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# Advances in the diagnosis and prevention of diabetic neuropathy

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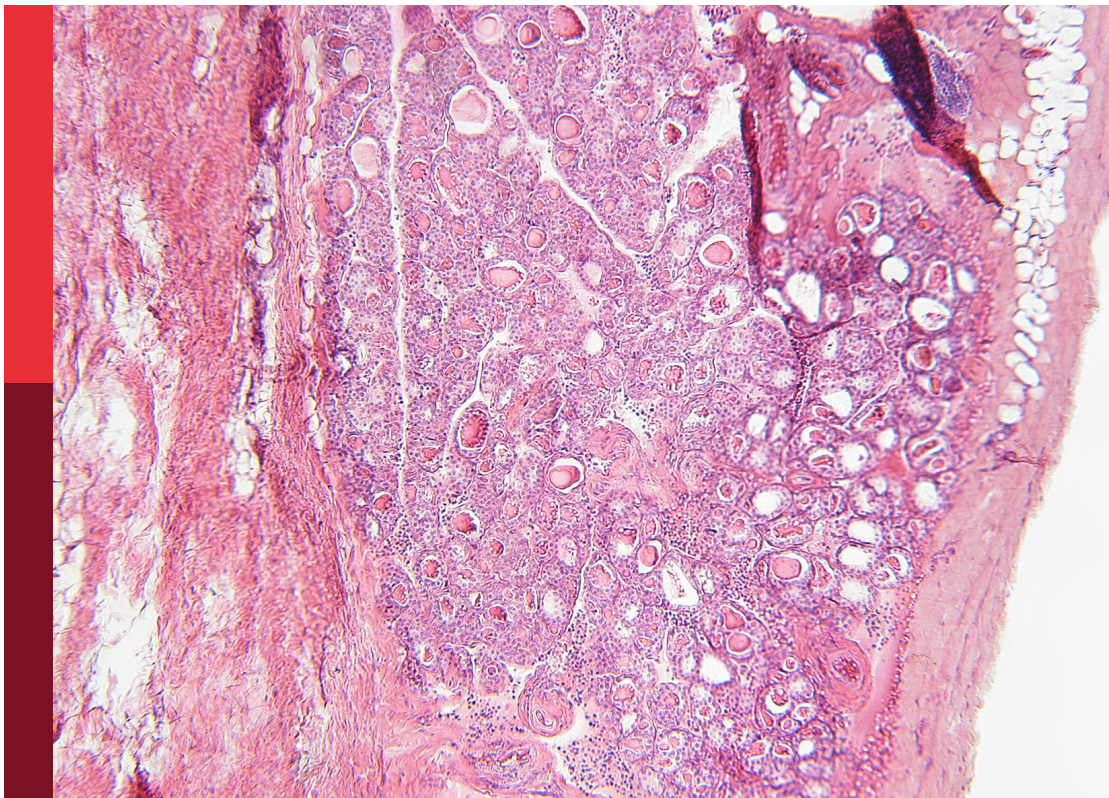

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# Advances in the diagnosis and prevention of diabetic neuropathy

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# Editorial: Advances in the diagnosis and prevention of diabetic neuropathy

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KEYWORDS

**Diabetic peripheral neuropathy, diagnosis, risk factor, biomarkers, recent advances**

Editorial on the Research Topic
Advances in the diagnosis and prevention of diabetic neuropathy

Diabetic Neuropathy (DN) is a common complication of type 1 and type 2 diabetes. Diabetic peripheral neuropathy (DPN) and cardiac autonomic neuropathy (CAN) lead to devastating complications like foot ulcers, lower limb amputations, major cardiovascular events, and sudden deaths (1). It is estimated that up to 50% of people with diabetes may be affected by some type of neuropathy making it a significant cause of disability and reduction in quality of life (2). Complications related to both DPN, and CAN are preventable if detected early and risk factors are optimally controlled through multidisciplinary care. However, due to the asymptomatic early stages of the disease progression, diagnosis is often a challenge. The issues are compounded by the threat of progression of disease to an irreversible stage if detection is delayed. This Research Topic collection aims to address these challenges by featuring and discussing the latest advances in diagnostic approaches to DN. In this collection, we have compiled 14 research articles from those submitted to the call for papers on "*Advances in the Diagnosis and Prevention of Diabetic Neuropathy*" topic and those received through regular submission route. The final collection of articles includes topics that summarize and evaluate the latest technologies in screening, diagnosis, and prediction of DPN.

Article type wise, the 14 research articles published under this collection include 2 Narrative Review, 1 Systematic Review and Meta-analysis, 1 Case Report, and 10 Original Research articles. These articles encompass four key areas related to recent advances in DPN:

1. Screening and diagnostic tests
2. Risk factors associated with DPN and prediction models for early detection of DPN
3. Potential biomarkers for diagnosing DPN
4. Novel techniques exploring mechanisms underlying DPN

## Screening and diagnostic tests

A narrative review by Yu gives an overview of the current screening technologies for detecting early DPN and summarises their advantages, disadvantages, and potential clinical application. The technologies assessed include quantitative sensory measurement, neurological function scoring system, corneal confocal microscopy (CCM), and high-frequency ultrasound. Carmichael et al. provide a comprehensive review of current knowledge and optimal approaches for screening and diagnosing DPN. Authors highlighted the limitations of the monofilament test, a commonly used screening test in primary care in terms of its inability to detect early-stage disease and high variability in sensitivity ranging from 43-93% compared to the gold standard, nerve conduction studies (NCS) for detecting late-stage DPN. They also assessed the performance of screening and diagnostic test currently available for early detection of DPN including the potential for several novel point of care devices (POCDs) such as Neuropad, DPNCheck and Sudoscan. Authors further highlighted the potential for CCM, a rapid non-invasive clinical assessment of corneal nerves in detecting small fibre neuropathy, the earliest manifestation of DPN and to assess its severity. Authors suggested that advancements in automated analysis softwares may improve clinical utility by overcoming the difficulty in manual analysis and technical expertise to quantify nerve pathology. Carmichael et al. also reported the results of a feasibility study using CCM to screen for DPN in patients with type 1 or type 2 diabetes attending primary care optometry settings in UK. Their study findings supported the current literature that CCM is a sensitive surrogate biomarker for DPN.

## Risk factors

Christensen et al. evaluated the association of glycemic variability (GV) assessed by means of continuous glucose monitoring (CGM) generated parameters and showed that GV was not associated with neither DPN nor CAN in a cohort of 133 young Danish adults with type 1 diabetes. Al-Saoudi et al. conducted a cross-sectional study assessing the association of advanced glycation end-products (AGEs) with CAN and distal symmetric polyneuropathy (DSPN) in 151 young adults with type 1 diabetes in Denmark. They concluded that higher levels of AGEs were associated with several measures of CAN and DSPN through diverse metabolic pathways including glycolytic dysfunction, lipid peroxidation and glucotoxicity. Liu et al. conducted a study comparing the prevalence and risk factors associated with DPN, peripheral artery disease (PAD) and foot deformity which are the common causes of diabetic foot using a large cohort of 3898 patients with diabetes from 11 hospitals in Beijing, China. They found the prevalence of foot deformities including callus to be higher (29.7%), followed by DPN (23.5%) while the prevalence of PAD to be lower with 11.6%. They found risk factors including higher systolic blood pressure, underweight, poor glycemic control, longer duration of diabetes, chronic kidney disease and cerebrovascular disease were commonly associated with 2 or more of the three conditions. Wang et al. developed and validated a nomogram model for predicting those at higher risk of developing diabetic foot using data collected from 1950 patients with type 2 diabetes attending a university hospital in China. The model based on traditional risk factors such as age, HbA1c, total and low-density lipoprotein cholesterol, smoking and drinking had good accuracy with area under the receive operating characteristic curve (AUC) of 0.857 in internal validation.

## Novel biomarkers

Three clinic-based studies reported cross-sectional association between novel biomarkers and DPN. Sun et al. reported higher levels of serum adiponectin to be positively associated with DPN independent of potential confounders in 219 Chinese type 2 diabetic patients aged 40-79 years. Jende et al. reported higher levels of troponin T (hsTNT) to be negatively correlated with markers of magnetic resonance peripheral nerve perfusion in a small case-control sample with and without diabetes suggesting that hsTNT may serve as a potential marker for assessing nerve perfusion in future studies of DN. Zhuang et al. demonstrated that plasma increased plasma levels of D-dimer to be independently associated with DPN in 393 patients with type 2 diabetes. Dong et al. performed a systematic review and meta-analysis of six studies that evaluated the performance of shear wave elastography (SWE) for tibial nerve stiffness as a quantitative biomarker complementary to neuroelectrophysiological examination for diagnosing DPN. They reported that SWE had good diagnostic accuracy for detecting DPN with summary sensitivity, and specificity of 75%, and 86% and area under the receiver operating characteristic curve (AUROC) of 0.86.

## Mechanistic studies

Chen et al. conducted a diffusion tensor imaging study to evaluate the differences in central neural mechanisms underlying erectile dysfunction due to type 2 diabetes (DM-ED) vs. psychological erectile dysfunction (pED) using the method of network-based statistic. Authors demonstrated differences in

structural connectivity patterns between the two types with increased connectivity in the fronto-parietal network in those with ED due to diabetes. Yang et al. compared the material properties (primary thickness, peak strain, peak stress, stiffness, viscous modulus etc. before and after continuous weight bearing) of heel pad in those with and without diabetes and investigated the impact of compressive loading history and the length of diabetes course on the material properties of heel pad. Authors demonstrated that patients with diabetes had altered material properties which may contribute to the vulnerability of heel pad to injury and ulceration.

Peng et al. presented a Case Report describing the clinical and genetic data of a Chinese patient with Werner syndrome with diabetic foot disease and myelodysplastic syndrome. They reported a novel pathogenic variation in the WRN gene in this patient with Werner syndrome.

In conclusion, this Research Topic make important contributions to our understanding of the recent advances in screening and diagnosis of DPN and may be of interest to a wider readership from the fields of Endocrinology, Neurology and General Medicine. Yet there remain important challenges in the prevention and diagnosis of DPN which future research studies could address, for e.g., novel technologies and biomarkers reported in this collection need further confirmation in large prospectively collected data, validation in external cohorts as well as in real-world settings. As suggested by Carmichael et al. such evaluation may also benefit more from fostering international collaborations rather than from the fragmented efforts of small, opportunistic studies.

## Author contributions

The author confirms being the sole contributor of this work and has approved it for publication.

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I would like to thank all authors for their contribution to this topic collection, the reviewers for ensuring the quality of accepted papers and the Editorial Team of Frontiers in Endocrinology for managing this Research Topic.

## Conflict of interest

The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Publisher's note



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ORIGINAL RESEARCH
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# Glycemic Variability and Diabetic Neuropathy in Young Adults With Type 1 Diabetes

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**Background:** Glycemic variability (GV) may attribute to the pathogenesis of diabetic neuropathy. The aim of this cross-sectional study was to investigate the association between GV and distal symmetric polyneuropathy (DSPN) and cardiovascular autonomic neuropathy (CAN) in a Danish population of young adults with type 1 diabetes.

**Methods:** Young adults between 18 and 24 years with type 1 diabetes were included in this cross-sectional study. CAN was assessed by cardiovascular autonomic reflex tests (CARTs) and heart rate variability (HRV). DSPN was assessed by light pressure, pain and vibration perception, electrochemical skin conductance, sural nerve conduction velocity (SNCV), and amplitude potential (SNAP). GV were obtained by continuous glucose monitoring including coefficient of variation (CV), SD, continuous overall net glycemic action (CONGA), and mean amplitude of glucose excursions (MAGE).

**Results:** The study comprised 133 young adults (43.6% males), mean age of 22 years (SD 1.6). Unadjusted, higher CV was associated with a decreased risk of sural nerve conduction ($P = 0.03$), abnormal SNAP ($P = 0.04$) and incidents of definite CAN ($P = 0.04$). Likewise, higher CONGA was associated with increasing incidents of subclinical DSPN ($P = 0.03$), abnormal SNAP ($P = 0.01$), and SNCV ($P = 0.02$). However, both associations were not statistically significant in the fully adjusted model. Higher MAGE was associated with slightly increasing measures of HRV ($P = 0.03$) but only when fully adjusted. When correcting for multiple tests significance was lost. A significant association was found between HbA1c and measures of both DSPN ($P < 0.02$) and HRV ($P < 0.03$) in fully adjusted models.

**Conclusions:** No significant associations between GV and diabetic neuropathy were found after adjusting for risk factors and multiple tests. This suggests that GV may not be a risk factor for diabetic neuropathy in young adults with type 1 diabetes. However, long-term effects of GV excursions may still play a role in the pathogenic mechanisms leading to neuropathy in later life.

**Keywords: type 1 diabetes, glycemic variability, young adults, cardiovascular autonomic neuropathy, distal symmetric polyneuropathy, continuous glucose monitoring**

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## INTRODUCTION

Distal symmetric polyneuropathy (DSPN) and cardiovascular autonomic neuropathy (CAN) are severe and common complications of type 1 diabetes (1, 2). DSPN and CAN are usually rare problems during childhood but may be present in adolescents and young adults (3, 4). Improved glycemic control in patients with type 1 diabetes may prevent and revert early stages of DSPN and CAN and slow the progression toward overt neuropathy (5–9). Thus, detecting and preventing diabetic neuropathy at an early stage is essential.

The Diabetes Control and Complications Trial (DCCT) (10) demonstrated that intensive glycemic control in type 1 diabetes monitored by HbA1c reduced the onset and progression of diabetic neuropathy. Glycated hemoglobin (HbA1c) is an integrated assessment marker of glycaemia in diabetes treatment (11) and is associated with increased risk of diabetic complications like neuropathy. However, some limitations arise when using measures of $HbA_{1c}$ to evaluate the role of glucose variability (GV): $HbA_{1c}$ depicts an average of blood glucose over 3 months and does not reflect incidents of hypo- and hyperglycemia on a daily basis (12). Hence $HbA_{1c}$ may be an insufficient tool to monitor and treat dysglycaemia. Diurnal GV may contribute to the risk of diabetic complications beyond HbA1c (13). The international consensus panel from the Advanced Technologies & Treatments for Diabetes Congress in February 2017 recommends using data from continuous glucose monitoring (CGM) and the coefficient of variation (CV) as the primary measure to assess GV.

Data on the association between GV and diabetic neuropathy is inconsistent, scarce, and has not previously been investigated in young adults with type 1 diabetes by applying CGM (14–19). Several studies point to glucose fluctuation as a risk factor for CAN in adults with type 1 diabetes (15–17). However, studies on the association between GV and peripheral neuropathy are conflicting (14, 15), possibly due to inconsistent use of various measures and definitions to assess GV and neuropathy in different studies. While large GV has been associated to oxidative stress, GV may attribute to the pathogenesis of diabetic neuropathy despite conflicting reports (13, 20). Studies on the association between GV and CAN and DSPN in type 2 diabetes are limited. However, they do demonstrate that variability of HbA1c in particular but also measures of GV assessed from CGM are associated with both CAN and DSPN (21–25).

**Abbreviations:** BPI, Brief Pain Inventory; CAN, cardiovascular autonomic neuropathy; CARTs, cardiovascular autonomic reflex tests; CONGA, continuous overall net glycemic action; CSII, continuous subcutaneous insulin infusion; CV, coefficient of variation; DSPN, diabetic symmetric polyneuropathy; E:I, deep breathing test; ESC, electrochemical skin conductance; HF, high frequency; HRV, heart rate variability; IQR, interquartile range; LF, low frequency; LF/HF, ratio low-frequency power/high-frequency power ratio; MAGE, mean amplitude of glucose excursions; MDI, multiple dose injections; MNSI, Michigan Neuropathy Screening Instrument; RMSSD, root mean square of the sum of the squares of differences between consecutive R–R intervals; SDNN, standard deviation of normal-to-normal intervals; SNAP, sural nerve amplitude potential; SNCV, sural nerve conduction velocity; VM, Valsalva Maneuver; VPT, vibration perception threshold; 30:15, lying-to-standing test.

The aim of this study was to investigate the association between modifiable glycaemic risk factors of neuropathy including GV and early and possibly reversible signs of DSPN and CAN early in the life of type 1 diabetes where preventive measures may have a substantial effect later in life. This was done in a Danish population of young adults with type 1 diabetes using the newest recommendations for assessing GV and both novel and established measures for DSPN and CAN.

## MATERIALS AND METHODS

### Study Population

The study was designed as a cross-sectional observational study. The structure has been described in detail previously (4).

In order to investigate a population with early signs of diabetic neuropathy a cohort of young adults was assessed. All participants (age between 18 and 24 years) were recruited from the outpatient clinic at Steno Diabetes Center Copenhagen, Gentofte, Denmark. Participants were included regardless of duration of type 1 diabetes. Three hundred and fifty participants received a written invitation to participate and were subsequently contacted by phone. Written informed consent was obtained prior to examination. Ethical approval was obtained from The Danish Research Ethics Committee (project id.: H-15006967) (26).

Participants were excluded from the present study if they were not able to wear the CGM or failed to measure and log their capillary blood glucose during the 5-days of CGM-monitoring. Moreover, examination of CAN was not performed if they were treated with beta blockers.

There was no basis for conducting sensible power calculations to estimate a sample size for the aim of the study, because the investigated associations have not previously been investigated in young adults with type 1 diabetes with the use of CGM. Moreover, previous studies in other patient groups have demonstrated conflicting results.

### Assessment of Diabetic Neuropathy

DSPN was assessed and categorized according to recommendations made by the Toronto Diabetic Neuropathy Expert Group (2). Questionnaires were used to assess symptoms of DSPN (see section "Questionnaires on peripheral neuropathy and exposures" for further details).

Signs of DSPN were evaluated by established measures: Light pressure perception was assessed by applying a 10-g monofilament until it buckled (Neuropen®, Owen Mumford Ltd, Oxford, UK) to three points at the distal bilateral foot pads: just proximal to the great, third and fifth toe (27, 28). Pain sensation was evaluated by using a 40-gram pin prick device (Neuropen®, Owen Mumford Ltd, Oxford, UK) applied at the dorsal side of the toes just proximal to the nail on the great, third, and fifth toe. Vibration perception threshold (VPT) was determined using a Bio-Thesiometer (Bio-medical instruments, Ohio, USA) at the distal end of the great toe on both feet, and age stratified perception thresholds were used to assess abnormal results (29).

Novel measures were used to objectively evaluate DSPN: Small autonomic fiber function was assessed using the non-invasive device Sudoscan™ (Impeto Medical, Paris, France) performing an electrochemical skin conductance (ESC) test on the hands and feet (30). Age and gender stratified ESC thresholds were applied (31). Sural nerve conduction velocity (SNCV) and amplitude potential (SNAP) were obtained by the handheld NC-Stat® DPNCheck™ (NeuroMetrix, Inc., Waltham, USA) (32). Age and height stratified SNAP and SNCV thresholds were used (33). Participants were examined for bilateral abnormalities in SNAP and SNCV. A composite measure, "sural nerve conduction" (SNC), was used when abnormalities in either SNAP, SNCV or both bilaterally. DSPN was defined according to four categories. The label "possible DSPN" was given if presence of symptoms of peripheral neuropathy as assessed by questionnaires, or signs as assessed by VPT, light pressure and pain perception were confirmed. If presence of symptoms and signs were confirmed the label "probable DSPN" was added. "Confirmed DSPN" was given if either the test for SNC or ESC was abnormal and if the participants had symptoms or signs. Ultimately, "subclinical DSPN" was defined as presence of abnormal SNC or ESC without symptoms or signs.

To evaluate CAN three standard cardiovascular autonomic reflex tests (CARTs) and measures of 5 min. resting heart rate variability (HRV) were performed in a quiet examination room. HRV was assessed after 5 min of supine rest and analyzed from 5-min resting heart rate (HR). HRV indices were analyzed in time- and frequency-domain. Time-domain included the root mean square of the sum of the squares of differences between consecutive R–R intervals (RMSSD) and standard deviation of normal-to-normal intervals (SDNN). Frequency-domain included low-frequency power band (LF) (0.04–0.15 Hz), high-frequency power band (HF) (0.15–0.4 Hz), total frequency power (Total) and the ratio low-frequency power/high-frequency power (LH/HF-ratio) (34).

The 5-min resting HRV test was followed by the three CARTs including the lying-to-standing test (30:15), the deep breathing test (E:I) and Valsalva Maneuver. "Early CAN" was defined as one out of the three CARTs was abnormal, "definite CAN" was defined as two or three were abnormal. Thresholds for abnormal results were age stratified (35). Resting HRV indices and CARTs were registered by using Vagus™ (Medicus Engineering, Aarhus, Denmark).

In line with the recommended criteria for examination of CAN (26), participants were asked to restrain from vigorous exercise 24 h before examination and from caffeine consumption on the specific day of examination.

## Questionnaires on Peripheral Neuropathy and Exposures

Each patient was asked to fill in the questionnaires Brief Pain Inventory (BPI) and Michigan Neuropathy Screening Instrument (MNSI) on the examination day. Participants were diagnosed with painful diabetic neuropathy if they in the BPI questionnaire answered having pain in both legs and/or both arms peripherally (36). A MNSI score of $\geq$7 was interpreted as presence of neuropathy (37).

Moreover, a questionnaire considering life style factors such as smoking status (current, former, or never) and weekly amount of exercise in hours (pooled light and moderate/vigorous exercise) was filled in.

## Assessment of GV Indices

The CGM sensor Enlite™ (Medtronic, Northridge, CA) was inserted into the subcutaneous tissue of the abdomen or alternatively the upper arm. Subsequently the iPro2™ (Medtronic, Northridge, CA) recorder was attached. The sensors should be worn for 5 days and the capillary finger blood glucose monitored four times daily for calibration. The software Medtronic CareLink™ iPro™ was used to generate data from the sensors. Participants were excluded from the study if there were not enough measurements of the capillary blood glucose to run the Medtronic CareLink™ iPro™ software. CV, standard deviation (SD), continuous overall net glycemic action (CONGA), and mean amplitude of glucose excursions (MAGE) were used to quantify GV (38). Time spent in hypo- ($<$3.0 mmol/l), eu- ($\geq$3.0; $\leq$10.0 mmol/l), and hyperglycemia ($>$10.0 mmol/l) were calculated (38) and presented in minutes and percentage.

## Blood Pressure and Anthropometric Measures

Blood pressure and heart rate (HR) were measured after 10 min of rest and calculated as the mean of three consecutive measures performed with intervals of 1 min. Automated oscillometric blood pressure recorders were used (AND UA-787plus, A&D medical, California, USA).

Height and weight were measured with clothes on but without shoes using a fixed rigid stadiometer (Seca, Chino, USA) and an electronic scale (Mettler Toledo, Glostrup, Denmark), respectively.

## Biochemical Measures

All biochemical measures were analyzed from venous blood samples except for urine albumin and creatinine. Blood and urine samples were collected on the same day as the examination. The participants were non-fasting.

$HbA_{1c}$ was analyzed by high performance liquid chromatography on a Tosoh G7 (Tosoh Cooperation, Japan). C-peptide was measured using a Cobas e411 (Roche Diagnostics, Mannheim, Germany). Triglycerides, HDL, and total cholesterol were analyzed by standard enzymatic colorimetry techniques on a Vitros 5600 (Ortho Clinical Diagnostics, France). Serum LDL cholesterol was calculated using the Friedewald equationTriglyceride level did not exceed 4.5 mmol/l in any subject. Hence, no other LDL assessments were deemed relevant. Plasma creatinine was analyzed by two-point rate enzymatic technique. The Chronic Kidney Disease Epidemiology (CKD-EPI) equation was used to estimate eGFR (39). Urinary albumin-to-creatinine ratio was analyzed by quantitative immunological turbidimetry.

## Medication

Data on medication were extracted from hospital electronic records and validated by the patient at examination day.

## Statistical Analysis

Patient characteristics are presented as means with standard deviation (SD) or in case of skewed distributions as medians with interquartile range [IQR].

Participants were excluded from the analysis of a specific test if the values were missing.

Both GV and $HbA_{1c}$ were examined as determinants for neuropathy. The associations were assessed by logistic regression for the categorical outcomes and presented as odds ratios (OR) with 95% confidence interval (CI). Linear regression analyses were applied for continuous outcomes and presented as estimates with 95% CI. To meet model assumptions outcomes were log-transformed prior to analysis and subsequently back transformed to original scale where appropriate. To avoid small-sample bias, determinants of DSPN and CAN were not included in the analyses if the number of affected participants were <5.

Four models of adjustments were applied: Model 1: Unadjusted; Model 2: Adjusted for age and gender; Model 3: Adjusted as model 2 + diabetes duration, BMI, exercise and $HbA_{1c}$; Model 4: Adjusted as model 3 + systolic blood pressure, triglycerides, LDL cholesterol and current smoking. HR was included as a confounder in models where HRV indices were determinants.

All analyses used 2-sided $P = 0.05$ as statistically significant and were adjusted for multiple tests by the Benjamini-Hochberg procedure (40).

Statistical analyses were performed in R version 3.3.3 (The R Foundation for Statistical Computing) and SAS, version 9.4 (SAS Institute, Cary, NC, USA).

# RESULTS

## Patient Characteristics

Overall, 133 young adults (43.6% male) were included in the study. Twenty-three participants were excluded due to missing or lacking CGM-monitoring including insufficient numbers of capillary finger blood glucose monitoring. Reasons for not wearing a CGM sensor were primarily irritative/allergic reactions to the bandage patches or fear of discomfort. Mean (SD) age was 22 years (1.6), diabetes duration 11 years (5.2), and median (IQR) $HbA_{1c}$ 65.5 mmol/mol (57;74). Mean (SD) BMI was 24.7 kg/m² (3.8) and 122 (92.4%) participants exercised regularly for an average of 9 h weekly. All participants were treated with insulin. Participant characteristics are presented in **Table 1**.

## Diabetic Neuropathy

The results of the prevalence of DSPN and CAN have been discussed elsewhere (4). In total, 51.1% ($n = 68$) were diagnosed with subclinical DSPN. One patient (0.8%) had confirmed DSPN, and two (1.5%) possible DSPN. None met the criteria for probable DSPN. Prevalence estimates of symmetric abnormal SNAP and SNCV were 20.3% ($n = 26$) and 34.4% ($n = 44$), respectively. Prevalence of the composite measure of SNAP/SNCV, SNC was 48.4% ($n = 62$). Abnormal ESC results on feet were found in 4.5% ($n = 6$) and 3% ($n = 4$) on hands. Symmetrically abnormal VPT was detected in 0.8% ($n = 1$) and likewise for symmetrical neuropathy diagnosed by the BPI questionnaire. No participants had abnormal results when light touch, pain perception or MNSI questionnaire were used.

Definite CAN was diagnosed in 6.1% ($n = 8$) and early CAN in 26.9% ($n = 35$).

Distribution and prevalence estimate of the outcomes are presented in **Table 2**.

## Glucose Variability

Mean (SD) CV was 40% (10) and median (IQR) SD was 3.9 mmol/l (3.2;4.7). Distribution of GV measures are presented in **Table 2**.

**TABLE 1 |** Characteristics of the study population.

| Clinical characteristics | Mean (*SD*)/Median [IQR]/*N* (%) |
|---|---|
| ***N*** | **133** |
| Age (yr) | 22 (1.6) |
| Males (%) | 58 (43.6) |
| CSII treatment (%) | 67 (50.4) |
| Diabetes duration (yr) | 11.0 (5.2) |
| BMI (kg/m$^2$) | 24.7 (3.8) |
| Exercise (%) / (hr/week) | 92.4 / 9.0 [5.0;15.5] |
| Current smoker (%) | 28 (21.2) |
| Systolic blood pressure (mmHg) | 125.9 (11.4) |
| Diastolic blood pressure (mmHg) | 81.2 (8.6) |
| Heart rate (bpm) | 76.6 (14.2) |
| **Biochemistry** | |
| $HbA_{1c}$ (mmol/mol) | 65.5 [57.0;74.0] |
| $HbA_{1c}$ (%) | 8.2 [7.4;9.0] |
| Cholesterol (mmol/l) | 4.4 (1.1) |
| Triglycerides (mmol/l) | 1.1 [0.8;1.6] |
| HDL (mmol/l) | 1.3 (0.4) |
| LDL (mmol/l) | 2.5 (0.9) |
| Urine albumin/creatinine ratio (mg/g) | 6.0 [4.0;11.0] |
| eGFR (ml/min/1.73m2) | 123.0 [115.9;127.1] |
| C-peptide (pmol/l) | 14.5 [7.0;101] |
| **Medication** | |
| Insulin treatment *n* (%) | 133 (100) |
| Metformin *n* (%) | 1 (0.8) |
| Other glucose-lowering drugs *n* (%) | 1 (0.8) |
| Antihypertensive treatment *n* (%) | 6 (4.5) |
| Beta blocker treatment *n* (%) | 2 (1.5) |
| Lipid lowering treatment *n* (%) | 1 (0.8) |
| Psychotropics *n* (%) | 5 (3.8) |

*Data are given in means (SD), medians [IQR] or proportions %.*
*eGFR, Estimated glomerular filtration rate; BMI, Body Mass Index; HDL, High-density lipoproteins; LDL, Low-density lipoproteins.*

**TABLE 2 |** Distribution of outcome and GV measures and prevalences of abnormal results.

| ***N*** | | **133** |
|---|---|---|
| **GV measures** | **Mean (*SD*)/median [IQR]** | **Prevalence *n* (%)** |
| CV (%) | 40 (10) | NA |
| SD (mmol/l) | 3.9 [3.2;4.7] | NA |
| MAGE (mmol/l) | 7.7 [5.9;9.9] | NA |
| CONGA (mmol/l) | 9.1 (2.2) | NA |
| Time spent in hypoglycaemia (min.) / (%) | 35 [0;120] / 1.0 [0.0;4.0] | NA |
| Time spent in euglycaemia (min.) / (%) | 3065 [2125;3895] / 52.2 (19.6) | NA |
| Time spent in hyperglycaemia (min.) / (%) | 2650 [1740;3480] / 44.7 (20.6) | NA |
| **Outcome Measures** | | |
| **CAN Measures** | | |
| CAN | NA | 8 (6.1) |
| Early CAN | NA | 35 (26.9) |
| Lying to standing ratio (30:15) | 1.4 (0.2) | 21 (15.9) |
| Deep breathing ratio (E:I) | 1.5 (0.2) | 10 (7.6) |
| Valsalva Maneuver ratio (VM) | 1.7 (0.4) | 22 (16.9) |
| SDNN (ms) | 48.1 [36.3;68.2] | NA |
| RMSSD (ms) | 38.9 [25.7;59.3] | NA |
| LF ($ms^2$) | 290.1 [130.1;670.0] | NA |
| HF ($ms^2$) | 251.3 [114.6;516.0] | NA |
| LF/HF ratio | 1.3 [0.8;2.8] | NA |
| Total | 779.5 [444.4;1570.5] | NA |
| **DSPN Measures** | | |
| Subclinical DSPN | NA | 68 (51.1) |
| Confirmed DSPN | NA | 1 (0.8) |
| Possible DSPN | NA | 2 (1.5) |
| Probable DSPN | NA | 0 |
| Monofilament (≥ 1 missing response) | NA | 0 |
| Pin prick (≥ 1 missing response) | NA | 0 |
| SNC | NA | 62 (48.4) |
| SNAP (μV) | 11.7 [8.7;15.0] | 26 (20.3) |
| SNCV (m/s) | 50.8 (4.2) | 44 (34.4) |
| VPT (V) | 4.5 [3.5;5.5] | 1 (0.8) |
| ESC—hands (μS) | 77.5 [69.5;83.5] | 4 (3.0) |
| ESC—feet (μS) | 82.3 [78.6;85.8] | 6 (4.5) |
| **Questionnaires** | | |
| BPI questionnaire: | | |
| *Painful neuropathy (% answered yes)* | NA | 1 (0.8) |
| MNSI questionnaire: | | |
| *MNSI neuropathy score (score ≥ 7 points)* | 1 [0;2] | 0 |

*Data are given in means (SD), medians [IQR], or proportions. NA, Not applicable. CAN, Cardiovascular autonomic neuropathy; RMSSD, root mean square of the sum of the squares of differences between consecutive R–R intervals; SDNN, standard deviation of normal-to-normal intervals; LF/HF ratio, low-frequency power/high-frequency power ratio; DSPN, distal symmetric polyneuropathy; VPT, vibration perception threshold; SNC, sural nerve conduction; SNAP, sural nerve amplitude potential; SNCV, sural nerve conduction velocity; ESC, Electrochemical skin conduction; BPI, Brief Pain Inventory; MNSI, Michigan Neuropathy Screening Instrument questionnaire; CV, coefficient of variation; MAGE, mean amplitude of glucose excursions; CONGA, continuous overall net glycemic action. Capillary blood glucose was divided into three groups: Hypoglycemia: <3.0 mmol/l, euglycemia: ≥3.0; ≤10.0 mmol/l, hyperglycemia: >10.0 mmol/l.*

## Association Between Glucose Variability and Diabetic Neuropathy

### Coefficient of Variation (CV)

Greater CV was associated with a decrease in incidents of symmetric abnormalities in SNAP, SNC, and definite CAN unadjusted and when adjusted for age and gender in model 2. Only for SNC significance remained in fully adjusted models. In addition, higher CV was inversely associated to incidents of subclinical DSPN in fully adjusted models (**Table 3**, **Figure 1**). However, both associations lost significance after applying the Benjamini-Hochberg procedure (40) to account for multiple testing.

### Standard Deviation (SD)

Higher SD was associated to a decreased risk of subclinical DSPN and increasing levels of the continuous outcome SNCV when adjusted for diabetes duration, BMI, and exercise and $HbA_{1c}$ in model 3. Again, no significance persisted after correcting for multiple tests (**Table 4**, **Figure 2**).

### Continuous Overall Net Glycemic Action (CONGA)

In the unadjusted model 1 higher CONGA was significantly associated to increasing incidents of subclinical DSPN, symmetric abnormalities SNAP, SNCV, and the composite measure SNC. Moreover, higher CONGA was associated to decreasing levels of the continuous measure of SNCV. When adjusted for gender and age in model 2 significance was kept for every outcome and in addition higher CONGA was associated with an increased risk of definite CAN. However, for every estimate significance was lost when adjusted for diabetes duration, BMI, and exercise and $HbA_{1c}$ in model 3 (**Table A1**).

### Mean Amplitude of Glucose Excursions (MAGE)

Greater MAGE became significantly associated with higher continuous measures of HRV only in the fully adjusted model 4 (**Table A2**) but when correcting for multiple tests significance was lost.

### Time Spent in Hypo-, Eu-, and Hyperglycemia

None of the determinants "time spent in hypo-, eu-, and hyperglycemia" were significantly associated with diabetic neuropathy (**Tables A3–A5**).

### Association Between $HbA_{1c}$ and Diabetic Neuropathy

An increase in $HbA_{1c}$ was associated with higher odds of subclinical DSPN when adjusted for diabetes duration, BMI, and exercise in model 3. Higher $HbA_{1c}$ was significantly associated with increasing incidents of symmetric abnormalities in SNAP, SNCV, and the composite measure SNC in the fully adjusted model 4. Congruently, higher $HbA_{1c}$ was associated to decreasing continuous values of SNAP and SNCV in model 4. Also, increasing levels of $HbA_{1c}$ were associated with an increase in heart rate (HR) in model 3 and decreasing measures of HRV in model 4 (**Table 5**, **Figure 3**).

## DISCUSSION

In this cross-sectional study 133 young adults with type 1 diabetes were identified with modest GV (38) with a mean coefficient of variation (CV) of 40% (38).

Modest associations between GV and measures of peripheral and autonomic diabetic neuropathy were found in the study. Higher CV was, against expectations, associated with decreased risk of DSPN and CAN, although not statistically significant when adjusting for relevant risk factors and multiple tests. Higher CONGA was associated with increasing incidents of both peripheral and autonomic neuropathy, but findings were confounded by relevant risk factors for diabetic neuropathy. After adjusting for risk factors higher MAGE was significantly associated with a slight increase in measures of HRV indicating an improvement of CAN. This may just be spurious findings—notably when the significant findings were attenuated after correcting for multiple tests. Overall only modest associations were found between GV and DSPN and CAN. Associations were confounded by known risk factors.

However, significant associations were found between higher levels of $HbA_{1c}$ and increased risks of both peripheral and autonomic neuropathy in fully adjusted models. This only supports earlier findings of high levels of $HbA_{1c}$ being an established and essential risk factor of diabetic neuropathy in type 1 diabetes (41).

Previous studies have revealed conflicting conclusions when examining the association between GV and diabetic neuropathy. Lachin et al. (17) evaluated 1,441 participants with type 1 diabetes and a mean age of 27 years from the DCCT. CARTs were

**TABLE 3 |** The association between CV and measures of diabetic neuropathy.

| CAN Measures | Model 1 | Model 2 | Model 3 | Model 4 |
|---|---|---|---|---|
| **Binary outcomes** | | **OR (95% CI)** | | |
| CAN | 0.0 (0.0;0.61)* | 0.0 (0.0;0.56)* | 0.0 (0.0;8.34) | 0.0 (0.0;6.18) |
| Early CAN | 0.96 (0.02;55.92) | 0.97 (0.02;57.80) | 0.87 (0.01;62.08) | 0.49 (0.01;38.15) |
| **Continuous outcomes** | | **Estimate (95% CI)** | | |
| Heart rate | −4.46 (−28.32;19.41) | −4.11 (−27.60;19.38) | 0.33 (−23.85;24.50) | 4.77 (−19.06;28.61) |
| Lying to standing (30:15) | 0.08 (−0.32;0.49) | 0.09 (−0.32;0.49) | 0.02 (−0.39;0.43) | −0.02 (−0.43;0.39) |
| Deep breathing (E:I) | 0.08 (−0.34;0.50) | 0.08 (−0.33;0.50) | −0.02 (−0.45;0.40) | 0.01 (−0.40;0.42) |
| Valsalva Maneuver (VM) | 0.07 (−0.57;0.71) | 0.06 (−0.57;0.70) | 0.15 (−0.51;0.80) | 0.13 (−0.54;0.79) |
| SDNN | 4.90 (−48.10;112.01) | 5.97 (−47.52;113.97) | −1.16 (−51.48;101.37) | −6.04 (−54.08;92.26) |
| RMSSD | −18.11 (−66.90;102.59) | −18.30 (−66.66;101.56) | −25.56 (−70.56;88.17) | −36.08 (−74.82;62.22) |
| LF | −6.83 (−85.63;505.08) | −4.61 (−85.25;516.84) | −11.44 (−86.55;483.07) | −21.28 (−88.13;422.14) |
| HF | −50.65 (−92.32;217.14) | −49.85 (−92.03;215.73) | −57.18 (−93.48;181.17) | −65.48 (−94.77;127.68) |
| LF/HF ratio | 88.76 (−55.36;698.18) | 90.20 (−53.19;672.85) | 106.85 (−48.65;733.21) | 128.09 (−42.70;808.01) |
| Total | −6.86 (−79.70;327.46) | −5.53 (−79.42;333.52) | −26.26 (−84.37;247.82) | −34.85 (−86.34;210.86) |
| **DSPN Measures** | | | | |
| **Binary outcomes** | | **OR (95% CI)** | | |
| Subclinical DSPN | 0.03 (0.0;1.03) | 0.02 (0.0;1.02) | 0.01 (0.0;0.48)* | 0.01 (0.0;0.78)* |
| SNC | 0.02 (0.0;0.69)* | 0.01 (0.0;0.58)* | 0.01 (0.0;0.48)* | 0.01 (0.0;0.81)* |
| SNAP | 0.01 (0.0;0.80)* | 0.0 (0.0;0.72)* | 0.01 (0.0;1.83) | 0.01 (0.0;1.97) |
| SNCV | 0.05 (0.0;2.29) | 0.04 (0.0;2.24) | 0.03 (0.0;2.73) | 0.05 (0.0;4.39) |
| ESC—feet | 10.75 (0.0;43428.88) | 14.86 (0.0;98876.15) | 1.67 (0.0;52896.65) | 2.61 (0.0;189015.80) |
| **Continuous outcomes** | | **Estimate (95% CI)** | | |
| VPT | −46.50 (−70.95;−1.48)* | −46.57 (−70.98;−1.62)* | −44.16 (−70.63;6.14) | −41.24 (−68.97;11.28) |
| SNAP | 59.59 (−27.44;251.0) | 60.87 (−20.46;225.35) | 49.27 (−23.81;192.44) | 54.88 (−20.55;201.92) |
| SNCV | 6.33 (−1.15;13.82) | 6.29 (−0.99;13.56) | 6.47 (−0.47;13.40) | 5.71 (−1.23;12.66) |
| ESC—hands | 1.38 (−19.65;27.92) | 1.13 (−19.63;27.27) | 3.97 (−18.15;32.07) | 3.72 (−18.46;31.94) |
| ESC—feet | 0.13 (−12.07;14.01) | 0.06 (−12.11;13.92) | 2.96 (−9.98;17.77) | 3.91 (−9.05;18.71) |

*Results are presented as odds ratios for binary outcomes based on logistic regression analyses and estimates for continuous outcomes based on linear regression analyses. Odds ratios show the change in odds for any increase of the GV determinants. Estimates show the percentage change in the outcomes for every 1-unit change of CV.*

*Model 1: unadjusted. Model 2: adjusted for age and gender. Model 3: adjusted for age, gender, HbA1c, diabetes duration, BMI and exercise. Model 4: adjusted for age, gender, HbA1c, diabetes duration, BMI, exercise, systolic blood pressure, triglycerides, LDL cholesterol and current smoking. The continuous outcomes of SDNN, RMSSD, LF, HF, LF/HF ratio, Total, VPT, SNAP, and ESC for hands and feet are log-transformed prior to analysis and subsequently back transformed to original scale. SDNN, RMSSD, LF, HF, LF/HF ratio, and Total are adjusted for HR in every model. Outcomes of DSPN are defined as presence of symmetric abnormal results. Binary outcomes were only included in the analyses if presence of 5 or more abnormal events.*

*CAN, Cardiovascular autonomic neuropathy; RMSSD, root mean square of the sum of the squares of differences between consecutive R-R intervals, SDNN, standard deviation of normal-to-normal intervals; LF/HF ratio, low-frequency power/high-frequency power ratio, DSPN, distal symmetric polyneuropathy; VPT, vibration perception threshold; SNC, sural nerve conduction; SNAP, sural nerve amplitude potential; SNCV, sural nerve conduction velocity; ESC, Electrochemical skin conduction.*

**P < 0.05.*

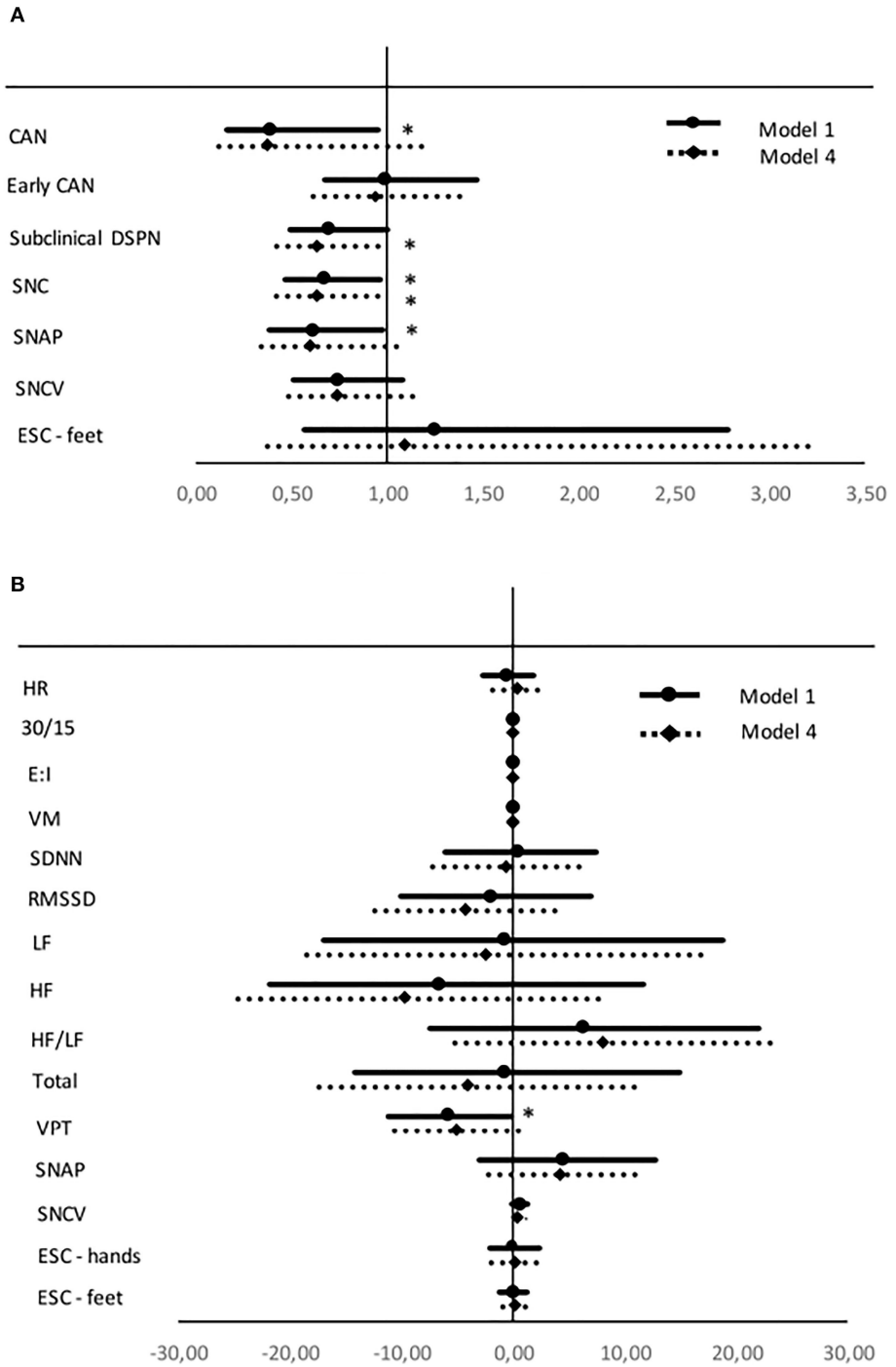


**FIGURE 1 |** Forest plot of the associations between standardized values of CV and both binary **(A)** and continuous **(B)** neuropathy endpoints. For binary outcomes results are presented as odds ratio and 95% confidence intervals. Odds ratio shows the change in odds for an increase of one deviation in the $HbA_{1c}$. For continuous outcomes results are presented as estimates and 95% confidence intervals. Estimates show the percentage change in the outcomes for an increase of one standard deviation in the SD. Studies with confidence interval crossing the vertical line are inconclusive. Model 1: unadjusted. Model 4: adjusted for age, gender, diabetes duration, BMI, exercise, systolic blood pressure, triglycerides, LDL cholesterol, and current smoking. SDNN, RMSSD, LF, HF, LF/HF ratio, and total are adjusted for HR in every model. Outcomes of DSPN are define as presence of symmetric abnormal results. Binary outcomes were only included in the analyses if presence of five or more abnormal events. CV, coefficient of variation; CAN, Cardiovascular autonomic neuropathy; SNC, sural nerve conduction; SNAP, sural nerve amplitude potential; SNCV, sural nerve conduction velocity; ESC, Electrochemical skin conduction; RMSSD, root mean square of the sum of the squares of differences between consecutive R–R intervals; SDNN, standard deviation of normal-to-normal intervals; LF/HF ratio, low-frequency power/high-frequency power ratio; VPT, vibration perception threshold. *$P < 0.05$.

**TABLE 4 |** The association between *SD* and measures of diabetic neuropathy.

| CAN measures | Model 1 | Model 2 | Model 3 | Model 4 |
|---|---|---|---|---|
| **Binary outcomes** | | **OR (95% CI)** | | |
| CAN | 0.79 (0.41;1.51) | 0.79 (0.41;1.53) | 0.54 (0.23;1.28) | 0.47 (0.17;1.32) |
| Early CAN | 1.02 (0.71;1.45) | 1.03 (0.72;1.48) | 1.14 (0.76;1.71) | 1.13 (0.75;1.72) |
| **Continuous outcomes** | | **Estimate (95% CI)** | | |
| Heart rate | 0.67 (−1.41;2.76) | 0.69 (−1.38;2.76) | −0.19 (−2.44;2.06) | 0.29 (−1.93;2.25) |
| Lying to standing (30:15) | −0.02 (−0.06;0.01) | −0.02 (−0.06;0.01) | −0.01 (−0.05;0.02) | −0.02 (−0.06;0.02) |
| Deep breathing (E:I) | 0.01 (−0.03;0.04) | 0.01 (−0.03;0.04) | 0.01 (−0.03;0.05) | 0.01 (−0.03;0.05) |
| Valsalva Maneuver (VM) | 0.03 (−0.02;0.09) | 0.03 (−0.03;0.08) | 0.02 (−0.04;0.08) | 0.02 (−0.04;0.08) |
| SDNN | −2.87 (−8.66;3.29) | −2.65 (−8.48;3.55) | 1.18 (−5.30;8.10) | 1.41 (−5.12;8.39) |
| RMSSD | −4.10 (−11.39;3.80) | −4.11 (−11.40;3.77) | −0.26 (−8.50;8.71) | −1.06 (−9.28;7.90) |
| LF | −5.42 (−19.70;11.38) | −4.87 (−19.29;12.11) | 2.77 (−13.74;22.46) | 3.97 (−12.80;23.97) |
| HF | −9.43 (−23.02;6.55) | −9.29 (−22.83;6.63) | −1.53 (−17.39;17.36) | −2.47 (−18.23;16.33) |
| LF/HF ratio | 4.43 (−7.98;18.52) | 4.87 (−7.33;18.68) | 4.38 (−8.33;18.86) | 6.61 (−6.27;21.26) |
| Total | −6.60 (−18.23;6.69) | −6.35 (−18.08;7.07) | 1.03 (−12.52;16.68) | 1.56 (−12.16;17.43) |
| **DSPN Measures** | | | | |
| **Binary outcomes** | | **OR (95% CI)** | | |
| Subclinical DSPN | 0.94 (0.69;1.28) | 0.93 (0.67;1.28) | 0.67 (0.45;0.99)* | 0.69 (0.46;1.04) |
| SNC | 0.93 (0.68;1.28) | 0.90 (0.65;1.26) | 0.68 (0.46;1.02) | 0.71 (0.47;1.08) |
| SNAP | 0.98 (0.66;1.45) | 0.94 (0.62;1.42) | 0.63 (0.37;1.07) | 0.59 (0.34;1.02) |
| SNCV | 1.00 (0.72;1.40) | 1.00 (0.71;1.40) | 0.76 (0.51;1.13) | 0.79 (0.52;1.19) |
| ESC – feet | 1.11 (0.52;2.38) | 1.16 (0.53;2.52) | 1.02 (0.41;2.55) | 0.99 (0.36;2.71) |
| **Continuous outcomes** | | **Estimate (95% CI)** | | |
| VPT | −0.15 (−5.44;5.43) | −0.16 (−5.47;5.44) | −0.78 (−6.62;5.43) | −0.63 (−6.46;5.55) |
| SNAP | 0.38 (−6.40;7.65) | 1.48 (−4.70;8.07) | 5.62 (−0.78;12.42) | 6.75 (0.35;13.56)* |
| SNCV | 0.12 (−0.55;0.79) | 0.06 (−0.59;0.72) | 0.65 (0.01;1.30)* | 0.64 (0.0;1.29) |
| ESC—hands | 0.51 (−1.52;2.58) | −0.01 (−0.02;0.01) | 0.29 (−1.93;2.55) | 0.48 (−1.75;2.77) |
| ESC—feet | 0.26 (−0.88;1.42) | 0.49 (−1.52;2.55) | 0.27 (−0.98;1.53) | 0.47 (−0.78;1.73) |

*Results are presented as odds ratios for binary outcomes based on logistic regression analyses and estimates for continuous outcomes based on linear regression analyses. Odds ratios show the change in odds for any increase of the GV determinants. Estimates show the percentage change in the outcomes for every 1-unit change of CV.*

*Model 1: unadjusted. Model 2: adjusted for age and gender. Model 3: adjusted for age, gender, $HbA_{1c}$, diabetes duration, BMI, and exercise. Model 4: adjusted for age, gender, $HbA_{1c}$, diabetes duration, BMI, exercise, systolic blood pressure, triglycerides, LDL cholesterol, and current smoking. The continuous outcomes of SDNN, RMSSD, LF, HF, LF/HF ratio, Total, VPT, SNAP, and ESC for hands and feet are log-transformed prior to analysis and subsequently back transformed to original scale. SDNN, RMSSD, LF, HF, LF/HF ratio, and Total are adjusted for HR in every model. Outcomes of DSPN are defined as presence of symmetric abnormal results. Binary outcomes were only included in the analyses if presence of five or more abnormal events.*

*CAN, Cardiovascular autonomic neuropathy; RMSSD, root mean square of the sum of the squares of differences between consecutive R–R intervals; SDNN, standard deviation of normal-to-normal intervals; LF/HF ratio, low-frequency power/high-frequency power ratio; DSPN, distal symmetric polyneuropathy; VPT, vibration perception threshold; SNC, sural nerve conduction; SNAP, sural nerve amplitude potential; SNCV, sural nerve conduction velocity; ESC, Electrochemical skin conduction.*

*$^*P < 0.05$.*

performed every 2 years to evaluate CAN. GV was assessed by SD, MAGE, and *M*-value (a hybrid of glucose exposure and glycemic variability) (42). No significant associations were found between GV and CAN after correcting for within-day and longitudinal mean blood glucose and multiple tests. However, in the DCCT the GV parameters were calculated from 7 fingerstick glucose levels per day and this may have been an inadequate metrics of GV to detect GV's association with CAN.

Jaiswal et al. (16) found modest associations between GV and CAN. The study included 44 participants with type 1 diabetes and a mean age of 34 years. CAN was assessed by CARTs and HRV. Five days CGM was used to compute GV measures: low blood glucose index (LBGI) and area under the curve (AUC) for hypoglycemia. Significant inverse associations were found between both LBGI and AUC and the HF and LF power which implicates impaired autonomic function when longer and more severe hypoglycemia. However, when adjusting for relevant risk factors significance was attenuated. No relationship was identified between GV and any of the CARTs. Moreover, Kwai et al. (14) found significant associations between MAGE, assessed from 6-days CGM, and median motor and sensory excitability assessment in a study comprising 17 participants with type 1 diabetes and a mean age of 28.6 years. The findings of the study point at impairment of peripheral neuropathy induced by higher GV.

Nyiraty et al. (18) investigated the association between GV and autonomic neuropathy (AN) in 20 participants with type 1 diabetes and a mean age of 39.5 years. Fifty percent of the participants were diagnosed with CAN. AN was evaluated by CARTs and orthostatic blood pressure assessment. GV was

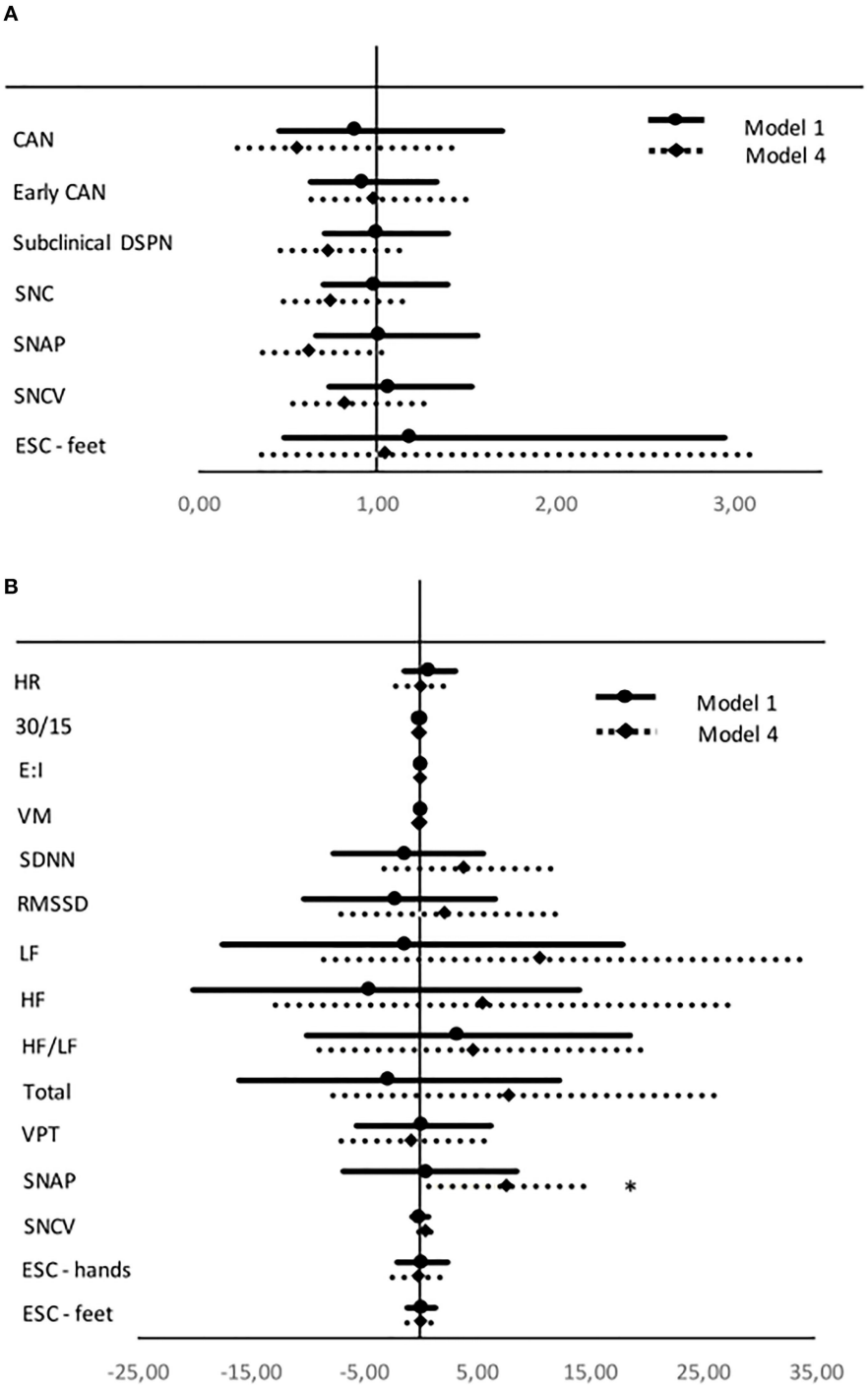


**FIGURE 2 |** Forest plot of the associations between standardized values of SD and both binary **(A)** and continuous **(B)** neuropathy endpoints. For binary outcomes results are presented as odds ratio and 95% confidence intervals. Odds ratio shows the change in odds for an increase of one deviation in the $HbA_{1c}$. For continuous outcomes results are presented as estimates and 95% confidence intervals. Estimates show the percentage change in the outcomes for an increase of one standard deviation in the SD. Studies with confidence interval crossing the vertical line are inconclusive. Model 1: unadjusted. Model 4: adjusted for age, gender, diabetes duration, BMI, exercise, systolic blood pressure, triglycerides, LDL cholesterol, and current smoking. SDNN, RMSSD, LF, HF, LF/HF ratio, and total are adjusted for HR in every model. Outcomes of DSPN are define as presence of symmetric abnormal results. Binary outcomes were only included in the analyses if presence of five or more abnormal events. CAN, Cardiovascular autonomic neuropathy; SNC, sural nerve conduction; SNAP, sural nerve amplitude potential; SNCV, sural nerve conduction velocity; ESC, Electrochemical skin conduction; RMSSD, root mean square of the sum of the squares of differences between consecutive R–R intervals; SDNN, standard deviation of normal-to-normal intervals; LF/HF ratio, low-frequency power/high-frequency power ratio; VPT, vibration perception threshold. *$P < 0.05$.

**TABLE 5 |** The association between $HbA_{1c}$ and measures of diabetic neuropathy.

| CAN measures | Model 1 | Model 2 | Model 3 | Model 4 |
|---|---|---|---|---|
| **Binary outcomes** | | **OR (95% CI)** | | |
| CAN | 1.02 (0.99;1.05) | 1.02 (0.99;1.05) | 1.01 (0.99;1.04) | 1.01 (0.97;1.05) |
| Early CAN | 1.0 (0.98;1.02) | 1.0 (0.98;1.02) | 1.0 (0.98;1.03) | 1.01 (0.98;1.03) |
| **Continuous outcomes** | | **Estimate (95% CI)** | | |
| Heart rate | 0.14 (0.03;0.26)* | 0.14 (0.03;0.26)* | 0.13 (0.02;0.25)* | 0.11 (−0.02;0.24) |
| Lying to standing (30:15) | 0.0 (0.0;0.0) | 0.0 (0.0;0.0) | 0.0 (0.0;0.0) | 0.0 (0.0;0.0) |
| Deep breathing (E:I) | 0.0 (0.0;0.0) | 0.0 (0.0;0.0) | 0.0 (0.0;0.0) | 0.0 (0.0;0.0) |
| Valsalva Maneuver (VM) | 0.0 (0.0;0.0) | 0.0 (0.0;0.0) | 0.0 (0.0;0.0) | 0.0 (0.0;0.01) |
| SDNN | −0.68 (−1.02;−0.34)* | −0.67 (−1.01;−0.32)* | −0.65 (−0.99;−0.30)* | −0.55 (−0.94;−0.17)* |
| RMSSD | −0.66 (−1.11;−0.20)* | −0.65 (−1.11;−0.20)* | −0.63 (−1.09;−0.18)* | −0.46 (−0.97;0.04) |
| LF | −1.48 (−2.39;−0.56)* | −1.44 (−2.36;−0.52)* | −1.38 (−2.29;−0.46)* | −1.19 (−2.21;−0.17)* |
| HF | −1.48 (−2.39;−0.55)* | −1.46 (−2.37;−0.54)* | −1.41 (−2.33;−0.49)* | −1.11 (−2.13;−0.08)* |
| LF/HF ratio | 0 (−0.72;0.72) | 0.02 (−0.69;0.73) | 0.03 (−0.65;0.72) | −0.08 (−0.83;0.67) |
| Total | −1.41 (−2.14;−0.68)* | −1.40 (−2.14;−0.66)* | −1.37 (−2.11;−0.63)* | −1.23 (−2.05;−0.40)* |
| **DSPN Measures** | | | | |
| **Binary outcomes** | | **OR (95% CI)** | | |
| Subclinical DSPN | 1.03 (1.01;1.05)* | 1.03 (1.01;1.05)* | 1.03 (1.01;1.05)* | 1.02 (1.0;1.05) |
| SNC | 1.03 (1.01;1.06)* | 1.04 (1.01;1.06)* | 1.03 (1.01;1.06)* | 1.03 (1.01;1.06)* |
| SNAP | 1.04 (1.01;1.06)* | 1.04 (1.01;1.06)* | 1.04 (1.02;1.07)* | 1.04 (1.02;1.07)* |
| SNCV | 1.04 (1.02;1.06)* | 1.04 (1.02;1.06)* | 1.04 (1.01;1.06)* | 1.03 (1.01;1.06)* |
| ESC—feet | 1.01 (0.97;1.05) | 1.01 (0.97;1.05) | 1.01 (0.96;1.05) | 1.01 (0.96;1.06) |
| **Continuous outcomes** | | **Estimate (95% CI)** | | |
| VPT | 0.09 (−0.22;0.40) | 0.09 (−0.23;0.40) | 0.10 (−0.22;0.42) | 0.16 (−0.19;0.51) |
| SNAP | −0.54 (−0.93;−0.14)* | −0.46 (−0.83;−0.10)* | −0.46 (−0.81;−0.11)* | −0.61 (−1.0;−0.22)* |
| SNCV | −0.09 (−0.12;−0.05)* | −0.09 (−0.13;−0.06)* | −0.09 (−0.13;−0.05)* | −0.08 (−0.13;−0.04)* |
| ESC—hands | 0.10 (−0.02;0.21) | 0.10 (−0.01;0.21) | 0.11 (−0.01;0.22) | 0.10 (−0.02;0.23) |
| ESC—feet | −0.02 (−0.10;0.07) | −0.02 (−0.10;0.07) | −0.01 (−0.10;0.07) | −0.03 (−0.13;0.07) |

*Results are presented as odds ratios for binary outcomes based on logistic regression analyses and estimates for continuous outcomes based on linear regression analyses. Odds ratios show the change in odds for any increase of the GV determinants. Estimates show the percentage change in the outcomes for every 1-unit change of CV.*

*Model 1: unadjusted. Model 2: adjusted for age and gender. Model 3: adjusted for age, gender, $HbA_{1c}$, diabetes duration, BMI, and exercise. Model 4: adjusted for age, gender, $HbA_{1c}$, diabetes duration, BMI, exercise, systolic blood pressure, triglycerides, LDL cholesterol, and current smoking. The continuous outcomes of SDNN, RMSSD, LF, HF, LF/HF ratio, Total, VPT, SNAP, and ESC for hands and feet are log-transformed prior to analysis and subsequently back transformed to original scale. SDNN, RMSSD, LF, HF, LF/HF ratio, and Total are adjusted for HR in every model. Outcomes of DSPN are defined as presence of symmetric abnormal results. Binary outcomes were only included in the analyses if presence of five or more abnormal events.*

*CAN, Cardiovascular autonomic neuropathy; RMSSD, root mean square of the sum of the squares of differences between consecutive R–R intervals; SDNN, standard deviation of normal-to-normal intervals; LF/HF ratio, low-frequency power/high-frequency power ratio; DSPN, distal symmetric polyneuropathy; VPT, vibration perception threshold; SNC, sural nerve conduction; SNAP, sural nerve amplitude potential; SNCV, sural nerve conduction velocity; ESC, Electrochemical skin conduction.*

*$^*P < 0.05$.*

assessed by 6 days of CGM and calculation of SD, MAGE, CONGA and mean absolute glucose (MAG). Only a correlation between SD and orthostatic hypotension was significant after adjusting for relevant risk factors. Again, the study does not provide a clear conclusion on the relationship between diabetic neuropathy and GV.

Studies investigating the association between GV and diabetic neuropathy in type 2 diabetes do not present uniform results. The number of studies investigating the association is limited and in particular studies assessing GV by CGM. However, CV in CGM was previously found to increase the risk of CAN in type 2 diabetes (23). Moreover, MAGE was significantly associated to DPN (24). Other studies have identified significant relations between variability in HbA1c and both DSPN and CAN (21–23). This may indicate an association between GV and diabetic neuropathy in type 2 diabetes however, like for studies concerning type 1 diabetes, there is a need for more studies with comparable measures of GV.

The results of our study are pointing at GV not being a risk factor for developing CAN and DSPN. However, the study has its limitations making causal conclusions difficult. We did find a significant association between higher levels of $HbA_{1c}$ and CAN and DSPN which to some extent shows that poor glycemic control is indeed a risk factor for diabetic neuropathy.

## STRENGTHS AND LIMITATIONS

The cross-sectional design of the study is not ideal when examining the relationship between GV and diabetic neuropathy in a causal manner. A prospective observational study design would have been more appropriate.

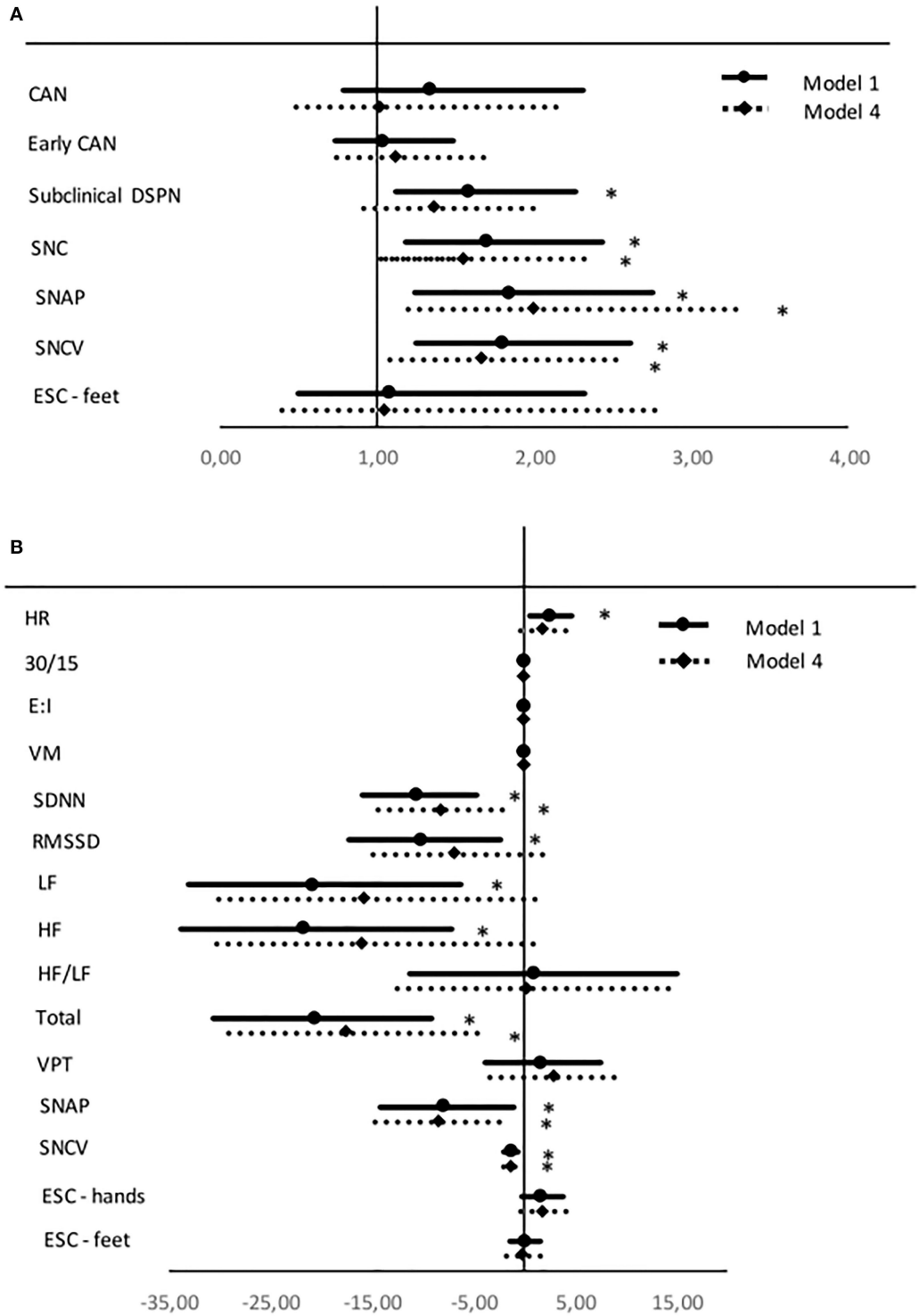


**FIGURE 3 |** Forest plot of the associations between standardized values of $HbA_{1c}$ and both binary **(A)** and continuous **(B)** neuropathy endpoints. For binary outcomes results are presented as odds ratio and 95% confidence intervals. Odds ratio shows the change in odds for an increase of one deviation in the $HbA_{1c}$. For continuous outcomes results are presented as estimates and 95% confidence intervals. Estimates show the percentage change in the outcomes for an increase of one standard deviation in the SD. Studies with confidence interval crossing the vertical line are inconclusive. Model 1: unadjusted. Model 4: adjusted for age, gender, diabetes duration, BMI, exercise, systolic blood pressure, triglycerides, LDL cholesterol, and current smoking. SDNN, RMSSD, LF, HF, LF/HF ratio, and total are adjusted for HR in every model. Outcomes of DSPN are define as presence of symmetric abnormal results. Binary outcomes were only included in the analyses if presence of five or more abnormal events. CAN, Cardiovascular autonomic neuropathy; SNC, sural nerve conduction; SNAP, sural nerve amplitude potential; SNCV, sural nerve conduction velocity; ESC, Electrochemical skin conduction; RMSSD, root mean square of the sum of the squares of differences between consecutive R–R intervals; SDNN, standard deviation of normal-to-normal intervals; LF/HF ratio, low-frequency power/high-frequency power ratio; VPT, vibration perception threshold. *$P < 0.05$.

As the study comprised 133 young adults identified with modest GV and prevalence of diabetic neuropathy there may not be enough power to extrapolate the results to the overall young population with type 1 diabetes. A larger sample size would have been beneficial in order to draw a more valid conclusion.

The aim of the study was to investigate the association between GV and early possible reversible neuropathy in a young cohort. Thus, conclusions on associations are limited to type 1 diabetes patients in the age-rage of the study cohort.

Novel and established methods of detecting DSPN and CAN (2, 35) were used which is a considerable strength in our study and may give a more detailed description of the nerve function.

CGM was used to assess GV as recommended (38) in order to detect periods of acute hypo- and hyperglycemia. However, the participants were only asked to wear the sensors for 5 days which may not be a sufficient duration to present a representable picture of daily fluctuations of blood glucose. Longer measuring durations may have given a more nuanced understanding.

It may be possible that more resourceful young adults chose to participate in the study after receiving the written invitation which could have caused selections bias.

It is recommended that participants avoid test confounders as smoking, use of several drugs, meals, and caffeine-containing liquids before testing for CAN (26). The participants were advised not to drink caffeine-containing liquids on the day before testing but the other recommendations were not met and may have affected CAN measures (4).

## CONCLUSION

After adjusting for relevant risk factors and multiple tests, no significant associations were found between GV and diabetic neuropathy in a cohort of young adults with type 1 diabetes. This finding is in line with some of the previous studies which have failed to provide consistent evidence that GV is a risk factor of development of CAN and DSPN.

This suggests that GV may not be a risk factor for early diabetic neuropathy in young adults with type 1 diabetes. However, the cross-sectional study approach including a relatively small sample size of young participants with modest GV and diabetic neuropathy make a strong conclusion difficult. Moreover, long-term effects of GV excursions may still play a role in the pathogenic mechanisms leading to neuropathy in later life. Increasing levels of $HbA_{1c}$ were significantly associated with both measures of DSPN and CAN which support earlier findings of high levels of $HbA_{1c}$ being an established and essential risk factor of diabetic neuropathy.

Previous studies addressing the aim of the present project have assessed GV and DSPN and CAN by heterogenic measuring modalities hampering comparability. To improve comparability there is a need for studies using recommended measures of GV and diabetic neuropathy. Furthermore, more studies on young adults with type 1 diabetes are needed to confirm our findings.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The Danish Research Ethics Committee (project id.: H-15006967). The patients/participants provided their written informed consent to participate in this study.

## AUTHOR CONTRIBUTIONS

MC has contributed to the design of the study, acquired, analyzed, interpreted data, drafted the article, and approved the final version to be published. EH, MJ, and JF has contributed to the design of the study, analyzed, interpreted data, revised the article critically, and approved the final version to be published. CH has contributed to the design of the study, acquisition, analysis, interpretation of data, revised the article critically, and approved the final version to be published. All authors contributed to the article and approved the submitted version.

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MC is the guarantor of this work and, as such had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2020.00644/full#supplementary-material

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The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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# Serum Adiponectin Levels Are Positively Associated With Diabetic Peripheral Neuropathy in Chinese Patients With Type 2 Diabetes

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**Objective:** To investigate the association between serum adiponectin levels and diabetic peripheral neuropathy (DPN) in Chinese type 2 diabetes (T2D) patients.

**Design and Methods:** Two hundred nineteen T2D patients aged 40–79 years were divided into two groups according to whether they had DPN. The systemic levels of five biomarkers were measured using a human adipokine multiplexed bead-based immunoassay. Diabetic peripheral neuropathy diagnostic criteria included both common DPN symptoms and neurological screening tests.

