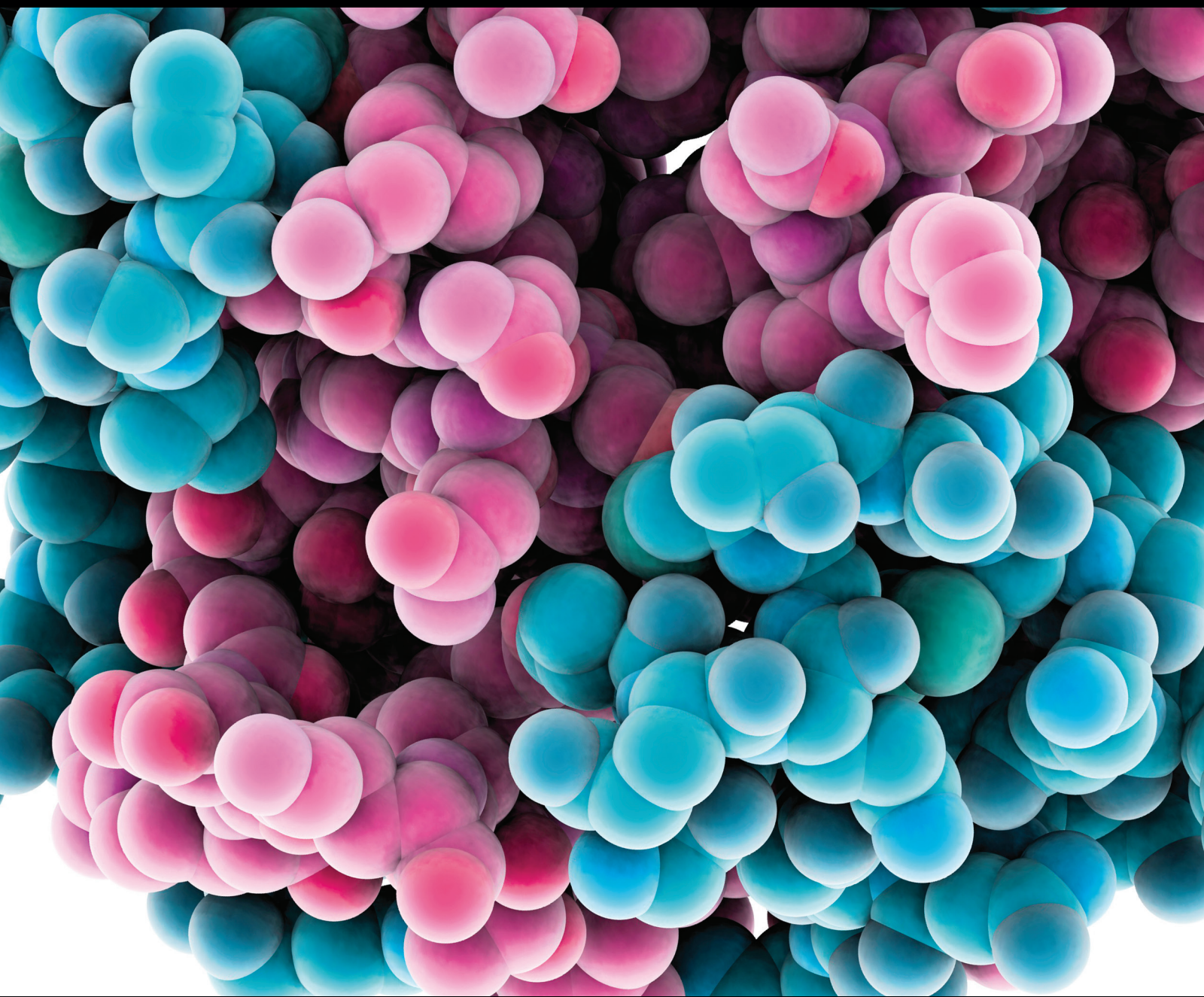
Journal of Diabetes Research

Diabetic Neuropathy: Current Status and Future Prospects

Lead Guest Editor: Mitra Tavakoli
Guest Editors: Dilek Gogas Yavuz, Abd A. Tahrani, Dinesh Selvarajah, Frank L. Bowling, and Hassan Fadavi



Diabetic Neuropathy: Current Status and Future Prospects

Journal of Diabetes Research

Diabetic Neuropathy: Current Status and Future Prospects

Lead Guest Editor: Mitra Tavakoli
Guest Editors: Dilek Gogas Yavuz, Abd A. Tahrani, Dinesh Selvarajah, Frank L. Bowling, and Hassan Fadavi

Editorial Board

Contents

Hindawi
Journal of Diabetes Research
Volume 2017, Article ID 5825971, 2 pages
https://doi.org/10.1155/2017/5825971

Editorial

Diabetic Neuropathy: Current Status and Future Prospects

Mitra Tavakoli,[1,2] Dilek Gogas Yavuz,[3] Abd A. Tahrani,[4] Dinesh Selvarajah,[5] Frank L. Bowling,[2] and Hassan Fadavi[2]

[1]*University of Exeter, Exeter, UK*
[2]*University of Manchester, Manchester, UK*
[3]*University of Marmara, Istanbul, Turkey*
[4]*University of Birmingham, Birmingham, UK*
[5]*University of Sheffield, Sheffield, UK*

Correspondence should be addressed to Mitra Tavakoli; m.tavakoli@exeter.ac.uk

Received 5 April 2017; Accepted 6 April 2017; Published 9 July 2017

Welcome to this special issue of the Journal of Diabetes research that is focussed on diabetic neuropathy (DN) and features a wide range of articles covering accepted topics related to the epidemiology, pathogenesis, and treatment of different aspects of DN. Diabetic peripheral neuropathy (DPN) and diabetic autonomic neuropathy (DAN) are the most common diabetes-related microvascular complications and can result in significant increase in morbidity, such as chronic pain, foot ulcerations and amputations, and mortality. But despite these significant consequences, current effective screening and treatment strategies are lacking unlike other diabetes-related microvascular complications such as retinopathy and nephropathy. This usually results in delay in the diagnosis of DN till it is well established and more difficult to treat while retinopathy and nephropathy can be detected early using current screening strategies such as retinal images and urinary assessments which allow early interventions to prevent the progression of the disease.

Glucose control is still the only main disease-modifying therapy for diabetic neuropathy, and several disease modification clinical trials for diabetic neuropathy have failed due to lack of sensitive biomarker. There exists an urgent need to identify the most accurate early biomarker of nerve damage to better diagnose DPN in the clinical care of patients and, in particular, to permit an accurate evaluation of future therapies in clinical trials.

This special issue aimed to provide a platform for advance in basic and clinical science in the field of DN.

In an elegant and well-constructed review, L. M. Román-Pintos et al. summarised the literature regarding the epidemiology, risk factors, pathophysiology, diagnosis, and treatments of DN. The review provides important insights into the mechanisms underlying the pathogenesis of DN particularly in relation to oxidative stress, inflammation, and mitochondrial dysfunction providing the experimental basis for each mechanism followed by its translational findings in patients.

N. A. Gavan et al. reported the outcome of the most recent epidemiological study performed in Romania. The study revealed a high prevalence of undisclosed DN, as well as a high prevalence of foot ulcers and amputations in the study population.

A paper by A. A. Tahrani et al. showed ethnic differences in microvascular function in the lower limbs in the South Asian patients with type 2 diabetes compared to White Europeans. In this interesting study, skin microvascular blood flow assessment demonstrated reduced heating flux but preserved acetylcholine response in South Asians. This might be related to the lower prevalence of DPN in South Asians [1].

Patients with diabetes have been reported to have a greater decline in cognitive function and a higher risk of developing dementia. In an interesting study, C.-W. Chang et al. showed in their large study population from Taiwan that regular uptake dosage of aspirin might decrease the risk of developing Alzheimer's disease in patients with type 2 diabetes.

M. C. Perez-Matos et al. reviewed the evidences about lipid-modifying therapies in DPN. The authors concluded that the future research should concentrate on targeting lipids with one or more aggressive interventions specifically in patients whose DPN is detectable but whose progression can still be largely prevented [2].

A. Ando et al. investigated the relationship between macroangiography and DPN applying cardio-ankle vascular index (CAVI) in patients with type 2 diabetes. Their study showed that the CAVI, arterial stiffness, and vascular damage marker have a close relationship with DPN [3].

F. Ishibashi et al. investigated whether the pupillary light reflex (PLR) mediated by intrinsically photosensitive retinal ganglion cells is impaired in type 2 diabetic patients without clinical evidences of autonomic neuropathy. The results showed that blue light induced a more intense and rapid PLR in control subjects and diabetic patients than did red light, and the PLR stimulated by blue light in patients with type 2 diabetes without DAN was more severely impaired than that caused by red light [4].

V. L. Newton et al. demonstrated the increased numbers of neutrophils and levels of L-selectin which is an adhesion molecule important for neutrophil transmigration, in the lumbar spinal cord after 8 weeks of STZ-induced diabetic rats. These findings suggest that dysregulated spinal L-selectin and neutrophil infiltration into the spinal cord could contribute to the pathogenesis of painful DPN [5].

The review by T. Kucera et al. summarised the current view on the etiology, diagnostics, and treatment of Charcot neuropathic osteoarthrophy in diabetes, with particular focus on preserving the extremity through surgical intervention.

Callus formation has long been an important factor to be considered as a predictor for ulceration and subsequent amputation. However, the role of vertical stress (pressure) and shear stress associated with callus has yet to be clarified. A. Amemiya et al. from the Department of Wound Care, Tokyo, Japan, looked into the role of hyperkeratosis (callus) and its link to frictional shear forces and subsequent tissue loss.

Acknowledgments

We would like to acknowledge the reviewers who carried out the task of critical appraisal of the articles and the authors for their valuable contributions. We sincerely hope that you will find these timely and insightful articles informative and intellectually motivating.

Mitra Tavakoli
Dilek Gogas Yavuz
Abd A. Tahrani
Dinesh Selvarajah
Frank L. Bowling
Hassan Fadavi

References

[1] A. A. Tahrani, Q. A. Altaf, M. K. Piya, and A. H. Barnett, "Peripheral and autonomic neuropathy in South Asians and White Caucasians with type 2 diabetes mellitus: possible explanations for epidemiological differences," *Journal of Diabetes Research*, vol. 2017, Article ID 1273789, 10 pages, 2017.

[2] M. C. Perez-Matos, M. C. Morales-Alvarez, and C. O. Mendivil, "Lipids: a suitable therapeutic target in diabetic neuropathy?" *Journal of Diabetes Research*, vol. 2017, Article ID 6943851, 9 pages, 2017.

[3] A. Ando, M. Miyamoto, K. Kotani, K. Okada, S. Nagasaka, and S. Ishibashi, "Cardio-ankle vascular index (CAVI) and indices of diabetic polyneuropathy in patients with type 2 diabetes," *Journal of Diabetes Research*, vol. 2017, Article ID 2810914, 8 pages, 2017.

[4] F. Ishibashi, R. Kojima, M. Taniguchi, A. Kosaka, H. Uetake, and M. Tavakoli, "The preferential impairment of pupil constriction stimulated by blue light in patients with type 2 diabetes without autonomic neuropathy," *Journal of Diabetes Research*, vol. 2017, Article ID 6069730, 11 pages, 2017.

[5] V. L. Newton, J. D. Guck, M. A. Cotter, N. E. Cameron, and N. J. Gardiner, "Neutrophils infiltrate the spinal cord parenchyma of rats with experimental diabetic neuropathy," *Journal of Diabetes Research*, vol. 2017, Article ID 4729284, 10 pages, 2017.

Hindawi
Journal of Diabetes Research
Volume 2017, Article ID 2810914, 8 pages
https://doi.org/10.1155/2017/2810914

Research Article

Cardio-Ankle Vascular Index and Indices of Diabetic Polyneuropathy in Patients with Type 2 Diabetes

Akihiko Ando,[1] Michiaki Miyamoto,[1,2] Kazuhiko Kotani,[3] Kenta Okada,[1] Shoichiro Nagasaka,[1] and Shun Ishibashi[1]

[1]*Division of Endocrinology and Metabolism, Department of Internal Medicine, Jichi Medical University, Tochigi 329-0498, Japan*
[2]*Department of Laboratory Medicine, Jichi Medical University, Tochigi 329-0498, Japan*
[3]*Division of Community and Family Medicine, Centre for Community Medicine, Jichi Medical University, Tochigi 329-0498, Japan*

Correspondence should be addressed to Akihiko Ando; aando27@jichi.ac.jp

Received 2 November 2016; Revised 20 February 2017; Accepted 14 March 2017; Published 9 May 2017

Academic Editor: Mitra Tavakoli

The cardio-ankle vascular index (CAVI) is used to test vascular function and is an arterial stiffness marker and potential predictor of cardiovascular events. This study aimed to analyze the relation between objective indices of diabetic polyneuropathy (DPN) and the CAVI. One hundred sixty-six patients with type 2 diabetes mellitus were included in this study. We used nerve conduction studies (NCSs) and the coefficient of variation of the R-R interval to evaluate DPN. We estimated arteriosclerosis by the CAVI. Simple and multiple linear regression analyses were performed between neuropathy indices and the CAVI. In univariate analysis, the CAVI showed significant associations with sural sensory nerve conduction velocity and median F-wave conduction velocity. Multiple linear regression analysis for the CAVI showed that sural nerve conduction velocity and median F-wave conduction velocity were significant explanatory variables second only to age. In multiple linear regression analysis for sural nerve conduction velocity among neuropathy indices, the CAVI remained the most significant explanatory variable. In multiple linear regression analysis for median nerve F-wave conduction velocity among neuropathy indices, the CAVI remained the second most significant explanatory variable following HbA1c. These results suggest a close relationship between macroangiopathy and DPN.

1. Introduction

A pandemic increase in type 2 diabetes leads to high morbidity and mortality because of its complications. A relation has been reported between macrovascular and microvascular impairment in type 2 diabetes [1–5]. There have been many studies concerning diabetic polyneuropathy (DPN) and its association with diabetic macroangiopathy [6–8]. Additionally, several studies have shown pathophysiological associations between DPN and macroangiopathy, as well as a similar onset time of DPN and macroangiopathy in the early phase of progression in diabetic complications [9–11]. Macrovascular impairment progresses through several phases, such as endothelial dysfunction, low vessel wall elasticity, and resulting structural sclerosis. Macrovascular damage together with microvascular change causes ischemia and hypoxia in neural tissues because of disordered endothelial function, arterial stiffness, and stenosis [12, 13]. This leads to dysregulated cardiovascular autonomic function. Loss of vessel wall elasticity and insufficiency of the peripheral circulation impair normal functioning of neurons. A prospective study showed that the incidence of DPN is associated with potentially modifiable cardiovascular risk factors, including elevated triglyceride levels and body mass index, smoking, and hypertension [14]. DPN as assessed by a 10 g monofilament was reported to be associated with an increased risk for cardiovascular events among individuals with diabetes [15]. Most previous studies on the relation between DPN and diabetic macroangiopathy were based on serum lipid markers, blood pressure, and neurological symptoms. Physiological assessment of arteriosclerosis, using pulse wave velocity (PWV) and intima-media thickness measurement of the carotid artery, is related to indices of diabetic autonomic neuropathy. These indices include the coefficient of variation

of the R-R interval ($CV_{R\text{-}R}$) [16–18] as well as indices of somatic neuropathy [19, 20].

Macrovascular impairment can be evaluated by arterial stiffness. PWV as a marker for arterial stiffness has become popular for its simple usability. However, application of PWV is limited by its dependence on blood pressure of the measurement point [21]. The cardio-ankle vascular index (CAVI) has been developed in Japan to overcome this limitation [22]. The CAVI can be determined from the stiffness parameter β, which represents the change in blood pressure required to expand the diameter of the artery. As a result of applying Bramwell-Hill's equation, the CAVI does not depend on blood pressure at measurement [23]. Recently, there have been two reports on the CAVI and DPN [24, 25]. One report showed that patients with DPN have a higher CAVI than those without DPN. Additionally, in multivariate analysis, this report showed that the CAVI was a significant determinant of DPN [24]. In the other report, the authors performed a retrospective, cross-sectional study of Korean patients with type 2 diabetes and showed that an increased CAVI was associated with DPN [25].

One of these previous studies defined DPN based on neuropathic symptoms, insensitivity of a 10 g monofilament, abnormal pinprick sensation, and the current perception threshold [24]. The nerve conduction study (NCS), which is the gold standard for objective diagnosis of diabetic neuropathy, was not used in this previous report [26]. In the other study, DPN was defined as a positive result of a neuropathy test (NCS, current perception threshold, and autonomic function test) [25]. The difference in CAVI values between those with DPN and those without DPN was examined. The current perception threshold used for DPN diagnosis was not totally objective and was dependent on patients' responses. A simultaneous change in the CAVI with NCS markers only was not demonstrated in this study [25].

In clinical practice, diagnosis and assessment of diabetic neuropathy depend on neuropathic symptoms and neurological examinations, which are subject to the patients' response, and vary according to the examiners. The most reproducible, reliable, and objective measures of DPN are NCSs [26]. However, NCSs can only assess large fibre neuropathy and they require some standardization [27].

In the present study, we aimed to determine the relationship between DPN and macroangiopathy by correlating neuropathy indices, especially NCS parameters, with the CAVI in patients with type 2 diabetes.

2. Materials and Methods

2.1. Study Design and Patient Population. We performed a cross-sectional study at Jichi Medical University in Japan. We recruited 207 inpatients and outpatients with type 2 diabetes from November 2011 to May 2015. Written consents were obtained from all of the patients. Finally, 166 patients were included in this study. Major exclusion criteria were as follows: age ≥ 75 years, renal failure requiring regular hemodialysis, those who had chemotherapy, alcohol or drug addicts, peripheral artery disease with an ankle-brachial index less than 0.9, neurodegenerative diseases (including Parkinsonism and dementia), cerebrovascular disease, and entrapment neuropathy. Patients who took α-blockers as an antihypertensive medicine were also excluded, because it was reported that α-blockers decreased CAVI value [28]. This study adhered to the tenets of the Declaration of Helsinki and was approved by the Institutional Review Board of Jichi Medical University.

The presence of neuropathy was determined by the Toronto consensus criteria of "probable neuropathy." The term "probable neuropathy" implies the presence of a combination of symptoms and signs of neuropathy, which include any two or more of the following: neuropathic symptoms, decreased distal sensation, and unequivocally decreased or absent ankle reflexes [29]. The definition of neuropathy above by the Toronto consensus criteria was with regard to typical DPN, and atypical DPN includes painful, autonomic, and nerve morphologic abnormalities (small fibre neuropathy). In typical DPN, autonomic dysfunction and neuropathic pain may also develop over time. Therefore, these symptoms were checked in all patients (see Section 2.3). In this paper, the term "DPN" principally signifies "diabetic symmetric sensorimotor polyneuropathy" but implies also part of diabetic autonomic neuropathy.

Diagnosis of diabetic retinopathy was made by an indirect ophthalmic examination based on the presence of clinical features in the fundus of both eyes. Diabetic retinopathy was subdivided as follows: no apparent diabetic retinopathy, simple diabetic retinopathy, preproliferative diabetic retinopathy, and proliferative diabetic retinopathy [30]. Those who had received panretinal photocoagulation were included in the category of proliferative retinopathy. Retinopathy was defined when simple diabetic retinopathy was present.

Diabetic nephropathy was classified into four groups (patients receiving dialysis therapy were excluded) as follows: (1) prenephropathy with normoalbuminuria (estimated glomerular filtration rate (eGFR) ≥ 30 mL/min/1.73 m^2), (2) incipient nephropathy with microalbuminuria (eGFR ≥ 30 mL/min/1.73 m^2), (3) overt nephropathy with macroalbuminuria or persistent proteinuria (eGFR ≥ 30 mL/min/1.73 m^2), and (4) kidney failure with any albuminuria/proteinuria status (eGFR < 30 mL/min/1.73 m^2), according to a new classification of diabetic nephropathy by a joint committee on diabetic nephropathy in Japan in 2014 [31]. Microalbuminuria was defined as an abnormally increased excretion rate of albumin in the urine in the range of 30–299 mg/g creatinine. Nephropathy was defined when microalbuminuria was present.

2.2. Physical Examination and Laboratory Measurements. For every patient, general medical assessments, such as body height, body weight, blood pressure, medical history taking, and blood sampling, were performed. The body mass index was derived from the following calculation: body weight (kg) divided by the square of body height (m). Blood pressure was measured in the supine position twice in a quiet room using an automated sphygmomanometer, and the mean levels were recorded [32]. Mean blood pressure was approximated by calculating the following formula:

(systolic blood pressure − diastolic blood pressure)/3 + diastolic blood pressure. Hypertension was defined as blood pressure above 140/90 mmHg or taking antihypertensive medication. In medical history taking, smoking status and the duration of diabetes were recorded. Blood samples were collected in the morning after 12 hours of fasting. Haemoglobin A1c (HbA1c) levels were measured by a high-performance liquid chromatography method. The present study presented HbA1c of the National Glycohaemoglobin Standardization Program (NGSP) equivalent value (%) together with the International Federation of Clinical Chemistry unit (mmol/mol) converted from NGSP value. Fasting plasma glucose, total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), and triglyceride (TG) levels were determined by enzymatic assays [33–36]. Low-density lipoprotein cholesterol (LDL-C) levels were calculated according to the Friedewald formula: LDL − C = TC − HDL − C − TG/5. Cystatin C levels were measured by latex agglutination turbidimetry [37].

2.3. Estimation of Diabetic Neuropathy. Every patient was asked about neuropathic symptoms. These symptoms included somatic symptoms, such as bilateral numbness, tingling, and a prickling sensation, and dysesthesia of the toes and soles of both of the feet. Additionally, autonomic symptoms, such as orthostatic hypotension, persistent constipation, and diarrhea, were recorded.

