackets are given for people with distal symmetric polyneuropathy/cardiovascular autonomic neuropathy.

Given the limited number of participants with T1DM, the modelling approach was modified for these persons: we entered potential covariates in the same 3 steps but using a backward stepwise approach.

Sensitivity analyses were conducted to investigate the robustness of our findings. First, we excluded participants with baseline malignancies. Second, we excluded participants if they had died during the first 2 years of follow-up.

All analyses were performed in SPSS version 21.0.

3. Results

3.1. Baseline Characteristics. Over a mean 9 (SD 5) years of follow-up, 28/131 (21%) T1DM participants died. Persons alive were 12 years younger, leaner, had 4 years shorter duration of diabetes, 5 mmHg lower systolic blood pressure, less likely to have known hypertension and to take antihypertensive or antianginal medications, and had a lower burden of comorbidities (simplified Charlson comorbidity index; all $p < 0.05$). No significant difference in sex distribution, height, lifestyles, diastolic blood pressure, heart rate, and use of other medications was found. While DSPN was less frequent among survivors, the frequency of CAN was similar in the groups (Table 1).

Over a mean 8 (SD 5) years of follow-up, 494/1,011 (44%) T2DM participants died. Similar differences to those in T1DM were found between participants deceased or alive for age, diabetes duration, antianginal medications, and burden of comorbidities. In addition, surviving persons with T2DM were more obese, taller, less likely to consume high amounts of alcohol, had a higher diastolic blood pressure, more likely to be on lipid-lowering medications, and less likely to take anticoagulants (all $p < 0.05$). Furthermore, the risk of hypertension or being on blood pressure-lowering medication was similar in those deceased or alive. Similarly to T1DM, DSPN was less frequent among the surviving persons, while the frequency of CAN was similar in the groups (Table 1).

3.2. Association between Neuropathy and All-Cause Mortality in T1DM. According to the Cox model adjusted for age, sex, anthropometrics, lifestyles, and diabetes duration, there was a nonsignificant 16% (HR 1.16 95% CI: 0.50-2.71) increased risk of mortality among participants with CAN at baseline. Given the wide confidence intervals, neither a true effect nor no difference can be excluded (Table 2).

According to the results of a similar Cox model, there was a markedly increased risk (HR 2.51 95% CI: 1.00-6.28) of mortality among participants with DSPN at baseline. When

Table 1: Baseline characteristics of participants by type of diabetes and survival status.

	Type 1 diabetes			Type 2 diabetes		
	Alive	Dead	p	Alive	Dead	p
n (%)	103 (78.6)	28 (21.4)		562 (55.6)	449 (44.4)	
Male	53 (51.4)	16 (56.3)	0.672	241 (42.9)	199 (44.2)	0.655
Age (years)	43.1 ± 12	55.9 ± 13	0.001	60.3 ± 9.9	67.4 ± 9.8	0.001
Height (cm)	169 ± 10	168 ± 9	0.879	166 ± 9	164 ± 10	0.002
Weight (kg)	79.5 ± 19.9	79.7 ± 17.8	0.014	86.7 ± 19.6	79.2 ± 15.8	0.001
BMI (kg/m^2)	27.9 ± 6.4	28.4 ± 6.5	0.006	31.3 ± 5.8	29.5 ± 5.3	0.001
High level of alcohol consumption, n (%)	NA	NA	1.000	31 (5.6)	47 (10.4)	0.004
Current smoker, n (%)	33 (32.1)	11 (37.5)	0.656	98 (17.5)	81 (18)	0.804
Duration of diabetes (years)	12.5 ± 9.8	16.8 ± 12	0.014	6.8 ± 6.9	7.9 ± 8.5	0.020
Systolic blood pressure (mmHg)	129 ± 16	133 ± 17	0.007	138 ± 17	137 ± 17	0.419
Diastolic blood pressure (mmHg)	80 ± 9	81 ± 10	0.099	81 ± 9	79 ± 9	0.005
Heart rate (beat/min)	78 ± 12	80 ± 12	0.792	76 ± 12	77 ± 14	0.127
Known hypertension, n (%)	53 (51.4)	23 (81.3)	0.005	479 (85.1)	387 (86.3)	0.718
Antihypertensive medication, n (%)	47 (45.9)	20 (71.9)	0.019	442 (78.7)	347 (77.3)	0.647
Lipid-lowering medication, n (%)	16 (15.6)	NA	1.000	221 (39.3)	84 (18.8)	≤0.0001
Antianginal treatment, n (%)	NA	NA	0.008	50 (9)	83 (18.6)	≤0.0001
Platelet aggregation inhibitors, n (%)	NA	NA	0.097	93 (16.6)	86 (19.2)	0.283
Anticoagulants, n (%)	NA	NA	0.115	24 (4.3)	41 (9.2)	0.002
Simplified Charlson comorbidity index	0.4 ± 0.7	1.4 ± 1.1	0.001	1.6 ± 1	2.7 ± 1.4	0.001
DSPN, n (%)	34 (33)	17 (60.7)	0.009	202 (36)	193 (42.9)	0.023
CAN, n (%)	45 (43.7)	10 (36.7)	0.521	170 (30.2)	139 (30.9)	0.837

Numbers are mean ± SD or n (%). p values are given for 2-sample t-tests or χ^2 tests as appropriate. For cells with $n \leq 10$, no values are given due to privacy protection regulations. DSPN: distal symmetric polyneuropathy; CAN: cardiovascular autonomic neuropathy.

Table 2: The association between CAN and DSPN (Cox proportional hazard models).

	Type 1 diabetes			Type 2 diabetes		
	HR	95% CI	p	HR	95% CI	p
CAN						
Model 1	1.16	0.5-2.71	0.727	1.31	1.07-1.61	0.009
Model 2				1.29	1.05-1.58	0.016
Model 3				1.33	1.08-1.63	0.007
Mutual model				1.32	1.07-1.64	0.01
DSPN						
Model 1	2.50	1-6.28	0.05	1.54	1.26-1.88	≤0.0001
Model 2				1.53	1.25-1.86	≤0.0001
Model 3				1.49	1.22-1.83	≤0.0001
Mutual model				1.44	1.17-1.76	≤0.0001
Backward stepwise model	2.99	1.04-8.63	0.043			

Model 1: adjusted for age, sex, height, BMI, current smoking, high level of alcohol consumption, diabetes duration; Model 2: as for Model 1+antihypertensive medication, known hypertension, and systolic and diastolic blood pressure; Model 3: as for Model 2+lipid lowering, antianginal, antiarrhythmic, platelet aggregation inhibitor, anticoagulant treatment, and simplified Charlson comorbidity index; Mutual model: as for Model 3 and both autonomic and sensory neuropathy; Backward stepwise model: adjusted for age, sex, and smoking; DSPN: distal symmetric polyneuropathy; CAN: cardiovascular autonomic neuropathy.

the covariables were selected by the backward stepwise method, the results remained (HR 2.99 95% CI: 1.03-8.63) (Table 2 and Figure 2).

3.3. Association between Neuropathy and All-Cause Mortality in T2DM. T2DM persons with CAN at baseline had a 31% increased hazard (95% CI: 1.07-1.61) of mortality compared to participants without CAN according to Model 1. This finding remained robust after adjustment for hypertension, blood pressure, medications, and comorbidities. Given that surviving and deceased persons had similar frequencies of CAN, people with CAN should have a strong mortality

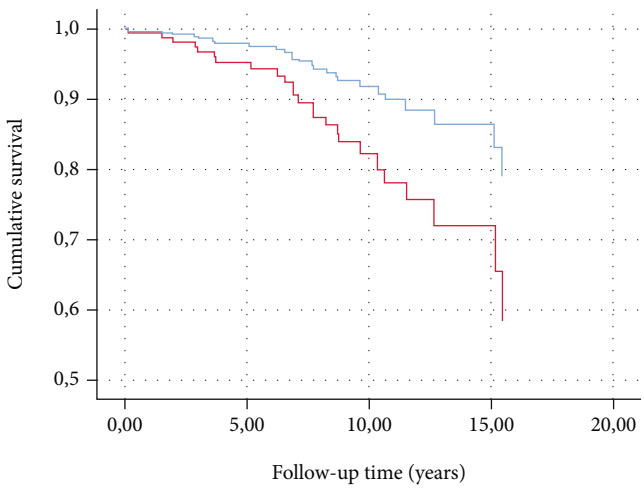

Figure 2: Cumulative survival by DSPN status at baseline in type 1 diabetes patients. Cox proportional hazard model adjusted for age, sex, and current smoking. Curves are fitted for populations with a mean age of 45 years, 53% male, and 36% smoker at baseline. Presence of DSPN at baseline—red line. Absence of DSPN at baseline—blue line. DSPN: distal symmetric polyneuropathy.

predictor from Model 1 that explained this finding. Indeed, these people were 4.0 (95% CI: 2.67-5.39) years younger than those without autonomic neuropathy suggesting a selection bias in the referral to our neuropathy service (Table 2 and Figure 3(a)).

The association of DSPN with all-cause mortality was even stronger (HR 1.54, 95% CI: 1.26-1.88) according to Model 1 and remained robust during further adjustments (Table 2 and Figure 3(b)).

To investigate whether the cooccurrence of the two types of neuropathy explained the observed strong associations, a mutual model was built where both CAN and DSPN were entered simultaneously. Effect sizes remained similar in this model, suggesting that the association of CAN and DSPN is mostly independent (Table 2).

3.4. Sensitivity Analyses. Both sensitivity analyses confirmed our main analyses showing similar effect sizes for the association between CAN and DSPN and all-cause mortality although the association between CAN and mortality became nonsignificant when we excluded the first 2 years of follow-up (data are available on request.)

4. Discussion

Based on the results of a retrospective cohort study from a secondary care centre with an almost complete follow-up, we found a markedly increased risk of mortality in participants with DSPN compared to controls both in T1DM and T2DM during an 8-9-year follow-up. While the confidence intervals for T1DM were wide, it is notable that the point estimate suggests a much stronger association in T1DM (2.51 to 2.99) compared to T2DM (1.54 to 1.44).

In contrast, CAN was not associated with mortality in T1DM, while there was a robust 30% increase in relative risk in T2DM. It should be noted that given the wide confidence intervals in T1DM, an effect similar to that in T2DM cannot be excluded.

According to a model adjusted for potential confounders and mutually including CAN and DSPN among T2DM persons, we found that CAN and DSPN were independent predictors of all-cause mortality.

A meta-analysis of observational studies found a strong association between *CAN* and mortality in both types of diabetes [7].

However, the comparison between our and previous findings in T1DM is hindered by aspects of design and methods of former cohorts. First, we had insufficient power to show modest effects in T1DM, meaning that our null finding is still compatible with even a doubling of risk in participants with CAN (similar to previous findings) [10, 14–18]. Second, the definition of autonomic neuropathy differed in the different cohorts that could largely affect the findings. As there is a clear dose-response association between the numbers of abnormal tests and risk of mortality [7, 15], our definition of CAN as ≥2/4 positive tests also includes "mild" CAN cases. Third, most of these cohorts are coming from secondary and tertiary care centres and thus are prone to selection/referral bias. Lastly, given that mortality predictors can be unequally distributed between participants with and without CAN, only studies that have multiple adjustments can be credibly compared. The point estimates in studies with multiple adjustments are much more homogenous and are in the range of 1.4 to 2.9 [10, 14, 17, 18]. Furthermore, in two of these, the association became nonsignificant in fully adjusted models [14, 18].

The comparisons between the published literature and our findings regarding the association between *CAN* and all-cause mortality are also limited in T2DM [7]. Older studies used one or two Ewing tests to define autonomic neuropathy [19–21], while newer studies used different measures of heart rate variability and QT interval changes with cut-off values not directly comparable to our results [12, 22, 23]. Altogether, these studies showed high relative risks and odds ratios in the range 2-4 in unadjusted analyses [12, 19–23]; however, these estimates were substantially inflated in models adjusted for mortality risk factors to 1.1-1.55 [12, 19–23]. These are close to our estimate of 1.3. These results altogether suggest a modest association between autonomic neuropathy and all-cause mortality after controlling for the effect of conventional risk factors of mortality.

There is strong evidence linking diabetic foot ulcers and all-cause mortality with an almost doubling of risk in both T1DM and T2DM [24, 25]. We found a 2- to 3-times increased all-cause mortality in T1DM with *DSPN* in adjusted models. These findings corroborate and extend those from previous cohorts in multiply adjusted models [10, 11]. While these reports used the vibration perception threshold in addition to absent reflexes and symptoms to define DSPN, we used the current perception threshold as the diagnostic test.