**Results:** Most features of DPN ($n$=98) and non-DPN patients ($n$=121) are similar, but the DPN patients were slightly older, had longer diabetes duration, higher hemoglobin (Hb) A1c, lower estimated glomerular filtration rates (eGFR), less exercise, and used lipid-lowering drugs more often. Serum adiponectin levels of DPN patients were higher than that of non-DPN patients (8.13 *vs*. 9.63 mg/ml, $P = 0.004$). Serum adiponectin levels were positively associated with DPN after adjusting for age, gender, body mass index, hypertension, HbA1c, alcohol intake, smoking status, physical activity, log-transformed low density lipoprotein cholesterol, lipid-lowering drug usage, eGFR, and diabetes duration {odds ratio (OR) 1.72 [95% confidence interval (CI) 1.02-2.89], $P = 0.041$}. The OR refers to a doubling in biomarkers.

**Conclusions:** Serum adiponectin levels were higher in DPN patients compared to non-DPN patients in this Chinese T2D population. Serum adiponectin levels were positively associated with DPN presence, independent of multiple confounders.

**Keywords: adiponectin, diabetic peripheral neuropathy, type 2 diabetes, Chinese patients, biomarker**

## INTRODUCTION

A 2017 survey by the International Diabetes Federation showed that approximately 425 million people suffer from diabetes mellitus (DM) worldwide, including about 114 million people in China (1). DM can cause a variety of chronic complications, among which diabetic peripheral neuropathy (DPN) is an important cause of disability or death. The study found that, with prolonged disease, DPN prevalence was as high as 30% or more (2). However, DPN pathogenesis remains unclear, thus limiting disease prevention and treatment.

Adiponectin is an adipocytokine that plays many roles in human metabolism, including lipid regulation (3), glucose metabolism, and mediating the bodily response to insulin (4). Unlike DPN in T1D patients, insulin resistance and dyslipidemia are important causes of DPN in T2D patients (5). In type 2 diabetes (T2D) patients, serum adiponectin concentrations have been found to be significantly lower than those non-T2D participants (6), but not as high as those of type 1 diabetic (T1D) patients (7). Previous studies have shown that in diabetic nephropathy (DN) patients, serum adiponectin levels are notably lower than those of non-DN patients; in diabetic retinopathy (DR) patients, serum adiponectin levels are notably higher compared with non-DR patients (8). Higher adiponectin levels are beneficial for autonomic cardiovascular function in T2D patients (9), but are positively correlated with microvascular complications (10). However, the mechanism by which serum adiponectin affects DPN remains controversial due to inconsistencies in reported outcomes (8, 11, 12). Some studies found significantly higher serum adiponectin levels in DPN patients compared to non-DPN patients (11), while others found low serum adiponectin to be significantly associated with DPN incidence (12, 13). Furthermore, one study suggests that adiponectin has no relationship with diabetic distal sensorimotor polyneuropathy (DSPN) (14).

At present, no study has investigated associations between serum adiponectin levels and DPN in Chinese T2D patients. Here, we assess the correlation between adiponectin and DPN in this population using serum adiponectin levels measured in Chinese T2D patients with and without DPN.

## PARTICIPANTS AND METHODS

### Study Population

This cross-sectional study recruited 246 T2D patients aged 40 to 79 years old who were being treated in-patient at Peking Union Medical College Hospital (PUMCH) between January 2015 and December 2017. Twenty-seven patients were excluded due to missing data in one or more of the study variables (age, sex, body mass index, HbA1c, diabetes duration, hypertension, lipid profile, alcohol intake, smoking, physical activity), resulting in a final sample size of 219 participants. The study was approved by the Institutional Review Boards of PUMCH, Peking Union Medical College, and the Chinese Academy of Medical Sciences (Beijing, China). Written informed consent was obtained from each study participant. Type 2 diabetes was either self-reported disease that was validated by a medical record review using World Health Organization diagnostic criteria (1999) or determined based on current usage of antidiabetic medications, or fasting and/or 2 h glucose levels in the diabetic range as measured by standardized 75 g oral glucose tolerance test (15).

### Measurement of Serum Biomarkers

Blood samples were collected from participants following overnight fasting. The serum was separated and stored at -80°C until analysis. Serum adiponectin was measured with a human adipokine multiplexed bead-based immunoassay (Millipore, Billerica, MA, USA) on a Luminex® 200™ Bioanalyzer (Austin, TX, USA) that concurrently measured serum leptin, lipocalin-2/NGAL, IL-6, and TNF-α levels. All other measurements were performed using routine laboratory tests and certified methods.

### Assessment of Diabetic Peripheral Neuropathy

Diagnostic criteria for DPN include the presence of common symptoms (foot sensation including pain, numbness, and paresthesia) and neurological screening examinations (temperature and pinprick sensation, 10-g monofilament test, vibration perception with 128-Hz tuning fork, and ankle reflexes). Diabetic peripheral neuropathy was assigned if there was at least one abnormal screening test in patients with DPN symptoms or at least two abnormal screening tests in patients without DPN symptoms (16). Patients with other forms of neuropathy, including chronic inflammatory demyelinating polyneuropathy, infections, malnutrition, exogenous toxins or drugs, hypothyroidism, and renal failure were excluded from the study (5).

### Assessment of Covariates

Participant height, weight, and blood pressure (BP) were measured by standardized methods. Body mass index (BMI) was calculated as $kg/m^2$. Hypertension was diagnosed if participant blood pressure was ≥140/90 mmHg or if participant was using antihypertensive medication. Trained medical interviewers collected participant medical history, smoking and alcohol habits, and physical activity. Participants with ≥1 h of sports activity per week during at least one athletic session were classified as physically active. Alcohol intake was defined as none (0 g/day), moderate (0–20 g/day for women, 0–40 g/day for men), or high (≥20 g/day for women, ≥40 g/day for men). Metabolic variables collected from medical records included serum fasting blood glucose, total cholesterol, high-density lipoprotein (HDL) and low-density lipoprotein-cholesterol (LDL-C), triglycerides, uric acid, and plasma hemoglobin A1c (HbA1c). Dyslipidemia was diagnosed in participants who either used lipid-lowering medication or had

a total cholesterol level ≥5.70 mmol/L, and/or LDL-C level ≥2.59 mmol/L, and/or HDL-C level ≤ 0.91 mmol/L, and/or triglycerides level ≥1.70 mmol/L (17). Estimated glomerular filtration rate (eGFR) was calculated using the Chronic Kidney Disease Epidemiology Collaboration equation (18). Those potential confounders were selected based on previous literature (12).

## Statistical Analysis

Continuous variables conforming to a normal distribution were presented as mean ± standard deviation (SD), otherwise they were presented in interquartile ranges with a median ($25^{th}$, $75^{th}$ percentiles). Binary variables were presented as percentages. Differences between DPN and non-DPN participants were analyzed by *t* test, Wilcoxon test, or $\chi 2$ test as appropriate. Associations between adipocytokines and DPN were analyzed separately using logistic regression after adjusting for age and sex, then subsequently adjusting for BMI, hypertension, HbA1c, smoking status, physical activity, and log-transformed LDL-C; data were further adjusted for the use of lipid-lowering medications, eGFR, and diabetes duration. In the sensitivity analyses, we examined the interaction between age groups (<60 *vs.* ≥60), sex, lipid-lowering medications, diabetic retinopathy, diabetic nephropathy and each adipocytokine, by adding appropriate cross-product term in the model. If there were statistically significant interaction effect, we would further conduct stratified analyses. All statistical analyses were performed using SAS 9.3 (SAS Institute, Cary, NC). All *P* values were two-sided and were considered statistically significant if <0.05.

# RESULTS

## Clinical and Biochemical Outcomes

Clinical and biochemical characteristics for the T2D patients in this study are summarized in **Table 1**. Patients with DPN (*n*=98) had higher serum adiponectin and leptin concentrations than those without DPN (*n*=121). Most of the characteristic data were similar between the two groups, although the participants with DPN were slightly older, had a longer diabetes duration, higher HbA1c levels, and lower eGFR levels. Furthermore, they tended to be less physically active and more often used lipid-lowering drugs (**Table 1**).

**TABLE 1** | Characteristics of Type 2 diabetic participants stratified by DPN status.

| Variable | Without DPN (*n* = 121) | With DPN (*n* = 98) | *P* value |
|---|---|---|---|
| Age (years) | 58.0 (52.0, 63.0) | 62.5 (54.0, 70.0) | **0.001** |
| Male (%) | 48.8 | 43.9 | 0.471 |
| Diabetes duration (years) | 10 (5, 15) | 16 (10, 21) | <0.001 |
| Height (cm) | 168 (160, 172) | 165 (160, 172) | 0.394 |
| BMI (kg/m$^2$) | 26.3 ± 3.4 | 26.4 ± 4.3 | 0.742 |
| Fasting blood glucose (mmol/L)* | 2.02 ± 0.31 | 2.10 ± 0.33 | 0.052 |
| HbA1c (%) | 7.4 (6.7, 8.8) | 8.0 (6.7, 9.4) | **0.047** |
| Hypertension (%)† | 62.0 | 67.4 | 0.410 |
| Systolic BP (mmHg) | 135.0 (120.0, 144.0) | 139.5 (120.0, 146.0) | 0.372 |
| Diastolic BP (mmHg) | 78.3 ± 10.3 | 76.9 ± 11.1 | 0.317 |
| Dyslipidemia (%)# | 85.1 | 86.7 | 0.734 |
| Total cholesterol (mmol/L) | 4.57 (3.73, 5.38) | 4.26 (3.58, 5.14) | 0.289 |
| Fasting triglycerides (mmol/L) | 1.62 (1.08, 2.26) | 1.64 (1.13, 2.36) | 0.684 |
| HDL-cholesterol (mmol/L)* | 0.008 ± 0.26 | 0.044 ± 0.30 | 0.345 |
| LDL-cholesterol (mmol/L)* | 0.953 ± 0.32 | 0.866 ± 0.40 | 0.080 |
| Use of lipid-lowering drugs (%) | 28.9 | 46.9 | **0.006** |
| Uric acid (umol/l) | 340.2 ± 82.3 | 351.8 ± 106.9 | 0.378 |
| eGFR (ml/min/1.73 m$^2$) | 93.6 ± 21.0 | 84.4 ± 30.0 | **0.011** |
| Smoking (%) | | | 0.547 |
| Current | 28.9 | 23.5 | |
| Former | 11.6 | 15.3 | |
| Never | 59.5 | 61.2 | |
| Alcohol intake (%)‡ | | | 0.064 |
| High | 24.8 | 12.5 | |
| Moderate | 4.1 | 5.1 | |
| None | 71.1 | 82.7 | |
| Physically active (%)§ | 34.7 | 17.4 | **0.004** |
| Biomarkers | | | |
| Adiponectin (mg/ml) | 8.13 (5.30, 11.13) | 9.63 (6.73, 14.13) | **0.004** |
| Leptin (ng/ml) | 5.87 (2.90, 11.87) | 7.94(2.98, 20.13) | **0.048** |
| NGAL (ng/ml) | 103.04(71.55, 200.64) | 101.75(79.13, 172.54) | 0.944 |
| IL-6 (pg/ml) | 7.00 (3.18, 22.82) | 7.55 (3.78, 14.88) | 0.794 |
| TNF-α (pg/ml) | 10.97 (6.31, 25.16) | 12.13 (7.16, 17.69) | 0.933 |
| hsCRP (mg/L)* | 0.19 ± 0.96 | 0.04 ± 1.10 | 0.271 |

*Data are given as mean ± SD, median ($25^{th}$, $75^{th}$ percentiles), or percentages.*
*BMI, body mass index; DPN, diabetic peripheral neuropathy; HbA1c, hemoglobinA1c; BP, blood pressure; HDL, high-density lipoprotein; LDL, low-density lipoprotein; eGFR, estimated glomerular filtration rate; NAGL, neutrophil gelatinase-associated lipocalin; IL, interleukin; TNF, tumor necrosis factor; hsCRP, high-sensitivity C-reactive protein; Fbg, fibrinogen.*
**Data were log2-transformed before analysis.*
*†Hypertension was assigned if blood pressure ≥140/90 mmHg or use of anti-hypertensive medication.*
*#Dyslipidemia was diagnosed in participants who used lipid-lowering drugs or had a total cholesterol level ≥5.70 mmol/L, and/or LDL-C level ≥2.59 mmol/L, and/or HDL-C level ≤1.81 mmol/L, and/or triglycerides level ≥1.70 mmol/L.*
*‡Alcohol intake was classified as none (0 g/day), moderate (≥0 to <20 g/day for women, ≥0 to <40 g/day for men), or high (≥20 g/day for women, ≥40 g/day for men)*
*§Physically active was defined as ≥1 h sports activity per week in at least one athletic season.*
*Boldface type indicates statistical significance (P<0.05).*

## Association Between Serum Adiponectin and Diabetic Peripheral Neuropathy

Correlation between serum adipocytokines and DPN was analyzed using multiple logistic regression analysis (**Table 2**). Results were adjusted for age, gender, BMI, hypertension, HbA1c levels, log-transformed LDL-C levels, alcohol intake, smoking status, and physical activity (models 1–2). The associations between serum adiponectin and leptin levels in DPN patients were statistically significant with an odds ratio (OR) for adiponectin of 1.91, 95% confidence interval (CI): 1.17–3.11, *P*=0.009; OR for leptin: 1.04, 95% CI: 1.00–1.07, *P*=0.040). The positive association with DPN persisted after adjustment for eGFR, diabetes duration, and use of lipid-lowering medications (model 3) for serum adiponectin (OR: 1.72, 95% CI: 1.02–2.89, *P*=0.041), but not for leptin (OR: 1.02, 95% CI: 0.99–1.06, *P*=0.220). In the sensitivity analyses, there were no statistically significant multiplicative interaction between age groups (<60 vs.

**TABLE 2 |** OR and 95% CI for the association between biomarkers and DPN.

| Variable | Model 1 | | Model 2 | | Model 3 | |
|---|---|---|---|---|---|---|
| | OR (95% CI) | *P* value | OR (95% CI) | *P* value | OR (95% CI) | *P* value |
| Adiponectin | **1.60 (1.06–2.43)** | **0.026** | **1.91 (1.17–3.11)** | **0.009** | **1.72 (1.02–2.89)** | **0.041** |
| Leptin | **1.04 (1.01–1.07)** | **0.007** | **1.04 (1.00–1.07)** | **0.040** | 1.02 (0.99–1.06) | 0.220 |
| NGAL | 1.06 (0.71–1.60) | 0.778 | 1.08 (0.69–1.67) | 0.739 | 0.90 (0.56–1.45) | 0.670 |
| IL-6 | 0.95 (0.83–1.09) | 0.493 | 0.97 (0.84–1.12) | 0.658 | 0.98 (0.84–1.15) | 0.831 |
| TNF-α | 0.92 (0.71–1.19) | 0.526 | 0.88 (0.66–1.16) | 0.349 | 0.83 (0.61–1.11) | 0.212 |
| hsCRP | 0.89 (0.68–1.17) | 0.405 | **0.69 (0.50–0.97)** | **0.030** | **0.62 (0.43–0.89)** | **0.009** |

*ORs, 95%CIs, and corresponding P values are given for a doubling in circulating of biomarkers.*
*Data were log2-transformed before logistic regression except for leptin. Serum adipocytokines were included in the models separately. OR was for per 1-unit increase in corresponding variables. Model 1: adjusted by age and gender. Model 2: adjusted by model 1 variables plus BMI, hypertension, HbA1c level, alcohol intake, smoking status, physical activity, and log-transformed LDL-C level. Model 3: adjusted by model 2 variables plus use of lipid-lowering medications, eGFR, and disease duration.*
*Boldface type indicates statistical significance (P < 0.05).*

≥60), sex, lipid-lowering medications, diabetic retinopathy, diabetic nephropathy, and each adipocytokine (*P* value for interaction >0.05 for all).

## DISCUSSION

This study found a positive association between serum adiponectin levels and the presence of DPN in T2D Chinese patients and, after adjusting for potential confounders, that association persisted.

Recently, many studies have investigated the relationship between serum adiponectin levels and DPN. A cross-sectional study from India tested serum adiponectin in 487 T2D patients, and the authors found that adiponectin levels are significantly higher in diabetic patients with neuropathy than in those without (11). The same results appeared in a similar study (8). Jung et al. measured adiponectin and evaluated DPN in 153 diabetic patients, and reported that high serum adiponectin levels are independently associated with a higher incidence of neuropathy. In the aforementioned studies, the diagnosis of neuropathy in the former was based on only one detection method, a biothesiometer to assess vibratory perception threshold (VPT). In contrast, the assessment of peripheral neuropathy of the latter was similar to this study, and was evaluated based on typical symptoms and neurological tests. Besides, the participants in these three studies were all Asians and had certain similarities in demographic information.

However, the KORA F4/FF4 study found that low serum adiponectin levels were related to DPN incidence (12). This difference with the results of the present study may be attributable to the following factors. KORA F4/FF4 was a population-based cohort study, with a DPN incidence of 133 DPN and 397 participants with no incidence of DPN, and investigated an elderly population aged 62 to 81 years. Our study is a cross-sectional study of an in-patient population with a relatively small sample size (*n*=219), however the age ranged from 40 to 79 years old. The ethnic compositions of participants in these two studies were significantly different, and adiponectin concentration levels were affected by different genetic backgrounds, potentially leading to different findings based on ethnic differences (19). In addition, the KORA F4/FF4 study used the examination part of the Michigan Neuropathy Screening Instrument (MNSI) and a 10-g monofilament as DPN diagnostic criteria, while our diagnostic criteria utilized common DPN symptoms and neurological screening tests. These confounding factors may have led to the different results observed in the two studies.

In addition, there were studies that differ from the above-mentioned research conclusions. A cross-sectional study from Japan analyzed the relationship between nerve conduction velocity (NCV) and plasma adipocytokines (TNFα, adiponectin, and leptin) in 105 T2D patients (20). The authors reported that there was no significant relationship between plasma adiponectin and NCV. Another study (21), also from Japan, showed that neither serum total nor high molecular weight adiponectin was correlated with DPN in 198 diabetic subjects. The inconsistency between the current research and the previous researches may be due to small sample sizes, If the sample size is appropriately expanded, perhaps the differences will be significant. What's more, these two studies diagnosed DPN by NCV or bilateral ankle reflex, leading to more advanced DPN patients, whose inflammation was less pronounced (22).

Adiponectin has multiple physiological functions in the human body. Adiponectin can improve insulin resistance induced by a high-fat diet, suggesting that adiponectin may have an insulin-sensitizing effect (23) that may be achieved by activating the AMP-activated protein kinase signaling pathway to promote glucose and fatty acid utilization (24). Another study found that adiponectin may be independent of insulin level, inhibiting glucose production, and promoting glucose assimilation (25). Thus, high concentrations of adiponectin are beneficial for patients with T2D. Adiponectin is the most important anti-inflammatory adipocytokine; it has been found to inhibit the NF-κB-signaling pathway in endothelial cells (26) and macrophages (27), and is involved in the transformation of macrophages from pro-inflammatory M1 to anti-inflammatory M2 cells (28). Adiponectin may alleviate the symptoms of DPN by inhibiting p38 mitogen-activated protein kinase (p38 MAPK) activation, as well as the transient receptor potential cation channel subfamily V member 1 (TRPV1) and calcitonin gene-related peptide (CGRP) signal pathways in dorsal root ganglions

neurons (29). Some studies indicate that high concentrations of leptin, the first adipocytokine to be identified, positively correlate with insulin resistance, T2D, and cardiovascular diseases (30). Leptin can upregulate pro-inflammatory cytokines, such as TNF-α and IL-6, while adiponectin has anti-inflammatory properties. However, the specific mechanism of adiponectin in DPN remains unknown. Adiponectin may play no role in the pathophysiology of DPN, and may be increased to counteract deleterious effects of pro-inflammatory cytokines, thus representing an indirect risk marker. It may also have a novel effect on DPN (31, 32).Strengths of this study are as follows: we explore the association between serum adiponectin levels and DPN prevalence in Chinese T2D patients. Additionally, our data allowed us to control multiple potential confounding variables between serum adiponectin levels and DPN. We used multivariate analyses to account for DPN risk factors as potential confounders.

However, our study has some limitations. First, the cross-sectional observational design may limit findings. Second, the statistical power of our conclusions is low, calling into question whether non-significance was due to a lack of relationship between the groups or due to lack of statistical power. Therefore, results should be interpreted with caution. Large-scale prospective trials are needed to properly evaluate the role of adiponectin in DPN.

## CONCLUSION

Our results show that serum adiponectin levels in DPN participants were higher than those of non-DPN participants in a Chinese T2D population. Serum adiponectin levels were positively associated with DPN, independent of multiple potential confounders. Future prospective studies are needed to evaluate whether adiponectin can be used as a biomarker and therapeutic target for DPN.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institutional Review Boards of PUMCH, Peking Union Medical College, and the Chinese Academy of Medical Sciences (Beijing, China). The patients/participants provided their written informed consent to participate in this study.

## AUTHOR CONTRIBUTIONS

XL and GT contributed to the concept and design of the study and supervised this research. QS, DY, CW, QZ, XS, and YS performed the clinical study. QS interpreted the data, drafted part of the manuscript and finally approved the submission of this research. BY wrote part of the manuscript and submitted the manuscript. JG conducted statistical analysis. GT and XL are responsible for the overall contents. All authors contributed to the article and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2020.567959/full#supplementary-material

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REVIEW
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# Advances in Screening, Early Diagnosis and Accurate Staging of Diabetic Neuropathy

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The incidence of both type 1 and type 2 diabetes is increasing worldwide. Diabetic peripheral neuropathy (DPN) is among the most distressing and costly of all the chronic complications of diabetes and is a cause of significant disability and poor quality of life. This incurs a significant burden on health care costs and society, especially as these young people enter their peak working and earning capacity at the time when diabetes-related complications most often first occur. DPN is often asymptomatic during the early stages; however, once symptoms and overt deficits have developed, it cannot be reversed. Therefore, early diagnosis and timely intervention are essential to prevent the development and progression of diabetic neuropathy. The diagnosis of DPN, the determination of the global prevalence, and incidence rates of DPN remain challenging. The opinions vary about the effectiveness of the expansion of screenings to enable early diagnosis and treatment initiation before disease onset and progression. Although research has evolved over the years, DPN still represents an enormous burden for clinicians and health systems worldwide due to its difficult diagnosis, high costs related to treatment, and the multidisciplinary approach required for effective management. Therefore, there is an unmet need for reliable surrogate biomarkers to monitor the onset and progression of early neuropathic changes in DPN and facilitate drug discovery. In this review paper, the aim was to assess the currently available tests for DPN's sensitivity and performance.

**Keywords: microvascular complications, Diabetic Neuropathy, Screening, Diagnosis, Early Detection, neuropathy biomarkers**

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## INTRODUCTION

Diabetes is one of the fastest-growing health challenges of the 21st century, with the number of adults living with diabetes having more than tripled over the past 20 years (1). The International Diabetes Federation reported that in 2019, the prevalence of diabetes was 9.3% (463 million people worldwide) with a predicted rise to 10.9% (700 million people) by 2045 (2). Furthermore, it has been shown that over 1.1 million children and adolescents below 20 years have type 1 diabetes. On top of

these staggering figures, are the number of people with impaired glucose tolerance (IGT) or metabolic syndrome with 373.9 million in 2019 (7.5%) and predicted rise to 548.4 million (8.6%) by 2045 (2).

In the UK alone, there were 4.8 million people with diabetes in 2019. Diabetes is on the rise. Figures from Diabetes UK shows that someone is diagnosed with diabetes every two minutes, with 5.3 million expected to be living with the condition by 2025 (3).

Diabetes is strongly associated with both microvascular and macrovascular complications. As a result, 10% of global health expenditure, equal to USD 760 billion, is directed toward diabetes and its complications (2). Microvascular changes lead to nephropathy, retinopathy and neuropathy. Among these complications, diabetic peripheral neuropathy (DPN) is the most common and costly diabetes-associated complication, occurring in around 50% of individuals with diabetes (4). Distal symmetric polyneuropathy (DSPN) (5) typically follows a distal-proximal course and results in symmetrical symptoms and signs between the body's left and right sides. Common symptoms include burning, numbness, tingling, pain and/or weakness starting in the distal lower extremities which progress into more extreme symptoms of neuropathic pain in around 10-30% of affected patients (6, 7). Symptoms may be sporadic or constant but can be debilitating and in many people lead to depression, sleep disorders and overall reduced quality of life (8).

The true prevalence of DPN is underestimated as its assessment is challenging. However, DPN is recognized as the most common complication of diabetes.

DPN is the strongest initiating risk factor for diabetic foot ulceration (neuropathic ulcer) (9, 10), and existing ulcers may be further exacerbated from damage to sensory neurones. Resultant limb numbness causes ulcers to remain undetected for longer periods (10); thus, corrective actions are not taken nor advice sought at early stages of the disease. Often the first sign that a person has diabetic peripheral neuropathy (DPN) is a foot ulcer, which may lead to irreversible tissue damage, lower limb amputation and significant morbidity.

In the UK, people with diabetes account for more than 40% of hospitalizations for major amputations and 73% of emergency admissions for minor amputations. A single diabetes related foot ulcer can take over 240 days to put into remission and costs £8,000 pa to treat. Ulcers frequently recur and eventually may require the amputation of a lower limb. DPN is hugely costly to our NHS (>£1.1 billion pa in direct medical costs) and to the wider UK economy (~£4 billion), is particularly debilitating and distressing for patients and their families and can lead to an untimely death (11, 12), with five-year mortality ranging from 52 to 80 percent after major amputation (13).

Furthermore, with diabetes related lower limb amputations increasing at the rate of almost 20% per annum in line with the increasing prevalence of diabetes, there is a huge strain on NHS budgets which are unable to keep up.

Autonomic neuropathies are a class of DPN which share similar diffuse pathophysiology with DSPN, but differ by being largely non-sensory (4). These typically affect the cardiovascular, urogenital and gastrointestinal systems. Patients may also suffer from sudomotor dysfunction, hypoglycemia obliviousness, and abnormal pupillary function (5). Rare forms of DPN include mononeuropathies, polyradiculopathies and treatment-induced neuropathies (5). These atypical forms are generally self-limiting and resolve with medical management and physical therapy, usually over several months (11).

In clinical settings, there are several different approaches to assess diabetic peripheral neuropathy (DPN), and the choice of the test will depend on the aim of testing. It is usually sufficient in a busy clinic to establish whether a patient is symptomatic, particularly of painful DPN (12), and whether or not they are at high risk of foot ulceration typically through monofilament testing. However, to fully assess damage and phenotype of DPN, sensory deficits must be detected early. Those accurate biomarkers are available for monitoring of DPN and for use in clinical trials of potential new treatments.

Currently, there are no simple markers for early detection of DPN in routine clinical practice. The measures we use are crude and detect the disease very late in its natural history. Even the benefits gained by standardizing clinical assessment with scored clinical evaluations remain subjective, heavily reliant on the examiners' interpretations.

This paper reviews the current knowledge and the optimal approaches for diagnosis and screening of diabetic peripheral neuropathy.

## TYPES OF NERVE FIBERS

Peripheral nerve fibers can be classified using Erlanger and Gasser's classification, which defines nerves based on diameter, conduction speed, and myelination level (**Table 1**). A-fibers have the largest diameter, with the thickest myelination and fastest conduction speed, and act as sensory and motor fibers within the somatic nervous system. They may be further divided into large nerve fibers that have sensory and motor functions (Aα and Aβ), and small nerve fibers (Aγ which has motor functions, and Aδ which may be autonomic or sensory fibers) (14).

Group B-fibers are small, with moderate myelination and slower conduction velocities than A-fibers. B-fibers act mainly as general visceral afferent and pre-ganglionic fibers and are found only in the autonomic nervous system.

Group C-fibers have a small diameter, low conduction velocity and are the only unmyelinated group. They act as somatic, afferent fibers that carry sensory information relating to temperature and pain, as well as having autonomic functions such as the stimulation of the sweat glands (14).

### Epidemiology

The prevalence of diabetic peripheral neuropathy (DPN) reported in various studies ranges from 6% to 51% depending on the population (15, 16). In the Diabetes Control and Complications Trial/Epidemiology of Diabetes Interventions and Complications (DDCT/EDIC) Study, the prevalence of DPN in adults with type 1 diabetes was 6% at baseline and increased to 30% after 13–14 years of follow-up (17). The prevalence of DPN among adults with type 1 diabetes in the

**TABLE 1** | Classification of nerve fibers in the peripheral nervous system according to modified Erlanger and Gasser.

| Classification | Myelination | Diameter (um) | Conduction Velocity (m/s) | Type | Function |
|---|---|---|---|---|---|
| **Aα (alpha)** | Yes | 12-22 | 70-120 | Sensory/motor | Proprioception, touch sensory, somatic motor to extrafusal muscles |
| **Aβ (beta)** | Yes | 5-12 | 30-70 | Sensory/motor | Proprioception, touch/pressure sensory, somatic motor to intrafusal muscles |
| **Aδ (delta)** | Yes | 1-5 | 5-30 | Sensory | Touch and cold thermoreceptors, nociception |
| **Aγ (gamma)** | Yes | 2-8 | 15-30 | Motor | Somatic motor to intrafusal muscles |
| **B** | Yes | <3 | 3-15 | Autonomic | Visceral afferent fibers and preganglionic efferent fibers |
| **C** | No | 0.1-1.3 | 0.6-2 | Sensory/ autonomic | Temperature (warm receptors), pain perception, nociception, itching |

Pittsburgh Epidemiology of Diabetes Complications was 34% and increased significantly with age (18–29 years: 18%; ≥30 years: 58%). It has been estimated that half of all children with diabetes with a duration of 5 years or longer already have diabetic neuropathy (18) and nearly 25% of pediatric patients with newly diagnosed diabetes have abnormal findings on nerve conduction studies (NCS), indicating nerve damage (19).

The prevalence of DPN is somewhat higher in patients with T2DM when compared to T1DM (4). The 'Action to Control Cardiovascular Risk in Diabetes' (ACCORD) (20) trial and the 'Veteran Affairs Diabetes Trial' (21) found that DPN was present in 42% and 39% of adults with type 2 diabetes, respectively, at baseline measurement. A study comparing magnetic resonance imaging (MRI) scans of the sciatic nerve in T1DM, and T2DM patients with DPN found that the predominant type of nerve lesion differed between the two (22). This study found that in T1DM, lesions were predominantly associated with poor glycemic control and loss of nerve conduction, whereas in T2DM lesions were associated with changes in lipid metabolism. This raises the question of whether damage to peripheral nerves results in different patterns of nerve damage, and thus would require different types of preventive treatment.

## Risk Factors

In both main types of diabetes, the prevalence and severity of DPN increases with disease duration and increasing age (16). A large study of 1172 patients with diabetes assessed for neuropathy at baseline reported that patients who had developed neuropathy by roughly ten-year follow-up were on average 3.8 years older and had diabetes for 3.3 years longer at baseline (16). Furthermore, the study found that in both T1DM and T2DM, higher hemoglobin A1c (HbA1c) level was a significant predictor of the development of diabetic neuropathy (16).

In cohorts of patients with T2DM, several metabolic syndromes such as hypertension, abdominal obesity, lower high-density lipoprotein (HDL) levels and hypertriglyceridemia have been consistently associated with DPN development (23), with additional independent risk factors including alcohol abuse and increased height (24). In a cohort of patients with T1DM, the EURODIAB prospective complications (25) study reported similar modifiable risk factors to those identified in T2DM, explicitly having an association with raised triglyceride level, obesity, smoking and hypertension. Several genes have also been linked to an increased risk of diabetic neuropathy. Still, only ACE (encoding angiotensin-converting enzyme) and MTHFR (encoding methylenetetrahydrofolate reductase) polymorphisms have been confirmed using large patient cohorts in multiple populations (24). Research into the role of genetics in diabetic neuropathy is currently limited, and many more studies are required.

Significantly lower levels of clinical neuropathy in South Asian patients have been reported compared to Europeans and Afro-Caribbean (26). A recent study found that in a population of people with type 2 diabetes, South Asians had significantly better-preserved small nerve fiber integrity than equivalent Europeans (27). However, this patient cohort was recruited from primary care, and most patients had no or mild neuropathy, so it was not representative of the diabetic population overall. A proposed explanation for the reduced risk was the differences in the transcutaneous partial pressure of oxygen (TCpO2) and height between the ethnicities (27). However, the study suggesting this explanation did not adjust for a range of possible confounders such as obesity, and alcohol intake, between ethnicities, all of which are established risk factors for developing DPN. A more recent study suggested that the variation may be due to differences in height and adiposity between the ethnic groups, as the adjustment for these factors rendered the difference insignificant (28).

## Prevention/Treatment

There is currently no Food and Drug Administration (FDA) approved therapy to prevent or reverse human DPN (4). The current management approach focuses on reasonable glycemic control, lifestyle modifications, and management of associated pain. The reasonable glycemic control consists of not only strict HbA1c control but also reduced glycemic variability, because glycemic variability has recently emerged as an another measure of glycemic control, which might constitute an additive, or even better predictor of microvascular complications including neuropathy than mean HbA1c levels (29, 30).

Previous studies have found that improving HbA1c levels does affect DPN progression in patients with T2DM (20, 31). The ACCORD study (20) found that intensive treatment caused delay in onset of albuminuria and it reduced neuropathy, MNSI socre, loss of ankle jerks, loss of light touch at end of the study. The veterens study (31) assessed whether new evidence of clinical neuropathy occurred during the period of intensive versus normal control and had quite severe criteria for definaitions. The Epidemiology of Diabetes Interventions and Complications (EDIC) trial reported that intensive glucose control significantly delayed the development and progression of diabetic neuropathy in T1DM patients over time (17).

Another study, following a cohort of T1DM patients over 24 years confirmed these findings. Patients who had stable, near-normal HbA1c levels (mean <7.0%) had significantly less deterioration in nerve fiber function when measured using electrophysiology and quantitative sensory methods ($p<0.05$ for all measures at 24 years follow-up) (32).

Attempts have been made to reduce DPN by lifestyle interventions (24). Several studies have demonstrated a potential for improved outcomes in patients with diagnosed DPN through exercise regimes put in place over ten weeks (33) to 12 months (34). Despite insignificant improvements in body mass index (BMI), these studies reported a significant improvement in objective nerve function measures and reduced neuropathy symptoms. Neither of these studies included a control group, which is essential to provide a measure of the change in neuropathy which could be expected over time without intervention but with the same amount of scrutiny, for example, additional contact time with healthcare professionals or individuals paying more attention to their health due to taking part in a study. In the absence of a control group, it is difficult to ensure that neuropathy improvements are genuinely due to modifications in exercise regimes alone.

A recent comprehensive study in Japanese patients with T2DM under poor glycemic control (HbA1c 9.6%, 81.6 mmol/mol) at baseline assessed the impact of intensive glucose control without hypoglycemia and found that normalizing A1c over the two years of follow-up resulted in significant improvement in neuropathy outcomes (35). This study also revealed that small glycemic variability assessed by SD and coefficient of variation (CV) of monthly measured HbA1c levels was associated with the improvement of neuropathy outcomes. In the follow-up study of T2DM (a median of 9.3 years), mean HbA1c levels were the main risk predictor for the composite outcome of developing or worsening diabetic neuropathy, whereas glycemic variability assessed by HbA1c variability was a better risk predictor for new incident of neuropathy (36). In the follow-up study of T1DM the long-term glycemic variability assessed by CV and SD of HbA1c levels was linked to DPN independent of mean HbA1c (37). These studies indicated that the strict HbA1c control as a long-term mean glycemic levels and suppressed glycemic variability are required for the prevention and slow neuropathy progression in patients with diabetes.

### Screening

The American Diabetes Association (ADA) and the International Working Group on the Diabetic Foot (IWGDF) recommends regular examination of people with DM for the diagnosis of DPN and loss of protective sensation using simple standard tests for the identification of those at risk for diabetic foot ulcer (38, 39). It is recommended that all patients with T2DM be screened for DPN at diagnosis, and for T1DM, the screening should begin five years post-diagnosis (40). After this initial screening, all patients should be reviewed annually.

Nerve conduction studies (NCS) are considered the gold standard for the diagnosis of large fibers neuropathy. The Toronto consensus (41) recommended the use of abnormal NCS with a symptom or sign to diagnose DPN. However, the need for specialist examiners and equipment renders NCS inappropriate as a screening test. Thus, it is used only to confirm any possible/probable DN picked up post-screening using other measures (40).

More commonly, screening for DPN involves history taking for neuropathic symptoms and examination of the feet, along with a screening test (40). Traditional screening tests benefit from being quick and easy; however, like NCS, these only assess larger fiber function and are unable to detect any early changes in small nerve fibers. Furthermore, two systematic reviews focusing on the use of monofilament testing, a commonly used screening test for DPN, described a variation of diagnostic value in the current literature and a lack of consistency in recommended test procedure and interpretation (42). Sensitivity for peripheral nerve fiber damage ranged from 43-93% when using NCS as a reference standard (43). Both review papers did not recommend the sole use of monofilament testing to diagnose peripheral neuropathy (43). This is just one example of the shortcomings of current screening tests.

## DIAGNOSTIC TESTS FOR DPN

While there is no single accurate definition of diabetic neuropathy, a simple definition for clinical practice is the presence of symptoms and/or signs of peripheral nerve dysfunction in people with diabetes after excluding other causes (5). Based on this, the diagnostic tests are focused on assessing the symptoms and signs of nerve dysfunctions.

There are numerous testing methods available to assess the peripheral nervous system's structure and function, with each test having its own advantages and disadvantages. Bedside tests used to aid diagnosis of DPN—including the 10g monofilament (**Figure 1D**), the Ipswich Touch Test, and vibration perception threshold testing with the Vibratip (**Figure 1E**), a tuning fork, or automated devices such as Neurobiothesiometer, are not only reliant on patients' subjective response but are also mainly used to identify the loss of protective foot sensation and risk of ulceration. As such, these tests tend to diagnose DPN when it is already well established. Late diagnosis hampers the potential benefits of intensified multifactorial intervention at an early stage of the disease, which could prevent the sequelae of DPN. Unfortunately, by the time DPN is detected with the crude tests currently used, it is often very well established and consequently impossible to reverse or halt the inexorable neuropathic process. Early diagnosis and timely intervention are thus essential in preventing the development of DPN.

Some of the most common tests and methods for diagnosis of DN have been summarized in **Table 2**.

### Symptoms

Various clinical scoring systems are available for DPN screening which involve symptom scoring, sign scoring or both (**Table 3**). These systems may enhance diagnostic accuracy through a composite score of different combined tests and are useful tools for aiding diagnosis of DPN, along with quantitative measures. Each questionnaire has a scoring system which can


**FIGURE 1** | Examples of nine different tests for diabetic peripheral neuropathy (DPN), **(A)** Physical examination and Neuropathy disability score (NDS), **(B)** Nerve Conduction studies being performed on the lower leg, **(C)** DPNcheck device to test sural nerve conduction performed on the lower leg, **(D)** Monofilament screening test sights and procedure, **(E)** Vibration perception threshold testing, **(F)** Medoc TSAII quantitative sensory testing device for thermal perception threshold, **(G)** Sudoscan equipment. Hand and feet sensor plates with displaying test results, **(H)** Neuropad test demonstrated original blue color, **(I)** A punch skin biopsy to collect samples needed for IENFD measurement, **(J)** An image of the corneal sub-basal nerves using corneal confocal microscopy and the CCM probe positioning during corneal scanning.

diagnose, and in some, stratify disease severity. **Table 3** presents a summary of the most commonly used questionnaires for assessing DPN.

The Neurological Symptom Score (NSS) is a 17 question, interview-based assessment of sensory, motor, and autonomic function used to screen DPN (87). Still, it is considered too extensive to be used efficiently in clinical practice. The diabetic neuropathy score (DNS) is an adaptation of the NSS that is a much quicker screening method, with only four questions and still offering moderate sensitivity (79%) and specificity (78%), but with slightly lower reliability for diagnosing DPN (45) when using a diagnostic score of 1 or more.

Other symptom scoring systems focus only on pain and differentiating neuropathic from other causes. Clinicians commonly recognize pain descriptors that are used by patients with neuropathic pain. The McGill pain questionnaire was the first questionnaire designed to offer a multidimensional assessment of pain, which included assessing severity or intensity, emotional impact, and significance to the pain sufferer (88). This questionnaire is one of the most commonly used multi-dimensional pain scales globally. A short-form is available for use in screening which has shown good agreement with the original version (89).

## Signs

The Neuropathy disability score (NDS) is a commonly used clinical examination method that assesses neuropathy signs (**Figure 1A**). Thirty-five items are used for both sides, evaluating cranial nerve damage, muscle strength, sensation loss and reflex delay/loss (90). However, some of the items have demonstrated a weak relation to DPN, and the full scoring system is too long to be used in clinical practice. Therefore a revised NDS has been created. This system is more commonly used and tests for four main neuropathy signs; ankle reflex, vibration, pinprick and temperature sensation at both sides of the largest toes. A maximum score is 10, and usually, more than 6 is considered abnormal (91).

## Composite Scoring Systems

The reliance on symptoms or signs alone may lead to low diagnostic accuracy for the presence of DPN, and a combination of both allows a more thorough assessment. Several scoring systems assess both signs and symptoms of DPN to produce a composite score. The Toronto clinical neuropathy score (TCNS) consists of three parts: symptom scores, reflex test scores and sensory test scores. The maximum score is 19, and the test is able to stratify patients into no DPN, mild DPN, moderate DPN and severe DPN depending on the overall score (92). Testing has proven validity and reliability for diagnosing and staging DPN compared to electrophysiology measures (92).

The Michigan neuropathy screening instrument (MNSI) is another commonly used composite scoring system that includes a questionnaire and a foot examination (77). Neuropathy can be defined as seven or more positive responses to this symptoms

**TABLE 2** | Diagnostic tests available for assessing DPN.

| | | Nerve Fibers Assessed | Advantages | Limitations |
|---|---|---|---|---|
| **Symptoms and Signs** | **Questionnaires** | Large (Aβ-fibers) and Small (Aδ and C-fibers) | Easy to administer. Used for monitoring symptoms (44) | Lack of Sensitivity, accuracy and reproducibility, Subjective (45) |
| | **NDS** | Large (Aβ-fibers) and Small (Aδ and C-fibers) | Does not require specialist equipment, Assesses large and small-fiber function (46) | Not sensitive or reproducible, Low correlation with small fiber quantitative tests (47) |
| | **10-gram Monofilament** | Large (Aβ-fibers) | Simple, quick and inexpensive (48). | No standardization of methods. Cannot detect early neuropathy (48). |
| | **Ipswich Touch Test** | Large (Aβ-fibers) | Simple. Requires no specialist equipment (49).Can test at home | Can only detect advanced neuropathy (48) |
| | **QST** (Thermal and Vibration thresholds) | Large (Aβ-fibers) and Small (Aδ and C-fibers) | Measures small and large fiber function (44) Good repeatability (50) | Unable to differentiate between peripheral and central abnormalities (51) High inter-operator variability (52) |
| **Large FiberTests** | **DPNCheck** | Large, sural nerve (Aβ-fibers) | Quick, Easy to perform,Good sensitivity (92-95%) compared to NCS (53, 54) | Relies on the accessibility of sural nerve (55). Validation studies had small patient numbers (53, 54) |
| | **NCS** | Large (Aβ-fibers) | A sensitive measure of large nerve function (56),Reproducible (57) | Doesn't assess small fibers, Uncomfortable (58), Does not assess early neuropathic changes |
| **Small Fiber Tests** | **Skin Biopsy (IENFD)** | Small (C-fibers) | Gold standard for SNF, Quantitative,Good sensitivity,Detects early nerve changes (59, 60) | Invasive, Risk of infection, Repeatability, Requires trained personnel and special labs (44) |
| | **CCM** | Small (Aδ and C-fibers) | Non-invasive,Good reproducibility (61), Rapid and objective (62, 63) | Relatively Expensive,Requires specialist equipment and personnel,manual analysis is time-consuming (44) |
| **AutonomicTests** | **Neuropad** | Small (C-fibers) | Can be self-administered,suitable for screeningNon-invasiveGood sensitivity (54, 64–69) | Varied interpretation of the results (54, 64–67) |
| | **Sudoscan** | Small (C-fibers) | Non-invasive,Easy to perform | Unclear if measuring sudomotor function Variable specificity (53-92%) (70–74) |
| | **QSART** | Small (C-fibers) | Sensitive for SFN (82%) (75) Gold standard for measuring sudomotor function | Time-consuming, Requires specialist equipment and trained personnel (76) Uncomfortable |

*IENFD, Intra-epidermal nerve fiber density; NCS, Nerve conduction studies; QSART, Quantitative sudomotor axon reflex test; CCM, Corneal confocal microscopy; NDS, Neuropathy disability score; QST, Quantitative sensory testing; SFN, Small fiber neuropathy.*

section alone (77). The foot examination is more frequently used and encompasses foot appearance (including ulcers), ankle reflex and the 128-Hz tuning fork test (77). One study (93) found a range of sensitivity (35-79%) and specificity (65-94%) in comparison to NCS depending on the cut-off value used for abnormality in MNSI. The higher specificity values indicate a potential high diagnostic impact for MNSI scoring; however, the lower sensitivity range indicates that milder DPN cases are likely not to get picked up.

Scoring of symptoms and signs is convenient and easy to perform as a method of screening for DPN. These tests are easily interpreted, making them a useful tool in supporting decisions on which patients should be referred on for specialist assessment. Quantitative, objective measures should be considered when the patient has signs and symptoms other than those rated by the scoring test.

## Large Fiber Tests

### Nerve Conduction Studies (NCS)

The current 'gold standard' for clinical diagnosis of DPN is through nerve conduction studies (NCS) by a trained neurophysiologist (**Figure 1B**). In 2010, the Toronto Consensus, by an expert panel recommended that one abnormal finding as part of NCS, combined with a symptom or sign of neuropathy should be used to confirm DPN (41). NCS has also demonstrated an ability to predict future DPN (94).

For reliable NCS results, close attention must be paid to factors such as filter setting, limb temperature, and recording location, as outcomes can be vulnerable to variations. Trials have demonstrated that NCS consistently demonstrate excellent intra-observer agreement (58, 95); however, a poor inter-observer agreement between expert clinical neurophysiologists is common (58) when no standardized, specific technique is followed. One study (95) assessed the results of 4 neurophysiologists, from 4 different centers. Specific assessment methods were provided in a specially prepared syllabus, and a training session was provided beforehand. The outcome was a significant improvement in inter-observer agreement with a standardized approach, and although not entirely eliminated, levels of disagreement were consequently considered clinically significant for medical practice (95).

Conversely, when considering the use of NCS in therapeutic clinical trials, even small inter-observer variability may be significant enough to impact results through impacting the statistical power of a study and thus the trial's outcomes. This may partially explain why previous clinical trials have used NCS as a primary outcome to detect treatment efficacy and have reported failed outcomes (96–98). Evidence supports the use of a single observer to repeat electrophysiological tests on each patient in these trials.

Furthermore, Standard NCS testing is not easily applicable as a screening tool for DPN since it is time-consuming, requires a specialist operator and can be uncomfortable for the patient (58).

**TABLE 3 |** Summary of questionnaires available for assessing DPN.

| | Questionnaire | Assessed | Type of Administration | Scoring |
|---|---|---|---|---|
| **Symptoms** | NSP | Symptoms of neuropathy | Clinician administered | **34** categories (women)<br>**36** categories (men) |
| | DNS | Symptoms of diabetic peripheral neuropathy | Clinician administered | 4 for Symptoms<br>**(Total 4)** |
| | NSS | Symptoms of neuropathy | Clinician administered | 8 for muscle weakness<br>5 for sensory disturbances<br>4 for autonomic symptoms<br>**(Total 17)** |
| | NPQ | Symptoms of neuropathic pain | Completed by the patient | **Total of 12** |
| | NPSI | Symptoms of neuropathic pain | Completed by patient | 10 descriptors, 2 duration<br>**(Total 12)** |
| | McGill Pain Questionnaire | Multidimensional symptoms of pain | Clinician administered | Subclass 1- Sensory<br>Subclass 2 - Affective<br>Subclass 3 - Evaluative<br>Subclass 4 - Miscellaneous<br>**(Total 78)** |
| | CNE | Signs of peripheral neuropathy | Clinician administered | 21 for sensory testing<br>8 for muscle strength<br>4 for ankle reflex<br>**(Total 33)** |
| **Signs** | NDS | Signs of peripheral neuropathy | Clinician administered | 2 for vibration sensation<br>2 for temperature sensation<br>2 for pinprick<br>4 for ankle reflex<br>**(Total 10)** |
| | DNE | Signs of peripheral neuropathy | Clinician administered | 4 for muscle strength<br>2 for reflex responses<br>10 for sensory testing<br>**(Total 16)** |
| | NIS-LL | Signs of neuropathy in the lower limbs | Clinician administered | 64 for muscle strength<br>16 for sensory testing<br>8 for reflex responses<br>**(Total 88)** |
| | MNDS | Signs of peripheral neuropathy | Clinician administered | 12 for sensory tests<br>18 for muscle strength<br>16 for reflex testing<br>**(Total 46)** |
| | UENS | Signs of peripheral neuropathy | Clinician administered | 11 for each side<br>**(Total 22)** |
| **Symptoms and Signs** | DN4 | Symptoms and signs of neuropathic pain | Clinician administered | 7 for symptoms<br>3 for signs<br>**(Total 10)** |
| | LANSS | Symptoms and signs of neuropathic pain | Clinician administered | 5 for symptoms<br>2 for signs<br>**(Total 7)** |
| | TCNS | Signs and symptoms of peripheral neuropathy | Clinician administered | 6 for symptoms<br>5 for sensory tests<br>8 for reflex tests<br>**(Total 19)** |
| | MNSI | Signs and symptoms of peripheral neuropathy | Symptoms by patient<br>Foot examination by a clinician | 15 for symptoms<br>8 for foot examination<br>**(Total 23)** |

*NSP, Neuropathy Symptoms Profile; NPQ, Neuropathic Pain Questionnaire; DNS, Diabetic Neuropathy Symptom; NSS, Neuropathy Symptom Score; NPSI, Neuropathic Pain Symptom Inventory; CNE, Clinical Neurological Examination; NDS, Neuropathy Disability Score; DNE, Diabetic Neuropathy Examination; NIS-LL, Neuropathy Impairment Score in the Lower Limbs; MNDS, Michigan Neuropathy Disability Score; UENS, Utah Early Neuropathy Scale; DN4, Douleur Neuropathique en 4; LANSS, Leeds Assessment of Neuropathic Symptoms and Signs; TCNS, Toronto Clinical Neuropathy Score; MNSI, Michigan Neuropathy Screening Instrument (47, 77–86).*

Electrodiagnostic studies have also been identified as one of the largest drivers of health care costs related to neuropathy evaluation (99). Results are often found to be normal in patients with diabetes who have early or small fiber predominant neuropathy.

## DPNCheck

To overcome some of the shortcomings of standard NCS testing, a novel point-of-care nerve conduction device (POCD), DPN-Check (Neurometrix Inc., Waltham, MA) has been developed with the potential to serve as an acceptable proxy to standard

NCS which are time-consuming, expensive, and often require patients to be seen in specialist clinics (**Figure 1C**). This test for sural nerve conduction velocity and amplitude is much quicker (3 minutes) to perform than conventional electrodiagnostic testing. It has been validated in type 1 and 2 diabetes populations through comparison with the Neuropathy Disability Score (NDS) (55) and standard NCS (53, 54). These studies have reported a high sensitivity of 92-95% for detecting abnormalities (**Table 4**). However, these studies' cohorts have been small, with two of the three studies assessing very low numbers of patients with type 1 diabetes (53, 54, 108). Furthermore, the DPNCheck device is dependent on the presence of an accessible sural nerve which can be anatomically absent in up to 9% of healthy subjects (55).

## Small Fiber Tests

### Punch Skin Biopsy

The evidence strongly suggests that in DPN, damage to small fibers precedes damage to large fibers (109, 110) and punch skin biopsy is currently considered the gold-standard single test for diagnosing small fiber neuropathy (111). A measure of intra-epidermal nerve fiber density (IENFD) can be quantified from these biopsies, which is a method of documenting the density of terminal branches of peripheral nerves within the epidermis ($no/mm^2$). The European Federation of Neurological Societies has published guidelines for its use in diagnosing peripheral neuropathies (112) (**Figure 1I**).

Two immuno-staining methods have become the most widely used in IENFD measurement: indirect immunofluorescence (IF) and bright-field immunohistochemistry (BFI). Although IF is considered a slightly more sensitive technique due to higher signal/noise ratio (113), the two methods have excellent correlation (114), and both can comparably detect SFN (113). At present, age-related normative values exist only for BFI, published by a multi-national group of 8 centers (115).

For both IF and BFI techniques, IENFs are typically counted directly through an epifluorescence microscope's oculars by focusing through the optical planes (113). For IF only, the more precise, but time-consuming technique confocal microscopy (CM) can analyze optical sections of 3-dimensional images using computer software (113). The two techniques have shown excellent correlation (113), and the latter is usually used when the more complex, second-level analysis is needed.

IENFD measurements have been shown to detect small fiber neuropathy with depletion of IENFD detected in patients with normal NCS and no clinical signs or symptoms of neuropathy (59, 60). A recent study reported low sensitivity of just 61% when using a cut off of 4.5fibers/mm IENFD to diagnose clinical DPN in T1DM patients (100). Earlier studies have published significantly higher values for sensitivity (80%) (116) and specificity (95%) (117), however, these studies were comparing healthy controls to DPN patients rather than the test's ability to identify DPN in a diabetic cohort. Other studies have found a decrease in IENFD correlating with the progression of neuropathy and duration of diabetes (118, 119) with reports that IENFD may also be lower in patients with painful DPN compared to painless DPN (120).

A 5-year follow-up study investigating the progression of DPN in T1DM and T2DM, reported a significant reduction of IENFD in T2DM patients, with IENFD measurement being the single most abnormal parameter (121). Overall, the reduction in IENFD was not significant in T1DM subjects. However, the lower number of patients in the T1DM group may explain this finding, as this would make it more challenging to prove statistically significant changes (121).

The main issue with IENFD measurements as a biomarker for small fiber neuropathy is that it is an invasive procedure. Obtaining a biopsy can cause side effects such as a mild infection due to improper wound management or, less commonly, excessive bleeding. Even though reported side effects are rare (1.9/1000) (115), the nature of this technique limits its practical use, particularly when a repeat biopsy is required in longitudinal studies or clinical intervention trials.

From a screening perspective, although intra-epidermal nerve fiber density measurement from a lower-limb skin biopsy is considered the gold standard for the diagnosis of small fiber neuropathy, it is invasive and therefore not suitable for routine screening (111, 122).

### Quantitative Sudomotor Axon Reflex Test QSART

The assessment of sudomotor nerve (sweat) function has also been used to assess small autonomic c-fibers, as anhidrosis can be characteristic of the presence of peripheral autonomic neuropathy.

The reference standard for measuring sudomotor function is the quantitative sudomotor axon reflex test (QSART). This test uses local sweat production, measured as a change of relative humidity over time, during and after skin activation. Special

**TABLE 4** | Summary studies for validity for four potential screening tests for DPN.

| Test | Fibers Assessed | Validated Against | Sensitivity | Specificity |
|---|---|---|---|---|
| **DPNCheck** (53, 54) | Large (Aβ-fibers) | NCS | 92-95% | 82-89% |
| **Neuropad** (64, 66–69) | Small (C-fibers) | NCS, NDS, VPT | 70-97.8% | 50-67% |
| **Sudoscan** (70–74) | Small (C-fibers) | NCS, Clinical Examination, UENS, VPT, NSS | 70-87.5% | 53-92% |
| **CCM** (62, 100–107) | Small (Aδ and C-fibers) | NCS, Clinical Examination,CASS | 59-86%(CNFL)<br>65-82%(CNFD)<br>17-100%(CNBD) | 61-84%(CNFL)<br>41-79%(CNFD)<br>45-96%(CNBD) |

software is used to digitalise, plot and analyze the temporal resolution, latency, magnitude and duration of the sudomotor response (123). However, due to highly technical demands and relative discomfort of the examination, QSART remains mostly limited to research centres and is not considered a potential screening tool for DPN (76).

## Neuropad

Neuropad® is a patented 10-minute screening test for the early detection of diabetic foot syndrome and can be used as a triage test (124). It is a unique, non-invasive, painless and simple diagnostic screening test employing a chemical reaction to minute quantities of sweat as a biomarker for much earlier signs of DPN.

The test has been created to assess the sweat function (small autonomic c-fibers) in the feet of patients with suspected neuropathy. An adhesive pad containing cobalt salts is stuck onto the foot's plantar aspect and changes color from blue to pink within 10 minutes if the sudomotor function is normal (67). If there is a decreased function, the pad remains blue or turns patchy in color. There is a strong association between skin dryness, sudomotor dysfunction and diabetic foot ulcer and the function of Neuropad. An abnormal Neuropad response is associated with sympathetic dysfunction and clinical neuropathy (**Figure 1H**).

This test's main advantage is that patients can self-administer at home, reducing clinical contact time and aiming to reinforce abnormal results visually. Instructions have been confirmed as clear for patients to follow, and the test is easy to use for most patients (64). However, due to older age, visual and kinetic problems, a fifth of patients still needed help when self-testing.

It has been reported as having good to excellent (70-97.8%) (64–69) sensitivity for DPN detection (**Table 4**). When comparing Neuropad to a range of different small and large fiber diagnostic tests, strong correlation between Neuropad and NDS (64, 69), IENFD (65), CCM (125), Sudoscan (126) and measures of sweat gland dysfunction (127) have been reported. It has also been identified as a useful tool for staging the severity of neuropathy in patients with type 2 diabetes demonstrating excellent agreement with the Michigan classification system (128). Another significant advantage of Neuropad is its high NPV, making it ideal to serve as a screening test primarily to exclude DPN (68, 129, 130).

However, studies are not consistent in terms of the position of the Neuropad on the foot and the NDS cut-off value chosen to indicate clinical DPN presence. Furthermore, some studies graded the Neuropad color change as a percentage (66) or score out of 1 (65), whereas others simply classified the results as normal or abnormal (64, 67). Standardization of elapsed time before test result analysis is also necessary as extending the observation period to 15 minutes may provide greater diagnostic usefulness (131). This highlights a need for software development that can consistently grade each test's color change over time to enable continuous and more accurate monitoring of sudomotor dysfunction.

In order to address these issues and increase both the sensitivity and particularly the specificity of Neuropad screening and create a continuous output, a smartphone software app and internet based image processing system has been developed. Neurometrics-Diab™ is a digital therapeutics (DTx) smartphone app which uses the Neuropad™ as a biomarker to produce a continuous record of a person's neuropathy to see if it is improving, is stable or is worsening with trend-lines helping to predict outcomes. Using a smartphone camera, patients can take a photo of their test result which is then automatically sent to a web server where the photo is run through a proprietary image processing algorithm resulting in a percentage score which is recorded. Over time a trend can be calculated. The DTx app is currently at the advanced prototype stage. Versions for other medical conditions are under development.

## Sudoscan

Sudoscan™ (Impeto Medical) is another quick, simple and non-invasive test that aims to assess sudomotor function using 'reverse iontophoresis' (132, 133) to measure electrochemical skin conductance (ESC) of sweat in the hands and feet. Compared to age-corrected standard data, a reduced ESC result may indicate degeneration of small c-fibers that innervate the sweat glands and, therefore, lead to reduced sweat gland function (71) (**Figure 1G**).

The ESC measurements from the feet are considered more sensitive for the detection of DPN than the hands (72), with less variation in results (134). This is likely due to a fluctuation in the hands' contact on the electrodes. In contrast, the feet are aided by gravity to maintain constant pressure on the electrodes throughout the test. Lower electrochemical skin conductance at the feet was also significantly associated with increasing symptoms in a large cohort of patients with T2DM (135).

Reference values in healthy subjects are available from a global collaborative analysis comparing different ethnic groups, age, and gender (136). This study noted a significantly lower hands and feet ESC for African-American, Indian, and Chinese populations than the Caucasian population, highlighting the need to match ethnicity groups in electrochemical skin conductance studies. The same study also observed no significant difference between women and men at the hands or feet and a weak decline in ESC with increased age.

ESC measurements may also be associated with subjects' weight (137), perhaps due to a weight-dependent change in sensitivity of the stainless-steel electrodes, or sweat gland density, when the subject is in the standing position. This could also be due to the correlation between higher weight and larger feet only (137). These hypotheses are yet to be assessed; however, these studies' findings emphasize the importance of profile matching different subject groups for a weight that did not occur in some validation studies (71, 74).

Validation studies have reported consistently good values for sensitivity (70-87.5%) (**Table 4**) when using foot ESC results to screen for DPN (70–72, 74, 132). However, there are inconsistencies in the ESC cut-off values used for identifying sudomotor dysfunction, ranging from 52uS (70) to 77uS (72). This variation and inconsistencies in the neuropathy tests being used as a reference standard are the likely cause of the extensive

range in reported specificity of between 53-92% (70–72, 74). It highlights the need for standardization of the classification criteria used. Patient cohorts also differed in their severity of DPN, with participants in one study (74) having significantly more advanced DPN than those in the study by Smith and colleagues (71). Therefore the test performed better in the former.

Overall, Sudoscan appears to be a promising DPN screening test that is non-invasive, easy to perform and eliminates the subjective component of clinician error, demonstrating good correlation with IENFD (137). However, there is some doubt as the current evidence does not strongly support ESC to distinguish between patients with DPN and control individuals (138). Therefore, longitudinal and more extensive cohort validation studies are needed, along with standardization of diagnostic criteria before Sudoscan can be used as a screening tool for small fiber neuropathy.

It is evident that progress has been made in developing point of care devices (POCDs) which may be capable of diagnosing DPN early before clinical signs are apparent. Neuropad, DPNCheck and Sudoscan are newer screening tests that have demonstrated potential for early detection, however validation studies, thus far, have reported a range of sensitivities, specificities depending on cohort and test used for comparison (**Table 3**).

## Quantitative Sensory Testing (QST)

Quantitative sensory testing (QST) has become a common method for evaluating small nerve fiber function using thermal threshold and thermal pain measurements and large fiber function using vibration thresholds (52). The most common commercial system is the Medoc TSA-II NeuroSensory Analyzer (Medoc Advanced Medical Systems, Israel) which is used to determine thermal thresholds, (**Figure 1F**). In recent years a cheaper, more portable device has been designed, NerveCheck (Phi Med Europe S.L., Barcelona, Spain), which has shown good reproducibility (ICC values = 0.79, 0.71 and 0.86 for vibration, warm and cold sensation respectively) and comparable diagnostic accuracy (86%, 72% and 79% for vibration, warm and cold sensation testing respectively) to established QST equipment for the diagnosis of DPN (139).

Cold thresholds can be used to evaluate myelinated A-delta fiber function, whereas warm thresholds are used to assess the function of unmyelinated C-fibers. Published normative data sets are available for heat threshold detection (140–145), and recommendations for conducting QST in both clinical practice and research have previously been published by The International Association for the Study of Pain (NeuPSIG) (146).

QST has been found to have reasonable repeatability (50); however, inter-operator and inter-patient variability depend on several factors. Training of both examiner and patient, the methodology of assessment, baseline skin temperature, stimulus characteristics, location and number of stimuli sites and duration of intervals between tests have all affected QST measurements (52). Using standardized methodology with extensive training has significantly reduced interobserver variability (147, 148). However, this may be too time-consuming to be implemented.

When it comes to the effects of body fat on thermal detection thresholds, there are conflicting findings. Malmström et al. (149) failed to detect differences between obese and other groups for cold and warm thresholds at the suprailiac site (149). In contrast, Pryce and colleagues (150) found that obese participants had significantly higher cold and warm detection thresholds than normal BMI participants on the abdomen.

Two psychophysical algorithms can be used to determine thermal thresholds. These are the method of limits and the method of level (described in detail elsewhere (50, 151), with the method of limits used more commonly due to it being less time-consuming (146). Measurements determined using limits have been reported as significantly higher than those measured by Level, irrespective of test location (52). However, the two methods correlate well with each other (52) and the 2013 consensus concluded that both were reliable (146). The major difference between these two methods is the effect of reaction time. For the method of limits, a patient has a longer reaction time due to age or height (causing a more extended sensory pathway) which may erroneously give a higher threshold.

Both warm and cold thresholds can be affected in patients with DPN, irrespective of how long the course of diabetes is, but the frequency of abnormal warm thresholds is significantly higher (141). A study found that cold detection thresholds significantly reduced in DM patients with no evidence of pre-clinical, sub-clinical and clinical DPN, respectively (152). A longitudinal study also found a significant positive correlation between deterioration of cold detection thresholds and pain intensity in painful DN, with warm detection thresholds also correlating at non-significant value (153).

One major issue with the use of QST is that it cannot differentiate between peripheral and central temperature perception causes. It involves sensory receptors, spinal cord pathways and termination sites in the thalamus. This means that if there is poor concentration, a language barrier or cognitive defect, subjects' results may affect their subjective nature (51).

# CORNEAL NERVES AS A BIOMARKER FOR DPN

Anatomically and developmentally, the eye can be considered an extension of the central nervous system (CNS). The cornea is the most densely innervated tissue in the body. It is richly supplied by a large number of sensory nerve fibers and a lesser number of autonomic fibers (154). The cornea possesses small unmyelinated C-fibers and myelinated Aδ-fibers for sensory innervation. These are derived from the trigeminal nerve's ophthalmic division and enter the corneal stroma at its periphery, in a radial fashion parallel to the corneal surface. As the fibers run forward toward the cornea center, they lose their myelin sheath; a necessary step to maintain corneal transparency (154).

Corneal C-fibers form a delicate three-dimensional network known as the 'sub-basal nerve plexus' (155), which is located beneath the basal layer of the corneal epithelium. Mapping of the cornea (156) has shown that this plexus forms a spiral or 'whorl like' pattern. The spiral center, often called the vortex, is located approximately 2-3 mm inferior and nasal to humans' corneal apex. Due to this arrangement, sub-basal nerves in the superior and human apical cornea are oriented vertically. In contrast, sub-basal nerves in other corneal regions may be orientated horizontally or obliquely, consistent with their locations within the whorl-like plexus (157)

Corneal confocal microscopy (CCM) is a non-invasive, *in vivo* ophthalmic imaging technique that allows a detailed examination of the cornea, at high magnification, on a cellular level (**Figure 1J**) (158). By capturing multiple two-dimensional images at different depths, CCM imaging can delineate the corneal layers of the cornea (158), providing superior magnification compared to standard slit-lamp biomicroscopy. These properties allow CCM to acquire high-quality images of the corneal C-fibers in the sub-basal nerve plexus. Considering the known relationship between damage to these fibers and diabetic peripheral neuropathy, the potential for their use as a surrogate biomarker for DPN has been identified.

When analyzing the sub-basal nerve plexus, most studies report results from four morphological parameters: Corneal nerve fiber density (CNFD) which is the total number of main nerve fibers per $mm^2$, corneal nerve fiber length (CNFL) which is the sum of the length of all nerve fibers and branches ($mm/mm^2$), tortuosity coefficient (TC) which is a unitless measurement that uses deviation from a straight line to measure the tortuosity of the main nerve fibers independent of their orientation, and corneal nerve branch density (CNBD) which is defined as the number of branches emanating from all main nerve fibers. There is, however, a discrepancy in how this can be quantified between studies with the established protocol for these parameters described elsewhere (62).

Of these four parameters, CNFL has been the most frequently used parameter for DPN, with one study reporting superior reliability than other parameters (159). Some studies have assessed the diagnostic performance of CCM for DPN and reported the results for CNFL only (101, 105). Hertz et al. (159) reported that CNFL produced the highest intra-observer and inter-observer reproducibility (ICC of 0.72 and 0.73 respectively), with TC demonstrating the lowest (0.23 and 0.29 respectively).

Two other parameters that have been reported in research studies are nerve reflectivity (160) and nerve fiber beading (number/100 μm) (161, 162). Nerve fiber reflectivity is usually assessed using grades as first outlined by Oliveira-Soto and Efron (160), whereby classification can be split into four grades according to a comparison with reference images. The number of beadings is defined as the number of beadings in a length of 100um of sub-basal nerves within a frame (163). Both parameters have demonstrated changes in dry eye conditions, where patients with Sjogren's syndrome have demonstrated significantly higher beading than dry eye patients of other primary causes (164). However, both measures require subjective judgment. Beading can be challenging to quantify and may require special software and may have poor repeatability and reproducibility (163).

More recently, newer corneal parameters have been investigated. These include inferior whorl length (IWL) (165) defined as the length of the nerves at the inferior whorl of the superficial nerve plexus, nerve fiber width (166) and nerve fiber area (167). These new measures have previously shown significant differences between the non-neuropathic and clinically neuropathic groups in DM (168) with CNFW and CNFA, demonstrating 74% and 66% sensitivity-specificity equal error rate point, respectively when identifying non-neuropathic patients compared to control subjects (168). This indicates that these new measures may have the capacity to identify individuals with early neuropathy; however, research into these new parameters is currently limited.

Another type of cell found in the sub-basal layer and has been of interest in DPN research are dendritic cells. These antigen-presenting cells of the cornea are of paramount importance. They play a critical role in activating other immune systems in the ocular surface, influencing both suppression and induction of inflammation (169, 170).

Langerhans cells are usually up to 15μm in diameter and can be seen in various forms (171). In their immature form, these cells have small dendritic processes or lack dendrites completely and are mainly located in the peripheral cornea's epithelium (172). In pathological states, Langerhans cells mature, form interlocking dendritic processes which may comprise a net-like structure, and migrate from the periphery into the central cornea (172).

Cross-sectional studies have shown an increase in the densities of Langerhans cells in the central cornea related to conditions such as dry eye with and without contact lens wear (171, 173) bacterial keratitis (174), thyroid eye disease (175) and diabetes (176, 177).

## CCM for the Detection of DPN

In the early 2000s, a novel study by Rosenberg and colleagues reported the correlation between increasing severity of DPN, corneal sensitivity and progressive loss of corneal sub-basal nerve fibers (178). This was closely followed by a similar small study published in 2003 (179) which found that CCM was able to detect abnormalities in the corneal nerves of 18 patients with diabetes deemed to have only mild neuropathy using conventional tests. Similarly, Midena and colleagues (180) reported a significant decrease in corneal nerve fiber and branch number, along with decreased beading in patients with diabetes. It should be noted that these three studies used a light corneal confocal microscope, which is the first commercially available generation of the confocal imaging device with inferior image quality in comparison to the methods now commonly used.

Since then, the use of corneal confocal microscopy (CCM) for rapid, noninvasive clinical assessment of corneal nerves has grown substantially, especially in recent years. It has proven to be particularly useful as a diagnostic marker for detecting

diabetic neuropathy and a range of other peripheral neuropathies (62, 100–106, 181–186). Some of them are reviewed in this paper.

## Diagnostic Performance for Clinical DPN

Several cross-sectional studies have evaluated the ability of CCM to diagnose clinical levels of DPN in comparison to a range of other diagnostic tests (**Table 5**). It must be noted that most of these studies assessed patients with T1DM only, meaning there is limited published data available for the diagnostic sensitivity and specificity values when assessing patients with T2DM.

These studies used a cut-off point for the reference neuropathy test/combination of tests to determine whether a patient had a DPN. However, the reference test and cut-off points varied between studies meaning there were no universal diagnostic reference criteria. Some studies validated CCM against a single test of nerve conduction studies (NCS) (100, 101) or neuropathy disability score (NDS) (62). In contrast, other studies used a combination of the two (102) or NCS and clinical examination (104, 106). A combination of diagnostic tests will likely increase the efficiency of detecting DPN compared to one test used alone. This is significant as some studies compare CCM to one single test, which is not the gold standard in the case of NDS. NCS only measures large fiber function, which is affected later than small nerve fibers in DPN. One study (187), demonstrated that diagnostic ability of CNFL measurement in DM patients is significantly worse if using clinical signs and symptoms as a reference standard in comparison to electrophysiology, plus one sign/symptom as per the Toronto consensus guidelines, which highlights the importance of a standardized diagnostic reference (187).

To explore which of the many measurements derived from CCM could best distinguish patients with and without clinical DPN, as part of each study, the same patients were examined using CCM, and all nerve parameters were derived. For each nerve parameters tested, ROC curves were plotted to determine a CCM cut-off point used to distinguish between patients with and without DPN in the diabetic cohort only. A range of cut-off points was studied to identify the best sensitivity/specificity value for diagnosing DPN for each nerve parameter.

CNFL was the most commonly reported nerve parameter for these studies, with all nine assessing its diagnostic ability and finding significant differences between patients with and without DPN. A range of sensitivity values between 59 and 86% was found and a specificity range of 61-84%, depending on the cut-off value used for diagnosis. The earliest of these studies (62), examined patients using a Tomey confoscan CCM. It is well known that these images are of more inferior quality, making it more challenging to identify nerve fibers during analysis. This is likely the explanation for the significantly lower diagnostic threshold value reported in this study compared to the others presented (**Table 5**).

For corneal nerve fiber density (CNFD) six of the cross-sectional studies (**Table 5**) reported a significant reduction in DM patients with DPN compared to both DM patients without DPN and healthy controls (62, 100, 102, 104, 106, 107). These studies reported sensitivity and specificity ranges as 65-82% and 41-79% respectively. A significantly higher cut off point of 39.2 CNFD no/mm$^2$ was defined in T2DM patients in the consortium study, resulting in an increased sensitivity value to 69% (104). This may explain why its specificity is the lowest value of only 41%, as a higher cut-off value may create more false-positive results. It is notable that based on their cohort, Scarr et al. (106) defined the lowest thresholds for diagnosis for both CNFD and CNFL out of the studies using the Heidelberg retinal tomograph (HRT) (III) CCM. This is likely due to their significantly older-aged cohort compared to the other cross-sectional studies as CNFD and CNFL have been shown to reduce with age (188).

For corneal nerve branch density (CNBD), six cross-sectional studies (62, 100, 102, 104, 106, 107) reported a significant reduction in DM with DPN than without DPN. For diagnostic value, the sensitivity (17-100%) (62, 102) and specificity (45-96%) (62, 102) values were significantly more varied, suggesting that this parameter has shown the least promise for DPN diagnosis until now.

There are several strengths to each of the cross-sectional studies. Three used profile-matched healthy controls (101, 102, 106), meaning that differences in measurements between the two groups due to age should have been accounted for, giving a better representation of changes that have occurred due to DPN. Ahmed et al. (101) also looked at the option of combining two corneal nerve parameters for the identification of neuropathy. Two of the studies looked at both manual, and automated software for DPN diagnosis (102, 106) which is significant as automated software for analysis would be required if CCM were to be introduced in large-scale screening.

Perkins et al. (104) in a consortium multi-center study funded by NIH assessed data from a large cohort of 998 subjects. This large cohort of different ethnicities and T1DM and T2DM gave a more accurate representation of the population of people with diabetes instead of focusing on one specific sub-group. Another strength of this NIH funded study was that it suggested an alternative approach of using one lower value chosen to more confidently rule in the presence of neuropathy (maximize specificity) and one higher value determined to simultaneously, more confidently rule out the presence of neuropathy (maximize sensitivity). This combination of decision criteria aims to minimize false positive and negative results. The study found that using this criterion increased their sensitivity to 88% and specificity to 89% using manual methods of analysis. However, this method caused 57.8% of the subjects to be unclassified as they fell between the two limits.