For neurological signs, the Achilles tendon reflex was checked. This reflex was regarded as decreased when there was a response for both legs in the knee-standing position (reinforcement method) with a percussion hammer or as disappeared when there was no response, even with such a reinforcement method. Vibratory perception was tested using a 128 Hz tuning fork. Shortening of the perceptible time for a fork vibration of less than 10 seconds was judged as attenuation of vibration perception. Semmes-Weinstein monofilament tests were performed to identify the presence of anesthesia. The patients lay down and closed their eyes. Monofilaments were then pressed onto the top and bottom of both feet until the tips of the filaments started to bend, changing the diameter of the filaments. When filaments that were thinner than number 3.84 were imperceptible, the patients were considered as having sensory attenuation. Muscle weakness and atrophy were estimated by a manual muscle test for the extensor digitorum brevis muscle in both feet. All these neurological symptoms and signs were arranged and fitted into 5 diabetic neuropathy stages by the Diabetic Neuropathy Study Group in Japan [38]: (I) no signs and symptoms or one of the following: bilateral foot symptoms, bilateral attenuation of Achilles tendon reflex, and bilateral attenuation of vibration perception; (II) bilateral attenuation of Achilles tendon reflex and bilateral attenuation of vibration perception; (III) II plus bilateral foot symptoms and bilateral attenuation of touch sensation; (IV) III plus autonomic symptoms (orthostatic hypotension, persistent diarrhea, and persistent constipation); and (V) IV plus muscle atrophy or weakness of extensor digitorum brevis (maybe without bilateral foot symptoms). Signs (Achilles tendon reflex > vibration test and monofilament) and the results of nerve conduction studies are given priority over somatic and autonomic symptoms when determining the stage due to the insufficient reliability of patients' statement about their symptoms.

The $CV_{R\text{-}R}$ at rest and in deep breathing was measured. After the patient had rested in the supine position for at least 10 minutes, a standard 12-lead electrocardiogram was recorded (Cardio StarFCP-7541; Nihon Kohden Corporation, Tokyo, Japan). The R-R intervals were measured for 100 heartbeats at resting breathing and then for 100 heartbeats while taking deep breaths (1 time for 5 seconds). The $CV_{R\text{-}R}$ was obtained by dividing the standard deviation (SD) by the mean (M): $CV(\%) = (SD/M) \times 100$ [39].

For sural nerve sensory conduction velocity, an active electrode was positioned on the middle point between the lateral malleolus and the tip of the little toe. A reference electrode was placed 3 cm distal to the active electrode. A ground electrode was placed on the distal lateral leg between stimulating and recording electrodes. For stimulation, a cathode was placed between the lateral malleolus and the Achilles tendon.

For peroneal nerve motor conduction velocity, an active electrode was positioned over the belly of the extensor digitorum brevis muscle. A reference electrode was placed distal to the active electrode over the tendon of the extensor digitorum brevis muscle. For stimulation, a cathode was placed distally at the middle point between the Achilles tendon and lateral malleolus and proximally above the head of the fibula.

For tibial nerve motor conduction velocity, an active electrode was positioned over the belly of the abductor hallucis muscle slightly beneath and in front of the navicular bone. A reference electrode was placed distal to the active electrode over the tendon of the abductor hallucis. For stimulation, a cathode was placed distally on the point proximal to the active electrode between the medial malleolus and the Achilles tendon and proximally slightly lateral to the center of the skin line in the popliteal fossa.

For median nerve F-wave conduction velocity, active electrodes were positioned over the belly of the abductor pollicis brevis muscle. A reference electrode was placed over the tendon of the abductor pollicis brevis at the base of the phalanx of the thumb. For supramaximal stimulation for the F-wave, a cathode was placed between the tendon of the palmaris longus muscle and that of the radial flexor muscle of the wrist. The distance was measured connecting the wrist, elbow, axillary line, middle point of the clavicle, and seventh cervical spinous process.

Sensory, motor, and F-wave conduction velocities of the nerves, of which action potentials could not be obtained, were omitted. Omitted numbers are 24, 19, 1, and 5 for sural sensory, peroneal motor, tibial motor, and median F-wave conduction velocities, respectively.

In all NCS measurements, a ground electrode was placed on the dorsum of the hand and foot between stimulating and active (recording) electrodes. The anode was 2 cm proximal to the cathode. All of the subjects were positioned in a quiet room and remained awake while testing. Skin temperature was maintained above 33°C in the upper limbs (at the midpoint of the forearm) and above 32°C in the lower limbs

(at the midpoint of the lower leg), as measured by a far infrared ray thermometer.

The measuring device that was used for the NCS was the NeuropackM1 KD-026A® (Nihon Kohden Corporation).

2.4. Measurement of the CAVI. The subjects were placed in the supine position. Blood pressure was measured at the brachial artery, and heart sounds were monitored using an electrocardiogram. The length from the aortic valve to the ankle and the time taken for the pulse wave to propagate from the heart to the ankle were measured. The pulse wave velocity from the heart to the ankle was obtained by dividing the length from the aortic valve to the ankle by the time taken for the pulse wave to propagate from the heart.

The principle of the CAVI formula and its calculation have been described previously [23]. For statistical evaluation of the CAVI, mean values of the left and right sides were used. The CAVI was measured by the VaSera VS-1000 (Fukuda Denshi, Tokyo, Japan).

2.5. Statistical Analysis. Statistical analysis was performed using Stata SE® ver. 12 (StataCorp LP, College Station, TX, USA). Clinical profiles of the recruited patients are shown as mean ± standard deviation. Simple and multiple linear regression analyses were performed to investigate determinants of neuropathy indices and the CAVI. Biologically plausible predictors were as follows: age, sex, presence of obesity, mean arterial pressure, presence of dyslipidaemia (defined as TG levels > 3.9 mmol/L, HDL-C levels < 1.0 mmol/L, LDL-C levels > 3.6 mmol/L, or taking medications for dyslipidaemia), current smoking, cystatin C, and HbA1c. Obesity was defined as body mass index (BMI) $\geq 25\,kg/m^2$ [40]. Cystatin C is a kidney function marker independent of sex, age, and muscle mass compared with serum creatinine [41]. Multilinearity was checked by the variance inflation factor.

3. Results

3.1. Clinical Profiles of the Patients. Most patients were middle-aged to older people, and 64.5% were male. The mean duration of diabetes was 13.0 ± 8.2 years. The rates of hypertension and dyslipidaemia were 48.2% and 73.5%, respectively. Those who had a habit of drinking, excluding chance drinkers, comprised 35.5% of the patients, but there were no alcohol addicts, leading to alcoholic neuropathy. Microangiopathies, such as retinopathy and nephropathy, were detected in approximately 40–50% of all patients (Table 1).

The CAVI value less than 8.0 was estimated as normal. The mean CAVI value of this population was 8.6 ± 1.4 and only 31.3% was within normal range. Somatosensory symptoms of both feet were observed in only 31.9% of patients, but the Achilles tendon reflex, vibration perception, and tactile sense of pressure as assessed by monofilaments were attenuated or lost in 54.5%, 41.6%, and 38.8% of patients, respectively. The effect of aging cannot be excluded when assessing these signs. Autonomic symptoms were detected in 56.6% of all patients, although these symptoms cannot clearly be attributable to DPN. Only 13.3% of the patients had atrophy and muscle weakness of the extensor digitorum brevis muscle. Neuropathy was diagnosed in 57.6% of all patients by the Toronto consensus criteria. There was a wide variation in the neuropathy stages in this population, thus considered suitable for the statistical analyses (Table 2).

Table 1: Clinical profile of the patients ($n = 166$).

Age (years)	59.1 ± 11.2
Male (%)	64.5
Body mass index (kg/m^2)	26.6 ± 5.0
Obesity (%)	56.6
Systolic blood pressure (mmHg)	136.0 ± 17.3
Diastolic blood pressure (mmHg)	81.3 ± 14.2
Mean arterial pressure (mmHg)	99.5 ± 13.7
Hypertension (%) (above140/90 mmHg or medication)	48.2
Smoking (%)	59.0
(Ex-smoker/current smoker)	37.9/21.1
Alcohol (%) (excluding chance drinker)	35.5
Duration of diabetes (years)	13.0 ± 8.2
Retinopathy (%)	47.2 (NDR, 52.8; SDR, 22.7; PPDR, 9.2; PDR, 15.3)
Nephropathy (%)	40.4 (stage 1, 59.6; stage 2, 27.1; stage 3, 10.8; stage 4, 2.4)
Cystatin C (mmol/L)	0.72 ± 0.25
Fasting plasma glucose (mmol/L)	8.1 ± 2.5
HbA1c (mmol/mol)	67.1 ± 1.3
TG (mmol/L)	1.4 ± 0.8
HDL-C (mmol/L)	1.4 ± 0.4
LDL-C (mmol/L)(Friedewald)	2.5 ± 0.8
Dyslipidaemia (%) (TG > 3.9, HDL < 1.0, LDL > 3.6, or medication)	73.5

Values are mean ± standard deviation.
BMI: body mass index; NDR: no diabetic retinopathy; PDR: proliferative diabetic retinopathy; PPDR: preproliferative diabetic retinopathy; SD: standard deviation; SDR: simple diabetic retinopathy; TG: triglycerides; HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol.

3.2. Simple and Multiple Linear Regression Analyses between Indices of Diabetic Neuropathy and Physiological Parameters for Arteriosclerosis. Simple linear regression showed that the CAVI was significantly associated with sural sensory nerve conduction velocity and median F-wave conduction velocity ($p < 0.001$; $p < 0.001$).

Multiple linear regression of other neuropathies and risk factors of arteriosclerosis (age, sex, presence of obesity, body height, mean arterial pressure, presence of dyslipidaemia, current smoking, cystatin C, and HbA1c) showed that the CAVI was significantly associated with sural sensory nerve conduction velocity and median F-wave conduction velocity (Table 3).

In multiple linear regression analysis for the CAVI, sural nerve conduction velocity remained a significant variable after the variable of age ($p < 0.001$). The same

Table 2: Clinical profile of the CAVI and DPN markers ($n = 166$).

CAVI	8.6 ± 1.4
Nerve conduction study	
Sural SCV (m/s)	42.3 ± 5.1
Peroneal MCV (m/s)	42.7 ± 4.6
Tibial MCV (m/s)	40.9 ± 4.5
Median FWCV (m/s)	61.9 ± 6.1
Neuropathy (rate of abnormality)	
Bilateral foot symptoms (%)	31.9
Decreased Achilles tendon reflex (%)	54.5
Decreased vibration perception (%)	41.6
Decreased touch sensation examined by Semmes–Weinstein monofilaments (%)	38.8
Autonomic symptoms (%)	56.6
Atrophy and muscle weakness of extensor digitorum brevis (%)	13.3
Neuropathy (by the Toronto consensus criteria) (%)	57.6
Neuropathy stage (by the Diabetic Neuropathy Study Group in Japan) (%)	1: 30.7; 2: 20.5; 3: 15.7; 4: 22.3; 5: 10.8
$CV_{R\text{-}R}$	
Resting (%)	2.4 ± 1.3
Deep breathing (%)	4.9 ± 2.5

Values are mean ± standard deviation.
CAVI: cardio-ankle vascular index; SCV: sensory nerve conduction velocity; MCV: motor nerve conduction velocity; FWCV: F-wave conduction velocity; $CV_{R\text{-}R}$: coefficient of variation of the R-R interval.

Table 3: Simple and multiple linear regression analyses of indices of diabetic neuropathy with the CAVI.

	CAVI		
	r	β	β'
Sural SCV	-0.41^{**}	-026^{**}	-0.36^{**}
Peroneal MCV	−0.16	−0.08	−0.10
Tibial MCV	−0.13	−0.13	−0.14
Median FWCV	-0.28^{**}	-0.25^{**}	-0.28^{**}
CVRR resting	−0.15	−0.00	−0.00
CVRR deep breathing	−0.07	0.05	0.07

Coefficients (standardized) of simple regression (r) and those of multiple regression (β) for the CAVI and (β') for neuropathy indices are shown. Please see the main text for details of the other explanatory variables. $^{**}p < 0.01$; SCV: sensory nerve conduction velocity; MCV: motor nerve conduction velocity; FWCV: F-wave conduction velocity; $CV_{R\text{-}R}$: coefficient of variation of the R-R interval.

analysis for sural nerve conduction velocity showed that the CAVI remained the most significant explanatory variable ($p < 0.001$). The same analysis for the CAVI showed that median nerve F-wave conduction velocity remained a significant explanatory variable following age ($p = 0.001$). The same analysis for median nerve F-wave conduction velocity showed that the CAVI remained a significant explanatory variable following HbA1c ($p = 0.001$).

There was no multilinearity between explanatory variables (Table 4).

Table 4: Standardized coefficients of multiple linear regression (β, β') of all of the explanatory variables.

CAVI	β
Sural SCV/median FWCV	$-0.26^{**}/-0.25^{**}$
Age	$0.41^{**}/0.37^{**}$
Gender	−0.02/0.02
Obesity	$-0.21^{**}/-0.13$
Mean arterial pressure	−0.02/−0.01
Dyslipidaemia	−0.06/−0.10
Current smoking	0.02/0.01
HbA1c	$-0.19^{**}/-0.18^{*}$
Cystatin C	0.03/0.15
Sural SCV/median FWCV	β'
CAVI	$-0.36^{**}/-0.28^{**}$
Age	−0.05/−0.10
Gender	−0.18/−0.03
Obesity	0.16/0.21
Mean arterial pressure	−0.03/0.07
Dyslipidaemia	0.01/−0.10
Current smoking	0.12/0.01
HbA1c	$-0.21^{*}/-0.34^{**}$
Cystatin C	−0.15/−0.11

β: independent variables were the CAVI, $^{*}p < 0.05$, $^{**}p < 0.01$; β': independent variables were sural SCV and median FWCV, $^{*}p < 0.05$, $^{**}p < 0.01$; SCV: sensory nerve conduction velocity; FWCV: F-wave conduction velocity; CAVI: cardio-ankle vascular index.

4. Discussion

In the current study, multiple linear regression analysis for sural sensory nerve conduction velocity and median nerve F-wave conduction velocity showed that the CAVI remained a significant explanatory variable in its relation to diabetic neuropathy and vice versa. Previous studies have shown that CAVI values differ between those with neuropathy and those without neuropathy [24, 25]. The present study evaluated and identified the relation of the CAVI with neuropathy by direct statistical assessment with objective measurement of DPN (using the NCS).

NCS markers of the lower extremities are primarily used for assessment of diabetic neuropathy. The most representative abnormalities of nerve conduction in DPN are peroneal motor nerve conduction velocity, compound muscle action potentials, distal latency, sural sensory nerve conduction velocity, sensory action potentials, and tibial distal latency [26]. Our study was conducted before normalization of nerve conduction measurements, such as the location of electrodes, in our institute. Therefore, distal latency and amplitude were not adopted. F-wave studies can be used to assess conduction of proximal nerve segments in contrast to conduction of only the distal segments in routine NCSs. Therefore, we measured

conduction velocity of the median nerve. F-wave conduction velocity of the tibial nerve was not measured because of the potential uncertainty of determination of distance. The F-wave is due to direct antidromic activation of spinal motor neurons. The same motor axon serves as the afferent and efferent arc. Sural sensory nerve conduction velocity, which was a determinant of the CAVI in our study, was selected among NCS parameters by a newly developed automated NCS device (NC-stat DPNCheck®; Waltham, Massachusetts, USA) for screening, early detection, and diagnosis of DPN together with sural nerve action potentials [42]. The reason why peroneal motor nerve conduction velocity had no relation with the CAVI may be partly due to the potential compression damage caused by the habit of sitting squarely in the elderly Japanese population, but the exact reason for this poor regression remains unknown.

A decrease in vascular elasticity (i.e., arterial stiffness) mainly depends on an abnormal increase and thickening of the intima. Subsequently, elastic fibres of the media in the elastic artery (not in muscular-type arteries) rupture, which leads to a loss of extensibility of the vascular wall. Elastic arteries play an auxiliary role in the pumping function of the heart. These arteries also help to alter intermittent cardiac ejection of continuous blood flow to muscular arteries and small vessels. Loss of vascular elasticity is due to a decrease in elastin and an increase in collagen, mainly caused by aging. This fact is supported by our finding that aging was the most significant explanatory variable in multiple regression for CAVI. Loss of vascular elasticity causes impairment of regulated continuous blood supply to the periphery and leads to ischemia and reperfusion-induced injury to neural tissues [15, 43]. This injury causes failure of autonomic drive and leads to further arteriosclerotic changes [15, 44].

In multiple regression analysis for CAVI, sural nerve conduction velocity and median nerve F-wave conduction velocity were the significant explanatory variables second to age. In the same analysis for sural nerve conduction velocity and median nerve F-wave conduction velocity, the CAVI remained the most significant explanatory variable for sural nerve conduction velocity and the second significant explanatory variable for median nerve F-wave conduction velocity following HbA1c. A study on patients with coronary artery disease showed that the CAVI could be used for predicting future cardiovascular events, although numbers of the participants were small and the included participants were limited to a specific disease [45]. These previous results suggest that the CAVI and the NCS may be related to future cardiovascular events [46].

This study has some limitations. In severe DPN cases, action potentials could not be obtained due to extinction of conducting nerve fibres. These cases are not included in this study, and analyses on NCSs, especially sural nerve sensory conduction velocity and peroneal nerve motor conduction velocity, were restricted to the cases whose conducting nerve fibres were still present. Another limitation is that this was a cross-sectional and observational study with a limited number of subjects. Therefore, a causal relationship between DPN and arteriosclerosis cannot be assumed. An interventional and large sample study is needed to further determine the pathophysiological relations between these variables. Lastly, NCSs can only assess large fibre neuropathy, and small fibre neuropathy was not fully examined. $CV_{R\text{-}R}$ can estimate diabetic small fibre impairment, but it only measures cardiac parasympathetic nerve function and cannot be applied to patients with arrhythmia.

5. Conclusions

In conclusion, multiple regression analysis indicated that the CAVI, a potential predictor of cardiovascular events, remains the most significant explanatory variable for sural sensory nerve conduction velocity, lower limb NCS markers that are essential for assessment of DPN. The CAVI, an arterial stiffness and vascular damage marker, has a close relationship with sural nerve conduction velocity and median F-wave conduction velocity, a marker of distal and proximal neuropathic impairment, implying that arterial stiffness and impairment of nerve function start at a similar point of time and progress at a similar rate in patients with diabetes. When considering management of neuropathy or arteriosclerosis, assessment of one of these two complications needs to be combined with understanding of the state of the other. The NCS is an established and objective measure of DPN, but it cannot assess small fibre neuropathy, including autonomic neuropathy. Repeated failure of past clinical trials of DPN was partly attributable to this limitation [47]. Future studies concerning the relations between direct functional vascular markers and noninvasive and reliable neuropathy markers covering small fibre neuropathy are expected for early diagnosis and for preventing the advancement in severity of DPN, amputation, and cardiovascular events.

Conflicts of Interest

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

Acknowledgments

The authors thank H. Yanagida and Professor T. Taniguchi of the Department of Laboratory Medicine, Jichi Medical University, for their assistance.

References

[1] M. Rema, V. Mohan, R. Deepa, and R. Ravikumar, "Association of carotid intima-media thickness and arterial stiffness with diabetic retinopathy," *Diabetes Care*, vol. 27, no. 8, pp. 1962–1967, 2004.

[2] M. Y. Malecki, G. Osmenda, M. Walus-Miarka et al., "Retinopathy in type 2 diabetes mellitus is associated with increased intima-media thickness and endothelial dysfunction," *European Journal of Clinical Investigation*, vol. 38, no. 12, pp. 925–930, 2008.

[3] Y. W. Yun, M. H. Shin, Y. H. Lee, J. A. Rhee, and J. S. Choi, "Arterial stiffness is associated with diabetic retinopathy in Korean type 2 diabetic patients," *Journal of Preventive Medicine and Public Health*, vol. 44, no. 6, pp. 260–266, 2011.

[4] M. Miyamoto, K. Kotani, K. Okada et al., "The correlation of common carotid arterial diameter with atherosclerosis and diabetic retinopathy in patients with type 2 diabetes mellitus," *Acta Diabetologica*, vol. 49, no. 1, pp. 63–68, 2012.

[5] S. W. Choi, W. J. Jun, H. Y. Kim et al., "Association between albuminuria, carotid atherosclerosis, arterial stiffness, and peripheral arterial disease in Korean type 2 diabetic patients," *Kidney & Blood Pressure Research*, vol. 33, no. 2, pp. 111–118, 2010.

[6] L. Kärvestedt, E. Mårtensson, V. Grill et al., "The prevalence of peripheral neuropathy in a population-based study of patients with type 2 diabetes in Sweden," *Journal of Diabetes and Its Complications*, vol. 25, no. 2, pp. 97–106, 2011.

[7] N. E. Cameron, S. E. Eaton, M. A. Cotter, and S. Tesfaye, "Vascular factors and metabolic interactions in the pathogenesis of diabetic neuropathy," *Diabetologia*, vol. 44, no. 11, pp. 1973–1988, 2001.

[8] N. E. Cameron and M. A. Cotter, "Metabolic and vascular factors in the pathogenesis of diabetic neuropathy," *Diabetes*, vol. 46, Supplement 2, pp. S31–S37, 1997.

[9] D. Ziegler, W. Rathman, T. Dickhaus, C. Meisinger, A. Mielck, and KORA study group, "Prevalence of polyneuropathy in pre-diabetes and diabetes is associated with abdominal obesity and macroangiopathy: the MONICA/KORA Augsburg Surveys S2 and S3," *Diabetes Care*, vol. 31, no. 3, pp. 464–469, 2008.

[10] M. T. Schram, R. M. Henry, R. A. van Dijk et al., "Increased central artery stiffness in impaired glucose metabolism and type2 diabetes: the Hoorn Study," *Hypertension*, vol. 43, no. 2, pp. 176–181, 2004.