Most studies report an association with point estimates similar to our findings in the range of 1.3 to 2.5 for unadjusted and 1.2 to 1.6 for multiply adjusted models between *DSPN* and all-cause mortality in populations of T2DM

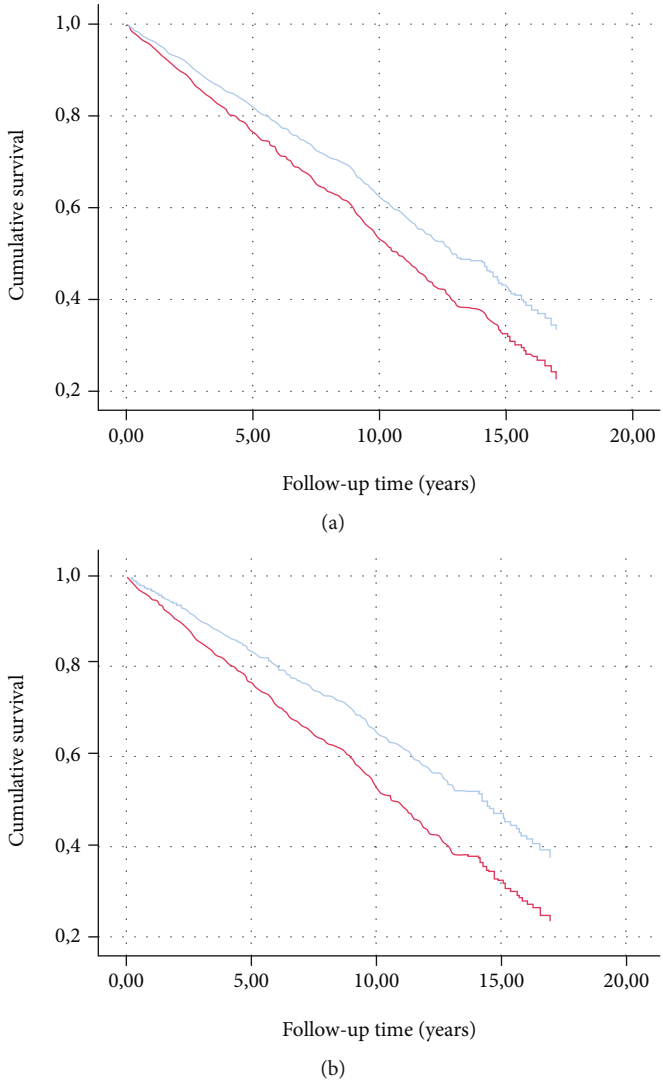

Figure 3: Cumulative survival by CAN (a) and DSPN (b) status in type 2 diabetes patients. Cox proportional hazard model adjusted for age, sex, height, BMI, current smoking, level of alcohol consumption, diabetes duration, known hypertension, systolic and diastolic blood pressure, medications, and burden of comorbidities. Curves are fitted for populations with covariates fixed to the population means. Presence of DSPN/CAN at baseline—red line. Absence of DSPN/CAN at baseline—blue line. DSPN: distal symmetric polyneuropathy; CAN: cardiovascular autonomic neuropathy.

irrespective of the diagnostic method [13, 26–29]. Our robust results together with the literature suggest a 30-50% increased risk of death in T2DM with DSPN that is not explained by conventional risk factors.

Diabetic microvascular complications have a similar set of predictors and frequently cooccur [30], suggesting that the effects of DSPN and CAN are not independent. To the best of our knowledge, our study is the first to show that the effect of DSPN and CAN on all-cause mortality is independent not only of conventional predictors but also from each other.

The observation that the association was much stronger between DSPN and mortality in T1DM compared to T2DM seems to be valid and in agreement with literature data. We suspect that this is not a consequence of the different pathophysiologies of T1DM and T2DM but relate to the large age difference between T1DM and T2DM. Younger people in general have better health than older people, and a significant risk factor (such as DSPN) may substantially increase their risk of mortality [31, 32].

A hallmark of CAN is resting tachycardia that is a well-known predictor of cardiovascular mortality [33], QT distance prolongation is frequently found in CAN and may lead to arrhythmias or sudden death [34]. In persons with CAN, symptoms of cardiovascular disease are frequently absent leading to delayed diagnosis and therapy, ultimately resulting in mortality [34]. The disturbed haemodynamic regulation associated with CAN could lead to diabetic cardiomyopathy and could increase the risk of cerebrovascular events [35]. Furthermore, if cardiovascular stressors such as infection or surgery are present, it may increase morbidity and mortality [34]. In addition to cardiovascular events, orthostatic hypotension increases the risk of falls and injuries, another potential cause of mortality [36].

Much less is known about the potential association between DSPN and mortality. In general, the pain caused by neuropathy is thought to be a factor that effects quality of life through disturbed sleep, recreation, and diminished physical and emotional well-being [37]. However, the neuroendocrine, proinflammatory, and neurodegenerative underpinnings of DSPN could also lead to cardiovascular disease, as well as increased oxidative stress and level of advanced glycation end products [38]. DSPN is also a risk factor for medial arterial calcification and balance impairment that could lead to falls and injuries [39]. DSPN is a leading factor of diabetic foot ulcers and amputations, both associated with mortality through infection and chronic inflammation [24, 25].

Alternatively, it is also possible that both CAN and DSPN are markers of other diseases that increase mortality. Indeed, microvascular diabetes complications show remarkable clustering [30]. Diabetic neuropathies may also be markers of a larger cumulative glycaemic exposure. Although we tried to adjust for most risk factors of mortality in participants with diabetes in our analysis, the role of residual confounding cannot be excluded, although the robustness of our findings argues against this.

Our population-based study sample may be representative of persons with diabetes seen in secondary care centres. The large sample size and long follow-up gives sufficient power to investigate even moderate associations between diabetic neuropathies and all-cause mortality. Our study benefits from the use of gold standard measures of diabetic neuropathies. Most important risk factors were collected at baseline that allow the investigation of diabetic neuropathies on top of established risk factors. The use of NHS data allows almost complete follow-up. The fact that all investigations were done using the same methodology in T1DM and T2DM allows comparisons of risks between diabetes types. The fact that our results were robust for adjustments and that the sensitivity analyses were confirmatory also supports the validity of our findings.

Our study has limitations that must be acknowledged. In spite the large number of participants, statistical power within T1DM is limited. Furthermore, as persons with T1DM and T2DM had different risk factor profiles, we were unable to include both types in the same model, limiting the interpretation of these comparisons. As our cohort includes a referred population, referral bias cannot be excluded. Indeed, unadjusted models showed no difference in the prevalence of CAN between deceased and surviving participants. However, we think that our adjusted models represent true differences. It is also likely that the included population has good generalizability to secondary care centres. While our measure of DSPN is noninvasive and probably identifies people with subclinical disease, it imperfectly correlates with the gold standard physiological measures and misses information on signs and symptoms of neuropathy [40, 41]. It should be noted that unless the imprecision of our measurement is directly related to all-cause mortality, it would bias our estimates toward the null. It should also be mentioned that most large-scale observational studies that investigated the association between DSPN and mortality did not use a comprehensive investigation for the diagnosis of DSPN [11, 42]. Our dataset is missing some potentially important confounders and risk factors of all-cause mortality, such as blood tests (lipids, glycaemic measures) and socioeconomic status that makes our results prone to residual confounding. Our results are hypothesis generating only, as we had no data on cause-specific mortality of participants. It could be hypothesized that point estimates could be even higher for those causes that are direct consequences of neuropathy, such as cardiovascular diseases, injuries.

5. Conclusion

Our study has clear public health ramifications. We confirmed that CAN is an important predictor of all-cause mortality on top of known cardiovascular and other mortality risk factors. Similarly, DSPN is independently associated with all-cause mortality even in persons without diabetic foot ulcer at baseline. Our results also suggest that the effects of these neuropathies are independent of each other. If these associations are not causal, the presence of any neuropathies still marks an increased risk that should lead to more stringent control of conventional risk factors (such as smoking, lipids, and blood pressure) in persons with neuropathy. If the association is causal, we can hope for better survival if not only symptomatic but etiological treatments become available for diabetic neuropathies. The finding that relative mortality is much higher in persons with sensory neuropathy and T1DM compared to T2DM is novel and suggests that younger age does not protect people from the most severe outcomes although this observation requires further confirmation in other cohort studies.

Abbreviations

ATC: Anatomical therapeutic chemical
CAN: Diabetic cardiovascular autonomic neuropathy
DSPN: Distal symmetric polyneuropathy
HR: Hazard ratio
NHS: National Health Service
SD: Standard deviation
T1DM: Type 1 diabetes mellitus
T2DM: Type 2 diabetes mellitus.

Data Availability

The datasets generated during and/or analysed during the current study are not publicly available due to data protection regulation related to reuse of NHS Hungary data but are available from the corresponding author upon reasonable request.

Ethical Approval

This research was approved by the local ethical committee (Semmelweis University Regional and Institutional Committee of Science and Research Ethics) under the number of SE TUKEB 36/2017.

Consent

Given that this is a retrospective analysis, no patient consent was required.

Conflicts of Interest

The authors declare that that they have no competing interests.

Authors' Contributions

Study design and conception were handled by V.J.H. and A.G.T. Analysis and interpretation were handled by all authors. Drafting of the article was handled by O.E.V, M.M.S., B.A.D, V.J.H., and A.G.T. Critical revision for intellectual content was handled by all authors. A.G.T. had full access to all the data used in these analyses and takes full responsibility for the integrity of the data and the accuracy of the data analysis. All authors reviewed and accepted the submitted version of this manuscript. OE Vági, MM Svébis, and BA Domján are co-first authors; VJ Horváth and AG Tabák are co-last authors of this paper.

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Supplementary Materials

Our paper has supplemental material that contains those details on materials that are only relevant to specialist readers who want to repeat the exact same analysis that we performed but not to the general readership of the journal. *(Supplementary materials)*

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Research Article

Network Pharmacology and Molecular Docking Study on the Potential Mechanism of Yi-Qi-Huo-Xue-Tong-Luo Formula in Treating Diabetic Peripheral Neuropathy

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Objective. To investigate the potential mechanism of action of Yi-Qi-Huo-Xue-Tong-Luo formula (YQHXTLF) in the treatment of diabetic peripheral neuropathy (DPN). *Methods*. Network pharmacology and molecular docking techniques were used in this study. Firstly, the active ingredients and the corresponding targets of YQHXTLF were retrieved using the Traditional Chinese Medicine Systems Pharmacology (TCMSP) platform; subsequently, the targets related to DPN were retrieved using GeneCards, Online Mendelian Inheritance in Man (OMIM), Pharmgkb, Therapeutic Target Database (TTD) and Drugbank databases; the common targets of YQHXTLF and DPN were obtained by Venn diagram; afterwards, the "YQHXTLF Pharmacodynamic Component-DPN Target" regulatory network was visualized using Cytoscape 3.6.1 software, and Gene Ontology (GO) enrichment analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis were performed on the potential targets using R 3.6.3 software. Finally, molecular docking of the main chemical components in the PPI network with the core targets was verified by Autodock Vina software. *Results*. A total of 86 active ingredients and 229 targets in YQHXTLF were screened, and 81 active ingredients and 110 targets were identified to be closely related to diabetic peripheral neuropathy disease. PPI network mapping identified TP53, MAPK1, JUN, and STAT3 as possible core targets. KEGG pathway analysis showed that these targets are mostly involved in AGE-RAGE signaling pathway in diabetic complications, TNF signaling pathway, and MAPK signaling pathway. The molecular docking results showed that the main chemical components of YQHXTLF have a stable binding activity to the core pivotal targets. *Conclusion*. YQHXTLF may act on TP53, MAPK1, JUN, and STAT3 to regulate inflammatory response, apoptosis, or proliferation as a molecular mechanism for the treatment of diabetic peripheral neuropathy, reflecting its multitarget and multipathway action, and providing new ideas to further uncover its pharmacological basis and mechanism of action.