There were several limitations to these cross-sectional studies. Some did not match the clinical profiles of their patients to the control subjects. For example, the patients' group in Alam et al. (100) being significantly older, with significantly longer disease duration than the T2DM group without neuropathy. Another limitation of two of these studies (101, 106) was that only 1 image per eye was used for analysis. One criterion for choosing this image in the Ahmed et al. (101) study was the most nervous frame. Using this method to choose 1 image per eye instead of calculating an average of 3 images or more may be less time

**TABLE 5 |** Summary of studies assessing the clinical utility of corneal nerve parameters for the diagnosis of clinical levels of diabetic neuropathy compared to chosen reference standards.

| | Study | Number subjects | Type of CCM device | Diabetes Type | Age (years) | Disease Duration (years) | Type of Neuropathy | Validated Against | Sensitivity (%) | Specificity (%) | CNFL Threshold (mm/mm²) | CNFD Threshold (no./mm²) | CNBD Threshold (no./mm²) | CNFT (TC) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Cross-sectional** | **Tavakoli et al. (62)** | 118 (101 DM, 17 HC) | Tomey ConfoScan P4 | 1,2 | 55 ± 4.8 (HC)<br>55 ± 1.9 (DPN-)<br>58 ± 2.1 (Mild DPN)<br>59 ± 2.5 (Mod DPN)<br>61 ± 2.05 (Sev DPN) | 10.7 ± 1.82 (DPN-)<br>15.5 ± 2.08 (Mild DPN)<br>18.6 ± 3.06 (Mod DPN)<br>19.3 ± 2.85 (Sev DPN) | DSPN | NDS | 64 (CNFL)<br>82 (CNFD)<br>91(CNBD) | 79 (CNFL)<br>52 (CNFD)<br>45 (CNBD) | 3.39 | 27.81 | 13.89 | – |
| | **Ahmed et al.** (101) | 153 (89 DM, 63 HC) | HRT (II) | 1 | 38.9 ± 17.6 (HC)<br>34.9 ± 14.8 (DPN-)<br>50.0 ± 14.3 (DPN+) | 17.6 ± 14.0 (DPN-)<br>31.4 ± 13.5 (DPN+) | DSPN | NCS, Clinical Examination | 85 | 84 | 14 | – | – | – |
| | **Tavakoli et al. (107)** | 52 (34 DM, 18 HC) | HRT (III) | 1,2 | 42 ± 0 (DAN-)<br>44 ± 3 (DAN+) | 12 ± 3 (DAN-)<br>26 ± 2 (DAN +) | DAN | Composite autonomic scoring scale (CASS) | 86 (CNFL)<br>86 (CNFD)<br>100 (CNBD) | 78(CNFL)<br>79(CNFD)<br>56 (CNBD) | 4.8 | 23.3 | 19.5 | – |
| | **Chen et al.** (102) | 89 (63 DM, 26 HC) | HRT (III) | 1 | 44 ± 15 (HC)<br>44 ± 13 (DPN-)<br>59 ± 11 (DPN+) | 23 ± 16 (DPN-)<br>39 ± 14 (DPN+) | DSPN | NCS, DNS/NDS | 59 (CNFL)<br>82 (CNFD)<br>17 (CNBD) | 74 (CNFL)<br>71 (CNFD)<br>96 (CNBD) | 16.5 | 24 | 15 | – |
| | **Alam et al.** (100) | 88 (61 DM, 27 HC) | HRT (III) | 1 | 41 ± 114.9 (HC)<br>38.8 ± 12.5 (DPN-)<br>53.3 ± 11.9 (DPN+) | 17.2 ± 12.0 (DPN-)<br>37.2 ± 13.1 (DPN+) | DSPN | NCS, Clinical Examination | 74 (CNFL)<br>77(CNFD)<br>67 (CNBD) | 61(CNFL)<br>79 (CNFD)<br>58 (CNBD) | 16.8 | 25 | 36.5 | – |
| | **Scarr et al.** (106) | 137 (67DM, 69HC) | HRT(III) | 1 | 64 ± 8 (HCs)<br>65 ± 7 (T1DM) | 52-58 (Median 54) | DSPN | NCS, Clinical Examination | 73 (CNFL)<br>76(CNFD)<br>44 (CNBD) | 75 (CNFL)<br>75(CNFD)<br>75 (CNBD) | 13.7 | 18.8 | 15.6 | – |

*(Continued)*

**TABLE 5** | Continued

| | Study | Number subjects | Type of CCM device | Diabetes Type | Age (years) | Disease Duration (years) | Type of Neuropathy | Validated Against | Sensitivity (%) | Specificity (%) | CNFL Threshold (mm/mm²) | CNFD Threshold (no./mm²) | CNBD Threshold (no./mm²) | CNFT (TC) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **Perkins et al.** (104) | 998 | Tomey Confoscan | 1 | 42 ± 19 | 21 ± 15 | DSPN | NCS, Clinical Examination | 71 (CNFL)<br>65 (CNFD)<br>67 (CNBD) | 67 (CNFL)<br>75 (CNFD)<br>72 (CNBD) | 16.4 | 28 | 37.6 | – |
| | (Consortium) | (516 T1DM, 482 T2DM) | P4, HRT (II),HRT (III) | 2 | 62 ± 10 | 12 ± 9 | | | 65 (CNFL)<br>69 (CNFD)<br>69(CNBD) | 69 (CNFL)<br>41(CNFD)<br>63(CNBD) | 16.3 | 39.2 | 44.8 | – |
| **Longitudinal** | **Pritchard et al.** (105) | 90 (T1 DM) | HRT (III) | 1 | 42 ± 16 (DPN-)<br>51 ± 14 (DPN+) | 15 ± 12 (DPN-)<br>29 ± 16 (DPN+) | DSPN | NCS, DNSS/NDS | 63 | 74 | 14.1 | – | – | – |
| | **Lovblom et al.** (103) | 65 (T1 DM) | HRT (III) | 1 | 34 ± 15 (DPN-)<br>38 ± 16 (DPN+) | 17 ± 12 (DPN-)<br>21 ± 9 (DPN+) | DSPN | NCS, TCNS | 82(CNFL)<br>55(CNFD)<br>82(CNBD)<br>73(CNFT) | 69 (CNFL)<br>59(CNFD)<br>50 (CNBD)<br>72(CNFT) | 14.9 | 41.7 | 36.1 | 15.9 |

*Studies presented are all published studies. Data presented as standard units or mean ± standard deviation.*

consuming for analysis; however, it is likely to give the false elevation of measurements per patient instead of representing an accurate average.

Another significant issue with these studies is that most of them use the Toronto consensus as to the diagnostic criteria for DPN (100–102, 104, 106), i.e. one abnormal finding as part of NCS, in combination with a symptom or sign of neuropathy (41). As mentioned previously, NCS assesses large fiber function whereas CCM assesses small fiber function.

Despite the variation in results and limitations of the studies, these findings supported the expanded role of CCM in the assessment of diagnosis DPN as a supplement to the vast array of neurological tests currently in use.

## Early Detection of Neuropathy

As there are currently no therapeutic agents approved for DPN treatment, early detection is essential to modify any risk factors. Several studies have specifically investigated CCM findings in early stages of DM and mild levels of DPN.

The published baseline characteristics of T1DM patients as part of the LANDMark study (189) were that corneal nerve fiber length was reduced in patients without clinical neuropathy, based on the Toronto criteria. Another paper written from the same study (190) assessed the use of CCM for distinguishing between control patients and DM patients (156 T1DM, 75 T2DM) with and without clinical DPN. For the patients with DPN, all cases were defined as mild (as defined by QST plus neurophysiology). This study reported a significant reduction in CNFL when comparing patients with and without mild neuropathy, suggesting that CNFL changes may occur early in the course of the disease.

One study (191) assessed the corneal sub-basal plexus in patients with recently diagnosed T2DM (mean duration 2.1± 1.6 years). This study reported significant differences between CNFD, CNBD and CNFL parameters when comparing the patient cohort to the control group, with CNFD emerging as the most sensitive in detecting corneal nerve pathology; indeed 21% of the patients fell below the 2.5$^{th}$ percentile of the control group. For this study, high-adapted software produced an image composed of an image stack. It reconstructed a combined mosaic image with an expanded field of view compared to standard imaging using CCM. This software is also able to correct for artefacts. As this method is not widely used, there is no direct comparison to other studies. To our knowledge, no other studies are assessing recently diagnosed patients with DM (<2 years duration). It must also be considered that in this study, even though patients were diagnosed newly, there may have been a delay in diagnosis, which could have varied between patients.

Another study assessing early nerve changes assessed patients with impaired glucose tolerance (IGT) (192). This study reported evidence that CCM may detect changes in nerve parameters before established diabetes. They reported that in patients with IGT, CNFD and CNBD were significantly reduced with 40.5% of subjects with IGT having significant small-fiber damage based on CNFD reduction compared to control subjects. This agreed with a decrease in IENFD and significantly higher warm thresholds and vibration perception thresholds in the same cohort (192).

## Langerhans Cells in DPN

The dominant antigen-presenting cells in the cornea and ocular surfaces are Langerhans cells (LCs) and Dendritic cells (DCs) which are derived from the bone marrow and can stimulate both primary and secondary T and B-cell responses (169). It has been shown that Corneal confocal microscopy provides a non-invasive means to readily demonstrate Langerhans cells (LCs) in the cornea of healthy subjects and a range of inflammatory ophthalmic conditions (**Figure 2**) (174, 193). Some studies demonstrated that the number of LCs increases in Diabetic Neuropathy (177, 194–196); however, the LCs activation mechanism is still unclear.

Zhivov et al. (176) assessed the corneal basal epithelial layer and the sub-basal nerve plexus for the presence of LCs in healthy subjects and found that 31% of subjects had LCs present. Tavakoli and colleagues (177) were the first to assess Langerhans cell density with differing severities of diabetic neuropathy (based on NDS scoring compared to controls). This study found a significant increase in the proportion of individuals with LCs in patients with T1DM and T2DM (73.8%) compared to control subjects (46.1%). The study also found that LC density was significantly increased in the patients with diabetes (17.73 ± 1.45) compared to control subjects (6.94 ± 1.58). However, with progression of neuropathy, patients with moderate and severe neuropathy showed a reduction in the LC density in comparison to patients with mild neuropathy and were not significantly different from control subjects. This may suggest that LCs have a role in the early phase of nerve damage. This study only focused on Bowman's layer which has been shown to have a lower density of LCs in comparison to the epithelial layer (197), so is not an accurate representation of overall LC density in the central cornea. Another limitation of the study was that the Tomey Confoscan CCM was used for imaging which has been shown to underestimate LCs density compared to newer the Heidelberg HRT III CCM (176) and cannot differentiate mature from immature LCs (176).

A more recent study, used the HRT (III) CCM to assess the density of LCs in a cohort of children and adolescents with diabetes and found a higher percentage of patients (85.9%) and controls (69.1%) with LCs present when compared to the previous two studies (176, 177). This study was also able to distinguish between mature and immature cells by classing LCs of less than 50 μm in length, without dendritic structures as immature cells and those greater than 50 μm with dendritic structures were considered as mature cells. A significant increase in mature and immature cells was found, and a correlation existed between CNFD and LCs density (198). However, this study only assessed a specific age-group of the diabetic cohort, so it does not represent the whole diabetic population. Overall, studies investigating LCs density in patients with diabetes are still limited, and more information is required to conclude the effect of diabetes on LCs.

## Comparing CCM and IENFD

Studies have found CCM to be comparable with measures of IENFD from biopsies in their diagnostic performance for detecting patients with clinical levels of DPN (100, 102) (**Figure 3**). Both studies found no significant difference in their diagnostic efficacy in patients with T1DM.

An older study using the Tomoscan confocal microscope (110) also concluded that both IENFD and CCM assessment accurately quantify small nerve fiber damage in patients with diabetes. Intraepidermal and corneal nerve fiber lengths were also both further reduced in patients with painful compared with painless diabetic neuropathy.

In comparison, one study's findings, using HRT (III) CCM were notably different (191). This study reported that CCM and IENFD were both able to detect nerve fiber loss in recently diagnosed type 2 diabetes, but mainly in different patients. They, therefore, suggested a possible patchy manifestation pattern of small fiber neuropathy. Only 2.7% of the patients had both abnormal CNFD and IENFD. Abnormal CCM with normal IEND was noted in 20.5% of the diabetic group and 11.0% for vice versa. No correlation between the CCM measures and IENFD was observed. There are possible explanations for these contradictory findings. Firstly, the cohort of patients in this study was all patients with T2DM, all of who had been newly diagnosed (known diabetes duration of ≤1 year). The disease duration was significantly less than that of Chen et al. (102) (DPN+ 39 ± 14 DPN- 23 ± 15 years) and Alam et al. (100) (DSPN+ 37.2 ± 13.1

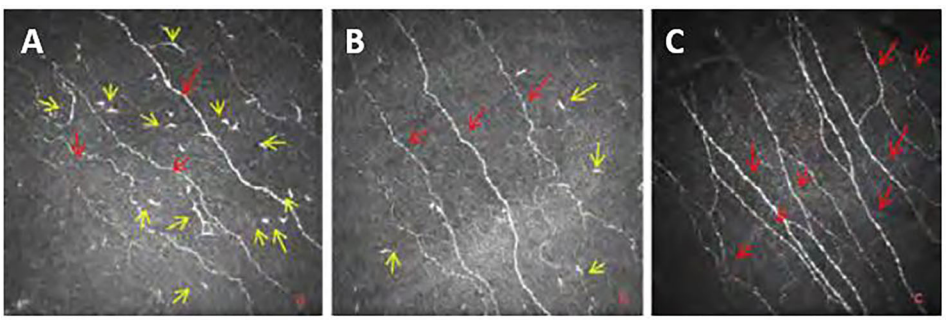


**FIGURE 2** | Images from Bowman's layer of the cornea at **(A)** T2DM with mild neuropathy; **(B)** moderate neuropathy; **(C)** Healthy Control Subject (yellow arrows show LCs and red arrows indicates main corneal nerve c nerve fibers).

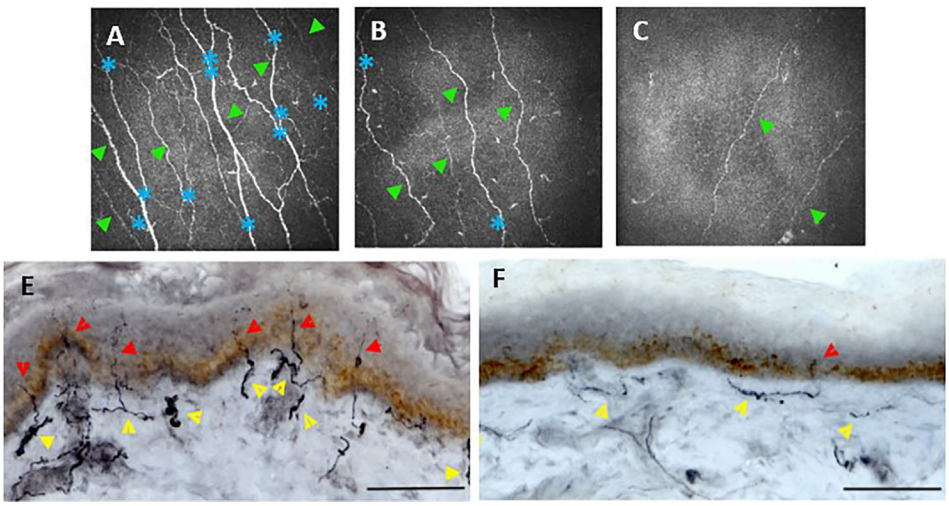


**FIGURE 3** | Corneal confocal microscopy images of the corneal, sub-basal nerves **(A–C)**. Healthy control **(A)** shows numerous corneal main nerve fibers (green arrowheads) with branching nerves (blue asterisks). CCM images of patients with diabetes and mild **(B)** or severe **(C)** neuropathy demonstrate reduced corneal nerves and branches. Skin biopsies **(E, F)** immunostained. Healthy control **(E)** shows numerous intraepidermal nerve fibers (red arrowheads) with a well-developed subepidermal nerve plexus (yellow arrowheads). A diabetic patient **(F)** demonstrates reduced subepidermal and minimal intraepidermal nerve fibers. Scale bar = 100 mm. **(E, F)** adapted from (186).

DSPN- 17.2 ± 12.0 years). These two studies also used comparisons between patients with and without clinical DPN to compare IENFD and CCM, whereas Ziegler et al. (191) only compared patients with T2DM to healthy controls. Lastly, Ziegler et al. used a different location for the IENFD biopsy. This was taken from the lateral calf in comparison to the dorsum of the foot. This more proximal site may have been at less risk IENFD changes or may present a different pattern of loss, as DSPN is known to follow a distal-proximal course.

One issue with the comparison of IENFD with analysis of the corneal sub-basal nerve plexus is that intra-epidermal nerves consist of both unmyelinated C-fibers (90%) and myelinated A-delta fibers (10%) (199), which are both included in the measurement for IENFD. In contrast, the sub-basal nerve plexus is made up of C-fibers only. This means that a direct comparison cannot be made between the two measurements. Although the A-delta fibers only make up 10% of the total number in the epidermal layer, they may be affected differently in DPN than the unmyelinated C-fibers, affecting the overall results.

## Longitudinal Studies for Application of CCM for Assessment DPN

Longitudinal studies suggest that CCM has good predictive value for subsequent DPN (187, 200). Longitudinal analysis of a T1DM cohort showed a mean 1-year change in CNFL was -1.6% in patients with unstable T1DM, while healthy volunteers showed a 5% increase per year (200).

As part of a 4-year follow up study, a study (103) (**Table 5**) found that three corneal nerve parameters were all significant predictors for the development of DPN, with a baseline CNFL of <14.9mm/mm$^2$ being the strongest single predictor when compared to 11 other small and large fiber tests. Other studies (105, 201) also reported an association between lower baseline CNFL and DPN development. Pritchard et al. (105) (**Table 5**) found a significant association with longer diabetes duration, higher triglycerides, worsening retinopathy and nephropathy, impaired sensation to temperature and vibration and slower peroneal and sural nerve conduction velocities. However, studies with larger cohorts and patients with type 2 diabetes are needed to confirm these studies' findings and a more extended period of monitoring. Studies should also ensure a set number of follow-ups over a set period as for Lovblom et al. (103) more than half of the patients had just one follow up visit, meaning that true progression is statistically challenging to prove.

Another prospective study specifically looked at a group of patients with IGT (202). They found that in subjects with IGT, lower baseline CNFD, CNBD, CNFL, and lower mean dendritic length of IENF were the strongest predictors of progression to T2DM over three years. Although significance was not recorded, there appeared to be very similar baseline HbA1c measures between those patients who remained IGT vs those developing T2DM over the three years follow up (42.8 ± 1.2 and 42.4 ± 1.0 respectively(mmol/mol), suggesting that corneal nerve parameters may have been stronger predictors of conversion to T2DM in comparison to baseline HbA1c. Those subjects who returned to normoglycemia showed a significant improvement in their CCM parameters while IENF length continued to decline during the same period. These findings may suggest that corneal nerve fibers regenerate quicker than IENF when glycemic control is improved.

Another observational follow up study (203), examined a small cohort of patients with diabetes (15 T1DM and 10 T2DM) at baseline and follow-up at two years. At follow up, an

improvement in glycemic control, cholesterol levels and blood pressure were found and increased CNFD, with a significant correlation between a decrease in HbA1c and CNFD. This demonstrated that improvements in HbA1c might lead to morphological repair of corneal nerve fibers, however, due to the small sample size and mixing of T1DM and T2DM in analysis, it is unclear if these differences are occurring in both types. It must also be noted that this was not planned as an interventional study, meaning there were no placebo controls or randomization, which would need to take place to confirm or reject these findings.

CCM has been used to investigate the sub-basal nerve plexus changes in patients with T1DM post-simultaneous pancreas and Kidney (SPK) transplant. Tavakoli et al. (186) assessed 15 patients at 6 and 12 months SPK transplant and found a significant improvement in all CCM parameters at 12 months. Symptoms, neurophysiology, quantitative sensory testing and skin biopsy results remained unchanged in the same patients. A similar, earlier study using an older CCM model also reported similar findings, with CNFD and CNFL increasing significantly after just six months (204). These studies may demonstrate that CCM can provide a novel non-invasive means to evidence early nerve repair missed by currently advocated assessment techniques. However, an alternative interpretation of this data could be that corneal nerves respond well to the restoration of insulin and normoglycemia. In contrast, other peripheral nerves do not; therefore, CCM may be measuring something unique that is not an accurate biomarker of the condition of peripheral nerves.

## CCM Application in Clinical Trials

Several DPN intervention trials have focused on large fiber function and have generally had ineffective outcomes. More recently, some studies have instead focused on CCM measures as markers for clinical trials of potential new treatments. In a recent pilot trial of seal oil omega-3, polyunsaturated fatty acid supplementation in patients with type 1 diabetes (disease duration 27 ± 14 years) over 12 months (205), there was a significant increase (30.4%) in corneal nerve fiber length, with no change found in NCS velocity or sensory function. Those subjects at high risk for future DPN and those with already diagnosed DPN (as determined by a Toronto clinical neuropathy score of ≥ 1) showed the best treatment response. This study was a single-arm, open-label, proof of concept trial; therefore, no placebo group was used, which is necessary to reduce a trial's bias.

Another study to determine whether the peptide, ARA 290, improves metabolic control and neuropathic pain in patients with type 2 diabetes used CCM measurements as a co-primary endpoint. This study found that ARA 290 treatment was associated with an increase in corneal nerve fiber density correlated with changes in neuropathic symptoms (206). This study was a double-blind, placebo-controlled investigator-initiated phase II clinical trial whose inclusion criteria were patients with T2DM who also had small fiber neuropathy symptoms. Whether allocation to the treatment and placebo groups was randomized was not discussed in the article. This study's limitation was that patients assigned to both groups generally had excellent metabolic control (HbA1c = 7.3 ± 0.4 and 6.9 ± 0.2 for treatment and placebo groups respectively), which does not truly represent the clinical population of patients with T2DM. It may be that this treatment is less or more effective for patients with poor metabolic control, comparatively. Finally, disease duration was also not mentioned, so it was unclear if there was a significant difference between the two groups.

These trials may be evidence that, like small fiber damage occurring before large fiber damage, small fibers are also the first to start regenerating after damage. Trials over a longer period, including other small fiber neuropathy measures, are required before these findings can be confirmed.

# CONCLUSIONS

There is an un-met need for a simple, reliable and accurate test for the early detection of diabetic peripheral neuropathy (DPN) which may help reduce the incidence of ulcers and amputations in people with diabetes which remains at an all-time high and increases by between 25-20% per annum. Current tests for DPN in primary care require HCPs to conduct and detect late neuropathy. Many people with diabetes do not have an annual foot check despite it being one of the most important of the 8 care processes mandated by NICE. In fact over 500,000 people in England alone never have an annual diabetes related foot examination. This places them at risk of having a first presentation with an active foot ulcer in A&E. Early diagnosis of DPN is critical since early damage to the small nerve fibers in the feet of people with diabetes can be stopped from progressing and even reversed while late signs of DPN and in particular lack of vital protective sensation in the feet cannot be reversed. Although a common and much feared complication, over 30% of people with diabetes remain unaware.

Diabetes UK consistently state that 80% of diabetes related foot complications are preventable yet no practical solution to this huge problem has been proposed. The only primary care test recommended by the National Institute of Health and Care Excellence (NICE) is the 10g Semmes Weinstein monofilament examination (SWME) which is a crude, inaccurate and subjective test for lack of sensation in the feet of people with diabetes - defined as 'late neuropathy' which cannot be reversed. Its evidence base is poor and outside NICE it is regarded as a particularly poor test for DPN.

Early diagnosis and timely intervention are thus essential in preventing its development. Whereas measurement of urinary albumin excretion and fundoscopic examinations serve as objective tests for early nephropathy and retinopathy respectively, a comparably objective, accurate test which is unbiased by the patient's subjective response is lacking for DPN.

Currently advocated diagnostic tests either focus on large nerve fibers, thus are not sensitive to early abnormalities, are too time-consuming and/or are too invasive to be used for repeated measures.

More recently, a number of non-invasive tests have been developed as surrogate measures of DPN. Of these, CCM has

shown great potential for the detection of small fiber neuropathy, the earliest manifestation of DPN. CCM has also demonstrated promising prognostic utility and has demonstrated early nerve regeneration post-SPK surgery and as part of several clinical trials.

Given that CCM is a rapid and non-invasive test, it is suitable for large-scale screening for DPN, and advancements in automated analysis software would further improve its promising potential.

In conclusion, there is no optimal biomarker and ideal endpoint available for DPN at the current time. Hence, there is an urgent need to identify the most accurate early biomarker of nerve damage to diagnose DPN in patients' clinical care better and, in particular, to permit a precise evaluation of future therapies in clinical trials. The global effort among scientists and clinicians, and researchers in the field should address these shortcomings to reduce incidence of complications and to achieve this; the search should continue for better and sensitive tests, screening, and early detection. We need to improve our systematic evaluation of the evidence and promote—from each translational step to the next—the biomarkers with the best evidence and performance at different populations. This will require evaluation of the wider biomarker research agenda. Such evaluation may also benefit more from fostering international collaborations rather than from the fragmented efforts of small, opportunistic studies. "We must learn to measure what we value rather than valuing what we can easily measure".

## AUTHOR CONTRIBUTIONS

JC wrote and revised the manuscript. HF, FI, and AS reviewed and revised the paper. MT designed, conceived the study, wrote the major revision and made comments, had full access to all data, and is the guarantor. MT and AS supervised JC. All authors contributed to the article and approved the submitted version.

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## GLOSSARY

| | |
|---|---|
| ACCORD | Action to Control Cardiovascular Risk in Diabetes |
| ACE | Angiotensin-Converting Enzyme |
| BFI | Bright Field Immunohistochemistry |
| BMI | Body Mass Index |
| CCM | Corneal Confocal Microscopy |
| CNBD | Corneal Nerve Branch Density |
| CNFA | Corneal Nerve Fiber Area |
| CNFD | Corneal Nerve Fiber Density |
| CNFL | Corneal Nerve Fiber Length |
| CNFW | Corneal Nerve Fiber Width |
| CNS | Central Nervous System |
| CTBD | Corneal Total Branch Density |
| DAN | Diabetic Autonomic Neuropathy |
| DN4 | Douleur Neuropathique en 4 |
| DNE | Diabetic Neuropathy Examination |
| DNS | Diabetic Neuropathy Score |
| DPN | Diabetic Peripheral Neuropathy |
| DSPN | Distal Symmetrical Polyneuropathy |
| EDIC | Epidemiology of Diabetes Interventions and Complications |
| ESC | Electrochemical Skin Conductance |
| HbA1c | Glycated Hemoglobin |
| HDL | High-Density Lipoprotein |
| HES | Hospital Eye Service |
| HRT | Heidelberg Retinal Tomograph |
| IENFD | Intra-epidermal Nerve Fiber Density |
| IF | Indirect Immunofluorescence |
| IGT | Impaired Glucose Tolerance |
| IWL | Inferior Whorl Length |
| LANSS | Leeds Assessment of Neuropathic Symptoms and Signs |
| LC | Langerhans Cell |
| LDL | Low-Density Lipoprotein |
| MNDS | Michigan Neuropathy Disability Score |
| MNSI | Michigan Neuropathy Screening Instrument |
| MRI | Magnetic Resonance Imaging |
| MTHFR | Methylenetetrahydrofolate Reductase |
| NCS | Nerve Conduction Studies |
| NDS | Neuropathy Disability Score |
| NeuPSIG | International Association for the Study of Pain |
| NEURODIAB | Diabetic Neuropathy Study Group of the European Association for the Study of Diabetes |
| NIS-LL | Neuropathy Impairment Score in the Lower Limbs |
| NPQ | Neuropathic Pain Questionnaire |
| NPV | Negative Predictive Value |
| NPSI | Neuropathic Pain Symptom Inventory |
| NSS | Neuropathy Symptoms Score |
| QSART | Quantitative Sudomotor Axon Testing |
| QST | Quantitative Sensory Testing |
| SFN | Small Fiber Neuropathy |
| T1DM | Type 1 Diabetes Mellitus |
| T2DM | Type 2 Diabetes Mellitus |
| TC | Tortuosity Coefficient |
| TCNS | Toronto Clinical Neuropathy Score |
| UENS | Utah Early Neuropathy Scale |

frontiers in Endocrinology

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# Gold Standard for Diagnosis of DPN

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Diabetic peripheral neuropathy (DPN) is a common complication of diabetes mellitus. It often causes symmetrical paresthesia, loss of sensation, and hyperalgesia. Without early intervention, it might lead to diabetic foot ulceration, gangrene, and subsequent amputation in people with diabetes. DPN is an insidious disease and often underdiagnosed. This paper reviews the current national and international prevalence of DPN, screening methods for early DPN, including quantitative sensory measurement, neurological function scoring system, confocal microscopy, and high-frequency ultrasound, and summarizes the related research progress, clinical application, and development prospects of these methods in recent years.

**Keywords: DPN, QST, neurological scoring system, CCM, HFUS**

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## INTRODUCTION

The global prevalence of diabetes in 2019 is close to 500 million, and is expected to increase by 51% in 2045 (1). According to the Chinese diabetes epidemiological survey, the total prevalence of diabetes and the prevalence of prediabetes are 9.7% (10.6% for men and 8.8% for women) and 15.5%, respectively (2). Diabetic peripheral neuropathy (DPN) is a common chronic complication of diabetes and mainly involves the small nerve fibers. It is diagnosed after the exclusion of other peripheral neuropathies, including autoimmune diseases (Sjogren's syndrome, lupus, rheumatoid arthritis), infections (HIV, hepatitis B and C), inherited (Charcot-Marie-Tooth), inflammatory (CIDP), tumors, vitamin B12 deficiency, hypothyroidism, alcoholism, and injury or pressure on the nerve. According to the Toronto DPN international consensus for DPN, a definite diagnosis requires at least one symptom and/or at least one sign of neuropathy and abnormality in NCS (3). However, the evaluation of abnormal myelinated nerve fibers in nerve conduction studies, including tactile, proprioceptive, vibration, and motor functions, is the late manifestation of DPN (4). On the contrary, small nerve fiber defects that affect C and A of the pulpless or thin pulp δ nerve fibers involved in thermal response, pain, and autonomic nervous function are thought to occur earlier in DPN (5). Prediabetes, persistent or sporadic pain is usually manifested in the absence of clinically detectable neuropathy, suggesting small nerve fiber defects. Intra-epidermal nerve fiber density from skin biopsy and corneal confocal microscopy (CCM) can detect small nerve fiber damage in prediabetes. Prediabetes is a risk factor for chronic axonal polyneuropathy, which is consistent with the initial involvement of small nerve fibers. This is the main cause of neuropathic pain and incidence rate, and also the starting factor of diabetic foot ulcers (6).

The onset of the disease is insidious and the progress is slow. A small number of patients show neuralgia, while most of the patients may not show symptoms in the early stage and progress to the

late stage. Late DPN can cause serious complications, such as diabetic foot ulcers, gangrene, and subsequent amputations, which seriously affect the quality of life of people with diabetes and brings a heavy economic burden.

DPN has a higher incidence in people with diabetes. Studies have shown that almost 50% of people with diabetes will have DPN (7), and in some areas, it can even be as high as 60% to 90% (8). In an epidemiological survey of patients with type 2 diabetes in eight communities in Wuhan and Changshu, China, it was found that the total prevalence of DPN was as high as 71.2% (9). With the improvement of social and economic level, the prevalence of diabetes in children and adolescents is also increasing, and the number of diabetes-related complications such as DPN has also increased (10). Some small cross-sectional studies have shown that the prevalence of DPN in children and adolescents with diabetes ranges from 5% to 62% (11, 12). In the United States, the prevalence of DPN in 1,734 patients with T1D and 258 patients with T2D was 7% and 22%, respectively (7). In Australia, the prevalence of DPN in 1,433 patients with T1D and 68 patients with T2D under 18 years old was 21% and 27% (13), respectively; In Denmark, the prevalence of DPN among 339 T1D adolescents was 62% (14).

The pathogenesis of DPN is complicated and has not yet been fully elucidated. The treatment is limited to intensive blood glucose control and symptomatic treatment. Studies have found that in the early stage of type 1 diabetes, paying attention to DPN and optimizing blood glucose management and control can effectively prevent or delay the occurrence of peripheral neuropathy (14). However, the progress of peripheral neuropathy can only be slowed down by management treatment for type 2 diabetes patients with DPN, but the loss of nerve cells is irreversible (15). Therefore, it is very important to screen and diagnose the peripheral neuropathy of people with diabetes in the early stage and take effective targeted measures in the treatment of DPN.

## DIAGNOSTIC GOLD STANDARD OF DPN

DPN is defined as "the presence of symptoms and/or signs of peripheral nerve dysfunction in people with diabetes after the exclusion of other causes". Neuropathy includes the manifestations of the somatic and/or autonomic parts of the peripheral nervous system. It is recommended that there are at least two abnormalities from symptoms, signs, abnormal nerve conduction, quantitative sensory test, or quantitative autonomic nerve test (16). Peripheral neuropathy includes all the conditions leading to the injury of peripheral nervous system and is classified according to the site of nerve injury. Distal symmetrical polyneuropathy (DSP), mononeuropathy, and lumbar/cervical radiculopathy are the most common peripheral neuropathies. The rare sites of peripheral neuropathy include diffuse, length-independent neuropathy, multiple mononeuropathy, multiple radiculopathy, plexopathy, and nerve root exocrine neuropathy. Currently, the diagnosis of DPN is mainly based on characteristic symptoms and signs. Nerve conduction studies (NCS) is one of the gold standard techniques for diagnosing DPN (17). It evaluates the occurrence and development of DPN by detecting the ability of peripheral nerve to transmit electrical signals in patients with DN. NCS has the characteristics of being quantifiable, objective, and sensitive, but it has the following disadvantages: time-consuming, high cost, poor experience, and the need for professional doctors to operate. It is difficult to implement in large sample screening (18). Moreover, NCS is also limited to evaluating large nerve fibers, while small nerve fibers are the first to be affected in DPN patients. These small fiber neuropathies cannot be evaluated by standard electrophysiological tests (19). The application of electrophysiology should be promoted only when the clinical presentation is atypical or the diagnosis is unclear (20). Therefore, it is necessary to apply some simple and effective methods to screen DPN for early intervention and control.

## EARLY SCREENING METHODS FOR DPN

### Quantitative Sensory Testing

Quantitative sensory testing (QST) is a technique for evaluating sensory neuropathy. It detects small fiber and large fiber neuropathy through standardized and quantified sensory differences such as stimulation, vibration, and temperature. Compared with NCS, this method is noninvasive and easy to operate. In people with diabetes, sensory dysfunction precedes symptoms such as painful neuropathy and foot ulcers (21). QST, which can reliably measure sensory loss and threshold, provides standardized and quantified stimuli and quantifies response levels. Brown et al. (22) found that cold and hot QST could detect the obvious changes of neurological function in patients with mild to moderate DPN after 12 months, while other indicators (vibration QST, neuropathy score, and monofilament examination) were not sensitive to the changes of neurological function in this population. However, the repeatability of QST has always been a difficult problem in clinical application, and there are significant differences between the treatment courses of the same patient. Relevant studies have shown that according to the severity of neuropathy, the sensitivity of the heat test is variable, with cold damage being 27%–98%, heat damage being 22%–98% (19), vibration testing sensitivity being 58%–84%, and the specificity being 51%–86% (23). A QST-based neural test is used to evaluate vibration perception threshold (VPT), cold perception threshold (CPT), warming perception threshold (WPT), and heat pain perception threshold (HPT). Its sensitivity to vibration test is as high as 84%, and its specificity is as high as 81%. Its sensitivity to heat test is high, and its specificity is medium, and has good repeatability and diagnostic accuracy in evaluating sensory loss (24). In China, Sun Yukai et al. believe that the sensitivity and specificity of VPT for DPN were 85.19% and 88.68%, respectively, and can be used for early screening of DPN lesions (25).

QST also has its limitations. First of all, QST can detect the integrity of the entire sensory nerve axis, which has no value in localization. Secondly, QST is a psychosomatic test, which lacks

the objectivity of NCS, and its results will change due to distraction, boredom, mental fatigue, drowsiness, or confusion. When patients consciously or unconsciously prefer abnormal QST results, no psychophysical test can reliably distinguish these patients from patients with organic diseases. Moreover, most of the QST measuring instruments are expensive, the cost is high, the test results are complex, and it is difficult to quantify sensory defects and other factors that hinder the wide application of QST in clinical practice (26).

## Clinical Neurological Function Scoring System

The clinical neurological function scoring system is mainly used in epidemiological surveys for early screening and evaluation of DPN, which helps to grade the severity of the disease and can quantitatively evaluate clinical indicators such as physical examination. Currently, commonly used neurological scoring scales include Michigan neuropathy screening instrument (MNSI), Toronto clinical scoring system (TCSS), neuropathy symptom score (NSS), and neuropathy disability score (NDS).

MNSI was first proposed in 1994 (27), including patient questionnaire and physical examination. The total score is 10 points, and more than 2 points are considered abnormal. It is widely used in clinical practice and large-scale clinical trials to evaluate distal symmetrical peripheral neuropathy, including action to control cardiovascular risk in diabetes (ACCORD) (28) and bypass angioplasty revascularization investigation 2 diabetes (BARI 2D) (29). MDNS includes three parts: toe sensation, distal muscle strength of limbs, and tendon reflex score. The total score is 46 points, and >6 points are considered abnormal. Domestic research has found that the abnormal detection rate of MDNS is 84.5%, the abnormal detection rate of TCSS is 62.0%, the abnormal detection rate of NCV is 76.0%, and the abnormal detection rate of the combination of the three is 91.5%. The sensitivity of MDNS and TCSS are 92.9% and 77.6%, the specificity are 51.6% and 87.1%, the accuracy are 82.9% and 79.8%, the positive predictive value are 85.8% and 95.0%, the negative predictive value are 69.6.% and 55.1%, Youden coefficients are 0.445 and 0.647, Kappa values are 0.488 and 0.539 ($p < 0.05$), and AUC are 0.837 and 0.875, respectively (30). The MNSI composite index has advantages in DPN mid-term screening. TCSS can be used for DPN classification, and its combined application can increase the detection rate of DPN. Some domestic studies believe that with an MNS score of 4 points being the segmentation point (31) and a TCSS score of 5 points being the segmentation point (32), neurological abnormalities can be better diagnosed in China, and the detection rate of DPN has been significantly improved. The limitation of MNS is that when it is used in type 1 people with diabetes with relatively young age, long course of disease, and low prevalence of distal symmetric peripheral neuropathy, the positive predictive value is low and the false positive is high (33).

NSS is scored according to the symptoms, location, and pain relief methods of lower limbs; NDS is scored according to ankle reflex, dorsalis pedis acupuncturing sensation, toe vibration sensation, and temperature sensation. DPN is a mainly axonal disease that gradually develops from the distal end to the proximal end. The nerve damage starts from the most distal end of the limb (34). Early DPN generally occurs in the upper limbs and other parts. In the early stage, sensory neuropathy is more obvious than motor nerve (35). Therefore, NSS and NDS assess lower limb neurosensory function and can be used as a screening tool for early DPN. Liu Wenqu used NSS/NDS to diagnose 679 patients with type 2 diabetes. The results showed that the sensitivity and specificity were 68.0% and 77.2%, respectively, and the positive predictive value, negative predictive value, and Youden index were 86.5%, 53.5%, and 45.2% respectively, suggesting that NSS/NDS has good application value for early screening of DPN patients (36).

## Corneal Confocal Microscope

Corneal confocal microscopy (CCM) is a non-invasive ophthalmic imaging technique for assessing the corneal nerve fiber morphology. With the use of an imaging software, the corneal nerve fiber morphology can be objectively quantified to the corneal nerve fiber density (CNFD), length (CNFL), and branch density (CNBD) of people with diabetes (37).

The application of CCM in diabetes was first proposed about 20 years ago (38), but at that time, due to the limited availability of equipment in general ophthalmology practice, the lack of experts with relevant professional knowledge in corneal scanning, the small field of vision of confocal microscope, the lack of clear consensus on the number of images needed for representative quantitative analysis, and other limitations, it was limited in clinical practice. In addition, severe dry eyes, severe corneal dystrophies, ocular trauma or surgery in the preceding 6 months, and glaucoma can affect the corneal nerve fiber morphology (39).

CCM has experienced three main development stages, namely, series scanning CCM, classified scanning CCM, and laser scanning CCM. At present, it is widely used in clinic. In the field of diabetes, the decrease of corneal nerve density was observed in diabetic animal models. After that, it was found that there were also some changes in the morphology of corneal nerve fibers in diabetic patients, and there was a certain correlation with the degree of peripheral neuropathy. Kallinikos and other scholars believe that this change in nerve fiber morphology is closely related to systemic neuropathy. Therefore, we can observe the changes of corneal nerve fibers through CCM to evaluate DPN.

The literature on the use of IVCM to quantify diabetic neuropathy shows that the density of the corneal basal nerve fibers and the curvature of nerve fibers in diabetic patients are increased. These nerve fiber changes are related to the stage or severity of peripheral neuropathy (39). In addition, ivcm can detect early peripheral neuropathy because the decrease of nerve density precedes the damage of corneal sensitivity. The significant correlation between corneal and cutaneous nerve degeneration in DPN strengthens the evidence that IVCM is a valuable tool for the diagnosis and assessment of DPN (40).

A meta-analysis on the value of CCM in the early diagnosis of DPN confirmed that compared with healthy controls and people with diabetes without DPN, CNFD, CNBD, and CNFL of DPN patients were significantly reduced, and CCM can detect corneal

nerve changes in DPN patients (41). In a longitudinal study, 89 patients with type 1 diabetes and 64 healthy subjects were subjected to CCM and clinical and electrophysiological examinations at the same time. Among them, the CCM parameters of DNP subjects were significantly reduced, and the threshold of CNFL ≤ 14.0 mm/mm (2) optimized the sensitivity and specificity of the diagnosis of DNP (sensitivity 85%, specificity 84%) (42).

However, CCM has the advantages of non-invasiveness, simple operation, short surface anesthesia time, and accurate and dynamic observation of corneal nerve changes, among others. In recent years, with the advancement of CCM technology including automatic scanning and analysis and wide-area imaging (43), it has a good application prospect for the evaluation of small nerve fiber damage in DPN, early diagnosis, and treatment effect evaluation.

## High-Frequency Ultrasound

High-frequency ultrasound was first used in the clinical diagnosis of DPN as a supplement to NCS. It measures the size, blood vessels, echo, and mobility of the diseased nerve to show the damage of the nerve tissue, which can effectively improve the diagnostic efficiency of DPN and reduce the missed diagnosis rate and the misdiagnosis rate (44). In patients with DPN, the cross-sectional area (CSA) and longitudinal section of the nerve are increased, and the echogenicity of the nerve, the boundary ambiguity, and the blood flow in the nerve are also significantly increased. The mean CSA in the examined nerves was higher in moderate to severe DPN than the mild DPN (45). High-resolution ultrasound has unique diagnostic advantages for early or subclinical neuropathy. High-frequency ultrasound can detect subclinical involvement of peripheral nerves and abnormalities in patients with normal electrical diagnosis (46).

Studies have shown that the maximum thickness of the median nerve and posterior tibial nerve tract, CSA, and hypoechoic area in people with diabetes are closely related to the degree of neuropathy ($p < 0.0001$) and are significantly greater than the control group ($p < 0.05$) (47), suggesting that high-frequency ultrasound can also be used to grade the severity of DPN. A study shows that the sensitivity and specificity of CSA and vascular proliferation in detecting DPN-induced carpal tunnel syndrome (CTS) are 90.9%, 94.0%, 93.4%, and 90.0%, respectively. The severity of CTS is significantly related to various stages of vascular proliferation, and high-frequency ultrasound can be used to diagnose CTS early and estimate its severity (48). However, some studies have found that NCS is more sensitive than ultrasound when determining the severity of ulnar neuropathy, especially in mild or moderate neuropathy (49).

The ultrasound image of DPN lacks a unified definition, and its results are easily affected by the subjective factors of the examiner. Therefore, it is necessary to establish a complete and unified ultrasound image quantitative scoring system. Some ultrasound scoring systems have been developed. For example, the Bochum ultrasound score (BUS) can effectively distinguish between subacute chronic inflammatory demyelinating polyneuropathy (CIDP) and acute inflammatory demyelinating polyneuropathy (AIDP) (sensitivity 90%, specificity 90.4%) (50). The DCEC scoring system uses the total scores of definition, CSA, echo, and nerve entrapment as quantitative evaluation indicators. The critical value of peripheral nerves in the arms and legs is 14.5, which is a good indicator of the presence or absence of DPN (the area under the curve is 0.85) (sensitivity is 0.81; specificity is 0.80) (51).

In addition, the newly developed ultrasound elastography can evaluate the neuroelasticity of DPN. As a new type of instrument, it can measure the hardness or elasticity of the tissue, reflecting the change of the elasticity of the compressed tissue with the degree of tissue deformation (52). Few studies have previously evaluated the changes in peripheral nerve elasticity in patients with DPN, but some studies have shown that this may be a new breakthrough point. The thickening of the peripheral nerve sheath fibers leads to changes in the elasticity of the tibial nerve in type 2 people with diabetes. The nerve stiffness of people with diabetes without clinical or electrophysiological signs of DPN was significantly higher than that of the control group, with a critical stiffness value of 51.05 kPa, a sensitivity of 90%, and a specificity of 85% (53, 54).

High-frequency ultrasound has the advantages of non-invasiveness and good repeatability, and has broad application prospects in the auxiliary diagnosis and prognosis judgment of DPN. The combination of ultrasound and nerve conduction examination can improve the diagnostic value of DPN and avoid missed diagnosis and misdiagnosis. It is helpful to evaluate the severity and prognosis of DPN with fuzzy boundary, enlarged CSA, and hypoechoic area, but it has certain limitations for the exploration of the deep neuromuscular plexus and lumbosacral plexus. With the popularity of high-resolution ultrasound, especially shear wave elastography, it is expected to replace neuroelectrophysiological examination in the diagnosis of DPN.

In summary, the above methods and technologies have their own advantages and disadvantages and need to be verified and further developed by large samples. They have not been widely used at present and are generally used as a supplement to traditional neuroelectrophysiological examinations. In clinical practice, it is still necessary to establish a unified, accurate, and convenient early screening method and diagnostic grading method for DPN, so as to quantitatively assess the degree of nerve damage, carry out early intervention and management, and improve the quality of life of DPN patients.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

## AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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# Troponin T Is Negatively Associated With 3 Tesla Magnetic Resonance Peripheral Nerve Perfusion in Type 2 Diabetes

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**Objective:** The pathogenesis of diabetic polyneuropathy (DN) is poorly understood and given the increasing prevalence of DN, there is a need for clinical or imaging biomarkers that quantify structural and functional nerve damage. While clinical studies have found evidence of an association between elevated levels of troponin T (hsTNT) and N-terminal pro brain natriuretic peptide (proBNP) with microvascular compromise in type 2 diabetes (T2D), their implication in mirroring DN nerve perfusion changes remains unclear. The objective of this study was, therefore, to investigate whether hsTNT and proBNP assays are associated with MRI nerve perfusion in T2D.

**Methods:** In this prospective cross-sectional single-center case-control study, 56 participants (44 with T2D, 12 healthy control subjects) consented to undergo magnetic resonance neurography (MRN) including dynamic contrast-enhanced (DCE) perfusion imaging of the right leg. Using the extended Tofts model, primary outcome parameters that were quantified are the sciatic nerve's microvascular permeability ($K^{trans}$), the extravascular extracellular volume fraction ($v_e$), and the plasma volume fraction ($v_p$), as well as hsTNT and proBNP values from serological workup. Further secondary outcomes were clinical, serological, and electrophysiological findings.

**Results:** In T2D patients, hsTNT was negatively correlated with $K^{trans}$ (r=-0.38; p=0.012) and $v_e$ (r=-0.30; p=0.048) but not with $v_p$ (r=-0.16; p=0.294). HsTNT, $K^{trans}$, and $v_e$ were correlated with peroneal nerve conduction velocities (NCVs; r=-0.44; p=0.006, r=0.42;

p=0.008, r=0.39; p=0.014), and tibial NCVs (r=-0.38;p=0.022, r=0.33; p=0.048, r=0.37; p=0.025). No such correlations were found for proBNP.

**Conclusions:** This study is the first to find that hsTNT is correlated with a decrease of microvascular permeability and a reduced extravascular extracellular volume fraction of nerves in patients with T2D. The results indicate that hsTNT may serve as a potential marker for the assessment of nerve perfusion in future studies on DN.

**Keywords: diabetic neuropathy, magnetic resonance neurography, dynamic contrast enhancement, perfusion, troponin T**

## INTRODUCTION

Diabetic polyneuropathy (DN) is one of the most frequent and most disabling complications of diabetes mellitus (1). Especially in type 2 diabetes (T2D), the complex pathophysiological mechanisms that cause DN have not been understood completely (1–4). Evidence from clinical and histological studies suggests that nerve ischemia related to macro- and microangiopathy as well as cardiac insufficiency is a major contributor to demyelination and axonal damage in T2D (5–8). Recent studies have found cardiac biomarkers, high sensitivity troponin T (hsTNT) and N-terminal pro brain natriuretic peptide (proBNP), to be associated with microvascular complications in patients with T2D (9). It remains to be determined, however, whether hsTNT and proBNP are also associated with the occurrence of DN and whether both hsTNT and proBNP codify parameters of nerve perfusion such as plasma volume or microvascular permeability (9). To date, is has not been possible to assess the perfusion of peripheral nerves directly in the context of clinical studies. Magnetic resonance neurography (MRN) at 3 Tesla (3T) is a non-invasive method that allows to visualize and quantify structural and physiological changes of peripheral nerves along the entire anatomical course (10, 11). Recent studies on MRN in patients with T2D have found that structural nerve damage associated with demyelination in T2D is related to elevated levels of hsTNT but not proBNP (12). While there are several animal experimental MRI studies on the vascular supply of peripheral nerves and monitoring changes in microvasculature (13–16), only a recent pilot study on dynamic contrast enhanced (DCE) MRN in patients focusing on inflammatory neuropathies could, for the first time, demonstrate that DCE sequences allow investigating the perfusion of peripheral nerves by assessing parameters related to plasma volume and microvascular permeability (17). These parameters can be obtained from DCE MRN using the extended Tofts model, which allows calculating the constant of the examined nerve's capillary permeability ($K^{trans}$), the volume fraction of the plasma space ($v_p$), and the volume fraction of the extracapillary extracellular space ($v_e$) (18, 19). The aim of this study was to combine DCE imaging of nerves at thigh level in patients with T2D with hsTNT and proBNP assays, and demographic, clinical, and electrophysiological data in order to investigate potential associations between hsTNT and proBNP with parameters of nerve microcirculation in patients with T2D.

## METHODS

### Study Design and Participants

This study was approved by the ethics committee of Heidelberg University Hospital (HEIST-DiC, clinicaltrials.gov identifier NCT03022721, local ethics number S-383/2016) and all participants gave written informed consent. Overall exclusion criteria were age <18, pregnancy, an estimated glomerular filtration rate (eGFR) <60ml/min, any contraindications for MR imaging or administration of MRI contrast agents. Further reasons for exclusion were history of myocardial infarction, coronary heart disease, heart surgery, spine surgery, lumbar disc extrusion, risk factors for sarcopenia or neuropathy other than diabetes such as malignant diseases, alcoholism, hypovitaminosis, previous or ongoing exposure to neurotoxic agents, chronic neurological diseases such as Parkinson's disease, restless legs syndrome, or multiple sclerosis. The sample size was based on the results of previous MRN studies on DN (12, 20) and 44 patients with T2D (17 women, 27 men) and 12 controls (7 women, 5 men) were enrolled in this prospective single-center study between June 2016 and March 2020 and underwent DCE MRN with subsequent clinical, electrophysiological, and serological assessments.

### Clinical and Electrophysiological Examination

For every participant, a detailed medical history was taken. Electrophysiological examinations (VikingQuest; Viasys Healthcare GmbH, Höchberg, Germany) included an assessment of nerve conduction velocities (NCVs) of the tibial, peroneal, and sural nerve, distal motor latencies (DMLs) of the right tibial and peroneal nerve, compound muscle action potentials (CMAPs) of the tibial and peroneal nerve, and sensory nerve action potentials (SNAPs) of the sural nerve of the right leg. Skin temperature was kept at 32°C throughout the examination. Electrophysiological studies were conducted by two specially trained medical technical assistants with more than 6 years of experience in electrophysiological assessments on patients with diabetes. An examination of neuropathic symptoms was performed comprising the neuropathy disability score (NDS) and the neuropathy severity scale (NSS) (21). In line with Gibbon's criteria for DN, patients with an NDS ≥ 3 were assigned to the DN group (22).

## MRI Imaging Protocol

All participants underwent high-resolution MRN of the right thigh in a 3.0 Tesla MR-scanner (Magnetom Tim TRIO, Siemens Healthineers, Erlangen, Germany). A 15-channel transmit-receive extremity coil was used and the following sequences were applied:

1) axial high resolution T2-weighted turbo spin echo (TSE) 2D sequence with spectral fat suppression; repetition time (TR) = 5970 ms, echo time (TE) = 55 ms, field of view (FOV) = 160 × 160 $mm^2$, matrix size = 512 × 512, slice thickness = 4 mm, no interslice gap, voxel size = 0.3 × 0.3 × 4.0 $mm^3$, 24 slices, 24 acquired images, total acquisition time = 4:42 min;
2) axial T1-weighted volume interpolated breathhold examination (VIBE) sequence; TR = 3.3 ms, TE = 1.11 ms, FOV = 160 × 160 $mm^2$, matrix size = 128 × 128, slice thickness = 4 mm, interslice gap = 0.8 mm, voxel size = 1.3 × 1.3 × 4.0 $mm^3$; single acquisition at a flip-angle of 5°, 8°, 11°, 14°, 17° (24 slices = 144 acquired images), total acquisition time = 30s;
3) axial T1-weighted volume interpolated breathhold examination (VIBE) sequence; TR = 3.3 ms, TE = 1.11 ms, FOV = 160 × 160 $mm^2$, matrix size = 128 × 128, slice thickness = 4 mm, interslice gap = 0.8 mm, voxel size = 1.3 × 1.3 × 4.0 $mm^3$ 50 repetitions (1200 acquired images) at a flip angle of 15°, contrast agent administration (Dotarem®, Guerbet, France, 0.1 mmol/kg, flow rate 3.5ml/s) after completion of the sixth repetition, total acquisition time = 4:09 min.

The sequence was centered on the sciatic nerve bifurcation at distal thigh level in every participant.

## MRI Data Analysis and Statistical Analysis

All images were pseudonymized and observers were blinded to all clinical data. For each patient, we segmented the sciatic nerve manually on all images of the T2-weighted sequence with ImageJ (23). T2-weighted images were also co-registered to the T1 VIBE sequence with affine transformations using custom-written code in Matlab (MathWorks, Natick, MA, R2020b) (24). The schematic process of image segmentation and co-registration is shown in **Figure 1**. We manually determined the arterial input function (AIF) by segmenting a region of interest of the femoral artery on a representative imaging slice. The average signal intensity of all artery voxels for consecutive imaging time points was then used to obtain the signal intensity curve during contrast administration. The signal intensity baseline end was determined as the last imaging time point before signal intensity would increase by more than 25% above the averaged signal intensity of all preceding imaging time points. The resulting AIF was smoothed with a moving average filter of 3 images.

We determined the relaxation time $T_{1,0}$ for the first imaging time point subsequently for each voxel in dependence on flip angle $\alpha$ by assigning $T_{1,x}(\alpha) = SI_0/\tan(\alpha)$ and $T_{1,y}(\alpha) = SI_0/\sin(\alpha)$, where $SI_0$ represents the initial signal intensity. Subsequent linear regression on $T_{1,x}$ and $T_{1,y}$ yields the slope $m$ (25). Spin-lattice relaxation time $T_{1,0}$ follows as $T_{1,0} = -TR/\log(m)$, with repetition

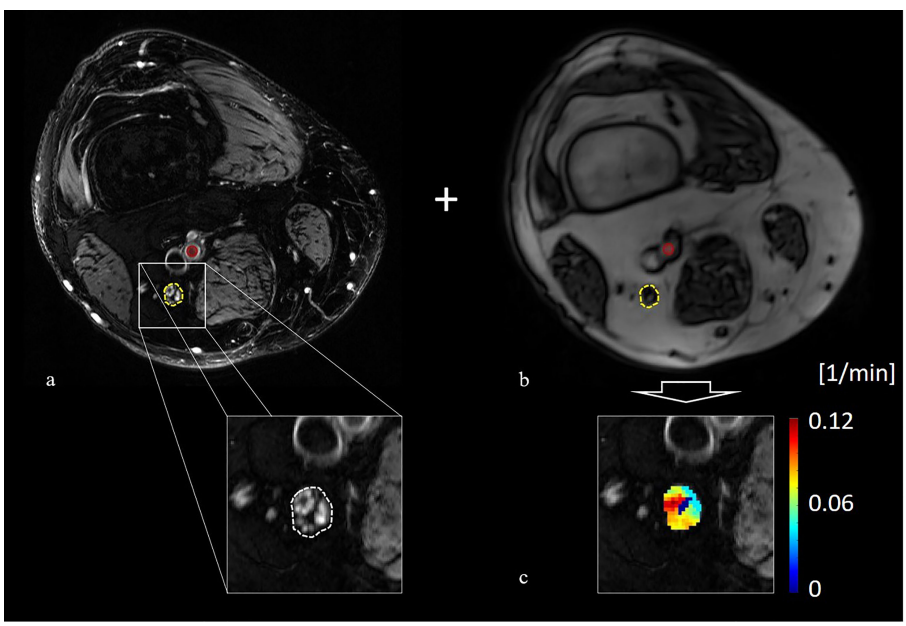


**FIGURE 1** | Principle of image co-registration and assessment of nerve perfusion parameters: **(A)** T2-weighted image of the distal right thigh showing the tibial compartment of the sciatic nerve (yellow circle) and the position of the femoral artery (red circle), **(B)** axial T1-weighted volume interpolated breathhold examination sequence of the same position a s in **(A)** with the co-registered position of the sciatic nerve (yellow circle) and the femoral artery (red circle), **(C)** color coded map of $K^{trans}$ values obtained from the extended Tofts model.

time $TR$. Using $\alpha_R$ = 15°, we obtain with signal intensity $SI_t$ and relaxation time $T_{1,t}$ at time $t$: $\frac{1}{T_{1,t}} = -\log\left(\frac{[1-C_a]}{1-\cos(\alpha_R)C_a}\right)/TR$, where $C_a = \frac{\frac{SI_t}{SI_0}[1-\exp(-\frac{TR}{T_{1,0}})]}{[1-\cos(\alpha_R)\exp(-\frac{TR}{T_{1,0}})]}$, c.f. Eq. (6) in (26). The tissue concentration $C(t)$ at time $t$ is then found as $C(t) = \frac{1}{r_1}\left[\frac{1}{T_{1,t}} - \frac{1}{T_{1,0}}\right]$ (26), where $r_1$ = 3.43 L/mmol/s represents the relaxivity of blood at 3 Tesla (27).

We chose the extended Tofts model (ETM) which is used as a default perfusion model in central nervous system imaging and diabetes, see e.g., (19). It consists of a plasma and an extravascular extracellular compartment. Simpler perfusion models, such as the Patlak model, assume a negligible diffusion of contrast agent from the extravascular space back to the intravascular space, however, this *a priori* assumption cannot be justified in diabetes where it was shown that the permeability of the blood-brain barrier is generally increased (28). In the ETM, we have (29): $C_M(t) = K^{trans}\int_0^t AIF(t')\exp\left(-\frac{K^{\text{trans}}[t-t']}{v_e}\right)dt' + v_p AIF(t)$, where $K^{trans}$ represents the volume transfer constant between plasma and the extravascular extracellular compartment, $v_e$ represents the volume fraction of extravascular extracellular space per unit volume of tissue, $v_p$ is blood plasma volume per unit volume of tissue, and $C_M(t)$ corresponds to the model tissue concentration at time $t$. To minimize the residual sum of least squares, $\Sigma_t[C_M(t) - C(t)]^2$, we used the Nelder-Mead simplex method for model parameters $K^{trans}$, $v_e$, and $v_p$ with starting values $K^{trans}$ = 0.007/s, $v_e$ = 0.15, $v_p$ = 0.025 (17, 30–32).

## Statistical Analysis

Statistical data analysis was carried out with MATLAB (R2020b) and GraphPad Prism 7. The D'Agostino-Pearson omnibus normality test was used to test for Gaussian normal distribution. If a Gaussian normal distribution was given, t tests were used for comparisons of two groups, one-way ANOVAs were used for comparisons of more than two groups, and Pearson correlation coefficients were used for correlation analysis. If data were not Gaussian distributed, the Mann-Whitney test was used for comparisons of two groups, the Kruskal-Wallis test with *post-hoc* Dunn correction was used for multiple comparisons of more than two groups, and nonparametric Bonferroni-corrected Spearman correlation was used for correlation analysis. In case of multiple significant correlations for one parameter, partial correlation analysis with controlling for confounding variables was performed.

# RESULTS

## Demographic and Clinical Data

This study comprised 44 patients with T2D (17 women, 27 men, mean age 66.14 ± 7.12) and 12 controls (7 women, 5 men mean age 61.58 ± 7.79). Between T2D patients and controls, there were no significant differences for age, gender, BMI, or glomerular filtration rate. In the T2D group, 26 patients suffered from DN. On electrophysiological examination, lower tibial and peroneal NCVs and CMAPs were found in T2D patients compared to controls. A summary and comparison of demographic and clinical data of patients with T2D and controls is provided in **Table 1**.

**TABLE 1** | Comparison of demographic, serologic, clinical, electrophysiological, and MRN imaging data of all study participants.

| | T2D | Controls | p |
|---|---|---|---|
| **$K^{trans}$ ($min^{-1}$)** | 0.040 ± 0.011 | 0.035 ± 0.011 | $0.207^{T}$ |
| **$v_p$ (%)** | 4.74 ± 0.82 | 4.63 ± 0.56 | $0.959^{M}$ |
| **$v_e$ (%)** | 3.28 ± 4.58 | 1.64 ± 1.71 | $0.118^{M}$ |
| **Age (years)** | 66.14 ± 7.12 | 61.58 ± 7.79 | $0.076^{T}$ |
| **Diabetes duration (years)** | 10.18 ± 9.53 | n.a. | n.a. |
| **Gender (w/m)** | 17 w/27m | 7w/5m | $0.229^{M}$ |
| **BMI ($kg/m^2$)** | 28.68 ± 4.11 | 27.47 ± 3.64 | $0.359^{T}$ |
| **hsTNT (pg/mL)** | 9.93 ± 4.04 | 7.25 ± 2.18 | $0.032^{T}$ |
| **proBNP (pg/mL)** | 115.30 ± 121.60 | 75.92 ± 52.20 | $0.646^{M}$ |
| **HbA1c %** | 6.88 ± 1.23 | 5.58 ± 0.55 | $<0.001^{M}$ |
| **GFR (ml/min)** | 87.55 ± 15.05 | 87.50 ± 13.88 | $0.992^{T}$ |
| **NDS** | 3.56 ± 3.08 | 1.33 ± 1.44 | $0.026^{M}$ |
| **NSS** | 4.14 ± 3.43 | 2.33 ± 3.60 | $0.144^{M}$ |
| **Sural nerve NCV (m/s)** | 45.13 ± 7.06 | 45.83 ± 4.82 | $0.758^{M}$ |
| **Sural nerve SNAP (μV)** | 5.54 ± 3.20 | 7.89 ± 4.71 | $0.070^{M}$ |
| **Peroneal NCV (m/s)** | 39.59 ± 5.40 | 45.08 ± 4.60 | $0.002^{T}$ |
| **Peroneal CMAP (mV)** | 5.02 ± 4.01 | 8.88 ± 6.96 | $0.020^{M}$ |
| **Peroneal DML (ms)** | 7.37 ± 13.13 | 3.94 ± 0.69 | $0.009^{M}$ |
| **Tibial NCV (m/s)** | 40.81 ± 5.02 | 44.75 ± 4.00 | $0.017^{T}$ |
| **Tibial CMAP (mV)** | 9.68 ± 6.41 | 16.58 ± 8.24 | $0.004^{T}$ |
| **Tibial DML (ms)** | 5.00 ± 3.01 | 3.62 ± 0.53 | $0.014^{M}$ |

*All values are displayed as mean ± standard deviation. $K^{trans}$, constant of permeability; $v_p$, plasma volume fraction; $v_e$, extracellular extravascular volume fraction; BMI, body-mass index; hsTNT, high sensitivity troponin T; proBNP, pro brain binatriuretic peptide; n.a., not applicable; NDS, neuropathy disability score; NSS, neuropathy severity scale; NCV, nerve comnduction velocity; CMAP, compound motor action potential; SNAP, sensory nerve action potential; m/s, meters per second; ms, miliseconds; mV, millivolts; μV, microvolts; $^{M}$, p value obtained from Mann-Whitney U test; $^{T}$, value obtained from t-Test.*

## Group Comparisons of hsTNT and proBNP Levels for T2D Patients and Controls

Patients with T2D showed higher levels of hsTNT compared to controls (9.93 ± 0.04 pg/mL versus 7.25 ± 2.18 pg/mL, respectively, p=0.032), see **Table 1**. Also, ANOVA revealed that hsTNT was higher in T2D patients with DN compared to T2D patients without DN (11.67 ± 3.50 vs. 8.65 ± 4.05; p=0.015) and compared to controls (vs. 7.25 ± 2.18; p=0.002), no such differences were found for proBNP. In T2D patients, hsTNT correlated negatively with tibial and peroneal NCVs and with tibial CMAPs. A positive correlation was found between hsTNT and age. No such correlations were found for proBNP. A summary of all correlations of hsTNT and proBNP in T2D patients and controls is provided in **Table 2**, and correlations of hsTNT with tibial and peroneal NCVs are illustrated in **Figures 2A, B**.

## MRN Perfusion Parameters

No significant differences were found for perfusion parameters $K^{trans,}$ $v_p$, and $v_e$ between T2D patients and the control group. ANOVA found lower $K^{trans}$ values in patients with DN compared to T2D patients without DN (0.037 ± 0.010 vs. 0.044 ± 0.010; p=0.042) but not compared to controls (0.037 ± 0.010 vs. 0.035 ± 0.011; p=0.882). In patients with T2D and in controls, $K^{trans}$ was strongly correlated with $v_e$ (r=0.75; p<0.001, and r=0.76; p=0.007, respectively). A summary of all correlations of perfusion parameters in T2D patients is provided in **Table 3**.

**TABLE 2** | Correlations of hsTNT and proBNP with MRN perfusion parameters and demographic, serologic, clinical, and electrophysiological data.

| | hsTNT T2D (pg/mL) | | proBNP T2D (pg/mL) | | hsTNT Co (pg/mL) | | proBNP Co (pg/mL) | |
|---|---|---|---|---|---|---|---|---|
| | r | p | r | p | r | p | r | p |
| **$K^{trans}$ ($min^{-1}$)** | -0.38 | 0.012 | -0.09 | 0.569 | 0.39 | 0.241 | 0.03 | 0.931 |
| **$v_p$ (%)** | -0.16 | 0.294 | -0.17 | 0.280 | -0.20 | 0.558 | -0.18 | 0.602 |
| **$v_e$ (%)** | -0.30 | 0.048 | -0.05 | 0.732 | 0.31 | 0.353 | 0.21 | 0.529 |
| **Age (years)** | 0.35 | 0.018 | 0.13 | 0.393 | 0.23 | 0.479 | 0.55 | 0.063 |
| **Diabetes duration (years)** | -0.07 | 0.694 | -0.12 | 0.499 | | | | |
| **Gender** | -0.16 | 0.291 | 0.41 | 0.006 | -0.38 | 0.217 | -0.23 | 0.463 |
| **BMI ($kg/m^2$)** | -0.03 | 0.824 | 0.17 | 0.274 | 0.06 | 0.860 | -0.34 | 0.286 |
| **hsTNT (pg/mL)** | | | 0.10 | 0.503 | | | 0.62 | 0.032 |
| **proBNP (pg/mL)** | 0.10 | 0.503 | | | 0.62 | 0.032 | | |
| **HbA1c %** | 0.24 | 0.120 | -0.03 | 0.850 | 0.27 | 0.398 | -0.06 | 0.849 |
| **GFR (ml/min)** | -0.25 | 0.123 | -0.24 | 0.137 | 0.33 | 0.295 | 0.31 | 0.325 |
| **NDS** | 0.45 | 0.003 | 0.09 | 0.556 | 0.17 | 0.588 | -0.02 | 0.941 |
| **NSS** | 0.17 | 0.276 | 0.12 | 0.431 | 0.27 | 0.403 | 0.54 | 0.068 |
| **Sural nerve NCV (m/s)** | -0.33 | 0.128 | <0.01 | 0.986 | 0.00 | 0.989 | -0.23 | 0.472 |
| **Sural nerve SNAP (μV)** | -0.19 | 0.320 | -0.16 | 0.398 | -0.10 | 0.757 | -0.16 | 0.616 |
| **Peroneal NCV (m/s)** | -0.53 | 0.001 | -0.03 | 0.850 | -0.31 | 0.326 | -0.58 | 0.049 |
| **Peroneal CMAP (mV)** | -0.19 | 0.259 | -0.18 | 0.284 | 0.15 | 0.637 | -0.23 | 0.482 |
| **Peroneal DML (ms)** | 0.12 | 0.468 | -0.27 | 0.101 | 0.31 | 0.330 | 0.47 | 0.121 |
| **Tibial NCV (m/s)** | -0.51 | 0.001 | 0.254 | 0.124 | 0.22 | 0.500 | 0.04 | 0.905 |
| **Tibial CMAP (mV)** | -0.55 | 0.001 | 0.19 | 0.276 | -0.70 | 0.012 | -0.55 | 0.065 |
| **Tibial DML (ms)** | 0.18 | 0.288 | -0.04 | 0.823 | 0.44 | 0.155 | 0.55 | 0.064 |

*$K^{trans}$, constant of permeability; $v_p$, plasma volume fraction; $v_e$, extracellular extravascular volume fraction; BMI, body-mass index; hsTNT, high sensitivity troponin T; proBNP, pro brain binatriuretic peptide; NDS, neuropathy disability score; NSS, neuropathy severity scale; NCV, nerve comnduction velocity; CMAP, compound motor action potential; SNAP, sensory nerve action potential; m/s, meters per second; ms, miliseconds; mV, millivolts; μV, microvolts; Co, controls.*

## Correlation of Perfusion Parameters With Demographic Data

In patients with T2D and controls, $K^{trans}$ correlated positively with the BMI. Another correlation was found between $v_e$ and BMI in patients with T2D. Parameter $v_p$ was negatively correlated with age, while no such correlation was found for $K^{trans}$ or $v_e$.

## Correlation of Perfusion Parameters With Serological, Clinical, and Electrophysiological Data

In T2D patients, $K^{trans}$ was positively correlated with NCVs of tibial, peroneal, and sural nerves. Parameter $v_e$ was positively correlated with tibial and peroneal NCVs and DMLs. Correlations of $K^{trans}$ with tibial and peroneal NCVs are illustrated in **Figures 2C, D**. $K^{trans}$ and $v_e$ were negatively correlated with hsTNT (**Figure 2E**). In a partial correlation analysis, double-controlled for confounding variables age and BMI, correlations remained significant between $K^{trans}$ and hsTNT (r=-0.42; p=0.005) and between $v_e$ and hsTNT (r=0.33; p=0.034). No such correlations were found for $K^{trans}$ and $v_e$ with proBNP, HbA1c, or the glomerular filtration rate. No correlations were found for $v_p$ with hsTNT or electrophysiological parameters. A detailed summary of all correlations of MRN perfusion parameters with clinical and electrophysiological data in T2D patients and controls is provided in **Table 3**. Using a modified classification score for diabetic neuropathy severity based on electrophysiological parameters as proposed in (33), we allocated a score of 1 to patients with a sural nerve SNAP amplitude ≥ 5 μV, and a score of 2, 3, and 4 to patients with a sural nerve SNAP amplitude < 5 μV and a tibial nerve CMAP amplitude ≥ 5 mV, ≥ 2 mV and ≤ 5 mV, and < 2 mV, respectively, where neuropathy severity increases with score value. We subsequently found significant negative correlations between neuropathy severity score value and $K^{trans}$ (r=-0.41; p=0.007), and $v_e$ (r=-0.37; p=0.017), indicating that neuropathy severity is associated with reduced nerve perfusion in agreement with previous animal experimental studies, possibly due to the development of abnormal microvasculature and capillary dysfunction (34, 35).

# DISCUSSION

This study used DCE 3T MRN to investigate potential associations of peripheral nerve perfusion with cardiac biomarkers hsTNT and proBNP in patients with T2D. The main findings were (i) in T2D patients, hsTNT was negatively correlated with $K^{trans}$ and $v_e$, while no such correlation was found for proBNP; (ii) in T2D, hsTNT, $K^{trans}$, and $v_e$ were correlated with electrophysiological parameters and an electrophysiology-based neuropathy severity score; and (iii) hsTNT was increased while $K^{trans}$ and $v_e$ were decreased in DN patients compared to patients without DN.