[11] F. S. Fang, M. Y. Liu, X. L. Cheng et al., "Insulin resistance correlates with the arterial stiffness before glucose intolerance," *Internal Medicine*, vol. 53, no. 3, pp. 189–194, 2014.

[12] S. Thrainsdottir, R. A. Malik, L. B. Dahlin et al., "Endoneurial capillary abnormalities presage deterioration of glucose tolerance and accompany peripheral neuropathy in man," *Diabetes*, vol. 52, no. 10, pp. 2615–2622, 2003.

[13] Z. Ram, M. Sadeh, R. Walden, and R. Adar, "Vascular insufficiency quantitatively aggravates diabetic neuropathy," *Archives of Neurology*, vol. 48, no. 12, pp. 1239–1242, 1991.

[14] S. Tesfaye, N. Chaturvedi, E. M. Simon et al., "Vascular risk factors and diabetic neuropathy," *The New England Journal of Medicine*, vol. 352, no. 4, pp. 341–350, 2005.

[15] J. R. W. Brownrigg, S. de Lusignan, A. Mcgovern et al., "Peripheral neuropathy and the risk of cardiovascular events in type 2 diabetes," *Heart*, vol. 100, no. 23, pp. 1837–1843, 2014.

[16] A. Gottsäter, A. Rydén-Ahlgren, B. Szelag et al., "Cardiovascular autonomic neuropathy associated with carotid arteriosclerosis in type 2 diabetic patients," *Diabetic Medicine*, vol. 20, no. 6, pp. 495–499, 2003.

[17] C. H. Jung, A. R. Baek, K. J. Kim et al., "Association between cardiac autonomic neuropathy, diabetic retinopathy and carotid arteriosclerosis in patients with type 2 diabetes," *Endocrinology and Metabolism*, vol. 28, no. 4, pp. 309–319, 2013.

[18] A. Bagherzadeh, A. Nejati-Afkham, Y. Tajallizade-Khoob et al., "Association of cardiac autonomic neuropathy with arterial stiffness in type 2 diabtes mellitus," *Journal of Diabetes and Metabolic Disorders*, vol. 12, no. 1, pp. 55–60, 2013.

[19] M. Suetsugu, K. Takebayashi, and Y. Aso, "Association between diabetic microangiopathy and vascular endothelial function evaluated by flow-mediated vasodilation in patients with type 2 diabetes," *International Journal of Clinical Practice*, vol. 61, no. 6, pp. 920–926, 2007.

[20] H. Yokoyama, Y. Yokota, J. Tada, and S. Kanno, "Diabetic neuropathy is closely associated with arterial stiffening and thickness in type 2 diabetes," *Diabetic Medicine*, vol. 24, no. 12, pp. 1329–1335, 2007.

[21] J. Ibata, H. Sasaki, T. Kakimoto et al., "Cardio-ankle vascular index measures arterial wall stiffness independent of blood pressure," *Diabetes Research and Clinical Practice*, vol. 80, no. 2, pp. 265–270, 2008.

[22] K. Kotani, M. Miyamoto, and N. Taniguchi, "Clinical significance of the cardio-ankle vascular index (CAVI) in hypertension," *Current Hypertension Reviews*, vol. 6, no. 4, pp. 251–253, 2010.

[23] K. M. Shirai, N. Hiruta, M. Song et al., "Cardio-ankle vascular index(CAVI) as a novel indicator of arterial stiffness: theory, evidence, and perspectives," *Journal of Atherosclerosis and Thrombosis*, vol. 18, no. 11, pp. 924–938, 2011.

[24] E. S. Kim, S. D. Moon, H. J. Kim et al., "Diabetic peripheral neuropathy is associated with increased arterial stiffness without changes in carotid intima-media thickness in type 2 diabetes," *Diabetes Care*, vol. 34, no. 6, pp. 1403–1405, 2011.

[25] K. J. Kim, B. W. Lee, H. M. Kim et al., "Associations between cardio-ankle vascular index and microvascular complications in type 2 diabetes," *Journal of Atherosclerosis and Thrombosis*, vol. 18, no. 4, pp. 328–336, 2011.

[26] P. J. Dyck, C. J. Overland, P. A. Low et al., "Signs and symptoms vs nerve conduction to diagnose diabetic sensorimotor polyneuropathy," *Muscle & Nerve*, vol. 42, no. 2, pp. 157–164, 2010.

[27] W. J. Litchy, J. W. Albers, J. Wolfe et al., "Proficiency of nerve conduction using standard methods and reference values (Cl. NPhys Trial 4)," *Muscle & Nerve*, vol. 50, no. 6, pp. 900–908, 2014.

[28] K. Shirai, M. Song, J. Suzuki et al., "Contradictory effects of β1- and α1- aderenergic receptor blockers on cardio-ankle vascular stiffness index (CAVI) - CAVI is independent of blood pressure," *Journal of Atherosclerosis and Thrombosis*, vol. 18, no. 1, pp. 49–55, 2011.

[29] S. Tesfaye, A. J. M. Boulton, P. J. Dyck et al., "Diabetic polyneuropathies: update on difinitions, diagnostic criteria, estimation of severity, and treatments," *Diabetes Care*, vol. 33, no. 10, pp. 2285–2293, 2010.

[30] M. D. Davis, F. L. Meyers, G. H. Bresnick, and G. deVenecia, "Current diagnosis and management of chorioretinal disease," in *Natural Evolution*, F. A. L'Esperance Jr, Ed., pp. 179–184, CV Mosby, St Louis, 1977.

[31] M. Haneda, K. Utsunomiya, D. Koya et al., "A new classification of diabetic nephropathy 2014: a report from joint committee on diabetic nephropathy," *Clinical and Experimental Nephrology*, vol. 19, no. 1, pp. 1–5, 2015.

[32] R. H. Fagard, J. A. Staessen, and L. Thijs, "Prediction of cardiac structure and function by repeated clinic and ambulatory blood pressure," *Hypertension*, vol. 29, no. 1 Pt 1, pp. 22–29, 1997.

[33] I. Miwa, J. Okuda, K. Maeda, and G. Okuda, "Mutarotase effect on colorimetric determination of blood glucose with -D-glucose oxidase," *Clinica Chimica Acta*, vol. 37, no. 1, pp. 538–540, 1972.

[34] C. C. Allain, L. S. Poon, C. S. Chan, W. Richmond, and P. C. Fu, "Enzymatic determination of total serum cholesterol," *Clinical Chemistry*, vol. 20, no. 4, pp. 470–475, 1974.

[35] T. Gordon, W. P. Castelli, M. C. Hjortland, W. B. Kannel, and T. R. Dawber, "High density lipoprotein as a protective factor against coronary heart disease. The Framingham Study," *The American Journal of Medicine*, vol. 62, no. 5, pp. 707–714, 1977.

[36] R. W. Spayd, B. Bruschi, B. A. Burdick et al., "Multilayer film elements for clinical analysis: applications to representative chemical determinations," *Clinical Chemistry*, vol. 24, no. 8, pp. 1343–1350, 1978.

[37] M. Tanaka, K. Matsuo, M. Enomoto, and K. Mizuno, "A sol particle homogeneous immunoassay for measuring serum cystatin C," *Clinical Biochemistry*, vol. 37, no. 1, pp. 27–35, 2004.

[38] A. Arimura, T. Deguchi, K. Sugimoto et al., "Intraepidermal nerve fiber density and nerve conduction study parameters correlate with clinical staging of diabetic polyneuropathy," *Diabetes Research and Clinical Practice*, vol. 99, no. 1, pp. 24–29, 2013.

[39] Y. Nakade, T. Takamura, M. Sakurai et al., "Association between coefficients of variation of the R-R intervals on electrocardiograms and post-challenge hyperglycemia in patients with newly diagnosed type 2 diabetes," *Journal of Diabetes Investigation*, vol. 2, no. 4, pp. 324–327, 2011.

[40] Y. Chen, W. K. Copeland, R. Vedanthan et al., "Association between body mass index and cardiovascular disease mortality in east Asians and south Asians: pooled analysis of prospective data from the Asia Cohort Consortium," *BMJ*, vol. 347, p. f5446, 2013.

[41] M. Yashiro, T. Kamata, H. Segawa, Y. Kadoya, T. Murakami, and E. Muso, "Comparisons of cystatin C with creatinine for evaluation of renal function in chronic kidney disease," *Clinical and Experimental Nephrology*, vol. 13, no. 6, pp. 598–604, 2009.

[42] G. Chatzikosma, K. Pafili, M. Demetriou, K. Vadikolias, E. Maltezos, and N. Papanas, "Evaluation of sural nerve automated nerve conduction study in the diagnosis of peripheral neuropathy in patients with type 2 diabetes mellitus," *Archives of Medical Science*, vol. 12, no. 2, pp. 390–393, 2016.

[43] M. Baba, H. Nukada, D. McMorran, K. Takahashi, R. Wada, and S. Yagihashi, "Prolonged ischemic conduction failure after reperfusion in diabetic nerve," *Muscle & Nerve*, vol. 33, no. 3, pp. 350–355, 2006.

[44] C. R. Cardoso, R. A. Moraes, N. C. Leite, and G. F. Salles, "Relationships between reduced heart rate variability and pre-clinical cardiovascular disease in patients with type 2 diabetes," *Diabetes Research and Clinical Practice*, vol. 106, no. 1, pp. 110–117, 2014.

[45] K. Otsuka, S. Fukuda, S. Shimada et al., "Serial assessment of arterial stiffness by cardio-ankle vascular index for prediction of future cardiovascular events in patients with coronary artery disease," *Hypertension Research*, vol. 37, no. 11, pp. 1014–1020, 2014.

[46] M. Paoloni, B. Volpe, M. Mangone, F. Ioppolo, and V. Santilli, "Peripheral nerve conduction abnormalities in nonparetic side of ischemic stroke patients," *Journal of Clinical Neurophysiology*, vol. 27, no. 1, pp. 48–51, 2010.

[47] V. Brill, "The perfect clinical trial," in *International Review of Neurobiology. Controversies in Diabetic Neuropathy*, N. A. Calcutt and P. Fernyhough, Eds., vol. 127pp. 27–44, Elsevier, Amsterdam, 2016.

Hindawi
Journal of Diabetes Research
Volume 2017, Article ID 6069730, 11 pages
https://doi.org/10.1155/2017/6069730

Research Article

The Preferential Impairment of Pupil Constriction Stimulated by Blue Light in Patients with Type 2 Diabetes without Autonomic Neuropathy

Fukashi Ishibashi,[1] Rie Kojima,[1] Miki Taniguchi,[1] Aiko Kosaka,[1] Harumi Uetake,[1] and Mitra Tavakoli[2]

[1]*Ishibashi Clinic, Hiroshima, Japan*
[2]*University of Exeter Medical School, Exeter, UK*

Correspondence should be addressed to Fukashi Ishibashi; ishiclic@urban.ne.jp and Mitra Tavakoli; m.tavakoli@exeter.ac.uk

Received 22 September 2016; Revised 12 December 2016; Accepted 23 February 2017; Published 22 March 2017

Academic Editor: Steven F. Abcouwer

The main aim of the present paper is to examine whether the pupillary light reflex (PLR) mediated by intrinsically photosensitive retinal ganglion cells (ipRGCs) is impaired in type 2 diabetic patients. One hundred and three diabetic patients without diabetic autonomic neuropathy (DAN) and 42 age-matched controls underwent a series of detailed neurological examinations. The patients were stratified into three groups: stage I, no neuropathy; stage II, asymptomatic neuropathy; stage III, symptomatic but without DAN. The PLR to 470 and 635 nm light at 20 cd/m^2 was recorded. Small fiber neuropathy was assessed by corneal confocal microscopy and quantifying corneal nerve fiber (CNF) morphology. The 470 nm light induced a stronger and faster PLR than did 635 nm light in all subjects. The PLR to both lights was impaired equally across all of the diabetic subgroups. The postillumination pupil response (PIPR) after 470 nm light offset at ≥1.7 sec was attenuated in diabetic patients without differences between subgroups. Receiver operating characteristic analysis revealed that the PIPR mediated by ipRGCs in patients with stage II and stage III neuropathy was different from that of the control subjects. Clinical factors, nerve conduction velocity, and CNF measures were significantly correlated with PLR parameters with 470 nm light. PLR kinetics were more impaired by stimulation with blue light than with red light in diabetic patients without DAN.

1. Introduction

Pupillary dysfunction is considered to be an early sign of systemic autonomic neuropathy [1]. The pupillary light reflex (PLR) is maintained in humans who are blind because of extrinsic outer retinal damage, indicating the presence of intrinsically photosensitive cells in the retina [2]. The rods-cones and melanopsin-expressing intrinsically photosensitive retinal ganglion cells (ipRGCs) operate together to regulate the PLR [3]. The ipRGCs are the third photoreceptor cells in the human and primate eye [4]. The primary function of ipRGCs is non-image-forming photoreception, for mediating the PLR via signaling to the pretectal olivary nucleus [5] and for the signaling of environmental irradiation to entrain the central body clock to the solar day to maintain the circadian rhythm. Red light produces pupil constriction mediated by cone input via transsynaptic activation of melanopsin-expressing retinal ganglion cells (RGCs), whereas blue light leads to pupil constriction mediated primarily by direct photoactivation of ipRGCs. Rods and cones have a physiological role in the PLR, and ipRGCs receive synaptic signals from the outer retina. The relative contributions of cones-rods and ipRGCs change depending on the stimulus wavelength, irradiance strength, and temporal profile [6, 7]. The contribution of melanopsin can only be isolated after the offset of a long duration of light irradiation as the postillumination pupil response (PIPR) [4]. Therefore, it is not possible to exactly determine the relative contributions of rods-cones and ipRGCs even when blue and red stimuli are employed for examining PLR kinetics. However, the PLR

to chromatic stimuli is the only measurable, noninvasive physiological response that directly reflects the cumulative behavior of the three types of retinal photoreceptor [8, 9]. Recently, Adhikari et al. reported that, after a 1 sec light pulse, PIPRs at <1.7 sec and ≥1.7 sec are best described by the actions of the combination of ipRGCs and rods and solely of ipRGCs, respectively [10].

The PLR has been used to diagnose diabetic autonomic neuropathy (DAN) [11, 12]. Apart from one case series [13], there have been no large-scale investigations in diabetic patients examining the relative contributions of the inner and outer photoreceptors selectively using the PLR induced by chromatic light. Of course, the PLR is the results of a neural reflex that is dependent upon pathways and synaptic events beyond the retina. The correlations between the parameters of PLR kinetics and clinical factors, neurophysiological tests, and CNF measures might indicate the disturbance of PLR arc beyond the retinal photoreceptors. The contribution of clinical factors other than hyperglycemia in impaired PLR in diabetic patients stimulated by blue and red light has never been investigated.

The present study aimed to measure the PLR stimulated by chromatic light as a differential assessment of the inner and outer retinal function and to clarify the possible causative role of the dysfunction of ipRGCs in the impaired PLR using commercially available equipment in a large number of type 2 diabetic patients without DAN.

2. Research Design and Methods

2.1. Subjects. Between June 2014 and November 2015, 103 Japanese patients with type 2 diabetes without clinical evidence of DAN as assessed by detailed examinations of diabetic neuropathy as defined in the Diabetic Neuropathy Study Group in Japan (DNSGJ) have been enrolled [14], at the Ishibashi Clinic, Hiroshima, Japan. 42 age-matched healthy subjects (HbA1c <5.7%, and fasting plasma glucose <5.5 mM or casual postprandial plasma glucose <7.7 mM) were recruited as control group. The exclusion criteria were as follows: being older than 55 years (because of the potential for yellowing of the crystalline lens [15]), color blindness, proliferative or preproliferative diabetic retinopathy, other retinal or ocular diseases, wearing hard (Rigid Gas Permeable) contact lenses, neurodegenerative diseases, and taking any drugs that affect autonomic nerve functions. Written informed consent was obtained from all subjects. The ethics committee of the Ishibashi Clinic approved the protocol of the study. All participants underwent detailed clinical, neurological, and ophthalmic assessments.

Gender and age were similar between the control group and the diabetic group and between the diabetic subgroups stratified by neuropathy severity (Table 1).

2.2. Ophthalmic Examinations

2.2.1. Pupillary Light Reflex (PLR). After dark adaptation for 10 min in a dark room, blue (470 nm) or red (635 nm) light of 20 cd/m^2 was emitted for 1 sec to the right or left eye in a random order, and changes in pupil diameter of bilateral eyes were recorded simultaneously using Iris corder Dual C10641 equipment (Hamamatsu Photonics Inc., Hamamatsu, Shizuoka, Japan) for 5 seconds. 470 nm light as a blue light and 635 nm light as a red light have the highest spectral powers. At 20 cd/m^2, 470 nm and 635 nm light are equivalent to 4.79×14 log and 2.14×14 log photons/cm^2/sec, respectively, when the pupil diameter is assumed to be 6.0 mm. We used red and blue stimuli in a random order but could not examine the influence of previous light exposure on the amplitude of pupil constriction caused by the next light exposure, because previous exposure to long wavelength light increases the amplitude of pupil constriction, whereas short wavelength light decreases it [16]. However, we used weak light irradiance for short periods (1 sec), so the influence of previous light exposure should have been small. The period between two stimuli was 15 min, to allow the pupil diameter to return to the baseline level. The light pulses were projected within the housing of a pair of goggles. The dark-adapted (baseline) pupil diameter (mm, $D1$), minimum pupil diameter (mm, $D2$) after light emission, pupil diameter constriction ($D1 - D2$, mm, PC), latency period (time required to start pupil constriction after light stimulus, msec, $T1$), period required for $D2$ (msec, $T3$), and velocity of constriction ($[D1 - D2]/T3$, μ/msec, CS) were calculated automatically by the apparatus. The PIPR was arbitrarily estimated from the area under the curve (AUC) by counting pixel numbers. According to a recent report by Adhikari et al. [10], AUCs of <1.7 sec and ≥1.7 to 3.0 sec are assumed to result from rod and melanopsin signaling and solely melanopsin signaling, respectively (Figure 1). Four measurements conducted for bilateral light-stimulated and consensual eyes were averaged. For eliminating the relative unilateral afferent pupil defects in patients, we excluded the patients whose PLR kinetic parameters were apparently different between bilateral eyes. All subjects were tested between 9 and 12 am [17].

2.2.2. Corneal Confocal Microscopy (CCM). All subjects were examined using a Heidelberg Retina Tomograph 3 equipped with a Rostock Cornea Module (Heidelberg Engineering, Heidelberg, Germany) [18]. Six high-clarity images per subject were analyzed to quantify the following parameters, to quantify the corneal nerve fibers (CNFs): CNF density (CNFD), the total number of major nerve fibers/mm^2; CNF length (CNFL), the total length of major nerve fibers (mm/mm^2); corneal nerve branch density (CNBD), the number of branches emanating from all major nerve trunks/mm^2; corneal nerve branch length (CNBL), the total length of the corneal nerve branch (mm/mm^2); tortuosity grade (TG); frequency/0.1 mm of beading (BF); and bead size (BS, μm^2). Except for the TG and BS, all measurements were performed using ImageJ (Texelcraft, Tokyo, Japan); the TG was measured using the criteria of Oliveira-Soto and Efron [19], and the BS was determined as previously reported [20].

2.3. Assessment of Neuropathy. Diabetic neuropathy was assessed in type 2 diabetic patients according to the simplified diagnostic criteria proposed by the DNSGJ [14], based on

Table 1: Clinical characteristics in control subjects and subgroups of type 2 diabetic patients stratified by the severity of diabetic neuropathy.

	Control subjects	Type 2 diabetic patients staged by neuropathy severity		
		Stage I	Stage II	Stage III
Number (M/F, M%)	42 (27/15, 64.3)	31 (20/11, 64.5)	38 (25/13, 65.8)	34 (22/12, 64.7)
Age (years)	41.1–46.2	42.9–47.6	43.5–48.5	43.4–49.1
BMI (kg/m^2)	21.8–23.6	24.1–29.8*	24.8–27.8†	26.8–29.8†
Systolic blood pressure (mmHg)	120.5–127.3	129.9–139.4‡	133.2–142.7†	136.6–147.3†
Diastolic blood pressure (mmHg)	75.5–79.0	78.2–84.7	78.9–84.5	81.8–89.4†
Number treated with angiotensin receptor blocker (%)	2 (4.8)	13 (41.9)†	15 (39.5)†	8 (23.5)‡
HbA1c (%, NGSP)	5.4–5.6	6.4–6.9†	6.9–8.3†	7.7–9.3†,§
HbA1c (mmol/mol)	35.4–37.5	46.7–52.0	52.4–67.5	60.7–78.6
Low density lipoprotein-cholesterol (mmol/L)	2.91–3.42	3.06–3.78	3.24–3.83	3.34–3.94
Number treated with statins (%)	3 (7.1)	2 (6.5)	4 (10.5)	5 (14.7)
High density lipoprotein-cholesterol (mmol/L)	1.59–1.84	1.17–1.48*	1.25–1.56*	1.23–1.52*
Triglycerides (mmol/L)	1.11–1.84	1.43–2.07	1.32–2.84	1.77–2.65*
ACR (mg/gCr)	5.1–12.2	0.0–31.5	10.0–24.2	19.4–193.8*
eGFR (ml/min)	78.6–88.2	78.9–92.1	80.0–91.9	78.8–90.5
Diabetic retinopathy (no/simple, %/%)		28/3, 90.3/9.7	33/5, 86.8/13.2	27/7, 79.4/20.6
Duration of diabetes (years)		4.1–8.1	5.4–9.6	5.3–10.6

Data are the 95% confidence intervals (CI) in control subjects and the subgroups of the type 2 diabetic patients stratified by the stages of the neuropathy according to the criteria of the Diabetic Neuropathy Study Group in Japan [14]. $^*p < 0.01$ compared with control subjects, $^\dagger p < 0.001$ compared with control subjects, $^\ddagger p < 0.05$ compared with control subjects, and $^\S p < 0.01$ compared with patients at stage I neuropathy.
ACR: urinary albumin/creatinine ratio; BMI: body mass index; eGFR: estimated glomerular filtration rate.