1. Introduction

Diabetic peripheral neuropathy (DPN) is one of the most common and serious microvascular complications of diabetes which is characterized by pain, sensory abnormalities, and loss of sensation [1, 2]. It has been shown that DPN affects approximately 40% to 60% of people with diabetes [3], and if not well treated, it increases the risk of disability and mortality [4]. Currently, there is no specific treatment for DPN in modern medicine, which consists mainly of improving metabolic disorders and pain management [5, 6]. With the continuous improvement in medical care and the increasing demand for health, the main issue facing us today is how to prevent and control the progression of DPN and improve the quality of survival of patients. Traditional Chinese Medicine (TCM) has a long history

of treating diabetes mellitus and its complications [7, 8]. In TCM, DPN is often classified as "paralysis," "impotence," and "blood paralysis" [9]. It is often caused by prolonged thirst, depletion of Qi and blood, deficiency of both yin and yang, and loss of nourishment for the tendons and veins, resulting in coldness, numbness, and muscle atrophy [10, 11]. Treatment is mostly based on benefiting Qi and nourishing Yin, invigorating blood circulation and relieving pain, emphasizing the treatment of both the symptoms and the root cause, and overall regulation [12]. A large number of studies have shown that Chinese medicine is effective in treating DPN, significantly improving the clinical symptoms of patients and delaying the development of the disease, with few toxic side effects [13]. Therefore, exploring the regulatory mechanisms of TCM can help develop new therapeutic approaches to improve the treatment of DPN.

Yi-Qi-Huo-Xue-Tong-Luo formula (YQHXTLF) is an in-hospital preparation for the prevention and control of diabetic peripheral neuropathy at Anhui Provincial Hospital of Traditional Chinese Medicine. It consists of 7 Chinese herbal medicines: Astragalus (Huangqi), Radix Angelicae Sinensis (Danggui), Radix et Rhizoma Dioscoreae (Dihuang), Radix et Rhizoma Yanhusuo (Yanhusuo), Radix et Rhizoma Puerariae (Gegen), Radix et Rhizoma Chrysanthemum (Jixueteng), and Radix et Rhizoma Weilingensis (Weilingxian). The combination of all the herbs in this formula can treat both the symptoms and the root cause of pain by treating Qi and blood together, which can benefit Qi and invigorate blood, as well as promote circulation and relieve pain. Preliminary clinical studies have shown that this formula can alleviate abnormal skin sensation, numbness, and tingling in the limbs and improve motor nerve conduction speed and sensory nerve conduction speed in DPN patients [14]. Basic research found that this formula can reduce islet cell damage in db/db mice, improve blood flow in the sciatic nerve area, and promote the repair and regeneration of damaged nerves, and the mechanism of action may be related to the improvement of diabetic inflammatory lesions and inhibition of excessive activation of the MAPK signaling pathway [15]. However, due to the multicomponent and multitarget nature of the Chinese medicine formula, the exact mechanism of action of the formula is still unclear.

Network pharmacology is a new discipline involving the analysis of drug-target-disease network associations [16]. It provides a systematic approach to the analysis of complex drug mechanisms of action and potential disease interventions by identifying the core targets shared by drugs and diseases [17, 18]. Through the use of network pharmacology, we can not only explore the complex active molecular components and potential molecular targets in Chinese medicine formulations but also understand the molecular relationships between different components in a compound formula and between the components and complex diseases and extract possible pathways for drug interventions to target diseases [19]. With the rapid development of network pharmacology, the mechanisms of TCM in the treatment of many serious diseases have been successfully predicted, and the multitarget integrated prevention and treatment approach has been applied to cancer [20], arthritis [21], diabetes, and other diseases with certain results [22, 23].

In this study, we use network pharmacology as a tool to further analyze the possible targets, molecular mechanisms, biological processes, and pathways of YQHXTLF for the treatment of DPN. We modeled the interrelationship between the targets of DPN and elucidated the synergistic mechanism between the active components of Chinese medicine, providing insights into the interrelationship and changes between Chinese medicine and diseases from the perspective of biological networks, which provided new possibilities and directions for the treatment of DPN. The flow chart of this study is shown in Figure 1.

2. Materials and Methods

2.1. Screening of Active Ingredients of YQHXTLF. The Traditional Chinese Medicine Systems Pharmacology (TCMSP, https://www.tcmspw.com) analysis platform [24] was used to screen the chemical components of YQHXTLF. TCMSP is a unique systemic pharmacology platform for Chinese herbal medicines that captures the relationship between drug, target, and disease. Oral bioavailability (OB) is one of the most important pharmacokinetic parameters in drug absorption, distribution, metabolism, and excretion (ADME) [25]. Drug-like properties (DL) refer to the similarity of a compound to a known drug. In drug development, drug-like studies are based on lead compounds, and drug-like molecules can be considered as high-quality lead compounds [26]. In this study, compounds with OB $\geq$ 30% and DL $\geq$ 0.18 were selected as potential active ingredients. The target information of these active ingredients was standardized by using the Uniprot database (https://www.uniprot.org/) with the species "homo sapiens."

2.2. Exploring Potential DPN Targets. We searched several important databases with the keyword "diabetic peripheral neuropathy", including the GeneCards database (https://www.genecards.org/), the OMIM database (https://www.omim), the Pharmgkb database (https://www.pharmgkb.org/), the TTD database (http://bidd.nus.edu.sg/group/cjttd/), and the Drugbank data (https://www.drugbank.ca/). For the targets in GeneCards, only those with a score $\geq$ 10 were chosen. The targets obtained from the above four databases were then integrated to construct a DPN-related target set.

2.3. Constructing a Regulatory Network of Targets for the Treatment of DPN with YQHXTLF. The overlapping targets were considered to be the common targets of YQHXTLF and DPN. Cytoscape (version 3.6.1; https://www.cytoscape.org/) [27] was then used to visualize the complex relationships between the active chemical components and the potential target genes to construct a regulatory network of targets for the treatment of DPN with YQHXTLF. The layout tool was used to quantify the degree of each node; the larger the node in the network, the higher the degree value of the node.

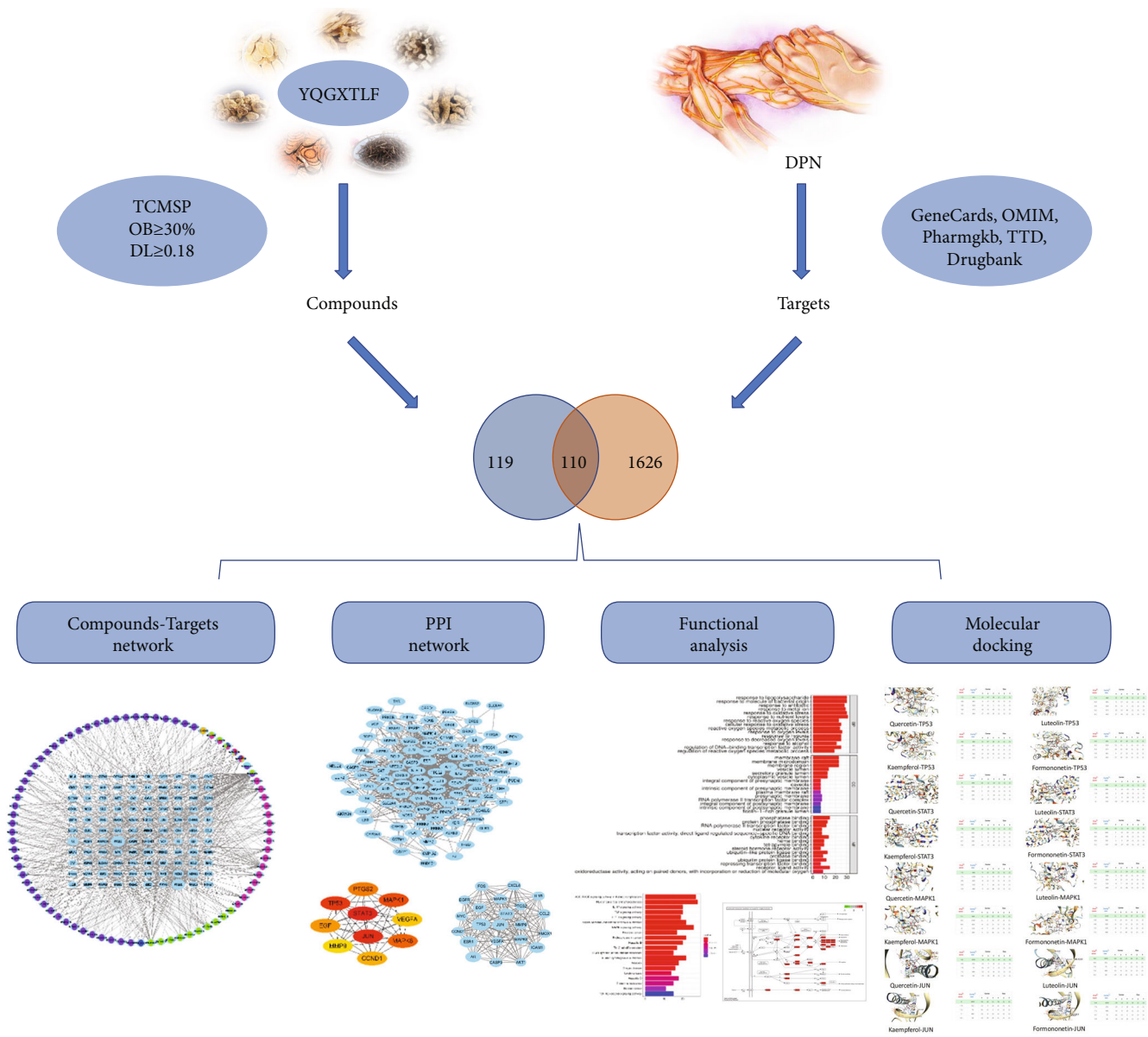


Figure 1: Workflow of the network pharmacology of YQHXTLF in the treatment of DPN. First, the effective active compounds of YQHXTLF were screened from the TCMSP. Relevant targets of DPN were summarized by searching databases. The intersection targets of compound targets and disease targets were obtained soon. Secondly, the interaction net between the compounds and the filtered targets was established. These key targets were analyzed by PPI analysis, functional analysis, and molecular docking verification. Finally, the key genes were used to find the biologic pathway and explain the therapeutic mechanism by network pharmacology analysis.

2.4. Protein-Protein Interaction Analysis and Core Gene Screening. The intersecting gene targets of YQHXTLF and DPN were imported into the STRING protein interaction analysis platform, and the protein classification was set to "homo sapiens" with a maximum confidence level of ≥0.7, hiding the unlinked nodes in the network. Protein interaction network analysis was performed, and the TSV file was downloaded. Cytoscape 3.6.1 software was then imported to construct protein-protein interaction (PPI) network maps. The hub targets in the PPI network were screened using the cytoHubba plugin, with darker node colors representing higher scores [28]. The Molecular Complex Detection (MCODE) plugin was used to discover closely linked regions in the PPI network [29]. The score value of a module reflects how dense the module is to the surrounding nodes, with higher scores indicating that the nodes are becoming more important.

2.5. Gene Ontology and KEGG Pathway Enrichment Analysis. Gene Ontology (GO) functional analysis is mainly used to describe the function of gene targets, including biological process, cellular component, and molecular function. KEGG enrichment analysis can obtain the signal pathways enriched by the common targets of YQHXTLF and DPN. By using R 3.6.3 software-related R packages (Colorspace, Stringi, GGPLOt2, BiocManager, Dose, clusterProfiler, Enrichplot,

Table 1: Detailed information of the 86 active compounds from YQHXTLF.

Mol Id	Mol name	Structure	OB (%)	DL
MOL000358	Beta-sitosterol		36.91	0.75
MOL000449	Stigmasterol		43.83	0.76
MOL000392	Formononetin		69.67	0.21
MOL002959	3′-Methoxydaidzein		48.57	0.24
MOL003629	Daidzein-4,7-diglucoside		47.27	0.67
MOL000211	Mairin		55.38	0.78
MOL000239	Jaranol		50.83	0.29
MOL000296	Hederagenin		36.91	0.75
MOL000033	(3S,8S,9S,10R,13R,14S,17R)-10,13-Dimethyl-17-[(2R,5S)-5-propan-2-yloctan-2-yl]-2,3,4,7,8,9,11,12,14,15,16,17-dodecahydro-1H-cyclopenta[a]phenanthren-3-ol		36.23	0.78
MOL000354	Isorhamnetin		49.6	0.31
MOL000371	3,9-Di-O-methylnissolin		53.74	0.48
MOL000378	7-O-Methylisomucronulatol		74.69	0.3
MOL000379	9,10-Dimethoxypterocarpane -3-O-β-D-glucoside		36.74	0.92
MOL000380	(6aR,11aR)-9,10-Dimethoxy-6a,11a-dihydro-6H-benzofurano[3,2-c]chromen-3-ol		64.26	0.42
MOL000387	Bifendate		31.1	0.67
MOL000417	Calycosin		47.75	0.24
MOL000422	Kaempferol		41.88	0.24
MOL000433	FA		68.96	0.71
MOL000439	Isomucronulatol-7,2′-di-O-glucosiole		49.28	0.62
MOL000442	1,7-Dihydroxy-3,9-dimethoxy pterocarpene		39.05	0.48
MOL000098	Quercetin		46.43	0.28
MOL000461	3,7-Dihydroxy-6-methoxy-dihydroflavonol		43.8	0.26
MOL000468	8-O-Methylreyusin		70.32	0.27
MOL000469	3-Hydroxystigmast-5-en-7-one		40.93	0.78
MOL000470	8-C-α-L-Arabinosylluteolin		35.54	0.66
MOL000471	Aloe-emodin		83.38	0.24
MOL000483	(Z)-3-(4-Hydroxy-3-methoxy-phenyl)-N-[2-(4-hydroxyphenyl)ethyl]acrylamide		118.35	0.26
MOL000490	Petunidin		30.05	0.31

Table 1: Continued.