The results of this study confirm the hypothesis that hsTNT codifies parameters of nerve perfusion in patients with T2D (9, 12, 36). Specifically, the correlation of hsTNT with $K^{trans}$ suggests that an increase in hsTNT is associated with a decrease in capillary permeability of peripheral nerves. The correlations of $K^{trans}$ with $v_e$ and between hsTNT and $v_e$ further indicate that a decrease in nerve capillary permeability accompanied by elevated hsTNT levels is associated with a reduction of the extracapillary extracellular volume (EEV) fraction, which may ultimately result in nerve ischemia and demyelination. This assumption is further

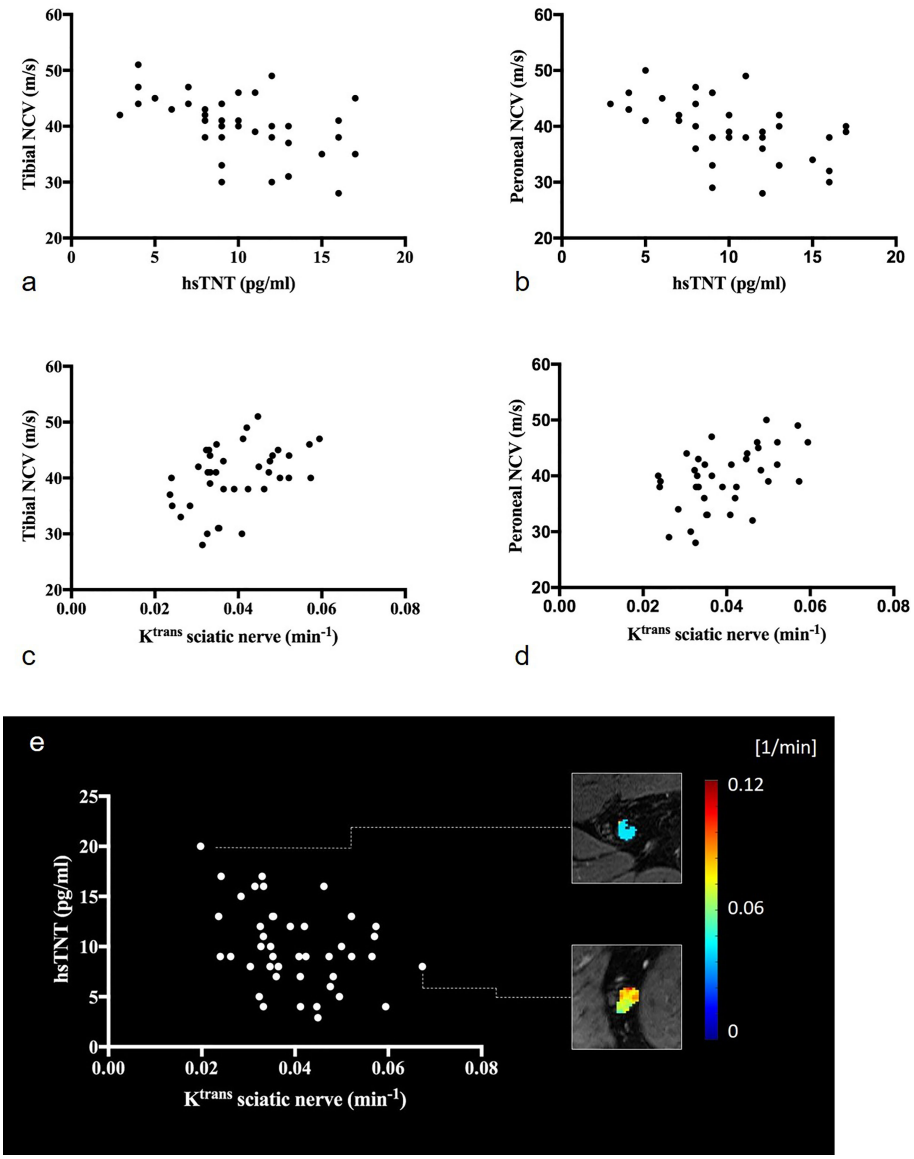


**FIGURE 2** | Correlations of the sciatic nerve's $K^{trans}$ and hsTNT with electrophysiologic parameters: **(A)** correlation of hsTNT with tibial nerve conduction velocities (r=-0.51; p=0.001), **(B)** correlation of hsTNT and peroneal nerve conduction velocities (r=-0.53; p=0.001), **(C)** correlation of $K^{trans}$ with tibial nerve conduction velocities (r=0.42; p=0.008), **(D)** correlation of $K^{trans}$ and peroneal nerve conduction velocities (r=0.49; p=0.002), **(E)** illustration of the correlation between $K^{trans}$ and hsTNT (r=-0.38; p=0.012). Representative color-coded $K^{trans}$ maps of the sciatic nerve are shown. Upper rectangle: T2D patient with hsTNT levels of 20pg/ml and a sciatic nerve's $K^{trans}$ of 0.020 ($min^{-1}$), lower rectangle: T2D patient with hsTNT levels of 8pg/ml and a sciatic nerve's $K^{trans}$ of 0.067 ($min^{-1}$).

supported by the finding that hsTNT was negatively correlated with nerve conduction velocities of tibial and peroneal nerves, while $K^{trans}$, and $v_e$ were positively correlated with nerve conduction velocities. Since a decrease in nerve conduction velocity is generally assumed to represent myelin damage (37), these correlations indicate that a decrease in capillary permeability and EEV fraction result in demyelination. In addition, the finding that there were no correlations for $v_p$ with hsTNT or any of the acquired electrophysiological parameters further implies that there is no relevant impact of the capillary plasma volume fraction on structural nerve damage in the examined patients. The absence of correlations between proBNP and any of the acquired clinical and serological parameters in T2D patients is of importance for an understanding of the origin of elevated hsTNT levels in patients with T2D, meaning, that while there was a correlation of hsTNT and proBNP in healthy controls, no such correlation was found in the T2D group. This finding is of particular interest since it indicates that the well-established correlation of hsTNT and proBNP (38) does not apply in the T2D group. Since proBNP is an indicator for myocardial insufficiency, the lack of a correlation between hsTNT and proBNP indicates that elevated hsTNT levels in T2D patients and correlations of MRN perfusion parameters

**TABLE 3 |** Correlations of MRN perfusion parameters with demographic, serological, clinical, and electrophysiological data.

| | $K^{trans}$ ($min^{-1}$) T2D | | $v_p$ (%) T2D | | $v_e$ (%) T2D | | $K^{trans}$ ($min^{-1}$) Co | | $v_p$ (%) Co | | $v_e$ (%) Co | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | r | p | r | p | r | p | r | p | r | p | r | p |
| **$K^{trans}$ ($min^{-1}$)** | | | 0.01 | 0.935 | 0.75 | <0.001 | | | -0.35 | 0.292 | 0.76 | 0.007 |
| **$v_p$ (%)** | -0.10 | 0.533 | | | 0.36 | 0.015 | -0.35 | 0.292 | | | 0.08 | 0.818 |
| **$v_e$ (%)** | 0.64 | <0.001 | 0.36 | 0.015 | | | 0.76 | 0.007 | 0.08 | 0.818 | | |
| **Age (years)** | -0.24 | 0.120 | -0.34 | 0.023 | -0.34 | 0.022 | 0.13 | 0.706 | 0.17 | 0.622 | 0.39 | 0.232 |
| **Diabetes duration (years)** | 0.05 | 0.790 | 0.20 | 0.249 | 0.00 | 0.998 | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. |
| **Gender** | 0.15 | 0.332 | -0.13 | 0.385 | 0.16 | 0.300 | 0.04 | 0.915 | 0.21 | 0.542 | -0.11 | 0.754 |
| **BMI ($kg/m^2$)** | 0.55 | <0.001 | 0.31 | 0.038 | 0.47 | 0.001 | 0.74 | 0.009 | -0.29 | 0.393 | 0.30 | 0.369 |
| **hsTNT (pg/mL)** | -0.38 | 0.012 | -0.16 | 0.294 | -0.30 | 0.048 | 0.39 | 0.241 | -0.20 | 0.558 | 0.31 | 0.353 |
| **proBNP (pg/mL)** | -0.09 | 0.569 | -0.17 | 0.280 | -0.05 | 0.732 | 0.03 | 0.931 | -0.18 | 0.602 | 0.21 | 0.529 |
| **HbA1c %** | -0.11 | 0.464 | 0.17 | 0.256 | -0.01 | 0.953 | 0.35 | 0.298 | -0.02 | 0.950 | 0.31 | 0.351 |
| **GFR (ml/min)** | -0.19 | 0.242 | 0.09 | 0.594 | -0.09 | 0.586 | -0.11 | 0.754 | 0.07 | 0.835 | 0.03 | 0.920 |
| **NDS** | -0.19 | 0.240 | -0.01 | 0.948 | -0.29 | 0.069 | 0.67 | 0.023 | -0.24 | 0.468 | 0.71 | 0.014 |
| **NSS** | -0.19 | 0.233 | -0.09 | 0.578 | -0.20 | 0.204 | 0.20 | 0.548 | 0.01 | 0.970 | 0.37 | 0.260 |
| **Sural nerve NCV (m/s)** | 0.45 | 0.030 | -0.23 | 0.283 | 0.22 | 0.314 | 0.35 | 0.293 | 0.17 | 0.616 | 0.25 | 0.457 |
| **Sural nerve SNAP (μV)** | 0.32 | 0.095 | -0.12 | 0.548 | 0.36 | 0.058 | 0.12 | 0.726 | 0.12 | 0.733 | -0.24 | 0.482 |
| **Peroneal NCV (m/s)** | 0.49 | 0.002 | -0.02 | 0.930 | 0.39 | 0.014 | 0.05 | 0.882 | 0.03 | 0.929 | -0.33 | 0.317 |
| **Peroneal CMAP (mV)** | 0.27 | 0.099 | 0.11 | 0.494 | 0.23 | 0.161 | 0.62 | 0.042 | -0.58 | 0.061 | 0.10 | 0.760 |
| **Peroneal DML (ms)** | -0.18 | 0.261 | -0.15 | 0.355 | -0.48 | 0.002 | -0.13 | 0.711 | -0.31 | 0.354 | -0.13 | 0.703 |
| **Tibial NCV (m/s)** | 0.42 | 0.008 | -0.123 | 0.650 | 0.40 | 0.014 | 0.07 | 0.842 | 0.44 | 0.172 | -0.02 | 0.949 |
| **Tibial CMAP (mV)** | 0.14 | 0.401 | 0.08 | 0.640 | 0.21 | 0.214 | -0.16 | 0.638 | 0.35 | 0.286 | -0.06 | 0.870 |
| **Tibial DML (ms)** | -0.33 | 0.044 | -0.24 | 0.156 | -0.48 | 0.003 | -0.12 | 0.724 | -0.27 | 0.414 | -0.05 | 0.893 |

*$K^{trans}$, constant of permeability; $v_p$, plasma volume fraction; $v_e$, extracellular extravascular volume fraction; BMI, body-mass index; hsTNT, high sensitivity troponin T; proBNP, pro brain binatriuretic peptide; n.a., not applicable; NDS, neuropathy disability score; NSS, neuropathy severity scale; NCV, nerve comnduction velocity; CMAP, compound motor action potential; SNAP, sensory nerve action potential; m/s, meters per second; ms, miliseconds; mV, millivolts; μV, microvolts.*

with hsTNT are not the consequence of myocardial insufficiency. Instead, our results are in line with previous studies suggesting that hsTNT represents myocardial damage due to microangiopathy that affects different organs in patients with T2D (9, 12). It remains to be determined, how much hyperglycemia or other metabolic factors, such as dyslipidemia, contribute to these microangiopathic changes (39–41).

One may of course argue, that hsTNT showed positive correlations with age while $K^{trans}$ showed positive correlations with BMI, therefore, the findings of this study only represent age- and obesity-related changes of perfusion in peripheral nerves. It should be considered, however, that negative correlations of $K^{trans}$ and $v_e$ with hsTNT remained significant in a partial correlation analysis which was controlled for both age and BMI as confounding variables.

This study only found differences in perfusion parameters $K^{trans}$ and ve between T2D patients with and without DN, while no such difference was found between controls and T2D patients with DN. This is in line with previous studies on animal models for diabetes that found an increased vascular permeability in nerves of diabetic rats without diabetic neuropathy, supposedly due to an increased permeability of the basement membrane in Schwann cells and a reduction of nerve permeability in rats with DN compared to rats without DN, supposedly due to microangiopathy (42, 43).

This study is limited by the fact that only patients without an impairment of renal function were included due to the administration of MRI contrast agent. Thus, we cannot draw conclusions on the impact of hsTNT levels on nerve perfusion in patients with impaired renal function, since hsTNT is usually elevated in those patients due to renal elimination. Another limitation is the cross-sectional nature of the study which does not allow any conclusions on the predictive value of hsTNT for the progression of DN. The study is further limited by the sample size of T2D patients and controls, which cannot rule out all potential confounders for the observed differences and correlations. It should be considered, however, that patients and controls were matched for age, BMI, and renal function to minimize confounding and that correlations between hsTNT and perfusion parameters remained stable in a double-controlled partial correlation analysis.

In summary, this study found correlations between hsTNT and parameters of nerve perfusion obtained from 3T DCE MRN. The results indicate that hsTNT codifies a decrease in capillary permeability of peripheral nerves which is associated with a decrease in extravascular extracellular volume that ultimately causes demyelination as a result of nerve ischemia. Further longitudinal studies on the predictive value of hsTNT for the progression of DN, including the effects of age and BMI on nerve perfusion, are warranted.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethikkommission der Medizinischen Fakultät

HeidelbergAlte Glockengießerei 11/169115 Heidelberg. The patients/participants provided their written informed consent to participate in this study.

## AUTHOR CONTRIBUTIONS

JMEJ Study design and coordination, organization of participants, collection of MR data, image segmentation, data analysis and interpretation, literature search, writing of manuscript, arrangement of figures. CM: Collection of MR data, data analysis and interpretation, literature search, writing of manuscript, arrangement of figures. ZK: Collection of clinical, electrophysiological, and serological data, organization of participants; LS: Collection of clinical, electrophysiological, and serological data, organization of participants; AJ: organization of participants, collection of MR data, data analysis; SH: Conception of MRN sequence protocol; PN: Study design and coordination; MB: Study design and coordination, development of MR sequence protocol, writing of manuscript; SK: Development of clinical and electrophysiological study protocol, collection of clinical, electrophysiological, and serological data; FTK Study design and coordination, programming of image analysis tools, image segmentation, data analysis and interpretation, literature search, writing of manuscript, arrangement of figures. All authors contributed to the article and approved the submitted version.

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# Risk Factors for Diabetic Peripheral Neuropathy, Peripheral Artery Disease, and Foot Deformity Among the Population With Diabetes in Beijing, China: A Multicenter, Cross-Sectional Study

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Diabetic peripheral neuropathy (DPN), peripheral artery disease (PAD), and foot deformity are the most common causes of diabetic foot, which can considerably worsen the patient's quality of life. In this study, we aimed to investigate the prevalence and risk factors associated with DPN, PAD, and foot deformity among patients with diabetes living in Beijing, China. In total, 3,898 diabetes patients from 11 hospitals in Beijing were evaluated using questionnaires and physical examinations, and 3,758 patients were included in the analysis. We compared the demographic, clinical, biological characteristics, and comorbidities of patients with and without DPN, PAD, or foot deformity, and used binary logistic regression analysis to identify potential factors associated with these outcomes. Overall, 882 patients (23.5%) had DPN, 437 patients (11.6%) had PAD, and 1,117 patients (29.7%) had foot deformities, including callus. The risk factors for DPN included: age ≥40 years, a ≥10+year duration of diabetes, a body mass index of <18.5 kg/m$^2$ or ≥24 kg/m$^2$, a systolic blood pressure (SBP) of ≥140 mm Hg, a hemoglobin $A_{1c}$ ($HbA_{1c}$) level of ≥7%, chronic kidney disease, and cerebrovascular disease. The risk factors for PAD included: a 15+ year diabetes duration, a body mass index of <18.5 kg/m$^2$, a SBP of ≥140 mm Hg, a $HbA_{1c}$ level of ≥7%, chronic kidney disease, coronary heart disease, and cerebrovascular disease. The risk factors for skeletal foot deformities included: women, age ≥40 years, a SBP ≥140 mm Hg, and hyperlipidemia. The risk factors for callus formation included: women, a SBP ≥140 mm Hg, and hyperlipidemia. In conclusion, the prevalence of foot deformities was higher than DPN and PAD in patients with diabetes. Managing the risk factors for DPN, PAD, and foot deformity is important for reducing the risk of diabetic foot.

**Keywords: peripheral neuropathy, peripheral artery disease, foot deformity, risk factors, diabetes**

## INTRODUCTION

Diabetic foot is a serious complication of diabetes, associated with a high prevalence and mortality, as well as an enormous medical burden. The annual incidence of diabetic foot is roughly 2%, with a lifetime incidence rate of 15–20% (1). Curing diabetic foot is difficult; even if the ulcer heals, the 1-year recurrence rate is 30–40% (2, 3). The early identification of at-risk patients is therefore crucial.

Intrinsic conditions, including neuropathy, vascular disease, and foot deformity, cause diabetic foot, as do extrinsic risk factors, such as unexpected trauma and infection. The most common extrinsic risk factors for diabetic foot are diabetic peripheral neuropathy (DPN) and peripheral artery disease (PAD) (4, 5). International consensus guidelines define DPN as the presence of symptoms or signs of peripheral nerve dysfunction in patients with diabetes after all other causes have been ruled out (6). The International Working Group on the Diabetic Foot (IWGDF) proposed the concept of graded at-risk foot screenings, which emphasizes the importance of screening for high-risk factors of foot disease among diabetic patients, including DPN, PAD, foot deformity, foot ulcers, and partial foot or leg amputation history (7). Early screening for PAD can reduce the occurrence of diabetic foot (8). In our previous research, through Delphi consultation, we developed the "Construction of the standardized process of at-risk foot screening, stratification and intervention for diabetic patients" (hereafter referred to as the Screening Process criteria), which provides a framework for the comprehensive management of diabetic foot in China (9). Overall, 47.1% of patients with diabetes who met the Screening Process criteria had foot diseases, including DPN, PAD, and foot deformity (10). Therefore, patients with diabetes who meet the Screening Process criteria have a higher risk of developing diabetic foot, and should be given special attention towards preventing this complication.

To the best of our current knowledge, there are limited data on this patient population. This study screened patients with diabetes in multiple outpatient clinics in Beijing, China using the Screening Process. We aimed to estimate the prevalence and risk factors associated with DPN, PAD, and foot deformity among patients with type 1 and 2 diabetes, and potentially enhance preventive measures and care for patients with diabetes.

## MATERIALS AND METHODS

### Subjects

The sample consisted of 3,898 consecutively enrolled outpatients who underwent a screening for diabetic foot between June 1, 2017 and January 14, 2019 and were diagnosed with diabetes across 11 hospitals located in Beijing, China. Peking University First Hospital, Dongzhimen Hospital Beijing University of Chinese Medicine, Beijing Jishuitan Hospital, Air Force General Hospital of PLA, Peking University Shougang Hospital, Aerospace Center Hospital, Beijing Pinggu Hospital, Beijing Miyun Hospital, Beijing Shijingshan Hospital, Beijing Shichahai Community Service Center, and the Beijing Xinjiekou Community Service Center participated in the study.

Men and women with type 1 or type 2 diabetes mellitus who were conscious, without language communication barriers, and willing to actively cooperate with the evaluation were eligible to participate. Diabetes was diagnosed based on the 1999 World Health Organization criteria (11). If the patient met any of the following criteria(Screening Process criteria) (9), he or she was included in this study: age > 60 years old; duration of diabetes > 8 years; history of PAD; history of DPN; history of diabetic nephropathy; history of diabetic retinopathy; history of foot deformity, including skeletal deformity of the foot and callus; history of diabetic foot and/or amputation. Exclusion criteria included: patients with existing foot ulcers, and patients whose medical records were missing relevant data (e.g., foot examination or the ankle-brachial index [ABI] results) (**Figure 1**).

In total, 3,758 patients with diabetes were included in the final sample. The institutional review board at each study site approved this study, and informed consent was obtained from all participants before the survey was conducted.

### Data Collection

Prior to study initiation, all doctors and nurses at each site were adequately trained in operations manual. Data on sex, age, diabetes duration, and accompanying disease history (e.g., hypertension, hyperlipidemia, chronic kidney disease, coronary heart disease, and cerebrovascular disease) were collected upon admission. Systolic blood pressure (SBP) and height and weight (excluding shoes and heavy clothes) were also measured on admission. Body mass index (BMI) was calculated as the weight in kilograms (kg) divided by the height in meters squared ($m^2$). The most recent hemoglobin $A_{1c}$ ($HbA_{1c}$) and

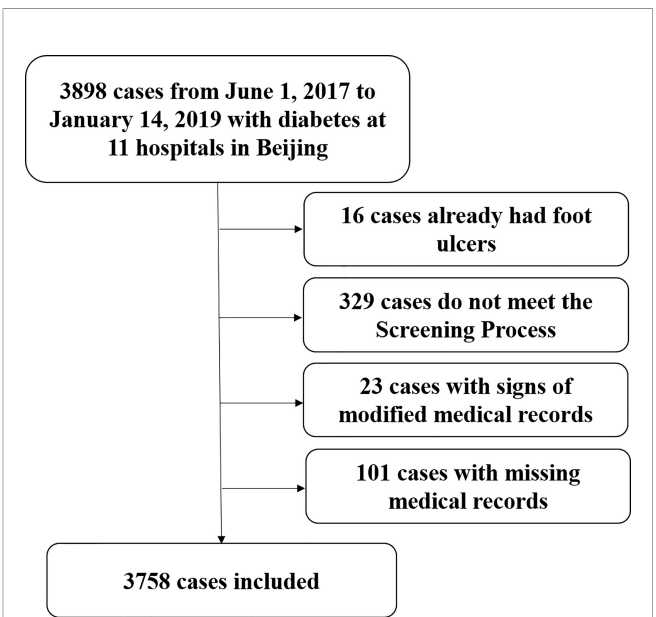


**FIGURE 1** | Flow chart of diabetic patient enrollment.

low-density lipoprotein cholesterol (LDL-c) levels were verified. $HbA_{1c}$ and LDL-c are uniformly tested by the central laboratory. The symptoms of DPN and PAD, including rest pain, intermittent claudication, numbness, and paresthesia, were collected by trained nurses. Physical examinations to diagnose DPN, PAD, or foot deformity were conducted by trained doctors. The examinations included measurement of the foot temperature sensation. For this procedure, a testing tool is placed onto the foot skin with its metal side (cooler) and polymer side (warmer); positivity was defined as inability to differentiate the two sides of the Tip Therm in either side of the foot. Besides examining the ABI and Achilles tendon reflex, a 10-g monofilament was gently bent for 1–2 s, and one side was pushed upon the arm skin to give the patient a reference for pressure sensation. The patient closed both eyes. For each foot, the pressure sensation was tested at the plantar surface of the great toe, the lateral side of the anterior sole, and medial side of the anterior sole. Positivity was defined as loss of pressure sensation in any tested sites of either foot. For vibration sensation, a 128 Hz tuning fork was placed onto the wrist or elbow to give the patient with a reference point for vibration or non-vibration. The patient closed both eyes. A vibrating tuning fork was then placed onto the dorsal surface of the metatarsal joint of the great toe. The patient was asked whether a vibration was felt. The test was repeated three times for both sides. Positivity was defined as two or three wrong answers for either side. Foot deformity included skeletal deformities of the foot (e.g., hallux valgus, toe deformity, Charcot foot) and callus formation. A history of hypertension, hyperlipidemia, chronic kidney disease, coronary heart disease and cerebrovascular disease were obtained from previous medical records.

## Diagnostic Criteria

Diagnostic criteria for DPN and PAD were based on the Guidelines for the Prevention and Control of Type 2 Diabetes in China (2017 Edition). Patients with clinical symptoms of neuropathy and one abnormal result from five examinations (ankle reflex, acupuncture pain sensation, vibration sensation, pressure sensation, and temperature sensation) were diagnosed with DPN. Patients without clinical symptoms of neuropathy and two abnormal results from the five examinations were also diagnosed with DPN. If the patient's symptoms were in line with a diabetes diagnosis and they had an ABI of ≤0.9 at rest, then PAD was diagnosed. Foot deformity included skeletal deformities of the foot (hallux valgus, hammer toe, claw toe, mallet toe, and Charcot foot), and callus. Hallux valgus was defined as deformity of the great toe by abduction valgus and pronation associated with bone prominence on the inner edge of the metatarsal (bunion) (12). Hammer toe was defined as plantar flexion of the distal and middle interphalangeal joint in comparison to the proximal phalanx. Claw toe was defined as the dorsal flexion of the metatarsophalangeal joint associated with hammer toe (13). Mallet toe was defined as flexion of the distal phalanx over the middle phalanx due to a contracture at the distal interphalangeal joint (14). Charcot foot was defined as non-infectious destruction of bone and joint tissue including loss of foot arches, i.e., rocker bottom deformity (15). Callus was defined as a broad, diffuse area of hyperkeratosis with a relatively even thickness (16).

## Statistical Analyses

Statistical analyses were performed using SPSS software (version 19.0; SPSS Inc., Chicago, IL, USA). Normally distributed continuous variables were expressed as means ± standard deviations (SD). First, we compared characteristics such as age, sex, diabetes duration, SBP, BMI, $HbA_{1c}$, and LDL-c levels between patients with and without DPN, PAD, or foot deformity. From a clinical point of view, we grouped age, duration of diabetes, BMI, SBP, $HbA_{1c}$ and $LDL\text{-}_C$ into categories. Categorical variables were compared using the Chi-squared test and reported as frequencies and proportions. Then we performed binary logistic regression analysis with the variables which show significant difference among the patients with and without DPN/PAD/foot deformity in order to identify potential risk factors for DPN, PAD, or foot deformity. All significant variables were added to the model simultaneously. Statistical significance was set at $p<0.05$.

# RESULTS

## Baseline Characteristics

**Table 1** presents the baseline characteristics of the study sample. In total, 3,758 patients were included in the analyses; 39 patients had type 1 diabetes, and 3,719 had type 2 diabetes. There were 2,024 (53.9%) men and 1,734 (46.1%) women, the median age was 62.97 years, and the median diabetes duration was 11.33 years. The only different in clinical characteristics between men and women is that the average age of women was higher than that of men (64.76 ± 10.67 years vs 61.40 ± 11.67 years, $p<0.001$). Overall, 882 (23.5%) patients had DPN, 437 (11.6%) had PAD, and 1,117 (29.7%) had a foot deformity (including callus) (**Table 2**). In patients with foot deformity (n=1117), 566 were diagnosed with skeletal deformity of the foot, accounting for 15.1% of the total patient sample overall. In addition, 904 patients were diagnosed with callus, accounting for 24.1% of the total patient sample overall. The other diagnoses in patients

**TABLE 1** | Characteristics of the study participants.

| Characteristics | Study participants (n = 3758) |
|---|---|
| Age (years) | 62.97 ± 11.40 |
| Women [n(%)] | 1734 (46.1%) |
| Duration of diabetes (years) | 11.33 ± 7.91 |
| BMI ($kg/m^2$) | 25.53 ± 3.73 |
| SBP (mmHg) | 131.58 ± 15.66 |
| $HbA_{1c}$(%) | 7.64 ± 1.74 |
| LDL-c (mmol/L) | 2.46 ± 0.81 |
| Hypertension [n(%)] | 2491(66.3%) |
| Hyperlipoidemia [n(%)] | 1990 (53.0%) |
| Chronic kidney disease [n(%)] | 149 (4.0%) |
| Coronary heart disease [n(%)] | 1077 (28.7%) |
| Cerebrovascular disease [n(%)] | 725 (19.3%) |

*BMI, body mass index; SBP, systolic blood pressure; $HbA_{1c}$, glycosylated hemoglobin; LDL-c, low density lipoprotein.*

**TABLE 2** | Prevalence of DPN, PAD and Foot Deformity in study participants.

| Foot screening | n (%) |
|---|---|
| DPN | 882 (23.5%) |
| PAD | 437 (11.6%) |
| Foot Deformity | 1117 (29.7%) |

*DPN, diabetic peripheral neuropathy; PAD, peripheral artery disease.*

with foot deformity were hallux valgus (n=503), toe deformity (n=95), and Charcot foot (n=3).

## DPN Risk Factors

We chose the variables which show significant difference among the patients with and without DPN (**Supplementary Table 1**). **Table 3** presents the logistic regression model outputs with DPN as the binary outcome. Age between 40-50, 50-60, 60-70, and ≥70 years, a 10-15 year and 10+ year diabetes duration, BMI <18.5 kg/m$^2$ or ≥24 kg/m$^2$, SBP ≥140 mm Hg, $HbA_{1c}$ level ≥7%, chronic kidney disease, and cerebrovascular disease were risk factors for DPN. Among these risk factors, the OR for BMI<18.5 kg/m$^2$ was the highest (**Table 3**). BMI levels appear to be non-linear in relation to DPN, so we further conducted smooth curve fitting(**Supplementary Figure 1**). Smooth curve fitting shows that there is a non-linear relationship between BMI and DPN. Further threshold effect analysis shows that the knee(K) is BMI=20.24kg/m$^2$ (**Supplementary Table 2**). That is, if BMI is less than 20.24 kg/m$^2$, BMI is negatively correlated with the prevalence of DPN. If BMI is greater than 20.24 kg/m$^2$, BMI is positively correlated with the prevalence of DPN. LDL-c seems to be a protective factor for DPN. We further conducted subgroup analysis and interaction testing. As we can see in the **Supplementary Table 3**, there were no interaction between age, duration of diabetes, BMI, Hyperlipoidemia, Chronic kidney disease, Coronary heart disease, Cerebrovascular disease and LDL-c.

**TABLE 3** | Logistic regression analysis for factors associated with DPN.

| | OR (95%CI) | P-value |
|---|---|---|
| Age (years) | | |
| <40 | Reference | |
| 40-50 | 1.960 (1.020, 3.766) | 0.043 |
| 50-60 | 2.537 (1.387, 4.642) | 0.003 |
| 60-70 | 2.549 (1.402, 4.636) | 0.002 |
| ≥70 | 2.627 (1.431, 4.822) | 0.002 |
| Duration of diabetes (years) | | |
| <5 | Reference | |
| 5-10 | 1.000 (0.763, 1.311) | NS |
| 10-15 | 1.629 (1.261, 2.106) | <0.001 |
| ≥15 | 1.898 (1.483, 2.429) | <0.001 |
| BMI (kg/m$^2$) | | |
| <18.5 | 3.560 (1.932, 6.562) | <0.001 |
| 18.5-23.9 | Reference | |
| 24-27.9 | 1.238 (1.024, 1.496) | 0.027 |
| ≥28 | 1.353 (1.083, 1.691) | 0.008 |
| SBP≥140mmHg | 1.684 (1.416, 2.003) | <0.001 |
| $HbA_{1c}$ (%) | | |
| <6.5 | Reference | |
| 6.5-6.9 | 0.930 (0.710, 1.217) | NS |
| 7-7.9 | 1.396 (1.095, 1.779) | 0.007 |
| ≥8.0 | 1.995 (1.590, 2.504) | <0.001 |
| LDL-c(mmol/L) | | |
| <1.8 | Reference | |
| 1.8-2.6 | 0.839 (0.680, 1.035) | NS |
| ≥2.6 | 0.751 (0.606, 0.931) | 0.009 |
| Hyperlipoidemia | 1.156 (0.981, 1.362) | NS |
| Chronic kidney disease | 2.278 (1.597, 3.250) | <0.001 |
| Coronary heart disease | 0.859 (0.712, 1.035) | NS |
| Cerebrovascular disease | 1.538 (1.267, 1.866) | <0.001 |

*DPN, diabetic peripheral neuropathy; BMI, body mass index; SBP, systolic blood pressure; $HbA_{1c}$, glycosylated hemoglobin; LDL-c, low density lipoprotein; NS, not significance.*

## PAD Risk Factors

We chose the variables which show significant difference among the patients with and without PAD (**Supplementary Table 4**). **Table 4** presents the logistic regression model outputs with DBN as the binary outcome. A 15+ year diabetes duration, BMI <18.5 kg/m$^2$, SBP ≥140 mm Hg, a $HbA_{1c}$ level of ≥7%, chronic kidney disease, coronary heart disease, and cerebrovascular disease were significant risk factors for PAD. Interestingly, the same as DPN, the OR value of BMI<18.5 kg/m$^2$ was the highest value. But BMI≥24 kg/m$^2$ were not risk factors for PAD.

## Foot Deformity Risk Factors

We chose the variables which show significant difference among the patients with and without foot skeletal deformity (**Supplementary Table 5**) or callus (**Supplementary Table 6**).

**TABLE 4** | Logistic regression analysis for factors associated with PAD.

| | OR (95%CI) | P-value |
|---|---|---|
| Age (years) | | |
| <40 | Reference | |
| 40-50 | 0.670 (0.308, 1.459) | NS |
| 50-60 | 0.768 (0.392, 1.504) | NS |
| 60-70 | 1.094 (0.571, 2.098) | NS |
| ≥70 | 1.507 (0.777, 2.922) | NS |
| Duration of diabetes (years) | | |
| <5 | Reference | |
| 5-10 | 0.879 (0.610, 1.267) | NS |
| 10-15 | 1.236 (0.872, 1.752) | NS |
| ≥15 | 1.565 (1.131, 2.167) | 0.007 |
| BMI (kg/m$^2$) | | |
| <18.5 | 2.464 (1.189, 5.104) | 0.015 |
| 18.5-23.9 | Reference | |
| 24-27.9 | 1.169 (0.912, 1.497) | NS |
| ≥28 | 1.188 (0.886, 1.592) | NS |
| SBP≥140mmHg | 1.261 (1.001, 1.588) | 0.049 |
| $HbA_{1c}$ (%) | | |
| <6.5 | Reference | |
| 6.5-6.9 | 1.016 (0.708, 1.457) | NS |
| 7-7.9 | 1.468 (1.063, 2.028) | 0.020 |
| ≥8.0 | 1.877 (1.385, 2.542) | <0.001 |
| LDL-c (mmol/L) | | |
| <1.8 | Reference | |
| 1.8-2.6 | 0.868 (0.663, 1.137) | NS |
| ≥2.6 | 0.850 (0.645, 1.119) | NS |
| Hypertension | 0.875 (0.683, 1.121) | NS |
| Hyperlipoidemia | 1.226 (0.984, 1.527) | NS |
| Chronic kidney disease | 1.793 (1.183, 2.717) | 0.006 |
| Coronary heart disease | 1.518 (1.205, 1.912) | <0.001 |
| Cerebrovascular disease | 1.511 (1.189, 1.921) | <0.001 |

*PAD, peripheral artery disease; BMI, body mass index; SBP, systolic blood pressure; $HbA_{1c}$, glycosylated hemoglobin; LDL-c, low density lipoprotein; NS, not significance.*

**Tables 5** and **6** present the logistic regression model outputs for foot skeletal deformity and callus as the binary outcomes, respectively. Women, aged ≥40 years, SBP ≥140 mm Hg, and hyperlipidemia were risk factors for skeletal deformities of the foot. The risk factors for callus included women, SBP ≥140 mm Hg and hyperlipidemia. As we can see in **Table 5** and **Table 6**, age significantly affects the risk of foot skeletal deformity, while age was not a risk factor for callus.

**TABLE 5** | Logistic regression analysis for factors associated with foot skeletal deformity.

| | OR (95%CI) | P-value |
|---|---|---|
| women | 1.815 (1.501, 2.195) | <0.001 |
| Age(years) | | |
| <40 | Reference | |
| 40-50 | 2.981 (1.114, 7.977) | 0.030 |
| 50-60 | 4.314 (1.711, 10.877) | 0.002 |
| 60-70 | 4.980 (1.993, 12.446) | <0.001 |
| ≥70 | 5.855 (2.323, 14.755) | <0.001 |
| Duration of diabetes(years) | | |
| <5 | Reference | |
| 5-10 | 0.637 (0.480, 0.847) | 0.002 |
| 10-15 | 0.792 (0.601, 1.045) | NS |
| ≥15 | 0.838 (0.643, 1.092) | NS |
| BMI(kg/m$^2$) | | |
| <18.5 | 1.285 (0.612, 2.697) | NS |
| 18.5-23.9 | Reference | |
| 24-27.9 | 0.876 (0.710, 1.079) | NS |
| ≥28 | 0.713 (0.545, 0.933) | 0.014 |
| SBP≥140mmHg | 1.556 (1.269, 1.907) | <0.001 |
| $HbA_{1c}$(%) | | |
| <6.5 | Reference | |
| 6.5-6.9 | 0.878 (0.672, 1.147) | NS |
| 7-7.9 | 0.903 (0.698, 1.167) | NS |
| ≥8.0 | 0.521 (0.400, 0.679) | <0.001 |
| LDL-c(mmol/L) | | |
| <1.8 | Reference | |
| 1.8-2.6 | 0.696 (0.543, 0.893) | 0.004 |
| ≥2.6 | 0.882 (0.690, 1.128) | NS |
| Hyperlipoidemia | 1.313 (1.086, 1.586) | 0.005 |
| Coronary heart disease | 0.580 (0.462, 0.728) | <0.001 |

*BMI, body mass index; SBP, systolic blood pressure; $HbA_{1c}$, glycosylated hemoglobin; LDL-c, low density lipoprotein; NS, not significance.*

**TABLE 6** | Logistic regression analysis for factors associated with callus.

| | OR (95%CI) | p-value |
|---|---|---|
| women | 1.344 (1.146-1.577) | <0.001 |
| Duration of diabetes (years) | | |
| <5 | Reference | |
| 5-10 | 0.565 (0.443-0.720) | <0.001 |
| 10-15 | 0.705 (0.555-0.896) | 0.004 |
| ≥15 | 0.935 (0.749-1.167) | NS |
| SBP≥140 mmHg | 1.577 (1.321-1.884) | <0.001 |
| $HbA_{1c}$ (%) | | |
| <6.5 | Reference | |
| 6.5-7.0 | 1.037 (0.822-1.308) | NS |
| 7.0-8.0 | 1.009 (0.806-1.263) | NS |
| ≥8.0 | 0.507 (0.404-0.636) | <0.001 |
| Hypertension | 0.673 (0.564-0.802) | <0.001 |
| Hyperlipoidemia | 1.662 (1.408-1.962) | <0.001 |
| Coronary heart disease | 0.828 (0.684-1.002) | NS |
| Cerebrovascular disease | 0.865 (0.697-1.074) | NS |

*$HbA_{1c}$, glycosylated hemoglobin; LDL-c, low density lipoprotein; NS, not significance.*

## DISCUSSION

DPN, PAD, and foot deformity are common in patients with diabetes, which brings physical and mental suffering to the patient and their family members, and negatively affects their quality of life. Understanding the prevalence and risk factors of DPN, PAD, and foot deformity are necessary to formulate a prevention strategy for diabetic foot in Chinese patients with diabetes. Therefore, we conducted a multicenter survey of 3,758 cases across 11 hospitals in Beijing to clarify the prevalence and risk factors of diabetic foot in a representative sample of Chinese patients with type I or type II diabetes.

DPN is an important risk factor in diabetic foot. The risk of diabetic foot ulcers is reported to be seven times greater in patients with DPN and sensory deficits than in patients without DPN and sensory deficits (17). A Chinese survey showed that 63.6% of diabetic foot ulcers were caused by DPN (18). DPN was defined as a major risk factor for diabetic foot by the IWGDF (1). In this study, we found that age ≥40 years, a ≥10-year duration of diabetes, underweight (BMI<18.5 kg/m$^2$) or overweight and obesity (BMI >24 kg/m$^2$), SBP ≥140 mm Hg, a $HbA_{1c}$ level of ≥7%, chronic kidney disease, and cerebrovascular disease were all risk factors of DPN. Consistent with our findings, The Action to Control Cardiovascular Risk in Diabetes (ACCORD) trial suggested that lowering blood sugar slowed the occurrence of DPN (19). Further, a meta-analysis identified diabetes duration, age, glycosylated hemoglobin, and diabetic retinopathy as risk factors for DBN (20). The results of our study showed that chronic kidney disease and underweight also increased the risk of DPN. Chronic kidney disease, especially renal insufficiency, can lead to systemic vascular diseases, including microvascular diseases (21, 22). Microvascular diseases lead to a decrease in peripheral nerve blood flow, causing DPN. Underweight is often accompanied by nutrient deficiency, which causes a decrease in neurotrophic factors and leads to DPN (23). Another interesting finding is that the DPN risk was lower in patients with an LDL-c level of ≥2.6 mmol/L than in patients with an LDL-c of <1.8 mmol/L. However, hyperlipidemia did not reduce the risk of DPN. We suspect that lipid-lowering drugs may influence neuropathy, but further research is needed to confirm this hypothesis.

In this study, we found that a diabetes duration of ≥15 years, underweight (BMI<18.5 kg/m$^2$), SBP of ≥140 mm Hg, $HbA_{1c}$ level of ≥7%, chronic kidney disease, and cardiovascular and cerebrovascular diseases were risk factors for PAD. Previous research has shown that age, hypertension, dyslipidemia, and smoking are PAD risk factors (24). Furthermore, patients aged >50 years with cardiovascular and cerebrovascular diseases, dyslipidemia, hypertension, smoking, or diabetes for more than 5 years are recommended to undergo screening for PAD every year for early detection (25, 26). Coronary heart disease or cerebrovascular disease indicates atherosclerosis in the blood vessels of the heart or brain. PAD is not a specific complication of diabetes, but one of the most common

manifestations of atherosclerosis in the lower extremities. Therefore, it is not difficult to understand that coronary heart disease or cerebrovascular disease were risk factors for PAD. Patients with chronic kidney disease are at increased risk of atherosclerosis (27), this could explain chronic kidney disease as a risk factor for PAD.

According to the results of this study, the prevalence of foot deformity was higher than DPN and PAD. However, IWGDF pays more attention to DPN than PAD and foot deformity. Therefore, foot deformities may be missed in many patients. In this study, we found both risk and protective factors for foot deformity in patients with diabetes. The risk of skeletal deformity of the foot increased with age, and was higher in women than in men, which could be attributed to walking and inappropriate shoes. However, coronary heart disease, obesity, and an elevated $HbA_{1c}$ level may be protective factors for skeletal foot deformity, potentially related to changes in activity habits. Foot deformity leads to a change in plantar pressure, increasing the incidence of foot ulcers (28). In the non-diabetic population, foot deformity is related to shoes or environmental factors (29). Angina pectoris and cardiac insufficiency caused by coronary heart disease lead to decreased activity tolerance, reducing the risk of skeletal foot deformity. Obesity and elevated blood sugar are also related to a sedentary lifestyle and inactivity. Thus, the protective effect of obesity and an elevated $HbA_{1c}$ level on skeletal foot deformities may also come from reduced activity. This kind of obesity paradox was also found in foot ulceration risk (30) and lower-extremity amputation risk (31), but lacked a valid explanation. In our study, lipids affected skeletal foot deformities in two ways. On one hand, hyperlipidemia increased the risk of skeletal deformity; on the other hand, an LDL-c level of ≤1.8 mmol/L did not reduce the risk of skeletal foot deformities, perhaps owing to cholesterol or statin use. Further research is needed in order to confirm this.

Callus increase plantar pressure, plantar pressure duration (32, 33), and the risk of diabetic foot ulcers. Callus are caused by excessive pressure and friction of the skin of the foot, often related to shoes. Similar to skeletal foot deformities, women had a greater risk of callus, likely resulting from shoe habits. An increased $HbA_{1c}$ level may be related to insufficient activity. Compared to a diabetes duration of fewer than 5 years, the risk of callus decreased when the diabetes duration was 5–15 years but was similar when the duration was more than 15 years. Thus, diabetes duration is a protective factor for callus. A history of hypertension reduced the risk of callus, but a higher SBP increased the risk, suggesting that some antihypertensive drugs may have a protective effect regarding callus, but further research is required.

There are certain limitations to our study. First, nerve conduction studies were not performed, potentially leading to missed diagnoses. Patients with PAD may have a non-compressible ABI due to medial calcinosis, which would be undetected using only the criterion of lower than 0.90. We also did not examine smoking history, which is a known risk factor for PAD. Second, our study focused on patients visiting clinics and did not cover a large number of patients who were unaware of their condition or unwilling to be treated. The cross-sectional nature of this study limits our ability to infer any causal effects. A prospective follow-up study is required to evaluate the associations between exposures and outcomes.

In conclusion, despite the high prevalence of DPN, PAD, and foot deformity in Chinese patients with diabetes, these complications were underdiagnosed and undertreated. Strengthening the management of risk factors for diabetic foot might improve the quality of life and lifespan of patients with diabetes and reducing their medical burden.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Biomedical Research Ethics Committee, Peking University First Hospital. The patients/participants provided their written informed consent to participate in this study.

## AUTHOR CONTRIBUTIONS

JYL, XY, and JL designed the study, and conducted the literature research. JYL and XY carried out the data interpretation, and drafted the manuscript. YS, DZ, and HL carried out data acquisition. GY, XQ, JZ, BW, and XG contributed to interpretation of the data and significantly improved the manuscript. All authors read and approved the final manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.824215/full#supplementary-material

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# Nomogram Prediction for the Risk of Diabetic Foot in Patients With Type 2 Diabetes Mellitus

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**Aims:** To develop and validate a nomogram prediction model for the risk of diabetic foot in patients with type 2 diabetes mellitus (T2DM) and evaluate its clinical application value.

**Methods:** We retrospectively collected clinical data from 1,950 patients with T2DM from the Second Affiliated Hospital of Xi'an Jiaotong University between January 2012 and June 2021. The patients were divided into training cohort and validation cohort according to the random number table method at a ratio of 7:3. The independent risk factors for diabetic foot among patients with T2DM were identified by multivariate logistic regression analysis. Then, a nomogram prediction model was developed using the independent risk factors. The model performances were evaluated by the area under the receiver operating characteristic curve (AUC), calibration plot, Hosmer–Lemeshow test, and the decision curve analysis (DCA).

**Results:** Multivariate logistic regression analysis indicated that age, hemoglobin A1c (HbA1c), low-density lipoprotein (LDL), total cholesterol (TC), smoke, and drink were independent risk factors for diabetic foot among patients with T2DM ($P < 0.05$). The AUCs of training cohort and validation cohort were 0.806 (95% CI: 0.775~0.837) and 0.857 (95% CI: 0.814~0.899), respectively, suggesting good discrimination of the model. Calibration curves of training cohort and validation cohort showed a favorable consistency between the predicted probability and the actual probability. In addition, the *P* values of Hosmer–Lemeshow test for training cohort and validation cohort were 0.826 and 0.480, respectively, suggesting a high calibration of the model. When the threshold probability was set as 11.6% in the DCA curve, the clinical net benefits of training cohort and validation cohort were 58% and 65%, respectively, indicating good clinical usefulness of the model.

**Conclusion:** We developed and validated a user-friendly nomogram prediction model for the risk of diabetic foot in patients with T2DM. Nomograms may help clinicians early screen and identify patients at high risk of diabetic foot.

**Keywords: type 2 diabetes mellitus (T2DM), diabetic foot, orthopedics, nomogram, individual risk prediction model**

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## INTRODUCTION

Type 2 diabetes mellitus (T2DM), previously referred to as noninsulin-dependent diabetes or adult-onset diabetes and accounting for 90%–95% of all diabetes, is a disease caused by a gradual decrease in insulin secretion from β cells in the context of insulin resistance (1, 2). T2DM is a disease involving the interaction between genetic and environmental risk factors leading to the underlying pathophysiology of beta cell dysfunction as well as insulin resistance in liver and muscle (3, 4). Poorly controlled T2DM can lead to chronic diabetic complications such as microangiopathy (retinopathy and kidney disease), atherosclerotic cardiovascular disease, peripheral neuropathy (sensory dysfunction), and diabetic foot (5–10). In the above complications, the diabetic foot negatively affects the quality of both work and life of patients. The decline in daily living activities of patients with diabetic foot results in substantial physical and psychological burdens on the patients.

Diabetic foot is one of the most serious and costly chronic complications of diabetes. It refers to foot ulcer, infection, or deep tissue destruction related to peripheral neuropathy in the lower extremity and peripheral vascular disease (11, 12). Mild diabetic foot patients usually present with foot deformities, hypoesthesia, skin dryness, and loss of skin elasticity. Patients with severe diabetic foot may develop foot ulcers and gangrene. Diabetic foot is the main reason for non-traumatic amputations in orthopedics. At present, there are many studies on the individual risk factors of diabetic foot in patients with T2DM, but no consensus has been reached. Although there are relevant clinical guidelines as the reference basis for the formulation of clinical treatment plans, how to predict the probability of diabetic foot according to risk factors and determine the timing of interventional treatment is an urgent problem to be solved at present.

The nomogram is drawn by the individual risk factors determined by multivariate logistic regression analysis. The nomogram can graphically represent the numerical relationship between specific disease and risk factors and intuitively predict the incidence of adverse events through a scoring system without any complicated calculation formula (13). The nomogram can provide accurate and individualized risk predictions for each individual. It is convenient for clinicians to effectively screen out high-risk patients and timely take interventions. Therefore, this study aimed to develop a nomogram prediction model for the risk of diabetic foot in patients with T2DM. Early screening and identification of high-risk patients can provide the reliable reference basis for early clinical intervention.

## METHODS

### Research Subjects

We retrospectively collected and analyzed clinical data from patients with diabetes mellitus from the Second Affiliated Hospital of Xi'an Jiaotong University between January 2012 and June 2021. Baseline-including criteria included (1) T2DM, diagnosis is made according to relevant criteria (fasting plasma glucose ≥ 7.0 mmol/L or 2-h plasma glucose ≥11.1 mmol/L or hemoglobin A1c ≥ 6.5%) (14); (2) both lower extremity arteries (femoral artery, superficial femoral artery, popliteal artery, anterior tibial artery, posterior tibial artery, and dorsalis pedis artery) of patients were examined by color Doppler ultrasonography for intima-media thickness, blood vessel diameter, and filling defects in blood flow; (3) orthopedic examination of the foot and ankle, including visual examination/palpation (skin condition and gait), mobility of foot and ankle (range of motion of the ankle joint, varus/valgus, and pronation/supination), and special examination (ankle anterior drawer test, varus/valgus stress test, and external rotation examination); and (4) patients gave oral informed consent. Baseline-excluding criteria included (1) type 1 diabetes mellitus, (2) gestational diabetes, (3) thromboangiitis obliterans, (4) combined with cancer, and (5) incomplete clinical data.

After the above screening, a total of 1,950 patients with T2DM were enrolled in the study. The patients were divided into training cohort ($n$ = 1,365) and validation cohort ($n$ = 585) according to the random number table method at a ratio of 7:3. The detailed flowchart is shown in **Figure 1**.

The study was approved by the medical ethics committee of the Second Affiliated Hospital of Xi'an Jiaotong University (Approval number: 2021234), which was consistent with medical ethics. The study was a retrospective cohort study and the data of included patients were anonymous. Oral informed consent was obtained from each enrolled patient before discharge.

### Observation Indexes

Clinical data including gender, age, course of disease, body mass index (BMI), oral glucose tolerance test (OGTT) 2-h plasma glucose, hemoglobin A1c (HbA1c), low-density lipoprotein (LDL), triglyceride (TG), total cholesterol(TC),smoke, drink, hypertension history, family history of T2DM, and exercise of patients were collected. The above data were collected and checked by three researchers to ensure the completeness and validity of the data.

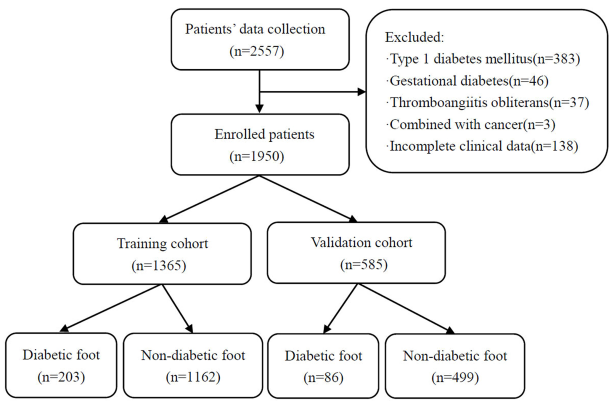


**FIGURE 1** | Flowchart of patients included in this study.

## Statistical Analysis

Statistical analysis was performed using SPSS software (Version 25.0, USA) and R software (Version 3.6.2, USA). Continuous variables were presented as means ± standard deviation or median (interquartile range). Categorical variables were presented using counts and percentages. Continuous variables were analyzed by *t* test or Mann–Whitney *U* test. Categorical variables were analyzed using the $\chi^2$ test or Fisher exact test. The independent risk factors for diabetic foot among patients with T2DM were identified by univariate and multivariate logistic regression analysis of the training cohort. Then, a nomogram prediction model was developed using the independent risk factors. The discrimination, calibration, and clinical usefulness of the nomogram prediction model were validated in training cohort and validation cohort. The area under the receiver operating characteristic curve (AUC) and C index were used to evaluate the discrimination. The calibration was evaluated by calibration plot and Hosmer–Lemeshow test. The clinical usefulness was evaluated by cutoff value combined with decision curve analysis (DCA) curve. $P < 0.05$ indicated statistically significant differences.

# RESULTS

## General Characteristics of Research Subjects

A total of 1,950 patients, including 1,365 patients in training cohort and 585 patients in validation cohort, were enrolled in this study (**Table 1**). In the training cohort, 203 patients developed diabetic foot, giving a frequency of 14.9%. In the validation cohort, 86 patients developed diabetic foot, giving a frequency of 14.7%. There were no statistically significant differences in gender, age, course of disease, BMI, etc. between training cohort and validation cohort ($P > 0.05$), indicating comparability between the two groups.

## Multivariate Logistic Regression Analysis

Univariate logistic regression analysis showed that the risk factors with statistically significant differences were age, course of disease, BMI, HbA1c, LDL, TC, smoke, and drink in training cohort ($P < 0.05$, **Table 2**). Then, the above risk factors were included in the multivariate logistic regression analysis. The results of multivariate logistic regression analysis showed that the independent risk factors for diabetic foot among patients with T2DM were age, HbA1c, LDL, TC, smoke, and drink ($P < 0.05$, **Table 3**).

**TABLE 1** | Characteristics of the patients in the training cohort and validation cohort.

| Characteristics | Training cohort (n=1365) | Validation cohort (n=585) | t/Z/$\chi^2$ | *P* |
|---|---|---|---|---|
| Gender [n(%)] | | | 0.845 | 0.358 |
| Male | 863 (63.2) | 357 (61.0) | | |
| Female | 502 (36.8) | 228 (39.0) | | |
| Age (year) | 46.79±2.71 | 45.12±2.70 | 0.533 | 0.601 |
| Course of disease (year) | 19.79±1.93 | 19.10±2.51 | 1.071 | 0.298 |
| BMI [n(%)] | | | 2.108 | 0.349 |
| <18.5kg/m$^2$ | 109 (8.0) | 53 (9.1) | | |
| 18.5-24 kg/m$^2$ | 846 (62.0) | 374 (63.9) | | |
| >24 kg/m$^2$ | 410 (30.0) | 158 (27.0) | | |
| OGTT 2h (mmol/L) | 14.05±1.85 | 13.90±1.57 | 0.441 | 0.664 |
| HbA1c (%) | 10.17±0.96 | 10.14±0.98 | 0.679 | 0.506 |
| LDL (mmol/L) | 3.73±1.04 | 3.79±0.90 | 1.341 | 0.180 |
| TG [n(%)] | | | 2.213 | 0.331 |
| <1.7 mmol/L | 478 (35.0) | 193 (33.0) | | |
| 1.7-2.3 mmol/L | 315 (23.1) | 153 (26.2) | | |
| >2.3 mmol/L | 572 (41.9) | 239 (40.8) | | |
| TC (mmol/L) | 5.56±1.00 | 5.58±0.97 | 0.478 | 0.633 |
| Smoke [n(%)] | | | 0.244 | 0.622 |
| No | 546 (40.0) | 241 (41.2) | | |
| Yes | 819 (60.0) | 344 (58.8) | | |
| Drink [n(%)] | | | 0.963 | 0.327 |
| No | 642 (47.0) | 261 (44.6) | | |
| Yes | 723 (53.0) | 324 (55.4) | | |
| Hypertension [n(%)] | | | 0.876 | 0.349 |
| No | 802 (58.8) | 357 (61.0) | | |
| Yes | 563 (41.2) | 228 (39.0) | | |
| Family history of type 2 diabetes [n(%)] | | | 0.702 | 0.402 |
| No | 328 (24.0) | 151 (25.8) | | |
| Yes | 1037 (76.0) | 434 (74.2) | | |
| Exercise [n(%)] | | | 1.222 | 0.269 |
| No | 892 (65.3) | 367 (62.7) | | |
| Yes | 473 (34.7) | 218 (37.3) | | |

*BMI, Body Mass Index; OGTT, Oral Glucose Tolerance Test; HbA1c, Hemoglobin A1c; LDL, Low-Density Lipoprotein; TG, Triglyceride; TC, Total Cholesterol.*

**TABLE 2 |** Univariate logistic regression analysis of patients in the training cohort.

| Characteristics | Diabetic foot group (n=203) | Non-diabetic foot group (n=1162) | t/Z/$\chi^2$ | *P* |
|---|---|---|---|---|
| Gender [n(%)] | | | 1.001 | 0.317 |
| Male | 122(60.1) | 741(63.8) | | |
| Female | 81(39.9) | 421(36.2) | | |
| Age (year) | 47.22±2.98 | 46.71±2.65 | 6.081 | 0.014 |
| Course of disease (year) | 20.09±2.00 | 19.73±1.91 | 5.848 | 0.016 |
| BMI [n(%)] | | | 6.426 | 0.011 |
| <18.5kg/m$^2$ | 15(7.4) | 94(8.1) | | |
| 18.5-24 kg/m$^2$ | 109(53.7) | 737(63.4) | | |
| >24 kg/m$^2$ | 79(38.9) | 331(28.5 ) | | |
| OGTT 2h (mmol/L) | 14.16±1.98 | 14.03±1.83 | 0.877 | 0.349 |
| HbA1c (%) | 10.86±0.97 | 10.05±0.91 | 112.052 | <0.001 |
| LDL (mmol/L) | 4.19±0.83 | 3.65±1.06 | 42.974 | <0.001 |
| TG [n(%)] | | | 2.193 | 0.139 |
| <1.7 mmol/L | 59(29.1) | 419(36.0) | | |
| 1.7-2.3 mmol/L | 54(26.6) | 261(22.5) | | |
| >2.3 mmol/L | 90(44.3) | 482(41.5) | | |
| TC (mmol/L) | 5.99±0.75 | 5.48±1.02 | 41.716 | <0.001 |
| Smoke [n(%)] | | | 6.271 | 0.012 |
| No | 65(32.0) | 481(41.4) | | |
| Yes | 138(68.0) | 681(58.6) | | |
| Drink [n(%)] | | | 7.032 | 0.008 |
| No | 78(38.4) | 564(48.5) | | |
| Yes | 125(61.6) | 598(51.5) | | |
| Hypertension [n(%)] | | | 1.263 | 0.261 |
| No | 112(55.2) | 690(59.4) | | |
| Yes | 91(44.8) | 472(40.6) | | |
| Family history of type 2 diabetes [n(%)] | | | 0.565 | 0.452 |
| No | 53(26.1) | 275(23.7) | | |
| Yes | 150(73.9) | 887(76.3) | | |
| Exercise [n(%)] | | | 1.132 | 0.287 |
| No | 126(62.1) | 766(65.9) | | |
| Yes | 77(37.9) | 396(34.1) | | |

*BMI, Body Mass Index; OGTT, Oral Glucose Tolerance Test; HbA1c, Hemoglobin A1c; LDL, Low-Density Lipoprotein; TG, Triglyceride; TC, Total Cholesterol.*

## Development of a Diabetic Foot-predicting Nomogram

A nomogram prediction model for the risk of diabetic foot in patients with T2DM was developed using above independent risk factors (**Figure 2**). The application of the nomogram prediction model was as follows. According to the nomogram, we could obtain the score corresponding to each predictor index, and then the sum of these score was recorded as the total score. The predicted probability corresponding to the total score was the risk of diabetic foot in patients with T2DM.

## Validation of a Diabetic Foot-Predicting Nomogram

### Discrimination

Receiver operating characteristic (ROC) curves of the training cohort and validation cohort were drawn (**Figure 3**). The AUC of the training cohort was 0.806 (95% CI: 0.775∼0.837). The cutoff value was 11.6% ($P < 0.05$). The C index was 0.806. The AUC of the validation cohort was 0.857 (95% CI 0.814∼0.899), ($P < 0.05$). The C index was 0.857. The C indexes of the nomogram prediction model in the training cohort and validation cohort were greater than 0.75, indicating good discrimination of the model.

**TABLE 3 |** Multivariate logistic regression analysis of patients in the training cohort.

| Variable | B | SE | Wald | OR | 95%CI | *P* |
|---|---|---|---|---|---|---|
| Age(year) | 0.098 | 0.031 | 9.855 | 1.103 | 1.038-1.173 | 0.002 |
| HbA1c (%) | 0.920 | 0.092 | 100.327 | 2.509 | 2.096-3.004 | <0.001 |
| LDL (mmol/L) | 0.585 | 0.096 | 37.078 | 1.796 | 1.487-2.168 | <0.001 |
| TC(mmol/L) | 0.524 | 0.098 | 28.877 | 1.690 | 1.395-2.046 | <0.001 |
| Smoke | 0.431 | 0.179 | 5.813 | 1.539 | 1.084-2.186 | 0.016 |
| Drink | 0.341 | 0.172 | 3.931 | 1.407 | 1.004-1.971 | 0.047 |
| Constant | -21.765 | 2.041 | 113.677 | 0.000 | – | <0.001 |

*HbA1c, Hemoglobin A1c; LDL, Low-Density Lipoprotein; TC, Total Cholesterol.*

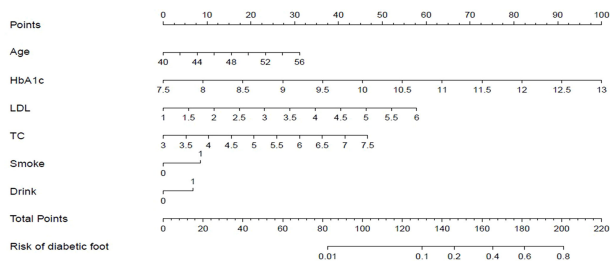


**FIGURE 2** | Nomogram prediction for the risk of diabetic foot in patients with T2DM.

### Calibration

Calibration curves of the nomogram prediction model in the training cohort and validation cohort showed a favorable consistency between the predicted probability and the actual probability (**Figure 4**). In addition, the results of Hosmer–Lemeshow test of the nomogram prediction model in training cohort and validation cohort were $\chi^2 = 4.336$ ($P = 0.826$) and $\chi^2 = 7.532$ ($P = 0.480$), respectively. The $P$ values of Hosmer–Lemeshow test of the nomogram prediction model in training cohort and validation cohort were greater than 0.05, suggesting no statistical significance. It indicated that the calibration of the model was high.

### Clinical usefulness

DCA curves of the training cohort and validation cohort were drawn (**Figure 5**). When the threshold probability was in the range of 3%∼62% and 3%∼99%, respectively, the net benefit of patients was higher than that of the other two extreme curves (The horizontal line indicated that no diabetic foot occurred in all patients and no treatment, and the net benefit was 0. The oblique line indicated that all patients developed diabetic foot and received treatment, and the net benefit was a negative slope backslash line.). Within the above range, the nomogram prediction model has good clinical usefulness. The cutoff value (11.6%) obtained from the ROC curve of the training cohort was within the threshold probability range of the above two DCA curves, indicating that the nomogram prediction model has good clinical usefulness. A further analysis of the DCA curves of the nomogram prediction model showed that the net clinical benefit of the training cohort and validation cohort was 58% and 65%, respectively, when 11.6% was set as the threshold probability value for diagnosing diabetic foot and taking intervention. In other words, 58 and 65 of every 100 patients with T2DM who were diagnosed with diabetic foot using the nomogram prediction model in the training cohort and validation cohort would respectively have clinical benefits.

## Visualization Application of a Diabetic Foot-Predicting Nomogram

Take a patient with T2DM as an example, the relevant clinical data of this patient were as follows: age 50, HbA1c 10.2%, LDL 4.7 mmol/L, TC 5.8 mmol/L, smoke, and drink. According to the nomogram prediction model (**Figure 6**), the predicted risk of diabetic foot for this patient was 22.5%, higher than the threshold probability (11.6%). At this time, according to the DCA curve, we should take intervention to reduce the risk of patients developing diabetic foot.

# DISCUSSION

Diabetic foot was one of the serious complications of diabetes mellitus. Diabetic foot imposed significant economic burdens on patients and society. T2DM accounted for a large proportion of diabetes mellitus. In patients with T2DM, the incidence of diabetic foot was as high as 12% to 15% (15–17). The overall incidence of diabetic foot in patients with T2DM in this study was approximately 14.9%. The incidence was similar to that reported in the above literature. Patients with T2DM who developed diabetic foot often had no obvious clinical symptoms and signs in the early stage, and sometimes only

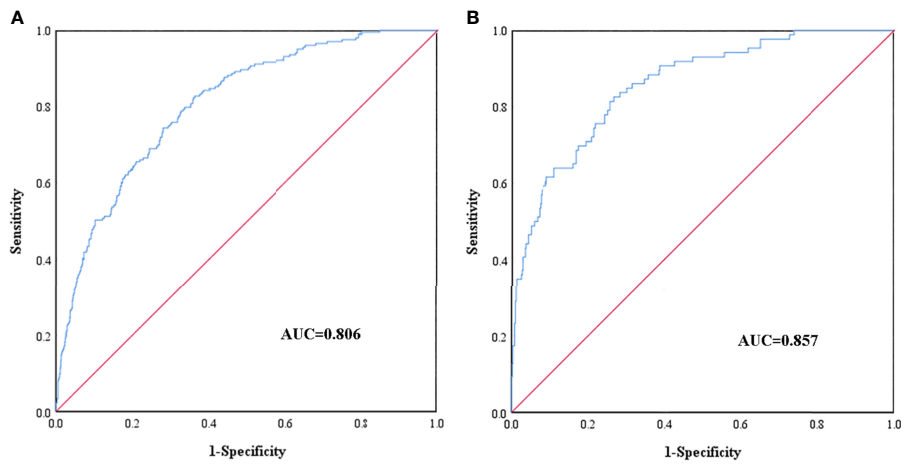


**FIGURE 3** | ROC curves of the nomogram prediction for the risk of diabetic foot in patients with T2DM in the training cohort **(A)** and validation cohort **(B)**.

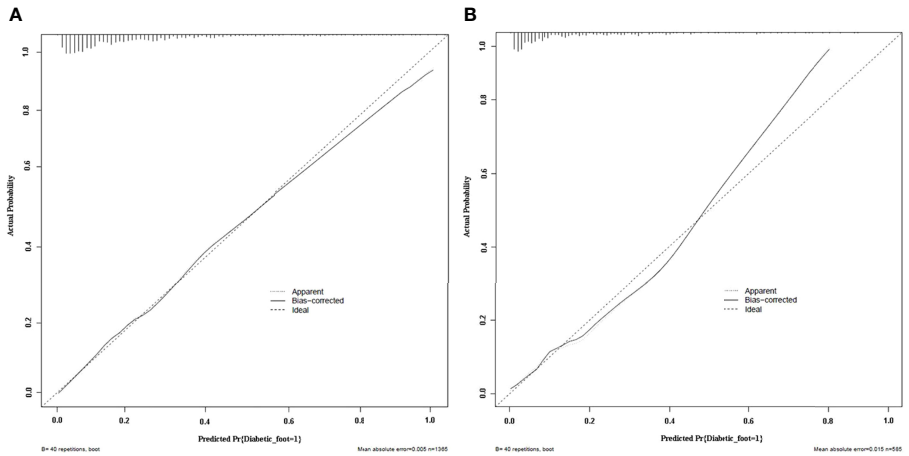


**FIGURE 4** | Calibration plots of the nomogram prediction for the risk of diabetic foot in patients with T2DM in the training cohort **(A)** and validation cohort **(B)**.

showed their decline of protective sensation. This made it easy to neglect the condition of the patient (18–20). Therefore, how to predict the risk of diabetic foot in patients with T2DM at an early stage and timely take intervention for high-risk patients is crucial.

Most of the current research on the relevant risk factors of diabetic foot has focused on intervening in the progression of diabetic foot to prevent severe ulcers and amputations (21–24). Although these studies are important, we believe that how to prevent diabetic patients from developing diabetic foot is more important and critical. On the one hand, there are few studies in this area. On the other hand, there is no consensus in this regard. In our study, univariate and multivariate logistic regression analysis found that the independent risk factors for diabetic foot in patients with T2DM were age, HbA1c, LDL, TC, smoke, and drink.

The pathogeneses of diabetic foot in patients with T2DM are as follows:

(1) Peripheral neuropathy

Patients with T2DM have various degree of neuropathy of distal lower extremities. Neuropathy of distal lower extremities mainly includes sensory, motor, and autonomic peripheral neuropathy (25). Sensory neuropathy is mainly characterized by the reduction or loss of vibration sensation (pallhypesthesia) and superficial sensation (pressure and touch) as well as subjective paresthesia. The sensation of pain in the foot is substantially declined as a consequence of chronic sensory neuropathy. As a result, the risk of foot trauma is significantly higher (26–29). Foot injuries and ulcers are neglected by patients and doctors due to the lack of pain symptoms (30, 31). Hence, foot injuries and ulcers often go undetected by doctors for weeks. Motor neuropathy of the foot is characterized by muscle atrophy,

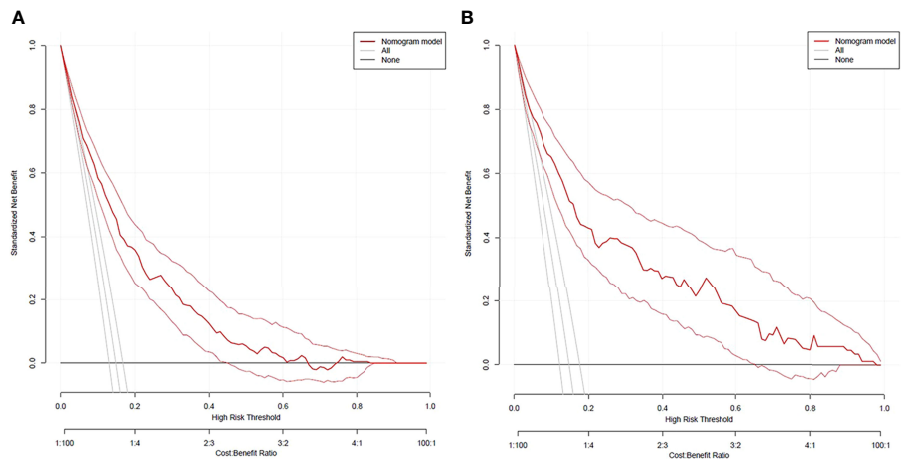


**FIGURE 5** | DCA curves of the nomogram prediction for the risk of diabetic foot in patients with T2DM in the training cohort **(A)** and validation cohort **(B)**.

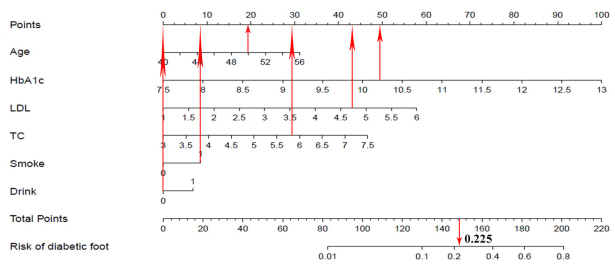


**FIGURE 6** | Visualization application of the nomogram prediction for the risk of diabetic foot in patients with T2DM.

motor paralysis, and loss of muscle self-reflexes. The combination of sensory and motor peripheral neuropathy results in severe foot load accompanied by gait abnormality. As the disease progresses, the lesions of the foot will worsen due to neuropathy and increased plantar pressure load. Secretion of sweat is dysfunctional by motor paralysis due to autonomic neuropathy. Perspiration dysfunction could lead to dry skin on the foot and a reduced protective skin function, which increases the risk of injury and ulcers. Through multivariate logistic regression analysis, our study found that age, HbA1c, and drink were related to the occurrence of diabetic foot, and their OR values were 1.103, 2.509, and 1.407, respectively. According to our analysis of the patients, the sensory function of distal lower extremities of elderly patients with T2DM was worse than that of young patients, so the risk of foot injuries and ulcers was higher than that of young patients. HbA1c reflects the level of recent plasma glucose control of patients. The increase of HbA1c beyond the normal range usually indicates that the patient's level of plasma glucose control is not ideal, leading to the occurrence of hyperglycemia. Previous studies showed that metabolic abnormalities due to hyperglycemia cause neuropathy (32). Hyperglycemia leads to nerve damage through four mechanisms, including increased levels of intracellular advanced glycation end products, activation of protein kinase C, hexosamine pathway, and polyol pathway (33). Alcohol has chronic neurotoxic effects (34), especially in patients with T2DM. This has a certain adverse effect on the sensory nerves of distal lower extremities of patients with T2DM, which leads to a decrease in the damage-sensing capacity of foot and increase the risk of diabetic foot in patients with T2DM.

(2) Peripheral vascular disease

Peripheral vascular disease is often present in the course of T2DM. Peripheral vascular disease is an atherosclerotic occlusive disease of the lower extremity. Patients with T2DM have a higher risk of peripheral vascular disease (35). In patients with T2DM, peripheral vascular disease is an important cause of the occurrence and development of diabetic foot (36). Patients with T2DM have a higher incidence of thickened basement membranes of the capillaries, atherosclerosis, endothelial cell hyperplasia, and arteriolosclerosis (37). As a result, patients with T2DM suffer from a lack of blood supply to their arteries. Poor peripheral blood supply can lead to poor wound healing of foot and worsen the condition. Patients with T2DM have reduced blood perfusion in the foot. As a result, the patients are at risk of ulcers and infections and eventually developing diabetic foot. Through multivariate logistic regression analysis, our study found that LDL, TC, and smoke were related to the occurrence of diabetic foot, and their OR values were 1.796, 1.690, and 1.539, respectively. After analyzing the patients, we believed that patients with T2DM would have a great risk of diabetic foot if their LDL and TC were higher than the normal range.LDL, a cholesterol-rich lipoprotein, is one of the risk factors for atherosclerosis (38). After chemical modification, LDL is ingested by phagocytes, forming foamy cells, and remaining in the vascular wall, resulting in a large amount of cholesterol deposition, which contributes to the formation of atheromatous plaque in the arterial wall (39–41). Thus, elevated LDL increases the risk of peripheral vascular disease in patients with T2DM, resulting in poor blood supply to the foot. In this context, diabetic foot ensues. TC is one of the risk factors for atherosclerosis in clinic (42). Therefore, increased TC will increase the risk of peripheral vascular atherosclerosis in patients. Increased TC has adverse effects on the blood supply to the foot of patients with T2DM, leading to the development of diabetic foot. Smoking can reduce the release of prostacyclin in patients, and then platelets tend to adhere to the arterial wall (43, 44). Smoking can also reduce high density lipoprotein cholesterol and increase TC in blood, resulting in atherosclerosis (45–47). Hence, smoking predisposes the patients with T2DM to atherosclerosis, which reduces peripheral blood supply to the foot. This increases the risk of diabetic foot in patients with T2DM.

At present, the preventive measures for diabetic foot mainly include the following. On the premise of glycemic control, the foot of the patients with T2DM should be checked regularly and protected preventatively (such as wearing loose-fitting shoes and socks). In clinical work, how to identify which patients need early clinical intervention is worth pondering. Meanwhile, there is a lack of related research on the nomogram prediction model for the risk of diabetic foot in patients with T2DM. Hence, we developed and validated a nomogram prediction model for the risk of diabetic foot in patients with T2DM and evaluated its clinical application value. In our study, when the cutoff value (11.6%) was taken as the threshold of DCA curve, we observed that the net clinical benefit of patients was higher than that of the other two extreme curves. This suggests that when the risk of diabetic foot is higher than 11.6% predicted by the nomogram prediction model, immediate intervention will benefit the patients clinically. When the risk of diabetic foot is lower than 11.6% predicted by the nomogram prediction model, doctors and patients can temporarily not take intervention and continue to pay attention to the dynamic changes of the disease. This facilitates clinical decision making in patients with T2DM.

There are some limitations to this study. First of all, it is a retrospective study, which requires further prospective studies in the later stage. Secondly, this study is a single-center study at the present stage. If the data of the patients from multiple centers can be included in the later stage to increase the sample size and the range of observed variables, we will further strengthen the nomogram prediction model.

## CONCLUSION

In conclusion, our study found that the independent risk factors for diabetic foot among patients with T2DM were age, HbA1c, LDL, TC, smoke, and drink. In addition, our study developed an individualized nomogram prediction model, which made the prediction model visualized and easy for clinical application. The nomogram prediction model has good discrimination, calibration, and clinical usefulness in both training cohort and validation cohort. This facilitates early prediction and identification of patients at high risk of developing diabetic foot.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The Medical Ethics Committee of the Second Affiliated Hospital of Xi'an Jiaotong University. The patients/participants provided their written informed consent to participate in this study.

## AUTHOR CONTRIBUTIONS

Study conception and design: JW and HL; data collection and data analysis: JW and TX; manuscript drafting: JW, TX, and SG. All authors were involved in the revision of the manuscript and approved the final version of the paper.

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# Case Report: A novel *WRN* mutation in Werner syndrome patient with diabetic foot disease and myelodysplastic syndrome

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Werner syndrome is an autosomal recessive rare disease caused by a *WRN* gene mutation, which is rarely reported in the Chinese population. We report the clinical and genetic data of a Chinese patient with Werner syndrome. The proband was a 40-year-old male patient who presented with diabetic foot ulcers, accompanied by short stature, cataracts, hypogonadism, and hair thinning, and myelodysplastic syndrome (MDS) occurred after 18 months. Genetic sequencing showed there were compound heterozygous mutations as c.3384-1G>C and c.3744dupA in the *WRN* gene. The c.3744dupA mutation is a novel pathogenic variation for Werner syndrome.