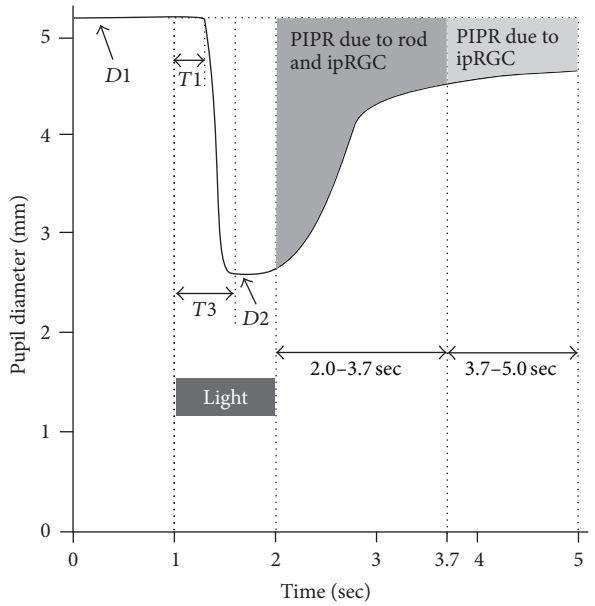


Figure 1: Pupillary light reflex waveform and kinetic parameters. *D*1, baseline average pupil diameters for one sec before light stimulus (470 nm or 635 nm); *T*1, period required to start pupil constriction after light stimulus; *D*2, the minimum pupil diameter; *T*3, period for *D*2 after light stimulus; PIPR (postillumination pupillary response) after light offset, due to rod and ipRGC at <1.7 sec and due to ipRGC at ≤1.7–3.0 sec after light offset.

the presence of two of the following three factors: subjective symptoms in the bilateral lower limbs or feet, absent or reduced ankle jerk, and decreased vibration perception. The diabetic patients were classified into one of five stages of diabetic neuropathy as defined in the DNSGJ criteria [14]: stage I, without diabetic neuropathy; stage II, asymptomatic diabetic neuropathy; stage III, symptomatic but either the ankle jerk reflex or vibration sensation was normal; stage IV, with autonomic neuropathy; and stage V, with motor neuropathy. Patients with stage IV or V neuropathy were excluded from the present study.

2.4. Neurophysiological Examinations. Electrophysiology and nerve conduction velocity (NCV) studies were performed using an electromyography instrument (Neuropack S1, Nihon Kohden, Tokyo, Japan) for the median nerve (motor) and the ulnar and sural nerves (sensory).

The vibration perception threshold (VPT) was measured at the left medial malleolus using a biothesiometer (Biomedical Instruments, Newbury, OH, USA). The warm and cold perception thresholds (PTs) at the dorsum of the foot were determined using a thermal stimulator (Intercross-200, Intercross Co., Tokyo, Japan). To assess cardiovagal function, the coefficient of variation in R-R intervals ($CV_{R\text{-}R}$) was calculated from the R-R intervals of 200 samples on an electrocardiogram.

Table 2: Neurophysiological functions in control subjects and subgroups of type 2 diabetic patients stratified by the severity of neuropathy.

	Control subjects	Type 2 diabetic patients staged by neuropathy severity		
		Stage I	Stage II	Stage III
MCV of median nerve (m/sec)	57.3–59.8	55.6–57.6	54.1–56.1*	50.4–53.6†,‡
Amplitude of median nerve (mV)	6.68–8.94	5.85–8.74	4.24–5.82*	2.82–5.15†,‡
SCV of ulnar nerve (m/sec)	62.8–65.1	61.0–63.5	60.1–62.3*	57.0–59.7†,‡
Amplitude of ulnar nerve (μV)	31.5–41.2	22.3–32.4	20.6–28.2*	15.5–22.8†,‡
SCV of sural nerve (m/sec)	47.0–49.1	46.5–49.9	46.4–49.3	45.2–48.0§
Amplitude of sural nerve (μV)	11.5–14.1	9.75–15.1	8.8–11.9	8.5–11.5
Vibration perception threshold (μ/120 c/sec)	1.56–2.64	1.76–3.62	1.91–3.15	2.30–4.00
$CV_{R\text{-}R}$ (%)	3.74–4.45	3.41–4.56	3.51–4.57	2.85–3.98
Warm perception threshold (W/m^2)	−602–−496	−582–−465	−619–−517	−616–−512
Cold perception threshold (W/m^2)	473–588	443–530	509–591	493–586

Data are the 95% confidence intervals in control subjects and the subgroups of the type 2 diabetic patients stratified by the stages of the neuropathy according to the criteria of the Diabetic Neuropathy Study Group in Japan [14]. * $p < 0.01$ compared with control subjects, † $p < 0.001$ compared with control subjects, ‡ $p < 0.01$ compared with patients at stage I neuropathy, and § $p < 0.05$ compared with control subjects.
CV: coefficient of variation; MCV: motor nerve conduction velocity; SCV: sensory nerve conduction velocity.

2.5. Medical and Laboratory Data. Body mass indexes (BMIs) and blood pressures were determined (Table 1). Glycated hemoglobin (HbA1c) levels were converted to National Glycohemoglobin Standardization Program (NGSP) units by adding 0.4% to the measured values [21]; they were subsequently converted to International Federation of Clinical Chemistry values by using the equation [(10.93NGSP)–23.50]. Serum creatinine levels, lipid profiles, urinary albumin creatinine ratio (ACR), and estimated glomerular filtration rate (eGFR) were also determined.

2.6. Statistical Analyses. All statistical analyses were performed using SPSS version 19 (SPSS, Chicago, IL, USA). All values are presented with 95% confidence intervals (CIs). All data sets were tested for normality using the Shapiro-Wilk test. Comparisons between subjects with and without diabetes were made by Student's t-test and Mann–Whitney U test for normally and nonnormally distributed continuous variables, respectively, and Fisher's exact test for categorical variables. PLR parameters obtained from using 470 nm and 635 nm light were compared in the controls and the patients with type 2 diabetes using Wilcoxon's signed-rank test. Comparisons of normally distributed variables between the control group and the diabetic subgroups were made using one-way analysis of variance (ANOVA) for continuous variables and Fisher's exact test for categorical variables, followed by Bonferroni corrections. For nonnormally distributed variables, the Kruskal-Wallis test was applied with subsequent Mann–Whitney's U test and Bonferroni corrections. The diagnostic value of a PIPR at ≥1.7 sec after blue light offset, for differentiating between the control group and the diabetic subgroups, was assessed using receiver operating characteristic (ROC) curve analysis. Multivariate regression analysis was used to determine the relationship between PLR parameters and clinical factors, neurophysiological tests, and CNF measures in the diabetic patients. A $p < 0.05$ was considered significant.

3. Results

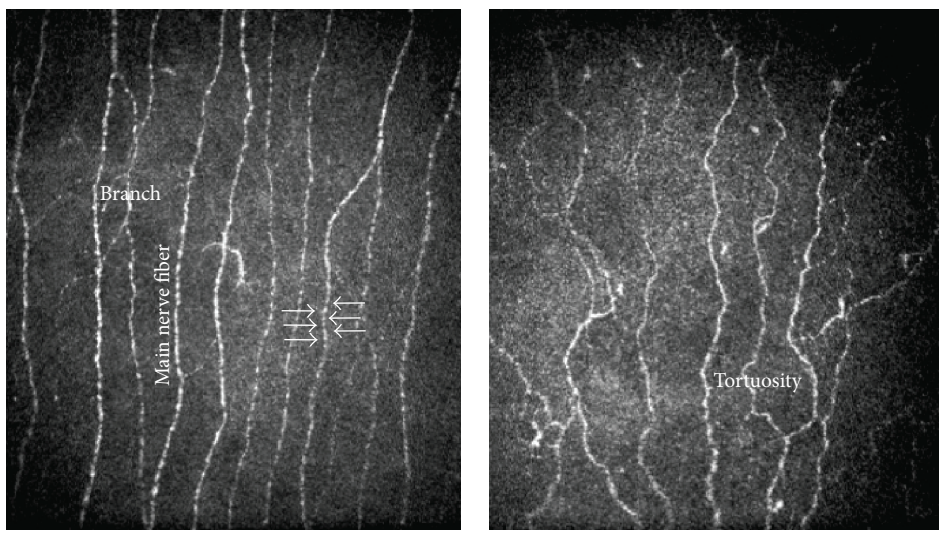
3.1. Clinical Characteristics of Control Subjects and Subgroups of Type 2 Diabetic Patients. The demographic data of the control subjects and diabetic patients are presented in Table 1. The BMIs of the diabetic patients were higher than those of the control subjects. The systolic blood pressure in all of the diabetic subgroups and the diastolic blood pressure in the subgroup with stage III neuropathy were higher than those of the control subjects. Angiotensin receptor blockers were prescribed more frequently for all diabetic patients than for the control subjects. The HbA1c levels in all of the diabetic subgroups were higher than those of the control group, and the HbA1c levels in patients with stage III neuropathy were higher than those in the patients without neuropathy. High-density lipoprotein- (HDL-) cholesterol levels in all of the diabetic subgroups were lower than those in the control group. The triglycerides levels in the subgroup of patients with stage III neuropathy were higher than those of the control subjects. The ACR in the subgroup with stage III neuropathy was higher than that of the control group. The incidence of simple diabetic retinopathy was similar among the diabetic subgroups.

3.2. Neurophysiological Tests. Neurophysiological test results in the patients without neuropathy were not different from those of the control subjects (Table 2). The NCV and amplitude of the median and ulnar nerves in the subgroup of patients with stage II neuropathy were lower compared with those of the control subjects and were even lower in patients with stage III neuropathy. The SCV of the sural nerve in patients with stage III neuropathy was slower than that in

Table 3: Corneal nerve fiber measures in control subjects and subgroups of type 2 diabetic patients stratified by the severity of neuropathy.

	Control subjects	Type 2 diabetic patients staged by neuropathy severity		
		Stage I	Stage II	Stage III
Corneal nerve fiber density (no/mm^2)	30.6–34.1	25.6–29.4*	24.9–28.1†	21.6–25.7†
Corneal nerve fiber length (mm/mm^2)	12.1–13.3	10.6–12.0	10.1–11.5*	8.95–10.4†,‡
Corneal nerve branch density (no/mm^2)	12.0–15.6	10.7–13.0	8.7–11.2*	8.4–11.0*
Corneal nerve branch length (mm/mm^2)	2.43–3.08	2.31–2.78	2.03–2.64	2.01–2.75
Tortuosity grade	1.86–2.04	2.40–2.64†	2.39–2.58†	2.47–2.68†
Beading frequency (no/0.1 mm)	23.5–24.6	19.3–20.7†	19.2–20.4†	19.5–20.8†
Bead size (μm^2)	7.89–8.25	9.80–10.2†	9.90–10.3†	10.0–10.4†

Data are the 95% confidence intervals in control subjects and the subgroups of the type 2 diabetic patients stratified by the stages of the neuropathy according to the criteria of the Diabetic Neuropathy Study Group in Japan [14]. $^*P < 0.01$ compared with control subjects, $^\dagger P < 0.001$ compared with control subjects, and $^\ddagger P < 0.05$ compared with patients at stage I neuropathy.



(a) (b)

Figure 2: Comparison of the morphology of corneal nerve fibers between a control subject (a) and a patient without neuropathy (b). White arrows indicated beads.

the control subjects. $CV_{R\text{-}R}$ and the temperature PTs in all diabetic subgroups were not significantly different from those in the control group (Table 2).

3.3. Corneal Nerve Fiber (CNF) Morphological Parameters in Control Subjects and Diabetic Subgroups. The CNFD and BF in patients without neuropathy were significantly lower, and the TG and BS were higher, compared with those of the control subjects (Table 3). In patients with stage II neuropathy, all CNFs measures except for CNBL were significantly different from those of the control subjects, and CNFL was smaller in patients with stage III neuropathy than in those with stage I neuropathy (Table 3). Figure 2 compares the CNF morphology between a control subject (a) and a diabetic patient without neuropathy (b). CNFD and CNBD were lower and tortuosity was higher in a patient without neuropathy.

3.4. Baseline Pupil Size and PLR Kinetic Parameters in Control Subjects, Total Type 2 Diabetic Patients, and Their Subgroups. The average PLR waveforms obtained from blue (Figure 3(a)) and red (Figure 3(b)) light exposure were compared among the control group and the diabetic subgroups. *D*1 of the diabetic patient group was smaller than that of the control group (Table 4). *T*1 after 470 nm light exposure was shorter than that after 635 nm light exposure in all subjects, and *T*1 after exposure to both lights was longer in diabetic patients than in control subjects. The PC caused by 470 nm light was larger than that caused by 635 nm light in all subjects, and the PC caused by both stimuli was smaller in diabetic patients than in control subjects. The CS caused by 470 nm light was faster than that caused by 635 nm light in all subjects, and the CS caused by 470 nm light was slower in diabetic patients than in the control subjects. The PIPRs at <1.7 sec and ≤1.7–3.0 sec after blue light offset were larger than those after red light offset in all subjects. The PIPRs at <1.7 sec and

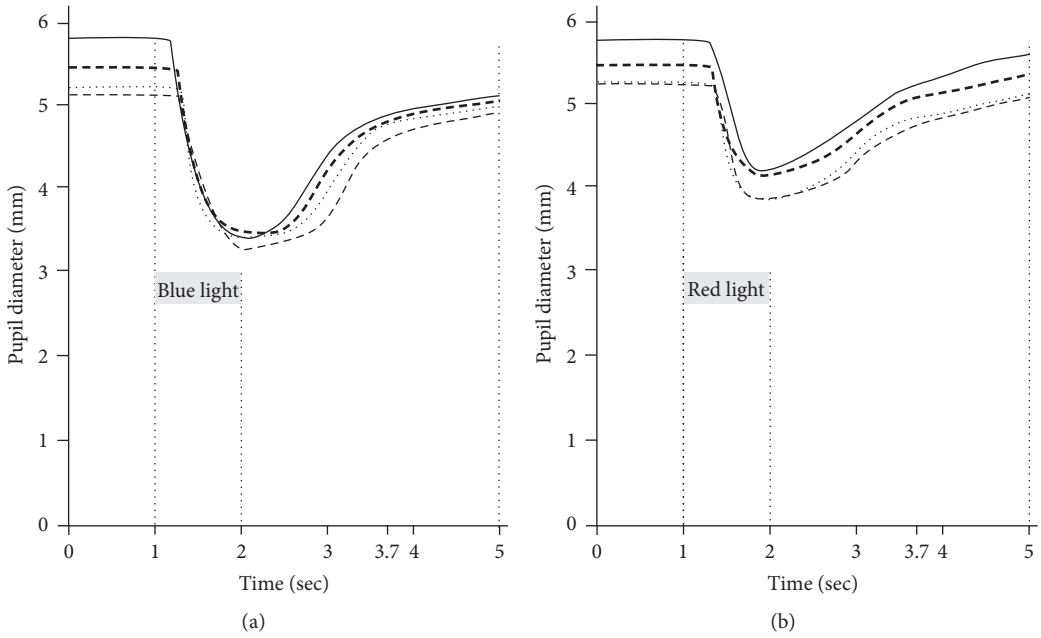


Figure 3: Comparison of average pupillary light reflex waveform on blue light (a) and red light (b) exposure between controls (—) and diabetic subgroups {I; without neuropathy (------), II; asymptomatic neuropathy (·····), and III; symptomatic but without diabetic autonomic neuropathy (- - -)} stratified by the severity of neuropathy.

≤1.7–3.0 sec after blue light offset were smaller in the diabetic patients than in the control subjects.

The PLR parameters obtained from exposure to 470 nm and 635 nm light were compared between the control subjects and the diabetic subgroups (Table 4). *D*1 before 470 nm and 635 nm light exposure was smaller in all diabetic subgroups than in the control group but was similar among the subgroups. *T*1 after exposure to 470 nm light was longer in all of the diabetic subgroups than in the control group, but there were no differences among the subgroups. *T*1 after exposure to 635 nm light of patients with stage I neuropathy was longer than that of the control subjects. In all subgroups, *T*1 after exposure to 635 nm light was longer than that after exposure to 470 nm light. The pupil constriction amplitudes in all diabetic patients and subgroups were smaller than those in the control group. *T*3s tended to be shorter in the diabetic patients than in the control subjects, but the differences did not reach significance ($p = 0.089$–0.341). PCs caused by 470 nm light were equally smaller in all subgroups than in the control group, whereas PCs caused by 635 nm light were similar across the control group and diabetic subgroups. Among the diabetic subgroups, the PCs caused by 470 nm light were more intense than those caused by 635 nm light. The CS caused by 470 nm light was slower in all of the diabetic subgroups than in the control group, whereas the CSs caused by 635 nm light were similar among the control group and the diabetic subgroups. The CSs caused by 470 nm light were faster in all of the diabetic subgroups than those caused by 635 nm light. The PIPRs at <1.7 sec and ≤1.7–3.0 sec after blue light offset were larger in the control group than in all of the diabetic subgroups.

3.5. ROC Curve Analysis of the PIPR Mediated by ipRGCs after Blue or Red Light Offset. According to ROC curves of the PIPR at ≥1.7 sec after blue (Figure 4(a)) or red light (Figure 4(b)) offset in the control group and the diabetic subgroups, the PIPRs after blue light offset of patients with stage II and stage III neuropathy had the diagnostic value for the dysfunction of ipRGCs in diabetic subgroup with stage II and III, while PIPR after red light offset did not.

3.6. Correlations between PLR Parameters and Clinical Factors, Neurophysiological Tests, and CCM Measures. Age was negatively correlated with the *T*1 and CS upon stimulation with 470 nm light and with the PC upon stimulation with lights of both wavelengths (Table 5). Blood pressure was negatively correlated with *D*1. HbA1c level was positively and HDL-cholesterol level was negatively correlated with *T*1 upon stimulation with 470 nm light. The amplitudes of the median nerve, CNFD, CNFL, CNBD, and CNBL were all positively correlated with *D*1 (Table 5). The PIPRs at <1.7 sec and ≥1.7 sec after blue light offset were not significantly correlated with any clinical factors, neurophysiological tests, or CNF measures ($p = 0.065$–0.983).

4. Discussion

It is becoming clear that neuroretinal cells [22, 23], especially RGCs [24, 25], are affected in the early stage of diabetes. Since the discovery of ipRGCs, their pivotal role in the PLR and circadian rhythm has been recognized [26, 27]. The ipRGCs project to the olivary pretectal nuclei, constituting

Table 4: Comparison of the parameters of pupillary light reflex between control subjects, type 2 diabetic patients, or their subgroups stratified by the severity of neuropathy.

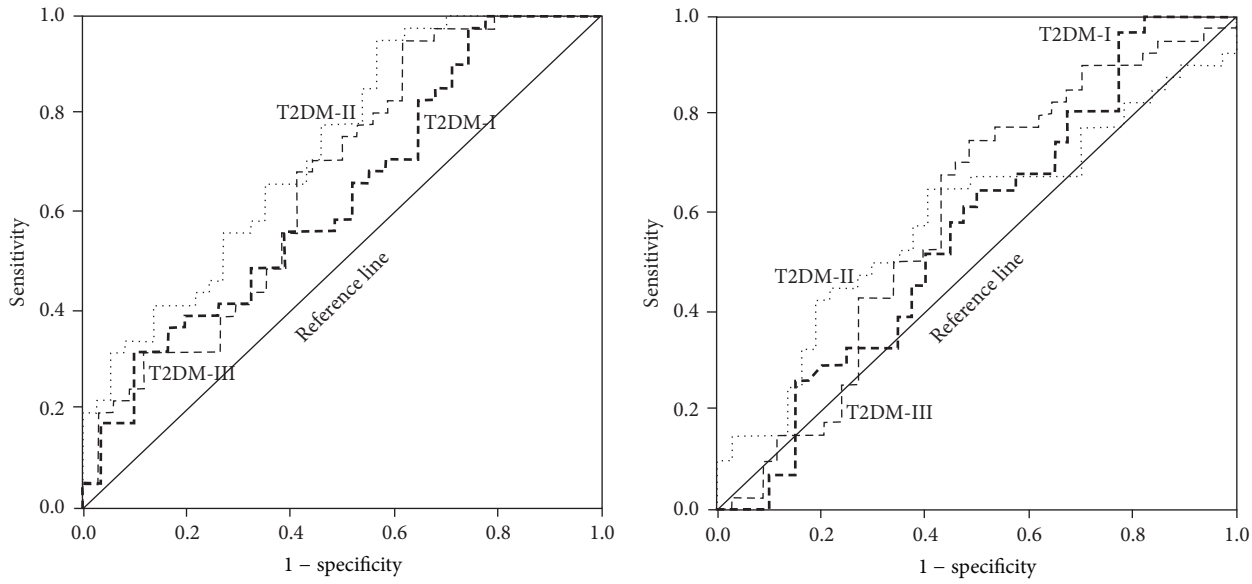
	Baseline pupil size (mm)	Latency period (msec)	Time for minimal pupil size (msec)	Pupil diameter-constriction (mm)	Pupil constric-tionvelocity (μ/msec)	PIPR (pixels) 0–1.7 sec	PIPR (pixels) ≥ 1.7–3.0 sec
Control subjects							
470 nm	5.70–5.92	247–260*	1096–1144	2.25–2.47*	2.01–2.18*	6862–7779*	2739–3407*
635 nm	5.63–5.90	285–297	848–971	1.43–1.72	1.62–1.85	3942–5009	1337–1920
Total type 2 diabetic patients							
470 nm	5.14–5.36†	267–276*,†	1049–1100	1.83–2.04*,†	1.69–1.85‡,†	5620–6301*,†	2047–2525*,†
635 nm	5.17–5.46†	301–313†	806–889	1.27–1.46†	1.53–1.70	3474–4178	1245–1614
Subgroups of neuropathy severity							
Stage I							
470 nm	5.29–5.63§	263–280§,*	1048–1132	1.81–2.22$^{\|,*}$	1.66–1.97$^{\|,\ddagger}$	5360–6693$^{\|}$	1958–2913§
635 nm	5.18–5.73§	300–324$^{\|}$	754–922	1.15–1.51	1.44–1.73	3022–4289	1023–1694
Stage II							
470 nm	5.02–5.37†	262–279§,*	1028–1108	1.67–2.01†,*	1.56–1.83†,‡	5375–6290†	1699–2452†
635 nm	5.04–5.51§	295–312	811–930	1.26–1.54	1.49–1.74	3483–4536	1176–1695
Stage III							
470 nm	4.88–5.35†	265–279§,*	1013–1120	1.77–2.16$^{\|,*}$	1.67–1.94$^{\|,\P}$	5333–6749$^{\|}$	1948–2811$^{\|}$
635 nm	4.94–5.52§	295–316$^{\|}$	748–911	1.17–1.54	1.45–1.82	3058–4508	1096–1880

Data are the 95% confidence intervals (CI) in control subjects, total type 2 diabetic patients, and their subgroups stratified by the stages of the neuropathy according to the criteria of the Diabetic Neuropathy Study Group in Japan [14]. $^{*}p < 0.001$ compared with 635 nm light, $^{\dagger}p < 0.001$ compared with control subjects, $^{\ddagger}p < 0.01$ compared with 635 nm light, $^{\S}p < 0.05$ compared with control subjects, $^{\|}p < 0.01$ compared with control subjects, and $^{\P}p < 0.05$ compared with 635 nm. PIPR: postillumination pupillary response.

the afferent arm of the PLR [28]. Although there were many methodological limitations, we were able to differentially evaluate the functions of cones-rods and ipRGCs using the PLR caused by red and blue light with the sensitive wave length and elucidate which photoreceptors were impaired by diabetes.