Mol Id	Mol name	Structure	OB (%)	DL
MOL000492	(+)-Catechin		54.83	0.24
MOL000493	Campesterol		37.58	0.71
MOL000497	Licochalcone a		40.79	0.29
MOL000500	Vestitol		74.66	0.21
MOL000501	Consume close grain		68.12	0.27
MOL000502	Cajinin		68.8	0.27
MOL000503	Medicagol		57.49	0.6
MOL000506	Lupinidine		61.89	0.21
MOL000507	Psi-baptigenin		70.12	0.31
MOL000006	Luteolin		36.16	0.25
MOL000359	Sitosterol		36.91	0.75
MOL005603	Diheptyl phthalate		42.26	0.31
MOL001454	Berberine		36.86	0.78
MOL001458	Coptisine		30.67	0.86
MOL001460	Cryptopine		78.74	0.72
MOL001461	Dihydrochelerythrine		32.73	0.81
MOL001463	Dihydrosanguinarine		59.31	0.86
MOL001474	Sanguinarine		37.81	0.86
MOL000217	(S)-Scoulerine		32.28	0.54
MOL002670	Cavidine		35.64	0.81
MOL002903	(R)-Canadine		55.37	0.77
MOL004071	Hyndarine		36.91	0.75
MOL004190	(-)-Alpha-N-methylcanadine		73.94	0.64
MOL004191	Capaurine		45.06	0.8
MOL004193	Clarkeanidine		62.91	0.69
MOL004195	CORYDALINE		86.65	0.54

Table 1: Continued.

Mol Id	Mol name	Structure	OB (%)	DL
MOL004196	Corydalmine		65.84	0.68
MOL004197	Corydine		52.5	0.59
MOL004198	18797-79-0		37.16	0.55
MOL004199	Corynoloxine		46.06	0.85
MOL004200	Methyl-[2-(3,4,6,7-tetramethoxy-1-phenanthryl)ethyl]amine		38.12	0.6
MOL004202	Dehydrocavidine		61.15	0.44
MOL004203	Dehydrocorybulbine		38.99	0.81
MOL004204	Dehydrocorydaline		46.97	0.63
MOL004205	Dehydrocorydalmine		41.98	0.68
MOL004208	Demethylcorydalmatine		43.9	0.59
MOL004209	13-Methyldehydrocorydalmine		38.99	0.54
MOL004210	(1S,8′R)-6,7-Dimethoxy-2-methylspiro[3,4-dihydroisoquinoline-1,7′-6,8-dihydrocyclopenta[g][1,3]benzodioxole]-8′-ol		35.94	0.63
MOL004763	Izoteolin		43.95	0.72
MOL004214	Isocorybulbine		39.53	0.51
MOL004215	Leonticine		40.18	0.66
MOL004216	13-Methylpalmatrubine		45.79	0.26
MOL004220	N-Methyllaurotetanine		40.97	0.63
MOL004221	Norglaucing		41.62	0.56
MOL004224	Pontevedrine		30.35	0.56
MOL004225	Pseudocoptisine		30.28	0.71
MOL004226	24240-05-9		38.97	0.86
MOL004228	Saulatine		53.75	0.83
MOL004230	Stylopine		42.74	0.79
MOL004231	Tetrahydrocorysamine		48.25	0.85

Table 1: Continued.

Mol Id	Mol name	Structure	OB (%)	DL
MOL004232	Tetrahydroprotopapaverine		34.17	0.86
MOL004233	ST057701		36.86	0.78
MOL004234	2,3,9,10-Tetramethoxy-13-methyl-5,6-dihydroisoquinolino[2,1-b]isoquinolin-8-one		30.67	0.86
MOL000785	Palmatine		78.74	0.72
MOL000787	Fumarine		32.73	0.81
MOL000790	Isocorypalmine		59.31	0.86
MOL000791	Bicuculline		37.81	0.86
MOL000793	C09367		32.28	0.54

Mol Id: molecule Id; Mol name: molecule name; OB: oral bioavailability; DL: drug-like.

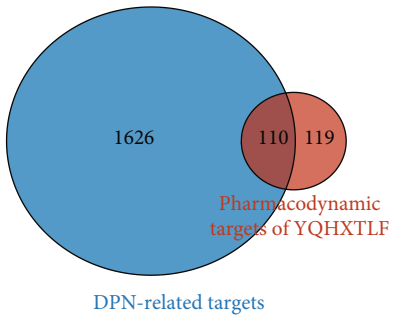


Figure 2: Venn diagram of targets of DPN treated by YQHXTLF.

Pathview), GO enrichment analysis and KEGG pathway enrichment analysis were conducted for the intersection target genes of YQHXTLF-DPN. According to Fisher's test, a p value < 0.05 and q value < 0.05 were considered statistically significant.

2.6. Molecular Docking Validation. Molecular docking validation is achieved through the CB-Dock server [30], a docking tool that predicts the binding site of a given protein and calculates the center and size using a novel curvature-based lumenal detection method. The 2D structure of the compound's small-molecule ligand was first obtained from the PubChem online database (https://pubchem.ncbi.nlm.nih.gov/) as the small-molecule ligand file for molecular docking. The core target protein receptor structure file was obtained from the PDB online database (https://www.rcsb.org). The CB-Dock software was used for molecular docking, and the score of the molecular docking complexes was calculated using the Vina program to evaluate their binding activity.

3. Results

3.1. Active Ingredients in YQHXTKF. The active ingredients were screened by searching the TCMSP database with a threshold value of OB ≥ 30% and DL ≥ 0 : 18. A total of 86 active ingredients derived from YQHXTLF were screened. Among them, 20 ingredients were from Astragalus, 2 from Radix Angelicae Sinensis, 4 from Radix Puerariae, 24 from Spatholobi Caulis, 2 from Rehmanniae Radix, 7 from Radix Clematidis, and 49 from Corydalis Rhizoma. The search for their corresponding targets showed that 86 compounds acted on 229 targets. The basic information of the 86 active compounds is shown in Table 1.

3.2. DPN-Related Gene Targets. Using the keyword "Diabetic peripheral neuropathy," the GeneCards website screened 1521 genes related to diabetic peripheral neuropathy, the OMIM database screened 118, the Pharmgkb database retrieved 198, the TTD online website retrieved 32, and the Drugbank database retrieved 20. After removing duplicate targets and standardized gene names, a total of 1736 DPN potential action targets were screened.

3.3. Intersection of TQHXTLF and DPN Target. The Hiplot research data visualization platform (https://hiplot.com.cn) was used to show the intersection of TQHXTLF and DPN targets, and Venn diagrams were drawn to obtain a total of 110 candidate targets for TQHXTLF and DPN, as shown in Figure 2.

3.4. Regulatory Network Analysis of YQHXTLF and DPN Targets. The "YQHXTLF Pharmacodynamic Component-DPN Target" regulatory network was mapped by using Cytoscape 3.6.1 software to match the YQHXTLF

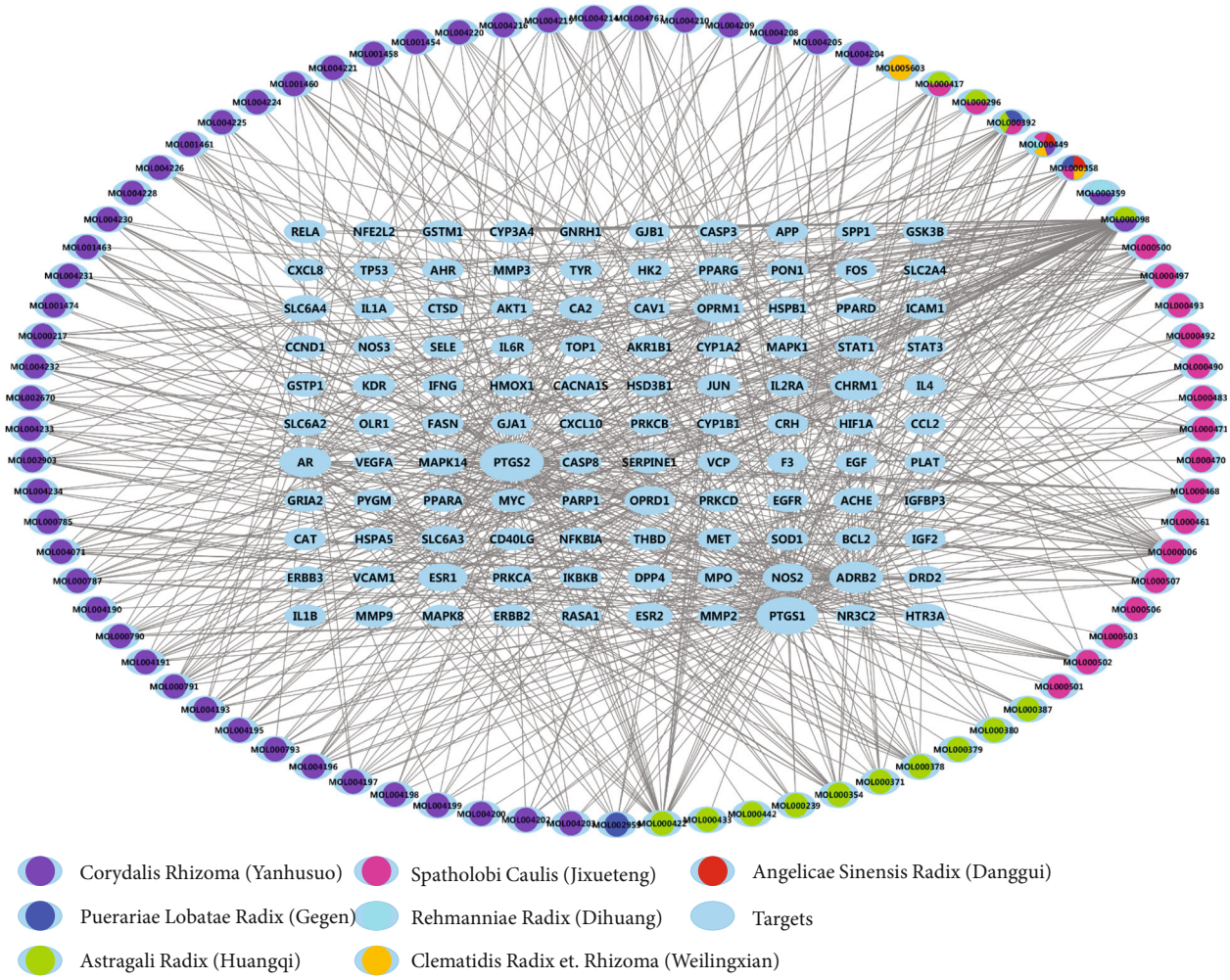


Figure 3: Construction of a target modulation network for DPN treatment with YQHXTLF. The blue ellipse represents the target site, the other coloured nodes represent the different herbal compounds, and the connecting lines represent the interaction between the compound and the target site.

pharmacodynamic components with the 110 action targets obtained from the screening. As shown in Figure 3, the network contained 191 nodes and 696 relationships. From the overall characteristics of the network, it can be found that among the 81 compounds in YQHXTLF, there is one compound corresponding to multiple targets and one target corresponding to multiple compounds; the larger the node, the larger the network value of the target. From the regulatory network, it can be seen that the chemical with the highest degree of connectivity is quercetin, which interacts with 75 targets, followed by luteolin, which interacts with 31 targets; kaempferol, which interacts with 28 targets; and formononetin, which interacts with 18 targets.