KEYWORDS

**Werner syndrome, myelodysplastic syndrome (MDS), diabetic foot disease, *WRN* gene, novel mutation**

## Introduction

WRN, belonging to the DNA helicases family, is associated with DNA glycosylase, nonhomologous end joining (NEIL1), base excision repair (BER), and homologous recombination (HR) (1). Werner syndrome (the Online Mendelian Inheritance in Man #277700) was caused by *WRN* gene mutation as autosomal recessive in humans, with mainly clinical symptoms as type 2 diabetes mellitus, hypogonadism, osteoporosis, atherosclerosis and malignancies, short stature, and other common age-related diseases (2), and the reports in a Chinese population were just a few (3). In this study, we report a 40-year-old man who

was hospitalized due to diabetic foot ulcers, with short stature, sparse hair, and uneven fat distribution, and with a history of cataracts, osteonecrosis of the femoral head, and supraventricular tachycardia and hypophysis. Genetic test results showed that there were compound heterozygous mutations of *WRN* c.3384-1G>C and c.3744dupA (p.Ala1248fs) in the proband. The proband was diagnosed with Werner syndrome, while during follow-up, 18 months later, the patient developed myelodysplastic syndrome (MDS) and was hospitalized again.

## Case

The proband, a 40-year-old man, was hospitalized with a diabetic foot ulcer as the main complaint. Physical examination showed that he was 147 cm tall, weighed 38 kg, had a body mass index (BMI) of 17.6 kg/m$^2$, a temperature of 36.7°C, a respiratory rate of 16 breaths/min, a blood pressure of 121/82 mmHg, a pulse rate of 97 beats/min, and had lost 5 kg in the previous 3 months. His hair, eyebrows, and beard were sparse (**Figure 1A**). The limbs were slender and out of proportion to the trunk, and with reduced subcutaneous fat (**Figures 1B, C**). The skin of the face, hands, and feet was waxy, thin, and with low elasticity (**Figures 1D, E**). The stretched penile length was 6 cm, the bilateral testicles were 2 ml, and the pubic hair Tanner stage was 1–2. There were 0.8 cm × 0.8 cm wounds with purulent secretion on the dorsal side of the left toe and 0.5 cm × 0.5 cm wounds at the lateral metatarsal joint of the left foot (**Figures 1E, F**). There was no deformity of the chest and spine. Clinical history (**Figure 1G**) was as follows: the height of the patient increased more slowly than their peers since childhood and delayed puberty. Nose bleeding was caused by trauma at age of 12 and then occurred intermittently for 2 years. Binocular cataracts occurred at the age of 20 with surgical treatment. Aseptic ischemia of the right femoral head was detected at the age of 35. At the age of 37, the right clavicle and knee joint were injured in a car accident and underwent surgical treatment. Thirsty, polydipsia, and polyuria occurred at the age of 39 and were not treated. Furthermore, consequential symptoms such as numbness, chills, and pain in both feet were not addressed and thus went untreated. Three months before this time of hospitalization, he received a test of fasting blood glucose (FBG) at 14.9 mmol/L (reference values 3.90–6.10 mmol/L) in the outpatient department, and metformin and Xiaoke pills were taken. Two months before this time of hospitalization, the foot skin was broken, accompanied by purulent secretion, and the pain was aggravated, so he went to the Endocrinology Department of our hospital. Family history has shown that there was no similar patient, and both parents and two older sisters were healthy.

### Laboratory tests

There were some abnormal indexes of glucose metabolism and islet function, serum lipids, thyroid function, adrenal function, liver and kidney function, and sexual hormones, and the results are shown in **Table 1**. Diabetes-related antibodies were negative, including insulin autoantibody, islet cell antibody, and glutamic acid decarboxylase autoantibody. The culture of secretion bacteria from feet wounds showed Shigella. Muck's routine examination demonstrated occult blood. Electrocardiogram results showed sinus rhythm with 88 beats/min and ventricular preshock type A. Peripheral nerve damage of the lower limbs was detected using an electromyogram, which showed the following (1): the sensory conduction velocity of the right sural nerve decreased (ankle-lower leg, 170.00 mm, 39.00 m/s) and the latency prolonged (4.40 ms); (2) the velocities of bilateral median and ulnar nerves were at the lower limit of normal value; (3) the amplitude of motor conduction of the left median (wrist-elbow, 190.00 mm, elbow 5.81 mV) and ulnar nerves (wrist-elbow, 220.00 mm, elbow 5.62 mV) decreased, and distal latency prolonged (median elbow 9.20 ms, ulnar elbow 8.20 ms); (4) the motor conduction velocity (36 m/s) and amplitude (ankle 0.22 mV) of the right peroneal nerve decreased, and the distal latency prolonged (fibula-head 13.30 ms); (5) left peroneal and tibial nerves motor conduction potential wave were not extracted. The results of color Doppler ultrasound showed that the patient had fatty liver, intrahepatic bile duct stones, testicular volume reduction with testicular microlithiasis, and no obvious abnormality was found in the heart and lower limb arteries.

The karyotype result for the first time showed a completely normal male karyotype as 46,XY from peripheral blood cells. The whole exome sequencing (WES) for proband (**Figure 2A, Ⅱ-5**) using peripheral blood demonstrated that there are heterozygous variants of the *WRN* gene (NM_000553) c.3384-1G>C (GRCh37, Chr8:31004568) and c.3744dupA p.Ala1248fs (**Figure 2B**). Family Sanger sequencing showed that the mother and younger sister (**Figures 2A, Ⅰ-2, Ⅱ-4**) had the same c.3384-1G>C variant. The c.3744dupA variants were not detected in the mother and two sisters. The father (**Figure 2A, Ⅰ-1**) refused any genetic testing. Although we do not know the genetic status of the patient's father, the c.3384-1G>C variant has been detected in two family members (mother and younger sister), so we believe that the patient carries compound heterozygous mutations of c.3384-1G>C and c.3744dupA in the *WRN* gene. The c.3384-1G>C was a splice mutation, and according to the American College of Medical Genetics and Genomics/ Association for Molecular Pathology (ACMG/AMP) variant pathogenicity guidelines (4), this mutation was judged to be pathogenic (PVS1+PM2+PP3). The c.3744dupA mutation, which was not included in the gnomAD database and the frequency in people was not known, was judged to be pathogenic (PVS1+PM2+PM3).

### Diagnosis, treatments, and follow-up

Combined with clinical manifestations and WES results, the patient was diagnosed with Werner syndrome caused by *WRN* gene

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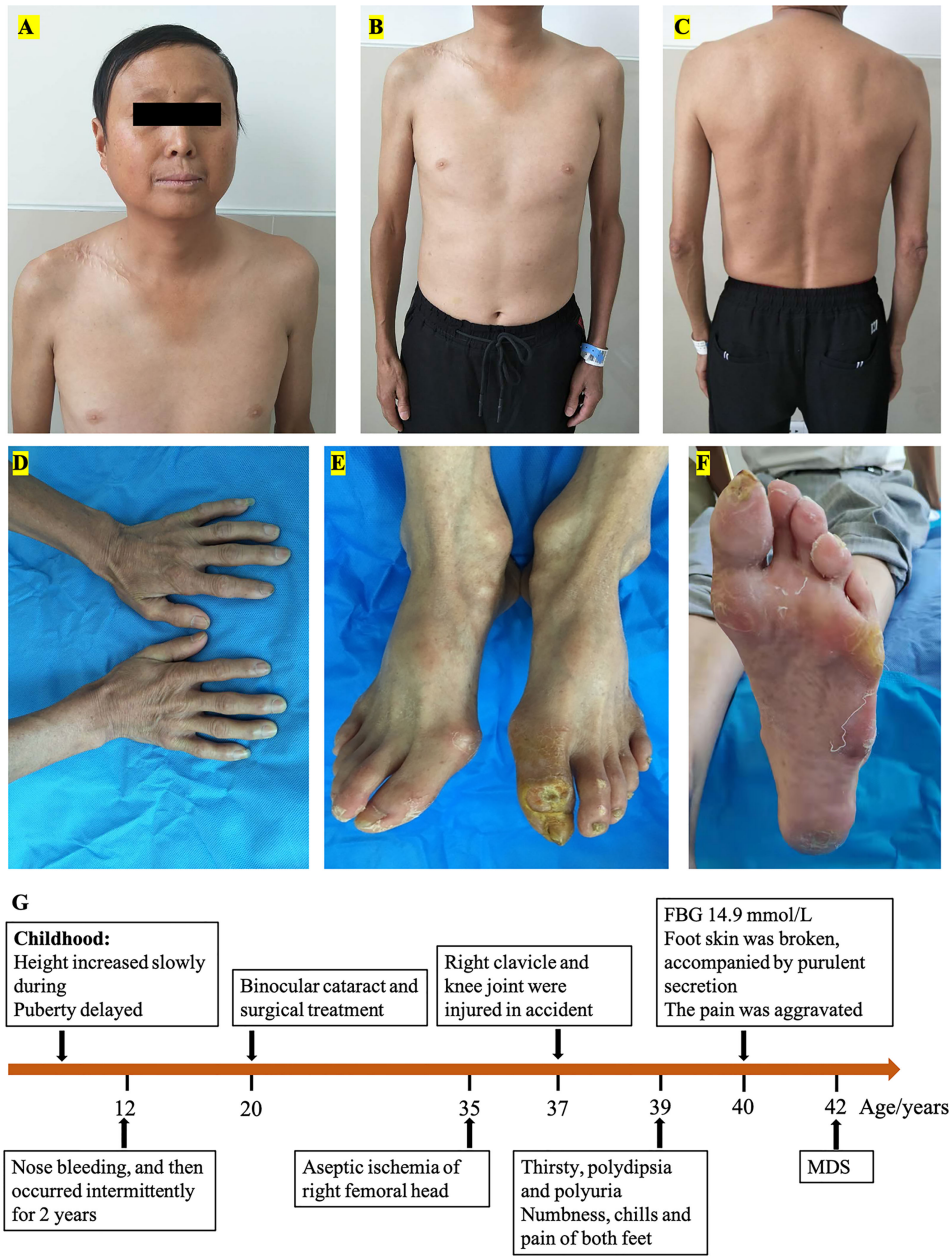


FIGURE 1
Patient's characteristics: **(A)** face, **(B)** ventral trunk, **(C)** back of the trunk, **(D)** hands, **(E)** feet, **(F)** sole, and clinical history diagram **(G)**.

mutations. For glucose control, insulin was used at 45 IU in total daily, metformin tablets at 0.5 g three times a day, pioglitazone dispersible tablets at 15 mg twice a day before breakfast and dinner, mecobalamin at 0.5 mg three times daily, and aspirin enteric-coated tablets at 0.1 g every night. Debridement and anti-infection treatments were carried out for diabetic foot disease, and the patient left the hospital after 40 days of treatments.

Eighteen months later, he was hospitalized in the Hematology Department, and the MDS was definitely diagnosed by bone marrow puncture morphology, biopsy, and chromosome karyotype analysis. The second time of karyotype analysis showed that the chromosomes had structural and numerical abnormalities in marrow cells as 45~46,XY,add(3)(p13),−5,del(7)(q22),+8,−20,+marl,+mar2[cp12]/46,XY[1]. The patient refused chemotherapy and instead received allogeneic red blood cells and erythropoietin to treat anemia, cefuroxime to treat infection, and an ibuprofen capsule as an analgesic. Patients have been informed of the severe adverse prognosis of Werner

10.3389/fendo.2022.918979

TABLE 1 Laboratory investigations of the proband.

| Items | Results | Reference values |
|---|---|---|
| Blood glucose (mmol/L) | | |
| Fasting | 7.13 | 3.90–6.10 |
| 30 min after OGTT | 12.39 | 3.90–11.10 |
| 60 min after OGTT | 17.75 | 6.70–9.40 |
| 120 min after OGTT | 18.33 | 3.90–7.80 |
| 180 min after OGTT | 18.04 | 3.90–6.70 |
| C-Peptide (ng/ml) | | |
| Fasting | 2.21 | 1.10–4.40 |
| 30 min after OGTT | 4.47 | – |
| 60 min after OGTT | 7.23 | – |
| 120 min after OGTT | 12.69 | – |
| 180 min after OGTT | 11.98 | – |
| HbA1c (%) | 8.00 | 4.50–6.50 |
| Urine glucose | +++ | – |
| Serum lipids | | |
| Triacylglycerol (mmol/L) | 11.19 | 0.90–1.72 |
| Total cholesterol (mmol/L) | 9.06 | 3.40–5.17 |
| LDL-c (mmol/L) | 3.05 | 2.59–3.34 |
| HDL-c (mmol/L) | 2.23 | 1.16–1.42 |
| Thyroid function test | | |
| TSH (μIU/ml) | 7.49 | 0.55–4.78 |
| FT3 (pg/ml) | 2.46 | 2.30–4.20 |
| FT4 (ng/dl) | 0.96 | 0.89–1.76 |
| TPOAb (IU/ml) | <28.00 | – |
| Adrenal function | | |
| ACTH (pg/ml) | 44.60 | – |
| Cor (8 am) (μg/dl) | 39.00 | 4.80–20.60 |
| Cor (16 am) (μg/dl) | 12.90 | 4.80–20.60 |
| Cor (0 am) (μg/dl) | 3.30 | 4.80–20.60 |
| Serum sexual hormones | | |
| FSH (mIU/ml) | 30.40 | 1.70–7.70 |
| LH (mIU/ml) | 8.17 | 2.10–14.70 |
| PRL (ng/ml) | 8.13 | 1.90–25.00 |
| T (ng/dl) | 53.54 | 262.00–1,593.00 |
| E2 (pg/ml) | 12.15 | (0.00–56.00) |
| P (ng/ml) | 0.47 | (0.28–1.22) |
| HCG excitation test (T (ng/dl)) | | |
| −15 min | 40.62 | – |
| 0 min | 41.56 | – |
| 24 h | 50.26 | – |
| 48 h | 62.12 | – |
| 72 h | 66.67 | – |
| Blood routine examination | | |
| Hb | 117.00 | 110.00–150.00 |
| Platelet ($\times 10^9$/L) | 352.00 | 125.00–350.00 |
| Serum GGT (U/L) | 77.00 | 10.00–60.00 |
| Serum creatinine (μmol/L) | 78.91 | 59.00–104.00 |
| Urine protein | + | – |

+, positive; +++, strongly positive.

syndrome and need to pay attention to how their condition changes over time.

## Discussion

The WRN protein contains an N-terminal 3′ to 5′ exonuclease domain, an ATP-dependent helicase domain, and a RecQ helicase domain in the central region and a helicase RNase D C-terminal domain and a nuclear localization signal (5). The c.3744dupA mutation, detected in this patient, might affect the nuclear localization signal of WRN helicase since it is a frameshift mutation. Also, this mutation was not reported before and is not contained in the gnomAD database. We wanted to say that WRN c.3744dupA was a novel pathogenic mutation for Werner syndrome.

An extremely high prevalence of diabetes mellitus was mentioned in Werner syndrome. Over half of the patients with the clinical features of diabetes mellitus or impaired glucose tolerance (67.5%, 27/40) ahd accumulated visceral fat, high insulin resistance, and low BMI (6). Metformin, thiazolidine, dipeptidyl peptidase-4 inhibitor, or glucagon-like peptide-1 receptor agonists could be carried out for diabetes treatment in Werner syndrome (7). Insulin, metformin tablets, and pioglitazone dispersible tablets were used in our patient, and for 18 months of treatment, there were no adverse reactions. The probability of skin ulcers in Werner syndrome was about 40%, and ulcers were often located at the distal one-third of the lower legs (8). Skin ulcers in Werner syndrome patients might be a double effect of the *WRN* gene abnormality and poor blood glucose control. Moreover, this may partly be because osteogenesis-related gene expression is upregulated while adipogenic and chondrogenic genes are downregulated in dermal fibroblasts from the foot in comparison with the trunk, resulting in uneven distribution of fat and skin ulcers in Werner syndrome (9). Nonsurgery, surgery, or invasive surgery should be considered for ulcer treatments under appropriate conditions, and anti-infection should be given more attention. The dressing of functional peptide (SR-0379) was proved safe, well-tolerated, and effective for leg ulcers in Werner syndrome, and the reduction rate of ulcer size was 22.9% in men after 4 weeks of treatment ($n = 4$) (10). In our patient, the ulcers in the foot were treated with skin flap repair, and some recovery was obtained after 40 days of dressing change cure.

MDS is a kind of clonal disorder in hematopoietic stem cell progenitors, with ineffective hematopoiesis, morphologic dysplasia, and a high risk of acute myeloid leukemia (AML). Almost 90% of MDS patients were detected carrying a somatic mutation in at least one gene, and the mutation genes were mainly implicated in the pathways of RNA splicing, DNA damage response, epigenetic regulators, signal transduction, and transcription factors (11). Werner syndrome patients with *WRN* mutations were easy to obtain *p53* gene mutations or related chromosomal abnormalities, and this was different from other MDS/AML patients' changes in cellular or molecular genetic material. In hematopoietic stem cells, WRN function

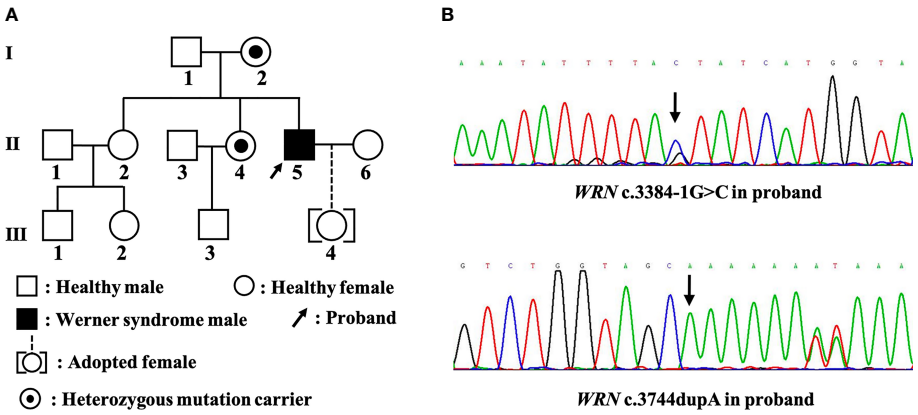


FIGURE 2
**(A)** Family map and **(B)** Sanger sequencing results.

loss could result in p53 inactivation and acquirer of competitive fitness and then develop into myeloid malignancies (12), which may increase chemosensitivity. CHK1-related homologous recombination repair (HRR) in WRN defection cells was the key approach for repairing double-strand breaks (DSB) caused by ionizing radiation, which resulted in hyper-radiosensitization (13). The specific molecular mechanism of tumorigenesis in Werner syndrome patients may lead to therapeutic differences.

The treatments for MDS in Werner syndrome were very limited. Chemotherapy could be difficult for Werner syndrome patients, and a 44-year-old male Werner syndrome patient with AML was treated using a combined chemotherapy regimen of cytarabine, mitoxantrone, and etoposide, while the abnormal toxicity occurred on day 5, which did not occur in other patients with the same treatment regimen (14). Cord blood transplantation (CBT) has some effects on the MDS of Werner syndrome. A 44-year-old male patient with MDS underwent CBT and obtained a 15-month survival period, during which time he had MDS remission and no treatment-related toxicity (15). Allogeneic hematopoietic cell transplantation (HCT) may be feasible for Werner syndrome-related AML, and an 18-year-old female patient with AML obtained a 5-year survival after HCT without severe chemotherapy or transplant-induced toxicities (16). Anemia is one of the major concerns in MDS patients. Transfusions and erythropoiesis-stimulating agents could be used. MDS accounted for 7.2% of all transfused patients, which was the second cause of hematologic prescriptions in Europe and the USA (17). In this study, the patient just received supportive treatment such as blood transfusion, and the effect was limited. In general, the *WRN* gene mutation made the treatment of MDS in Werner syndrome very difficult, and the effects of supportive or chemotherapy were very few, while CBT and HCT may have better effects.

In conclusion, we report a Chinese Werner syndrome male patient with diabetic foot ulcers and MDS. The c.3744dupA mutation of the *WRN* gene in our patient was a novel pathogenic variation for Werner syndrome.

## Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding authors.

## Ethics statement

The studies involving human participants were reviewed and approved by the ethics committee of the First Affiliated Hospital of Henan University of Science and Technology. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

## Author contributions

HP, HJ, and HZ contributed to the conception and design of the study. HP and JW wrote the draft of the manuscript. YL, HY, LL, and YM collect data. All authors contributed to manuscript revision and read and approved the submitted version.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Publisher's note



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# Shear wave elastography as a quantitative biomarker of diabetic peripheral neuropathy: A systematic review and meta-analysis

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**Background:** Diabetic peripheral neuropathy (DPN) is one of the most common chronic complications of diabetes and the strongest initiating risk factor for diabetic foot ulceration. Early diagnosis of DPN through screening measures is, therefore, of great importance for diabetic patients. Recently, shear wave elastography (SWE) has been used as a method that is complementary to neuroelectrophysiological examination in the diagnosis of DPN. We aimed to conduct a meta-analysis based on currently available data to evaluate the performance of tibial nerve stiffness on SWE for diagnosing DPN.

**Methods:** Both PubMed, EMBASE, the Cochrane Library, and Web of Science were searched for studies that investigated the diagnostic performance of SWE for DPN up to March 1th, 2022. Three measures of diagnostic test performance, including the summary area under receiver operating characteristics curve (AUROC), the summary sensitivity and specificity, and the summary diagnostic odds ratios were used to assess the diagnostic accuracy of SWE. All included studies were published between 2017 and 2021.

**Results:** Six eligible studies (with 170 DPN patients, 28 clinically defined DPN patients, 168 non-DPN patients, and 154 control participants) that evaluated tibial nerve stiffness were included for meta-analysis. The summary sensitivity and specificity of SWE for tibial nerve stiffness were 75% (95% confidence interval [CI]: 68–80%) and 86% (95% CI: 80–90%), respectively, and the summary AUROC was 0.84 (95% CI: 0.81–0.87), for diagnosing DPN. A subgroup analysis of five two-dimensional SWE studies revealed similar diagnostic performance, showing the summary sensitivity and specificity of 77% (95% CI: 69–83%) and 86% (95% CI: 79–91%), respectively, and a summary AUROC value of 0.86 (95% CI: 0.83–0.89).

**Conclusions:** SWE is found to have good diagnostic accuracy for detecting DPN and has considerable potential as an important and noninvasive adjunctive tool in the management of patients with DPN.

KEYWORDS

**biomarker, diabetic peripheral neuropathy, diagnosis, stiffness measurement, shear wave elastography**

# Introduction

Diabetes is one of the most common chronic diseases worldwide and has become an important public health problem recently (1). According to the data of International Diabetes Federation (IDF) (2), ∼463 million adults around the world were suffering from diabetes in 2019, and the diabetic population is expected to reach 700 million people by 2045 (about 10% of the global population).

Diabetic peripheral neuropathy (DPN), the main type of diabetic neuropathy, is one of the most common and serious complication of diabetes and the strongest initiating risk factor for diabetic foot ulceration, occurring in about 50% of patients with diabetes (3, 4). This percentage is even higher, up to 60–90% in some areas (5, 6). DPN is the leading cause of lower-limb amputation and disabling neuropathic pain (7), which has a devastating effect on the quality of life and long-term survival of patients with diabetes and brings a heavy economic burden. It is worth noting that major amputations in patients with diabetes are associated with a low life expectancy, with a 5-year mortality ranging from 52 to 80% (8). Therefore, early diagnosis of the DPN in people with diabetes is of great importance for taking effective targeted measures, thereby preventing the development of foot ulcers and amputations.

However, in clinical settings, the assessment of DPN can be challenging and is mainly based on characteristic symptoms and signs (3, 9). At present, nerve conduction studies (NCS) is widely considered to be one of the gold standard methods for evaluating DPN (10). NCS is a quantifiable, objective, and sensitive method. Nevertheless, there are some limitations of this technique, such as invasiveness, time-consuming, high cost, and the need for qualified professionals to perform (9), which has largely restricted their practical applications. Notably, NCS is limited to evaluating large nerve fibers, while small nerve fibers are the first to be affected in DPN patients (9). So this technique does not assess early neuropathic changes (3). Moreover, NCS has usage difficultly for screening in large sample sizes (11). Therefore, there is a pressing need for portable, reliable, and valid tools to detect DPN.

Over the past few years, shear wave elastography (SWE) has gathered considerable attention. SWE is a non-invasive imaging technique that maps the elastic properties of tissues by assessing the velocity of shear wave propagation in the particular tissue (12). The shear wave speed is directly related to tissue stiffness (12). This modality offers a new type of high-quality ultrasound examination and has been widely applied in many organs such as the liver (13, 14), thyroid (15, 16), and the breast (17, 18). As an exciting and rapidly evolving adjunctive diagnostic tool to conventional ultrasound, SWE provides more quantitative information of tissue properties that used in the routine clinical evaluation of various traumatic and pathological conditions of the musculoskeletal system, which may contribute to diagnosis (19). Interestingly, a recent study in which SWE technique was used as a method that is complementary to neuroelectrophysiological examination in the diagnosis of DPN has been found that the stiffness of the affected nerves of diabetic patients with DPN was significantly greater than that of diabetic patients without DPN and healthy control individuals (20).

Although several studies have evaluated the diagnostic performance of SWE in detecting DPN, most have included a relatively small sample size (20–23). Furthermore, a consensus for the value of SWE that used as a biomarker in the diagnosis of DPN has not been reached. Generating an evidence-based summary of the SWE performance characteristics would be of high clinical importance for improved management of DPN in diabetic patients. Given this, in the present study, we aimed to conduct a meta-analysis based on currently available data to evaluate the diagnostic accuracy of SWE for the detection of DPN.

# Methods

## Literature search strategy

The Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) 2020 guidelines were followed for reporting this systemic review and meta-analysis (24). Both PubMed, EMBASE, the Cochrane Library, and Web of Science were systematically searched from inception to March 1th, 2022 using the following keywords: ((“diabetes”) OR (“diabetic peripheral neuropathy”) OR (DPN) OR (“diabetic foot”) OR (“diabetic foot ulcers”) OR (DFU) OR (“diabetic complications”)) AND ((elastography)). In addition, references of the identified articles were manually examined for other relevant publications. In our study, the references were managed using EndNote X9 software (Clarivate Analytics, Philadelphia, PA, United States).

## Inclusion and exclusion criteria

The original research articles were included in the present study if they conformed the following criteria: (1) the study examined the diagnostic performance of SWE for detecting DPN; (2) the SWE was included as an index test; (3) all the research patients were patients with diabetes; (4) the study enrolled at least 10 patients with diabetes, and; (5) at least one 2 × 2 table (i.e., true-positive, false-positive, false-negative, and true-negative) of test performance can be constructed using the data extracted from the study. Studies fulfilling any of the following criteria were excluded: (1) studies were not relevant to SWE diagnosis (e.g., studies that used only strain elastography); (2) reviews, guidelines, conference abstracts, and author comments; (3) animal studies; (4) data incomplete; (5)

duplicate publications, and; (6) studies published in non-English or non-Science Citation Index (SCI) journals.

## Data extraction

Two investigators (B.T. Dong and X.C. Yang) read the articles, and checked the study eligibility and quality independently. In our meta-analysis, Microsoft Excel 2019 was used to pre-design the data extraction form. The patients' data including number of patients, age, sex, and body mass index (BMI) were collected from each included article. Moreover, the outcome indicators including cut-off values, sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and area under the receiver operating characteristic curve (AUROC) values were also extracted from the included studies.

As for the technical characteristics of SWE, the various aspects of this technique were assessed as follows: (1) vendors; (2) type of elastography; (3) probes; (4) target nerve; (5) the number of repeated measurements performed per patient; (6) the representative value of elasticity (mean or median); (7) number of readers; (8) blinding to the reference standard, and; (9) time interval between SWE and reference.

## Quality assessment

The quality of the included studies was assessed by the Quality Assessment of Diagnostic Accuracy Studies 2 (QUADAS-2) tool (25). The four steps of searching literatures, selecting studies, extracting data, and checking the study quality were separately performed by B.T. Dong and X.C. Yang in this meta-analysis. All discrepancies were resolved by consensus of these three authors (B.T. Dong, X.C. Yang, and G.R. Lyu).

## Data synthesis and analysis

Stata version 15.0 (STATA Corp., TX, USA) was selected to perform all statistical analyses. Review Manager (version 5.4.1; Cochrane Collaboration, https://training.cochrane.org/online-

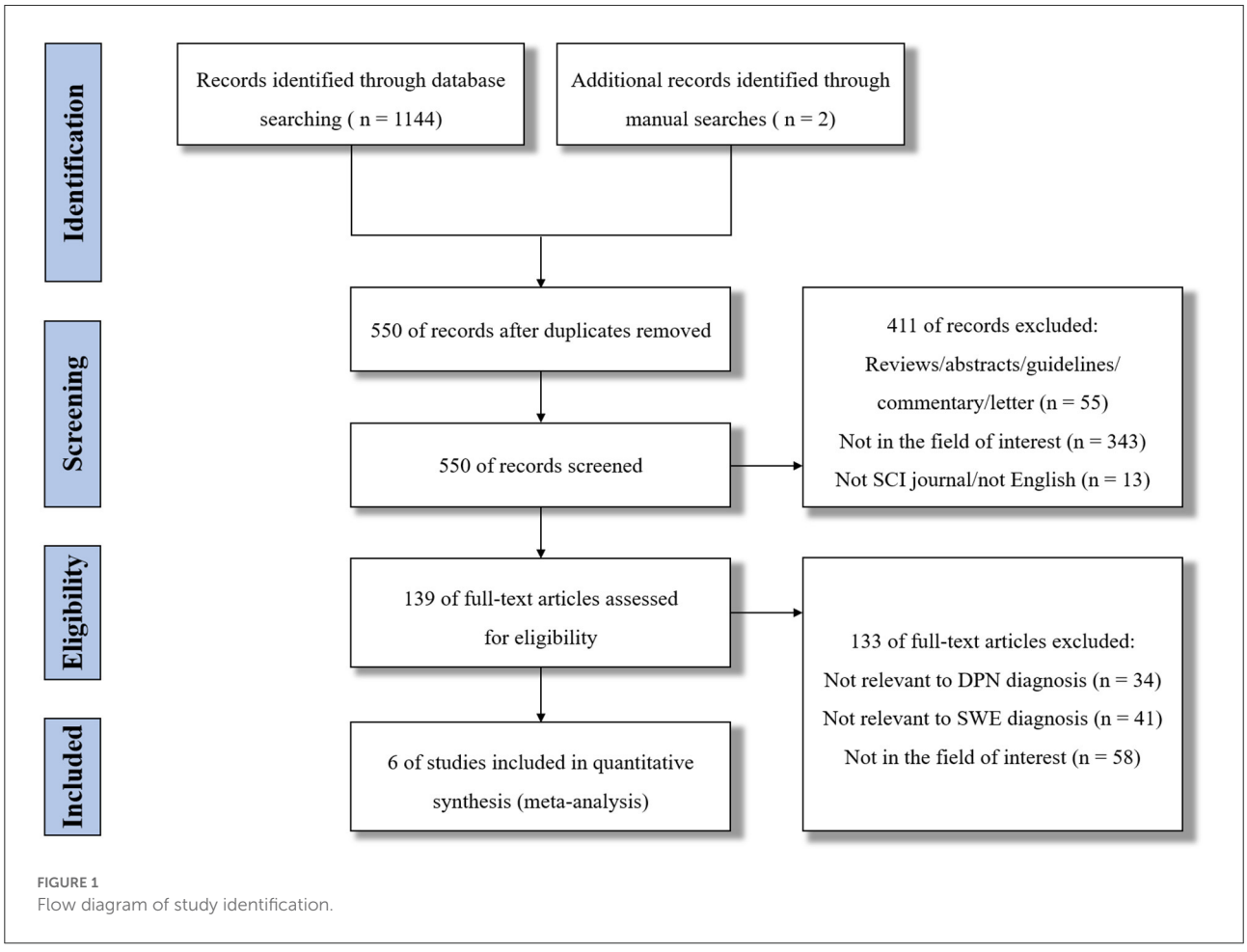


FIGURE 1
Flow diagram of study identification.

TABLE 1 Basic characteristics of the included studies.

| References, region | Study period | Center | Study design | Subject | Size | Median/mean age, years | BMI, kg/m$^2$ | Male, % |
|---|---|---|---|---|---|---|---|---|
| Dikici et al. (20), Turkey | Nov 2013–Jul 2014 | One center | Prospective | DPN | 20 | 60.0 | 31.4 | 50.0 |
| | | | | Non-DPN | 20 | 61.0 | 29.8 | 40.0 |
| | | | | CG | 20 | 58.0 | 28.7 | 45.0 |
| Jiang et al. (21), China | Nov 2017–May 2018 | One center | Prospective | DPN | 25 | 66.2 | 24.3 | 44.0 |
| | | | | CDDPN[a] | 25 | 60.9 | 24.2 | 24.0 |
| | | | | Non-DPN | 20 | 57.1 | 25.4 | 40.0 |
| | | | | CG | 20 | 57.8 | 24.2 | 50.0 |
| He et al. (12), China | Nov 2016–Jul 2017 | One center | NA | DPN | 40 | 60.43 | 25.11 | 42.5 |
| | | | | Non-DPN | 40 | 58.63 | 24.72 | 55.0 |
| | | | | CG | 40 | 55.20 | 22.38 | 60.0 |
| Wei et al. (22), China | Jun 2017–Sep 2017 | One center | Prospective | Type 2 DM | 30[b] | 60.10 | 23.43 | 60.0 |
| | | | | CG | 20 | 57.35 | 22.95 | 76.7 |
| Chen et al. (23), China | Oct 2018–Aug 2019 | One center | Prospective | DPN | 30 | 54.43 | 25.67 | 60.0 |
| | | | | Non-DPN | 33 | 54.85 | 26.19 | 45.5 |
| | | | | CG | 33 | 51.51 | 23.28 | 42.4 |
| Wang et al. (27), China | Dec 2017–Dec 2019 | One center | NA | DPN | 41 | 59.05 | 24.72 | 68.3 |
| | | | | Non-DPN | 42 | 58.50 | 24.75 | 64.3 |
| | | | | CG | 21 | 56.05 | 23.46 | 38.1 |

BMI, body mass index; CDDPN, clinically defined diabetic peripheral neuropathy; CG, control group; DM, diabetes mellitus; DPN, diabetic peripheral neuropathy; NA, not available; Non-DPN, non-diabetic peripheral neuropathy.
[a]CDDPN, clinically defined DPN, which is defined as diabetic patients with clinical signs or symptoms of DPN but normal nerve conduction study (NCS).
[b]The 30 patients with type 2 diabetes mellitus included 14 patients with a diagnosis DPN and 13 patients without a diagnosis of DPN, and 3 patients had positive symptoms or signs of neuropathy, but the NCS results were negative.

learning/core-software/revman/revman-5-download) software was used to assess the methodological quality of the included studies (26). First, we extracted the raw data from all the included studies, and then 2 × 2 tables were reconstructed for further analysis. In our meta-analysis, three measures of diagnostic test performance, including the summary AUROC, the summary diagnostic odds ratio (DOR), and the summary sensitivity and specificity, were used with the aim of examining the accuracy of SWE for diagnosing DPN. Positive likelihood ratio (LR) and negative LR were also calculated. For each summary statistic, we computed the 95% confidence intervals (CIs). In addition, we also conducted a subgroup analysis in order to evaluate the diagnostic performance of the typical type of SWE technique (two-dimensional SWE). Further, the summary receiver operating characteristic (SROC) curve was constructed using the data from the studies included in our meta-analysis to calculate the summary AUROC of SWE for detecting DPN.

## Assessment of heterogeneity and publication bias

The Spearman correlation coefficient was calculated to evaluate the threshold effect of the included studies. The existence of threshold heterogeneity was considered when the $P < 0.05$. To evaluate the non-threshold heterogeneity of included studies, the Cochran's $Q$-test and inconsistency index ($I^2$) statistic was used. $I^2$ value was calculated in our analysis, and then used to describe the amount of non-threshold heterogeneity. Using the Cochran's $Q$-test and $I^2$ statistic, $P < 0.05$ indicated statistically significant heterogeneity; an $I^2$ value >50% may be considered as substantial heterogeneity. Furthermore, the Deeks' funnel plot was used to assess the potential publication bias of the SWE studies with regard to their performance in detecting DPN, with a $P < 0.05$ suggested significant bias.

# Results

## Characteristics of the retrieved studies

The flow diagram of study identification is shown in Figure 1. Using the search strategies presented, a total of 1,146 records were retrieved. After removal of 596 duplicates, 550 studies were initially screened. However, 544 studies were excluded for some reasons, such as reviews, only abstract, not relevant to DPN diagnosis, or not relevant to SWE diagnosis, etc. Finally, six studies were included for evaluation and meta-analysis (12, 20–23, 27).

Table 1 displays the basic characteristics of the studies included in this review. Most of the studies were published between 2019 and 2021, except one study that was published in 2017. One study cohort was from the Turkey, while the others were from the China. In addition, all six studies were conducted in a single-center setting. Among the included studies, there were 4 prospective studies. In terms of a reference standard used for diagnosis of DPN, three studies used the NCS, two used the electrophysiology examination, and one used the NCS and positive symptoms or signs of neuropathy. The articles included in this meta-analysis were published in six different journals, the mean impact factor of these journals was 4.638 (range: 1.889–11.105).

## Characteristics of the study populations

In total, 520 subjects were included in this meta-analysis. Specifically, they were 170 DPN patients, 28 clinically defined DPN patients (i.e., patients with clinical signs or symptoms of DPN but normal NCS), 168 non-DPN patients, and 154 control participants. In the DPN group, the study populations were all patients with type 2 diabetes mellitus (T2DM). Moreover, all the included subjects were adults. Figure 2 shows the distribution of research population of the included studies.

## Technical characteristics of shear wave elastography

The technical characteristics of the SWE technique used in the included studies are summarized in Table 2. Among the included studies, SWE was performed using two types of devices, including Aixplorer in five studies and Acuson S2000

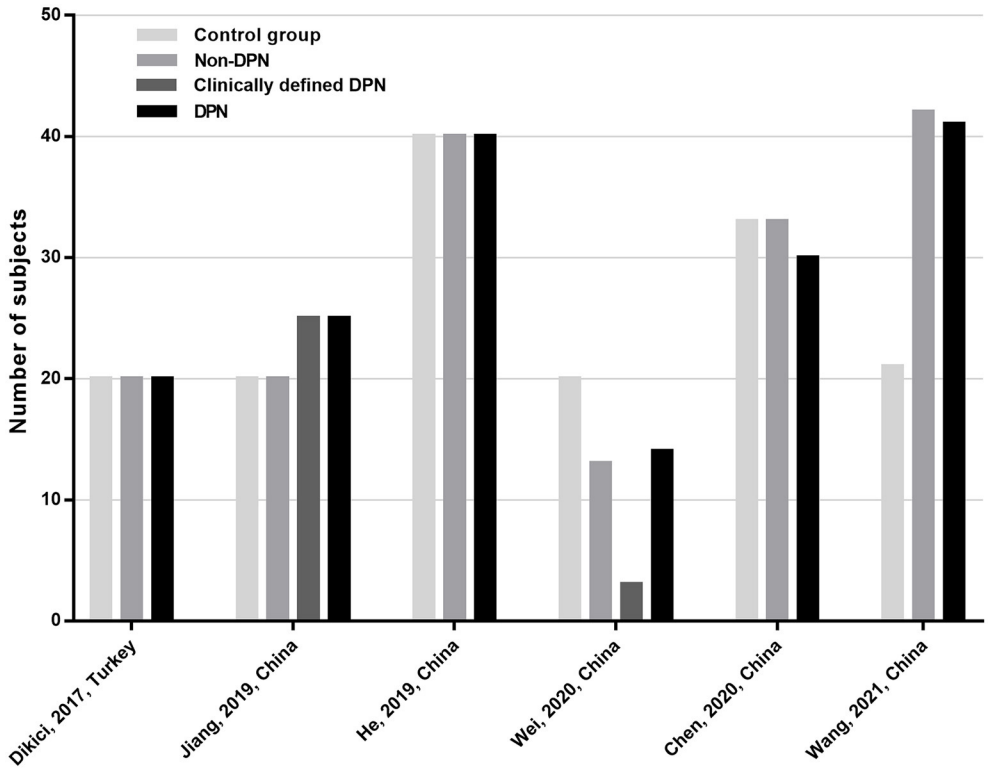


FIGURE 2
Distribution of research population of the included studies.

TABLE 2 Technical characteristics of the elastography/reference standard used in the included studies.

| References, region | Technique | Elastography systems | Probe | No. of measurements | Representative values | Readers | Blinding | Time between Reference Standard and SWE | Reference standard |
|---|---|---|---|---|---|---|---|---|---|
| Dikici et al. (20), Turkey | 2D-SWE | Aixplorer[a] | 4–15 MHz LAT | 3 | Mean | 2 | Yes | <1 week | NCS |
| Jiang et al. (21), China | 2D-SWE | Aixplorer[a] | 4–15 MHz LAT | 4 | Mean | 2 | Yes | NA | NCS |
| He et al. (12), China | 2D-SWE | Aixplorer[a] | 4–15 MHz LAT | 3 | Mean | 2 | Yes | NA | NCS |
| Wei et al. (22), China | p-SWE | Virtual Touch Q[b] | 9L4 LAT | 3 | Mean | 2 | Yes | NA | NCS and positive symptoms or signs of neuropathy |
| Chen et al. (23), China | 2D-SWE | Aixplorer[a] | 4–15 MHz LAT | 5 | Mean | NA | NA | NA | Electrophysiology test |
| Wang et al. (27), China | 2D-SWE | Aixplorer[a] | 4–15 MHz LAT | 3 | Mean | 1 | Yes | <1 week | Electrophysiology test |

LAT, linear array transducer; NA, not available; NCS, nerve conduction study; p-SWE; point shear wave elastography; SWE, shear wave elastography; 2D-SWE, two-dimensional shear wave elastography.
[a]SuperSonic Imagine, Aix-en-Provence, France.
[b]Siemens AG, Erlangen, Germany.

in one study. Based on the technique used in this meta-analysis, SWE can be categorized as either point SWE, i.e., Virtual Touch Q (Siemens AG, Erlangen, Germany), or as two-dimensional SWE, i.e., Aixplorer (SuperSonic Imagine, Aix-en-Provence, France). The SWE technique used in examination of nerves is illustrated in Figure 3. Of note, all the 6 included studies measured tibial nerve stiffness, and two of these reports also included median nerve stiffness and common peroneal nerve stiffness measurements, respectively. Furthermore, as the measure of nerve stiffness, four two-dimensional SWE studies used elasticity, expressed in kilopascals (kPa), and one two-dimensional SWE study and one point SWE study used the shear wave speed, which expressed in meters per second.

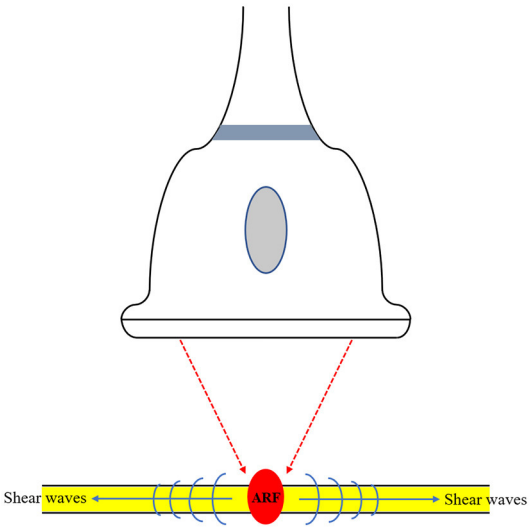


FIGURE 3
Illustration of the shear wave elastography (SWE) technique used in examination of nerves. SWE generates shear waves via acoustic radiation force (ARF). This technique provides more quantitative information in relation to the elasticity of the tissue.

## Diagnostic accuracy

Six studies investigated SWE diagnostic performance for the prediction of DPN. As is shown in Table 3, in the 6 studies evaluating the tibial nerve stiffness using SWE for diagnosing DPN, the mean AUROC value was 0.864 (range: 0.712–0.941). Figure 4 demonstrates that, for the tibial nerve stiffness using SWE, the summary sensitivity and specificity were 75% (95% CI: 68–80%) and 86% (95% CI: 80–90%), respectively. For diagnosing DPN, the summary AUROC of SWE for tibial nerve stiffness was 0.84 (95% CI: 0.81–0.87), as shown in Table 4. The summary DOR was 18 (95% CI: 10–33), when tibial nerve stiffness was used to diagnose DPN. It is worth mentioning that tibial nerve stiffness measured by SWE had a sensitivity and specificity value of 90.0 and 85.0%, respectively, and an AUROC value of 0.941, at a cut-off value of 51.1 kPa, for predicting DPN.

TABLE 3 Summary of diagnostic accuracy of SWE for diagnosing DPN.

| Reference, region | Target nerve | Optimal EI outcome | Cut-off value | AUROC | Sensitivity, % | Specificity, % | PPV, % | NPV, % |
|---|---|---|---|---|---|---|---|---|
| Dikici et al. (20), Turkey | TN | Mean | 51.1 kPa | 0.941 | 90.0 | 85.0 | 75.0 | 94.4 |
| Jiang et al. (21), China | TN | Min | 45.7 kPa | 0.867 | 74.0 | 87.6 | 88.2 | 72.9 |
| He et al. (12), China | TN | Mean | 4.1 m/s | 0.927 | 81.3 | 88.7 | 78.3 | 90.5 |
| | MN | Mean | 4.1 m/s | 0.899 | 80.0 | 85.0 | 72.7 | 89.5 |
| Wei et al. (22), China | TN | Mean | 2.6 m/s | 0.836 | 63.3 | 92.5 | 92.7 | 62.7 |
| Chen et al. (23), China | TN | Mean | 32.7 kPa | 0.902 | 73.3 | 90.9 | 78.6 | 88.2 |
| | CPN | Mean | NA | 0.653 | NA | NA | NA | NA |
| Wang et al. (27), China | TN | Mean | 71.3 kPa | 0.712 | 68.3 | 73.8 | 62.9 | 78.2 |

AUROC, area under the receiver operating characteristic curve; CPN, common peroneal nerve; EI, elasticity indices; MN, median nerve; NA, not available; NPV, negative predictive value; PPV, positive predictive value; SWE, shear wave elastography; TN, tibial nerve.

Furthermore, a subgroup analysis of five two-dimensional SWE studies revealed similar diagnostic performance, showing the summary sensitivity and specificity of 77% (95% CI: 69–83%) and 86% (95% CI: 79–91%), respectively, and a summary AUROC value of 0.86 (95% CI: 0.83–0.89) (Figure 5). Table 5 shows that the summary DOR of two-dimensional SWE for tibial nerve stiffness was 20 (95% CI: 10–39), for predicting DPN.

Notably, there was one study in which SWE measurements were performed on both the median nerve and tibial nerve; this study simultaneously reported the diagnostic performance of median nerve stiffness (cut-off value, 4.1 m/s; sensitivity, 80.0%; specificity, 85.0%; AUROC, 0.899) and tibial nerve stiffness (cut-off value, 4.1 m/s; sensitivity, 81.3%; specificity, 88.7%; AUROC, 0.927). Additionally, another study also reported that common peroneal nerve measured by SWE had an AUROC of 0.653 for diagnosing DPN.

## Heterogeneity and publication bias

No threshold effect was found in the present meta-analysis, as depicted by the Spearman correlation coefficient value of 0.257 ($P = 0.623$). When SWE technique was used to diagnose DPN, the Cochran's Q-test (sensitivity, $P = 0.23$; specificity, $P = 0.06$) showed no statistically significant heterogeneity evidence both with regard to the summary sensitivity and specificity. This can be seen in Figure 4.

Deeks' funnel plot of SWE used to assess publication bias is illustrated in Figure 6. In this meta-analysis, there was no evidence of publication bias among the six included studies with a $P$ value of 0.64.

# Discussion

DPN is the most important risk factor for the occurrence of diabetic foot ulcers, which seriously affect the quality of life and survival of patients with diabetes (3, 9). Early diagnosis of DPN is, therefore, of increasing importance. Unfortunately, in routine clinical practice, there are currently no simple biomarkers for early detection of DPN (3). In this regard, a novel diagnostic tool known as SWE technique may provide valuable alternatives, which has recently been introduced for the detection of DPN. In view of this, we wondered whether SWE can be used as a biomarker for the diagnosis of DPN. Our meta-analysis presents a comprehensive summary of the SWE performance characteristics for DPN, and, to our knowledge, this is the first meta-analysis of published studies that provides evidence of SWE being a novel and non-invasive tool for diagnosing DPN.

In this systematic review and meta-analysis, we identified six (170 DPN patients, 28 clinically defined DPN patients, 168 non-DPN patients, and 154 control participants) original articles with enough data to assess the performance of SWE for the prediction of DPN. Our study has revealed that tibial nerve stiffness measurement by SWE has good diagnostic performance for DPN, showing a summary sensitivity and specificity of 75 and 86%, respectively, and a summary AUROC of 0.84. A subgroup analysis of five two-dimensional SWE studies revealed similar diagnostic performance. The summary sensitivity and

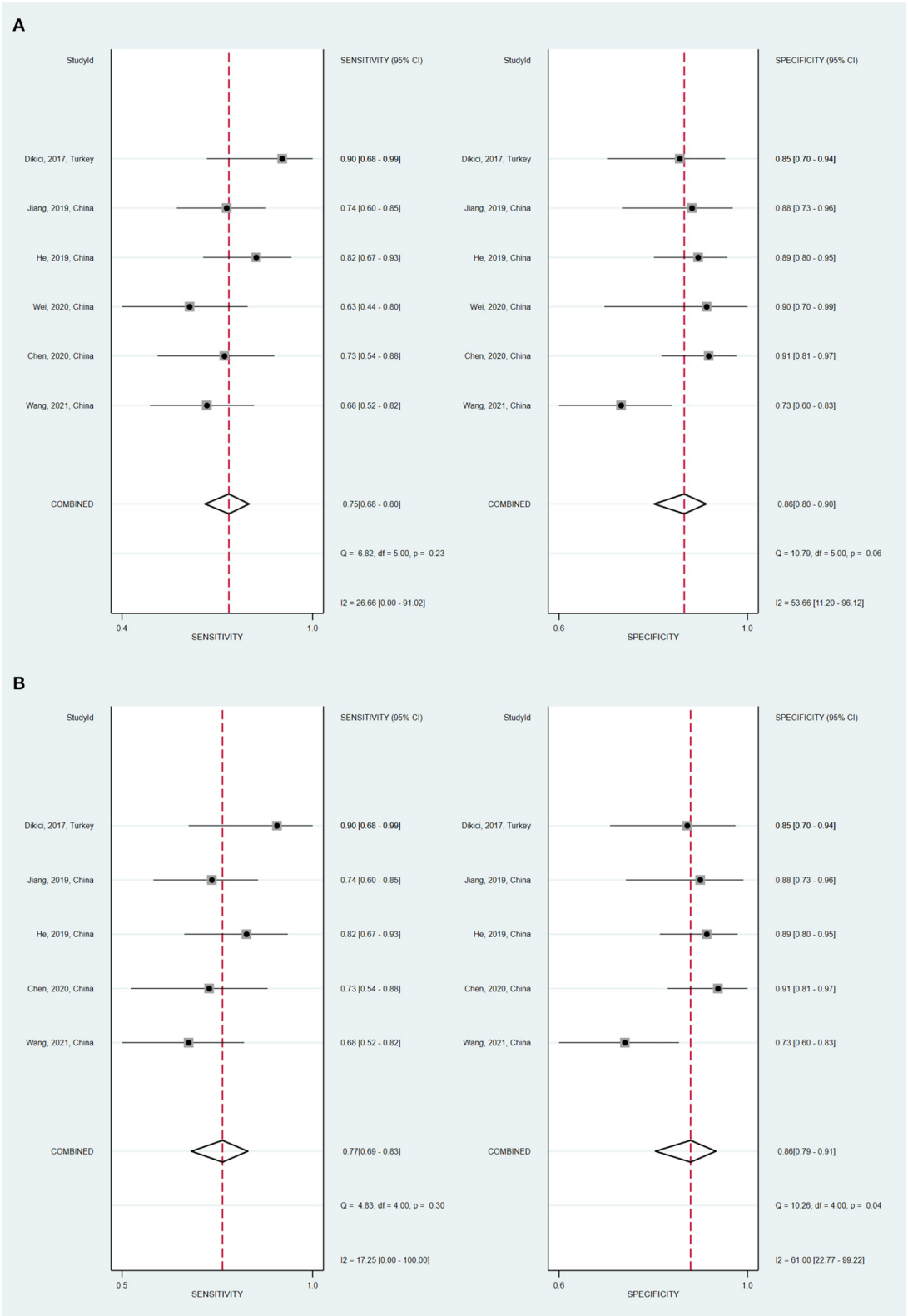


FIGURE 4
Coupled forest plots of the summary sensitivity and specificity of tibial nerve stiffness using shear wave elastography (SWE) **(A)** and two-dimensional SWE **(B)** for the diagnosis of diabetic peripheral neuropathy (DPN).

TABLE 4 Meta-analysis results of the stiffness of the tibial nerve using SWE for prediction of DPN.

| | No. of studies (Subjects) | Summary sensitivity (95% CI, %) | Summary specificity (95% CI, %) | Summary LR+ (95% CI) | Summary LR– (95% CI) | Summary AUROC (95% CI) | Summary DOR (95% CI) |
|---|---|---|---|---|---|---|---|
| **Target nerve** | | | | | | | |
| Tibial nerve | 6 (DPN, 170; CDDPN, 28; Non-DPN, 168; CG, 154) | 75 (68–80) | 86 (80–90) | 5.3 (3.5–7.9) | 0.30 (0.22–0.39) | 0.84 (0.81–0.87) | 18 (10–33) |

AUROC, area under the receiver operating characteristic curve; CDDPN, clinically defined diabetic peripheral neuropathy; CG, control group; CI, confidence interval; DOR, diagnostic odds ratio; DPN, diabetic peripheral neuropathy; LR+, positive likelihood ratio, LR–, negative likelihood ratio; SWE, shear wave elastography.

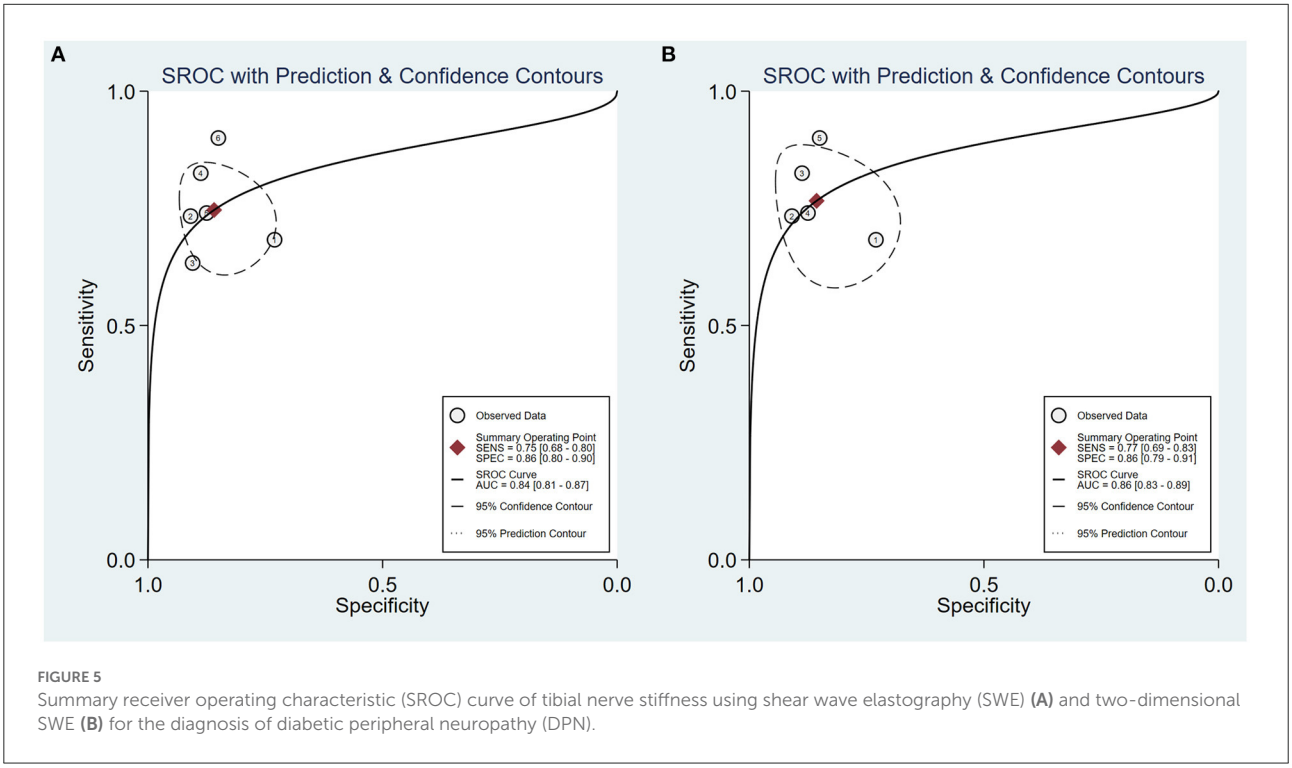


FIGURE 5
Summary receiver operating characteristic (SROC) curve of tibial nerve stiffness using shear wave elastography (SWE) **(A)** and two-dimensional SWE **(B)** for the diagnosis of diabetic peripheral neuropathy (DPN).

TABLE 5 Meta-analysis results of the stiffness of the tibial nerve using 2D-SWE for prediction of DPN.

| | No. of studies (Subjects) | Summary sensitivity (95% CI, %) | Summary specificity (95% CI, %) | Summary LR+ (95% CI) | Summary LR– (95% CI) | Summary AUROC (95% CI) | Summary DOR (95% CI) |
|---|---|---|---|---|---|---|---|
| **Target nerve** | | | | | | | |
| Tibial nerve | 5 (DPN, 156; CDDPN, 25; Non-DPN, 155; CG, 134) | 77 (69–83) | 86 (79–91) | 5.3 (3.5–8.3) | 0.27 (0.20–0.38) | 0.86 (0.83–0.89) | 20 (10–39) |

AUROC, area under the receiver operating characteristic curve; CDDPN, clinically defined diabetic peripheral neuropathy; CG, control group; CI, confidence interval; DOR, diagnostic odds ratio; DPN, diabetic peripheral neuropathy; LR+, positive likelihood ratio, LR–, negative likelihood ratio; SWE, shear wave elastography; 2D-SWE, two-dimensional shear wave elastography.

specificity were 77 and 86%, respectively, and the summary AUROC was 0.86. These results thus indicate that SWE can be used as a potential novel, useful, and quantitative tool for diagnosing DPN.

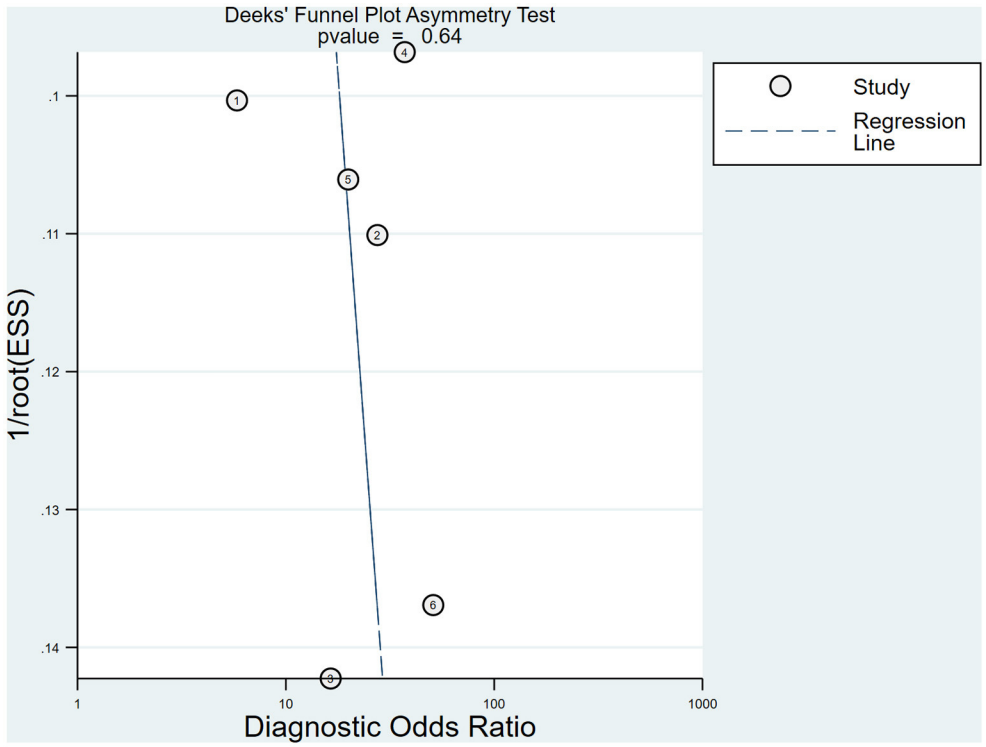


FIGURE 6
Deeks' funnel plot used to assess publication bias.

Tibial nerve is the most frequently involved site in diabetic polyneuropathy (20). Indeed, previous work has clearly shown that, when measured with SWE, the tibial nerve is stiffer in patients with diabetes. Several pathophysiological mechanisms may be used to explain changes in nerve stiffness as reflected by SWE measurements. The DPN developed because of the metabolic disorders associated with chronic hyperglycemia; edema within the nerve fascicle can increase intraneural pressure and then make the nerve stiffer (21, 27).

Of interest, even in diabetic patients without DPN, the stiffness of the tibial nerve was significantly higher than that of healthy control subjects (20). However, both neuroelectrophysiological examination and cross-sectional area (CSA) at ultrasound examination did not reflect such a change between diabetic patients without DPN and healthy control subjects (20). Notably, in the previous original study conducted by Jiang et al. (21), the results had shown that clinically defined DPN patients, i.e., patients with clinical signs or symptoms of DPN but normal NCS which often occurs in the early stages of DPN (28), had significantly greater tibial nerve stiffness than both diabetic patients without DPN and control subjects. These diabetic patients may have already suffered some nerve damage, although normal electrophysiological examination results exists (23). Which suggests that the SWE technique may be able to detect DPN before it becomes evident clinically or on NCS (29). This finding also shows that SWE, compared to electrophysiology test, exhibits a better correlation with clinical findings (21). Therefore, SWE technique has more potential value in early subelectrophysiological DPN detection. Early diagnosis of DPN is important, as early treatment at the earliest stages of DPN decreases both short-term and long-term morbidity (30, 31). Nevertheless, in view of the limited sample size, future studies using the SWE technique to assess the nerve stiffness in patients with clinically defined DPN are clearly needed.

In our meta-analysis, five of the six included studies used two-dimensional SWE technique for nerve stiffness assessment, and the other used point SWE technique. Both of these techniques rely on the acoustic radiation force impulse (ARFI) technique, which generates shear waves using focused, short-duration acoustic pulses (32). In contrast, two-dimensional SWE represents a relatively new ultrasound elastography technology for quantitative estimation of tissue stiffness (33). Two-dimensional SWE is a real-time and noninvasive imaging technique, which has distinct strengths for the evaluation of peripheral neuromuscular disorders. Surprisingly, one of the earliest studies using this technique showed that two-dimensional SWE displayed high sensitivity (90%) and

specificity (85%) in the diagnosis of DPN, outperforming the CSA measurements (20). Thus, two-dimensional SWE has the considerable potential to be a promising non-invasive tool for diagnosing DPN. Considering the limited number of included studies, however, we were unable to compare these two SWE techniques in this meta-analysis. Additional studies are therefore needed to further investigate the performance differences between two-dimensional SWE and point SWE.

Notably, across the studies included in our meta-analysis, the cut-off values used to diagnose DPN varied. Several factors, such as imaging plane (longitudinal or axial) and the size of the region of interest while performing the elastography, limb position, different anatomic regions, and sometimes a variable distribution of the severity of diabetes, may have contributed to these differences. A previous study has examined the effect of limb position on the stiffness of the tibial nerve measurement by SWE technique (34). There may also be other factors that may simultaneously affect the cut-off value. In actual clinical practice, determining the optimal cut-off value for nerve SWE measurements is very important in order to ensure its general clinical applicability. Additionally, if used in combination with other methods such as the Toronto clinical scoring system (TCSS), SWE technique could potentially further improve the diagnostic value for DPN (27).

This meta-analysis has some limitations that should be noted. First, of the six included studies, five were from China. Therefore, the generalization of the present meta-analysis findings is relatively limited. Second, DPN is a multiple peripheral nerve disease; we only summarized the diagnostic value of SWE in the detection of DPN of the tibial nerve. It is worth stating that, in two of the six included studies (12, 23) that also reported the diagnostic performance of median nerve and common peroneal nerve, respectively, the results showed that the tibial nerve on SWE had better performance for diagnosing DPN. Third, our meta-analysis maybe have several intrinsic heterogeneities, such as techniques, reference standards, and SWE measurements. Furthermore, the optimal thresholds were not determined in this meta-analysis. Therefore, further studies with a larger sample size are needed. Finally, we only focused on the full-articles published with English, which may bias the results.

In conclusion, our meta-analysis demonstrated that SWE shows good performance in diagnosing DPN and has considerable potential as an important and noninvasive adjunctive tool in the management of patients with DPN. Further studies focusing on the identification of optimal cut-off value for nerve SWE measurements are required in order to ensure its general clinical applicability.

## Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding authors.

## Author contributions

BD, YC, and GL contributed to the study design and literature search. XY, HW, and YC completed the data analysis. BD, HW, and YC generated and improved the figures and tables. BD completed the manuscript. BD and GL proofread the manuscript. All authors contributed to the article and approved the submitted version.

## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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# Differences in structural connectivity between diabetic and psychological erectile dysfunction revealed by network-based statistic: A diffusion tensor imaging study

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**Introduction:** Type 2 diabetes mellitus (T2DM) has been found to be associated with abnormalities of the central and peripheral vascular nervous system, which were considered to be involved in the development of cognitive impairments and erectile dysfunction (ED). In addition, altered brain function and structure were identified in patients with ED, especially psychological ED (pED). However, the similarities and the differences of the central neural mechanisms underlying pED and T2DM with ED (DM-ED) remained unclear.

**Methods:** Diffusion tensor imaging data were acquired from 30 T2DM, 32 ED, and 31 DM-ED patients and 47 healthy controls (HCs). Then, whole-brain structural networks were constructed, which were mapped by connectivity matrices (90 × 90) representing the white matter between 90 brain regions parcellated by the anatomical automatic labeling template. Finally, the method of network-based statistic (NBS) was applied to assess the group differences of the structural connectivity.

**Results:** Our NBS analysis demonstrated three subnetworks with reduced structural connectivity in DM, pED, and DM-ED patients when compared to HCs, which were predominantly located in the prefrontal and subcortical areas. Compared with DM patients, DM-ED patients had an impaired subnetwork with increased structural connectivity, which were primarily located in the parietal regions. Compared with pED patients, an altered subnetwork with increased

structural connectivity was identified in DM-ED patients, which were mainly located in the prefrontal and cingulate areas.

**Conclusion:** These findings highlighted that the reduced structural connections in the prefrontal and subcortical areas were similar mechanisms to those associated with pED and DM-ED. However, different connectivity patterns were found between pED and DM-ED, and the increased connectivity in the frontal–parietal network might be due to the compensation mechanisms that were devoted to improving erectile function.

KEYWORDS

type 2 diabetes mellitus, erectile dysfunction, diffusion tensor imaging, network-based statistic, psychological erectile dysfunction

## Introduction

Type 2 diabetes mellitus (T2DM) is a chronic metabolic disorder characterized by hyperglycemia, insulin secretion dysfunction, and insulin resistance, which can lead to inflammation, oxidative stress, and endothelial dysfunction (1–3). T2DM has been identified to be associated with a variety of nervous system-related diseases and macro- and microvascular-related complications (4–6). The population-based studies suggested that the incidence of mild cognitive impairment in diabetic patients was around 21.8% in China and varied from 28 to 31.5% worldwide (7–9). An epidemiological study suggested that the prevalence of diabetes was increasing rapidly, and the prevalence of erectile dysfunction (ED) among diabetic patients varied from 35 to 90% (10). Compared with the general population, patients with T2DM have a higher risk for cognitive decline, which is one of the central nervous system complications associated with abnormalities of brain function and structure (11–13). In addition, T2DM patients are at higher risk of developing male sexual dysfunction, including ED and retrograde ejaculation, which are two common peripheral microvascular and neurological complications associated with oxidative stress-induced penile vascular endothelial cell injury and peripheral neuropathy (14–17).

ED is defined, in the Diagnostic and Statistical Manual of Mental Disorders (DSM-V) (18), as the inability to achieve and/or maintain an adequate erection until the completion of a sexual activity on 75% of attempts at a partnered sexual activity for satisfactory sexual intercourse or a marked decrease in turgidity ≥6 months with unsatisfactory sexual intercourse. Normal penile erection and detumescence is a complex neurovascular event that is regulated by the balance between the contraction and relaxation of cavernous smooth muscles (19). The etiological factors of ED can be classified as organic (neurogenic, arterial/venous, hormonal, and drug-induced) and psychological (20). Diabetes mellitus is considered as an important cause of organic ED (21, 22), while psychogenic ED (pED) is predominantly and exclusively attributed to psychological or interpersonal factors, such as performance anxiety and relationship stress (23). Hyperglycemia was considered to be associated with the development of impaired vasodilatory signaling, smooth muscle cell hypercontractility, and veno-occlusive disorder, which were all the mechanisms causing ED in T2DM patients and often led to resistance to current therapy (24, 25). Endothelial dysfunction was an important mechanism for the development of T2DM-related ED, and chronic hyperglycemia might lead to inflammation and contribute to the formation of reactive oxygen species, which were related to the development of endothelial dysfunction in T2DM-related ED (24). In addition, pED has been found to be related to impaired activity/functional connectivity and abnormal gray matter/white matter of the brain in recent functional and structural magnetic resonance imaging (MRI) studies (26–29). However, the neural mechanisms underlying T2DM, T2DM with ED (DM-ED), and pED remain unclear.

Diffusion tensor imaging (DTI) is a noninvasive MRI method that can be used to detect microstructural alterations of the white matter, which cannot be revealed by conventional structural MRI scan (30, 31). The integrity of nerve fibers can be measured by the parameter of fractional anisotropy (FA), which indicates the strength and direction of water molecules' motion within the nerve fibers (32). Decreased FA values (values range from 0 to 1) indicate impaired microstructural tissue integrity of the white matter (33). A variety of white matter regions with microstructural alterations were found in T2DM patients by the technique of DTI (34). In addition, the structural brain networks [two elements: nodes defined by automated anatomical labeling

(AAL) template; edges defined by white matter] of pED were constructed by the method of graph theory analysis, and the topological measures were compared with healthy controls (HCs) in our previous DTI study (26). The results showed that white matter fiber tracts connected with the left inferior frontal gyrus(triangular), amygdale, right inferior temporal gyrus, and rolandic operculum exhibited decreased strength of structural connectivity in pED patients, which was measured by FA value-weighted edges in the structural brain network (26).

Network-based statistic (NBS) is a validated nonparametrical statistical approach for elucidating the organization of brain while controlling family-wise error. It is frequently applied to clinical applications, which can reveal altered connective strength in the brain network. To further identify different structural connections between pED and DM-ED, DTI data were acquired, and the approach of NBS was used in this study. We hypothesized that these patients would show a different structural connectivity located in key regions for sexual behavior regulation of the brain.

# Materials and methods

## Participants

In this cross-sectional study, a total of 93 patients, including 30 T2DM, 32 pED, and 31 DM-ED patients, were enrolled in this study. In addition, 47 age- and education-matched HCs were recruited by local advertisements. The protocol and informed consent document were approved by the Medical Ethics Committee of Jiangsu Province Hospital of Chinese Medicine, Affiliated Hospital of Nanjing University of Chinese Medicine. Written informed consents were obtained from all individuals before their participation in this study.

The inclusion criteria for all subjects were as follows: (1) right-handed, (2) educated for at least 9 years, and (3) aged between 20 and 60 years. The level of HbA1c was measured for the diagnosis of T2DM, and all participants were asked to fill in the five-item version of the international index of erectile function (IIEF-5) questionnaire to determine the presence of ED (35). T2DM patients met the diagnosis of T2DM according to the latest criteria published by the American Diabetes Association (ADA) (2014) (36): (1) fasting plasma glucose (FPG) level ≥7.0 mmol/L or (2) 2-h oral glucose tolerance test glucose level ≥11.1 mmol/L. Patients with DM met the diagnosis of T2DM based on ADA criteria with IIEF-5 scores >21. Patients with pED met the diagnosis of ED based on DSM-V criteria with IIEF-5 scores ≤21 and normal erection during sleeping (normal morning erection) or masturbation (the penis could maintain an erection until ejaculation during masturbation) was reported by themselves as well as normal penile hemodynamics rated by the color duplex doppler ultrasonography combined with intracavernous injection. DM-ED patients met the diagnosis of T2DM (within 2 years) with the presence of ED (IIEF-5 scores ≤21; abnormal erection during sleeping and masturbation without obvious psychological factors, such as depression, anxiety, *etc.*). HCs were defined as individuals with normal FPG (<7.0 mmol/L), HbA1c (<6.5) level, and IIEF-5 scores >21.

The exclusion criteria for all individuals were as follows: (1) other types of diabetes, (2) history of severe hyperglycemia coma and hypoglycemia, (3) major medical illnesses or complications, such as severe liver, kidney, or cardiovascular disease or tumors, (4) psychiatric or neurologic disorders, (5) alcohol or other substance abuse, (6) organic brain lesions, such as brain injury, cerebrovascular lesions, or tumors, and (7) any MRI contraindication.

## MRI data acquisition

The MRI data were obtained with a 3.0-T MRI scanner (Siemens, Germany). All participants were instructed to relax with their eyes closed, keeping their heads still an avoid deliberate thinking and falling asleep during scanning. High-resolution sagittal three-dimensional T1-weighted images and DTI images were acquired with the parameters that have been described in our previous studies (37–40).

## Data preprocessing and network construction

T1-weighted and DTI data were preprocessed using the diffusion toolbox of Functional MRI of the Brain software library (41). Then, whole-brain tractography was performed for the definition of edges in the brain network using the software of Diffusion Toolkit. In addition, the AAL template was used to define the nodes of the brain network (42). The detailed steps of preprocessing and construction of the whole-brain white matter network were performed (Figure 1) as reported in our previous studies (37–40).