When the PLR kinetics following long-standing blue and red light irradiation were compared, the onset of pupil constriction caused by blue light was slower than that caused by red light, and the PIPR after the offset of blue light persisted longer than that after red light, to which the ipRGCs solely contribute [4, 6]. However, the relative contributions of cones-rods and ipRGCs change depending on the stimulus wavelength, irradiation strength, and temporal profile [6, 7]. Therefore, many human PLR studies of ipRGCs have employed stimuli with long durations (>10 sec) [4, 29, 30], which robustly established the contribution of ipRGCs to PLR kinetics. Using 10 sec stimulus with a 30 sec follow-up period after light offset, Feigl et al. demonstrated impaired ipRGC function in diabetic patients for the first time [13]. Park et al. [31] reported that blue light stimulation for 1 sec elicited a prolonged PIPR after light offset more effectively than 10 sec stimulation. Recently, Adhikari et al. [10] elegantly showed that, after a 1 sec light pulse, PIPR spectral sensitivity at ≥1.7 sec after light offset is best described by ipRGC contribution, and at times <1.7 sec the effect is mediated by rods and ipRGCs. These two reports enabled us to use 1 sec chromatic light stimulation to clinically elicit the impaired contribution of ipRGCs to the PLR using a large sample size. We used 470 nm blue light because it has the highest sensitivity in the short wavelength region of the spectrum of light that ipRGCs respond to. Therefore, the present study employed light at 470 nm and 635 nm for 1 sec with a follow-up period after light offset of 3 sec, and the PIPR after light offset was assessed by the area within the redilatation curve at ≥1.7 sec and <1.7 sec separately [10]. This enabled the elucidation of the kinetic differences in PLR caused by two different light stimuli between control subjects, type 2 diabetic patients, and the subgroups of diabetic patients stratified by the severity of neuropathy. Since the melanopsin PIPR can persist for as long as 80 sec [32], our follow-up period of 3 sec was too short to fully elucidate the contribution of ipRGCs. In addition, because we did not perform spectral analysis, the relative contributions by cones-rods and ipRGCs to the early metrics of the PLR could not be assessed. As previously reported [11], $D1$ of the diabetic patients was smaller than that of the control subjects. We did not dilate the pupils and used a low level of light irradiance close to the threshold for eliciting a melanopsin signal. These methodological limitations may have influenced the results of the present study.

In this study, a more rapid and intense pupil constriction was caused by blue light than by red light in control subjects as well as in diabetic patients. Irrespective of the severity of neuropathy, pupil constriction caused by blue and red light



PIPR (≥1.7 sec)	Control subjects versus T2DM-I	Control subjects versus T2DM-II	Control subjects versus T2DM-III
AUC	0.631	0.729	0.666
p value	0.058	0.001	0.014
Cut-off (pixels)	2801	2535	2428
Sensitivity	0.561	0.659	0.683
Specificity	0.613	0.649	0.588

(a)

PIPR (≥1.7 sec)	Control subjects versus T2DM-I	Control subjects versus T2DM-II	Control subjects versus T2DM-III
AUC	0.562	0.591	0.594
p value	0.376	0.172	0.140
Cut-off (pixels)	1782	1464	1298
Sensitivity	0.523	0.654	0.674
Specificity	0.550	0.514	0.559

(b)

Figure 4: The receiver operating characteristic (ROC) curve analysis of postillumination pupillary response (PIPR) after the offset of blue (a) and red (b) light at ≥1.7–3.0 sec between the control subjects and diabetic subgroups stratified by the severity of neuropathy {I; without neuropathy (------), II; asymptomatic neuropathy (·····), and III; symptomatic but without diabetic autonomic neuropathy (- - -)}.

in diabetic patients was slower and less pronounced than that in the control subjects. *T*1 after stimulation by both kinds of light was longer in the diabetic patients than in the control subjects. The PIPRs at ≥1.7 sec and <1.7 sec after blue light offset in the control subjects were larger than those after red light offset. The PIPRs at ≥1.7 sec and <1.7 sec after blue light offset were smaller in the diabetic group than in the control subjects, irrespective of the severity of neuropathy. These results indicate that 1 sec irradiation with blue or red light was clinically useful for eliciting different PLR kinetics in the control subjects and patients with type 2 diabetes. As reported previously [11, 12], *D*1 of diabetic patients was significantly smaller than that of control subjects. This has been considered to be due to sympathetic nerve dysfunction [33, 34]. The good correlations observed between *D*1 and CNFD, CNFL, CNBD, CNBL, and the amplitude of the median nerve seem to be compatible with changes in sympathetic nerve function. Hypertension has been reported as a risk factor for diabetic neuropathy [34] and has been associated with small pupil size in type 1 diabetic patients [35]; this is consistent with the present study in that high blood pressure was related to a small *D*1.

Although we employed 470 nm light for elucidating the function of ipRGCs, the ipRGCs have synapses with bipolar and amacrine cells for signaling between the outer and inner retina [36, 37], which are thought to modulate light detection in the PLR. Therefore, the PLR stimulated by 470 nm light should be considered to represent the function of ipRGCs modified by outer retinal signals. However, because we did not perform spectral analysis of PLR kinetics, it was not possible for the present study to exactly determine the relative contributions of cones-rods and ipRGCs to the parameters of the PLR.

*T*1 obtained with blue light of a high intensity and long irradiation period (>5–10 sec) is longer than that occurring with red light [4, 8]. There has been no study comparing *T*1 with blue or red light for 1 or 2 sec or comparing healthy people and diabetic patients. *T*1 of diabetic patients occurring with nonchromatic light irradiation was reported to be significantly longer than that occurring in control subjects and correlated with thermal PTs and glycemic control [38]. In the present study, in control subjects and diabetic patients, *T*1 with 470 nm light was shorter than that with 635 nm light, and in the diabetic patients, the *T*1 with blue light was positively related to age and HbA1c level and inversely associated with HDL-cholesterol level. As the efferent loop of the PLR (sympathetic and parasympathetic nerve) is the same, 470 nm light seemed to evoke a stronger signal than 635 nm light in ipRGCs transmitting to the olivary pretectal nucleus. HDL-cholesterol plays an important role in the functions of RGCs [39], so the HDL-cholesterol levels appeared to favorably influence the PLR kinetics.

Table 5: Relationship between the parameters of pupillary light reflex and clinical factors, neurophysiological tests, or corneal nerve fiber measures in total type 2 diabetic patients.

	Baseline pupil diameter		Latency period				Pupil diameter constriction				Pupil constriction velocity			
			470 nm		635 nm		470 nm		635 nm		470 nm		635 nm	
	St. β	p	St. β	p	St. β	p	St. β	p	St. β	p	St. β	p	St. β	p
Age	−0.177	0.100	0.245	**0.025**	0.063	0.570	−0.255	**0.020**	−0.236	**0.032**	−0.237	**0.041**	−0.087	0.435
SBP	−0.263	**0.010**	0.034	0.739	0.126	0.232	−0.185	0.072	−0.109	0.287	−0.140	0.178	−0.009	0.933
DBP	−0.324	**0.002**	−0.039	0.707	0.088	0.411	−0.118	0.262	−0.149	0.153	−0.083	0.433	−0.072	0.502
HbA1c	−0.157	0.159	0.238	**0.035**	−0.030	0.797	−0.057	0.614	0.028	0.808	0.003	0.977	0.071	0.545
HDL-C	−0.116	0.282	−0.275	**0.013**	−0.166	0.143	0.060	0.580	0.201	0.070	0.121	0.275	0.200	0.078
Amplitude of MN	0.231	**0.024**	−0.027	0.794	0.012	0.912	0.180	0.084	0.066	0.530	0.145	0.172	0.024	0.822
CNFD	0.267	**0.006**	0.156	0.115	0.105	0.306	0.077	0.438	0.102	0.308	0.079	0.435	0.102	0.323
CNFL	0.242	**0.014**	0.158	0.113	0.084	0.418	0.072	0.472	0.112	0.265	0.076	0.457	0.105	0.310
CNBD	0.310	**0.001**	−0.057	0.578	0.090	0.395	0.118	0.252	0.182	0.077	0.076	0.468	0.071	0.506
CNBL	0.272	**0.005**	−0.012	0.911	0.070	0.512	0.106	0.308	0.163	0.117	0.064	0.544	0.102	0.339

CNBD: corneal nerve branch density; CNBL: corneal nerve branch length; CNFD: corneal nerve fiber density; CNFL: corneal nerve fiber length, CV: coefficient of variation; DBP: diastolic blood pressure; HDL: high density lipoprotein; LDL: low density lipoprotein; MCV: motor nerve conduction velocity; MN: median nerve; SBP: systolic blood pressure; St.: standard.

Park et al. [31] compared PLRs using blue and red stimuli of different intensities for 1 sec in normal subjects and reported that low-intensity blue stimulation induced larger PC, which was sustained longer after light offset compared with that caused by red stimulus. These results were similar to those of the present study. Feigl et al. [13], using 10 sec chromatic stimulation, reported that the transient kinetics of PC caused by blue stimulation were within the normal range in type 2 diabetic patients. In contrast, the present study revealed that the constriction kinetics caused by blue light in diabetic patients were significantly smaller and slower than those caused in control subjects. This difference may be due to the duration and intensity of light used. Furthermore, Feigl et al. matched the intensity of irradiance between control subjects and diabetic patients [13], while we did not. The present study revealed that the PIPR after blue light offset (mediated solely by ipRGCs at ≥1.7 sec and by rods and ipRGCs at <1.7 sec) in diabetic patients, irrespective of neuropathy severity, was significantly smaller than that of the control subjects. However, there was no difference in PIPR after red light offset at <1.7 sec and ≥1.7 sec between the control subjects and diabetic patients. This might reflect the compromised function of ipRGCs in patients with diabetes. However, baseline pupil size influences the amplitude of pupil constriction and PIPR after light offset [40]. We normalized pupil diameter constriction ([$D1 - D2$]/$D1$ [%]) and PIPR after light offset to $D1$. The percentage constriction caused by blue light in control subjects was significantly larger than that in all diabetic patients ($p = 0.005$) and in the stage II subgroup ($p = 0.016$). After normalization to $D1$, the PIPR at ≥1.7 sec in the control group was larger than that in the diabetic patient group ($p = 0.015$) and the stage II subgroup ($p = 0.029$), indicating that $D1$ appears to influence the parameters of PLR kinetics to some extent. Therefore, the irradiation level should be equalized between control subjects and diabetic patients to prevent possible false differences due to small pupil size and accelerated lenticular yellowing in diabetic patients [41].

Although we used 20 cd/m^2 light at 470 nm and 635 nm to assess PLR, photoreceptors (cones, rods, and ipRGCs) respond to light by sensing photon density. According to the equation by the manufacturer, 20 cd/m^2 light at 470 nm is equivalent to 4.79 × 14 log photons/cm^2/sec, and 635 nm is equivalent to 2.14 × 14 log photons/cm^2/sec, when the pupil diameter is assumed to be 6.0 mm. As blue light contains twice the amount of photons as red light, the more intense and rapid PLR kinetics caused by blue light may be due to the larger numbers of photons of the blue light. We therefore also compared the kinetic parameters of the PLR induced by 20 cd/m^2 blue and 100 cd/m^2 red lights in control subjects. The latter red light comprises 1.07 × 15 log photons/cm^2/sec, more than twice the number of 20 cd/m^2 blue light. The kinetic parameters resulting from this were as follows (blue versus red, resp.): $T1$, 247–260 versus 257–269 msec, $p < 0.05$; PC, 2.32–2.38 versus 1.95–2.17 mm, $p < 0.01$; CS, 2.01–2.18 versus 1.84–1.99 μ/msec, $p < 0.05$; PIPR at ≥1.7 sec, 2778–3450 versus 2241–2762 pixels, $p < 0.01$. The 20 cd/m^2 blue light had better kinetic parameters than did 100 cd/m^2 red light. It therefore seemed unlikely that the differences in PLR kinetics were caused by the higher photon density of the blue light.

The present study focused on the dysfunction of the retinal inner and outer photoreceptors as an etiology of impaired PLR caused by diabetes. Of course, the PLR is the results of a neural reflex that is dependent upon pathways and synaptic events beyond the retina. The correlations between the parameters of PLR kinetics and clinical factors, neurophysiological tests, and CNF measures might indicate the disturbance of PLR arc beyond the retinal photoreceptors. Since some CNF measures and the parameters of PLR kinetics were impaired even in diabetic patients without

neuropathy, CNF morphology and PLR may be an early clinical predictor of the onset of clinical diabetic neuropathy. Although the size and frequency of beading change in the early stages of hyperglycemia, a fact that is related to impaired peripheral nerve function [20], these factors were not related to the PLR parameters.

We acknowledge that the present study has limitations, which may affect the interpretation of the results. First, although we used 470 nm and 635 nm light to differentially elucidate the functions of ipRGCs and the outer retina, the ipRGCs receive synaptic signals from the outer retina. The relative contributions of cones-rods and ipRGCs to PLR parameters change depending on the magnitude and period of irradiation. We did not perform spectral analysis. Therefore, the present study was not able to assess the relative contribution of ipRGCs to blue light (470 nm) induced PLR kinetic parameters. Second, we did not dilate the pupils, and the pupil diameter of the diabetic patients was smaller than that of the control subjects. We excluded subjects older than 55 years because of potential age-related yellowing of the lens, but we did not perform any specific ophthalmological examinations for diseases of the cornea or lens. The lenses of diabetic patients become yellow at an accelerated rate compared with that of healthy people [41]. It was not possible to exclude the potential influence of these experimental conditions on the attenuation of blue light, resulting in an impaired PLR evoked by blue light in diabetic patients. Third, even though 20 cd/m^2 blue light induced better PLR kinetics than did 100 cd/m^2 red light, the photon densities of 470 nm and 635 nm light stimuli would ideally be matched.

We acknowledge that the pupillometry protocol that applied at this work is not a sensitive measure of melanopsin function, and the protocol has limited capacity to detect melanopsin dysfunction in the diabetic patients.

Finally, although some clinical factors were significantly associated with PLR parameters, clarifying the meaning of these relationships was beyond the scope of the present study. Unfortunately, we were not able to find clinical factors that contributed to impaired ipRGC function in the PIPR in diabetic patients.

5. Conclusions

In conclusion, this study confirmed in a large population of patients with type 2 diabetes that PIPR is impaired after blue light offset, as previously reported by Feigl et al. [13]. The novel finding from this study is that blue light induced a more intense and rapid PLR in control subjects and diabetic patients than did red light, and the PLR stimulated by blue light in type 2 diabetic patients without DAN was more severely impaired than that caused by red light. It is therefore possible to detect ipRGCs dysfunction before the development of DAN. However, refined methods are required to confirm these results.

Disclosure

Fukashi Ishibashi and Mitra Tavakoli are the guarantors of this work and as such had full access to all data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis and interpretation.

Conflicts of Interest

This study received no financial support. The authors have reported no conflicts of interest.

Authors' Contributions

Fukashi Ishibashi designed the study, researched data, and wrote the entire manuscript. Rie Kojima and Miki Taniguchi performed a PLR and CCM examination and neurophysiological tests. Aiko Kosaka and Harumi Uetake gathered the clinical and laboratory data and statistically analyzed all data. Mitra Tavakoli advised on the statistical analysis, interpreted the results, and reviewed and revised the whole paper.

References

[1] F. Bremner and S. Smith, "Pupil findings in a consecutive series of 150 patients with generalised autonomic neuropathy," *Journal of Neurology, Neurosurgery and Psychiatry*, vol. 77, no. 10, pp. 1163–1168, 2006.

[2] C. A. Czeisler, T. L. Shanahan, E. B. Klerman et al., "Suppression of melatonin secretion in some blind patients by exposure to bright light," *New England Journal of Medicine*, vol. 332, no. 1, pp. 6–11, 1995.

[3] R. J. Lucas, "Mammalian inner retinal photoreception," *Current Biology*, vol. 23, no. 3, pp. R125–R133, 2013.

[4] P. D. R. Gamlin, D. H. McDougal, J. Pokorny, V. C. Smith, K.-W. Yau, and D. M. Dacey, "Human and macaque pupil responses driven by melanopsin-containing retinal ganglion cells," *Vision Research*, vol. 47, no. 7, pp. 946–954, 2007.

[5] J. J. Gooley, J. Lu, D. Fischer, and C. B. Saper, "A broad role for melanopsin in nonvisual photoreception," *Journal of Neuroscience*, vol. 23, no. 18, pp. 7093–7106, 2003.

[6] D. H. McDougal and P. D. Gamlin, "The influence of intrinsically-photosensitive retinal ganglion cells on the spectral sensitivity and response dynamics of the human pupillary light reflex," *Vision Research*, vol. 50, no. 1, pp. 72–87, 2010.

[7] D. S. Joyce, B. Feigl, D. Cao, and A. J. Zele, "Temporal characteristics of melanopsin inputs to the human pupil light reflex," *Vision Research*, vol. 107, pp. 58–66, 2015.

[8] J. J. Gooley, I. H. Mien, M. A. St Hilaire et al., "Melanopsin and Rod-cone photoreceptors play different roles in mediating pupillary light responses during exposure to continuous light in humans," *Journal of Neuroscience*, vol. 32, no. 41, pp. 14242–14253, 2012.

[9] B. Feigl and A. J. Zele, "Melanopsin-expressing intrinsically photosensitive retinal ganglion cells in retinal disease," *Optometry and Vision Science*, vol. 91, no. 8, pp. 894–903, 2014.

[10] P. Adhikari, B. Feigl, and A. J. Zele, "Rhodopsin and melanopsin contributions to the early redilation phase of the post-illumination pupil response (PIPR)," *PLoS ONE*, vol. 11, no. 8, Article ID e0161175, 2016.

[11] S. A. Smith and S. E. Smith, "Reduced pupillary light reflexes in diabetic autonomic neuropathy," *Diabetologia*, vol. 24, no. 5, pp. 330–332, 1983.

[12] M. Dütsch, H. Marthol, G. Michelson, B. Neundörfer, and M. J. Hilz, "Pupillography refines the diagnosis of diabetic autonomic neuropathy," *Journal of the Neurological Sciences*, vol. 222, no. 1-2, pp. 75–81, 2004.

[13] B. Feigl, A. J. Zele, S. M. Fader et al., "The post-illumination pupil response of melanopsin-expressing intrinsically photosensitive retinal ganglion cells in diabetes," *Acta Ophthalmologica*, vol. 90, no. 3, pp. e230–e234, 2012.

[14] H. Yasuda, M. Sanada, K. Kitada et al., "Rationale and usefulness of newly devised abbreviated diagnostic criteria and staging for diabetic polyneuropathy," *Diabetes Research and Clinical Practice*, vol. 77, no. 3, pp. S178–S183, 2007.

[15] L. Kessel, G. Siganos, T. Jørgensen, and M. Larsen, "Sleep disturbances are related to decreased transmission of blue light to the retina caused by lens yellowing," *Sleep*, vol. 34, no. 9, pp. 1215–1219, 2011.

[16] L. S. Mure, P.-L. Comut, C. Rieux et al., "Melanopsin bistability: a fly's eye technology in the human retina," *PLoS ONE*, vol. 4, no. 6, Article ID e5991, 2009.

[17] A. J. Zele, B. Feigl, S. S. Smith, and E. L. Markwell, "The circadian response of intrinsically photosensitive retinal ganglion cells," *PLoS ONE*, vol. 6, no. 3, Article ID e17860, 2011.

[18] F. Ishibashi, M. Okino, M. Ishibashi et al., "Corneal nerve fiber pathology in Japanese type 1 diabetic patients and its correlation with antecedent glycemic control and blood pressure," *Journal of Diabetes Investigation*, vol. 3, no. 2, pp. 191–198, 2012.