3.5. PPI Network Construction and Core Module Analysis. We imported common targets into the STRING 11.0 platform and visualized the PPI network using Cytoscape 3.6.1 software, as shown in Figure 4(a). 105 nodes and 797 edges were included in the network. Five targets did not interact with any other targets and were therefore not included in the PPI network. The cytoHubba plugin was applied to calculate the degree of degree connectivity for each target (Figure 4(b)). The top 10 hub target genes ranked by node degree were AKT1, MAPK8, TP53, MAPK1, STAT3, VEGFA, JUN, EGFR, and EGF. These target genes may play a key role in the network. The MCODE plugin was analyzed to filter out the most significant modules, with an MCODE score of 17.238, containing 22 nodes and 181 edges. The top ten targets in the MOCDE score were TP53, ESR1, ICAM1, JUN, AR, STAT3, CXCL8, MAPK1, and FOS (Figure 4(c)). The results showed that TP53, MAPK1, JUN, and STAT3 were the key targets under different algorithms.

3.6. Gene Ontology and KEGG Pathway Enrichment Analysis. We intercepted the first 15 terms from the smallest to the largest according to the adj. p value. The results of the biological process analysis showed that the intersection targets were mostly enriched in response to lipopolysaccharide, response to molecule of bacterial origin, response to antibiotic, response to metal ion response to oxidative stress, and response to nutrient levels. The results of cell composition showed that the intersection targets were mostly enriched

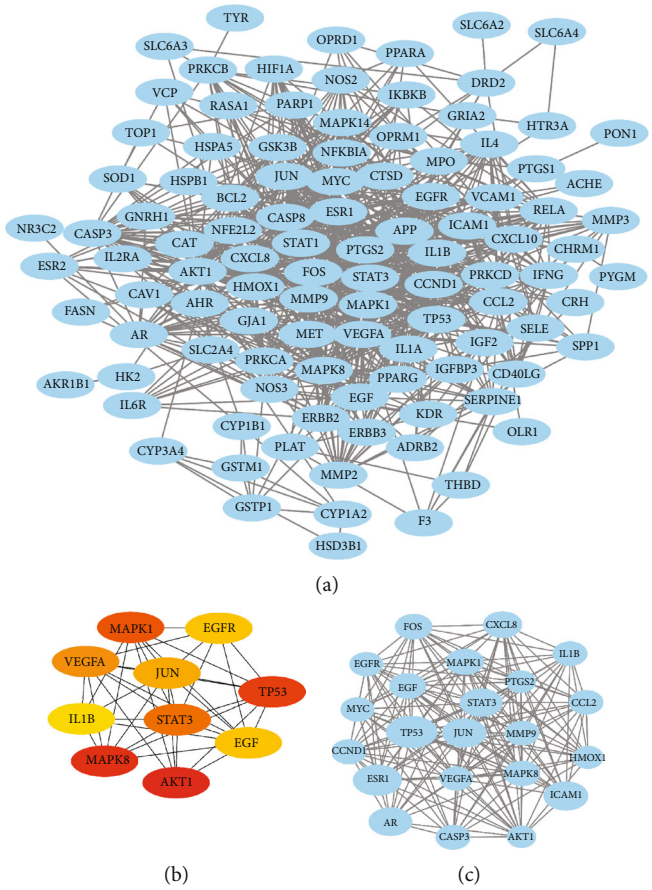


Figure 4: PPI network construction and core module: (a) YQHXTLF pharmacodynamic composition-DPN target PPI network; (b) top 10 hub target genes ranked by node degree; (c) the most significant modules analyzed by the MCODE plugin.

in membrane raft, membrane microdomain, membrane region, vesicle lumen, secretory granule lumen, cytoplasmic vesicle lumen, etc. Molecular functions mainly include phosphatase binding, protein phosphatase binding, RNA polymerase II transcription factor binding, nuclear receptor activity, transcription factor activity, direct ligand regulated sequence-specific DNA binding, and cytokine receptor binding, as shown in Figure 5(a).

We intercepted the top 20 KEGG pathways from the smallest to the largest based on the p value. The analysis showed that these targets were mostly enriched in the AGE-RAGE signaling pathway in diabetic complications, fluid shear stress and atherosclerosis, TNF signaling pathway, MAPK signaling pathway, etc., as shown in Figure 5(b).

3.7. Molecular Docking Validation. The four active ingredients quercetin, luteolin, kaempferol, and formononetin, which have a high number of targets in YQHXTKF, were selected for molecular docking validation with the core targets TP53, MAPK1, JUN, and STAT3 in the PPI network map. The binding energy (Vina score) was used to evaluate the bonding strength between the docked molecules, and the value of the Vina score indicated some binding activity between the proteins and the compounds. The smaller the binding energy (Vina score), the higher the affinity of the receptor and ligand and the more stable the binding of the compound to the target site. The binding energies of the four active ingredients and the corresponding target proteins were all less than -5.0, indicating good docking and high binding activity [31]. Molecular docking simulations showed a stable point docking structure for the binding of small-molecule ligands and protein receptors (Figure 6).

4. Discussion

In this study, we used network pharmacology and molecular docking techniques to analyze the potential molecular biological mechanisms of YQHXTLF for the treatment of DPN. We firstly screened the active ingredients of YQHXTLF using the criteria of OB ≥ 30% and DL ≥ 0.18 and obtained 94 compounds, 229 potential targets, and 110 targets overlapping with DPN to construct a compound-disease target regulatory network. Through the network analysis, 81 components in this prescription may act on 110 targets to exert the therapeutic effect. These identified compounds, particularly the four compounds including quercetin, luteolin, kaempferol, and formononetin, were linked to more than 10 targets, indicating that these compounds might play a vital role in the process of DPN treatment. Quercetin is a flavonoid which is widely found in nature and has been proved to have a great effect on antibacterial, anti-inflammatory, and antiallergic therapy [32].

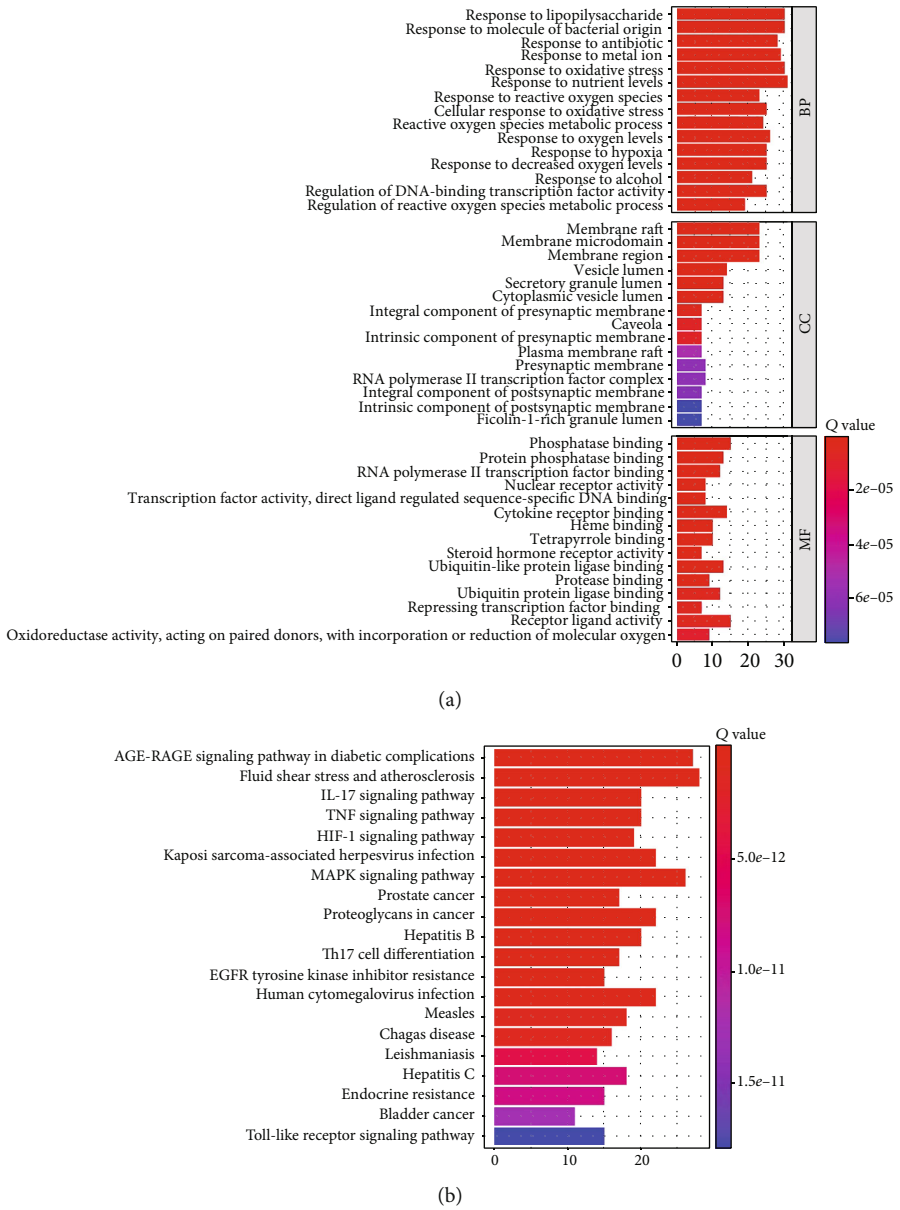
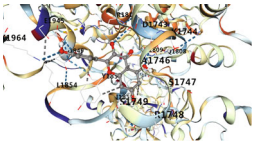


Figure 5: GO and KEGG pathway enrichment analysis: (a) the top 15 significantly enriched GO terms of BP, CC, and MF; (b) the top 20 significantly enriched KEGG pathways.

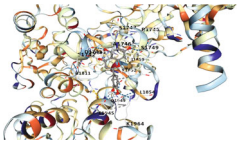
Quercetin reduces neuronal loss and inhibits neuronal apoptosis by improving neurotrophic factor levels [33, 34]. It has been reported that quercetin exerts neuroprotective effects on DRG neuronal cells in a high-glucose environment, possibly by activating Nrf-2/HO-1 and inhibiting NF-κB to reduce apoptosis [35]. Quercetin also has neuroprotective effects in diabetic peripheral neuropathy by inducing autophagy to reduce the damage to neuronal cells from high glucose and improving the antioxidant status [36, 37]. Luteolin significantly upregulates the protein levels of NRF2 and HO-1 in diabetic nerves and improved nerve conduction velocity and nerve blood flow [38]. Luteolin has been shown to improve blood glucose, glycosylation, insulin, and HOMR-IR levels in diabetic model mice, with positive effects against diabetes and its complications [39]. Kaempferol has excellent antioxidant properties and can correct hyperglycemia in DM rats by regulating oxidative stress and reducing AGE accumulation, thus preventing the risk of complications [40, 41]. It reduces the expression of IL-1β and TNF-α, thereby inhibiting the neuroimmune activation of microglia and alleviating the progression of diabetic neuropathy [42]. Formononetin is an isoflavone that is known to prevent and slow the progression of long-term diabetic complications by reducing hyperglycemia and highlighting neuroprotective effects [43]. Formononetin protects diabetic animals from hyperglycemia-induced neuronal damage by increasing the expression of SIRT1 and NGF in neural tissue [44, 45]. Therefore, quercetin, luteolin, kaempferol, and formononetin may be the most important components of YQHXTLF for the treatment of DPN, as shown in Table 2.