## NBS analysis

NBS is a statistical method based on the graph theory and is often used to explore differences of the structural connectivity in the brain white matter network, which may be related to the diagnostic statue (43). NBS analysis is usually conducted to identify subnetworks comprising pairs of nodes and connections for which the strength of structural connectivity is significantly different between groups (44, 45). Firstly, two-sample *t*-tests were performed for all pairs ($90 \times 89/2 = 4{,}005$) of nodes to test the null hypothesis of equality between groups in mean structural connectivity with respect to the size of interconnected subnetwork/component of edges rather than individually at

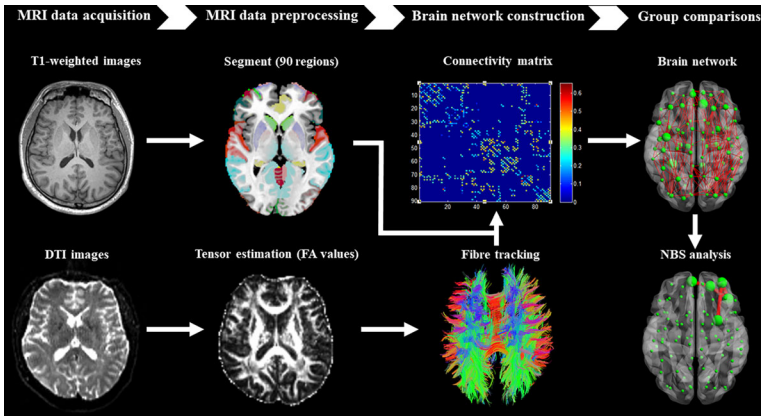


FIGURE 1
Brief flow chart showing MRI data acquisition, preprocessing, construction of structural brain network, and network-based statistical analysis between groups.

each connection. Among the structural connections exceeding 2.5 (test–statistic threshold), the search was performed to identify any connected subnetwork, including a collection of regions and a set of suprathreshold connections. The size of the identified subnetwork was determined by the number of suprathreshold connections it comprised. Secondly, permutation tests were conducted to calculate the corrected *P*-value for each network. The size of the largest subnetwork was recorded, and the null distribution was generated for calculating the family-wise error-corrected statistical threshold across the set of all connections. Finally, the corrected *P*-value for the identified subnetwork (size = *K*) in the un-permuted/actual data was computed as the proportion of permutations for which the size of the subnetwork was equal or greater than *K*. Therefore, NBS is a statistical approach that controls the family-wise error rate across all connections of the brain network, which offers more power than the method of false discovery rate.

## Statistical analysis

The group differences of demographic and clinical variables were compared by using the SPSS software package (IBM, USA). The data normality was evaluated by Kolmogorov–Smirnov test, and the variance homogeneity was measured by Levene's test. The one-way ANOVA was used to detect demographic and clinical differences among the three groups, while two sample *t*-test was performed to reveal differences of variables between two groups. The statistical significance threshold was set at $P < 0.05$.

One-way ANOVA and *post-hoc* analysis with two-sample *t*-test were applied to identify the group differences of structural connectivity in the white matter brain network by the method of NBS. The connected subnetworks were considered to be significantly different if the corrected $P < 0.05$ at the whole-network level with the preliminary statistic threshold 2.5 (50,000 permutations).

# Results

## Demographic and clinical characteristics

The demographic and clinical characteristics of the three groups are presented in Table 1. No significant differences were found in the age and educational level. Patients with pED and DM-ED had decreased IIEF-5 scores when compared to those with DM and HCs. In addition, there were no significant differences in the level of HbA1c between patients with DM and DM-ED.

TABLE 1 Demographic and clinical characteristics.

| Variables | DM ($n$ = 30) | pED ($n$ = 32) | DM-ED ($n$ = 31) | HCs ($n$ = 47) | $F/t$ | $P$ |
|---|---|---|---|---|---|---|
| Age (years) | 44.30 ± 8.03 | 42.69 ± 3.95 | 43.55 ± 9.82 | 43.19 ± 7.34 | 0.25 | 0.86 |
| Education level (years) | 14.80 ± 2.68 | 14.47 ± 2.51 | 14.48 ± 2.68 | 14.45 ± 1.60 | 0.17 | 0.92 |
| IIEF-5 scores | 23.53 ± 1.11 | 10.56 ± 5.07 | 15.23 ± 3.15 | 22.72 ± 0.68 | 153.63 | <0.00 |
| HbA1c (%) | 8.24 ± 2.61 | – | 9.52 ± 2.52 | – | -1.94 | 0.06 |

P <0.05 was considered to be statistically significant.
DM, diabetes mellitus; pED, psychological erectile dysfunction; DM-ED, diabetic erectile dysfunction; HCs, healthy controls; IIEF, international index of erectile function.

## Differences of structural connectivity revealed by NBS analysis

As shown in Table 2 and Figure 2, the subnetworks that showed significant differences between groups were identified. Compared to HCs, DM patients showed significantly decreased structural connectivity in a subnetwork comprising four brain regions (four right and zero left) and three connections (zero interhemispheric and three intrahemispheric). This subnetwork involved right middle frontal gyrus (orbital part), thalamus, putamen, and caudate nucleus. A subnetwork comprising five brain regions (four right and one left) and four reduced structural connections (one interhemispheric and three intrahemispheric) was identified in patients with pED when compared with HCs. In this subnetwork, the five well-connected brain regions were the left superior frontal gyrus (medial orbital) and right superior frontal gyrus (orbital part), inferior frontal gyrus (orbital part), middle frontal gyrus (orbital part), and putamen. The NBS analysis also revealed that a subnetwork was significantly different between DM-ED patients and HCs. The subnetwork consisted of four brain regions, including the right middle frontal gyrus (orbital part), thalamus, putamen, pallidum (four right and zero left) and four reduced structural connections (zero interhemispheric and four intrahemispheric).

In addition, the DM-ED patients demonstrated a subnetwork with five brain regions (five right and zero left) and four increased connections (zero interhemispheric and four intrahemispheric) when compared with DM patients. The regions of this subnetwork were located in the right superior parietal gyrus, inferior parietal gyrus, postcentral gyrus, angular gyrus, and superior occipital gyrus. Moreover, DM-ED patients had a different subnetwork comprising five brain regions (two right and three left) and four increased connections (one interhemispheric and three intrahemispheric) when compared with pED patients. The subnetwork consisted of the left middle

TABLE 2 Subnetworks identified to be significantly different among the DM, pED, DM-ED, and HC groups using network-based statistical analysis.

| Subnetwork | Edge | | *t* | *P* |
|---|---|---|---|---|
| | Node 1 | Node 2 | | |
| DM < HCs | Right middle frontal gyrus (orbital part) | Right thalamus | 2.76 | <0.05 |
| | Right middle frontal gyrus (orbital part) | Right putamen | 3.43 | <0.05 |
| | Right middle frontal gyrus (orbital part) | Right caudate nucleus | 3.30 | <0.05 |
| DM > HCs | | No significant edge was found | | |
| pED < HCs | Left superior frontal gyrus (medial orbital) | Right superior frontal gyrus (orbital part) | 2.53 | <0.05 |
| | Right superior frontal gyrus (orbital part) | Right inferior frontal gyrus (orbital part) | 3.21 | <0.05 |
| | Right inferior frontal gyrus (orbital part) | Right middle frontal gyrus (orbital part) | 4.22 | <0.05 |
| | Right middle frontal gyrus (orbital part) | Right putamen | 2.52 | <0.05 |
| pED > HCs | | No significant edge was found | | |
| DM-ED < HCs | Right middle frontal gyrus (orbital part) | Right thalamus | 2.81 | <0.05 |
| | Right middle frontal gyrus (orbital part) | Right putamen | 3.78 | <0.05 |
| | Right thalamus | Right putamen | 3.74 | <0.05 |
| | Right thalamus | Right pallidum | 3.12 | <0.05 |
| DM-ED > HCs | | No significant edge was found | | |
| DM < DM-ED | Right superior parietal gyrus | Right inferior parietal gyrus | 3.26 | <0.05 |
| | Right inferior parietal gyrus | Right postcentral gyrus | 3.16 | <0.05 |
| | Right superior parietal gyrus | Right angular gyrus | 3.18 | <0.05 |
| | Right superior parietal gyrus | Right superior occipital gyrus | 2.91 | <0.05 |
| DM > DM-ED | | No significant edge was found | | |
| pED < DM-ED | Left middle frontal gyrus | Left caudate nucleus | 4.21 | <0.05 |
| | Left middle frontal gyrus | Left anterior cingulate gyrus | 3.16 | <0.05 |
| | Left anterior cingulate gyrus | Right median cingulate gyrus | 2.70 | <0.05 |
| | Right median cingulate gyrus | Right postcentral gyrus | 2.90 | <0.05 |
| pED > DM-ED | | No significant edge was found | | |

To identify the significance of each subnetwork, nonparametric permutation statistic (test statistic threshold = 2.5; 5,000 permutations; $P < 0.05$) was performed with network-based statistical correction, and network size was measured with intensity.
DM, diabetes mellitus; pED, psychological erectile dysfunction; DM-ED, diabetic erectile dysfunction; HC, healthy controls.

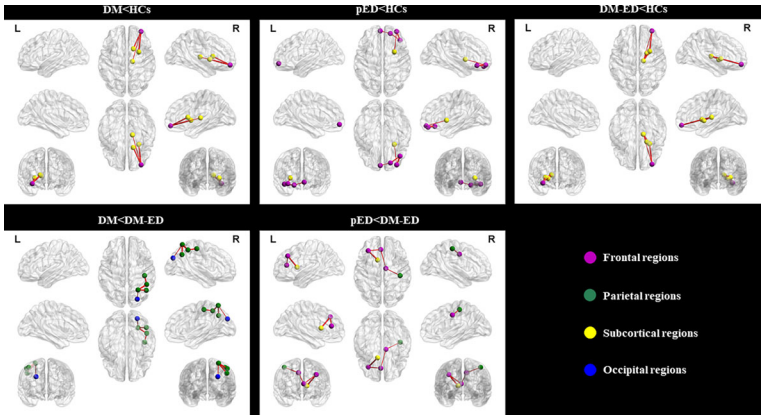


FIGURE 2
Subnetworks showing differences among the DM, pED, DM-ED, and HC groups using network-based statistical analysis. L, left; R, right; DM, diabetes mellitus; pED, psychogenic erectile dysfunction; DM-ED, diabetic erectile dysfunction; HCs, healthy controls.

frontal gyrus, anterior cingulate gyrus, caudate nucleus, right median cingulate gyrus, and postcentral gyrus.

## Discussion

To the best of our knowledge, this is the first study to explore the differences of structural connectivity between patients with pED and DM-ED by the method of NBS analysis. The findings demonstrated that decreased structural connectivity was found in patients with DM, pED, and DM-ED when compared with HCs. The abnormal brain regions were mainly distributed in the prefrontal and subcortical areas. In addition, DM-ED patients presented increased subnetworks consisting of parietal regions and prefrontal–cingulate areas when compared with DM patients and pED patients, respectively. These findings highlighted the importance of structural network analysis in understanding the different central neural mechanisms underlying diabetic and psychological ED.

In this study, we used DTI data to investigate the different topological properties of brain network between pED and DM-ED. Abnormal structural connectivity of white matter in the brain network were found in DM, pED, and DM-ED patients. The microstructural changes of white matter were speculated to be caused by the compromise of myelin sheath and the impairment or decrement of axons, which might lead to decreased neuronal signal transmission (34). The measure of FA, representing white matter integrity, is more sensitive than structural MRI metrics (33). DTI can detect and quantify subtle abnormalities of white matter before those are detectable by conventional structural MRI scans (30). Therefore, these findings might serve as imaging biomarkers for early diagnosis, monitoring disease progression, and response to therapy of brain disorders (46–48).

In recent years, DTI has been actively used in the investigation of brain structural connectivity alterations in sexual dysfunction patients including ED and premature ejaculation to understand the neuropathophysiology of these two disorders related to some psychological factors (26, 40). Our previous study showed that pED patients had damaged white matter in the left prefrontal and limbic cortex by the method of graph theoretical analysis (26). In addition, white matter microstructural changes were also found in pED patients by the method of tract-based spatial statistics based on DTI data (49). In this study, both pED and DM-ED patients showed lower structural connectivity in the prefrontal and subcortical areas when compared with HCs. Reduced structural connectivity was identified in the left superior frontal gyrus, right frontal regions, and putamen in pED patients, while DM-ED patients exhibited decreased structural connectivity in the right middle frontal gyrus, thalamus, putamen, and pallidum. This finding suggested that pED patients had more impairments in the frontal regions; however, DM-ED patients had more abnormalities in the subcortical areas. Previous studies demonstrated that the subcortical areas and, in particular, the thalamus seemed to be susceptible to T2DM. In addition, pED, owing predominantly to psychological factors including anxiety, depression, and introversion, was found to be more vulnerable to structural and functional changes in the prefrontal regions (26, 27, 38). Therefore, our findings were in agreement with the central neural mechanisms of pED and DM in previous neuroimaging studies (11, 38, 50).

The putamen was a key subcortical region receiving inputs from the prefrontal regions and projecting to other portions of the subcortical areas (51). The putamen, a critical component of the reward network, was considered to facilitate the integration of information from different brain areas and played an important role in reward-related behaviors (52). Sexual

behavior was a subjectively pleasurable experience and activity which, in the putamen, could be triggered by visual sexual stimuli, which acted like rewarding stimuli (53–55). In previous neuroimaging studies, activation in the putamen was found to be associated with male sexual arousal and penile turgidity (56, 57). The interactions between the prefrontal and putamen were known to be important for reward and sexual behavior (58, 59). Impaired gray matter of the putamen was found in pED patients (60). The structural connectivity between the prefrontal and putamen might be abnormal and associated with the underlying neural mechanisms of pED. With the exception of the putamen, more subcortical regions, including thalamus and pallidum, were found to have reduced structural connectivity in DM-ED patients. The thalamus was considered as an integration center for different brain regions, and it was found to be a critical structure for cognitive dysfunction in T2DM patients (61, 62). Decreased FA value was found in the thalamus in diabetes mellitus patients when compared with HCs, and the decreased FA was associated with worse neurocognitive performance of patients (63). Both the putamen and pallidum were two important components of the striatum, which played a key role in various brain functions, including cognitive function and reward, through the cortico-striato-thalamo-cortical pathway (64–66). T2DM was often accompanied with ED, which might be also associated with the structural abnormalities in the brain as manifested by decreased structural connectivity in the striato-thalamo-frontal circuit.

In this study, increased structural connectivity was found in the frontal–parietal network of DM-ED when compared with DM and pED. The frontal–parietal network played a vital role in cognitive function, including attention, executive function, and working memory, and it was often activated by executive function-related tasks (67). In previous studies, the inferior parietal lobule was activated in response to visual sexual stimuli, and the regional cerebral blood flow of this region was found to be positively correlated with the level of penile tumescence (56, 68). The initiation and level of penile tumescence in response to visual sexual stimuli was controlled by the frontal–network (69). In addition, increased activation was found in the frontal–parietal network in youth with type 1 diabetes when compared with HCs (70). Therefore, the increased structural connectivity in the frontal–parietal network might indicate compensatory changes for DM-ED patients. However, the complex mechanisms underlying the compensatory changes needed to be explored in further studies with a larger sample size.

In addition, several limitations should be taken into consideration in this study. Firstly, the relatively small sample size and cross-sectional study might limit the generalizability of these findings. Secondly, more demographic and clinical characteristics should be obtained, and their relationships with altered structural connectivity in the brain network should also be explored in our future studies. Finally, future studies entailing longitudinal studies with treatment were needed to evaluate the alterations in brain structural connectivity under treatment and might provide new insight into the treatment strategy of ED.

## Conclusion

In summary, this might be the first study to investigate the differences of structural connectivity between diabetic and psychological ED by the method of NBS analysis based on DTI data. Our results showed that both DM-ED and pED had decreased structural connectivity in the frontal-subcortical regions. In addition, DM-ED patients presented increased structural connectivity in the frontal–parietal network, which might be a compensatory mechanism. These findings provided the first evidence of the common and different central neural mechanisms between diabetic and psychological factors related ED.

## Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## Ethics statement

The studies involving human participants were reviewed and approved by the medical ethics committee of Jiangsu Province Hospital of Chinese Medicine, Affiliated Hospital of Nanjing University of Chinese Medicine. The patients/participants provided their written informed consent to participate in this study.

## Author contributions

JC, YC, JY, and JW designed the experiments. JC, JY, JW, XH, RS, ZX, YX, SC, and WX contributed to clinical data collection and assessment. JC, XH, ZX, YX, JW, and JY analyzed the results. JC, JY, JW, and XH wrote the manuscript. JC, YC, JY, and JW approved the final manuscript. All authors contributed to the article and approved the submitted version.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Publisher's note



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# Comparison of material properties of heel pad between adults with and without type 2 diabetes history: An *in-vivo* investigation during gait

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**Objective:** This study was aimed to compare the material properties of heel pad between diabetes patients and healthy adults, and investigate the impact of compressive loading history and length of diabetes course on the material properties of heel pad.

**Methods:** The dual fluoroscopic imaging system (DFIS) and dynamic foot-ground contact pressure-test plate were used for measuring the material properties, including primary thickness, peak strain, peak stress, stiffness, viscous modulus and energy dissipation ratio (EDR), both at time zero and following continuous loading. Material properties between healthy adults and DM patients were compared both at time zero and following continuous weight bearing. After then, comparison between time-zero material properties and properties following continuous loading was performed to identify the loading history-dependent biomechanical behaviour of heel pad. Subgroup-based sensitivity analysis was then conducted to investigate the diabetes course (<10 years vs. ≥10 years) on the material properties of heel pad.

**Results:** Ten type II DM subjects (20 legs), aged from 59 to 73 (average: 67.8 ± 4.9), and 10 age-matched healthy adults (20 legs), aged from 59 to 72 (average: 64.4 ± 3.4), were enrolled. Diabetes history was demonstrated to be associated with significantly lower primary thickness (t=3.18, p=0.003**), higher peak strain (t=2.41, p=0.021*), lower stiffness (w=283, p=0.024*) and lower viscous modulus (w=331, p<0.001***) at time zero, and significantly lower primary thickness (t=3.30, p=0.002**), higher peak strain (w=120, p=0.031*) and lower viscous modulus (t=3.42, p=0.002**) following continuous loading. The continuous loading was found to be associated with significantly lower primary thickness (paired-w=204, p<0.001***) and viscous modulus (paired-t=5.45, p<0.001***) in healthy adults, and significantly lower primary thickness

(paired-w=206, p<0.001***) and viscous modulus (paired-t=7.47, p<0.001***) in diabetes group. No any significant difference was found when conducting the subgroup analysis based on length of diabetes course (<10 years vs. ≥10 years), but the regression analysis showed that the length of diabetes history was positively associated with the peak strain, at time zero (r=0.506, p<0.050) and following continuous loading (r=0.584, p<0.010).

**Conclusions:** Diabetes patients were found to be associated with decreased primary thickness and viscous modulus, and increased peak strain, which may contribute to the vulnerability of heel pad to injury and ulceration. Pre-compression history-dependent behaviour is observable in soft tissue of heel pad, with lowered primary thickness and viscous modulus.

KEYWORDS

material properties, diabetes, heel pad, dual fluoroscopic system, gait, loading history

## Introduction

Diabetes mellitus (DM) is a 21st century epidemic, affecting about 9.3% of the world's population at 2019 (1). This figure is estimated to be as high as 10.2% and 10.9% by 2030 and 2045 respectively, according to the 9th International Diabetes Federation (IDF) Conference (1). Diabetic foot ulcer (DFU), as one of the most common, serious and destructive complications, would be experienced by about 25% of the DM patients over their course of disease (2, 3). Despite an advanced team cooperation, many ulcerous foots must be amputated within 4 years due to unsatisfactory treatment effect, comprising up to one-third of the direct expense of diabetic care (4–6). It is estimated that 10% and 19% of the DFU patients would encounter major amputation and minor amputation respectively, within 1 year of diagnosis (7). Although the heel ulcerations are less common than forefoot ulcerations, they are generally more challenging to treat, difficult to heal and associated with larger morbidity and higher costs (8, 9). It is reported that the limb salvage success rate of heel ulcers is 2~3 times less than that of the forefoot ulcers (8). As the first point of contact between the foot and ground during human locomotion, the heel acts as an efficient shock absorber to dampen the impact stress transferred to the skeletal system (10–12). The heel fat pad is histologically composed of honeycombed fat globules formed by clustered fat cells in whorls of fibroelastic septa (13). The intact configurations of the adipocyte cluster and fibrous envelop are necessary for heel pad to resist the external compressive loads during the stance phase of gait cycle (11, 13, 14). However, the histomorphology of the heel pad would be significantly altered, including breaking of collagen bundles and fragmentation of elastin strands in septa, and relative shrinking of adipocytes, in pathological condition of diabetes (10, 11, 15–17). These alterations, subsequently, would lead to further modifications on the material properties, causing increased stiffness of septa, decreased damping ability, and increased vulnerability of tissue to injury (10, 13, 15–18).

To date, researchers have developed plenty of footwear and custom insole designs to prevent the ulceration of the plantar soft tissue in diabetes. These designs, predominately, depended on the premise that the elevated barefoot pressure (or peak stress) is the main cause of diabetic plantar ulceration (19–21). Subsequently, the major goal of the footwear and insole is to reduce or redistribute the pressure on the plantar surface at locations with risk of ulceration. Additionally, the pressure relieving effect has been widely adopted as the primary index to evaluate the treatment efficacy (19–21). However, diabetes patients sometimes develop ulcers at the locations with normal pressure, or the elevated peak pressure is available in some healthy adults (22, 23). Veves et al. (24) reported that only 38% of the DFU locations matched with the peak pressure areas in plantar surface. Healy et al. (25) indicated that over 42% of the diabetic amputations were caused by improper footwear. To accurately identify the comprehensive mechanical parameters more than peak pressure, therefore, is essential for guiding the designing of footwear and insole to compensate the atrophy and degeneration of diabetic plantar tissue. Several methods have been established to investigate the viscoelastic material properties of heel pad through either *in vitro* cadaver or *in vivo* volunteer experiments (14, 26–37). The uniaxial compression and stress-relaxation experiments with *in-vitro* tissue cut from cadaveric plantar pad was firstly performed to test the material properties (14, 27, 28). These tests, nevertheless, have been proven to overestimate the stiffness (six times higher)

and underestimate the energy dissipation ratio (EDR, three times lower) compared to *in-vivo* testing, because of the inclusion of entire lower leg for *in vivo* tests (29–31). More recently, some novel *in vivo* methods, such as indentation test (26, 32), drop impact test (29), ballistic pendulum (33) and ultrasound elastography approach (34, 35), have been widely applied to quantify the static or quasi-static material properties of plantar soft tissue. These methods, nevertheless, are not able to replicate the actual loading conditions experienced by the heel when contacting with the ground during dynamic gait cycle, as the heel pad is loaded at a fixed position without movement. To overcome this limitation, De Clercq et al. (36) and Gefen et al. (37) developed an innovative method by incorporating of the fluoroscopy (cine-radiography) and simultaneous pressure test plate beneath the foot to measure the *in vivo* material properties during dynamic gait (36, 37). Up until then, it was possible to dynamically investigate the material properties of heel pad. However, this method was based on two-dimensional fluoroscopy, which would lead to bias on measured strain due to varying shooting angle. Instead, the dual fluoroscopic imaging system (DFIS) could capture two perpendicularly intersected images for reconstructing a three-dimensional structure, and has been recently applied in many situations to help investigate the structural and locational indexes. In a previous preliminary study, we have established a novel system for testing the material properties of heel pad, and performed pilot investigation within several healthy adults (38).

In this study, we set out to: (i) identify the material properties of heel pad in DM patients; (ii) compare the material properties of heel pad between DM patients and healthy adults; (iii) perform subgroup analysis according to diabetes history with the aim of comparing the material properties between DM patients with <10 and ≥10 years of DM history; (iv) investigate the effect of continuous compression load on the material properties of heel pad.

# Materials and methods

## Inclusion of subjects

In accordance with the Declaration of Helsinki, and upon attaining the ethical approval from the Institutional Review Board of Huashan Hospital, Fudan University, 10 type II DM subjects and 10 age-matched healthy adult subjects were enrolled. The DM subjects fulfill the Guidelines for the Prevention and Control of Type 2 Diabetes in China (2017 edition) (39). All subjects received their informed consent at the time before examination. All subjects had palpable pulse at their dorsalis pedis and posterior tibial arteries. Participants with pathological conditions other than diabetes that could affect the properties of heel pad, including heel pain, rheumatoid arthritis, foot deformity, dysvascula of foot, history of foot surgery, and trauma of foot, were excluded. Each subject received CT scan on foot before the experiment, for building models of calcaneus that were used in 2D-3D registration with Mimics Medical 21.0 (Materialize, Belgium).

## Instrumentation and experiment design

A diagram of the equipment is presented in Figure 1A. Two C-arm fluoroscopes (BV Pulsera, Phillips Medical, USA) placed orthogonally were utilized for capturing the *in vivo* compressive strain of the heel pad, with pre-set frame rate of 50 Hz, resolution of 1024×1024 pixels and beam energy setting of 75kV•40mA. At beginning of the experiment, a single cubic and a pair of retiform custom-made calibrators were used for obtaining calibration images, which were then used for calibrating the distortions of fluoroscopies before model-image registration. At the same time, a dynamic foot-ground contact pressure-test plate (zebris PDM-XS, 570*400*15mm, Germany) was embedded in the custom gait platform (with length of 3.5m and width of 0.8m) to continuously record the evolution of compressive pressure.

A cube calibrator and a pair of retiform calibrators matching the biplane were placed at the intersection of the two fluorescent projections, and placed on the two receivers of the fluoroscopes, respectively. Following gathering of the calibration images with the aid of software XMAlab (https://bitbucket.org/xromm/xmalab/), subjects were trained to walk barefooted on the gait platform with the aid and protection of researchers, until they could locomote deftly and stably without any aid. To eliminate the impact of strain rate on the material properties of heel fat pad, the subjects were required to move with an approximate gait velocity of 1.0 m/s by visual inspection. A "two-step" gait cycle was performed for all subjects, with the second step striking at a marked position on the force plate with the heel to maintain the location of the tested foot to be in the view of fluoroscopes. Both the left and right heels were measured using the identical procedures. After all subjects were familiar with the experiment procedure, their foots were kept in relaxation and free of load for one hour. Then, the subjects were taken back to test the time-zero (i.e., the status without continuous loading on the heel pad) material properties. Next, with the aim of investigating the impact of loading history on material properties, the subjects were required to keep their foots on continuous loading by sustaining standing or roaming in the laboratory room for 15 minutes, after which the material properties of the heel pad were measured.

## Data processing

The data obtained by DFIS were handled with software Rhinoceros 5.0 (Robert McNeel & Associates, Washington,

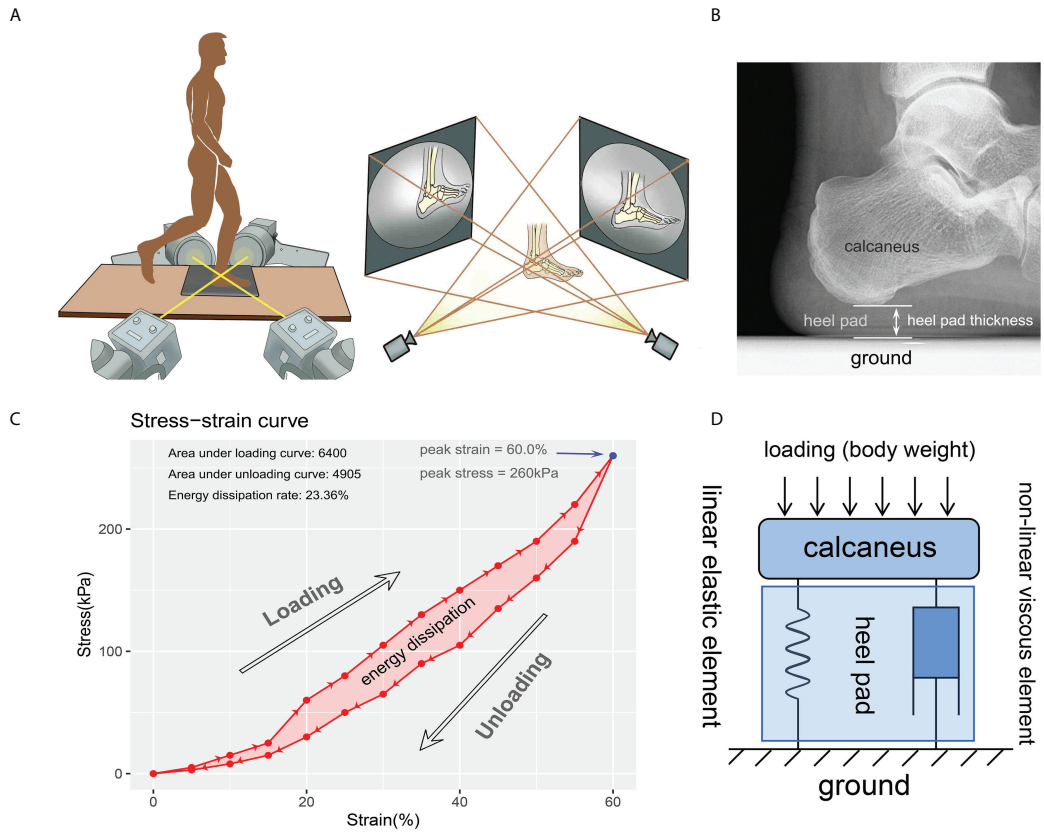


FIGURE 1
Diagrams for the experiment instrument and data process. **(A)** instrumentation including two orthogonally placed fluoroscopes and dynamic foot-ground contact force plate embedded in the custom gait platform. Using this instrumentation, two 2-D images at each time were obtained for reconstructing the 3-D model and investigate the heel pad thickness. **(B)** thickness of the heel pad was defined as the perpendicular distance between the ground and the base of the calcaneus. **(C)** a representative stress-strain curve depicting a cycle of loading and unloading. The most-right blue point indicates the peak stress (260kPa) and peak strain (60.0%). The energy dissipation was defined as the area between the loading and unloading curves (the light red area), and the energy dissipation rate (23.36%) was defined as the ratio between energy dissipation and area under loading curve. **(D)** modified Kelvin-Voigtviscoelastic model composed of parallelly connected linear elastic element and non-linear viscous element representing the viscoelastic characteristic of heel pad.

USA), to get the time-dependent strain rate of heel pad. The time-matched data recorded by contact force plate were exported as xml file to calculate the heel-ground contact stress. As shown in Figure 1B, the thickness of the heel pad was measured on each continuous frame, to figure out the perpendicular distance between the ground and the base of the calcaneus. The "primary thickness" was defined as the thickness measured on the frame where skin of heel initially contacts with force plate. Strain ($\varepsilon_c$) was change in thickness divided by primary thickness. Stress ($\sigma_c$) was defned as heel-ground contact force of heel area divided by acreage in each frame. Stress-strain curve, depicting a cycle of loading and unloading process of heel pad, was then generated using strain values and stress values matched according to time points. A case of stress-strain curve is shown in Figure 1C. The "peak stress" and "peak strain" were defined as the stress and strain at the most right point in stress-strain curve. The stress-strain data of each foot were fitted to the Kelvin-Voigt model ($\sigma_c = -E_c - \eta\varepsilon_c\dot{\varepsilon}_c$), and the Young's modulus (E) and viscous modulus ($\eta$) were obtained according to the least squares method (Figure 1D). The energy dissipation was defined as the area in the hysteresis loop bounded by the loading and unloading curves, and the EDR was expressed as the ratio of energy dissipation and the area under the loading curve.

## Statistical analyses

Continuous variables were presented as mean ± SD (standard deviation) in case of meeting the normality distribution, or presented as median and range in case of not

meeting normality distribution. Age and BMI between healthy and diabetes groups were compared with student-t test as fulfilling the normality and homoscedasticity.

When comparing the material properties (time zero, following continuous loading, and difference between two statuses) between healthy adults and DM patients, student-t test (meet the normal distribution and homoscedasticity assumptions), Welch-t test (meet the normal distribution and but not homoscedasticity) or Wilcoxon rank sum test (not meet the normal distribution assumption) were selected for statistical analyses. When comparing the material properties (healthy adults and DM patients) between two loading statuses, paired-t test (difference between two statuses must meet normal distribution) or paired-Wilcoxon test (difference between two statuses not meet normal distribution) were selected for statistical analyses.

Subgroup analysis was performed according to the diabetes history, including the groups with diabetes course of <10 years and ≥10 years. One-way analysis of variance (ANOVA, meet the normal distribution and homoscedasticity assumptions), Brown-Forsythe test (meet the normal distribution and but not homoscedasticity), or Kruskal-Wallis test (H test, not meet the normal distribution assumption) were performed to compare the difference among healthy group and two diabetes groups. After that, *post hoc* analyses, including SNK-q (for ANOVA), Tamhane's T2 (for Brown-Forsythe test) and Dunn's (for H test) tests were selected to conduct pair-wise comparison for the three groups. For the two subgroups of diabetes subjects and the subgroups of left and right legs, the material properties at time-zero and following continuous loading were compared with paired-t test (difference between two statuses meet normal distribution) or paired-Wilcoxon test (difference between two statuses not meet normal distribution) were selected for statistical analyses.

In healthy adults, correlation matrices were generated for properties measured at time zero and following continuous loading, and for the differences of properties measured at two statuses. In diabetes subjects, correlation matrices were generated for properties measured at time zero and following continuous loading, and for the differences of properties measured at two statuses, with subgroup analysis for the length of diabetes course (either <10 or ≥10 years). Pearson's correlation analysis was used to detect the correlation between continuous variables in matrices, and correlation efficient (R) was calculated to depict the magnitude of the correlativity.

The above statistical analyses were conducted with R version 4.0.5 (Foundation for Statistical Computing, Vienna, Austria). Significance level was defined as p value of less than 0.050. The normality test and homogeneity test of variances were conducted with Shapiro-Wilk test (W test, $p<0.05$ indicates significant non-normality) and modified Bartlett's test ($p<0.05$ indicates significant non-homoscedasticity).

# Results

## Baseline characteristics

The baseline characteristics of the enrolled patients are available in Table 1. A total of 10 type II DM subjects (9 male and 1 female, 20 legs), aged from 59 to 73 (average: 67.8 ± 4.9), and 10 age-matched healthy adult subjects (9 male and 1 female, 20 legs), aged from 59 to 72 (average: 64.4 ± 3.4), were enrolled. The median value of the length of diabetes course was 9.5 (range: 2~25) years in diabetes group, with five subjects have a diabetes history of <10 years (mean: 4.8 ± 2.8 years) and five ≥10 years (mean: 16.2 ± 5.9 years). No significant difference was presented for age ($t=1.80$, $p=0.088$) and BMI ($t=0.43$, $p=0.676$) between healthy group and diabetes group. Between the subgroups with diabetes courses of <10 and ≥10 years, similar age ($t=0.122$, $p=0.906$) and BMI ($t=0.11$, $p=0.917$) were demonstrated.

## Impact of diabetes and loading history on material properties of heel pad

Using the DFIS incorporated with force plate, time-dependent strain and stress applied to heel pad were concurrently obtained. The summaries of the material properties of heel pad, including the mean/median values of properties at time zero and following continuous loading and the differences between two loading statuses, are presented in Table 2. Figure 2 shows the rain-cloud plot depicting the material properties of heel pad in healthy and diabetes subjects. Diabetes history was demonstrated to be associated with significantly lower primary thickness (Figure 2A, $t=3.18$, $p=0.003^{**}$), higher peak strain (Figure 2B, $t=2.41$, $p=0.021^{*}$), lower stiffness (Figure 2D, $w=283$, $p=0.024^{*}$) and lower viscous modulus (Figure 2E, $w=331$, $p<0.001^{***}$) at time zero, and significantly lower primary thickness (Figure 2A, $t=3.30$, $p=0.002^{**}$), higher peak strain (Figure 2B, $w=120$, $p=0.031^{*}$) and lower viscous modulus (Figure 2E, $t=3.42$, $p=0.002^{**}$) following continuous loading. The continuous loading history was found to be associated with significantly lower primary thickness (Figure 2A, paired-$w=204$, $p<0.001^{***}$) and viscous modulus (Figure 2E, paired-$t=5.45$, $p<0.001^{***}$) in healthy adults, and significantly lower primary thickness (Figure 2A, paired-$w=206$, $p<0.001^{***}$) and viscous modulus (Figure 2E, paired-$t=7.47$, $p<0.001^{***}$) in diabetes group. There was no any significant difference when comparing the difference of material properties between two groups of subjects.

TABLE 1 Baseline characteristics of the enrolled patients.

| Variables | Normal group (n=10) | Diabetic foot group | | | p value (normal vs. whole diabetic foot) | p value (diabetes history <10y vs. diabetes history ≥10y) |
|---|---|---|---|---|---|---|
| | | Whole group (n=10) | Diabetes history (<10 y, n=5) | Diabetes history (≥10 y, n=5) | | |
| **Age** | 64.4±3.4 | 67.8±4.9 | 67.6±3.7 | 72 (range: 59~73)[#] | T=1.80, p=0.088 | T=0.122, p=0.906 |
| **BMI** | 24.1±2.2 | 24.7±3.9 | 25.4±3.5 | 25.1±4.3 | T=0.43, p=0.676 | T=0.11, p=0.917 |
| **Length of diabetes history** | NA | 9.5 (range: 2~25)[#] | 4.8±2.8 | 16.2±5.9 | NA | NA |
| **Sex** | | | | | | |
| Male-n(%) | 9 | 9 | 4 | 5 | NA | NA |
| Female-n(%) | 1 | 1 | 1 | 0 | | |

[#]data are presented with the median values as well as the minimum-to-maximum ranges, as they don't follow a normal distribution. NA, not applicable; BMI, body mass index.

## Subgroup analyses basing on course of diabetes and side of leg

Subgroup analyses were performed basing on length of diabetes course (<10 and ≥10 years, marked as diabetes A and diabetes B groups respectively), and the rain-cloud plot is available in Figure 3. Generally, significantly different primary thickness (Figure 3A, ANOVA: F=5.81, p=0.006**; ANOVA: F=5.68, p=0.007**), and viscous modulus (Figure 3E, Kruskal-Wallis: H=14.05, p<0.001***; ANOVA: F=6.17, p=0.005**) were demonstrated both at time zero and following continuous loading. *Post hoc* analyses demonstrated significantly higher primary thickness (Figure 3A, time zero: SNK, p=0.014*/p=0.027*; following continuous loading: SNK, p=0.029*/p=0.038*) and viscous modulus (Figure 3E, time zero: Dunn's, p=0.001**/p=0.048*; following continuous loading: SNK,

TABLE 2 Summaries of the material properties of plantar soft tissue at heels of normal adults and adults with diabetic foot.

| Properties | Normal group (n=20) | Diabetic foot group | | |
|---|---|---|---|---|
| | | Whole group (n=20) | Diabetes history (<10 y, n=10) | Diabetes history (≥10 y, n=10) |
| **Time zero** | | | | |
| -Primary thickness (mm) | 14.85±2.81 | 12.25±2.34 | 12.69±2.16 | 12.54±2.62 |
| -Peak strain (%) | 52.30±10.09 | 60.65±11.73 | 58.80±10.44 | 62.50±13.18 |
| -Peak stress (kPa) | 144.80±25.56 | 138.95±31.93 | 135.70±35.40 | 142.20±29.59 |
| -Young's modulus (kPa) | 265.50 (range: 154.79~306.28)[#] | 214.39±48.04 | 206.50±43.08 | 221.30±53.69 |
| -Viscous modulus (kPa·s) | 66.59 (range: 36.70~137.97)[#] | 42.28±18.93 | 36.20±14.37 | 47.60±21.74 |
| -EDR (%) | 19.45±13.49 | 19.05±10.29 | 18.00±8.35 | 20.10±12.30 |
| **Following continuous loading** | | | | |
| -Primary thickness (mm) | 14.55±2.74 | 11.95±2.21 | 11.90±2.13 | 12.00±2.40 |
| -Peak strain (%) | 51.55 (range: 41.09~71.85)[#] | 58.61 (range: 40.64~92.92)[#] | 57.23±8.72 | 58.96 (range: 48.12~92.92)[#] |
| -Peak stress (kPa) | 153.74 (range: 90.80~178.55)[#] | 141.82±30.95 | 138.10±32.63 | 144.60±30.71 |
| -Young's modulus (kPa) | 236.40±47.21 | 209.95±49.04 | 204.60±34.58 | 215.30±61.79 |
| -Viscous modulus (kPa·s) | 46.30±19.99 | 26.45±16.55 | 23.10±11.12 | 29.80±20.72 |
| -EDR (%) | 16.00±10.23 | 14.44 (range: 1.20~47.52)[#] | 14.20±8.28 | 21.30±14.41 |
| **Difference between time-zero and continuously loaded heel pads** | | | | |
| -Primary thickness (mm) | 0.19 (range: -0.06~2.80)[#] | 0.13 (range: -0.02~2.33)[#] | 0.17 (range: 0.04~2.33)[#] | 0.09±0.11 |
| -Peak strain (%) | 0.39±3.57 | 1.57 (range: -19.53~10.65)[#] | 2.36±5.79 | 1.11 (range: -19.53~6.99)[#] |
| -Peak stress (kPa) | 0.80±25.41 | -2.35±30.72 | -2.22±38.40 | -2.48±23.86 |
| -Young's modulus (kPa) | 10.75±25.97 | 3.95±31.48 | 1.86±33.37 | 6.25±31.84 |
| -Viscous modulus (kPa·s) | 19.85±16.72 | 15.00±9.23 | 12.92±9.89 | 17.72±8.71 |
| -EDR (%) | 3.59±17.83 | 5.10 (range: -19.56~12.48)[#] | 3.98±8.74 | -1.10±10.88 |

[#]data are presented with the median values as well as the minimum-to-maximum ranges, as they don't follow a normal distribution.

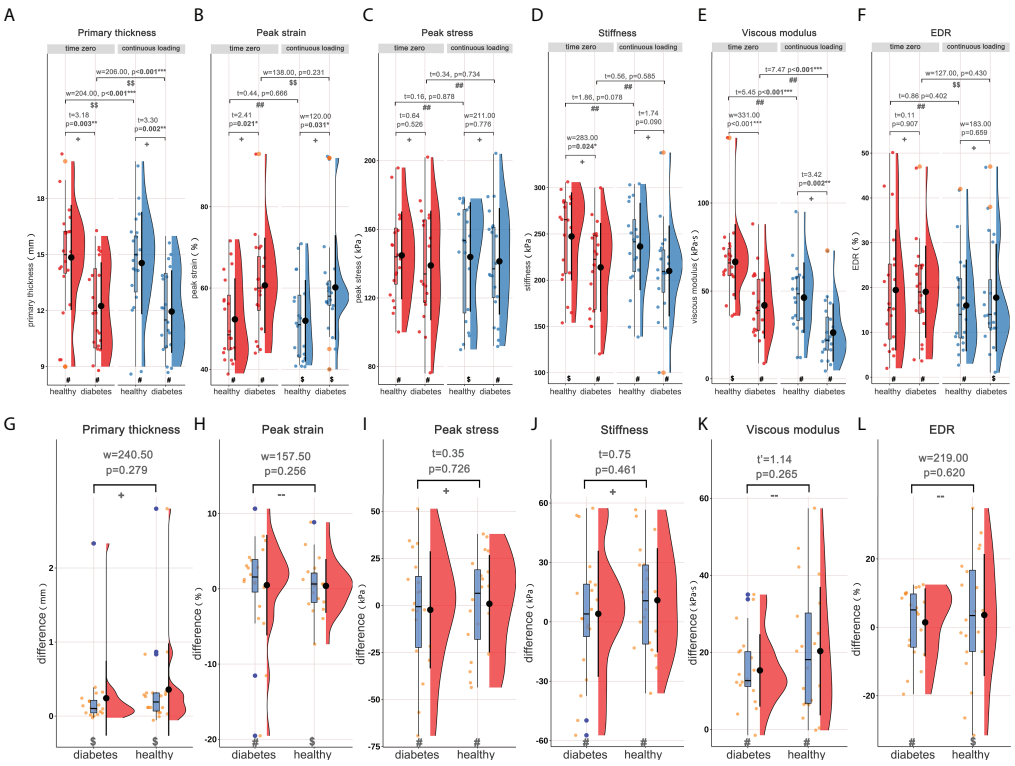


FIGURE 2
Rain-cloud plot depicting the material properties of heel pad, including primary thickness, peak strain, peak stress, stiffness, viscous modulus and EDR. **(A–F)** the material properties of heel pad in healthy and diabetes groups at time zero and following continuous loading. **(G–L)** the differences of material properties of heel pad between two loading statuses in healthy and diabetes groups. $^{\#}$, data follow the normality distribution; $^{\$}$, data didn't follow the normality distribution; $^{\#\#}$, the difference between two loading statuses follow the normal distribution; $^{\$\$}$, the difference between two loading statuses didn't follow the normal distribution; +, meet the homoscedasticity assumption; -, didn't meet the homoscedasticity assumption. "t", "t'", and "w" represent the statistical effect sizes for T test, Welch's T test, and Wilcoxon test, respectively. Error bar represents the mean value and standard deviation; box plot represent the median value and quartiles. EDR= energy dissipation rate. *, p<0.050; **p<0.010; ***p<0.001.

p=0.010*/p=0.034*) both at time zero and following continuous loading, for healthy subjects when compared with diabetes A and diabetes B groups. When comparing the material properties between two loading statuses, the continuous loading was found to be associated with significantly lower primary thickness (Figure 3A, healthy adults: paired-w=55, p=0.002**; diabetes subjects: paired-w=51, p=0.014*) and viscous modulus (Figure 3E, healthy adults: paired-t=4.32, p=0.002**; diabetes subjects: paired-t=6.45, p<0.001***) both for healthy adults and diabetes subjects. No any significant difference was found when comparing the difference of material properties among three subgroups of subjects.

The rain-cloud plot depicting the subgroup analysis comparing the material properties of left and right sides is available in Supplementary Figure 1. The material properties at time zero (A-F) and following continuous loading (G-L) were compared, and no any significant difference was detected between two groups.

## Correlation analysis for material properties of heel pad

The correlation matrices for BMI, age, and the properties of heel in healthy adults at time zero and following continuous loading are available in Figures 4, 5, respectively. As a result, at time zero, primary thickness was shown to be significantly correlated with BMI (R=0.509, p<0.050*) and age (R=-0.505, p<0.050*); peak strain was significantly correlated with age (R=0.467, p<0.050*) and primary thickness (R=-0.827, p<0.001***); stiffness was significantly correlated with BMI (R=0.524, p<0.050*), primary thickness (R=0.589, p<0.010**), peak strain (R=0.-0.633, p<0.010**) and peak stress (R=0.534, p<0.050*); viscous modulus was significantly correlated with primary thickness (R=0.468, p<0.050*), peak strain (R=-0.468, p<0.050*) and stiffness (R=0.600, p<0.010**). Following continuous loading, primary thickness was shown to be significantly correlated with BMI (R=0.462, p<0.050*) and age

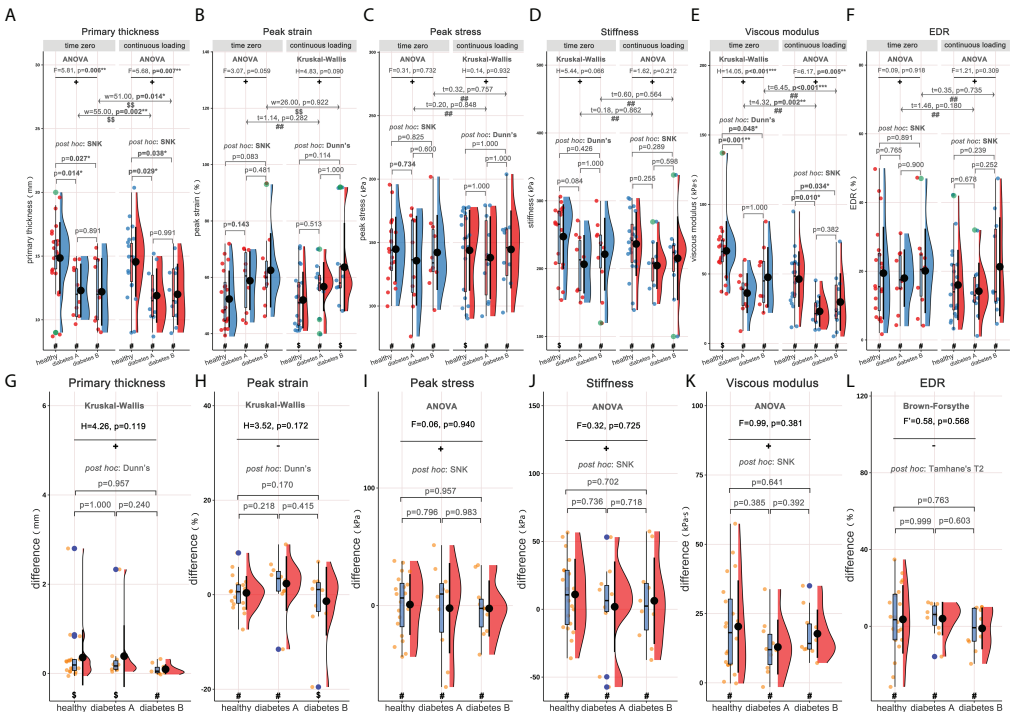


FIGURE 3
Rain-cloud plot for subgroup analysis based on length of diabetes course (diabetes A: <10years; diabetes B: ≥10years), depicting the material properties of heel pad, including primary thickness, peak strain, peak stress, stiffness, viscous modulus and EDR. **(A–F)** the material properties of heel pad in three subgroups (healthy, diabetes A and diabetes B) at time zero and following continuous loading. **(G–L)** the differences of material properties of heel pad between two loading statuses in three subgroups. $^{\#}$, data follow the normality distribution; $^{\$}$, data didn't follow the normality distribution; $^{\#\#}$, the difference between two loading statuses follow the normal distribution; $^{\$\$}$, the difference between two loading statuses didn't follow the normal distribution; +, meet the homoscedasticity assumption; -, didn't meet the homoscedasticity assumption. "F", "F'", "H", "t" and "w" represent the statistical effect sizes for ANOVA, Brown-Forsythe test, Kruskal-Wallis test, T test, and Wilcoxon test, respectively. Error bar represents the mean value and standard deviation; box plot represent the median value and quartiles. ANOVA= analysis of variance; EDR= energy dissipation rate. *, p<0.050; **p<0.010; ***p<0.001.

(R=-0.471, p<0.050*); peak strain was significantly correlated with age (R=0.521, p<0.050*) and primary thickness (R=-0.705, p<0.001***); peak stress was significantly correlated with BMI (R=0.540, p<0.050*); stiffness was significantly correlated with BMI (R=0.592, p<0.010**), primary thickness (R=0.525, p<0.050*), peak strain (R=0.-0.497, p<0.050*) and peak stress (R=0.610, p<0.010*); viscous modulus was significantly correlated with primary thickness (R=0.629, p<0.010**), peak strain (R=-0.619, p<0.010**) and stiffness (R=0.601, p<0.010**); EDR was significantly correlated with viscous modulus (R=-0.521, p<0.050*).

The correlation matrices for length of diabetes course, BMI, age, and the properties of heel in diabetes subjects at time zero and following continuous loading are available in supplementary Figures 2, 3, respectively. The regression analysis results showed that the length of diabetes history was positively associated with the peak strain, at time zero (r=0.506, p<0.050) and following continuous loading (r=0.584, p<0.010). The correlation matrices for BMI, age, and the properties of heel in diabetes subjects at time zero and following continuous loading, with subgroup analyses based on course of diabetes, are available in Figures 6, 7, respectively. At time zero, stiffness was significantly correlated with primary thickness (R=0.578, p<0.010**), peak stress (R=0.711, p<0.001***) and EDR (R=-0.473, p<0.050*); primary thickness was significantly correlated with viscous modulus (R=0.482, p<0.050*). Following continuous loading, stiffness was significantly correlated with primary thickness (R=0.648, p<0.010**), peak strain (R=-0.545, p<0.050*) and peak stress (R=0.607, p<0.010**); viscous modulus was significantly correlated with primary thickness (R=0.570, p<0.010**), peak strain (R=-0.526, p<0.050*) and stiffness (R=0.661, p<0.010**).

# Discussion

## Main findings

In this study, using our previously reported novel method, further investigation of the material properties of heel pad in diabetes patients was conducted, with combination of the DFIS

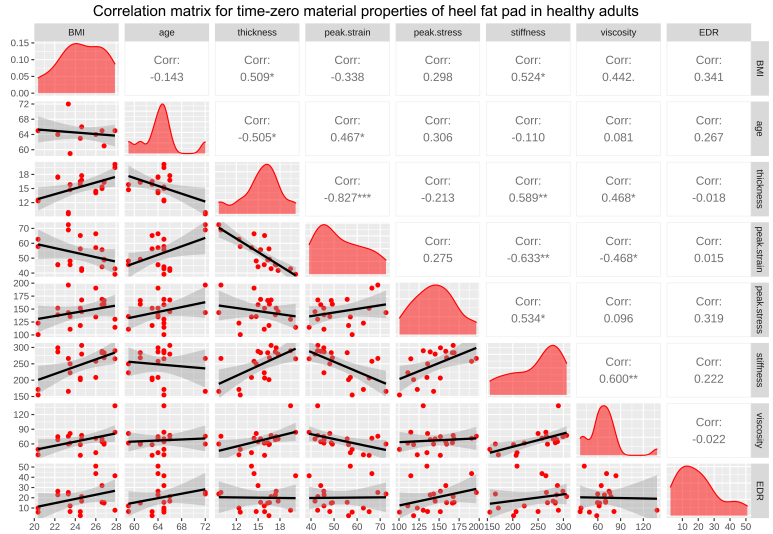


FIGURE 4
The correlation matrix for time-zero BMI, age, primary thickness, peak strain, peak stress, stiffness, viscous modulus, and EDR of heel in healthy adults. The values displayed in the right-upper triangle represent the Pearson's correlation coefficients (R values). The lower-left triangle displays the scatter plots and regression lines. The plots on the diagonal line present the distribution density of the variables in the matrix. BMI: body mass index; EDR= energy dissipation rate. P values:.p<0.100, *p<0.050, **p<0.010, ***p<0.001.

and force plate. The main findings of the current study include: (i) diabetes disease was associated with decreased primary thickness and viscous modulus, and increased peak strain both at time zero and following continuous loading; (ii) loading history (continuous weight-bearing) was related to decreased primary thickness and viscous modulus both for healthy adults and diabetes subjects; (iii) length of diabetes course has no obvious impact on the material properties of heel pad.

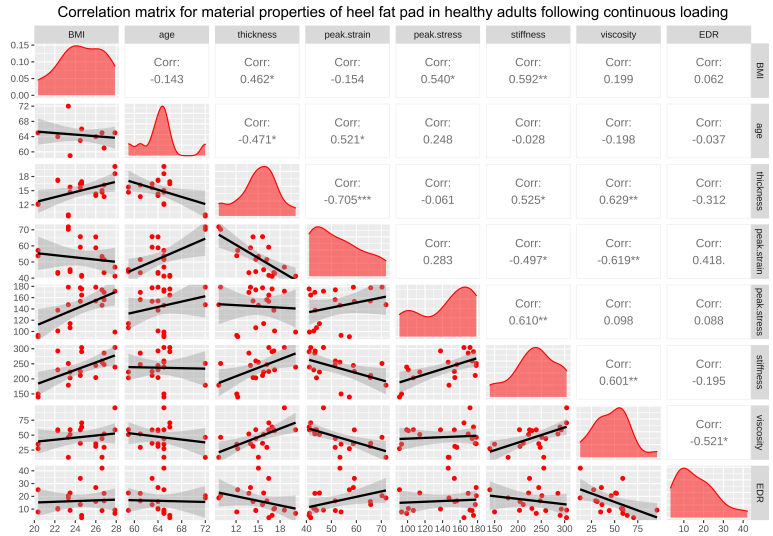


FIGURE 5
The correlation matrix for BMI, age, primary thickness, peak strain, peak stress, stiffness, viscous modulus, and EDR of heel in healthy adults following continuous loading. The values displayed in the right-upper triangle represent the Pearson's correlation coefficients (R values). The lower-left triangle displays the scatter plots and regression lines. The plots on the diagonal line present the distribution density of the variables in the matrix. BMI: body mass index; EDR= energy dissipation rate. P values:.p<0.100, *p<0.050, **p<0.010, ***p<0.001.

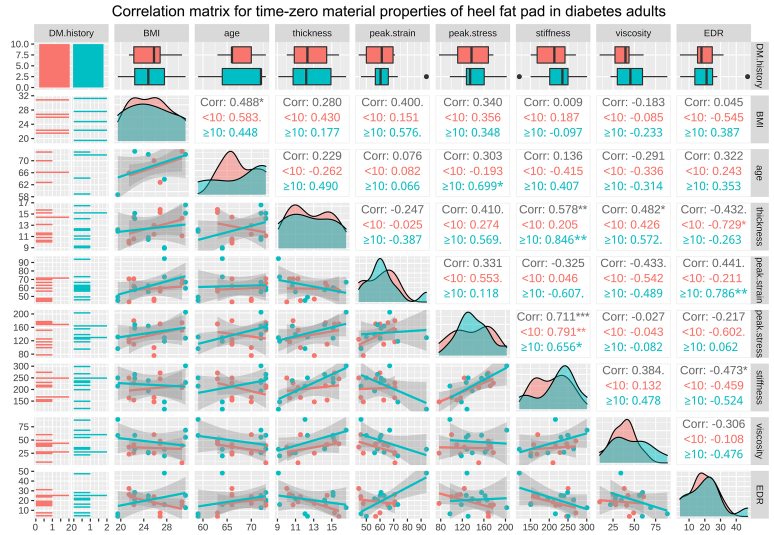


FIGURE 6
The correlation matrix for time-zero BMI, age, primary thickness, peak strain, peak stress, stiffness, viscous modulus, and EDR of heel in diabetes subjects. The values displayed in the right-upper triangle represent the Pearson's correlation coefficients (R values). The lower-left triangle displays the scatter plots and regression lines. The plots on the diagonal line present the distribution density of the variables in the matrix. Subgroup analysis was conducted basing on the diabetes course (<10 years and ≥10 years), and red colour represents subgroup of <10 years while cyan colour represents subgroup of ≥10 years. BMI: body mass index; EDR= energy dissipation rate. P values:.p<0.100, *p<0.050, **p<0.010, ***p<0.001.

## Material properties of heel pad in DM patients

The biomechanical changes in DM patients have been widely investigated with various methods (9, 28, 35, 40–46). However, it remains a difficulty to accurately measure the material properties of heel pad during normal gait with non-invasive approach. To replicate the stance phase of normal gait as far as possible, "two step method" was adopted in this study. Although "midgait method" has been widely used in the past, it is limited when applied for diabetes subjects, with the following considerations: (i) diabetic patients have difficulty on accurately reaching the relatively small force plate in a longer step due to impaired coordination caused by neuropathy and possibly associated visual impairment; (ii) many attempts are required to achieve an accurate measurement, which can lead to fatigue that changes the normal gait and testing results; (iii) extensive gait testing also increases the risk of plantar soft tissue damage in diabetic foot patients. McPOIL et al. (47) indicated that through 3~5 times of repeated tests, similar local peak pressure and pressure-time integration could be obtained using the "two-step method" as that measured by "mid-gait method". Additionally, the DFIS helps the researcher dynamically monitor the thickness change in a 3-dimensional perspective, which could also improve the precision of thickness measuring.

In diabetes patients, a series of chemical reactions would take place between reducing glucose and cellular proteins, which generates advanced glycation end products (AGEs) (48, 49). The accumulation of the AGEs is the major cause of pathophysiologic changes of diabetes tissue (22, 50). Many histomorphometric studies demonstrated thicker, and fragmented fibrous septa, and smaller adipocyte area and diameter in diabetic plantar tissue (10, 16, 17). In this study, we found decreased thickness in diabetes subjects both at time zero (12.25 ± 2.34 mm vs. 14.85 ± 2.81 mm) and following continuous loading (11.95 ± 2.21 mm vs. 14.55 ± 2.74 mm), compared with that of the healthy adults. It could be speculated that this phenomenon is caused by the atrophy of the adipose tissue and degeneration of septa that form the specific honeycomb structure. The peak strain was found to be significantly increased in diabetes patients in our results. This may also be related with the breaking of collagen bundles and fragmentation of elastin strands in septa, which provide elastic constraining force for the fatty chambers. Undoubtedly, the increased deformation during gait makes the heel pad more vulnerable to mechanical trauma and is associated with higher risk of ulceration. Thus, some authors have developed novel methods, such as 3D digital image correlation (3D-DIC) (51, 52), optical coherence tomography (OCT) (53), 3D scanning technology (54), multi-view stereoscopic technology (55) and motion capture system (56), to investigate the surface deformation and strain of plantar tissue. However, these instruments were primarily designed to recorded the surface displacement and strain, and

cannot be used for investigating the viscoelastic mechanical characteristics for heel pad.

The cushioning ability of plantar soft tissue is largely depended on the tissue's viscoelastic characteristic. Thus, if the tissue losses the viscoelasticity due to the continuous accumulation of the AGEs, its capacity of absorbing the shock and uniformly distributing loads during weight bearing activity will be reduced (36). In the previous studies, stiffness has always been the mostly concerned material property of heel pad, and most of them demonstrated increased stiffness for plantar tissue of diabetes patients (9, 28, 35, 40–46). However, in our results, we detected minor decrease of stiffness in diabetes patients compared with that of healthy subjects at time zero, and similar stiffness was found following continuous loading. The slope of loading line in stress-strain curve represents the stiffness, according to modified Kelvin-Voigt viscoelastic model. Thus, the increased magnitude of strain in diabetes patients would be the cause of the decreased stiffness. The significantly negative correlation between stiffness and peak strain in Figures 4–7 could further verify this assumption. What's more, to eliminate the impact of the age, the subjects in healthy group were matched according to age. As a result, the age in healthy group was relatively older, which consequently would increase the stiffness of this group (57). It is of particular importance to evaluate the time-dependent behaviour (i.e., viscous properties) of heel fat pad, as it has been widely recognized as the major origin of the ability of shock absorption at heel strike (58). What's more, the modifications on viscous properties may be even more sensitive to pathological conditions, such as diabetes, than other commonly evaluated material properties (such as stiffness) (40). Using our novel system, in consequence, clinicians could easily obtain the viscous parameter to assist the diagnoses and interventions of pathological states at heel. In our results, we demonstrated significantly lower viscous modulus for diabetes patients both at time zero and following continuous loading. Thus, the change on viscous constant can be used as an instructive factor to predict the risk of ulceration., 3

## Loading history and diabetes course on material properties of heel pad

The history-dependent viscoelastic properties have been widely reported in other soft tissues (59–61). Sommer et al. (59) determined the biaxial extension and triaxial shear properties of the passive human ventricular myocardium, and found that under quasi-static and dynamic multiaxial loadings it is a nonlinear, anisotropic, viscoelastic and history-dependent soft biological material. This study showed clearly higher Cauchy stresses at the same stretch level, caused by history-dependent behaviour (strain softening). The similar strain softening behaviour was also observed during shearing whenever the shear level was increased. Weickenmeier et al. (60) performed

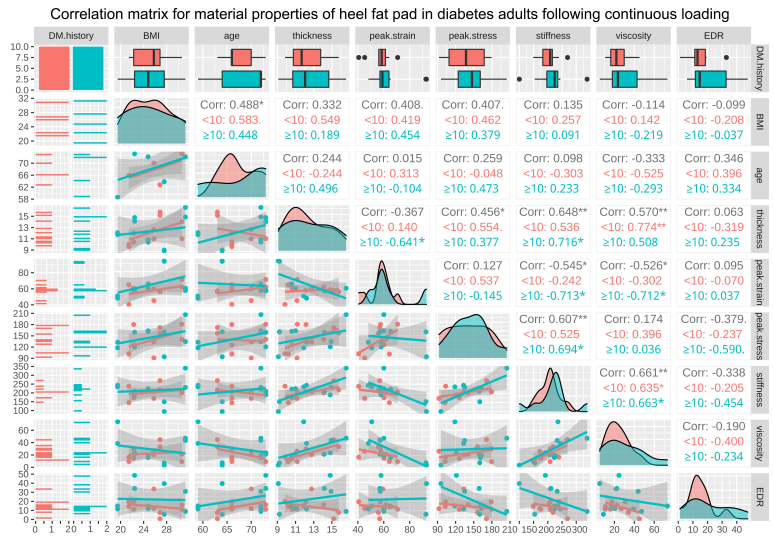


FIGURE 7
The correlation matrix for BMI, age, primary thickness, peak strain, peak stress, stiffness, viscous modulus, and EDR of heel in diabetes subjects following continuous loading. The values displayed in the right-upper triangle represent the Pearson's correlation coefficients (R values). The lower-left triangle displays the scatter plots and regression lines. The plots on the diagonal line present the distribution density of the variables in the matrix. Subgroup analysis was conducted basing on the diabetes course (<10 years and ≥10 years), and red colour represents subgroup of <10 years while cyan colour represents subgroup of ≥10 years. BMI: body mass index; EDR= energy dissipation rate. P values:.p<0.100, *p<0.050, **p<0.010.

a combined experimental and numerical investigation of the mechanical response of superficial facial tissues, and demonstrated location, time and loading history dependent material properties of the facial skin and superficial musculoaponeurotic system. In plantar soft tissue, unfortunately, to date the experiments observing the history-dependent material properties are seldom available. In the present study, we investigated the impact of loading history (continuous weight bearing) on the material characteristics of heel pad, showing decreased primary thickness and viscous modulus both in healthy and diabetes groups. This phenomenon indicates that fatigue status would be related to lowered capacity of shock absorbing with the decreased viscosity, which in turn leads to higher vulnerability to injury and ulceration.

No any significant difference was found when subgroup analysis was perform based on length of diabetes course, however, the regression analysis demonstrated significant positive correlation bewteen the peak strain and length of diabetes history. In study of Sacco et al. (62), they also introduced obvious heterogeneity on the findings about biomechanical characteristics in diabetes, and they speculated that the inconsistent findings may be due to the divergent classification/grouping criteria adopted. Hence, material properties of heel pad in diabetes patients is not uniquely dependent on the length of diabetes course, but many other factors, such as diabetic polyneuropathy, history of ulceration (63) and blooding sugar value (35), should be taken into consideration at the same time. Concerning the impact of dominate leg on the material properties, Flanagan et al. (64) proposed that the dominate leg was related with decreased deformity than non-dominated leg, and speculated that the dominate legs of subjects may contributed to the differences between two legs. While in study of Ugbolue et al. (52), no significant different displacement was evident when comparing the non-dominant and dominant heels. The current study did not found significant differences on material properties between two sides. However, the predominate leg of the participants may be either the left or the right leg, which may cause potential bias on the results. Thus, future researches with large sample size focusing on the influence of dominate leg on heel pad properties is desired.

### Limitations

This study, nevertheless, has some limitations that must be pointed out. Firstly, the strain rate applied to the heel pad has been widely proven to obviously impact the material properties of heel pad (14, 26, 28). While in the stance phase of gait, it is non-possible to precisely control the strain rate as that performed in *in-vitro* machine-based loading. To overcome this problem, subjects were trained prior to measurement to ensure an approximate gait velocity of 1.0 m/s. Then, with the aim of investigating the impacts of diabetes course and side of legs on the material properties, subgroup analyses were conducted. However, the sample sizes (10 legs) in the subgroups are relatively small, which may increase the risk of type II error. Thus, the results of the subgroup analyses should be interpreted with caution at this stage, and some future studies with larger sample size are required to further investigate the impact of diabetes course.

## Conclusions

Utilizing the novel measurement approach, the material properties of heel pad in healthy and diabetes subjects were investigated, in the stance phase of normal gait. As a result, diabetes patients were found to be associated with decreased primary thickness and viscous modulus, and increased peak strain, which may contribute to the vulnerability of heel pad to injury and ulceration. Pre-compression history by continuous weight bearing could significantly lower the primary thickness and viscous modulus of heel pad. Thus, the fatigue status is a risk factor that decrease the cushioning ability of heel pad, which in turn causes ulceration. Among diabetes patients, the length of diabetes course could not be used as the single factor to predict the degree of degeneration, but multiple conditions in diabetes should be taken into consideration.

## Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## Ethics statement

The studies involving human participants were reviewed and approved by Institutional Review Board of Huashan Hospital, Fudan University. The patients/participants provided their written informed consent to participate in this study.

## Author contributions

XGY, ZLT and XG: methodology, validation, formal analysis, investigation, data curation, writing-original draft, writing-reviewing and editing, and project administration. ZMZ, KW, RH and WMC: investigation, and data processing. CW, LC, CZ, JZH and XW: validation, writing-reviewing and editing. XG, and XM: project administration. All authors contributed to the article and approved the submitted version.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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## Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.894383/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Paired box plot comparing the material properties between left and right foots, before **(A–F)** and after **(G–L)** continuous loading. ##, the difference between two loading statuses follow the normal distribution; $$, the difference between two loading statuses didn't follow the normal distribution; "t" and "w" represent the statistical effect sizes for paired-T test, and paired-Wilcoxon test, respectively. EDR, energy dissipation rate.

SUPPLEMENTARY FIGURE 2
The correlation matrix for duration of diabetes history, BMI, age, primary thickness, peak strain, peak stress, stiffness, viscous modulus, and EDR of heel in diabetes subjects at time zero. The values displayed in the right-upper triangle represent the Pearson's correlation coefficients (R values). The lower-left triangle displays the scatter plots and regression lines. The plots on the diagonal line present the distribution density of the variables in the matrix. BMI: body mass index; EDR, energy dissipation rate. P values:.p<0.100, *p<0.050, **p<0.010, ***p<0.001.

SUPPLEMENTARY FIGURE 3
The correlation matrix for duration of diabetes history, BMI, age, primary thickness, peak strain, peak stress, stiffness, viscous modulus, and EDR of heel in diabetes subjects following continuous loading. The values displayed in the right-upper triangle represent the Pearson's correlation coefficients (R values). The lower-left triangle displays the scatter plots and regression lines. The plots on the diagonal line present the distribution density of the variables in the matrix. BMI: body mass index; EDR, energy dissipation rate. P values:.p<0.100, *p<0.050, **p<0.010, ***p<0.001.

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# Increased plasma D-dimer levels may be a promising indicator for diabetic peripheral neuropathy in type 2 diabetes

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**Background:** Increased plasma D-dimer levels have been reported to be associated with a range of adverse health outcomes. This study aimed to determine whether plasma D-dimer is connected to diabetic peripheral neuropathy (DPN) in patients with type 2 diabetes (T2D).

**Methods:** This study was part of a series exploring the potential risks for DPN. All patients were questioned for neurologic symptoms, examined for neurologic signs, and received nerve conduction studies to collect nerve action potential onset latency, amplitude, and nerve conduction velocity (NCV). Composite *Z* scores of latency, amplitude, and NCV were calculated. DPN was confirmed as both at least a neurologic symptom/sign and an abnormality of nerve conduction studies. Coagulation function indices, such as plasma D-dimer levels, were also synchronously detected.

**Results:** We finally recruited 393 eligible patients for this study, of whom 24.7% ($n$ = 97) were determined to have DPN. The plasma D-dimer level was found to be closely associated with the composite *Z* score of latency, amplitude, and NCV after adjusting for other coagulation function indices and clinical covariates (latency: $\beta$ = 0.134, $t$ = 2.299, $p$ = 0.022; amplitude: $\beta$ = −0.138, $t$ = −2.286, $p$ = 0.023; NCV: $\beta$ = −0.139, $t$ = −2.433, $p$ = 0.016). Moreover, the prevalence of DPN in the first, second, third, and fourth quartiles (Q1, Q2, Q3, and Q4) of the D-dimer level was 15.2%, 15.9%, 26.4%, and 42.7%, respectively ($p$ for trend < 0.001). The corresponding adjusted odds ratios and 95% CIs for DPN in D-dimer quartiles were 1, 0.79 (0.21–2.99), 1.75 (0.49–6.26), and 5.17 (1.38–19.42), respectively. Furthermore, the optimal cutoff value of the plasma D-dimer level to discriminate DPN was ≥0.22 mg/L (sensitivity = 67.01%,

specificity = 58.78%, and Youden index = 0.26) after analysis by the receiver operating characteristic curve.

**Conclusions:** Increased plasma D-dimer levels may be a promising indicator for DPN in patients with T2D.

KEYWORDS

D-dimer, neuropathy, type 2 diabetes, risk, diagnosis

# Introduction

Diabetic peripheral neuropathy (DPN), a primary complication of diabetes, is the main facilitative factor for falls, fractures, foot ulcerations, and amputation (1, 2). Moreover, rather than a purely peripheral neuropathy, DPN is also associated with central nervous system structural alterations (such as reduction in cervical cord cross-sectional area and somatosensory cortex gray matter volume) (3, 4) and functional abnormalities (such as abnormal thalamocortical connectivity) (5). Hence, patients with DPN are more susceptible to suffer from disability, encounter a poor quality of life, and experience reduced psychosocial wellbeing (6, 7). The pathogenesis of DPN is not very clear, but it involves the interaction between multiple factors. Therefore, it is worthwhile to explore additional risk factors for DPN, which may help develop approaches to prevent or ameliorate DPN.

D-dimer is a degradation product from fibrinolysis, and its increment in plasma serves as a traditional biomarker of hypercoagulability (8). Plasma D-dimer levels were reported to be associated with an increased risk of coronary events (8), ischemic and hemorrhagic stroke (9, 10), cardiovascular disease (CVD)-specific mortality, and cancer incidence and prognosis in the general population (11), let alone be indicative of venous thrombus formation (12). Moreover, increased plasma D-dimer levels were also documented to be partly responsible for angiopathic complications in patients with diabetes, such as microalbuminuria (13), renal dysfunction (14), diabetic retinopathy (15), atherosclerotic plaque (16), and poor cardiovascular outcomes (17). Furthermore, plasma D-dimer can be used to reflect the progressive nature of diabetes and cardiovascular complications (18). However, the relationship between plasma D-dimer and DPN in type 2 diabetes (T2D) has not been well studied. In light of the above, we hypothesized that increased plasma D-dimer levels may be a potential risk factor for DPN in T2D.

Therefore, we designed the present study to determine whether increased plasma D-dimer levels are connected to DPN in T2D.

**Abbreviations:** T2D, type 2 diabetes; DPN, diabetic peripheral neuropathy; lnD-dimer, natural logarithm transformed D-dimer; NCV, nerve conduction velocity; SBP/DBP, systolic/diastolic blood pressure; BMI, body mass index; TZDs, thiozolindiones; AGIs, α-glucosidase inhibitors; DPP-4Is, dipeptidyl peptidase-4 inhibitors; SGLT-2Is, sodium-glucose cotransporter-2 inhibitors; GLP-1RAs, glucagon-like peptide-1 receptor agonists; ALT, alanine aminotransferase; TG, triglycerides; TC, total cholesterol; HDLC, high-density lipoprotein cholesterol; LDLC, low-density lipoprotein cholesterol; UA, uric acid; eGFR, estimated glomerular filtration rate; HbA1c, glycosylated hemoglobin A1c; PT, prothrombin time; APTT, activated partial prothrombin time; MN, median nerve; UN, ulnar nerve; CPN, common peroneal nerve; PTN, posterior tibial nerve; SN, sural nerve; SPN, superficial peroneal nerve.

# Methods

## Participant recruitment

This study was part of a series we designed to explore the potential risks for DPN. We recruited participants for the study from First People's Hospital of Nantong City and Second People's Hospital of Nantong City between November 2017 and December 2021. The inclusion criteria for participants were as follows: (1) between 20 and 80 years of age; (2) met the diagnostic criteria of T2D (2015 Edition, American Diabetes Association) (19); (3) normal coagulation function; (4) plasma D-dimer level < 2.0 mg/L; and (5) voluntarily agreed to take part in the study. The exclusion criteria for participants were described below: (1) positive for insulin antibody; (2) thyroid hormonal abnormality; (3) history of cancer; (4) severe cardio-cerebral vascular diseases (e.g., myocardial infarction); (5) chronic kidney disease, and estimated glomerular filtration rate (eGFR) < 60 ml/min/1.73 $m^2$; (6) acute or chronic infectious diseases; (7) autoimmune diseases; (8) connective tissue diseases; (9) use of drugs with side effects of neurotoxicity; (10) deficiencies of folate or vitamin B12; (11) spinal or foraminal stenosis; (12) neurodegenerative diseases; (13) inflammatory demyelinating neuropathies; (14) suffered from trauma in the last 3 months; (15) history of vascular interventional surgery; (16) use of anticoagulants or antiplatelet drugs in the last 3 months; (17) thrombotic diseases; and (18) coagulopathy and platelet

dysfunction. We finally recruited 393 eligible patients for this study. The study was initiated and academically supported by First People's Hospital of Nantong, so the study protocol was reviewed and approved by the First People's Hospital of Nantong. In addition, the processes of the study followed the Declaration of Helsinki, and all participants provided informed consent when recruited into the study.