[19] L. Oliveira-Soto and N. Efron, "Morphology of corneal nerves using confocal microscopy," *Cornea*, vol. 20, no. 4, pp. 374–384, 2001.

[20] F. Ishibashi, R. Kojima, M. Taniguchi, A. Kosaka, H. Uetake, and M. Tavakoli, "The expanded bead size of corneal c-nerve fibers visualized by corneal confocal microscopy is associated with slow conduction velocity of the peripheral nerves in patients with Type 2 diabetes mellitus," *Journal of Diabetes Research*, vol. 2016, Article ID 3653459, 9 pages, 2016.

[21] A. Kashiwagi, M. Kasuga, E. Araki et al., "International clinical harmonization of glycated hemoglobin in Japan: from Japan diabetes society to national glycohemoglobin standardization program values," *Journal of Diabetes Investigation*, vol. 3, no. 1, pp. 39–40, 2012.

[22] A. A. F. Sima, W.-X. Zhang, P. V. Cherian, and S. Chakrabarti, "Impaired visual evoked potential and primary axonopathy of the optic nerve in the diabetic BB/W-rat," *Diabetologia*, vol. 35, no. 7, pp. 602–607, 1992.

[23] X.-X. Zeng, Y.-K. Ng, and E.-A. Ling, "Neuronal and microglial response in the retina of streptozotocin-induced diabetic rats," *Visual Neuroscience*, vol. 17, no. 3, pp. 463–471, 2000.

[24] T. S. Kern and A. J. Barber, "Retinal ganglion cells in diabetes," *Journal of Physiology*, vol. 586, no. 18, pp. 4401–4408, 2008.

[25] H. W. van Dijk, P. H. B. Kok, M. Garvin et al., "Selective loss of inner retinal layer thickness in type 1 diabetic patients with minimal diabetic retinopathy," *Investigative Ophthalmology and Visual Science*, vol. 50, no. 7, pp. 3404–3409, 2009.

[26] T. M. Schmidt, M. T. H. Do, D. Dacey, R. Lucas, S. Hattar, and A. Matynia, "Melanopsin-positive intrinsically photosensitive retinal ganglion cells: from form to function," *The Journal of Neuroscience*, vol. 31, no. 45, pp. 16094–16101, 2011.

[27] C. M. Altimus, A. D. Güler, K. L. Villa, D. S. McNeill, T. A. LeGates, and S. Hattar, "Rods-cones and melanopsin detect light and dark to modulate sleep independent of image formation," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 105, no. 50, pp. 19998–20003, 2008.

[28] S. Hattar, M. Kumar, A. Park et al., "Central projections of melanopsin-expressing retinal ganglion cells in the mouse," *Journal of Comparative Neurology*, vol. 497, no. 3, pp. 326–349, 2006.

[29] R. Kardon, S. C. Anderson, T. G. Damarjian, E. M. Grace, E. Stone, and A. Kawasaki, "Chromatic pupil responses: preferential activation of the melanopsin-mediated versus outer photoreceptor-mediated pupil light reflex," *Ophthalmology*, vol. 116, no. 8, pp. 1564–1573, 2009.

[30] M. Münch, L. Léon, S. V. Crippa, and A. Kawasaki, "Circadian and wake-dependent effects on the pupil light reflex in response to narrow-bandwidth light pulses," *Investigative Ophthalmology and Visual Science*, vol. 53, no. 8, pp. 4546–4555, 2012.

[31] J. C. Park, A. L. Moura, A. S. Raza, D. W. Rhee, R. H. Kardon, and D. C. Hood, "Toward a clinical protocol for assessing rod, cone, and melanopsin contributions to the human pupil response," *Investigative Ophthalmology and Visual Science*, vol. 52, no. 9, pp. 6624–6635, 2011.

[32] P. Adhikari, A. J. Zele, and B. Feigl, "The post-illumination pupil response (PIPR)," *Investigative Ophthalmology & Visual Science*, vol. 56, no. 6, pp. 3838–3849, 2015.

[33] D. Pittasch, R. Lobmann, W. Behrens-Baumann, and H. Lehnert, "Pupil signs of sympathetic autonomic neuropathy in patients with type 1 diabetes," *Diabetes Care*, vol. 25, no. 9, pp. 1545–1550, 2002.

[34] K. Y.-Z. Forrest, R. E. Maser, G. Pambianco, D. J. Becker, and T. J. Orchard, "Hypertension as a risk factor for diabetic neuropathy: a prospective study," *Diabetes*, vol. 46, no. 4, pp. 665–670, 1997.

[35] K. Karavanaki, A. G. Davies, L. P. Hunt, M. H. Morgan, and J. D. Baum, "Pupil size in diabetes," *Archives of Disease in Childhood*, vol. 71, no. 6, pp. 511–515, 1994.

[36] J. Østergaard, J. Hannibal, and J. Fahrenkrug, "Synaptic contact between melanopsin-containing retinal ganglion cells and rod bipolar cells," *Investigative Ophthalmology and Visual Science*, vol. 48, no. 8, pp. 3812–3820, 2007.

[37] M. A. Belenky, C. A. Smeraski, I. Provencio, P. J. Sollars, and G. E. Pickard, "Melanopsin retinal ganglion cells receive bipolar and amacrine cell synapses," *Journal of Comparative Neurology*, vol. 460, no. 3, pp. 380–393, 2003.

[38] P. Lanting, J. J. Heimans, J. P. H. Reulen, J. Nauta, and E. A. van der Veen, "Pupillary light reflex and quantitative sensory and motor neural function tests in diabetic patients," *Journal of Neurology*, vol. 235, no. 4, pp. 245–247, 1988.

[39] N. Tserentsoodol, N. V. Gordiyenko, I. Pascual, J. W. Lee, S. J. Fliesler, and I. R. Rodriguez, "Intraretinal lipid transport is dependent on high density lipoprotein-like particles and class B scavenger receptors," *Molecular Vision*, vol. 12, pp. 1319–1333, 2006.

[40] D. S. Joyce, B. Feigl, and A. J. Zele, "The effects of short-term light adaptation on the human post-illumination pupil response," *Investigative Opthalmology & Visual Science*, vol. 57, no. 13, pp. 5672–5680, 2016.

[41] M. Lutze and G. H. Bresnick, "Lenses of diabetic patients 'yellow' at an accelerated rate similar to older normals," *Investigative Ophthalmology and Visual Science*, vol. 32, no. 1, pp. 194–199, 1991.

Hindawi
Journal of Diabetes Research
Volume 2017, Article ID 1273789, 10 pages
https://doi.org/10.1155/2017/1273789

Research Article

Peripheral and Autonomic Neuropathy in South Asians and White Caucasians with Type 2 Diabetes Mellitus: Possible Explanations for Epidemiological Differences

Abd A. Tahrani,[1,2,3] Q. A. Altaf,[1,2] Milan K. Piya,[3,4] and Anthony H. Barnett[1,2]

[1]*Institute of Metabolism and Systems Research, University of Birmingham, Birmingham, UK*
[2]*Department of Diabetes and Endocrinology, Heart of England NHS Foundation Trust, Birmingham, UK*
[3]*Centre of Endocrinology, Diabetes and Metabolism, Birmingham Health Partners, Birmingham, UK*
[4]*Department of Diabetes and Endocrinology, Derby Teaching Hospitals NHS Foundation Trust, Derby, UK*

Correspondence should be addressed to Abd A. Tahrani; abd.tahrani@nhs.net

Received 21 November 2016; Revised 1 February 2017; Accepted 19 February 2017; Published 20 March 2017

Academic Editor: Patrizio Tatti

Objectives. To compare the prevalence of diabetic peripheral neuropathy (DPN) and that of cardiac autonomic neuropathy (CAN) between South Asians and White Caucasians with type 2 diabetes and to explore reasons for observed differences. *Methods.* A cross-sectional study of casually selected South Asian and White Caucasian adults attending a hospital-based diabetes clinic in the UK. DPN and CAN were assessed using the Michigan Neuropathy Screening Instrument (MNSI) and heart rate variability testing, respectively. *Results.* Patients ($n = 266$) were recruited (47.4% South Asians). DPN was more common in White Caucasians compared to South Asians (54.3% versus 38.1%, $p = 0.008$). Foot insensitivity as assessed by 10 g monofilament perception was more common in White Caucasians (43.9% versus 23.8%, $p = 0.001$). After adjustment for confounders, White Caucasians remained twice as likely to have DPN as South Asians, but the impact of ethnicity became nonsignificant after adjusting for adiposity measures or height. No difference in prevalence of standardized CAN test abnormalities was detected between ethnicities. Skin microvascular assessment demonstrated that South Asians had reduced heating flux but preserved acetylcholine response. *Conclusions.* South Asians with type 2 diabetes have fewer clinical signs of DPN compared to White Caucasians. Differences in adiposity (and its distribution) and height appear to explain these differences.

1. Introduction

Diabetic neuropathy (DN) is the most common form of neuropathy in the Western world and one of the most troublesome complications of diabetes mellitus, resulting in great morbidity (e.g., it is the leading cause of nontraumatic lower limb amputation), mortality, and significant economic burden [1–3]. The reported prevalence of diabetic peripheral neuropathy (DPN) and diabetic autonomic neuropathy (DAN) varies significantly depending on the population studied and the methods used [2–9]. Many factors have been implicated in the pathogenesis of DN including non-metabolic factors such as age and height [3, 6, 10, 11], as well as metabolic factors such as hyperglycemia, hypertension, dyslipidemia, waist circumference, and obesity [3, 12–14]. These metabolic factors stimulate multiple pathways resulting in direct cellular damage and functional and/or structural defects involving the extracellular matrix and/or microvasculature [15, 16]. Disease-modifying treatments are still lacking (with the exception of improved glycaemic control) [12, 15]. Hence, improved understanding of DN pathogenesis is important in order to identify new treatment targets [15].

South Asians with type 2 diabetes seem to be at lower risk of developing DPN resulting in a lower incidence of foot ulcerations and amputations [17–19]. Such a difference is surprising as diabetes-related complications have predominantly a vascular aetiology, with South Asian patients at higher risk of cardiovascular disease compared to White Caucasians with type 2 diabetes [20]. However, the previous reports that identified this difference either conducted a very

limited assessment of DPN [17, 20] or utilized a patient population with a low prevalence of diabetes complications and could not detect a difference in DPN prevalence using routine clinical examination techniques [18]. Critically, the underlying factors that could explain such differences in DPN prevalence have not been fully explored. One previous report, based on a primary care population in the UK, suggested that differences in height and transcutaneous partial pressure of oxygen (TCpO2) were responsible for the lower prevalence of DPN in South Asians [18]. This report, however, did not adjust for a wide range of possible confounders such as obesity, alcohol intake, and triglycerides (amongst others), all of which are well established risk factors for neuropathy.

In contrast to DPN, the impact of ethnicity on CAN in patients with type 2 diabetes has received little attention, with the exception of a single report that compared the E/I ratios between South Asians and White Caucasians [18]; hence there is need for further studies utilizing more sensitive and detailed assessments of CAN.

The primary aim of this study was therefore to explore the possible metabolic, demographic, and microvascular differences that might explain the lower prevalence of DPN in South Asians with type 2 diabetes compared to White Caucasians. Secondary aims included comparing the prevalence of clinically detectable DPN and that of cardiac autonomic neuropathy (CAN) in South Asians and White Caucasians with type 2 diabetes receiving hospital-based diabetes care.

2. Materials and Methods

This is a secondary analysis of an ongoing project with the aim of exploring the mechanisms contributing to the development of microvascular complications in patients with type 2 diabetes [21–24]. We have conducted a cross-sectional study of casually selected South Asian or White Caucasian adults with type 2 diabetes recruited from the clinic of a hospital-based diabetes centre in the UK. Subjects excluded were those under the age of 18, not of either South Asian or White Caucasian ethnicity, or known to have neuropathy for other reasons apart from diabetes (<1%).

Patients were recruited casually from the outpatient diabetes departments of two different hospitals in the UK. The majority of participants (86%) were recruited from the Heart of England NHS Foundation Trust in Birmingham, UK, which has a large South Asian population with type 2 diabetes (approximately 40% of the total clinic). These factors suggest that our sample is representative and the two ethnicities are comparable. Patients who originated from India, Pakistan, Sri Lanka, or Bangladesh were considered to be of South Asian origin. Ethnicity was determined using the UK decennial census by the study participants. The project was approved by the Warwickshire Research Ethics Committee (REC number 08/H1211/145). Patients were consented prior to participation in the study.

DPN was assessed using the Michigan Neuropathy Screening Instrument (MNSI) which is a validated, 2-component tool designed to facilitate the early diagnosis of DPN [4, 25–29]. The questionnaire component (MNSIq) comprises 15 questions seeking to characterize sensory disturbance but also peripheral vascular disease and general asthenia [25]. The examination component (MNSIe) comprises a limited foot inspection to identify deformity, skin abnormalities, and ulceration, coupled with an assessment of the vibratory perception and ankle tendon reflexes [25]. For the purpose of this study, DPN was diagnosed if the MNSIe score was >2 and/or MNSIq was ≥7 [27]. We have also used perception to a 10 g monofilament (applied to 10 positions, the tip of each toe, under 3 metatarsal heads, the plantar surface of the foot, and the dorsal space between the first and second toe) as a test for foot insensitivity; an abnormal monofilament test was defined as < 8 correct responses [30]. The relationship of ethnicity to neuropathic symptoms was also assessed using the MNSIq.

CAN was assessed using heart rate variability (HRV) and was analyzed using the continuous wavelet transform methods to generate numerical and graphical data using the ANX-3.0 software, ANSAR Inc., Philadelphia, PA. The R-R intervals were recorded and the HRV was plotted in the frequency domain to separate the high-frequency (Rfa, 0.15 to 0.4 Hz) from the low-frequency (Lfa, 0.04 to 0.15 Hz) components by spectral analysis. HRV and BP were recorded with the patient in sitting position during resting, deep breathing, Valsalva maneuver, and standing position [31]. Data recorded included the E/I (expiratory/inspiratory) ratio, Valsalva ratio, 30 : 15 ratio, frequency domain analysis with respiratory adjustment (adjusted low frequency (Lfa), adjusted respiratory frequency (Rfa), and Lfa/Rfa), and time domain analysis including standard deviation of beat-to-beat intervals (sdNN), root mean square of successive differences (rmsSD), and the proportion of NN50 (pNN50). For the purpose of this study, a diagnosis of CAN was made when 2 or more of the following tests were abnormal: E/I ratio, Valsalva ratio, 30 : 15 ratio, and postural drop in BP (drop of 20 mmHg in systolic or 10 mmHg in diastolic BP) [2]. Age-related normal values for E/I, Valsalva, and 30 : 15 ratios were defined as previously reported [32].

Microvascular and endothelial assessment were performed on a casually chosen subset of patients ($n = 71$) using Laser speckle contrast imaging (Moor Instruments Ltd., Devon, UK) which has been shown to be reproducible [33]. All patients were approached to have the vascular/endothelial assessment and the test was performed on all those who agreed. The South Asians and White Caucasians in this subset had similar characteristics to those in the total study population reported in Table 1. Microvascular blood flow (measures in arbitrary perfusion units (APU)) was assessed at the left mid-thigh level. This site was chosen to minimise the impact of any peripheral vascular disease in the lower limbs. Measurements were taken with the patient in a semirecumbent position in a room temperature of 22-23°C following 15 minutes of patients acclimatisation [34]. Imaging was performed over 20 minutes, and all measurements were taken simultaneously. Blood flow was measured at baseline (for 20 minutes) and following maximal dilatation following heating to 44°C. Endothelial function was measured following the iontophoresis of 1% acetylcholine (Ach) and 2% sodium nitroprusside (SNP) (5 pulses over 5 minutes). Data are presented as absolute values, conductance (measured as flux

Table 1: Summary of baseline characteristics in relation to ethnicity. Data are presented as median (IQR) or mean ± 1SD depending on data distribution. The percentages represent % of participants in the ethnic group. STR: sight threatening retinopathy defined as preproliferative or proliferative retinopathy or maculopathy or previous laser treatment. TIA: transient ischaemic attack. PVD: peripheral vascular disease.

	South Asians ($n = 126$)	White Caucasians ($n = 140$)	p value
Age (years)	54.9 ± 12.5	59.2 ± 10.8	0.003
Male (%)	60.3	56.4	0.521
Smoking (current or ex-smoker)	30.2	50.7	0.001
Alcohol (%)	2.4	47.9	<0.001
Diabetes duration (years)	11.0 (7.0–18.0)	10.5 (5.0–16.0)	0.153
BMI (kg/m^2)	30.1 (26.5–33.6)	35.3 (31.8–41.4)	<0.001
Waist circumference (cm)	103.2 (96.9–113.1)	117.2 (109.2–129.4)	<0.001
Hip circumference (cm)	102.5 (97–112.25)	120.0 (108.6–131.75)	<0.001
Waist/hip ratio	1.00 ± 0.06	0.99 ± 0.11	0.276
Neck circumference (cm)	39.0 (36.9–41.8)	43.0 (39.6–47.0)	<0.001
Height (cm)	164.5 ± 8.9	167.1 ± 12.3	0.051
Neck height ratio	0.24 ± 0.02	0.26 ± 0.03	<0.001
Systolic blood pressure (mmHg)	126.0 (115.5–138.5)	130.0 (124.5–140.0)	0.008
Diastolic blood pressure (mmHg)	77.5 (70.0–84.5)	79.0 (73.6–85.9)	0.032
HbA1c (%)	8.0 (7.16–9.20)	8.0 (7.23–9.19)	0.868
Total cholesterol (mmol/L)	3.7 (3.3–4.3)	3.7 (3.3–4.4)	0.637
Triglycerides (mmol/L)	1.8 (1.1–2.4)	1.7 (1.3–2.5)	0.550
HDL (mmol/L)	1.1 (0.9–1.2)	1.1 (0.9–1.3)	0.217
TSH (mU/L)	1.6 (1.0–2.3)	1.9 (1.4–2.3)	0.024
Estimated GFR (mL/min/1.73 m^2)	90.0 ± 25.5	83.7 ± 27.1	0.053
Oral antidiabetes agent (%)	91.3	92.9	0.632
Sulphonylureas (%)	42.1	30.7	0.054
Insulin (%)	50.0	57.9	0.199
Insulin dose (units)	80.0 (44.0–118)	67.0 (52.0–97.5)	0.649
GLP-1 analogues (%)	4.0	17.1	0.001
Lipid lowering treatment (%)	81	85	0.379
Antihypertensive therapy	74.6	84.3	0.05
Antiplatelet agents (%)	65.6	64.5	0.847
Albuminuria (%)	38.6	34.6	0.536
STR (%)	36.0	36.8	0.908
Ischaemic heart disease (%)	23.0	17.1	0.231
CABG (%)	13.5	10.0	0.375
Stroke/ TIA (%)	9.5	10.7	0.748
PVD (%)	2.4	8.6	0.029

divided by mean arterial pressure in order to account for differences in BP) and as percentage of maximal dilatation flow as recommended by previous reports [34]. Heating response represented maximum dilatation [34].

Data analysis was performed using SPSS 15.0 software (SPSS Inc., Chicago, USA). Data are presented as mean ± 1SD or median (IQR) depending on data distribution. Normality testing was performed using histograms and the Shapiro-Wilk test. Independent continuous variables were compared using the independent t-test or the Mann–Whitney test. Categorical variables were compared using the Chi square test. The Bonferroni correction was applied to define statistical significance when the components of the MNSIe and MNSIq were compared between the ethnicities. In order to identify independent predictors of DPN and CAN (separately), logistic regression models were applied. Variables included in logistic regression models were tested for multicollinearity using the tolerance test and the variance inflation factor; no evidence of multicollinearity was found in the logistic regression models used in this paper. Adiposity measures (BMI, waist, and neck circumferences) were included in the regression models separately due to the high correlation between these variables. A p value <0.05 was considered significant throughout the manuscript except when stated otherwise.

3. Results

Patients ($n = 266$) were included in this analysis, 47.4% of whom were South Asian. South Asians were shorter and younger but had similar duration of known diabetes. South Asians also had lower adiposity measurements, BP, TSH levels, smoking, and alcohol intake (Table 1). The prescription of antihypertensive and GLP-1 analogue treatment was also lower in South Asians (Table 1). The prevalence of other microvascular complications and past medical history of coronary artery disease was similar between ethnicities, while South Asians had lower prevalence of peripheral vascular disease (PVD) (Table 1) which is consistent with other previous reports [17].

DPN was more common in White Caucasians compared to South Asians (54.3% versus 38.1%, $p = 0.008$). Foot insensitivity, as assessed by abnormal monofilament perception, was also more common in White Caucasians (43.9% versus 23.8%, $p = 0.001$) (Table 2). Analysis of patient symptom scores demonstrated that symptoms consistent with sensory deficit were not different between ethnic groups whereas pain/discomfort symptoms related to were nonsignificantly more common in South Asians (Table 2). White Caucasian patients had more abnormalities on all aspects of neuropathy examination and, consistent with our findings with the monofilament, reported more open sores on the foot (Table 2).

To determine the potential influence of demographic and metabolic differences between ethnicities on the prevalence of DPN, logistic regression models with increasing complexity were used (Table 3). The association between ethnicity and DPN remained significant, despite adjusting for a wide range of possible confounders. This association was attenuated by age and a history of PVD and was abolished after adjusting for height and adiposity measures (with the exception of waist/hip ratio) (Table 3), suggesting that ethnic differences in DPN prevalence can be mainly explained by the differences in height and adiposity between the ethnic groups. All adiposity measures were independently associated with DPN despite adjusting for all possible confounders listed in Table 3 (except height and BMI which were not used in the same model) [BMI: odds ratio (OR) 1.076 (95% CI 1.026 to 1.130), $p = 0.003$; waist circumference: OR 1.040 (95% CI 1.016 to 1.064), $p = 0.001$; neck circumference: OR 1.143 (95% CI 1.045 to 1.250), $p = 0.003$]. Other independent associations included previously reported measures such as age, diabetes duration, and insulin treatment [13, 14, 35].