Quercetin-TP53

Vina score	Cavity size	Center			Size		
		x	y	z	x	y	z
−7.2	442	15	47	38	21	21	21
−6.8	461	9	17	22	21	21	21
−6.4	826	53	31	3	21	21	21
−6.4	476	30	33	45	21	21	21
−6.1	402	27	39	23	21	21	21

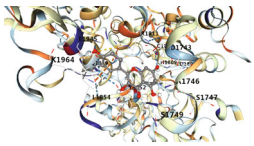
(a)



Luteolin-TP53

Vina score	Cavity size	Center			Size		
		x	y	z	x	y	z
−7.3	442	15	47	38	21	21	21
−6.9	461	9	17	22	21	21	21
−6.4	826	53	31	3	21	21	21
−6.2	473	30	33	45	21	21	21
−6.1	402	27	39	23	21	21	21

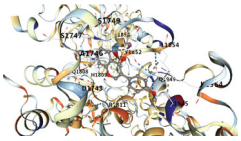
(b)



Kaempferol-TP53

Vina score	Cavity size	Center			Size		
		x	y	z	x	y	z
−6.8	442	15	47	38	21	21	21
−6.5	461	9	17	22	21	21	21
−6.3	473	30	33	45	21	21	21
−6.3	402	27	39	23	21	21	21
−6.2	826	53	31	3	21	21	21

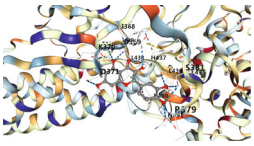
(c)



Formononetin-TP53

Vina score	Cavity size	Center			Size		
		x	y	z	x	y	z
−6.6	442	15	47	38	21	21	21
−6.2	826	53	31	3	21	21	21
−5.9	473	30	33		21	21	21
−5.9	461	9	17		21	21	21
−5.9	402	27	39		21	21	21

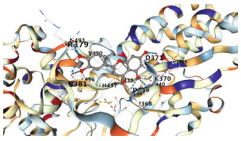
(d)



Quercetin-STAT3

Vina score	Cavity size	Center			Size		
		x	y	z	x	y	z
−8	458	16	12	18	21	21	21
−7.4	565	2	26	29	21	21	21
−7.2	846	5	32	20	21	21	21
−6.9	390	−1	9	26	21	21	21
−6.1	339	−29	−27	62	21	21	21

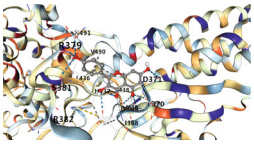
(e)



Luteolin-STAT3

Vina score	Cavity size	Center			Size		
		x	y	z	x	y	z
−7.8	458	16	12	18	21	21	21
−7.4	846	5	32	20	21	21	21
−7.4	565	2	26	29	21	21	21
−7	390	−1	9	26	21	21	21
−6	339	−29	−27	62	21	21	21

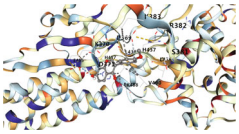
(f)



Kaempferol-STAT3

Vina score	Cavity size	Center			Size		
		x	y	z	x	y	z
−7.8	458	16	12	18	21	21	21
−7.2	565	2	26	29	21	21	21
−6.9	846	5	32	20	21	21	21
−6.7	390	−1	9	26	21	21	21
−5.9	339	−29	−27	62	21	21	21

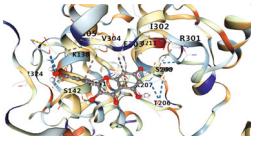
(g)



Formononetin-STAT3

Vina score	Cavity size	Center			Size		
		x	y	z	x	y	z
−7.3	458	16	12	18	21	21	21
−6.6	846	5	32	20	21	21	21
−6.6	565	2	26	29	21	21	21
−6.4	390	−1	9	26	21	21	21
−5.1	339	−29	−27	62	21	21	21

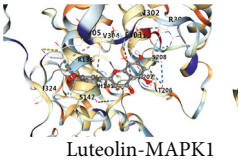
(h)



Quercetin-MAPK1

Vina score	Cavity size	Center			Size		
		x	y	z	x	y	z
−7.3	664	−5	23	25	21	21	21
−7.1	2340	11	2	16	21	21	21
−6.6	426	−6	12	13	21	21	21
−6.4	529	−4	7	26	21	21	21
−5.6	446	−5	13	46	21	21	21

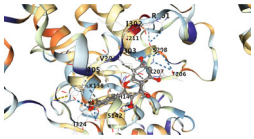
(i)



Luteolin-MAPK1

Vina score	Cavity size	Center			Size		
		x	y	z	x	y	z
−7.4	2340	11	2	16	27	21	21
−7.4	664	−5	23	25	21	21	21
−6.7	426	−6	12	13	21	21	21
−6.2	529	−4	7	26	21	21	21
−5.5	446	−5	13	46	21	21	21

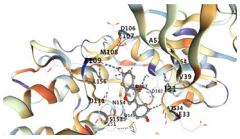
(j)



Kaempferol-MAPK1

Vina score	Cavity size	Center			Size		
		x	y	z	x	y	z
−7.1	664	−5	23	25	21	21	21
−6.8	2340	11	2	16	27	21	21
−6.6	426	−6	12	13	21	21	21
−6	529	−4	7	26	21	21	21
−5.8	446	−5	13	46	21	21	21

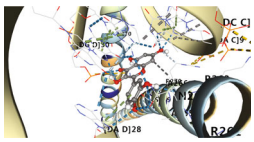
(k)



Formononetin-MAPK1

Vina score	Cavity size	Center			Size		
		x	y	z	x	y	z
−7.2	2340	11	2	16	27	21	21
−6.8	664	−5	23	25	21	21	21
−6.8	426	−6	12	13	21	21	21
−5.5	529	−4	7	26	21	21	21
−5.3	446	−5	13	46	21	21	21

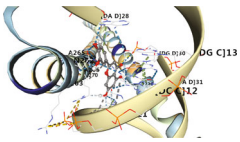
(l)



Quercetin-JUN

Vina score	Cavity size	Center			Size		
		x	y	z	x	y	z
−9.2	3850	−34	16	13	34	21	33
−8.4	252	−23	−3	18	21	21	21
−7.4	76	−41	13	2	21	21	21
−7.2	486	−32	−6	16	21	21	21
−6.4	64	−16	18	28	21	21	21

(m)

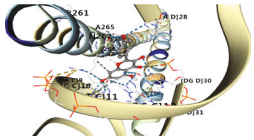


Luteolin-JUN

Vina score	Cavity size	Center			Size		
		x	y	z	x	y	z
−8.8	3850	−34	16	13	34	21	33
−8.6	252	−23	−3	18	21	21	21
−7.8	76	−41	13	2	21	21	21
−7.4	486	−32	−6	16	21	21	21
−6.2	64	−16	18	28	21	21	21

(n)

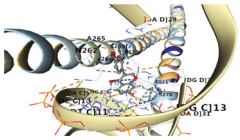
Figure 6: Continued.



Vina score	Cavity size	Center			Size		
		x	y	z	x	y	z
−9	3850	−34	16	13	34	21	33
−7.9	252	−23	−3	18	21	21	21
−7.2	76	−41	13	2	21	21	21
−7	486	−32	−6	16	21	21	21
−6.1	64	−16	18	28	21	21	21

Kaempferol-JUN

(o)



Vina score	Cavity size	Center			Size		
		x	y	z	x	y	z
−8.4	3850	−34	16	13	34	21	33
−7.6	252	−23	−3	18	21	21	21
−7.1	486	−32	−6	16	21	21	21
−6.7	76	−41	13	2	21	21	21
−5.6	64	−16	18	28	21	21	21

Formononetin-JUN

(p)

Figure 6: The docking model diagram of the active ingredient of the drug and the core target molecule: (a–d) the action mode of TP53 and quercetin, luteolin, kaempferol, and formononetin; (e–h) the action mode of STAT3 and quercetin, luteolin, kaempferol, and formononetin; (i–l) the action mode of MAPK1 and quercetin, luteolin, kaempferol, and formononetin; (m–p) the action mode of JUN and quercetin, luteolin, kaempferol, and formononetin.

Table 2: The potential mechanisms of the four main compounds in the treatment of DPN.

Compound	Mechanism	Model	Reference
Quercetin	Increased BDNF, NGF, and Bcl-2, inhibited caspase-3	Diabetic rats	[33]
	Maintained the density of the general neuronal population, reduced the loss of interosseous neurons, antioxidant	Diabetic rats	[34]
	Activated the Nrf-2/HO-1 pathway, inhibited the NF-κB pathway, and inhibited iNOS, COX-2, IL-6, and TNF-α	DRG cells	[35]
	Upregulated Beclin-1 and LC3 protein expression levels, increased cell proliferation, and upregulated autophagy	Schwann cells	[36]
	Reduced total cholesterol and TBARS levels, increased HDL-cholesterol, SOD, CAT, and GSH-Px activity	Db/db mice	[37]
Luteolin	Upregulated protein levels of Nrf2 and HO-1, improved nerve conduction velocity and nerve blood flow	Diabetic rats	[38]
	Improved the levels of blood glucose, HbA 1c, insulin, and HOMR-IR Reduced mRNA expression of SREBP-1c, TNF-α	KK-A^y mice	[39]
	Regulated oxidative and nitrosative stress and reduced the formation of AGEs	Diabetic rats	[40]
Kaempferol	Reduced ROS production and inhibited caspase-3 activation	PC12 cells	[41]
	Reduction IL-1β, TNF-α, IC, and ROS and inhibited neuroimmune activation of microglia	Diabetic mice	[42]
Formononetin	Inhibited islet B cell apoptosis and promoted islet B cell regeneration, insulin secretion, hepatic glycogen synthesis, and hepatic glycolysis	Diabetic mice	[43]
	Controlled hyperglycemia and increased expression of SIRT1 and NGF	Diabetic rats	[44]
	Increased SIRT1 expression and reduced blood glucose	Diabetic rats	[45]

The exact etiology of DPN is still unclear, and its pathogenesis may be related to mitochondrial dysfunction and oxidative stress, polyol pathway activation, advanced glycosylation end products, and endoplasmic reticulum stress due to prolonged and severe hyperglycaemia [46, 47]. In addition, metabolic inflammation, neurotrophic vasculopathy, insulin resistance, and neurotrophic factors are all involved, creating a complex and interrelated pathogenesis [48]. Studies have shown that elevated levels of RAGE expression have been found in skin biopsy specimens from DPN patients [49]. Advanced glycosylation end products (AGEs) are a complex group of compounds. The primary receptor for AGEs (RAGE or AGER), which belongs to the immunoglobulin superfamily, has been described as a pattern recognition receptor [50]. AGE/RAGE signaling causes activation of multiple intracellular signaling pathways involving NADPH oxidase, protein kinase C (PKC), and MAPKs [51] and promotes the expression of multiple proinflammatory cytokines, leading to segmental demyelination of peripheral neurons (Figure 7). The interaction between AGE and RAGE leads to increased diacylglycerol (DAG) synthesis and excessive activation of PKC, which increases NF-κB and tissue-type fibrinogen activator inhibitor-1 (PAI-1) expression, further activating inflammatory factors, transforming growth factor-β (TGF-β), and vascular endothelial growth factor (VEGF), leading to altered vascular function and peripheral neurological microangiopathy [52, 53]. Numerous studies have shown that AGEs bound to their receptors rapidly activate NADPH oxidase, increasing the level of mitochondrial oxidative stress, generating large amounts of ROS, and promoting a signal transduction cascade to induce apoptosis [54, 55]. STAT3 and MAPK pathways are important signaling pathways involved in inflammatory responses, cell proliferation, and apoptosis. STAT3, signal transducer

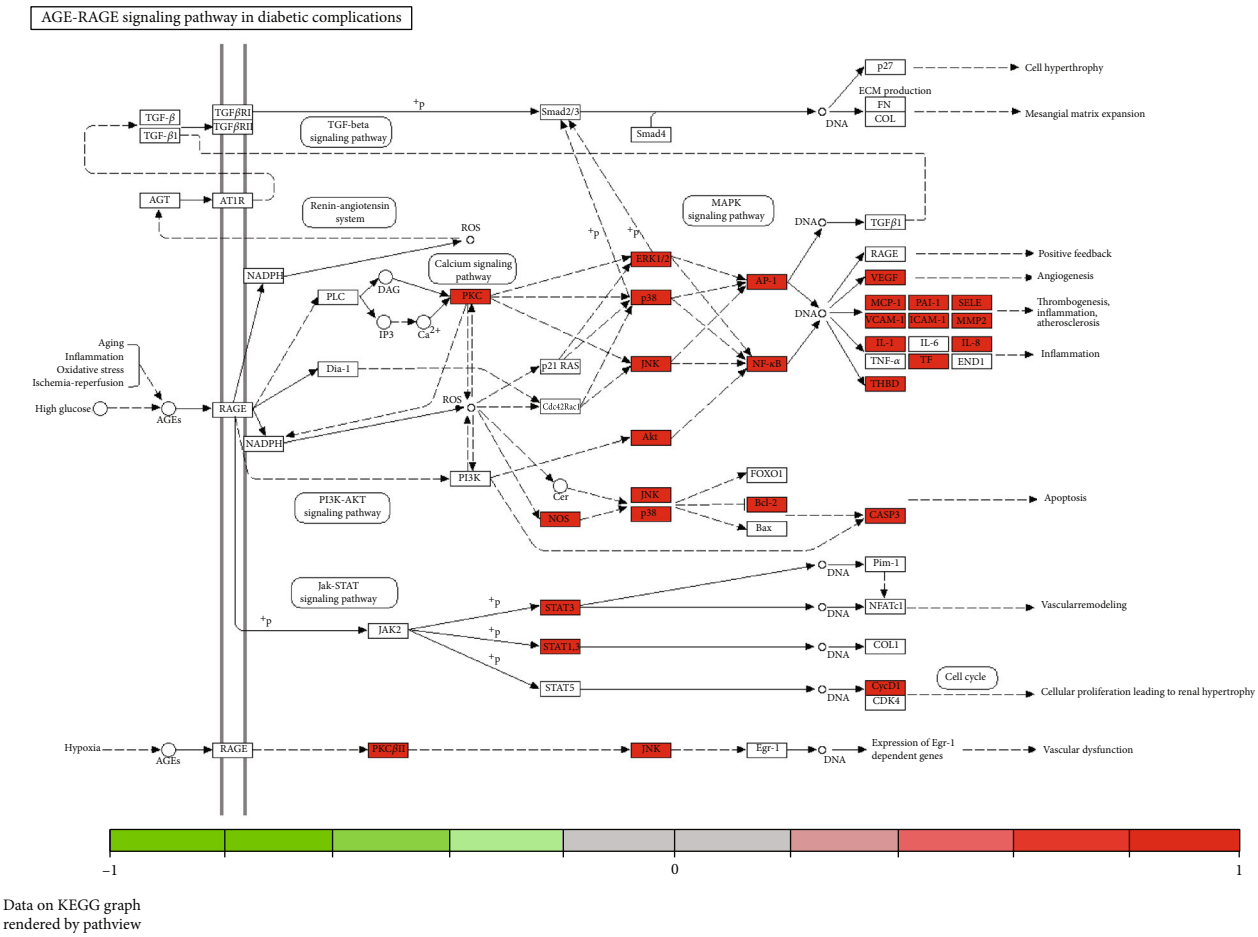


Figure 7: Pathway map of YQHXTLF in the treatment of DPN. AGE/RAGE signaling causes activation of multiple intracellular signaling pathways involving NADPH oxidase, protein kinase C (PKC), and MAPKs and promotes the expression of multiple proinflammatory cytokines, leading to segmental demyelination of peripheral neurons.