## Clinical data collection

Clinical data from all participants were collected by trained clinical staff. These data included age, sex, body mass index (BMI), systolic/diastolic blood pressure (SBP/DBP), diabetes duration, hypertension status, statin use, and antidiabetic treatments. Hypertension was identified as reported in our previous studies (20, 21). Antidiabetic treatments in our study were divided into nine categories: drug naïve, insulin, secretagogues, metformin, thiozolindiones (TZDs), α-glucosidase inhibitors (AGIs), dipeptidyl peptidase-4 inhibitors (DPP-4Is), sodium-glucose cotransporter-2 inhibitors (SGLT-2Is), and glucagon-like peptide-1 receptor agonists (GlP-1RAs).

Plasma was isolated from blood specimens (stored by tubes with 3.2% sodium citrate solution) to detect coagulation function indices, such as international normalized ratio (INR), prothrombin time (PT), activated partial prothrombin time (APTT), fibrinogen, and D-dimer. PT, APTT, and fibrinogen were measured by the solidification method, and D-dimer was measured by the immunoturbidimetric method in a fully automated blood coagulation analyzer (CS5100, Sysmex, Japan). Serum was isolated from fasting blood samples to detect alanine aminotransferase (ALT), triglycerides (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDLC), low-density lipoprotein cholesterol (LDLC), uric acid (UA), and C-peptide. Whole blood specimens were drawn to detect glycosylated hemoglobin (HbA1c). Plasma was also isolated to detect glucagon. Serum creatinine was also detected to calculate the estimated glomerular filtration rate (eGFR) using the Modification of Diet in Renal Disease equation (22).

## Screening for DPN and nerve conduction studies

Screening for DPN was carried out as reported in our previous studies (20, 21, 23). Confirmation of DPN is dependent on both at least a neurologic symptom/sign and an abnormality of peripheral nerve conduction studies (24).

Neurological symptoms and signs were collected by detailed history taking and physical examinations. Neurologic symptoms included numbness, pain (such as tingling, stabbing, burning, shooting, and electrical shock pain), and paresthesia (such as abnormal cold or heat sensation, allodynia, and hyperalgesia), and neurologic signs were defined as reduced ankle reflexes or reduced distal sensation (such as touch sensation, thermal discrimination, nociception, vibration perception, equilibrioception, and proprioception).

Nerve conduction studies were implemented by an experienced neurological technician using an electromyogram (MEB-9200K, Nihon Kohden). The nerve conduction parameters included the onset latency, nerve action potential amplitude, and nerve conduction velocity (NCV). Motor nerve studies were conducted on two sides of the median nerve (MN), ulnar nerve (UN), common peroneal nerve (CPN), and posterior tibial nerve (PTN). Sensory nerve studies were conducted on the sides of the MN, UN, sural nerve (SN), and superficial peroneal nerve (SPN). Data of nerve latency, amplitude, and NCV were then $Z$ score transformed. Furthermore, the composite $Z$ score of latency was calculated by taking the average value of the latency $Z$ score of all motor and sensory nerves of the upper and lower limbs, which was also described in our previous study (23). In the same way, the composite $Z$ scores of amplitude and NCV were calculated.

## Statistical analysis

We used SPSS for Windows (Version 25.0, IBM Corp.) to pool and analyze clinical data, and statistical significance was identified when the $p$-value $< 0.05$.

First, descriptive statistics were performed for all patients and four subgroups categorized by the quartiles of plasma D-dimer levels. Means and standard deviations were for normally distributed quantitative data, medians and interquartile ranges were for skew-distributed quantitative data, and frequencies and percentages were for qualitative data. To analyze the changes in trends of clinical data among the four subgroups, one-way analysis of variance (ANOVA) with linear polynomial contrasts ($F$ value), the Jonckheere–Terpstra test (standard $Z$ value), and the chi-squared test with linear-by-linear association ($\chi^2$ value) were used as appropriate. The plasma D-dimer level was skew-distributed and was natural-logarithm transformed (lnD-dimer) for further correlation and regression analysis.

Second, Pearson's correlation was used to assess univariate correlation between plasma D-dimer and nerve conduction indices. Moreover, given that HbA1c or fibrinogen may exert an effect on these correlations, partial correlation was used to adjust the effect of HbA1c or fibrinogen on these correlations.

Third, to determine the independent effects of plasma D-dimer on nerve conduction indices, we used multivariable linear regression analyses to control for other coagulation function indices and clinical covariates. Meanwhile, we used multivariable logistic regression analyses to determine unadjusted and adjusted odds ratios (ORs) and 95% confidence intervals (CIs) for DPN in four subgroups of plasma D-dimer quartiles.

Finally, we used receiver operating characteristic (ROC) curves to assess the diagnostic capability of plasma D-dimer to confirm

DPN and to determine the optimal cutoff value of plasma D-dimer to discriminate DPN. HbA1c and plasma fibrinogen were well-established risk factors for DPN in our previous studies and other previous studies (21, 25, 26). To explore whether the predictive ability of plasma D-dimer is beyond traditional risk factors, we set a reference model including age, sex, diabetes duration, BMI, SBP, DBP, hypertension, statin treatment, ALT, lipid profiles, UA, eGFR, fasting C-peptide, fasting glucagon, PT, APTT, and antidiabetic treatments. Then, we used ROC analysis to compare the performance of the reference model adding D-dimer with the reference model alone, the reference model adding fibrinogen, and the reference model adding HbA1c (methods by DeLong et al.).

# Results

## Clinical features of recruited patients

The clinical features of all eligible patients are exhibited in Table 1. The range of plasma D-dimer levels of all patients was 0.10–1.65 mg/L, and those of the first, second, third, and fourth quartiles (Q1, Q2, Q3, and Q4) were 0.10–0.14 mg/L, 0.15–0.22 mg/L, 0.23–0.45 mg/L, and 0.46–1.65 mg/L, respectively. In addition, with quartiles of ascending plasma D-dimer, age, SBP, diabetes duration, plasma fibrinogen, and PT were increased, while ALT, TC, LDLC, fasting C-peptide, and glucagon were decreased. However, sex distribution, DBP, hypertension history, statin use, TG, HDLC, UA, eGFR, HbA1c, and plasma APTT displayed no differences among the four subgroups. Regarding antidiabetic treatments, insulin use tended to increase plasma D-dimer within a certain range ($p$ = 0.041), while SGLT-2Is use tended to decrease plasma D-dimer, but this failed to reach statistical significance ($p$ = 0.052). However, drug naïve, uses of secretagogues, metformin, TZDs, AGIs, DPP-4Is, and GLP-1RAs were comparable among the four subgroups.

## Correlations between plasma D-dimer and nerve conduction indices

With increasing quartiles of plasma D-dimer, the composite $Z$ score of latency was increased, while the composite $Z$ score of amplitude and NCV were markedly decreased (Table 1). Univariate correlation analysis demonstrated that the plasma D-dimer level was linked to the composite $Z$ score of nerve latency, amplitude and NCV ($r$ = 0.210, −0.209, and −0.270, respectively, $p$ < 0.001) (Figure 1). After controlling for the potential effect of HbA1c on these correlations by partial correlation analysis, plasma D-dimer levels remained linked to composite $Z$ scores of nerve latency, amplitude, and NCV ($r$ = 0.188, −0.192, and −0.256, respectively, $p$ < 0.001) (Figure 2). Meanwhile, after controlling for the potential effect of fibrinogen on these correlations, plasma D-dimer levels remained linked to composite $Z$ scores of nerve latency, amplitude and NCV ($r$ = 0.170, −0.175, and −0.217, respectively, $p$ < 0.001) (Figure 3).

Moreover, we used multivariable linear regression analyses to determine the effects of plasma D-dimer on nerve conduction indices (Table 2). After gradually adjusting for other coagulation function indices and clinical covariates (from model 0 to model 4), plasma D-dimer levels remained independently associated with nerve conduction indices. The fully adjusted model 4 demonstrated that plasma D-dimer level was independently and positively related to the composite $Z$ score of latency ($\beta$ = 0.134, $t$ = 2.299, $p$ = 0.022) and was independently and negatively related to the composite $Z$ score of amplitude ($\beta$ = −0.138, $t$ = −2.286, $p$ = 0.023) and NCV ($\beta$ = −0.139, $t$ = −2.433, $p$ = 0.016), respectively.

## Risks for DPN at differential levels of plasma D-dimer quartiles

After DPN assessment, 24.7% ($n$ = 97) of recruited patients were determined to have DPN. The prevalence of DPN in Q1, Q2, Q3, and Q4 of the D-dimer level was 15.2%, 15.9%, 26.4%, and 42.7%, respectively ($p$ for trend < 0.001). Moreover, the ORs and 95% CIs for DPN in Q1, Q2, Q3, and Q4 of the D-dimer level were 1, 1.06 (0.50–2.25), 2.01 (0.98–4.12), and 4.18 (2.11–8.26), respectively (Table 3). Furthermore, after adjusting for other coagulation function indices and clinical covariates by multivariable logistic regression analyses, the corresponding ORs and 95% CIs for DPN in the Q1, Q2, Q3, and Q4 plasma D-dimer quartiles were 1, 0.79 (0.21–2.99), 1.75 (0.49–6.26), and 5.17 (1.38–19.42), respectively (Table 3).

## Potential capability of plasma D-dimer to discriminate DPN

Figure 4 exhibits the capability of plasma D-dimer to discriminate DPN after ROC curve analysis. The area under the ROC curve (AUC) of plasma D-dimer was 0.659 (95% CI: 0.610–0.706). Additionally, ROC analysis also determined that the optimal cutoff value of plasma D-dimer to discriminate DPN was ≥0.22 mg/L, with a Youden index of 0.26, a sensitivity of 67.01%, and a specificity of 58.78%.

## Performance of plasma D-dimer to discriminate DPN after adjustment for traditional risk factors

Moreover, to explore whether the predictive ability of plasma D-dimer to discriminate DPN is beyond traditional risk factors, we used ROC analysis to compare the performance of the reference model adding D-dimer with the reference model alone, the

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TABLE 1 Clinical features of the recruited patients.

| Variables | Total | Quartiles of plasma D-dimer levels | | | | Test statistic | *p* for trend |
|---|---|---|---|---|---|---|---|
| | | Q1 | Q2 | Q3 | Q4 | | |
| Plasma D-dimer (mg/L) (range) | 0.36 ± 0.32 (0.10–1.65) | 0.11 ± 0.02 (0.10–0.14) | 0.19 ± 0.02 (0.15–0.22) | 0.32 ± 0.07 (0.23–0.45) | 0.86 ± 0.28 (0.46–1.65) | – | – |
| ln D-dimer | −1.33 ± 0.76 | −2.18 ± 0.13 | −1.69 ± 0.11 | −1.16 ± 0.20 | −0.20 ± 0.32 | – | – |
| *n* | 393 | 99 | 107 | 91 | 96 | – | – |
| Age (years) | 51.4 ± 8.9 | 49.3 ± 6.4 | 50.5 ± 8.9 | 53.2 ± 9.1 | 53.0 ± 10.4 | 11.767[a] | 0.001 |
| Female, *n* (%) | 157 (39.9) | 39 (39.4) | 40 (37.4) | 33 (36.3) | 45 (46.9) | 0.938[c] | 0.333 |
| BMI (kg/m$^2$) | 25.1 ± 3.2 | 25.3 ± 3.0 | 25.4 ± 3.0 | 25.2 ± 3.4 | 24.5 ± 3.3 | 3.152[a] | 0.077 |
| SBP (mmHg) | 132.8 ± 16.3 | 129.9 ± 17.8 | 133.0 ± 15.4 | 133.2 ± 16.5 | 135.2 ± 15.4 | 4.849[a] | 0.028 |
| DBP (mmHg) | 79.7 ± 10.5 | 78.8 ± 9.9 | 81.7 ± 11.4 | 79.8 ± 9.1 | 78.1 ± 11.0 | 0.714[a] | 0.398 |
| Diabetes duration (years) | 5.0 (1.0–10.0) | 5.0 (1.0–9.5) | 4.0 (1.0–8.3) | 8.0 (1.0–12.0) | 7.5 (1.3–10.0) | 2.486[b] | 0.013 |
| Antidiabetic treatments | | | | | | | |
| Drug naive, *n* (%) | 41 (10.4) | 11 (11.1) | 13 (12.1) | 9 (9.9) | 8 (8.3) | 0.591[c] | 0.442 |
| Insulin, *n* (%) | 166 (42.2) | 36 (3.4) | 43 (40.2) | 38 (41.8) | 49 (51.0) | 4.160[c] | 0.041 |
| Secretagogues, *n* (%) | 172 (43.8) | 45 (45.5) | 37 (34.6) | 46 (50.5) | 44 (45.8) | 0.596[c] | 0.440 |
| Metformin, *n* (%) | 192 (48.9) | 52 (52.5) | 53 (49.5) | 48 (52.7) | 39 (40.6) | 2.067[c] | 0.151 |
| TZDs, *n* (%) | 73 (18.6) | 19 (19.2) | 16 (15.0) | 18 (19.9) | 20 (20.8) | 0.314[c] | 0.571 |
| AGIs, *n* (%) | 54 (13.7) | 12 (12.1) | 15 (14.0) | 10 (11.0) | 17 (17.7) | 0.778[c] | 0.378 |
| DPP-4Is, *n* (%) | 60 (15.3) | 18 (18.2) | 19 (17.8) | 12 (13.2) | 11 (11.5) | 2.311[c] | 0.128 |
| SGLT-2Is, *n* (%) | 16 (4.1) | 7 (7.1) | 5 (4.7) | 2 (2.2) | 2 (2.1) | 3.774[c] | 0.052 |
| GLP-1RAs, *n* (%) | 31 (7.9) | 6 (6.1) | 8 (7.5) | 6 (6.6) | 11 (11.5) | 1.577[c] | 0.209 |
| Hypertension, *n* (%) | 143 (36.4) | 33 (33.3) | 36 (33.6) | 40 (44.0) | 34 (35.4) | 0.572[c] | 0.449 |
| Statins uses, *n* (%) | 117 (29.8) | 28 (28.3) | 23 (21.5) | 34 (37.4) | 32 (33.3) | 2.266[c] | 0.132 |
| ALT (U/L) | 19 (13–28) | 22 (13–28) | 21 (14–31) | 13 (20–29) | 15 (11–22) | −2.738[b] | 0.006 |
| TG (mmol/L) | 1.64 (1.04–2.51) | 1.73 (1.19–2.44) | 1.72 (1.14–2.78) | 1.61 (0.86–2.87) | 1.45 (1.05–2.34) | −1.421[b] | 0.155 |
| TC (mmol/L) | 4.38 ± 0.96 | 4.52 ± 1.06 | 4.54 ± 0.85 | 4.17 ± 0.92 | 4.26 ± 0.98 | 6.575[a] | 0.011 |
| HDLC (mmol/L) | 1.17 ± 0.36 | 1.17 ± 0.28 | 1.18 ± 0.53 | 1.15 ± 0.29 | 1.18 ± 0.26 | 0.008[a] | 0.927 |
| LDLC (mmol/L) | 2.73 ± 0.85 | 2.87 ± 0.95 | 2.89 ± 0.74 | 2.50 ± 0.77 | 2.63 ± 0.87 | 7.840[a] | 0.005 |
| UA (μmol/L) | 298 ± 88 | 303 ± 101 | 299 ± 82 | 296 ± 79 | 294 ± 92 | 0.423[a] | 0.516 |
| Fasting C-peptide (ng/ml) | 1.44 (0.86–2.19) | 1.73 (0.97–2.21) | 1.62 (0.86–2.41) | 1.37 (0.85–2.05) | 1.14 (0.69–1.73) | −3.042[b] | 0.002 |
| Fasting glucagon (pg/ml) | 148.1 (113.3–202.0) | 161.0 (115.1–208.6) | 161.0 (119.1–222.5) | 134.9 (111.8–210.2) | 136.2 (111.8–177.5) | −2.180[b] | 0.029 |
| eGFR (ml/min/1.73 m$^2$) | 120 ± 34 | 119 ± 29 | 125 ± 32 | 122 ± 44 | 114 ± 30 | 1.124[a] | 0.290 |
| HbA1c (%) | 8.09 ± 1.17 | 7.87 ± 1.10 | 8.12 ± 1.20 | 8.22 ± 1.26 | 8.15 ± 1.10 | 3.243[a] | 0.073 |
| Plasma fibrinogen (g/L) | 2.50 ± 0.78 | 2.22 ± 0.57 | 2.47 ± 0.56 | 2.54 ± 0.68 | 2.78 ± 0.85 | 33.037[a] | <0.001 |
| Plasma PT (s) | 11.36 ± 0.88 | 11.32 ± 1.00 | 11.10 ± 0.75 | 11.41 ± 0.89 | 11.65 ± 0.81 | 11.325[a] | 0.001 |
| Plasma APTT (s) | 29.46 ± 5.16 | 29.97 ± 5.58 | 28.73 ± 4.38 | 29.83 ± 5.71 | 29.41 ± 4.95 | 0.057[a] | 0.811 |
| Composite *Z* score of latency | 0.03 ± 0.61 | −0.08 ± 0.57 | −0.11 ± 0.55 | 0.06 ± 0.59 | 0.27 ± 0.68 | 20.255[a] | <0.001 |
| Composite *Z* score of amplitude | −0.02 ± 0.67 | 0.19 ± 0.64 | 0.02 ± 0.58 | −0.11 ± 0.70 | −0.21 ± 0.67 | 20.598[a] | <0.001 |
| Composite *Z* score of NCV | −0.03 ± 0.74 | 0.19 ± 0.67 | 0.13 ± 0.58 | −0.11 ± 0.85 | −0.34 ± 0.77 | 32.079[a] | <0.001 |
| DPN, *n* (%) | 97 (24.7) | 15 (15.2) | 17 (15.9) | 24 (26.4) | 41 (42.7) | 22.855[c] | <0.001 |

[a]Linear polynomial contrasts of ANOVA (F value), [b]Jonckheere–Terpstra test (Z value), and [c]linear-by-linear association of chi-squared test ($\chi^2$ value) were performed as appropriate.

reference model adding fibrinogen, and the reference model adding HbA1c (Figure 5). After comparing the AUC for these models, we found that the performance of the reference model adding D-dimer to discriminate DPN was superior to that of the reference model alone (Table 4). However, the performance of the reference model adding D-dimer, the reference model adding HbA1c, and the reference model adding fibrinogen was comparable to discriminate DPN (Table 4).

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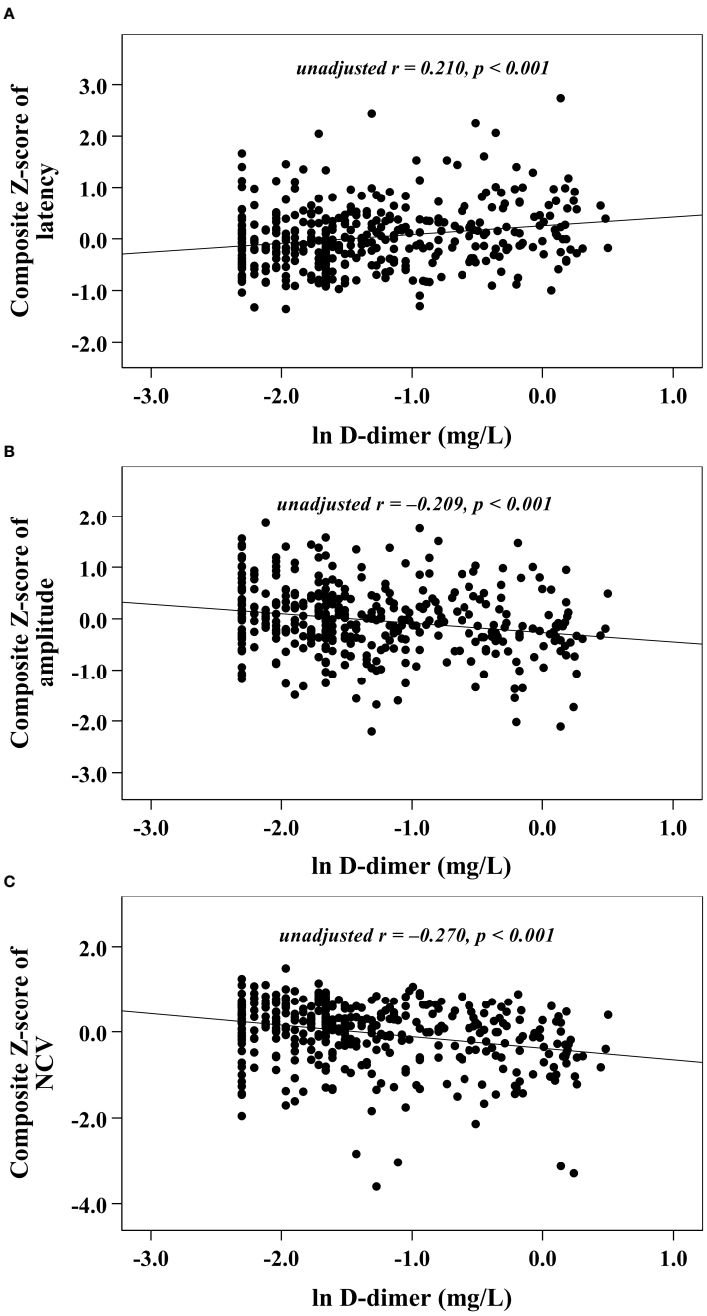


FIGURE 1
Graphically exhibited correlations between D-dimer and nerve conduction indices (**A:** composite Z score of latency; **B:** composite Z score of amplitude; **C:** composite Z score of NCV).

## Discussion

DPN is extremely complex and debilitating due to its pathogenesis involving a complex interaction between multiple factors. We initiated a series to find additional risk factors for DPN. The present study aimed to determine whether plasma D-dimer is an independent risk factor for DPN in T2D. The primary contributions of the study are described as follows: first, plasma D-dimer was closely connected with the nerve action potential onset latency, amplitude, and conduction velocity, even after correcting for clinical covariates; second, the risk of DPN was estimated to be fivefold (OR 5.17, 95% CI:

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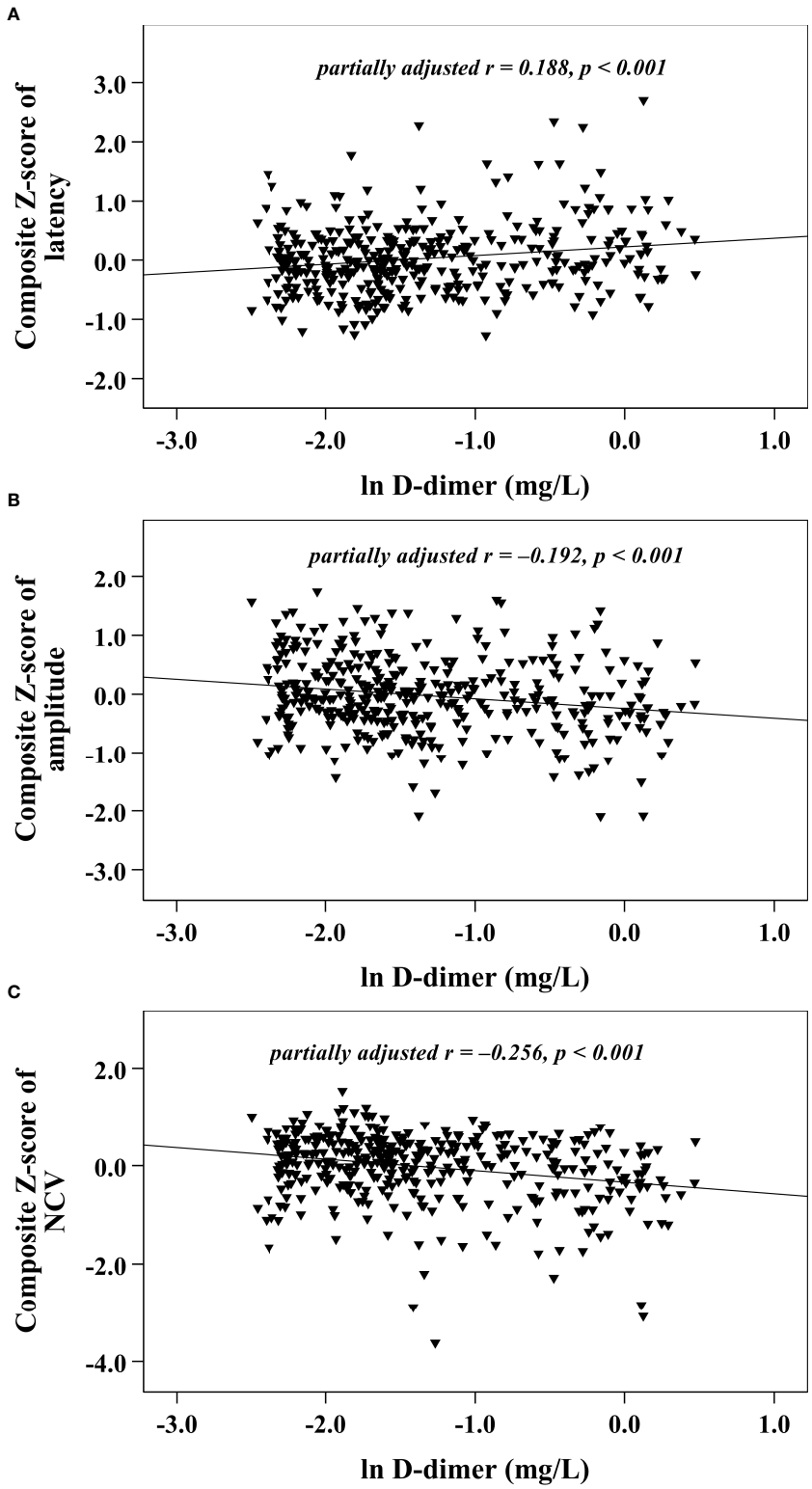


FIGURE 2
Graphically exhibited correlations between D-dimer and nerve conduction indices after adjusting for HbA1c (**A:** composite Z score of latency; **B:** composite Z score of amplitude; **C:** composite Z score of NCV).

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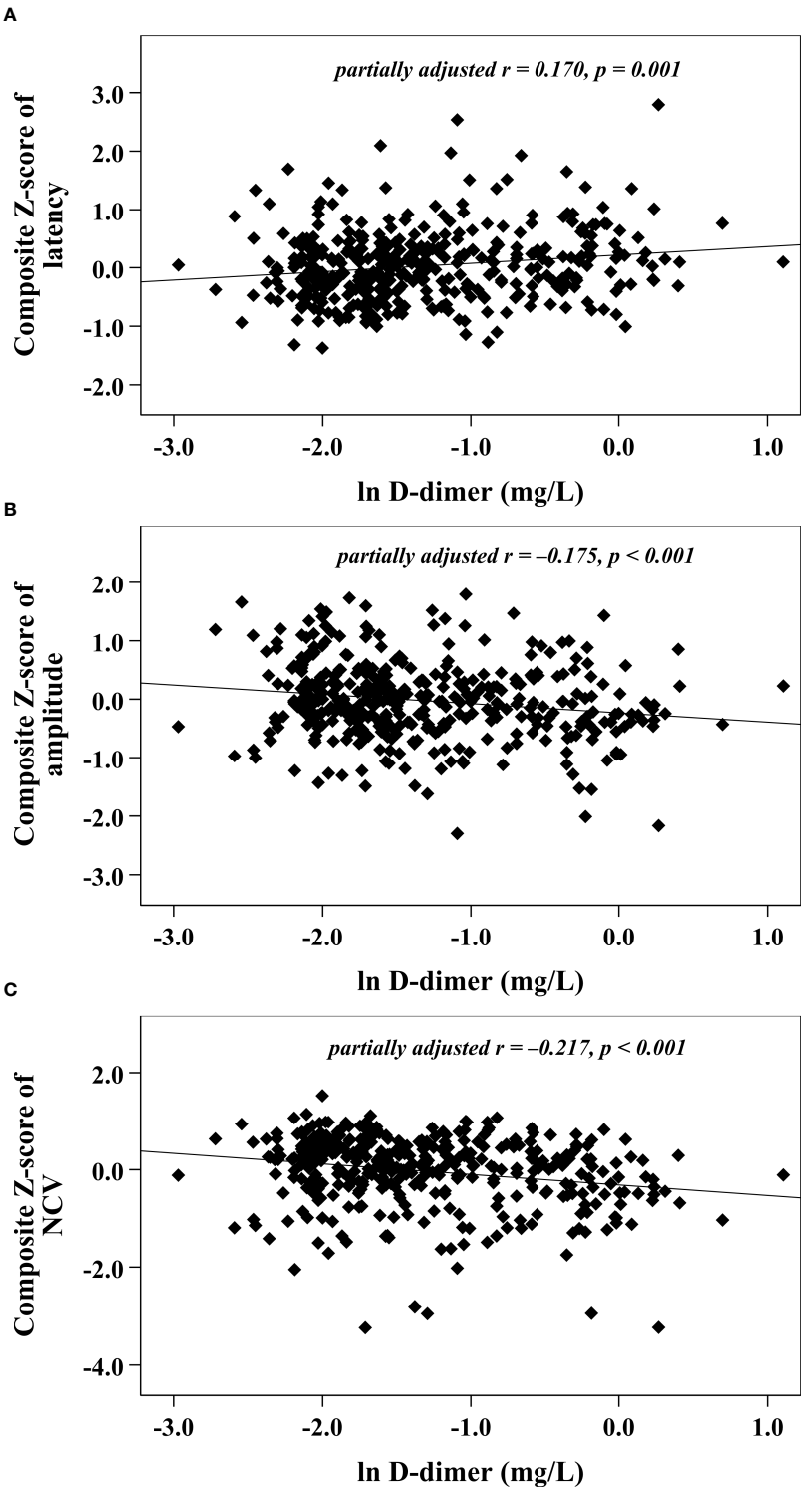


FIGURE 3
Graphically exhibited correlations between D-dimer and nerve conduction indices after adjusting for fibrinogen (**A:** composite Z score of latency; **B:** composite Z score of amplitude; **C:** composite Z score of NCV).

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TABLE 2 Impacts of plasma D-dimer on outcomes of nerve conduction indices by multivariable linear regression analysis.

| Models | B (95% CI) | $\beta$ | $t$ | $p$ | Adjusted $R^2$ |
|---|---|---|---|---|---|
| **Composite *Z* score of latency** | | | | | |
| Model 0 | 0.169 (0.091 to 0.248) | 0.210 | 4.234 | <0.001 | 0.044 |
| Model 1 | 0.150 (0.077 to 0.223) | 0.185 | 4.022 | <0.001 | 0.236 |
| Model 2 | 0.149 (0.065 to 0.243) | 0.183 | 3.478 | 0.001 | 0.400 |
| Model 3 | 0.151 (0.065 to 0.237) | 0.184 | 3.452 | 0.001 | 0.432 |
| Model 4 | 0.109 (0.016 to 0.203) | 0.134 | 2.299 | 0.022 | 0.443 |
| **Composite *Z* score of amplitude** | | | | | |
| Model 0 | −0.182 (−0.267 to −0.097) | −0.209 | −4.129 | <0.001 | 0.044 |
| Model 1 | −0138 (−0.217 to −0.059) | −0.158 | −3.440 | 0.001 | 0.237 |
| Model 2 | −0.147 (−0.244 to −0.049) | −0.160 | −2.970 | 0.003 | 0.368 |
| Model 3 | −0.134 (−0.233 to −0.035) | −0.147 | −2.677 | 0.008 | 0.402 |
| Model 4 | −0.127 (−0.235 to −0.018) | −0.138 | −2.286 | 0.023 | 0.405 |
| **Composite *Z* score of NCV** | | | | | |
| Model 0 | −0.269 (−0.363 to −0.175) | −0.274 | −5.639 | <0.001 | 0.075 |
| Model 1 | −0.234 (−0.325 to −0.143) | −0.239 | −5.040 | <0.001 | 0.191 |
| Model 2 | −0.209 (−0.316 to −0.103) | −0.206 | −3.888 | <0.001 | 0.389 |
| Model 3 | −0.186 (−0.293 to −0.079) | −0.183 | −3.429 | 0.001 | 0.430 |
| Model 4 | −0.142 (−0.256 to −0.027) | −0.139 | −2.433 | 0.016 | 0.461 |

D-dimer was natural logarithmically transformed for the regression analysis.Model 0: unadjusted.
Model 1: adjusted for age, sex, diabetic duration, BMI, SBP, DBP, hypertension and statins treatment.
Model 2: additionally adjusted for ALT, lipid profiles, UA, eGFR, HbA1c, fasting C-peptide and glucagon.
Model 3: additionally adjusted for antidiabetic treatments.
Model 4: additionally adjusted for plasma fibrinogen, PT and APTT.

1.38–19.42) higher in patients in the highest quartile of plasma D-dimer than in those in the lowest quartile; third, plasma D-dimer ≥0.22 mg/L was the optimal cutoff value to discriminate DPN (sensitivity = 67.01%, specificity = 58.78%); fourth, compared to the well-established risk factors (reference model adding HbA1c and reference model adding plasma fibrinogen), we found that plasma D-dimer, HbA1c, and fibrinogen were comparable in the ability to discriminate DPN.

DPN incidence and progression result from the accumulation of a complex interaction of multiple cardiometabolic risk factors in the background of diabetes (27, 28). Previous well-conducted studies have shown that glucose burden is a central risk factor underlying the pathogenesis of diabetic neuropathy (25). Other risk factors have also been identified to play an important role in diabetic neuropathy, such as long-term diabetes, aging, abdominal obesity, hypertension, smoking, dyslipidemia (raised TG level and decreased HDLC level), hypoalbuminemia, and anemia (2, 29–32). Of course, glucose burden is the most notable risk factor for DPN, and our previous series demonstrated that short-term glycemic fluctuation estimated by the mean amplitude of glycemic excursions (MAGE) (21) and plasma 1,5-anhydro-d-glucitol (23) and long-term glycemic fluctuation estimated by HbA1c variability (20) were involved in DPN. Alongside an excessive glucose burden, a hypercoagulability state was also

TABLE 3 Risks for DPN at differential levels of plasma D-dimer quartiles (ORs [95% CIs]).

| Models | Q1 | Q2 | Q3 | Q4 | $p$ value for trend |
|---|---|---|---|---|---|
| *n* | 99 | 107 | 91 | 96 | – |
| DPN, *n* (%) | 15 (15.2) | 17 (15.9) | 24 (26.4) | 41 (42.7) | – |
| Model 0 | 1–reference | 1.06 (0.50 to 2.25) | 2.01 (0.98 to 4.12) | 4.18 (2.11 to 8.26) | <0.001 |
| Model 1 | 1–reference | 1.04 (0.48 to 2.25) | 1.65 (0.78 to 3.50) | 3.78 (1.86 to 7.70) | <0.001 |
| Model 2 | 1–reference | 1.14 (0.34 to 3.79) | 1.60 (0.50 to 5.18) | 6.10 (1.98 to 18.84) | 0.001 |
| Model 3 | 1–reference | 1.06 (0.30 to 3.78) | 2.15 (0.62 to 7.43) | 7.05 (2.10 to 23.63) | <0.001 |
| Model 4 | 1–reference | 0.79 (0.21 to 2.99) | 1.75 (0.49 to 6.26) | 5.17 (1.38 to 19.42) | 0.005 |

Model 0: unadjusted.
Model 1: adjusted for age, sex, diabetes duration, BMI, SBP, DBP, hypertension, and statin treatment.
Model 2: additionally adjusted for ALT, lipid profiles, UA, eGFR, HbA1c, fasting C-peptide, and glucagon.
Model 3: additionally adjusted for antidiabetic treatments.
Model 4: additionally adjusted for plasma fibrinogen, PT, and APTT.

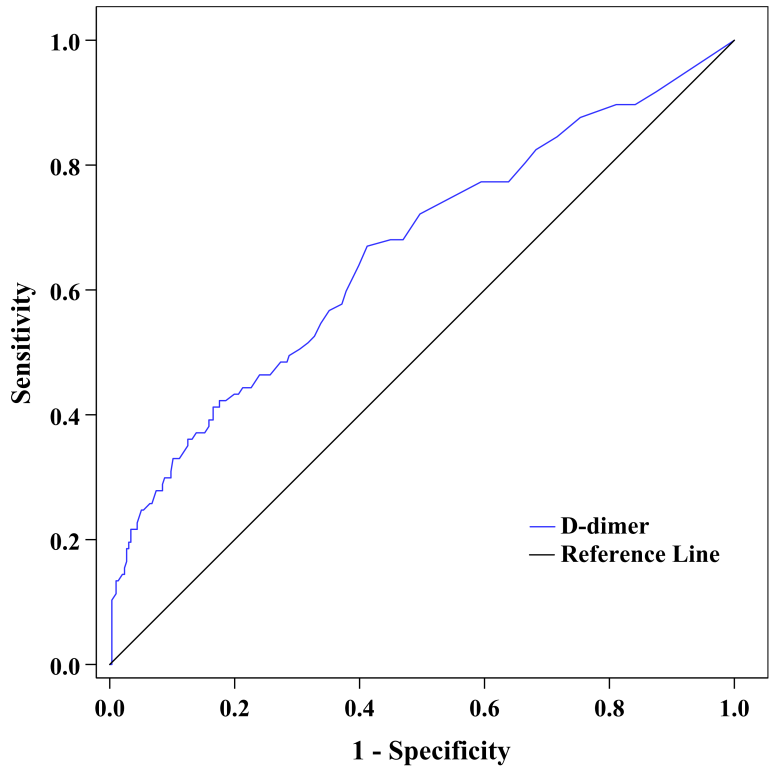


FIGURE 4
ROC curve exhibited the capability of plasma D-dimer to discriminate DPN (AUC was 0.659 [95% CI: 0.610–0.706], optimal cutoff value was ≥0.22 mg/L, Youden index was 0.26, sensitivity was 67.01%, and specificity was 58.78%).

found to take part in the vascular complications of diabetes (33). As a coagulation factor, an increased level of plasma fibrinogen was revealed to be closely related to DPN (26). In our present study, plasma D-dimer, a degradation product of fibrinogen, was found to be associated with DPN. We also found that plasma D-dimer, HbA1c, and fibrinogen were comparable in their ability to indicate DPN.

Increased plasma D-dimer levels not only can predict a range of adverse health outcomes in the general population (11, 34) but also can partly account for metabolic diseases and vascular complications with a background of insulin resistance. When compared to the healthy controls, the level of plasma D-dimer was obviously elevated in the first-degree relatives of T2D (35), prediabetes (18), gestational hypertension (36), polycystic ovary syndrome (37), and metabolic syndrome (38), let alone in overt T2D. Moreover, plasma D-dimer was observed to be independently associated with inflammatory cytokines (39), oxidized LDL (40), poor glycemic control (hyperglycemia, glycemic variability, and hypoglycemia) (41–43), diabetic retinopathy and nephropathy (14, 15), and CVD (17) in patients with T2D. In addition, plasma D-dimer levels were also increased in parallel with the differential stages from family history of diabetes to prediabetes to T2D with CVD complications (18), which suggested that plasma D-dimer could be applied to indicate the progressive nature of diabetes and diabetes-related complications. Furthermore, in our present study, increased plasma D-dimer levels were revealed to be associated with increased nerve action potential onset latency and decreased nerve amplitude and NCV in T2D. In addition, patients in the highest quartile of plasma D-dimer presented with a fivefold higher DPN risk than those in the lowest quartile. We also calculated that plasma D-dimer ≥0.22 mg/L was the optimal cutoff value to discriminate DPN, with a sensitivity of 67.01% and a specificity of 58.78%. Our study added evidence to support increased plasma D-dimer in the pathogenesis of diabetic microvascular complications.

Several underlying mechanisms may explain the correlation of increased plasma D-dimer with DPN in T2D. In the context of T2D, hyperglycemic exposure and dyslipidemia together with microvascular disease trigger detrimental downstream oxidative stress (44), mitochondrial damage, and inflammation in peripheral neurons, glial cells, and vascular endothelial cells, all of which may result in impaired nerve function and neuropathy (27, 28). Increased plasma D-dimer reflects a hypercoagulability state (45), which may potentiate microvascular disease and endothelial dysfunction, leading to

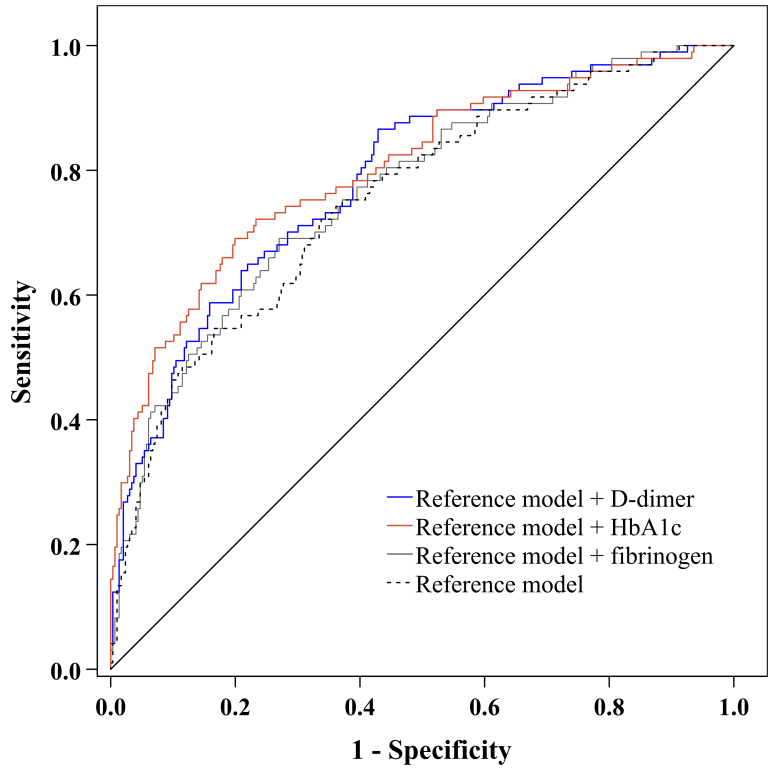


FIGURE 5
ROC curve exhibited the performance of plasma D-dimer to discriminate DPN after adjustment for traditional risk factors.

subsequent insufficient neural blood flow, endoneurial hypoxia, and neuronal damage (28, 33). Plasma D-dimer is also a thromboinflammatory factor (45). A previous study showed that D-dimer can stimulate monocytes *in vitro* to release inflammatory cytokines (such as IL-1β and IL-6), which indicates that D-dimer may facilitate localized coagulation (46). Moreover, increased plasma D-dimer was also found to be independently associated with an inflammatory state (assessed by IL-6) and dyslipidemia in T2D (39, 40), which could contribute to nerve dysfunction and neuropathy. Furthermore, increased plasma D-dimer was also a marker of psychosocial distress (47, 48), which has been shown to participate in the maladaptations of the peripheral and central nervous systems (49, 50).

The present study should be addressed in light of a few limitations. First, our study was cross-sectionally conducted and may not conclude a causal relationship between elevated plasma D-dimer and DPN. We need a longitudinal study to compensate for this defect. Second, we did not find a relationship between increased plasma D-dimer and the severity of DPN evaluated by the Michigan neuropathy screening instrument (MNSI). The possible causes are that the sample size is small (97 cases with DPN), and the MNSI tends to be influenced by subjective factors. Third, the duration of DPN was difficult to clearly define, so the

TABLE 4 Performance of plasma D-dimer to discriminate DPN after adjustment for traditional risk factors.

| Models | AUC (95% CI) | AUC differences (95% CI) | *Z* for AUC differences | *p* for AUC differences |
|---|---|---|---|---|
| Reference model + D-dimer | 0.786 (0.742 to 0.825) | – | | |
| Reference model | 0.754 (0.708 to 0.795) | 0.0324 (0.0008 to 0.0640)[a] | 2.010 | 0.041 |
| Reference model + fibrinogen | 0.767 (0.722 to 0.808) | 0.0193 (–0.0095 to 0.0482)[b] | 1.313 | 0.189 |
| Reference model + HbA1c | 0.802 (0.759 to 0.841) | 0.0164 (–0.0316 to 0.0644)[c] | 0.670 | 0.503 |

The reference model includes age, sex, diabetes duration, BMI, SBP, DBP, hypertension, statin treatment, ALT, lipid profiles, UA, eGFR, fasting C-peptide, fasting glucagon, PT, APTT, and antidiabetic treatments.
[a]Reference model + D-dimer vs. reference model.
[b]Reference model + D-dimer vs. reference model + fibrinogen.
[c]Reference model + D-dimer vs. reference model + HbA1c.

data were not presented and analyzed in our study. Fourth, plasma D-dimer levels were determined by one sampling. However, the plasma D-dimer level could fluctuate during the course of T2D and might be affected by antidiabetic medications. It would be more convincing to present the average data of plasma D-dimer collected within weeks or months. Fifth, the level of plasma D-dimer can be affected by hypoglycemia (43), and we did not assess the effect of hypoglycemic events on the relationship between plasma D-dimer and DPN. Sixth, our results may be affected by the heterogeneities of T2D patients who received multiple antidiabetic agents. We tried our best to compensate for this limitation by adjusting for antidiabetic agents during the statistical analysis.

## Conclusion

In summary, increased plasma D-dimer levels were closely connected to dysfunction of peripheral nerve conduction and prevalence of DPN in T2D and could serve as a promising indicator for DPN in T2D.

## Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

## Ethics statement

The studies involving human participants were reviewed and approved by First People's Hospital of Nantong. The patients/participants provided their written informed consent to participate in this study.

## Author contributions

J-bS and X-qW initiated and acquired funding for the series; LZ, CY, and J-bS designed this study; D-mZ coordinated and supervised the study; CY, LZ, FX, L-hZ, X-hW, C-hW, L-yN, and X-lZ recruited patients and collected the data; LZ, CY, and J-bS analyzed the data and interpreted the results; LZ and CY drafted the manuscript; all authors read, modified, and approved the manuscript.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Publisher's note



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# Advanced glycation end-products are associated with diabetic neuropathy in young adults with type 1 diabetes

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**Aims/hypothesis:** Advanced glycation end-products (AGEs) may contribute to the development of diabetic neuropathy. In young adults with type 1 diabetes, we aimed to investigate the association between AGEs and cardiovascular autonomic neuropathy (CAN) and distal symmetric polyneuropathy (DSPN).

**Methods:** This cross-sectional study comprised 151 young adults. CAN was assessed by cardiovascular autonomic reflex tests; lying-to-standing test, deep breathing test (E/I), Valsalva manoeuvre, and heart rate variability indices; and the mean square of the sum of the squares of differences between consecutive R-R intervals and standard deviation of normal-to-normal intervals (SDNN), high- (HF) and low-frequency (LF) power, total frequency power, and the LF/HF ratio. DSPN was assessed by light touch, pain and vibration perception threshold (VPT), neuropathy questionnaires, and objective measures. AGEs were analysed in four groups using z-scores adjusted for relevant confounders and multiple testing: i) "glycolytic dysfunction", ii) "lipid peroxidation", iii) "oxidative stress", and iv) "glucotoxicity".

**Results:** A higher z-score of "glycolytic dysfunction" was associated with higher VPT (4.14% (95% CI 1.31; 7.04), p = 0.004) and E/I (0.03% (95% CI 0.01; 0.05), p = 0.005), "lipid peroxidation" was associated with higher LF/HF ratio (37.72% (95% CI 1.12; 87.57), p = 0.044), and "glucotoxicity" was associated with lower SDNN (–4.20% (95% CI –8.1416; –0.0896), p = 0.047). No significance remained after adjustment for multiple testing.

**Conclusions/interpretations:** In young adults with type 1 diabetes, increased levels of AGEs involving different metabolic pathways were associated with

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several measures of CAN and DSPN, suggesting that AGEs may play a diverse role in the pathogeneses of diabetic neuropathy.

KEYWORDS

advanced-glycation end-products, AGEs, type 1 diabetes, cardiovascular autonomic neuropathy, peripheral neuropathy, distal symmetric polyneuropathy

# Introduction

Diabetic neuropathy is a leading cause of morbidity and mortality in both type 1 and type 2 diabetes (1, 2). The progressive nature with insidious onset and varying symptoms and clinical manifestations often lead to delayed diagnosis with severe and irreversible symptoms (1). Cardiovascular autonomic neuropathy (CAN) and distal symmetric polyneuropathy (DSPN) are the most prevalent types of diabetic neuropathy with varying prevalence reaching up to 35% and 41%, respectively, in adults with type 1 diabetes (3–7). Previous studies have identified a high prevalence of definite and subclinical manifestations of diabetic neuropathy in young adults with type 1 diabetes (8–12). This suggests that early screening in adolescence may detect early stages of CAN and DSPN, where prevention of progression may still be possible. Although diagnostic methods for both types of neuropathy are available, it remains unknown which underlying mechanisms are involved and lead to painful versus insensate symptoms (1).

Hyperglycaemia and metabolic derangements are suggested to play an important role in the progression of neurological complications (1, 13). One of the pathogenic pathways described is the formation of reactive metabolites leading to increased levels of advanced glycation end-products (AGEs) as a direct consequence of hyperglycaemia and lipid peroxidation (13–15). The accumulation of AGEs tends to alter protein function and thereby the structure of the nerve tissue, contributing to the development of neuropathy (15).

In the serum of diabetic patients, AGEs are elevated alongside levels of their main precursors: glucose and the dicarbonyls methylglyoxal (MG), glyoxal, and 3-deoxyglucosone (3-DG) (13, 16).

While studies regarding the dicarbonyls 3-DG and glyoxal and their corresponding AGEs are scarce (13, 17, 18), MG and MG-derived AGEs (i.e., methylglyoxal-derived hydroimidazolone 1 (MG-H1)) have been associated with the progression of diabetic neuropathy, retinopathy, and nephropathy in type 1 diabetes. Likewise, increased plasma levels of MG and MG-H1 have been associated with diabetic painful peripheral neuropathy in both type 1 and type 2 diabetes (16, 17).

Also, the glucose-derived AGEs, fructose lysine (FL) and glucosepane, have been identified as strong predictors of diabetic neuropathy, supporting that high glucose levels may generally associate with the risk of complications and therefore have been targeted for treatment (1, 17, 19). However, good glycaemic control does not necessarily prevent the progression of diabetic neuropathy (20). Some studies have found increased levels of MG in both type 1 and type 2 diabetes independent of blood glucose levels, suggesting that factors other than hyperglycaemia are involved in late diabetes complications (14).

Although several AGEs have been associated with diabetic neuropathy, identifying specific pathways involved in the pathogeneses of neuropathy is missing. Furthermore, diabetic neuropathy represents a collection of syndromes, and therefore, subtypes of neuropathy symptoms may be related to different dysfunctional pathways.

In this cross-sectional study, we aim to define the metabolic pathways leading to the formation or accumulation of AGEs and relate them to measures of CAN and DSPN and thereby to specify potential pathways associated with distinct neuropathy signs and/or symptoms. This will be performed in a Danish population of young adults with type 1 diabetes using objective sensitive age-matched measuring methods to detect even early signs of neuropathy.

# Methods

## Study design and study population

The study was designed as a cross-sectional observational study and has previously been described in detail (8). In brief, inclusion criteria were type 1 diabetes and age >17 and <25 years. The patients were recruited from the outpatient clinic at Steno Diabetes Center Copenhagen, Gentofte, Denmark. Of the 340 eligible participants who received an invitation to participate, 156 were accepted. Five participants were excluded due to missing biochemical measures, leaving 151 included in the study. Ethical approval was obtained from the Danish Research Ethics Committee (ID No. H-15006967), and written

informed consent was obtained from all patients prior to examination.

## Measures of diabetic neuropathy

Measures of DSPN were assessed and categorized according to recommendations made by the Toronto Diabetic Neuropathy Expert Group (2) and included the following:

- -Symptoms of DSPN were assessed by the questionnaires Brief Pain Inventory (BPI) and Michigan Neuropathy Screening Instrument (MNSI). Diabetic neuropathy was, in the BPI questionnaire, defined as the presence of pain in both legs and/or both arms peripherally and, in the MNSI questionnaire, as a score of ≥7 (21).
- -Signs of DSPN were assessed by established measures including light touch perception using a 10-g monofilament, pain perception using a pinprick device, and vibration perception threshold (VPT) determined by using a biothesiometer. To assess abnormal VPT tests, age-stratified perception thresholds were used (12). The tests were pathological if the results were abnormal bilaterally.
- -Objective measuring methods using the non-invasive device Sudoscan™ to test for electrochemical skin conductance (ESC) on hands and feet and the handheld NC-stat® to measure sural nerve conduction velocity (SNCV) and sural nerve amplitude potential (SNAP).

To identify abnormal results for the ESC test and measures of SNCV and SNAP, age- and gender-stratified thresholds (22) and age- and height-stratified thresholds were applied, respectively. When abnormalities in SNCV, SNAP, or both bilaterally were found, the measure of sural nerve conduction (SNC) was used.

The definition of DSPN was stratified into four categories: "possible DSPN" if the patient had symptoms or signs according to the abovementioned measures, "probable DSPN" in the presence of symptoms and signs, and "confirmed DSPN" if the patient had a combination of either abnormal test for SNC or ESC and the presence of either symptoms or signs. The presence of abnormal SNC or ESC without symptoms or signs was defined as "subclinical DSPN".

CAN was evaluated by three standard cardiovascular autonomic reflex tests (CARTs) and measures of 5-min resting heart rate variability (HRV) using the device Vagus™ and performed in a quiet examination room in the afternoon.

After 5 min of supine rest, HRV measures were analysed in time and frequency domain from 5-min resting heart rate (HR). Time domain included the mean square of the sum of the squares of differences between consecutive R-R intervals (RMSSD) and standard deviation of normal-to-normal intervals (SDNN). Frequency-domain analyses included low-frequency power band (LF), high-frequency power band (HF), total frequency power (Total), and the ratio of low-frequency power/high-frequency power (LF/HF ratio).

After the 5-min resting HRV test, the three CARTs for diagnosing CAN were performed and included the lying-to-standing test (30:15), the deep breathing test (E/I), and the Valsalva manoeuvre (23). The participants were asked to restrain from vigorous exercise 24 h prior to the examination and intake of caffeine on the specific day of examination.

The diagnosis of "early CAN" and "definite CAN" was given according to age-stratified thresholds (3) if one of the three CARTs was abnormal and if two or three tests were abnormal, respectively.

## Dicarbonyls and advanced glycation end-products

The plasma levels of AGEs were grouped according to the main pathways of AGE formation (13) that proceed *via* glucose and the reactive dicarbonyls MG, glyoxal, and 3-DG:

i. "Glycolytic dysfunction" includes the AGEs derived from methylglyoxal: MG-H1, $N^{\epsilon}$-(carboxyethyl)-lysine (CEL), methylglyoxal-lysine dimers (MOLD), and argpyrimidine. As methylglyoxal comes from glycolysis, increased methylglyoxal and methylglyoxal-derived AGEs will represent a dysregulation of glycolysis.
ii. "Lipid peroxidation" includes the glyoxal-derived AGEs: glyoxal-derived hydroimidazolone 1 (G-H1) and $N^{\epsilon}$-(carboxymethyl)-lysine (CML). Glyoxal and glyoxal-derived AGEs derive from the degradation of lipids, and therefore increased levels of glyoxal and glyoxal-derived AGEs will represent increased oxidation of lipids.
iii. "Oxidative stress" includes AGEs generated from the direct interaction of reactive oxygen species (ROS) with methionine: methionine sulfoxide.
iv. "Glucotoxicity" includes AGEs and dicarbonyls derived directly from the modification of amino acids by glucose: fructose lysine (FL), glucosepane, and 3-DG.

v. "Dicarbonyls": MG, glyoxal, and 3-DG. The dicarbonyls were also analysed separately in a fifth group:

## Clinical data collection

As described previously, data on medication were extracted from hospital electronic records, and lifestyle factors including smoking status and weekly amount of exercise were recorded in a questionnaire filled in by the patients.

Blood pressure and heart rate were measured after 10 min of rest using automated oscillometric blood pressure recorders and calculated as the mean of three consecutive measures performed with intervals of 1 min. Height and weight were measured with clothes on and without shoes using a fixed rigid stadiometer and an electronic scale, respectively.

## Biochemical measures

$HbA_{1c}$, serum total cholesterol, serum high-density lipoprotein (HDL) cholesterol, serum triglycerides, and plasma creatinine were measured from non-fasting venous blood samples and collected on the examination day. $HbA_{1c}$ was analysed by high-performance liquid chromatography on a Tosoh G7 (Tosoh Corporation, Tokyo, Japan). Triglycerides, HDL, and total cholesterol were analysed by standard enzymatic colorimetry techniques on a Vitros 5600 (Ortho Clinical Diagnostics, Illkirch-Graffenstaden, France). Serum LDL cholesterol was calculated using the Friedewald equation.

All biochemical measures were analysed in the laboratory at Steno Diabetes Center, Denmark, except for the plasma samples used for the analysis of AGEs and dicarbonyls. The samples were stored at −80°C at Steno Diabetes Center and transferred on dry ice to the laboratory at the University of Heidelberg, Germany, for analysis.

## Assessment of glycaemic variability indices

As described previously, the continuous glucose monitoring (CGM) sensor Enlite™ (Medtronic, Northridge, CA, USA) was inserted into the subcutaneous tissue of the abdomen or the upper arm and was worn for 5 days (24). The capillary finger blood glucose was monitored four times daily for calibration. To generate data from the sensors, the software Medtronic Carelink™ iPro™ was used. To quantify glycaemic variability, coefficient of variation (CV), standard deviation (SD), continuous overall net glycaemic action, and mean amplitude of glucose excursions were used. Time spent in hypoglycaemia (<3.0 mmol/L), euglycaemia (≥3.0; ¾10.0 mmol/L), and hyperglycaemia (>10.0 mmol/L) were calculated and presented in minutes and percentage.

## Measurement of dicarbonyls

The dicarbonyl content in plasma was determined by isotope dilution and tandem mass spectroscopy, following derivatization with 1,2-diaminobenzene. Briefly, 20 µl of serum was precipitated by addition of 10 µl of ice-cold 20% (wt/vol) trichloroacetic acid in 0.9% (wt/vol) sodium chloride (20 µl) and water (40 µl) (25). An aliquot (5 µl) of the internal standard (400 nM of [$^{13}C_2$]-Glyoxal, [$^{13}C_3$]-methylglyoxal, and [$^{13}C_6$]-3-deoxyglucosone) was then added, and the samples vortex-mixed. Following centrifugation (14,000 rpm; 5 min at 4°C), 35 µl of the supernatant was transferred to high-resolution mass spectrometry (HPLC) vials containing a 200-µl glass interest. An aliquot (5 µl) of 3% sodium azide (wt/vol) was then added to each sample followed by 10 µl of 0.5 mM of DB in 200 mM of HCl containing 0.5 mM of diethylenetriaminepentaacetic acid (DETAPAC) in water. The samples were then incubated for 4 h at room temperature, protected from the light. Samples were then analysed by liquid chromatography–tandem mass spectrometry (LC-MS/MS) using an ACQUITY™ ultra-high-performance liquid chromatography system with a Xevo-TQS LC-MS/MS mass spectrometer (Waters, Manchester, UK). The column was a Waters BEH C18 (100 × 2.1 mm) and a guard column (5 × 2.1 mm). The mobile phase was 0.1% formic acid in water with a linear gradient of 0%–100% 0.1% formic acid in 50% acetonitrile:water over 0–10 min; the flow rate was 0.2 ml/min, and the column temperature was 5°C. The capillary voltage was 0.5 kV; the cone voltage was 20 V; the interscan delay time was 100 ms; the source and desolvation gas temperatures were 150°C and 350°C, respectively; the cone gas and desolvation gas flows were 150 and 800 L/h, respectively. Mass transitions (parent ion > fragment ion; collision energy), retention time, limit of detection, and recoveries were as follows: 3-deoxyglucosone, 235.0 > 199.1; 14 eV, 4.09 min, 0.36 pmol, 95%, glyoxal, 130.9 > 77.1; 22 eV, 5.28 min, 1.15 pmol, 97%, methylglyoxal, 145.0 > 77.1; 24 eV, 5.93 min, 0.52 pmol, 98%. Acquisition and quantification were completed with MassLynx 4.1 and TargetLynx 2.7 (Waters®).

## Measurement of protein-free advanced glycation end-products

Protein-free AGEs in the plasma were determined by isotope dilution and tandem mass spectroscopy, as previously

described (26). Briefly, an aliquot of plasma (20 µl) was diluted to 500 µl with water and filtered by microspin ultrafiltration (10 kDa cutoff) at 14,000 rpm for 30 min at 4°C. The ultrafiltrate was then retained for the free adduct analysis. An aliquot of the sample (ca. 30 µl) was spiked with an equal volume of 0.2% trifluoroacetic acid (TFA) in water containing the isotopic standards (5–25 pmol). Normal and isotopic standards were either purchased (Cambridge Isotope, Polypeptide Laboratories, Iris Biotech) or prepared in-house, as described previously. Samples were then analysed by LC-MS/MS using an ACQUITY ultra-high-performance liquid chromatography system with a Xevo-TQS LC-MS/MS spectrometer (Waters). Two 5-µm Hypercarb™ columns (Thermo Scientific, Waltham, MA, USA) in series were used: 2.1 × 50 mm, fitted with a 5 × 2.1 mm pre-column, and 2.1 × 250 mm. The mobile phases were 0.1% TFA in water and 0.1% TFA in 50% water. The column temperature and flow rates were 30°C and 0.2 ml/min, respectively. Analytes were eluted using a two-step gradient, and the columns were washed after each sample with 0.1% TFA in 50% tetrahydrofuran (THF), as described previously (26). AGEs, including oxidation and nitration markers, were detected by electrospray positive ionization with multiple reaction monitoring (MRM). The ionization source temperature was 150°C, and the desolvation temperature was 500°C. The cone gas and desolvation gas flows were 150 and 1,000, L/h, respectively. The capillary voltage was 0.5 kV. Molecular ion and fragment ion masses, as well as cone voltage and collision energy, were optimized to ±0.1 Da and ±1 eV for MRM detection of the analytes. Acquisition and quantification were completed with MassLynx 4.1 and TargetLynx 2.7 (Waters®).

## Statistical analysis

Patient characteristics are represented as means with SD for normally distributed continuous data, as medians with interquartile range (IQR) for skewed distributed data, and as numbers (%) for categorical data.

A standardized z-score was calculated for each of the following groups of AGEs and dicarbonyls and was examined as a determinant for neuropathy: i) "glycolytic dysfunction" (MG-H1, CEL, and MOLD), ii) "lipid peroxidation" (G-H1 and CML), iii) "oxidative stress" (methionine sulfoxide), iv) "glucotoxicity" (FL, glucosepane, and 3-DG), and v) "dicarbonyls" (MG, glyoxal, and 3-DG).

Outcomes were all continuous, assessed using linear regression analyses, and presented as estimates of a one-unit difference in z-score with 95% CI.

Three models of adjustments were applied: model 1 was adjusted for age and gender, model 2 was adjusted as model 1 + for diabetes duration and $HbA_{1c}$, and model 3 was adjusted as model 2 + for current smoking, total cholesterol, triglycerides, systolic blood pressure, and the use of beta blockers.

All analyses used two-sided p = 0.05 as statistically significant and were adjusted for multiple tests by the Benjamini–Hochberg procedure (27).

Statistical analyses were performed in SAS 9.4 (SAS Institute, Cary, NC, USA).

# Results

## Patient characteristics

The study population included 151 patients (42.4% men) with a mean age of 22 years (SD 1.6). The mean diabetes duration was 11 years (SD 5.1), and $HbA_{1c}$ was 66.5 mmol/mol (IQR 58.0; 77.0). Seventy-two patients (47.7%) were treated with continuous subcutaneous insulin infusion (CSII), and the rest were treated with multiple-dose injections (MDIs). All patients were on insulin (Table 1).

Previously, we investigated the association between glycaemic variability and diabetic neuropathy in the same study population (24). Of the included patients, 133 had CGM. During the 5-day CGM monitoring, mean (SD) CV was 40% (10), median (IQR) SD was 3.9 mmol/L, and time spent on hypoglycaemia was 35 min (0, 120)/1.0% [0.0; 4.0].

The prevalence of subclinical and possible DSPN was 54.3% and 3.3%, respectively. DSPN was diagnosed in 2.7%, and none met the criteria for probable DSPN. The prevalence of CAN and early CAN was 8.7% and 28.4%, respectively.

The characteristics of the study population are shown in Table 1.

## Dicarbonyls and advanced glycation end-products

The mean plasma level of MG, glyoxal, and 3-DG was 116.3 nmol/L (111.3; 127.6), 215.7 nmol/L (186.1; 253.4), and 57.8 nmol/L (54.3; 62.6), respectively.

The associations between groups of AGEs and measures of CAN and DSPN are presented in Figures 1–5 with forest plots as estimates and 95% CI.

## Cardiovascular autonomic neuropathy measures

A higher z-score of "glycolytic dysfunction" was associated with a higher E/I also when adjusted in model 3 (0.03% (95% CI 0.01; 0.05), p = 0.005) (Figure 1).

"Lipid peroxidation" was associated with a higher LF/HF ratio. Further adjustment in model 3 did not affect the association (37.72% (95% CI 1.12; 87.57), p = 0.044) (Figure 2).

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TABLE 1 Characteristics of the study population.

| Clinical characteristics | Mean (SD)/median (IQR)/N (%) |
|---|---|
| N | 151 |
| Age (years) | 22 (1.6) |
| Male (%) | 64 (42.4) |
| Diabetes duration (years) | 11.3 (5.1) |
| CSII treatment (%) | 72 (47.7) |
| Current smoker (%) | 33 (21.9) |
| Systolic blood pressure (mmHg) | 125.3 (11.5) |
| $HbA_{1c}$ (mmol/mol) | 67.0 (58.0;77.0) |
| $HbA_{1c}$ (%) | 8.3 (7.4; 9.2) |
| Cholesterol (mmol/L) | 4.5 (1.2) |
| Triglycerides (mmol/L) | 1.1 (0.8; 1.6) |
| LDL (mmol/L) | 2.6 (0.9) |
| Urine albumin/creatinine ratio (mg/g) | 6 (4.0; 14.0) |
| eGFR (ml/min/1.73 $m^3$) | 123.9 (116.6; 129.3) |
| *Medication* | |
| Insulin (%) | 151 (100) |
| Metformin (%) | 1 (0.7) |
| Other glucose-lowering drugs (%) | 1 (0.7) |
| Antihypertensive treatment (%) | 6 (4.0) |
| Beta-blocker treatment n (%) | 2 (1.3) |
| Lipid-lowering treatment (%) | 2 (1.3) |
| Psychotropics (%) | 6 (4.0) |
| *CAN measures* | |
| Definite CAN (%) | 13 (8.7) |
| Early CAN (%) | 42 (28.4) |
| *DSPN measures* | |
| Confirmed DSPN (%) | 4 (2.7) |
| Subclinical DSPN (%) | 82 (54.3) |
| Probable DSPN (%) | 0 (0) |
| Possible DSPN (%) | 5 (3.3) |
| *BPI questionnaire* | |
| Painful neuropathy (% answered yes) | 3 (2.0) |
| *MNSI questionnaire* | |
| MNSI neuropathy (score ≥ 7 points) | 0 (0) |
| *Serum dicarbonyls levels* | |
| Methylglyoxal (nmol/L) | 116.3 (111.3; 127.6) |
| Glyoxal (nmol/L) | 215.7 (186.1; 253.4) |
| 3-Deoxyglucosone (nmol/L) | 57.8 (54.3; 62.6) |

Data are given in means (SD), medians (IQR), or proportions %.
SD, standard deviation; IQR, interquartile range; CSII, continuous subcutaneous insulin infusion; LDL, low-density lipoproteins; eGFR, estimated glomerular filtration rate; CAN, cardiovascular autonomic neuropathy; DSPN, distal symmetric polyneuropathy; BPI, Brief Pain Inventory; MNSI, Michigan Neuropathy Screening Instrument.

A higher z-score of "glucotoxicity" was associated with lower values of the HRV indices SDNN, RMSSD, LF, HF, and total power in model 1 (Figure 3). Only for SDNN did significance remain after further adjustment in model 3 (−4.20% (95% CI −8.14; −0.09), p = 0.047). "Dicarbonyls" was also associated with lower values of the HRV indices. However, significance was lost in models 2 and 3 (Figure 4). In addition, higher z-scores of "glucotoxicity" and "dicarbonyls" were both associated with higher HR when fully adjusted in model 3 (1.31% (95% CI 0.16; 2.47), p = 0.027) and 1.51% (95% CI 0.54; 2.47), p = 0.003), respectively).

When applying the Benjamini–Hochberg procedure to account for multiple testing, all associations found between the groups of AGEs and dicarbonyls and measures of CAN lost significance.

No associations were found between measures of "oxidative stress" and any measures of cardiovascular autonomic neuropathy (Figure 5).

## Peripheral neuropathy measures

A higher z-score of "glycolytic dysfunction" was associated with a higher VPT in all three models (4.14% (95% CI 1.31; 7.04), p = 0.004) (Figure 1), but not when correcting for multiple tests. "Lipid peroxidation" was also associated with a higher VPT in models 1 and 2, but not when fully adjusted in model 3 (12.90% (95% CI −2.30; 30.45), p = 0.102) (Figure 2).

A higher z-score of "glucotoxicity" was associated with higher SNCV. However, the associations lost significance after adjustment in models 2 and 3 (Figure 3).

No associations were found between measures of peripheral neuropathy and "oxidative stress" or "dicarbonyls".

# Discussion

In this cross-sectional study of 151 young adults with type 1 diabetes, we found that a number of pathways leading to AGE formation are activated in diabetic neuropathy. However, the relative contribution of glucose- and lipid-derived AGEs varied between the different measures of diabetic neuropathy, suggesting that both CAN and DSPN represent multiple symptoms involving more than one dysfunctional pathway. We found that increased levels of methylglyoxal- and glyoxal-derived AGEs representing changes in glycolytic function and lipid peroxidation, respectively, were associated with higher vibration perception thresholds. For CAN measures, we found that increased "glucotoxicity",

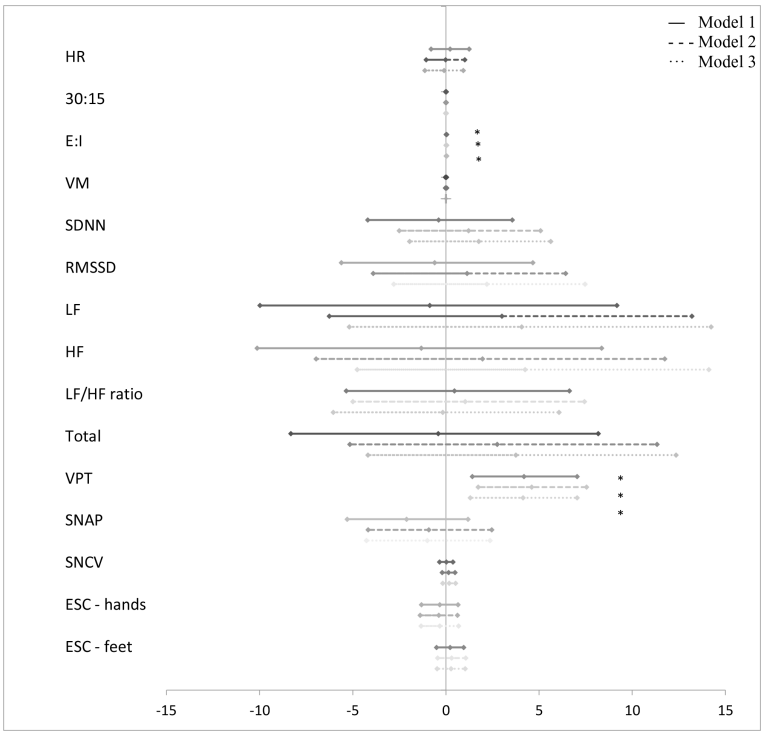


FIGURE 1
Forest plot of the associations between "glycolytic dysfunction" and measures of diabetic neuropathy. Results are presented as estimates and 95% confidence intervals. Estimates show the percentage change in the outcomes for an increase of one unit of "glycolytic dysfunction". Studies with confidence interval crossing the vertical line are inconclusive. Model 1 adjusted for age and gender, model 2 adjusted as model 1 + diabetes duration and $HbA_{1c}$, and model 3 adjusted as model 2 + current smoking, total cholesterol, triglycerides, systolic blood pressure, and the use of beta blockers. CAN, cardiovascular autonomic neuropathy; HR, heart rate; 30:15, lying-to-standing test; E:I, deep breathing test; VM, Valsalva Manoeuvre; SDNN, standard deviation of normal-to-normal intervals; RMSSD, root mean square of the sum of the squares of differences between consecutive R-R intervals; LF, low-frequency power; HF, high-frequency power; DSPN, distal symmetric polyneuropathy; VPT, vibration perception threshold; SNAP, sural nerve amplitude potential; SNCV, sural nerve conduction velocity; ESC, electrochemical skin conduction. *p < 0.05.

representing glucose-derived AGEs, was associated with higher HR and lower SDNN. "Dicarbonyls" was also associated with higher HR. However, after adjustments for multiple testing, no significant associations remained.

Previous studies in experimental models of diabetes as well as in diabetic humans have emphasized the importance of AGEs and their precursors, the reactive dicarbonyls, for the development of diabetic complications (13, 28). In diabetic neuropathy, disturbances in metabolic pathways caused by hyperglycaemia are believed to play a causative role. The higher glucose flux leads to increased formation of dicarbonyls mainly from glycolysis intermediates and lipid peroxidation (13). The formation and accumulation of reactive dicarbonyls are expected to be higher in neuronal tissue, as its primary source of energy is glucose (28). Moreover, increased formation of reactive oxygen species (ROS) leading to oxidative stress may contribute to neuronal dysfunction as ROS indirectly inhibits glyoxalase 1 (GLO-1) activity, which plays a role in the metabolization of dicarbonyl species (28).