Unlike clinically apparent DPN, we found no difference in prevalence of abnormalities of standardized cardiovascular autonomic function tests between ethnicities (40.9% versus 40.9%, $p = 0.995$). Frequency and time domain analysis, however, revealed more extensive deficits in the White Caucasian compared to the South Asian cohort (Table 4). However, these differences were not significant after adjusting for possible confounders (such as age). Diabetes duration [OR 1.084 (95% CI 1.028 to 1.144), $p = 0.003$] and the use of calcium channel blockers [OR 0.292 (95% CI 0.123 to 0.694), $p = 0.005$] were found to be independently associated with CAN after adjusting the data, but not ethnicity [OR 1.077 (95% CI 0.473 to 2.452), $p = 0.860$].

Microvascular and endothelial assessment demonstrated significant differences between the two ethnicities. South Asians had similar baseline skin flux and conductance and markedly lower maximal dilatation following heating despite a similar SNP response but had preserved endothelial-dependant vasodilatation (Table 5). Indeed, endothelial-dependent vasodilatation significantly exceeded that measured in the White Caucasian population when expressed as the ratio of Ach-induced to heating-induced flux [34] (Table 5).

4. Discussion

We have compared the prevalence of DPN and that of CAN between South Asians and White Caucasians with type 2 diabetes from a hospital-based care setting. We then tried to determine whether specific metabolic and/or demographic factors could explain observed differences in the prevalence of clinically detectable DPN and CAN between these groups. Our study clearly demonstrates that DPN and foot insensitivity are less common in South Asian patients and implicates height and adiposity as potential factors underlying this difference. Furthermore, we found that total body (BMI), visceral (waist circumference), and upper body (neck circumference) fat were independently associated with DPN after adjusting for a range of confounders.

Our diabetes centre serves an area that has a high proportion of South Asians, although the majority of the general population are White Caucasians (a reflection of the much higher prevalence of type 2 diabetes in South Asians in the UK and elsewhere). Both ethnicities live in the same geographical area, have similar deprivation scores, and are referred to our clinic by primary care physicians using the same referral guidelines which are preagreed with our centre. Furthermore, there are no differences in clinic attendance rates between the ethnicities at our centre. Moreover, our population of South Asians and White Europeans with type 2 diabetes has similar characteristics (such as age, gender, smoking, alcohol, BMI, and HbA1c) to those described in previous reports in primary care in other localities in the UK. As our study population was hospital-based and with consequently more advanced disease, our findings are not necessarily generalizable to the general population of patients with type 2 diabetes (please see below) [18].

Our data demonstrating a lower prevalence of DPN in South Asian subjects are consistent with other reports [17–19] but extend these findings by demonstrating significant differences detectable by routine clinical examination and the novel finding that obesity (and its distribution) explains, at least in part, the ethnic differences in DPN prevalence. In addition, previous study populations exploring the relationships of DPN and ethnicity were drawn from Primary Care Practices in the UK which was reflected in the shorter duration of diabetes, the lower prevalence of microvascular, and macrovascular complications and the less frequent use of insulin and lipid lowering treatments [18, 19], while our study extends the findings to hospital-based population. Our study patients were drawn from a population receiving hospital-based diabetes care and are therefore representative of a more

Table 2: Ethnic differences in components of the MNSI and monofilament perception. Data are presented as % of abnormal test/response in the particular ethnic groups. MNSIe: the examination component of MNSI. MNSIq: the questionnaire component of MNSI. *These questions are not scored as part of the MNSIq. $p < 0.01$ and < 0.0033 were considered significant for differences in the components of MNSIe and MNSIq, respectively, following the Bonferroni correction. Statistical analysis in this table represents univariate analysis with no adjustments.

	South Asian ($n = 126$)	White Caucasian ($n = 140$)	p values
MNSIe			
Inspection	51.6	63.3	0.054
Ulcers	1.6	4.3	0.195
Ankle reflexes	35.7	56.1	0.001
Vibration	32.5	54.7	<0.001
10 g monofilament	23.8	43.9	0.001
MNSIq			
Are your legs and/or feet numb?	46.8	41.4	0.376
Do you ever have any burning pain in your legs and/or feet?	55.6	43.6	0.051
Are your feet too sensitive to touch?	24.6	26.4	0.733
Do you get muscle cramps in your legs and/or feet?*	62.7	70.7	0.165
Do you ever have any prickling feelings in your legs or feet?	59.5	44.3	0.013
Does it hurt when the bed covers touch your skin?	14.3	11.4	0.486
When you get into the tub or shower, are you able to tell the hot water from the cold water?	7.9	10.0	0.558
Have you ever had an open sore on your foot?	11.1	25.7	0.002
Has your doctor ever told you that you have diabetic neuropathy?	20.6	27.1	0.215
Do you feel weak all over most of the time?*	53.2	33.6	0.001
Are your symptoms worse at night?	43.7	36.4	0.230
Do your legs hurt when you walk?	59.5	60.7	0.843
Are you able to sense your feet when you walk?	8.7	17.1	0.043
Is the skin on your feet so dry that it cracks open?	38.9	46.4	0.215
Have you ever had an amputation?	2.4	8.6	0.029

Table 3: Assessing the impact of possible confounders on the association between ethnicity and DPN (based on MNSI) using logistic regression models with increasing complexity. The odds ratios reported are the odds for having DPN in White Caucasians to South Asians. BP: blood pressure; eGFR: estimated glomerular filtration rate; PVD: peripheral vascular disease; BMI: body mass index.

Model	Nagelkerke R^2	Odds ratio	95% confidence interval	p value
Unadjusted: ethnicity	0.035	1.930	1.182–3.149	0.009
Model 1: ethnicity + age + gender	0.091	1.684	1.016–2.793	0.043
Model 2: ethnicity + age + gender + alcohol intake + smoking + BP + diabetes duration + HbA1c + lipids[*] + TSH + eGFR + glucose lowering treatments[$] + antihypertensives[£] + antiplatelets[@] + lipid lowering therapy[^]	0.224	1.987	1.069–3.693	0.030
Model 3: Model 2 + PVD	0.237	1.887	1.008–3.530	0.047
Model 4: Model 3 + height	0.244	1.766	0.932–3.348	0.081
Model 5: Model 3 + BMI	0.279	1.169	0.581–2.352	0.661
Model 6: Model 3 + waist circumference	0.290	1.077	0.532–2.180	0.837
Model 7: Model 3 + neck circumference	0.278	1.087	0.528–2.237	0.822

[*] Adjustment for lipids included adjustment for total cholesterol, triglycerides, and HDL individually.
[$] Adjustment for glucose lowering treatments included adjustment for metformin, sulphonylurea, glitazones, DPP-4 inhibitors, insulin, and GLP-1 analogues individually. No other glucose lowering treatment was used in our study population.
[£] Adjustment for antihypertensives included adjustment for ACE inhibitors, angiotensin 2 blockers, beta blockers, alpha blockers, calcium antagonists, and diuretics individually.
[@] Antiplatelets included aspirin and clopidogrel.
[^] Adjustment for lipid lowering therapy included adjustment for statins, ezetimibe, and fibrates individually.

Table 4: Differences in heart rate variability, spectral analysis, and time-domain and frequency-domain parameters between South Asians and White Caucasians with diabetes. Nonsignificant comparisons from the HRV and frequency and time domain analysis have not been included. Data are presented as median (IQR).

	South Asians (n = 115)	White Caucasians (n = 110)	p value
30 : 15 ratio	1.26 (1.11–1.60)	1.19 (1.08–1.37)	0.027
Baseline Lfa	0.95 (0.42–2.14)	0.60 (0.27–1.38)	0.041
Baseline Rfa	0.54 (0.21–1.19)	0.35 (0.15–0.80)	0.032
Deep breathing Lfa	0.70 (0.29–1.84)	0.44 (0.16–1.35)	0.035
Standing Lfa	1.13 (0.41–2.73)	0.69 (0.23–1.43)	0.018

Table 5: Assessment of microvascular blood flow and endothelial function in South Asians and White Caucasians with type 2 diabetes. Data presented as median (IQR) or ratios. Blood flux was measured in arbitrary perfusion units (APU). Conductance is the measure of dividing flux by the mean arterial pressure.

	South Asians (n = 33)	White Caucasians (n = 38)	p value, unadjusted
	Flux		
Baseline	23.10 (17.30–35.15)	24.20 (15.70–35.22)	0.986
Heating	140.10 (107.30–169.05)	174.00 (143.12–207.62)	0.006
Ach	112.00 (84.80–135.50)	107.40 (73.62–142.77)	0.991
SNP	102.80 (66.70–141.10)	127.30 (81.60–172.60)	0.170
	Conductance		
Baseline	0.27 (0.18–0.40)	0.27 (0.17–0.37)	0.653
Heating	1.58 (1.22–1.90)	1.77 (1.50–2.19)	0.029
Ach	1.26 (0.88–1.38)	1.07 (0.77–1.53)	0.612
SNP	1.12 (0.75–1.51)	1.30 (0.88–1.84)	0.273
	Flux in relation to maximum vasodilatation		
Baseline	0.17 (0.12–0.24)	0.15 (0.10–0.21)	0.074
Ach	0.78 (0.62–0.91)	0.63 (0.43–0.76)	0.01
SNP	0.78 (0.46–1.01)	0.75 (0.51–0.94)	0.596

advanced, complicated disease, which has not previously been assessed. This difference in population characteristics may underlie the previously reported inability to detect significant differences in DPN prevalence using the Neuropathy Disability Score (NDS), instead relying upon findings from nerve electrophysiology [18]. Moreover, although not directly assessed, preservation of the microcirculation was implicated as a potential contributor to the lower risk of DPN and foot ulceration observed in South Asian patients [18]. There was, however, no adjustment performed for adiposity.

Our study confirms previous reports that foot ulceration is lower in South Asian compared to White Caucasian patients with type 2 diabetes [17]. This finding is consistent with our observation of relative preservation of 10 g monofilament perception in South Asian patients. Interestingly, the overall prevalence of neuropathic symptoms ascribable to DPN, however, was comparable across ethnicities with similar percentages reporting symptoms consistent with sensory loss but (nonsignificantly) more South Asian patients reporting neuropathic pain. It is known that South Asians have a higher prevalence of vitamin D deficiency [36], which might

contribute to differences in some symptoms, particularly generalized weakness.

Our results showed that age, diabetes duration, and insulin treatment were independently associated with DPN, as has been previously reported [13, 14, 35]. The relationship between DPN and insulin is interesting, but due to the cross-sectional nature of this study, causation cannot be proven. Intensive insulin treatment by improving metabolic control has been shown to have beneficial effects on DPN [12]. Patients might have been started on insulin because of the presence of DPN and other microvascular complications in order to slow progression. On the other hand, our data do not show a relationship between DPN and other risk factors such as triglycerides [37]; this could be related to our sample size, the different characteristics of our study population, or the high proportion of patients that were on lipid lowering therapy.

South Asians have different body fat distribution to White Europeans and tend to have greater fat mass than White Europeans for the same BMI level [38–40]. In order to address this issue, we used different measures of adiposity including BMI, waist circumference, and neck circumference, all of which were greater in White Europeans compared to South Asians in our study. All of these adiposity measures removed the impact of ethnicity on DPN when added to the regression model.

Our data, demonstrating BMI, waist circumference, and neck circumference as independent predictors of DPN, is potentially of mechanistic importance. Neck circumference is associated with cardiometabolic risk factors [41] and with obstructive sleep apnoea (OSA) [42], a disease that is very common in type 2 diabetes (up to 86% prevalence) [43] and is known to be associated with endothelial dysfunction and abnormal blood flow regulation [44]. Interestingly, neck circumference has recently been identified as an independent predictor of diabetic retinopathy [45].

Maximal skin blood flow of the lower limb on heating was reduced but endothelial function preserved in South Asian compared to White Caucasians with type 2 diabetes. In contrast, there were no significant differences in the response to SNP. To our knowledge, this is the first evaluation of skin microvascular flow regulation in South Asian patients with diabetes. Heating to 44°C leads to maximal vasodilatation which corresponds to the maximal vasodilatory capacity [34]. Deficits in heating responses have been associated with increased prevalence of cardiovascular disease [46, 47] and so our findings might have relevance for the higher cardiovascular disease risk observed in South Asians with type 2 diabetes [20].

In contrast to the heating response, the response to Ach (when measured as a ratio of maximal vasodilatation) was greater in South Asians, suggesting relative preservation of endothelial-dependent vasodilatation. The response to Ach is thought to reflect both an axon reflex and prostaglandin-mediated component [34] which might, therefore, be of relevance to the lower prevalence of DPN and foot complications in these subjects. In patients *without* diabetes, heating response was not different [47] while SNP was lower [48] in South Asians compared to White Caucasians when measured in the forearm. Our data are also consistent with the finding of relative preservation of TCpO2 in South Asian subjects with diabetes [18].

Unlike DPN, there was no difference in the prevalence of abnormalities of standardized CAN tests between South Asian and White Caucasian patients, although differences in spectral analysis suggested more preserved autonomic function in South Asians. This highlights the fact that the pathogenesis of DPN and CAN may not be the same and understanding such differences will be important to develop targeted treatments. Differences in CAN prevalence between South Asian and White Caucasian patients with type 2 diabetes have been previously assessed using the E/I ratio and the postural drop in BP as markers of CAN [18]. In this previous report, there was no difference in the postural drop between ethnicities (consistent with our findings), but there was a significant difference in the change in heart rate associated with deep breathing between ethnicities. It is difficult to directly compare our results with the results from this report, as we performed a more extensive assessment of CAN and we have presented our E/I ratio data as categorical variable rather than an absolute change in heart rate.

There are some limitations of our study, particularly the differences in baseline parameters such as age, height, BP, smoking, and alcohol consumption between the ethnic groups which might have affected the associations observed in our study. However, the principal findings of our study remained significant after adjusting for a wide range of possible confounders. Matching ethnicities for some of the baseline parameters might have been preferred, but such precise matching is difficult (particularly regarding age and adiposity measures) due to the significant demographic differences that exist between our local South Asian and White Caucasian population with type 2 diabetes. The relatively small size of our sample might have obscured the relationship between CAN and ethnicity. Nonetheless, this sample size was adequate to detect a difference in the prevalence of DPN consistent with the construct that differences in the pathogenesis of peripheral and autonomic neuropathy may exist. In addition, this is a cross-sectional study and hence causation cannot be proven. Additionally, there is the possibility of self-selection bias (i.e., patients with microvascular complications preferentially agreeing to participate), which cannot be entirely excluded. However, all patients attending a general diabetes clinic were approached about willingness to enrol in the study and we are unaware of an ethnicity-mediated self-selection bias which would affect our conclusions. This is further supported by the similar prevalence of diabetic retinopathy between the two ethnicities. Finally, the MNSI is not the "gold standard" for diagnosing DPN but it has been validated against nerve conduction studies [25, 29] and has been used widely in landmark studies [4, 26, 27, 30]. We chose to use the MNSI (in concert with the 10 g monofilament) since it offers the advantage of providing robust, meaningful, clinically detectable end-points.

In summary, clinical signs consistent with DPN are reduced in South Asian compared to White Caucasian patients with type 2 diabetes receiving hospital-based diabetes care. In contrast, the overall prevalence of neuropathic

symptoms ascribable to DPN was similar in these ethnic groups. Differences in height and adiposity appear to explain the ethnic differences in DPN prevalence; and obesity was an independent determent of DPN in these populations. We have also identified a novel association between neck circumference and DPN which might be of pathogenic importance as neck circumference is associated with cardiometabolic risk factors and OSA. Endothelial-dependent microvascular function, but not the response to heating, was also relatively preserved in South Asian subjects. Future studies are warranted to characterize small nerve fibre loss and dysfunction in different populations, address the possible role of OSA in the pathogenesis of DPN, and further explore differences in microvascular blood flow regulation between different ethnicities.

Abbreviations

DN: Diabetic neuropathy
DPN: Diabetic peripheral neuropathy
DAN: Diabetic autonomic neuropathy
CAN: Cardiac autonomic neuropathy
TCpO2: Transcutaneous partial pressure of oxygen
MNSI: Michigan Neuropathy Screening Instrument
HRV: Heart rate variability
VLF: Very low frequency
LF: Low frequency
HF: High frequency
NDS: Neuropathy Disability Score
AGE: Advanced glycation end-product.

Disclosure

Dr. Abd Tahrani is a NIHR Clinician Scientist. The views expressed in this publication are those of the authors and not necessarily those of the NHS, the National Institute for Health Research, or the Department of Health.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Abd A. Tahrani contributed to conception, design, analysis, interpretation, writing first draft, and final approval. Q. A. Altaf contributed to reviewing draft and final approval. Milan K. Piya contributed to conduct of the study, data collection, reviewing the draft, and final approval. Anthony H. Barnett contributed to design, reviewing draft, and final approval.

Acknowledgments

Dr. Abd Tahrani is a Clinician Scientist supported by the National Institute for Health Research. The authors acknowledge Dr. Fahmy Hanna, Mrs. Helen Hodgson, and Mrs. Rebecca Barakam from the University Hospital of North Staffordshire for their help in recruitment.

References

[1] A. I. Vinik, T. S. Park, K. B. Stansberry, and G. L. Pittenger, "Diabetic neuropathies," *Diabetologia*, vol. 43, no. 8, pp. 957–973, 2000.

[2] A. I. Vinik and D. Ziegler, "Diabetic cardiovascular autonomic neuropathy," *Circulation*, vol. 115, no. 3, pp. 387–397, 2007.

[3] D. Ziegler, K. Dannehl, H. Muhlen, M. Spuler, and F. A. Gries, "Prevalence of cardiovascular autonomic dysfunction assessed by spectral analysis, vector analysis, and standard tests of heart rate variation and blood pressure responses at various stages of diabetic neuropathy," *Diabetic Medicine*, vol. 9, no. 9, pp. 806–814, 1992.

[4] The DCCT Research Group, "Factors in development of diabetic neuropathy. Baseline analysis of neuropathy in feasibility phase of Diabetes Control and Complications Trial (DCCT)," *Diabetes*, vol. 37, no. 4, pp. 476–481, 1988.

[5] M. Toeller, A. E. Buyken, G. Heitkamp, G. Berg, and W. A. Scherbaum, "Prevalence of chronic complications, metabolic control and nutritional intake in type 1 diabetes: comparison between different European regions," *Hormone and Metabolic Research*, vol. 31, no. 12, pp. 680–685, 1999.

[6] R. E. Maser, A. R. Steenkiste, J. S. Dorman et al., "Epidemiological correlates of diabetic neuropathy. Report from Pittsburgh epidemiology of diabetes complications study," *Diabetes*, vol. 38, no. 11, pp. 1456–1461, 1989.

[7] C. N. De Wytt, R. V. Jackson, G. I. Hockings, J. M. Joyner, and C. R. Strakosch, "Polyneuropathy in Australian outpatients with type II diabetes mellitus," *Journal of Diabetes and Its Complications*, vol. 13, no. 2, pp. 74–78, 1999.

[8] M. J. Stevens, F. Dayanikli, D. M. Raffel et al., "Scintigraphic assessment of regionalized defects in myocardial sympathetic innervation and blood flow regulation in diabetic patients with autonomic neuropathy," *Journal of the American College of Cardiology*, vol. 31, no. 7, pp. 1575–1584, 1998.

[9] M. J. Stevens, D. M. Raffel, K. C. Allman et al., "Cardiac sympathetic dysinnervation in diabetes: implications for enhanced cardiovascular risk," *Circulation*, vol. 98, no. 10, pp. 961–968, 1998.

[10] J. Partanen, L. Niskanen, J. Lehtinen, E. Mervaala, O. Siitonen, and M. Uusitupa, "Natural history of peripheral neuropathy in patients with non-insulin- dependent diabetes mellitus," *New England Journal of Medicine*, vol. 333, no. 2, pp. 89–94, 1995.

[11] J. P. Töyry, L. K. Niskanen, M. J. Mäntysaari, E. A. Länsimies, and M. I. J. Uusitupa, "Occurrence, predictors, and clinical significance of autonomic neuropathy in NIDDM: ten-year follow-up from the diagnosis," *Diabetes*, vol. 45, no. 3, pp. 308–315, 1996.

[12] The Diabetes Control and Complications Trial Research Group, "The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus," *The New England Journal of Medicine*, vol. 329, no. 14, pp. 977–986, 1993.

[13] S. Tesfaye, N. Chaturvedi, S. E. M. Eaton et al., "Vascular risk factors and diabetic neuropathy," *New England Journal of Medicine*, vol. 352, no. 4, pp. 341–431, 2005.

[14] J. Elliott, S. Tesfaye, N. Chaturvedi et al., "Large-fiber dysfunction in diabetic peripheral neuropathy is predicted by cardiovascular risk factors," *Diabetes Care*, vol. 32, no. 10, pp. 1896–1900, 2009.

[15] A. A. Tahrani, T. Askwith, and M. J. Stevens, "Emerging drugs for diabetic neuropathy," *Expert Opinion on Emerging Drugs*, vol. 15, no. 4, pp. 661–683, 2010.

[16] M. Brownlee, "The pathobiology of diabetic complications," *Diabetes*, vol. 54, no. 6, pp. 1615–1625, 2005.

[17] C. A. Abbott, A. P. Garrow, A. L. Carrington, J. Morris, E. R. Van Ross, and A. J. Boulton, "Foot ulcer risk is lower in South-Asian and African-Caribbean compared with European diabetic patients in the U.K.," *Diabetes Care*, vol. 28, no. 8, pp. 1869–1875, 2005.