and activator of transcription 3, a member of the signal transducer and activator of transcription family, is closely associated with central cell growth, proliferation and survival, and immune response [56]. Activated STAT3 can be implicated in the activity of downstream mediators such as p27kip1, P16INK4A, and p21kip1 proteins, which regulate cell growth, differentiation, and angiogenesis and are involved in the pathogenesis of diabetes [57]. MAPK is a mitogen-activated protein kinase involved in a variety of cellular functions, including cell proliferation, differentiation, and migration [58]. MAPKs cause neuronal apoptosis, impaired neuronal regeneration, and neuropathy through the direct action of glucose and glucose-induced oxidative stress [59]. Tumor necrosis factor-α (TNF-α) is a proinflammatory factor involved in peripheral nerve injury. It stimulates monocytes and endothelial cells to secrete IL-1β and IL-6 and other inflammatory factors, which have toxic effects on neurons and glial cells and lead to demyelination [60]. TNF-α inhibits nitric oxide synthase (NOS) activity in vascular endothelial cells, resulting in reduced NO-induced vasodilation, which leads to endothelial dysfunction and neurotrophic vascular damage and induces neuropathy [61]. In addition, TNF-α can activate the c-jun amino-terminal kinase JNK signaling pathway, leading to apoptosis [62]. TP53, cellular tumor antigen p53, is a stress-sensitive transcription factor responsible for controlling cell survival and death to prevent tumor formation [63]. TP53 has been found to inhibit glycolysis and to be involved in oxidative stress, the TP53 gene polymorphism marker Pro72Arg has been associated with DPN pathogenesis [64], and TP53 serum levels are significantly increased in patients with T2DM [65], so it is hypothesized that TP53 may play an important role in metabolic diseases such as diabetes. The molecular docking results showed that the active ingredients of the key compounds in YQHXTLF were able to bind stably to TP53, MAPK1, JUN, and STAT3. Thus, these results also confirm that our screened targets are consistent with literature reports, suggesting that YQHXTLF can play a therapeutic role in DPN by regulating apoptosis or proliferation and mediating inflammatory responses or oxidative stress.

5. Conclusion

In summary, this study analyzed the potential molecular biological mechanisms of YQHXTLF in the treatment of DPN throughnetwork pharmacology and molecular docking

approach. The results showed that the active components of YQHXTLF in DPN treatment were composed of 81 compounds, among which quercetin, luteolin, kaempferol, and formononetin were the important active components. Moreover, a total of 110 target genes were screened, among which TP53, MAPK1, JUN, and STAT3 are possible core targets. The pathways involved in the treatment of DPN by YQHXTLF may be related to the AGE-RAGE signaling pathway, TNF signaling pathway, and MAPK signaling pathway, reflecting the multicomponent, multitarget, and multipathway biological properties of YQHXTLF. However, the network pharmacology is only a reasonable prediction of the mechanism of action of the herbal compound for the treatment of DPN based on the data mining perspective, which can provide a reference for the study of its therapeutic mechanism. We will be followed by animal and clinical experiments to validate the screened targets based on this prediction analysis, to provide more scientific evidence for its clinical application.

Data Availability

We have presented all our main data in the form of figures and additional files. The data used to support the conclusions of this study are available from the authors.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

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Supplementary Materials

Supplementary 1. Ingredients: potential active ingredients and action targets of the herbal compound were obtained through screening.

Supplementary 2. Diseases: DPN-related targets were obtained.

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Research Article

Designing a Logistic Regression Model for a Dataset to Predict Diabetic Foot Ulcer in Diabetic Patients: High-Density Lipoprotein (HDL) Cholesterol Was the Negative Predictor

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Objectives. Although the risk factors for diabetic neuropathy and diabetic foot ulcer have been detected, there was no practical modeling for their prediction. We aimed to design a logistic regression model on an Iranian dataset to predict the probability of experiencing diabetic foot ulcers up to a considered age in diabetic patients. *Methods*. The present study was a statistical modeling on a previously published dataset. The covariates were sex, age, body mass index (BMI), fasting blood sugar (FBS), hemoglobin A1C (HbA1C), low-density lipoprotein (LDL), high-density lipoprotein (HDL), triglyceride (TG), insulin dependency, and statin use. The final model of logistic regression was designed through a manual stepwise method. To study the performance of the model, an area under receiver operating characteristic (AUC) curve was reported. A scoring system was defined according to the *beta* coefficients to be used in logistic function for calculation of the probability. *Results*. The pretest probability for the outcome was 30.83%. The final model consisted of age ($\beta 1 = 0.133$), BMI ($\beta 2 = 0.194$), FBS ($\beta 3 = 0.011$), HDL ($\beta 4 = -0.118$), and insulin dependency ($\beta 5 = 0.986$) ($P < 0.1$). The performance of the model was definitely acceptable (AUC = 0.914). *Conclusion*. This model can be used clinically for consulting the patients. The only negative predictor of the risk is HDL cholesterol. Keeping the HDL level more than 50 (mg/dl) is strongly suggested. Logistic regression modeling is a simple and practical method to be used in the clinic.

1. Introduction

Diabetes mellitus (DM) is one of the major causes of morbidity and mortality in the world characterized by a rising blood glucose level. The prevalence of DM is estimated to be increased by 2050 [1]. This increasing prevalence is an alarm, because it imposes a high burden on the surveillance about DM complications. DM has many macrovascular and microvascular complications. A high glucose level results in both its conversion to sorbitol *via* the polyol pathway and formation of advanced glycosylated end products (AGE). The microvascular complications are attributed to many pathophysiological mechanisms that one of which is sorbitol accumulation in cells [2, 3]. Diabetic neuropathy is one of the microvascular complications of DM and is one of the most important causes of diabetic foot ulcers (other than the neuropathic ulcers, the ulcers can also be ischemic) [4].

A diabetic foot ulcer is a severe complication of DM that consists of damage to deep tissues of usually lower limbs along with neurological and peripheral vascular injures. Its incidence is globally increasing due to the increased prevalence of DM and increased life expectancy of DM patients.

Table 1: Logistic regression modeling for prediction of diabetic foot ulcer.

Predictor (unit)	*Beta* coefficient (*P* value)		
	Step 1	Step 2	Step 3
Sex (male)	0.314 (0.582)		
Age (year)	0.146 (<0.001*)	0.136 (<0.001*)	0.133 (<0.001*)
BMI (kg/m^2)	0.229 (0.003*)	0.206 (0.002*)	0.194 (0.004*)
FBS (mg/dl)	0.014 (0.026*)	0.010 (0.026*)	0.011 (0.015*)
HbA1C (%)	0.010 (0.641)		
LDL (mg/dl)	-0.174 (0.174)		
HDL (mg/dl)	-0.124 (0.012*)	-0.124 (0.007*)	-0.118 (0.010*)
TG (mg/dl)	-0.323 (0.323)		
Insulin dependency (yes)	1.285 (0.038*)	1.217 (0.039*)	0.986 (0.074*)
Statin use (yes)	-1.181 (0.092*)	-0.929 (0.166)	
Model properties and performance			
Constant (β0)	-13.692	-12.497	-12.987
Number cases	133	133	133
Pseudo-*R* square	0.488	0.465	0.453
AIC	106.09	101.93	101.87
BIC	137.88	122.16	119.21
AUC	0.924	0.920	0.914

*$P < 0.1$. AIC: Akaike information criterion; BIC: Bayesian information criterion.

Therefore, it has a high global burden. Its lifetime prevalence is about 25% in DM patients, and it is estimated that one lower extremity is amputated due to DM every 30 seconds worldwide. In addition, its financial burden is of great importance. The average annual expenditure of diabetic foot ulcers is more than $8000 US per patient [5].

There are many risk factors and protecting factors for diabetic foot ulcers. A population-based cohort study showed that the risk factors were history of diabetic foot ulcer or amputation, insulin usage, gender, distal neuropathy, and foot deformity [6]. Researches were used to find the predictors of diabetic foot ulcers based on the risk and protecting factors. The attentions were to the commonly available clinical information [7].

Although the risk factors for diabetic neuropathy and diabetic foot ulcers have been detected, there was no simple practical modeling with a finally given formula for prediction. Hereby, we intend to perform a secondary analysis using binary logistic regression on an Iranian dataset to predict the probability of experiencing diabetic foot ulcers up to a specific age in diabetic patients.

2. Material and Methods

The present study was a statistical modeling on a previously published dataset in Lur and Lak populations of Iran. The dataset had been collected according to ethical guidelines, and it had an ethical registration number [8]. No further ethical registration was needed for this secondary analysis. The outcome variable was history of diabetic foot ulcers (as a binary variable). The covariates were sex, age, body mass index (BMI), fasting blood sugar (FBS), hemoglobin A1C (HbA1C), low-density lipoprotein (LDL), high-density lipoprotein (HDL), triglyceride (TG), insulin dependency, and statin use.

Stata 14 (StataCorp LLC, Texas, US) software was used for statistical modeling. To perform logistic regression, *-logit-* command was used and adjusted *beta* coefficients were reported. The model was repeated in 3 steps; step 1, all the covariates were imported to the model; step 2, the covariates with *P* value less than 0.1 in step 1 were imported to the model; and step 3, the covariates with *P* value less than 0.1 in step 2 were imported to the model. Then, the covariates of step 3 were considered as the final modeling. To study the performance of the model, the area under receiver operating characteristic (ROC) curve (AUC) was reported using *-lroc-* postestimation command. In addition, *-lsens-* postestimation command was used to report sensitivity and specificity at each cutoff point of the probability. A scoring system was defined using *-generate-* command according to the *beta* coefficients of step 3 of the model (equation (1)). Then, the scoring system was used in a univariable model to predict the outcome. This scoring system was imported to the sigmoid (logistic) function to predict the exact probability of the outcome variable (equation (2)). Marginal analysis on the sigmoid function was performed using *-margins*, *at* (score = (−8(1)5)) *plot-* postestimation command.

$$y = \beta 1.x1 + \beta 2.x2 + \beta 3.x3 + \cdots + \beta 0 = \text{score}, \quad (1)$$

$$y = \frac{\exp\,(\text{score})}{1 + \exp\,(\text{score})} = \text{probability}. \quad (2)$$

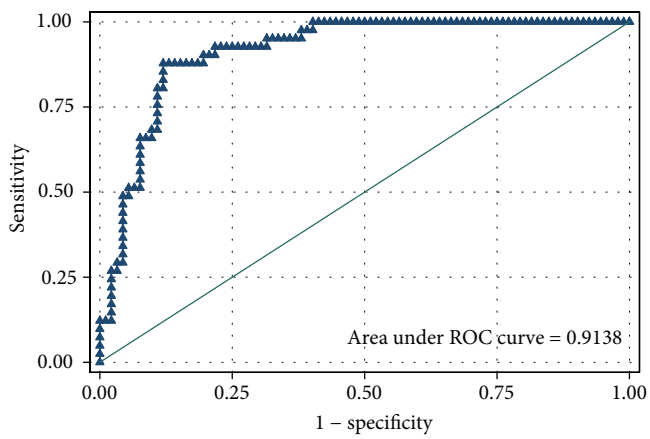

Figure 1: Postestimation ROC curve for the final step of the model (the unit of the predictor is the scoring system).