In type 2 diabetes, studies have not presented uniform results when investigating the association between the reactive dicarbonyl, methylglyoxal, and diabetic neuropathy. Increased levels of plasma methylglyoxal have been associated with diabetic painful peripheral neuropathy by modifications of the voltage-gated nociceptor-specific sodium channel Nav1.8, which is associated with enhanced electrical excitability and facilitates firing of nociceptive neurons, thereby resulting in hyperalgesia (16). A recent prospective observational study of 1,256 new-onset type 2 diabetes patients found that higher levels of methylglyoxal are a risk factor for the development of diabetic polyneuropathy as assessed by the MNSI questionnaire (29). However, no association between serum methylglyoxal and

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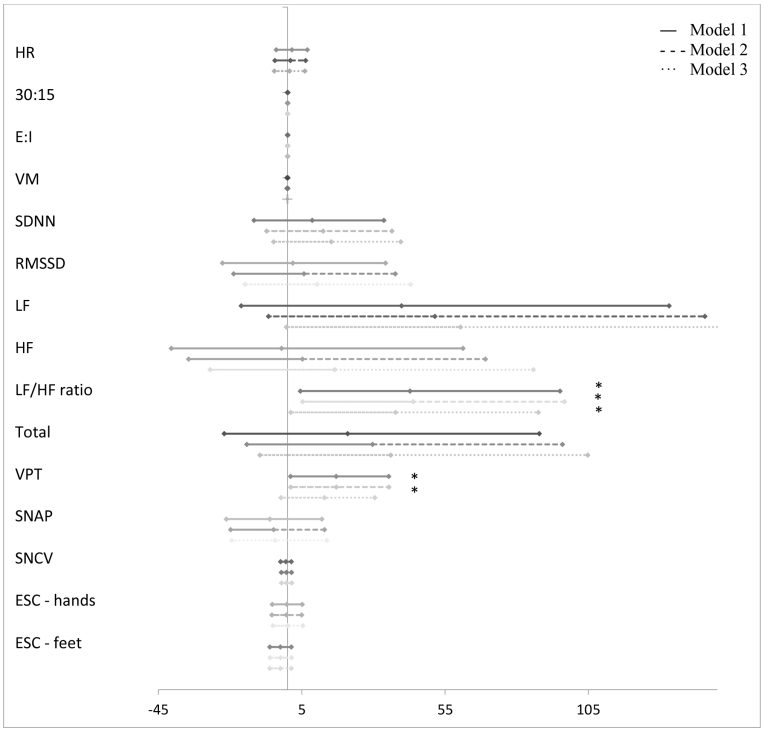


FIGURE 2
Forest plot of the associations between "lipid peroxidation" and measures of diabetic neuropathy. Results are presented as estimates and 95% confidence intervals. Estimates show the percentage change in the outcomes for an increase of one unit of "lipid peroxidation". Studies with confidence interval crossing the vertical line are inconclusive. Model 1 adjusted for age and gender, model 2 adjusted as model 1 + diabetes duration and $HbA_{1c}$, and model 3 adjusted as model 2 + current smoking, total cholesterol, triglycerides, systolic blood pressure, and the use of beta blockers. CAN, cardiovascular autonomic neuropathy; HR, heart rate; 30:15, lying-to-standing test; E:I, deep breathing test; VM, Valsalva Manoeuvre; SDNN, standard deviation of normal-to-normal intervals; RMSSD, root mean square of the sum of the squares of differences between consecutive R-R intervals; LF, low-frequency power; HF, high-frequency power; DSPN, distal symmetric polyneuropathy; VPT, vibration perception threshold; SNAP, sural nerve amplitude potential; SNCV, sural nerve conduction velocity; ESC, electrochemical skin conduction. *p < 0.05.

diabetic autonomic and peripheral neuropathy was found in the cross-sectional study at the 6-year follow-up in the same cohort (30). Thus, increased methylglyoxal levels may play a causal role in painful neuropathy but not necessarily the other symptoms of diabetic neuropathy.

In our study, "dicarbonyls" were only associated with higher HR, but no other measures of CAN or DSPN. However, plasma levels of dicarbonyls might not represent dicarbonyl levels in nervous tissue, as it is shown in animal models that the activity of GLO-1 is low in nervous tissue compared to others (28). In addition, AGEs derived from methylglyoxal as well as other dicarbonyl species might play a major role. In line with this, we found that increased "lipid peroxidation", although not statistically significant when fully adjusted, and "glycolytic dysfunction" representing glyoxal- and methylglyoxal-derived AGEs, respectively, were positively associated with worse vibration perception threshold. Previous clinical studies support the role of methylglyoxal-derived AGEs in diabetic neuropathy. A prospective cohort study of 216 humans with type 1 diabetes found that skin levels of AGEs were associated with the progression of diabetic complications including neuropathy (17, 19). The correlation of MG-H1 with diabetic neuropathy remained strongly associated when adjusted for all other risk factors. In addition, increased serum levels of MG-H1 have been associated with foot heat and pain detection threshold in patients with long-standing type 1 diabetes (18). Furthermore, increased serum levels of the glyoxal-derived AGE, CML, were associated with the development of small-fibre dysfunction. Despite studies supporting the role of methylglyoxal- and glyoxal-derived AGEs in diabetic neuropathy (17–19), it remains unknown which pathway is causing which symptom, and it does not

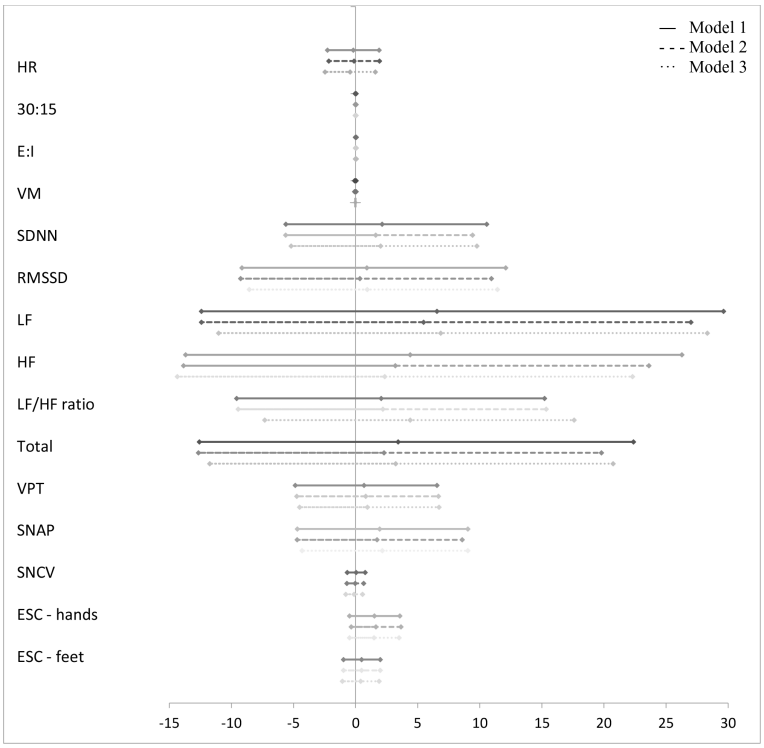


FIGURE 3
Forest plot of the associations between "glucotoxicity" and measures of diabetic neuropathy. Results are presented as estimates and 95% confidence intervals. Estimates show the percentage change in the outcomes for an increase of one unit of "glucotoxicity". Studies with confidence interval crossing the vertical line are inconclusive. Model 1 adjusted for age and gender, model 2 adjusted as model 1 + diabetes duration and $HbA_{1c}$, and model 3 adjusted as model 2 + current smoking, total cholesterol, triglycerides, systolic blood pressure, and the use of beta blockers. CAN, cardiovascular autonomic neuropathy; HR, heart rate; 30:15, lying-to-standing test; E:I, deep breathing test; VM, Valsalva Manoeuvre; SDNN, standard deviation of normal-to-normal intervals; RMSSD, root mean square of the sum of the squares of differences between consecutive R-R intervals; LF, low-frequency power; HF, high-frequency power; DSPN, distal symmetric polyneuropathy; VPT, vibration perception threshold; SNAP, sural nerve amplitude potential; SNCV, sural nerve conduction velocity; ESC, electrochemical skin conduction. *p < 0.05.

explain why one symptom of neuropathy is more distinct than the others in patients with diabetes, as the studies do not discriminate the pathways involved; thus, the differences observed may be spurious.

In our study, "glucotoxicity" was inversely associated with several HRV indices, indicating autonomic dysfunction. However, most associations disappeared once adjusted for relevant confounders. Only a significant and consistent association between lower SDNN and higher resting HR remained. Also, "dicarbonyls" was associated with higher resting HR, while "lipid peroxidation" was associated with a higher LF/HF ratio. These findings support our hypothesis that a number of dysfunctional pathways are involved in CAN and implicate that the reactive metabolites are detrimental to the autonomic nervous system. However, all the associations were insignificant when adjusted for multiple testing.

Thus, our study indicates that AGE formation involving different dysfunctional pathways seems to play a diverse role in the pathogeneses of early diabetic neuropathy in young adults with type 1 diabetes in line with the relatively few previous studies in both type 1 and type 2 diabetes. This supports the hypothesis that metabolic derangements, rather than hyperglycaemia per se, may be the main cause contributing to diabetic complications.

## Strengths and limitations

A limitation of this study is the relatively low prevalence of definite CAN and DSPN in this population of young adults with type 1 diabetes, which might affect the ability to detect associations between AGEs and measures of neuropathy. However, objective measures of neuropathy assessed by novel and established methods of detecting CAN and DSPN have been applied, which is a strength of our study, as we have detected early stages of neuropathy. Further, we have grouped the AGEs

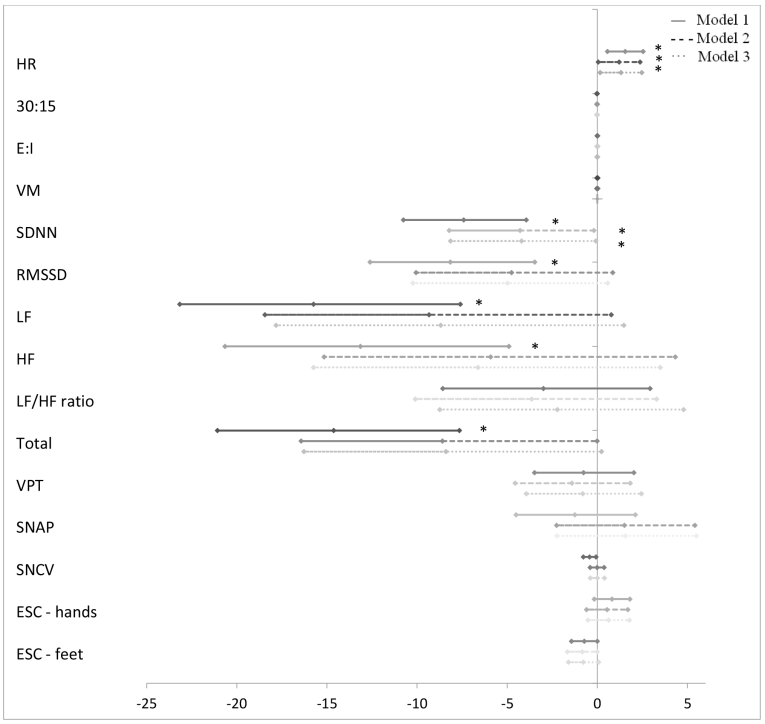


FIGURE 4
Forest plot of the associations between "dicarbonyls" and measures of diabetic neuropathy. Results are presented as estimates and 95% confidence intervals. Estimates show the percentage change in the outcomes for an increase of one unit of "dicarbonyls". Studies with confidence interval crossing the vertical line are inconclusive. Model 1 adjusted for age and gender, model 2 adjusted as model 1 + diabetes duration and $HbA_{1c}$, and model 3 adjusted as model 2 + current smoking, total cholesterol, triglycerides, systolic blood pressure, and the use of beta blockers. CAN, cardiovascular autonomic neuropathy; HR, heart rate; 30:15, lying-to-standing test; E:I, deep breathing test; VM, Valsalva Manoeuvre; SDNN, standard deviation of normal-to-normal intervals; RMSSD, root mean square of the sum of the squares of differences between consecutive R-R intervals; LF, low-frequency power; HF, high-frequency power; DSPN, distal symmetric polyneuropathy; VPT, vibration perception threshold; SNAP, sural nerve amplitude potential; SNCV, sural nerve conduction velocity; ESC, electrochemical skin conduction. *$p < 0.05$.

with respect to their main precursors and thereby being able to specify potential pathways involved in the pathogeneses of neuropathy. Causal conclusions are, however, difficult to make due to the cross-sectional design of our study. The small sample size is another limitation and might affect the power of our study, thereby not making it generalizable. In addition, the significance of associations was lost when adjusting for multiple testing, indicating that the population size limited the power of analyses. Also, a healthy control group in our study would have enabled a comparison with age- and gender-matched non-diabetic young adults. However, this was not in the scheme of the study.

It is also recommended that participants avoid smoking, several drugs, and meals before the CARTs and HRV test, but we did not meet these recommendations, which may therefore affect CAN measures.

## Conclusion

In young adults with type 1 diabetes, we found that "glycolytic dysfunction", "lipid peroxidation", and "glucotoxicity", all pathways leading to AGE formation, are involved in both CAN and DSPN. However, the involvement of the pathways varied between measures of CAN and DSPN, which reflects the diverse nature of both types of neuropathy. Thus, our study indicates that AGEs mainly derived from changes in glycolytic function and lipid peroxidation may contribute to decreased vibration sensation, while AGEs derived from glucose are related to autonomic dysfunction.

Despite the lost associations after adjustments for multiple testing, our results indicate a possible association between some AGEs and different measures of neuropathy in a relatively young

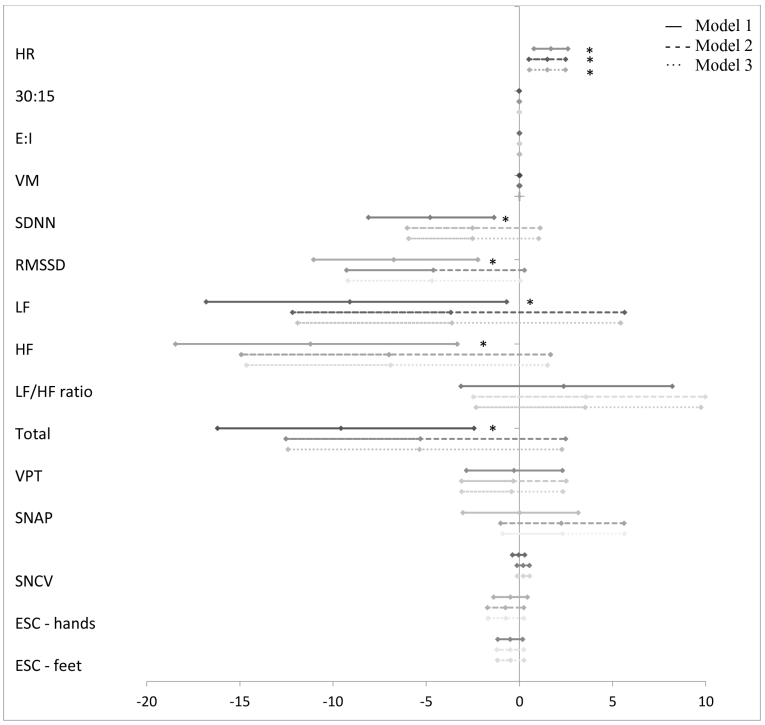


FIGURE 5
Forest plot of the associations between "oxidative stress" and measures of diabetic neuropathy. Results are presented as estimates and 95% confidence intervals. Estimates show the percentage change in the outcomes for an increase of one unit of "oxidative stress". Studies with confidence interval crossing the vertical line are inconclusive. Model 1 adjusted for age and gender, model 2 adjusted as model 1 + diabetes duration and $HbA_{1c}$, and model 3 adjusted as model 2 + current smoking, total cholesterol, triglycerides, systolic blood pressure, and the use of beta blockers. CAN, cardiovascular autonomic neuropathy; HR, heart rate; 30:15, lying-to-standing test; E:I, deep breathing test; VM, Valsalva Manoeuvre; SDNN, standard deviation of normal-to-normal intervals; RMSSD, root mean square of the sum of the squares of differences between consecutive R-R intervals; LF, low-frequency power; HF, high-frequency power; DSPN, distal symmetric polyneuropathy; VPT, vibration perception threshold; SNAP, sural nerve amplitude potential; SNCV, sural nerve conduction velocity; ESC, electrochemical skin conduction. *p < 0.05.

population with a modest prevalence of neuropathy. This suggests that AGEs even in the early stages of diabetes may play a diverse role in the pathogeneses of different types of diabetic neuropathy.

## Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## Ethics statement

This study was reviewed and approved by The Danish Research Ethics Committee (Id. No.:H-15006967). The patients/participants provided their written informed consent to participate in this study.

## Author contributions

EA-S analysed and interpreted the data and drafted the article. MC contributed to the design of the study, acquired and interpreted data, drafted the article, and approved the final version to be published. PN analysed blood samples for AGEs, interpreted data, revised the article critically, and approved the final version to be published. TF analysed blood samples for AGEs, interpreted data, revised the article critically, and approved the final version to be published. EH contributed to the design of the study, analysed and interpreted data, revised the article critically, and approved the final version to be published. MJ contributed to the design of the study, analysed and interpreted data, revised the article critically, and approved the final version to be published. JF contributed to the design of the study, analysed and interpreted data, revised the article critically, and approved the final version to be published. CH contributed to the design of the study and acquisition, analysis, and interpretation of data. He revised the article critically and

approved the final version to be published. All authors contributed to the article and approved the submitted version.

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EA-S is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

## Conflict of interest

JF holds stocks in Medicus Engineering. EH, MC and MJ hold shares in Novo Nordisk AS. MJ has received research grants from AMGEN, Astra Zeneca, Boehringer Ingelheim, Novo Nordisk, and Sanofi Aventis.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Publisher's note



## Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.891442/full#supplementary-material

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# Implementation of corneal confocal microscopy for screening and early detection of diabetic neuropathy in primary care alongside retinopathy screening: Results from a feasibility study

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**Objective:** Screening for diabetic peripheral neuropathy (DPN) is essential for early detection and timely intervention. Quantitative assessment of small nerve fiber damage is key to the early diagnosis and assessment of its progression. Corneal confocal microscopy (CCM) is a non-invasive, in-vivo diagnostic technique that provides an accurate surrogate biomarker for small-fiber neuropathy. In this novel study for the first time, we introduced CCM to primary care as a screening tool for DPN alongside retinopathy screening to assess the level of neuropathy in this novel cohort.

**Research design and methods:** 450 consecutive subjects with type 1 or type 2 diabetes attending for annual eye screening in primary care optometry settings underwent assessment with CCM to establish the prevalence of sub-clinical diabetic peripheral neuropathy. Subjects underwent assessment for neurological and ocular symptoms of diabetes and a history of diabetic foot disease, neuropathy and diabetic retinopathy (DR).

**Results:** CCM examination was completed successfully in 427 (94.9%) subjects, 22% of whom had neuropathy according to Diabetic Neuropathy Symptom (DNS) score. The prevalence of sub-clinical neuropathy as defined by abnormal corneal nerve fiber length (CNFL) was 12.9%. In the subjects with a short duration of type 2 diabetes, 9.2% had abnormal CNFL. CCM showed significant abnormalities in corneal nerve parameters in this cohort of subjects with

reduction of corneal nerve fiber density (CNFD, p<0.001), CNFL (p<0.001) and corneal nerve branch density (CNBD, p<0.001) compared to healthy subjects. In subjects who had no evidence of DR (67% of all subjects), 12.0% had abnormal CNFL.

**Conclusions:** CCM may be a sensitive biomarker for early detection and screening of DPN in primary care alongside retinopathy screening.

KEYWORDS

screening, diabetic neuropathy, corneal confocal microscopy, early detection, diagnosis

## Introduction

Screening for microvascular complications of diabetes is essential if we are to tackle its devastating complications through early detection and timely intervention. Diabetic retinopathy (DR) screening in the UK has been rated one of the most successful screening programs, in which the NHS offers annual digital fundus photography to all people with diabetes over the age of 12 years (1). This has resulted in diabetes no longer being the leading cause of blindness in the working population among all developed countries (2).

Diabetic peripheral neuropathy (DPN) is the most common and costly diabetes-associated complication, occurring in around 50% of individuals with diabetes (3). It is the strongest initiating risk factor for diabetic foot ulceration. In the UK, people with diabetes account for more than 40% of hospitalizations for major amputations and 73% of emergency admissions for minor amputations (4).

Currently, there is no Food and Drug Administration (FDA) approved therapy to prevent or reverse human DPN (3). The management approach focuses on reasonable glycaemic control, lifestyle modifications, and management of associated pain. Thus, it is important that DPN is detected early in its course. It is recommended that all subjects with type 2 diabetes are screened for DPN at diagnosis, and for type 1 diabetes, the screening should begin five years post-diagnosis (5). After this initial screening, all subjects should be reviewed annually. However, screening for DPN is challenging. There are currently no simple markers for early detection of DPN in routine clinical practice. The measures used are crude and detect the disease late in its natural course.

According to current guidelines for accurate assessment of DPN, a combination of tests may apply which mainly focus on medical history and simple clinical tests such as pinprick and temperature sensation, vibration perception and 10-g monofilament test (6, 7)

While symptoms and neurological deficits have direct relevance to patients, the assessment is excessively variable with poor reproducibility. Similarly, Quantitative Sensory Testing (QST) is subjective, is highly variable, and has limited reproducibility. Neurophysiological tests including Nerve Conduction Studies (NCS) is objective and reproducible but does not assess small fibers, which are the earliest to be damaged and show repair. Small fibers can be assessed objectively by quantifying intra-epidermal nerve fiber density (IENFD) in skin biopsies; however, this is an invasive procedure that requires expert laboratory assessment and results are considerably variable even in healthy controls (8). Therefore, effective treatments may have failed not because of a lack of efficacy, but because of an inability of the currently advocated end points to detect improvement in clinical trials of DN. The use of corneal confocal microscopy (CCM) for rapid, non-invasive clinical assessment of corneal nerves has grown substantially in recent years. It has proven to be particularly useful as a diagnostic marker for the detection of diabetic neuropathy. Numerous studies have confirmed its good sensitivity and specificity (8–14), demonstrating good reproducibility (15) for identifying DPN, proving that it can be particularly useful as a diagnostic marker for screening and stratification of DPN (14, 16–18) as well as a range of other peripheral neuropathies (19–21).

Currently, in the United Kingdom (UK) and globally, there is a lack of robust screening programmes for early detection of DPN and diabetic foot disease, resulting in increasing rates of foot ulcers and amputations among patients with diabetes (22). Previous studies have aimed to establish a screening model for diabetic foot diseases alongside eye disease. These include a recent study in a cohort of patients attending the retinal

**Abbreviations:** CCM, Corneal Confocal Microscopy; CNBD, Corneal Nerve Branch Density; CNFA, Corneal Nerve Fiber Area; CNFD, Corneal Nerve Fiber Density; CNFL, Corneal Nerve Fiber Length; CNFW, Corneal Nerve Fiber Width; CTBD, Corneal Total Branch Density; DNS, Diabetic Neuropathy Score; DPN, Diabetic Peripheral Neuropathy; HbA1c, Glycated Hemoglobin; LC; Langerhans Cells; NIHR, National Institute for Health Research; TC, Tortuosity Coefficient.

screening service in a hospital and primary care setting that showed combined eye, foot and renal screening is feasible (23).

The current paper presents the results of a large cohort of subjects screened in primary care using CCM alongside a retinopathy screening service. In comparison to previous CCM studies, this cohort is more representative of the community population for whom CCM could be utilised in the future as a monitoring and screening tool. This group represents subjects, mostly with type 2 diabetes, who are not under hospital care and have less severe complications of diabetes than those who have previously been investigated with CCM. It thus provided an opportunity to explore CCM as a biomarker for DPN in a novel cohort.

# Materials and methods

## Study group

The current dataset was originally collected as part of a study for investigating the feasibility and acceptability of implementing CCM for screening of DPN in primary care alongside the diabetic retinopathy screening program in South Manchester Diabetic Retinopathy Screening Service (SMDRSS) at four primary care optometry practices (24). During the six-month study (2014–2015), 450 consecutive subjects with type 1 or type 2 diabetes attended annual diabetes eye screening. Of those who consented and enrolled on the study, 95.5% had type 2 diabetes, 38% were female, and the median age of the whole study population was 68 years (range 21-93). The median duration since diabetes diagnosis was 6 years (0.1-51).

Data were successfully collected to an acceptable standard from 427 subjects (Figure 1 and Table 1) by four experienced and trained optometrists. The composition of the study population was compared against the UK population with diabetes as reported in the National Diabetes Audit (NDA) (25). Overall, apart from ethnicity, the composition of the study population was similar to the UK population with diabetes for age, gender, type of diabetes and duration of diabetes.

The research adhered to the tenets of the Declaration of Helsinki, and ethical approval was granted by the NRES East Midlands committee (REC: 15/EM/0079). All subjects provided written informed consent before participating in this study. Inclusion criteria were subjects aged 16 years and over with type 1 or 2 diabetes participating in NHS diabetes retinopathy screening programme. The exclusion criteria were subjects under 16, subjects who were unable to provide written consent, concurrent ocular disease, ocular infection or inflammation which may affect the cornea, a history of ocular disease or systemic disease that has affected the cornea (e.g. keratoconus, corneal dystrophies, refractive surgery), and wearing a hard contact lens. Full details of the study methods can be found in the National Institute for Health Research (NIHR) report (24).

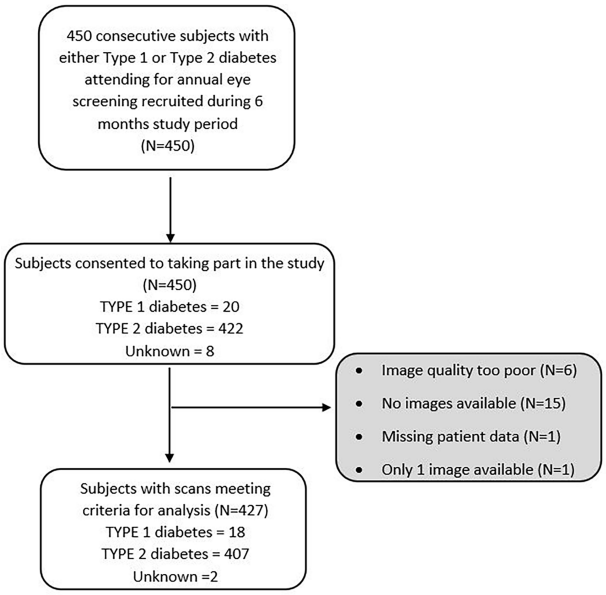


FIGURE 1
Study flow diagram.

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TABLE 1 Characteristics of the cohort with diabetes.

| | | Subgroups of Diabetes subjects | | | |
|---|---|---|---|---|---|
| | All subjects with Diabetes (N = 427) | Type 1 Diabetes (N = 18) | Type 2 Diabetes (N = 407) | Type 2 Diabetes ≤2 yrs. (N = 98) | No Retinopathy (R0) or Maculopathy (M0)(N = 241) |
| **Gender** | | | | | |
| Female n (%) | 167 (39%) | 8 (44%) | 158 (39%) | 39 (40%) | 94 (39%) |
| Male n (%) | 260 (61%) | 10 (56%) | 249 (61%) | 59 (60%) | 147 (61%) |
| **Age, years** | 67.9 (21-93) | 67.9 (21-93) | 68.30 (21-93) | 60.85 (21-89) | 68.40 (34-92) |
| **Type of Diabetes** | | | | | |
| Type 1 n (%) | 18 (4%) | | | | 3 (1%) |
| Type 2 n (%) | 407 (95%) | – | – | – | 236 (98%) |
| Unknown | 2 (<1%) | | | | 2 (1%) |
| **Duration of diabetes, years** | 6 (0.1-51) | 6 (0.1-51) | 6 (0.1-30) | 1 (0.1-2) | 5 (0.10-51) |
| **Ethnicity** | | | | | |
| White n (%) | 347 (81%) | 16 (89%) | 328 (81%) | 79 (81%) | 202 (83.5%) |
| Black n (%) | 65 (15%) | 2 (11%) | 63 (15%) | 15 (15%) | 31 (13%) |
| Asian n (%) | 12 (3%) | 0 | 12 (3%) | 3 (3%) | 5 (2%) |
| Mixed | 2 (1%) | 0 | 2 (1%) | 1 (1%) | 1 (0.5%) |
| Other/unknown | 3 (1%) | 0 | 2 (1%) | 0 | 2 (1%) |
| **History of DN** | | | | | |
| Yes n (%) | 28 (6.5%) | 3 (17%) | 25 (6%) | 4 (4%) | 8 (3%) |
| No n (%) | 397 (93%) | 15 (83%) | 380 (93%) | 94 (96%) | 231 (96%) |
| Unknown | 2 (0.5%) | 0 | 2 (1%) | 0 | 2 (1%) |
| **History of Foot Ulcer** | | | | | |
| Yes n (%) | 16 (4%) | 3 (17%) | 13 (3%) | 2 (2%) | 4 (1.5%) |
| No n (%) | 410 (96%) | 15 (83%) | 393 (97%) | 96 98%) | 236 (98%) |
| Unknown | 1 (<0.5%) | 0 | 1 (<0.5%) | 0 | 1 (0.5%) |
| **History of Retinopathy** | | | | | |
| Yes n (%) | 146 (34%) | 5 (28%) | 133 (33%) | 20 (20%) | 0 |
| No n (%) | 273 (64%) | 13 (72%) | 266 (65%) | 77 (79%) | 241 (100%) |
| Unknown | 8 (2%) | 0 | 8 (2%) | 1 (1%) | 0 |
| **History of Laser Treatment for Retinopathy** | 9 (2%) | 2 (11%) | 7 (2%) | 1 (1%) | 0 |
| Yes n (%) | 417 (98%) | 16 (89%) | 399 (98%) | 97 (99%) | 241 (100%) |
| No n (%) | 1 (<0.5%) | 0 | 1 (<0.5%) | 0 | 0 |
| Unknown n (%) | | | | | |
| **DNS Score** | | | | | |
| 0 n (%) | 262 (61%) | 14 (78%) | 246 (60.5%) | 71 (73%) | 163 (68%) |
| 1 n (%) | 73 (17%) | 3 (16.5%) | 70 (17%) | 13 (13%) | 34 (14%) |
| 2 n (%) | 49 (12%) | 1 (5.5%) | 48 (12%) | 7 (7%) | 22 (9%) |
| 3 n (%) | 21 (5%) | 0 | 21 (5%) | 3 (3%) | 11 (4.5%) |
| 4 n (%) | 22 (5%) | 0 | 22 (5.5%) | 4 (4%) | 11 (4.5%) |
| **Retinopathy Grading** | | | | | |
| R0 n (%) | 288 (67%) | 5 (28%) | 281 (69%) | 78 (80%) | |
| R1 n (%) | 132 (31%) | 10 (55.5%) | 122 (30%) | 20 (20%) | |
| R2 n (%) | 4 (1%) | 1 (5.5%) | 3 (1%) | 0 | |
| R3 n (%) | 3 (1%) | 2 (11%) | 1 (<0.5%) | 0 | – |
| **Maculopathy Grading** | | | | | |
| M0 n (%) | 414 (97%) | 17 (94%) | 395 (97%) | 95 (97%) | |
| M1 n (%) | 13 (3%) | 1 (6%) | 12 (3%) | 3 (3%) | – |
| **CNFD** (no./mm$^2$) | | | | | |
| Semi-automated | 25.84 (± 7.08) | 23.79 (± 9.75) | 25.94 (± 6.95) | 27.65 (± 6.96) | 26.28 ( ± 7.02) |
| Automated | 21.63 (± 7.10) | 18.31(± 9.72) | 21.76 (± 6.95) | 23.59 (± 6.91) | 21.98 ( ± 7.00) |
| **CNBD** (no./mm$^2$) | | | | | |
| Semi-automated | 75.00 (0-212.50) | 64.58 (5-119.79) | 75.00 (0-212.50) | 79.16 (0-194.79) | 78.12 (0-210.42) |
| Automated | 29.16 (0-82.29) | 22.39 (2.08-49.96) | 29.16 (0-82.29) | 31.25 (4.17- 82.29) | 30.21 (0-82.29) |

*(Continued)*

TABLE 1 Continued

| | All subjects with Diabetes (N = 427) | Subgroups of Diabetes subjects | | | |
|---|---|---|---|---|---|
| | | Type 1 Diabetes (N = 18) | Type 2 Diabetes (N = 407) | Type 2 Diabetes ≤2 yrs. (N = 98) | No Retinopathy (R0) or Maculopathy (M0)(N = 241) |
| **CNFL** (mm/mm²) | | | | | |
| Semi-Automated | 19.37 (± 5.68) | 17.29 (± 6.73) | 19.48 (± 5.63) | 20.78 (± 5.45) | 19.78 ( ± 5.53) |
| Automated | 13.62 (± 3.55) | 12.80 (± 4.32) | 13.68 (± 3.52) | 14.45 (± 3.66) | 13.80 ( ± 3.46) |
| **TC** (TC) | 16.90(9.60-32.66) | 15.39(9.46-32.66) | 17.00 (8.18-31.67) | 16.42(10.86-31.33) | 16.73 (8.85 - 29.72) |
| **CNFW** (mm/mm²) | 0.021 (0.019-0.030) | 0.022 (0.020-0.028) | 0.021 (0.019-0.030) | 0.022 (0.021-0.023) | 0.021 (0.019-0.030) |
| **CNFA** (mm²/mm²) | 0.006 (0.001-0.013) | 0.005 (0.003-0.008) | 0.006 (0.001-0.013) | 0.005 (0.002-0.010) | 0.006 (0.002-0.011) |
| **CTBD** (no./mm²) | 46.87 (0-138.5) | 34.50 (12.50-78.12) | 46.87 (0-138.5) | 62.50 (59.37-65.62) | 47.91 (6.25-123.95) |
| **LCs Presence** | | | | | |
| Yes n (%) | 417 (98%) | 417 (98%) | 397 (98%) | 97 (99%) | 234 (97%) |
| No n (%) | 10 (2%) | 10 (2%) | 10 (2%) | 1 (1%) | 7 (3%) |
| **LCs Density** (no./mm²) | 22.92 (0-225) | 40.31 (2.08-126.04) | 22.92 (0-225) | 28.65 (0-225) | 20.83 (0-170.38) |

Summary of the characteristics of the whole cohort with diabetes (Subjects), the subjects with type 1 diabetes, subjects with type 2 diabetes, subjects with type 2 diabetes and less than, or equal to, two years since disease diagnosis, and subjects with no evidence of retinopathy (R0) or maculopathy (M0). Age and duration of diabetes are represented by median (range) due to non-normal distribution. Retinopathy grading based on the ETDRS criteria: 0 = no retinopathy, 1 = background, 2 = pre-proliferative, 3 = proliferative. Maculopathy grading: 0 = no maculopathy 1 = maculopathy. 'Unknown' represents subjects for whom information was not available. Excluded cases were those with image quality that was deemed unacceptable, or there were < 2 images available for analysis.

## History of neuropathy, foot ulcer and retinopathy

All attending subjects completed a detailed questionnaire on their history of diabetic complications. Specifically, they were asked about any previously diagnosed neuropathy, diabetic foot disease, foot ulceration, gradable retinopathy, and previous laser treatment for DR.

## Assessment of neuropathy

The Diabetic Neuropathy Symptom Score (DNS) was used to assess each subject with diabetes for clinically evident DPN. The DNS score is a four-item symptom score for assessing DPN, developed by an expert panel and has been described previously (26). A score of 1 or more, out of a maximum of 4, indicates clinically detectable DPN (26).

## Retinopathy screening

All subjects underwent retinal screening in one of the four primary care optometry practices. A detailed description of the Diabetic Retinopathy Screening Service (DRSS) (1) and the retinopathy grading criteria (27) have been published previously. In summary, retinopathy is graded as one of four grades from R0 to R3. R0 represents no DR, R1 represents background DR, R2 represents pre-proliferative DR, and R3 represents proliferative DR. Mydriatic, 2-dimensional fundus photography was carried out on each patient. Images were graded by the appropriately qualified, attending optometrist for the level of DR with reference to previously established criteria (27).

## Examination with CCM

Each subject underwent assessment using in-vivo CCM (Heidelberg Retinal Tomograph III Rostock Cornea Module; Heidelberg Engineering GmbH, Heidelberg, Germany) by a trained optometrist, based on established methodology (28). Each optometrist selected eight (4 per eye) high-quality, non-overlapping images from Bowman's layer and sub-basal nerve plexus in a masked, randomized order. Through a secure server that was established and supported by the University of Manchester, the fully anonymized images were shared on a daily basis with the PI (MT). An in-depth protocol of this imaging technique has been described previously (24).

## CCM analysis

Images of the corneal sub-basal nerve plexus were evaluated for quality and artefacts by the attending optometrist. Six non-

overlapping masked images were analysed per subject using semi-automated CCMetrics software (MA Dabbah; Imaging Science and biomedical engineering, University of Manchester, Manchester, UK). It is recognised that semi-automated analysis can be a time consuming, resource intensive procedure as it involves manual tracing of nerve fibres (29). Considering this, fully automated, algorithmic defined, software such as ACCMetrics (University of Manchester, Manchester, UK) has been developed to eliminate the manual input. However, some small cohort studies have previously reported problems of automated software such as false positive and false negative identification of nerve structures (30, 31) and systematically lower measures of CNFL compared to semi-automated methods, despite good correlation (29). We therefore included both methods of analysis.

The following semi-automated CCM parameters were quantified: (i) Corneal nerve fiber density (CNFD) – the total number of major nerves/mm$^2$ of corneal tissue, (ii) corneal nerve branch density (CNBD) – the number of branches emanating from all major nerve trunks/mm$^2$ of corneal tissue, (iii) corneal nerve fiber length (CNFL) – the total length of all nerve fibers and branches (mm/mm$^2$), (iv) tortuosity coefficient (TC) and (v) Langerhans cells (LCs) presence and density within the area of corneal tissue.

The ACCMetrics automated software produces three parameters which are comparable to the semi-automated software (CNFL, CNFD and CNBD), as well as three additional parameters: corneal total branch density (CTB) - the total number of branch points from the main nerve/mm$^2$, corneal nerve fiber area (CNFA) - total nerve fiber area (mm$^2$/mm$^2$), and corneal nerve fiber width (CNFW) - the average nerve fiber width (mm/mm$^2$).

## Statistics

Statistical analysis was performed in Microsoft Excel for Office 365 (Microsoft Corp, Seattle, WA, USA) and SPSS for Windows version 26 (SPSS Inc., Chicago, IL, USA). Data were tested for normal distribution, and appropriate statistical tests were carried out accordingly.

For agreement analysis of automated vs semi-automated software, a two-way, mixed-effects model intraclass correlation test was conducted for absolute agreement between patient values. This was expressed as intraclass correlation coefficient (ICC) and 95% confidence interval.

A sample size of 400 subjects with a 95% confidence interval of ±5% was originally calculated to allow estimations of the proportions on study outcomes. This decision was made in consideration of equipment availability and the feasibility for the practices to recruit enough participants during the timeframe of the original study.

# Results

## Demographics and clinical information

The demographics, relevant patient history and clinical information for the subjects are presented in Table 1. The patient population of this study purposefully included wide ethnic and socio-economic mix factors, according to the NIHR deprivation index. This was broadly comparable to the adult national population with diabetes.

From the 450 subjects recruited for the study, 23 (5.1%) were excluded. Figure 1 displays reasons for exclusion.

## Correlation with age

Overall, there was a significant negative age-related correlation for semi-automated CNFD, CNFL and CNBD ($p<0.001$ for all 3) (Table 2). Similarly, there was a significant negative correlation between age and all three parameters measured using automated software ($p<0.001$ for CNFL and CNFD, $p=0.002$ for CNBD). The only other parameter with a significant negative correlation to age was corneal total branch density (CTBD) derived using automated software. There was no correlation of tortuosity (using either coefficient), LCs density, corneal nerve fiber width (CNFW) and corneal nerve fiber area (CNFA) with age.

## Comparison of corneal nerve data derived using semi-automated and automated analysis of the same CCM images

In order to compare agreement between automated and semi-automated corneal nerve analysis, values for CNFD, CNFL and CNBD for each method were assessed (Table 3). For most of the subjects examined (88.3%), a higher measurement for CNFD was obtained using semi-automated analysis in comparison to the automated method (Figure 2A). Overall, the mean difference between the two measurements was 4.26 (no/mm$^2$), and the mean percentage difference was 16.49%, with a mean higher value for semi-automated analysis. ICC values gave moderate agreement (ICC=0.75) between the two measures. There was no correlation between the mean CNFD number and the difference between the two measurements (Figure 2A).

For most subjects (97.0%), a higher measurement for CNFL was obtained using semi-automated analysis in comparison to automated software (Figure 2B). Overall, the mean difference between the two measurements was 5.73(mm/mm$^2$), Overall, the mean difference between the two methods for CNBD was 46.34 (no/mm2) (Figure 2C) and the mean percentage difference

TABLE 2 Correlations of nerve fiber parameters with age.

| Statistic | | | Age (Years) | |
|---|---|---|---|---|
| | | n | Rs | p-value |
| **CNFD**: Corneal nerve fiber density | Semi-automated | 427 | -0.26 | <0.001 |
| (no/mm$^2$) | Automated | 427 | -0.27 | <0.001 |
| **CNBD**: Corneal nerve branch density | Semi-automated | 427 | -0.2 | <0.001 |
| (no./mm$^2$) | Automated | 427 | -0.15 | 0.002 |
| **CNFL**: Corneal nerve fiber length | Semi-automated | 427 | -0.24 | <0.001 |
| (mm/mm$^2$) | Automated | 427 | -0.2 | <0.001 |
| **TC**: Tortuosity coefficient | (0-1) | 427 | 0.07 | 0.10 |
| | (0-20) | 427 | 0.07 | 0.20 |
| **LCs: Langerhans cells Density** (no./mm$^2$) | | 427 | 0.09 | 0.06 |
| **CTBD**: Corneal total branch density (no./mm$^2$) | | 427 | -0.12 | 0.02 |
| **CNFA**: Corneal nerve fiber area (mm$^2$/mm$^2$) | | 427 | 0.002 | >0.90 |
| **CNFW**: Corneal nerve fiber width (mm/mm$^2$) | | 426 | 0.02 | 0.70 |

Spearman's correlation (Rs) and statistical significance (p-value) of semi-automated and automated CCM image analysis with age. Data analyzed from patient cohort and included subjects with type 1 and type 2 diabetes. Significant correlations are highlighted in red.

between the two measurements was 29.61%, with an overall higher mean for semi-automated analysis. ICC values again gave moderate agreement (ICC =0.63) between the two measures; however, confidence intervals for ICC values were broad (Table 3). There was a modest positive correlation between mean CNFL and the difference between the two measurements (Figure 2B), indicating that the discrepancy between the methods becomes greater as the fiber length increases.

Overall, the mean difference between the two methods for CNBD was 46.34 (no/mm$^2$), and the mean percentage difference was 59.95%, making CNBD the parameter with the largest % disagreement between the two methods. ICC values gave poor agreement (ICC=0.41) between the two methods, and confidence intervals for ICC values were broad (Table 3). There was modest positive correlation between mean CNBD and the difference between the two measurements (Table 3).

## Prevalence of small fiber neuropathy

As the results demonstrated age-related negative correlations, we assessed whether subjects' CCM data differed from those

TABLE 3 Comparison of automated and semi-automated analysis.

| | | CNFD (no./mm$^2$) | CNFL (mm/mm$^2$) | CNBD (no./mm$^2$) |
|---|---|---|---|---|
| **Semi-automated Analysis** | Mean | 25.83 | 19.35 | 77.3 |
| | ±SD | 7.08 | 5.69 | 37.92 |
| | SEM | 0.34 | 0.28 | 1.83 |
| **Automated Analysis** | Mean | 21.57 | 13.62 | 30.96 |
| | ±SD | 7.11 | 3.56 | 16.45 |
| | SEM | 0.34 | 0.17 | 0.79 |
| | Mean Difference | 4.26 | 5.73 | 46.34 |
| | Mean % Difference | 16.49 | 29.61 | 59.95 |
| | P-value | <0.001 | <0.001 | <0.001 |
| | ICC | 0.75 | 0.63 | 0.41 |
| | 95% Confidence Interval | 0.039-0.912 | -0.183-0.876 | -0.204 - 0.723 |

Comparison of automated and semi-automated quantified measurements for CNFD, CNFL, and CNBD. Analysis conducted on the cohort with diabetes (n = 427). Results reportedas mean, standard deviation (SD) and standard error of the mean (SEM). Mean difference value represents the mean difference between results from each method. Mean % difference represents the average difference expressed as a percentage of the semi-automated analysis result (i.e. if the semi-automated CNFL result for a parameter was 20mm/mm2 and for automated software is was 10mm/mm2 then the % difference would be 50% as the difference is 50% of the semi-automated CNFL). Two-way mixed models for ICC are shown with 95% confidence intervals and statistical significance reported to represent agreement between semi-automated and automated software. All p-values calculated with a paired samples T-test. Statistical significance determined by p ≤0.05.

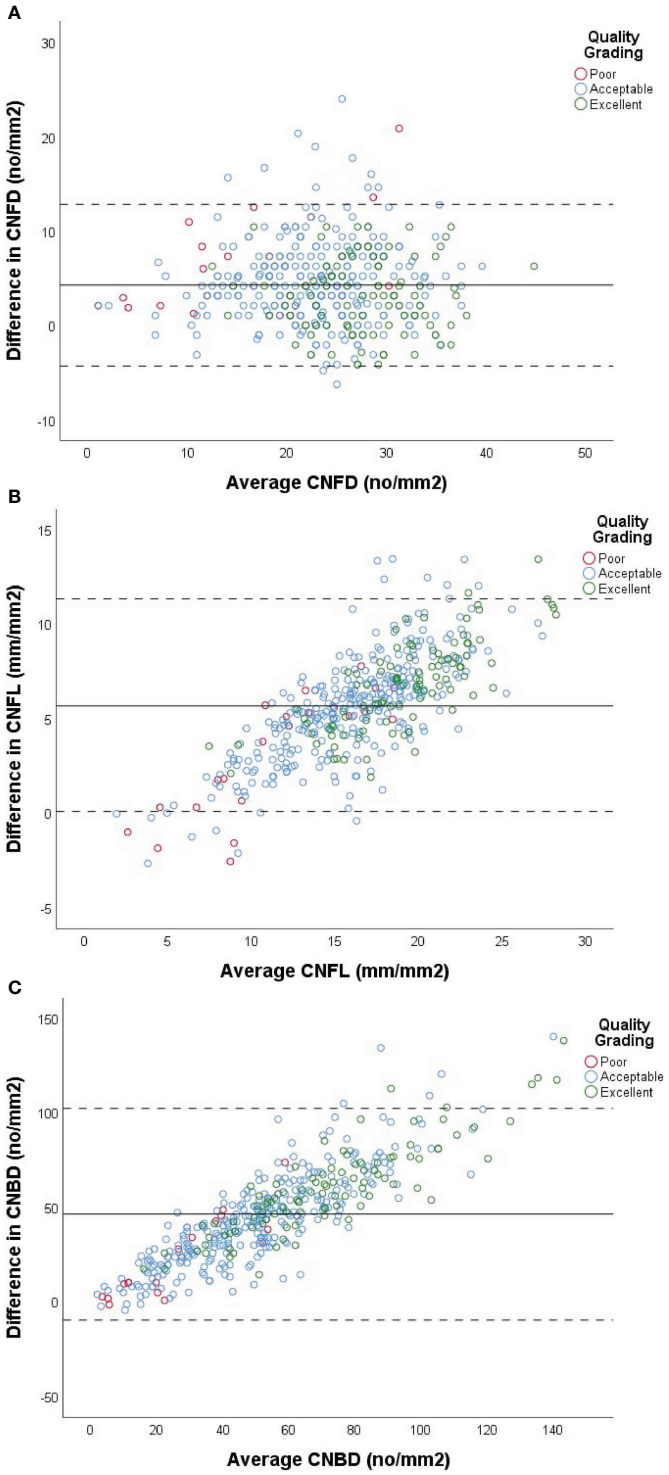


FIGURE 2
Comparison of automated vs semi-automated analysis of CNFD **(A)**, CNFL **(B)** and CNBD **(C)** using Bland-Altman plots. X-axis of each plot represents the mean measurement between the two methods of analysis for each subject. Y-axis represents the difference in values between the two methods for each subject, calculated by (semi-automated value - automated value) for each patient. Data from cohort with diabetes used in analysis (n=427). Solid line represents the mean difference between the two methods. Dashed lines represent +/- (1.96 x 2SD). Red, blue and green markers represent images graded as poor, acceptable and excellent by the investigator, respectively.

expected in healthy individuals, using data from a published age-segregated normative range by an international consortium (32). Due to the large differences between our automated and semi-automated results, we used only our semi-automated for comparison to the semi-automated derived published normative ranges. Table 4 displays the median values for the male and female subjects with diabetes, separated into six groups, based on age at the point of examination. Data are presented for semi-automated analysis derived CNFD and CNFL, compared to each age group's published normative median values. All age groups in the cohort of male individuals with diabetes demonstrate a median value for CNFL less than the normative published data. This was also true for all but one of the age groups in the female cohort, where the median of the 56-65 age group was 0.57 mm/mm$^2$ higher than that of the published normative median. Due to very small numbers of subjects under the age of 45, we assessed the number of subjects whose CNFL was less than the normally published median for their age group. For the female cohort, 7 of the 8 subjects under the age of 45 had a CNFL length that was less than the median. For the male cohort, 10 of the 13 subjects under the age of 45 had a CNFL length that was less than the median.

For both males and females, the median CNFD was lower than the normative published median CNFD for the three youngest age groups; 16-25, 26-35 and 36-45. Again, due to small numbers of subjects under the age of 45, we assessed the number of subjects whose CNFD was less than the normally published median for their age group. For the female cohort, 6 of the 8 subjects under the age of 45 had a CNFD that was less than the median. For the male cohort, 9 of the 13 subjects under the age of 45 had a CNFD that was less than the median. In contrast, the median CNFD was higher than the normative published median CNFD for males and females in the three oldest age groups; 46-55, 56-65 and over 65.

Age-corrected values at which CNFL may be considered abnormal have previously been published (32). When compared to these values, 20 (11.98%) females were below the CNFL cut-off (Table 4) and classified as abnormal. Of these 20 females, 2 had type 1, and 18 had type 2 diabetes. Overall, 25% of subjects with type 1 diabetes and 11.39% with type 2 diabetes were classified as abnormal.

A slightly higher proportion of 35 (13.46%) males in the patient cohort were below the CNFL cut-off. Of these 35 males, 2 had type 1 diabetes, and 33 had type 2 diabetes. Overall, 20% of males with type 1 diabetes and 13.25% of males with type 2 diabetes were classified as abnormal using CNFL alone.

## CNFL in subjects based on diabetes disease duration

In order to assess whether there may be corneal nerve alterations early in the course of diabetes for this cohort, a sub-group of the subjects who were diagnosed with diabetes ≤ 2 years ago were compared with age-corrected normal published values for CNFL (Tables 1, 5). Due to the very small number of subjects with type 1 diabetes (n=2), these subjects were excluded, and those with type 2 diabetes were considered alone for these analyses (n=98). When assessing the group of subjects with ≤ 2 years duration of diabetes from the time of diagnosis, 9.18% of subjects were classified as below the age-corrected published cut-off point for CNFL (32) and would have been considered abnormal for this parameter alone.

When considering CNFL in participants with a longer duration of diabetes (Table 5), the percentage of patients falling below the normative published cut-off value were similar across the middle three duration groups (12.05-13.74%). The group with the shortest duration (≤ 2 years) had a smaller percentage of patients falling below the cut-off value (9.18%) and the group with the longest duration of diabetes (>20 years) had the highest percentage of patients falling below the cut-off value (15.38%) however due to the small number of patients in each group, we were unable to test for statistical significance.

## CCM parameters in subjects with different grades of retinopathy

CNFD was compared across four groups according to DR grading (Figure 3 and Table 6). When testing for significance between groups R0 and R1, a non-significant difference was found (p=0.37). Three of the four subjects (75%) in the R2 group had a CNFD level less than the published normative median value for their age group. For group R3 subjects, all three subjects (100%) had a CNFD level which was less than the published median normative value for their age group. As the number of subjects in groups R2 and R3 was very small, we could not perform statistical tests.

Similarly, CNFL demonstrated no significant difference between the R0 and R1 subjects (p=0.19). As published, suggested cut-off points are available for CNFL; these were used to assess each group. In the R0 group (Tables 1, 6), 11.81% of subjects with R0 fell below the cut-off point. This increased to 13.64% in the R1 group and 100% in the R3 group; however, none of the R2 group fell below the CNFL cut-off point.

According to the Early Treatment Diabetic Retinopathy Study (ETDRS), grading of DR (31), R0 and M0 represent no detectable retinopathy and maculopathy, respectively. Therefore, the group of subjects meeting these criteria and with no previous history of retinopathy, maculopathy or laser was compared with age-corrected normal published values for CNFL to assess any significant changes prior to detectable retinopathy (Table 1). The patient group consisted of mainly subjects with type 2 diabetes (98%). The majority of the patient group had a DNS score of 0 (68%), with 32% scoring positively on the DNS score for at least one symptom of neuropathy. Based on CNFL length alone,

TABLE 4 Comparison to normative age-related median and cut-off values.

| | | | CNFD | | | CNFL | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Age | n | Cohort median | Normative median | Difference | Cohort median | Normative median | Difference | CNFL Cut-off value (mm/mm2) | < CNFL Cut-off (no) | < CNFL Cut-off (%) |
| **FEMALES** | 16-25 | 2 | 22.40 | 31.85 | 9.45 | 16.07 | 26.43 | 10.36 | 15.08 | 1 | 50.00 |
| **(n = 167)** | 26-35 | 1* | 20.83 | 30.20 | 9.37 | 13.24 | 25.45 | 12.21 | 13.17 | 0 | 0.00 |
| | 36-45 | 5 | 26.04 | 28.56 | 2.52 | 21.84 | 24.37 | 2.53 | 12.48 | 1 | 20.00 |
| | 46-55 | 29 | 30.21 | 26.91 | **-3.3** | 21.87 | 23.28 | 1.41 | 12.48 | 1 | 3.45 |
| | 56-65 | 36 | 27.08 | 25.27 | **-1.81** | 22.77 | 22.20 | **-0.57** | 12.9 | 3 | 8.33 |
| | >65 | 94 | 23.96 | 23.54 | **-0.42** | 18.86 | 21.11 | 2.25 | 13.67 | 14 | 14.89 |
| **Total** | | **167** | | | | | | | | **Type 1: 2**<br>**Type 2: 18**<br>**Overall: 20** | **Type 1: 25**<br>**Type 2: 11.39**<br>**Overall: 11.98** |
| **MALES** | 16-25 | 1* | 23.96 | 32.44 | 8.48 | 17.73 | 23.16 | 5.43 | 15.93 | 0 | 0 |
| **(n =260)** | 26-35 | 3 | 25.00 | 30.56 | 5.56 | 18.81 | 22.92 | 4.11 | 14.05 | 1 | 33.33 |
| | 36-45 | 9 | 26.04 | 28.68 | 2.64 | 17.57 | 23.34 | 5.77 | 13.20 | 1 | 11.11 |
| | 46-55 | 26 | 29.69 | 26.80 | **-2.89** | 22.94 | 23.63 | 0.69 | 13.01 | 3 | 11.54 |
| | 56-65 | 66 | 28.04 | 24.92 | **-3.12** | 20.23 | 23.03 | 2.80 | 13.12 | 7 | 10.61 |
| | >65 | 155 | 25.00 | 22.95 | **-2.05** | 18.66 | 20.61 | 1.95 | 13.15 | 23 | 14.84 |
| **Total** | | **260** | | | | | | | | **Type 1: 2**<br>**Type 2: 33**<br>**Overall: 35** | **Type 1: 20**<br>**Type 2: 13.25**<br>**Overall: 13.46** |

Comparison of 2 semi-automated corneal nerve parameters (CNFD and CNFL) with age-matched published normative values (32) for females and males. 'Cohort Median' represents the median value in each age group of the patient cohort. 'Normative Median' represents the published median values for females in each age group (32). The difference between the normative and cohort medians was calculated as (Normative median – Cohort median). Positive values are represented in red, whereas negative values are shown in black. (* unable to calculate median as n = 1). Classification of females and males within as having pathological CNFL when compared to published cut-off values (25) (0.05th quantile of normative database). The number and % of subjects classified as having pathological CNFL is given for each age group.



10.3389/fendo.2022.891575

TABLE 5 Comparison of groups based on years since diagnosis.

| | ≤ 2 Years | 2-5 Years | 5-10 Years | 10-20 Years | >20 Years | p-value |
|---|---|---|---|---|---|---|
| **n** | 98 | 83 | 131 | 82 | 13 | - |
| **Age (years)** | 60.85 (21-89) | 63.30 (34-87) | 69.10 (45-92) | 72.45 (46-93) | 77.20 (57-86) | <0.001 |
| **Gender** | | | | | | |
| F | 39 (40%) | 32 (39%) | 53 (40%) | 27 (33%) | 7 (54%) | |
| M | 59 (60%) | 51 (61%) | 78 (60%) | 55 (67%) | 6 (46%) | |
| **Ethnicity** | | | | | | |
| White | 79 (81%) | 65 (78%) | 110 (84%) | 65 (79%) | 9 (69%) | |
| Black | 15 (15%) | 15 (18%) | 16 (12%) | 13 (16%) | 4 (31%) | |
| Asian | 3 (3%) | 3 (4%) | 2 (1.5%) | 4 (5%) | 0 | – |
| Mixed | 1 (1%) | 0 | 1 (1%) | 0 | 0 | |
| Other | 0 | 0 | 2 (1.5%) | 0 | 0 | |
| **DNS Score** | | | | | | |
| 0 | 71 (72.5%) | 51 (61.5%) | 70 (53.5%) | 45 (55%) | 9 (69%) | |
| 1 | 13 (13.5%) | 12 (14.5%) | 29 (22.5%) | 15 (18%) | 1 (8%) | |
| 2 | 7 (7%) | 6 (7%) | 20 (15%) | 13 (16%) | 2 (15%) | – |
| 3 | 3 (3%) | 10 (12%) | 3 (2%) | 4 (5%) | 1 (8%) | |
| 4 | 4 (4%) | 4 (5%) | 9 (7%) | 5 (6%) | 0 | |
| **Retinopathy Grade** | | | | | | |
| R0 | 78 (80%) | 63 (76%) | 94 (72%) | 41 (50%) | 5 (38%) | |
| R1 | 20 (20%) | 20 (24%) | 36 (27%) | 39 (48%) | 7 (54%) | |
| R2 | 0 | 0 | 1 (1%) | 2 (2%) | 0 | – |
| R3 | 0 | 0 | 0 | 0 | 1 (8%) | |
| **Maculopathy Grade** | | | | | | |
| M0 | 95 (97%) | 82 (99%) | 130 (99%) | 77 (94%) | 11 (85%) | |
| M1 | 3 (3%) | 1 (1%) | 1 (1%) | 5 (6%) | 2 (15%) | – |
| **No of subjects < CNFL cut-off** | 9 (9.18%) | 10 (12.05%) | 18 (13.74%) | 11 (12.20%) | 2 (15.38%) | |

Summary of the known characteristics and clinical grading information for subjects with type 2 diabetes, split into 5 age groups and control subjects (Controls). Age is represented by median (range) due to a non-normal distribution. Retinopathy grading: 0 = no retinopathy, 1= background, 2 = pre-proliferative, 3 = proliferative. Maculopathy grading: 0 = no maculopathy 1 = maculopathy. See methods section for detailed grading characteristics. 'Unknown' represents subjects for which information was not available. Number of subjects <cut-off was calculated using published age-corrected values (32).

12.0% of subjects were below the age-dependent published cut-off point, suggesting that 12.0% of subjects with no evidence of retinopathy may have significant CNFL reduction.

## Discussion

This study assessed the implementation of CCM to screen for DPN in clinical practice outside of the research environment and to our knowledge, is the first study of this type. As a measure of the corneal sub-basal nerve plexus, CCM provides a potential surrogate biomarker for assessing small nerve fiber changes in subjects with diabetes. Several studies that recruited subjects from hospital clinics have confirmed CCM's ability to detect nerve alterations in people with diabetes compared to healthy controls (5, 6, 8, 11, 15) and distinguish between subjects with and without clinical DPN (9, 12). CCM has shown promise for predicting future neuropathy from baseline measurements (11) and has detected nerve regeneration post-therapeutic intervention (33).

Automated software is significantly quicker when analysing images in comparison to semi-automated software. It is likely the only viable option for analysis if using CCM to screen for neuropathy in the future. In our study, when comparing automated and semi-automated analysis, the results for automated CNFD, CNFL and CNBD were all significantly lower. This is in agreement with previous studies, also finding an underestimation when using ACCMetrics automated software (29, 31) and was the reason we focused mainly on semi-automated methods for the most accurate analysis. If automated software is to be used for DPN screening, software needs to be improved and updated to resolve the measurements bias. As we await these technological advances, adjustment factors must be put in place to compare to semi-automated analysis.

In this study, we found that CNFL, CNFD and CNBD significantly decreased with increasing age (Table 2), in line with previously published literature (32, 34). Thus, we referenced CCM published normative age values for CNFL and CNFD (32). Twenty females (11.98%) and 35 (13.46%) males were classified as having abnormal CNFL that could be considered clinically significant (12.88% overall). This implies that in our cohort, 12.9% of subjects may be deemed to have small fiber neuropathy if using CNFL as a single diagnostic measure. This percentage is less than that of Anderson et al. (2018) (35), who found a prevalence of 19% DPN when using the Toronto consensus for diagnosis in subjects with type 2 diabetes. CCM identifies small fiber damage, which has been

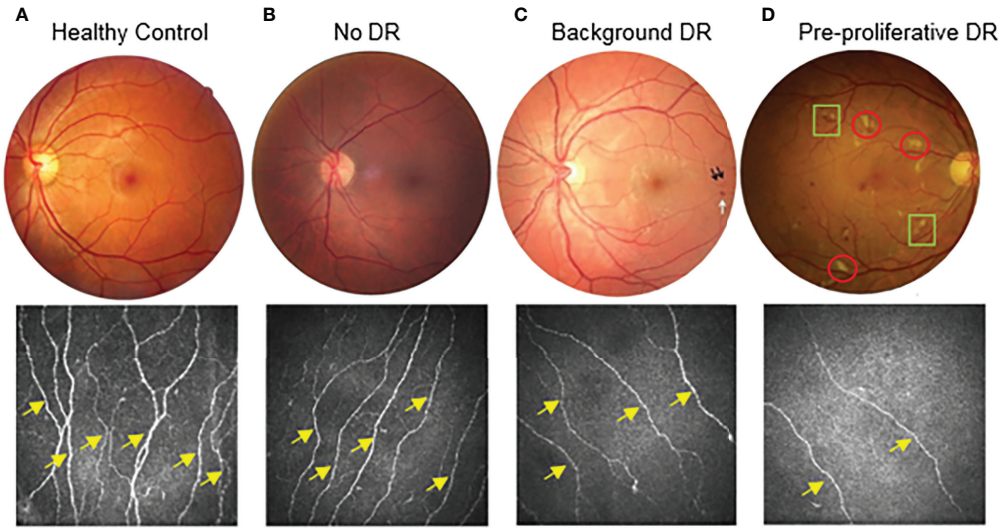


FIGURE 3
Stages of diabetic retinopathy linked to CCM findings from healthy control to pre-proliferative diabetic retinopathy (DR grade 2). Proliferative (DR grade 3) not displayed. **(A)** demonstrates a healthy control subject. **(B)** demonstrates a patient with diabetes but no diabetic retinopathy (DR grade 0). **(C)** demonstrates a patient with background diabetic retinopathy (DR grade 1). Dot haemorrhages (black arrows) and a blot haemorrhage(white arrow) can be seen. **(D)** demonstrates a patient with diabetes and pre-proliferative diabetic retinopathy (DR grade 2). As well as multiple dot and blot haemorrhages, there are also numerous cotton wool spots (red circles) and intra-retinal microvascular abnormalities (IRMA)(green squares). For the corresponding examples of CCM images, the nerve fibre density of the main nerves (yellow arrows) decreases as diabetic retinopathy progresses. There is also a clear decrease in the overall nerve fibre length.

shown to precede large fiber changes (36, 37); thus, we expected a higher percentage of abnormality in our study. This highlights the problematic nature of comparing DPN prevalence across studies using a range of definitions for classification. The Diabetes Control and Complications Trial (DCCT) data exemplified the impact of varying diagnostic testing procedures. In their cohort, the prevalence of DPN at baseline varied from 0.3% (abnormalities of reflexes, sensory examination and neuropathic symptoms) to 21.8% (abnormal nerve conduction in at least two nerves) depending on the criteria used for detection (38).

A recent study of 236 people attending retinal screening showed that combined eye, foot and renal screening is feasible. In that study, the authors reported a prevalence of DPN, assessed using the Toronto Clinical Neuropathy Score, of 30.9%, which was underestimated by the 10-g monofilament test (14.4%). The clinical characteristics of the cohorts might explain the differences between their findings and our results, as studies are never identical with respect to the demographics of their subjects and risk factors for DPN. The authors do not report the duration of diabetes of their patient group (23), and additionally, subjects attended screening either in primary care or within a hospital setting. Subjects that attended the secondary care setting may have been at higher risk of diabetic complications. An important aspect of our study was that subjects were tested during community screening. Although this would need to be confirmed with further studies, it is likely that the relative stability of subjects attending community retinopathy screening would make them less susceptible to developing diabetic complications such as DPN and associated reduction in corneal nerve fibers.

To assess the potential role of CCM to identify early nerve changes, it was important to evaluate subjects with diabetes of duration ≤ 2 years since diagnosis. This was to determine if corneal changes were occurring early in diabetes.

When assessing subjects with diabetes within early stages since diagnosis, Ziegler and colleagues (18) concluded, using their own control cohort, that CNFD was the most sensitive parameter for detecting neuropathy, as it detected 21% of subjects falling below the 2.5th percentile of the control group. CNFL was the second most sensitive, with 17% falling below the 2.5th percentile (18). This percentage for CNFL abnormality is significantly higher than that of abnormal CNFL in subjects with diabetes, found in our study (9.18%). It is likely that the significantly lower comparative percentage is largely due to a difference in percentile cut-off points used to define an abnormality. In comparison, we used the $0.5^{th}$ percentile as a cut-off point from age-corrected published values (32), therefore identifying fewer subjects as outside of this range.

It is difficult to confidently compare the results of these two studies as although sample sizes were similar (86 vs 98), our study evaluated only subjects with ≤ 2 years disease duration, whereas the mean disease duration of the subjects in the Ziegler et al. (2014) study was 2.1± 1.6 years. The longer duration of

TABLE 6 Comparison of different retinopathy grades.

| Retinopathy grade | R0 | R1 | R2 | R3 | p-value |
|---|---|---|---|---|---|
| n | 288 | 132 | 4 | 3 | - |
| **Age (years)** | 68.35 (23-92) | 67.50 (21-93) | 65.90 (30-69) | 50.40 (41-77) | 0.50 |
| **Type of diabetes** | | | | | |
| Type 1 | 5 (2%) | 10 (7.5%) | 1 (25%) | 2 (66.5%) | |
| Type 2 | 281 (97.5%) | 122 (92.5%) | 3 (75%) | 1 (33.5%) | |
| Unknown | 2 (0.5%) | 0 | 0 | 0 | |
| **Duration of diabetes** | 6 (0.10-51) | 9 (0.20-35) | 14 (6-20) | 21 (11-35) | <0.001 |
| **Gender** | | | | | |
| F | 109 (38%) | 54 (41%) | 3 (75%) | 1 (33.5%) | 0.50 |
| M | 179 (62%) | 78 (59%) | 1 (25%) | 2 (66.5%) | |
| **Ethnicity** | | | | | |
| White | 233 (81%) | 106 (80.5%) | 4 (100%) | 3 (100%) | |
| Black | 42 (14.5%) | 23 (17.5%) | 0 | 0 | |
| Asian | 9 (3%) | 3 (2%) | 0 | 0 | 0.70 |
| Mixed | 2 (0.5%) | 0 | 0 | 0 | |
| Other | 2 (0.5%) | 0 | 0 | 0 | |
| **DNS Score** | | | | | |
| **0** | 186 (64.5%) | 72 (55%) | 2 (50%) | 2 (66.5%) | |
| **1** | 44 (15%) | 29 (22%) | 0 | 0 | |
| **2** | 30 (10.5%) | 18 (13.5%) | 0 | 1 (33.5%) | 0.20 |
| **3** | 14 (5%) | 7 (5%) | 0 | 0 | |
| **4** | 14 (5%) | 6 (4.5%) | 2 (50%) | 0 | |
| **Maculopathy Grade** | | | | | |
| M0 | 286 (99.5%) | 125 (94.5%) | 0 | 3 (100%) | – |
| M1 | 2 (0.5%) | 7 (5.5%) | 4 (100%) | 0 | |
| **CNFD (no/mm$^2$)** | 26.18 (± 7.03) | 25.53 (± 6.90) | 23.70 (± 6.66) | 11.04 (± 6.38) | – |
| **CNBD (no/mm$^2$)** | 77.08 (0-212.50) | 71.87 (0-161.46) | 43.23 (25.0-122.92) | 28.12 (5.0-50.0) | – |
| **CNFL (mm/mm$^2$)** | 19.70 (± 5.65) | 18.93 (± 5.57) | 18.06 (± 5.44) | 8.36 (± 4.23) | – |
| **No of subjects < CNFL cut-off** | 34 (11.81%) | 18 (13.64%) | 0 | 3 (100%) | |

Summary of the known characteristics and clinical grading information for subjects, assorted into 4 groups, based on retinopathy grade, as well as controls. The CCM parameters are calculated with Semi-automated software. Age and duration of diabetes are represented by median (range) due to a non-normal distribution. Retinopathy grading: 0 = no retinopathy, 1 = background, 2 = pre-proliferative, 3 = proliferative. Maculopathy grading: 0 = no maculopathy 1 = maculopathy. See methods section for detailed grading characteristics. 'Unknown' represents subjects for which information was not available. The number of subjects below cut-off was calculated using published age-corrected values (32).

diabetes in some of their cohort may have caused more significant corneal nerve changes.

One recently published study (39) found that there was no significant difference in CNFL between patient groups with type 2 diabetes duration <10 years (mean age 5 ± 3) and control subjects. This contradicts the findings of our study and that of Ziegler and colleagues (18); however, it may be attributed to the study's strict inclusion/exclusion criteria - subjects with glycated haemoglobin (HbA1c) levels of >7.8% or a history of proliferative retinopathy were excluded.

Despite limited research into subjects during the very early stages of diabetes, our findings suggest that corneal nerve fiber changes may occur early and may be an indicator of changes in the sensory nervous system.

At present, retinal photography is a successful screening method for DR and can detect early microvascular changes. Our findings suggest that changes in corneal nerves may precede detectable retinopathy.

These findings confirm those of Bitirgen et al. (40), who reported, in subjects with type 2 diabetes and no DR, a significant reduction in CNFD (p<0.001), CNFL (p=0.02) and CNBD (p =0.001) compared to healthy subjects when assessed using automated software. An earlier study (41) also found a significant difference in all three parameters; however, this study used their own custom-written routines in MATLAB rather than a commonly used software such as CCMetrics.

When assessing subjects with type 1 diabetes, two similar studies (42, 43) reported a reduction in CNFD, CNFL and CNBD, prior to any retinopathy. However, Szalai et al. (2016) only assessed young subjects (mean age 22.86 ± 9.05 years), which was not representative of the type 1 diabetes population overall. This cohort was very different to ours, which was (1) mainly in people with type 2 diabetes and (2) of significantly older age.

Our study into this area is novel in that we assessed subjects in primary care along with DR screening. This has allowed us to evaluate a larger cohort of 241 subjects with no retinopathy or history of retinopathy compared to previous studies. Of these subjects, 29 (12.0%) had a CNFL measurement less than the published age-corrected reference value. This may suggest that

several subjects do not meet the referral criteria into the hospital eye service (HES) based on retinopathy but may require further investigation and closer monitoring of peripheral nerve changes. More studies are needed to investigate the cost-effectiveness of this increase in referrals and the benefits to the subjects.

Although our study demonstrates good agreement with the current literature, the four previous studies discussed were completed in a hospital setting by a trained expert, thus were not representative of a cohort attending community DR screening. There was also a significant lack of recently diagnosed subjects (< 2 years), most notably in one of these studies (41). Nevertheless, the findings of these and our studies challenge the current screening strategies deployed to detect the complications of diabetes. Using CCM to identify corneal nerve changes may be the earliest window of opportunity to intervene and prevent the progression of the triad of microvascular complications; nephropathy, neuropathy and retinopathy.

There were some limitations to this study: first, there was no available information regarding height, triglyceride levels or HbA1c levels, which were previously associated with increased risk of neuropathy (44). A recent study by Wang et al. (2020) (45) found that subjects with type 2 diabetes and DPN had significantly higher levels of HbA1c (p=0.035), high-density lipoprotein (p=0.003) and fasting blood glucose (p=0.026). We are unable to confidently conclude that any significant/non-significant changes between subgroups of subjects were down to the grouping factor and no other independent factors.

Second, our cohort was made up of mainly older subjects with type 2 diabetes. This may be considered partially as a limitation, as we were unable to perform statistical testing on data from younger subjects and subjects with type 1 diabetes. However, the composition of the study population was compared against the UK population with diabetes; thus, our cohort mirrors the demographic of subjects attending the retinal screening service in the UK and therefore adequately acts as a representative population of this specific group.

Due to frequent delays in diabetes diagnosis in primary care, the exact time of disease onset is uncertain. One study previously reported a delay of at least 4-7 years before diagnosing type 2 diabetes (46). Subjects in our study classed as having the disease duration of ≤ 2 years may be wildly different from the precise time since disease onset, thus erroneously suggesting more significant changes to corneal nerve fibers early in the course of diabetic disease.

Finally, due to the nature of this study, we were unable to assess the neuropathy in detail, including nerve conduction studies (NCS) to use as an objective assessment of DPN and comparator to determine the sensitivity and specificity of CCM to diagnose DPN. However, the sensitivity and specificity of CCM for said measurement has been previously validated in a number of studies (8–12, 14, 47–49).

To our knowledge, this study has been the first to use CCM to assess a large cohort of subjects with diabetes in a primary care screening service in which CCM images were taken by primary care clinicians. Our study presents evidence that CCM can be used in primary care to accurately detect corneal nerve abnormalities prior to evident retinopathy and in the early years since diagnosis. Overall, the findings support the current literature that CCM is a sensitive surrogate biomarker for DPN. Further research should focus on developing software for automated analysis and validating its diagnostic validity for detecting early DPN in larger, age-matched cohorts in primary care.

## Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## Ethics statement

The studies involving human participants were reviewed and approved by NRES East Midlands committee (REC: 15/EM/0079). The patients/participants provided their written informed consent to participate in this study.

## Author contributions

JC and MT wrote the manuscript. JC, HF, FI, MT contributed to data analysis and image analysis of the study subjects. JC, HF, FI, AB, SH, AS, MT reviewed and revised the paper. MT and AS supervised JC. MT was the PI study. MT designed, conceived the study, wrote the article, major revisions, made comments, had full access to all data, and is the guarantor. All authors provided important intellectual input and approved the final version of the manuscript. All authors contributed to the article and approved the submitted version.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Publisher's note



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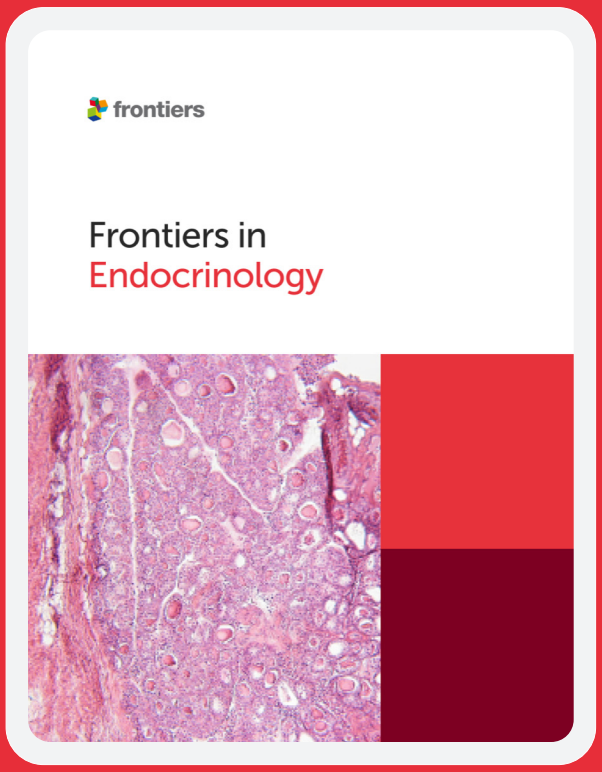


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