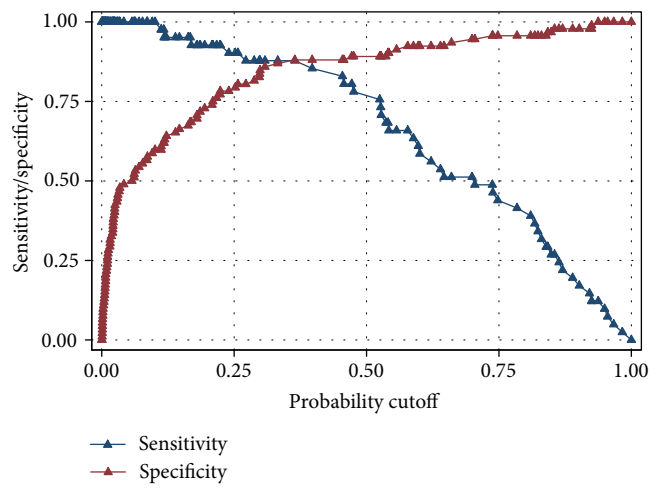

Figure 2: Postestimation sensitivity/specificity plot for the final step of the model (the unit of the predictor is the scoring system).

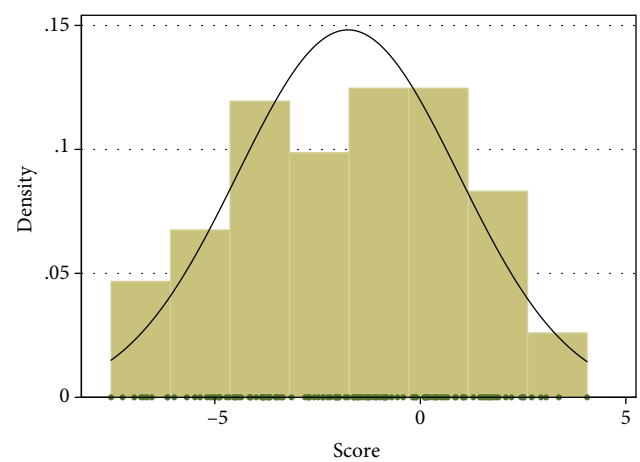

Figure 3: Distribution of diabetic foot ulcer predicting score in the studied cases. Mean = −1.759, standard deviation = 2.692, minimum = −7.525, maximum = 4.057.

3. Results

The pretest probability for the outcome was 30.83% in the samples of the present study. Logistic regression was performed in three steps. Among the covariates, sex, HbA1C, LDL, and TG could not pass step 1 ($P > 0.1$). Then, the covariate statin use could not pass step 2 ($P > 0.1$). The final model consisted of age ($\beta 1 = 0.133$), BMI ($\beta 2 = 0.194$), FBS ($\beta 3 = 0.011$), HDL ($\beta 4 = -0.118$), and insulin dependency ($\beta 5 = 0.986$) ($P < 0.1$). The performance of the model was definitely acceptable (AUC = 0.914) with an acceptable preservation of sensitivity at higher cutoff points of the probability (Table 1, Figures 1 and 2).

The final formula for the scoring system is shown (equation (3)). Distribution of the score for the samples of the study is also shown (Figure 3). The score of each diabetic patient should be replaced in logistic function (equation (2)) for prediction of experiencing diabetic foot ulcers up to a specific age. We imagined 10 examples for prediction of the probability (Table 2). The graph of this marginal prediction based on logistic function is shown (Figure 4). The number needed to treat (NNT) was calculated for improving HDL from 40 (mg/dl) to 50 (mg/dl) (equation (4)) for the 10 mentioned examples (Table 2). For instance, in a 70-year-old insulin-dependent patient with BMI = 30 (kg/m^2) and FBS = 160 (mg/dl), at HDL = 40 (mg/dl), the probability of the outcome is 54.0% whereas at HDL = 50 (mg/dl), the probability of the outcome is 26.3% (NNT = 4):

$$\begin{aligned} y = {} & 0.1331305 \times \text{age} + 0.1944625 \times \text{BMI} + 0.0108864 \times \text{FBS} \\ & + (-0.1184257) \times \text{HDL} + 0.9855977 \times \text{insulin (0 or 1)} \\ & + (-12.98725), \end{aligned} \tag{3}$$

$$\text{NNT} = \frac{1}{\text{Absolute Risk Reduction}} = \frac{1}{P(\text{at HDL1}) - P(\text{at HDL2})}. \tag{4}$$

4. Discussion

This study was aimed at estimating the probability of experiencing diabetic foot ulcers at least for one time from the time of diagnosing DM up to the considered age. Accordingly, age, BMI, FBS, and insulin dependency were positive predictors while HDL was a negative predictor. This model seemed to be accurate enough, and since the P values of the final model were low, this model seemed to be repeatable for prospective use. Generally, in each regression model, reducing the number of covariates results in reduction of goodness of fit criteria such as R squared, pseudo-R squared, and AUC. Nevertheless, it is inevitable to remove covariates with high P values because of making nuisance in the model. In other words, although a chock-a-block with covariates model is more accurate for retrospective prediction of a currently studied sample, this model will not be repeatable for prospective prediction in another sample of population.

Table 2: Examples of diabetic foot ulcer prediction.

Example	Age	BMI	FBS	Insulin dependence	Probability (%) if HDL = 40	HDL = 45	HDL = 50	NNT (HDL 40➔50)
1	50	25	140	0	1.0	0.5	0.2	157
2	50	25	140	1	2.4	1.3	0.8	60
3	70	25	140	0	11.7	6.8	3.9	13
4	70	25	140	1	26.2	16.4	9.8	6
5	50	30	140	0	2.4	1.3	0.7	61
6	50	30	140	1	6.2	3.5	2.0	24
7	70	30	140	0	26.0	16.3	9.7	6
8	70	30	140	1	48.5	34.2	22.3	4
9	70	30	160	0	30.3	19.4	11.8	5
10	70	30	160	1	54.0	39.2	26.3	4

Examples 1 and 2 indicate a middle-aged patient; examples 3 and 4 indicate an old-aged patient; examples 5 and 6 indicate a middle-aged patient with a high BMI; examples 7 and 8 indicate an old-aged patient with a high BMI; and examples 9 and 10 indicate an old-aged patient with a high BMI and a high FBS. The amounts of NNT have been rounded and calculated based on the exact amounts of probability.

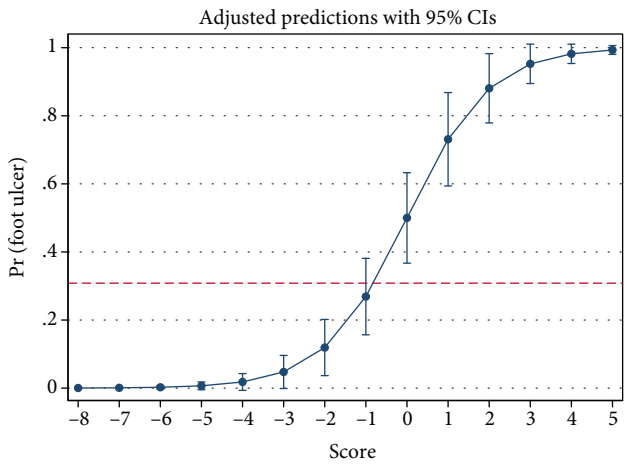

Figure 4: Marginal analysis shows the probability of diabetic foot ulcer at each score. The reference line shows the prevalence of diabetic foot ulcers in the studied samples 30.83% (pretest probability).

Before us, two other studies had used this dataset of ours for statistical modeling. Alfian et al. designed a deep neural network to predict diabetic retinopathy. Their model showed better performance than the previous modelings with accuracy of 82.03% [9]. Reddy et al. designed a neural network to predict diabetic neuropathy. Their aim was to compare different modeling methods [10].

Previously, there were not enough studies that modeled the predictors of diabetic foot ulcers. Boyko et al. tried to design a model using commonly available clinical information. They used Cox regression modeling. The advantages of their study in comparison to the study of ours were the cohort approach, higher sample size, and access to the time to events. Nevertheless, the disadvantages of their study in comparison to the study of ours were more complexity of the model, lack of studying lipid profile, lack of finding a protecting factor, and lack of reporting a final practical formula. The performance of our model was better according to the AUCs [7].

In many studies, low HDL and high TG were associated with increased diabetic peripheral neuropathy while LDL did not show any association. Smith et al. showed that there was an association between low HDL and elevated triglycerides with diabetic neuropathy. They aimed to determine whether the characteristics of metabolic syndrome other than hyperglycemia increased the risk of diabetic neuropathy [11]. Tesfaye et al., with the aim of investigating the risk factors for neuropathic modification, showed that the incidence of diabetic neuropathy was associated with high TG levels in addition to blood sugar [12]. A study conducted by Pai et al. was aimed at investigating the risk factors for peripheral neuropathy in patients with type 2 DM. They concluded that low levels of HDL increased the risk of diabetic peripheral neuropathy [13]. Rosales-Hernandez et al. after examining oxidized LDL (OxLDL) in diabetic peripheral neuropathy concluded that there was no association between its level and occurring peripheral neuropathy [14].

In contrast to the studies with positive results, Zhu et al. showed that the number of monocytes and HDL levels were similar between healthy individuals and patients with type 2 diabetes with or without diabetic peripheral neuropathy [15]. Interestingly, Li et al. in a study in China aimed at investigating the incidence of amputation in patients with diabetic foot ulcers and risk factors for amputation showed that low levels of TG were an independent risk factor for lower limb amputation in patients with diabetic foot ulcers [16].

Our study supported the results of previous studies for susceptibility to diabetic foot ulcers. Ikura et al. examined this issue of whether HDL levels predict the incidence of lower limb amputation and wound-related death in patients with diabetic foot ulcers or not. They concluded that low HDL levels in patients with diabetic foot ulcers were associated with the incidence of minor and major extremity amputation or wound-related death. But triglyceride and LDL levels did not predict them [17]. Pei et al. in a meta-analysis aimed to investigate the effect of lipids and lipoproteins on diabetic

foot ulcer risk in patients with type 2 DM. They showed that decreased HDL was an associated factor [18]. Dai et al. conducted a study to investigate the relationship between vitamin D and risk of diabetic foot ulcer in patients with type 2 DM. The results of that study showed that low serum 25-OH-vitamin D levels were associated with the risk of diabetic foot ulcer. Although vitamin D levels showed higher diagnostic accuracy, the protective effect of HDL was greater based on logistic regression after adjusting the *beta* coefficients. The protective effects of HDL might be due to its anti-inflammatory effects on immune cells [19].

A high-fat diet results in hyperlipidemia. Cholesterol and other substances of lipid metabolism accumulate in neurons. The deposition of these substances causes oxidative stress, followed by increased expression of proinflammatory cytokines and neuronal apoptosis. An animal study showed that dyslipidemia was an independent risk factor for the development of diabetic neuropathy [20].

The strength of this study was achieving an acceptable performance (AUC > 0.90) and an acceptable goodness of fit (McFadden pseudo-$R^2 > 0.40$) to be used in clinics. It seems that it was the first time that a practical formula for direct calculation of probability was reported for the prediction of diabetic foot ulcers. However, the study had some limitations. The most important one was lack of access to the time of event for this complication, and therefore, we did not consider time to event and could not perform Cox regression.

5. Conclusion

This model can be used clinically for consulting and managing diabetic patients who are at risk for diabetic foot ulcers. Among the predictors, age is not changeable and insulin dependency is usually inevitable; however, BMI and FBS can be controlled. The only negative predictor of the risk is HDL cholesterol. Keeping the HDL level more than 50 (mg/dl) is strongly suggested. Although statin use was not a significant predictor for diabetic foot ulcers, it should be regarded that its administration might be necessary for other indications and it can ameliorate lipid profile of the patients. Logistic regression modeling is a method in machine learning and data mining, but nevertheless, it is very practical and easy to interpret and use in daily clinic.

Data Availability

The raw data are available from https://data.mendeley.com/datasets/k62fdsnwkg/1.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

All the authors participated in design and conceptualization and drafting or editing. Specifically, SAYA performed statistical analysis, NM collected the samples for the main data source and prepared data for the current study, and MJ was the supervisor and biochemistry consultant. All the authors approved the manuscript.

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