[18] C. A. Abbott, N. Chaturvedi, R. A. Malik et al., "Explanations for the lower rates of diabetic neuropathy in Indian Asians versus Europeans," *Diabetes Care*, vol. 33, no. 6, pp. 1325–1330, 2010.

[19] N. Chaturvedi, C. A. Abbott, A. Whalley, P. Widdows, S. Y. Leggetter, and A. J. M. Boulton, "Risk of diabetes-related amputation in South Asians vs. Europeans in the UK," *Diabetic Medicine*, vol. 19, no. 2, pp. 99–104, 2002.

[20] A. H. Barnett, A. N. Dixon, S. Bellary, M. W. Hanif, J. P. O'Hare, and N. T. Raymond, "Type 2 diabetes and cardiovascular risk in the UK south Asian community," *Diabetologia*, vol. 49, no. 10, pp. 2234–2246, 2006.

[21] A. A. Tahrani, A. Ali, N. T. Raymond et al., "Obstructive sleep apnea and diabetic neuropathy: a novel association in patients with type 2 diabetes," *American Journal of Respiratory and Critical Care Medicine*, vol. 186, no. 5, pp. 434–441, 2012.

[22] A. A. Tahrani, A. Ali, N. T. Raymond et al., "Obstructive sleep apnea and diabetic nephropathy: a cohort study," *Diabetes Care*, vol. 36, no. 11, pp. 3718–3725, 2013.

[23] A. A. Tahrani, K. Dubb, N. T. Raymond et al., "Cardiac autonomic neuropathy predicts renal function decline in patients with type 2 diabetes: a cohort study," *Diabetologia*, vol. 57, no. 6, pp. 1249–1256, 2014.

[24] A. A. M. Tahrani, *Microvascular complications in patients with type 2 diabetes: the impact of ethnicity, sleep and oxidative stress [Ph.D. thesis]*, University of Birmingham, 2013.

[25] E. L. Feldman, M. J. Stevens, P. K. Thomas, M. B. Brown, N. Canal, and D. A. Greene, "A practical two-step quantitative clinical and electrophysiological assessment for the diagnosis and staging of diabetic neuropathy," *Diabetes Care*, vol. 17, no. 11, pp. 1281–1289, 1994.

[26] Epidemiology of Diabetes Interventions and Complications (EDIC), "Design, implementation, and preliminary results of a long-term follow-up of the Diabetes Control and Complications Trial cohort," *Diabetes Care*, vol. 22, no. 1, pp. 99–111, 1999.

[27] C. L. Martin, J. Albers, W. H. Herman et al., "Neuropathy among the diabetes control and complications trial cohort 8 years after trial completion," *Diabetes Care*, vol. 29, no. 2, pp. 340–344, 2006.

[28] O. Boyraz and M. Saracoglu, "The effect of obesity on the assessment of diabetic peripheral neuropathy: a comparison of Michigan patient version test and Michigan physical assessment," *Diabetes Research and Clinical Practice*, vol. 90, no. 3, pp. 256–260, 2010.

[29] A. Moghtaderi, A. Bakhshipour, and H. Rashidi, "Validation of Michigan neuropathy screening instrument for diabetic peripheral neuropathy," *Clinical Neurology and Neurosurgery*, vol. 108, no. 5, pp. 477–481, 2006.

[30] G. Pambianco, T. Costacou, E. Strotmeyer, and T. J. Orchard, "The assessment of clinical distal symmetric polyneuropathy in type 1 diabetes: a comparison of methodologies from the Pittsburgh Epidemiology of Diabetes Complications Cohort," *Diabetes Research and Clinical Practice*, vol. 92, no. 2, pp. 280–287, 2011.

[31] J. P. Colombo, W. C. M. Shoemaker, H. M. Belzberg, G. M. Hatzakis, P. M. Fathizadeh, and D. M. Demetriades, "Noninvasive monitoring of the autonomic nervous system and hemodynamics of patients with blunt and penetrating trauma," *Journal of Trauma—Injury, Infection & Critical Care*, vol. 65, no. 6, pp. 1364–1373, 2008.

[32] D. Ziegler, G. Laux, K. Dannehl et al., "Assessment of cardiovascular autonomic function: age-related normal ranges and reproducibility of spectral analysis, vector analysis, and standard tests of heart rate variation and blood pressure responses," *Diabetic Medicine*, vol. 9, no. 2, pp. 166–175, 1992.

[33] M. Roustit, C. Millet, S. Blaise, B. Dufournet, and J. L. Cracowski, "Excellent reproducibility of laser speckle contrast imaging to assess skin microvascular reactivity," *Microvascular Research*, vol. 80, no. 3, pp. 505–511, 2010.

[34] J.-L. Cracowski, C. T. Minson, M. Salvat-Melis, and J. R. Halliwill, "Methodological issues in the assessment of skin microvascular endothelial function in humans," *Trends in Pharmacological Sciences*, vol. 27, no. 9, pp. 503–508, 2006.

[35] R. Pop-Busui, J. Lu, N. Lopes, and T. L. Z. Jones, "Prevalence of diabetic peripheral neuropathy and relation to glycemic control therapies at baseline in the BARI 2D cohort," *Journal of the Peripheral Nervous System*, vol. 14, no. 1, pp. 1–13, 2009.

[36] A. A. Tahrani, A. Ball, L. Shepherd, A. Rahim, A. F. Jones, and A. Bates, "The prevalence of vitamin D abnormalities in South Asians with type 2 diabetes mellitus in the UK," *International Journal of Clinical Practice*, vol. 64, no. 3, pp. 351–355, 2010.

[37] T. D. Wiggin, K. A. Sullivan, R. Pop-Busui, A. Amato, A. A. F. Sima, and E. L. Feldman, "Elevated triglycerides correlate with progression of diabetic neuropathy," *Diabetes*, vol. 58, no. 7, pp. 1634–1640, 2009.

[38] A. H. Barnett, A. N. Dixon, S. Bellary et al., "Type 2 diabetes and cardiovascular risk in the UK south Asian community," *Diabetologia*, vol. 49, no. 10, pp. 2234–2246, 2006.

[39] L. J. Gray, T. Yates, M. J. Davies et al., "Defining obesity cut-off points for migrant South Asians," *PLoS ONE*, vol. 6, no. 10, Article ID e26464, 2011.

[40] F. Razak, S. S. Anand, H. Shannon et al., "Defining obesity cut points in a multiethnic population," *Circulation*, vol. 115, no. 16, pp. 2111–2118, 2007.

[41] S. R. Preis, J. M. Massaro, U. Hoffmann et al., "Neck circumference as a novel measure of cardiometabolic risk: the framingham heart study," *Journal of Clinical Endocrinology and Metabolism*, vol. 95, no. 8, pp. 3701–3710, 2010.

[42] B. Caffo, M. Diener-West, N. M. Punjabi, and J. Samet, "A novel approach to prediction of mild obstructive sleep disordered breathing in a population-based sample: the sleep heart health study," *Sleep*, vol. 33, no. 12, pp. 1641–1648, 2010.

[43] G. D. Foster, M. H. Sanders, R. Millman et al., "Obstructive sleep apnea among obese patients with type 2 diabetes," *Diabetes Care*, vol. 32, no. 6, pp. 1017–1019, 2009.

[44] S. Yim-Yeh, S. Rahangdale, A. T. D. Nguyen et al., "Vascular dysfunction in obstructive sleep apnea and type 2 diabetes mellitus," *Obesity*, vol. 19, no. 1, pp. 17–22, 2011.

[45] M. Dirani, J. Xie, E. Fenwick et al., "Are obesity and anthropometry risk factors for diabetic retinopathy?: the diabetes management project," *Investigative Ophthalmology and Visual Science*, vol. 52, no. 7, pp. 4416–4421, 2011.

[46] P. Dandona, H. Ghanim, A. Chaudhuri, and P. Mohanty, "Thiazolidinediones—improving endothelial function and potential long-term benefits on cardiovascular disease in subjects with type 2 diabetes," *Journal of Diabetes and Its Complications*, vol. 22, no. 1, pp. 62–75, 2008.

[47] W. D. Strain, A. D. Hughes, J. Mayet et al., "Attenuation of microvascular function in those with cardiovascular disease is similar in patients of Indian Asian and European descent," *BMC Cardiovascular Disorders*, vol. 10, no. 1, article 3, 2010.

[48] A. Misra and L. Khurana, "Obesity-related non-communicable diseases: South Asians vs White Caucasians," *International Journal of Obesity*, vol. 35, no. 2, pp. 167–187, 2011.

Hindawi
Journal of Diabetes Research
Volume 2017, Article ID 4729284, 10 pages
https://doi.org/10.1155/2017/4729284

Research Article

Neutrophils Infiltrate the Spinal Cord Parenchyma of Rats with Experimental Diabetic Neuropathy

Victoria L. Newton,[1] Jonathan D. Guck,[1] Mary A. Cotter,[2] Norman E. Cameron,[2] and Natalie J. Gardiner[1]

[1]*Division of Diabetes, Endocrinology and Gastroenterology, School of Medical Sciences, Faculty of Biology, Medicine and Health, University of Manchester, Oxford Road, Manchester M13 9PT, UK*
[2]*School of Medical Sciences, University of Aberdeen, Aberdeen, UK*

Correspondence should be addressed to Natalie J. Gardiner; natalie.gardiner@manchester.ac.uk

Received 24 October 2016; Accepted 15 January 2017; Published 15 February 2017

Academic Editor: Dilek Yavuz

Spinal glial cell activation and cytokine secretion have been implicated in the etiology of neuropathic pain in a number of experimental models, including diabetic neuropathy. In this study, streptozotocin- (STZ-) induced diabetic rats were either untreated or treated with gabapentin (50 mg/kg/day by gavage for 2 weeks, from 6 weeks after STZ). At 8 weeks after STZ, hypersensitivity was confirmed in the untreated diabetic rats as a reduced response threshold to touch, whilst mechanical thresholds in gabapentin-treated diabetic rats were no different from controls. Diabetes-associated thermal hypersensitivity was also ameliorated by gabapentin. We performed a cytokine profiling array in lumbar spinal cord samples from control and diabetic rats. This revealed an increase in L-selectin, an adhesion molecule important for neutrophil transmigration, in the spinal cord of diabetic rats but not diabetic rats treated with gabapentin. Furthermore, we found an increase in the number of neutrophils present in the parenchyma of the spinal cord, which was again ameliorated in gabapentin-treated diabetic rats. Therefore, we suggest that dysregulated spinal L-selectin and neutrophil infiltration into the spinal cord could contribute to the pathogenesis of painful diabetic neuropathy.

1. Introduction

The prevalence of diabetes mellitus is increasing worldwide, which has significant health and economic implications. Associated costs include both primary treatment and also treating the associated secondary complications such as retinopathy, nephropathy, and neuropathy [1]. Distal sensory polyneuropathy (DPN) is the most common of the peripheral nerve disorders associated with diabetes [2]. DPN may be accompanied by paresthesia such as tingling and burning sensations, heightened sensitivity to normally innocuous stimuli, and spontaneous pain. Neuropathic pain serves no useful function [3] and is a debilitating condition [4]. There is currently no effective treatment, with single analgesics usually failing to adequately treat the pain, meaning that patients are offered available therapies in a stepwise and often combinatorial fashion [5, 6]. One of the first-line treatments is gabapentin [7], an analogue of the inhibitory neurotransmitter γ-aminobutyric acid (GABA), an anticonvulsant drug which is also antiallodynic in neuropathic and inflammatory pain states [8]. The underlying mechanisms of analgesia provided by this drug are not thoroughly understood. However, gabapentin can interact with several targets, including the $\alpha 2$-δ subunit of voltage-activated calcium channels [9] and the L-amino acid transporters (LAT-1 and LAT-2; [10]) and has been shown to reduce spinal microglial activation and allodynia in rats with experimental diabetes [11, 12].

The pathogenesis of neuropathic pain involves changes in neuronal activity in the peripheral and central nervous systems and also activation of glial and immune cells [13, 14]. Glial cells are rapidly activated in response to peripheral nerve injury [15–17] and are responsible for the release of many inflammatory mediators, including chemokines and cytokines important for the establishment and maintenance

of neuropathic pain [13, 18]. In rodent models of diabetes, microglia are activated in the dorsal horn of the lumbar spinal cord [11, 19, 20] and their activation has been associated with neuropathic pain. Whilst the intact blood-brain barrier largely prevents circulating immune cells from crossing into the parenchyma, permeability can be modulated by trauma or inflammation, enabling recruitment and infiltration of immune cells into the central nervous system (CNS). Peripheral injection of carrageenan into the hind paw, for example, causes an increase in ICAM and VCAM expression in spinal epithelium, changes in tight junction proteins, an increase in brain-barrier permeability, and the consequent migration of neutrophils into the CNS [21]. In both diabetic nephropathy and retinopathy, increased leukostasis and infiltration have been observed and are detrimental to symptom severity [22, 23]. However, to our knowledge, no information exists regarding the transmigration of neutrophils in the spinal cord in painful diabetic neuropathy.

In this study, we identify an increase in L-selectin and in the number of neutrophils present in the parenchyma of the spinal cord of untreated diabetic rats, which could represent a novel therapeutic target to explore in future studies.

2. Methods

2.1. Induction of Diabetes and Drug Delivery. All experiments were carried out using mature adult male Sprague-Dawley rats (495–570 g, bred at The Institute of Medical Science, Aberdeen University) in accordance with the UK Animals (Scientific Procedures) Act 1986. Diabetes was induced using streptozotocin (STZ in sterile saline; 40 mg/kg i.p.; $n = 12$). Age- and weight-matched rats were used as nondiabetic controls ($n = 6$). Diabetic rats were either untreated or treated daily for 2 weeks with gabapentin (50 mg/kg/day by gavage; Spectrum Chemical Manufacturing Corp, Gardena) starting 6 weeks following STZ injection.

2.2. Behavioural Testing. After 8 weeks of diabetes and within 2 hours following gabapentin administration, the behavioural response to mechanical and thermal stimulation of the hind paw was assessed using an automatic Von Frey probe and Hargreaves apparatus, respectively (Ugo Basile, Italy). All animals were habituated to the tester and the environment prior to testing. The Von Frey probe was set to deliver an initial 2 g force over 8 seconds. The stimulus was then increased by a 0.2 log unit increment if there was no response or decreased by the same increment if the animal responded. Testing was performed on each foot and a 50% threshold was determined [24, 25]. An average value for the 2 feet was then calculated. For thermal testing, the time taken for the animal to remove its hind paw from the infrared heat stimulus was recorded 3 times for each foot. The first reading from each foot was discarded, and the mean response time was then calculated.

2.3. Tissue Processing. Rats were killed by isoflurane overdose 1 day after their final behavioural assessment and dose of gabapentin. Blood glucose levels were measured using an Ascensia Breeze 2 machine (Bayer, UK) from blood obtained by cardiac puncture. The lumbar (L3–L6) spinal cord region was rapidly dissected and postfixed in ice-cold 4% paraformaldehyde for 4 hours or frozen on dry-ice.

2.4. Immunohistochemistry. Fixed lumbar spinal cord was cryoprotected at 2–8°C in 10% sucrose in 0.1 M phosphate buffer for 18–24 hours followed by 30% sucrose in 0.1 M phosphate buffer for a further 18–24 hours. Tissue was then embedded in OCT embedding matrix media (Thermo Shandon Ltd., UK) and frozen on dry-ice. Transverse sections were cut by cryostat (16 μm) and thaw-mounted onto Superfrost Plus Slides (Fisher Scientific, UK).

For staining of microglia, one group of slides were first incubated twice for 15 minutes each (0.1 M PBS, 20% (v/v) methanol, 1.5% (v/v) hydrogen peroxide) for peroxidase-conjugated studies. After washing (phosphate buffered saline (PBS)), nonspecific binding in all slides was blocked for an hour (5% goat serum in 0.2% triton-X in PBS at room temperature) and sections were incubated overnight with primary antibody for ionized calcium binding adaptor molecule 1 (rabbit anti-Iba1; Wako, Germany; 1/1000 in 5% goat serum in 0.2% triton-X PBS at 2–8°C;). Biotinylated or fluorescein conjugated anti-rabbit secondary antibody (1/500 in 5% goat serum in 0.2% triton-X PBS; Vector, USA) was allowed to bind primary antibody for 1.5 hours at room temperature. Fluorescent sections were washed then either mounted in Vectorshield containing DAPI (Vector, USA) or incubated for 1 hour at room temperature with AvidinBiotin solution as per manufacturer's instructions (VECTASTAIN® ABC system, Vector, USA). Immunoreactivity was then visualised using DAB as per manufacturer's instructions (Vector, USA). Sections were dehydrated through a 50, 70, 90%, and absolute ethanol series followed by xylene washes, before mounting with Pertex mounting media (CellPath, UK).

For staining of neutrophils and blood vessels, sections were washed and nonspecific binding and autofluorescence were reduced (0.1 M glycine, 5% goat serum, 5% donkey serum in 0.3% triton-X in PBS; for 2 hours at room temperature). Primary antibodies for rabbit anti-neutrophil serum (SJC; 1/10,000; gift A. Dénes, (University of Manchester, UK) from D. Anthony and S. Campbell, University of Oxford, UK, produced [26]) and mouse anti-human laminin (1/1000; Millipore, UK) were applied overnight (in 2.5% goat serum, 2.5% donkey serum 0.3% triton-X in PBS, 2–8°C). After washing, primary antibodies were visualised with donkey anti-rabbit secondary Cyanine- 3-(Cy3-) conjugated antibody (1/1000; Jackson ImmunoResearch, USA) and goat anti-mouse conjugated Alexa Fluor™ 488 (1/1000; Invitrogen Life Technologies, UK) for 2 hours at room temperature (5% goat serum, 5% donkey serum in 0.3% triton-X in PBS).

Negative control sections were produced with each batch of immunostaining, with the absence of primary antibody. Staining was visualised on a Leica DMR microscope and images captured using a Hamamatsu digital C4742-95 digital camera (Japan). Higher power fluorescence images were taken using an Olympus BX51 upright microscope and Coolsnap EZ (Photometrics, USA) camera running MetaVue Software (Molecular Devices, USA), or a Delta Vision (Applied Precision, USA) restoration microscope using a

60x objective, captured using a Coolsnap HQ (Photometrics, USA) camera with a Z optical spacing of 0.2 μm. Raw images were then deconvolved using Softworx (Applied Precision, USA). High magnification images of microglia were captured on a Panoramic 250 slide scanner (3DHISTECH Ltd., Hungary), using a 20x objective and visualised using Pannoramic Viewer 1.15 software (3DHISTECH Ltd., Hungary).

2.5. Cytokine Profile Array. Spinal cords were homogenized in ice-cold lysis buffer (25 mM Tris HCl pH 7.4, 15 mM NaCl, 10 mM NaF, 10 mM Na Pyrophosphate, 2 mM EDTA, 0.2 mM Na_4OV_3, 1 mM PMSF, Protease Inhibitor Cocktail (1 : 200; Sigma Aldrich, UK)) using the FastPrep® bead beater system (MP Biomedicals, USA). The protein concentration of each sample was determined using a BCA assay (Pierce Biotechnology, UK) and 350 μg of lysate was loaded per membrane (Rat Cytokine Array Panel A, R&D Systems, UK), as per manufacturer's instructions. Cytokine levels were visualised and quantified using IRDye® 800CW Streptavidin secondary antibody (1/2000 in assay buffer 6; Li-Cor, UK) and captured on an Odyssey® Infrared Imaging System. Results were analysed using Odyssey 2.1 Software (Li-Cor, UK) and all absorbance values were corrected for background.

2.6. L-Selectin ELISA. Spinal cord lysates from control, untreated diabetic and gabapentin-treated rats (75 μg of protein) were loaded in duplicate and analysed for L-selectin content using a commercial ELISA kit (rat L-selectin/CD62L DuoSet, R&D Systems, UK).

2.7. Spinal Cord Neutrophil Analysis. To determine the number of infiltrating neutrophils, dual labelling was used to visualise both blood vessels and neutrophils present within the parenchyma. Only neutrophils outside of blood vessels were measured, that is, not intraluminal neutrophils. Parenchymal cell counts were performed on 4-5 lumbar spinal cord sections per animal ($n = 6$) and expressed as mean number of neutrophils per section.

2.8. Microglia Analysis. Iba1-immunoreactive cell morphology was analysed from DAB-labelled sections using the method described by Calvo et al. [27]. Briefly, SigmaScan Pro software (SPSS) was used to overlay a grid with squares measuring 10000 μm^2 on regions of the dorsal horn. A minimum of 5 randomly selected squares from a minimum of 4 spinal cord sections per animal were analysed. Only cells with a clearly visible cell body were analysed. Microglia whose processes were greater than double the cell-body length were deemed "surveying." Those cells where processes were less than double the cell-body length were categorised as "effector." Results are presented as the percentage of microglia displaying an "effector" morphology. Iba-1-immunoreactive cells present in the superficial dorsal horn were counted from 3–5 sections per animal and expressed as number of cells per 1×10^5 μm^2.

2.9. Statistical Analysis. Data are expressed as mean values ± standard deviation, unless otherwise stated. A Kruskal-Wallis ranking test was carried out on the Von Frey, and L-selectin ELISA data and 1-way ANOVA with Bonferroni post hoc tests were used to analyse all other data using GraphPad Prism 4 software (GraphPad software, USA).

3. Results

3.1. Gabapentin Reduces Hypersensitivity in STZ-Diabetic Rats. Rats with STZ-induced diabetes displayed mechanical allodynia at 8 weeks compared with age-matched controls (control: 37.0 g ± 3.2 versus untreated diabetic: 17.4 g ± 4.1, $p < 0.001$; Figure 1(a)). Gabapentin-treated diabetic rats (50 mg/kg/day for 2 weeks from 6 weeks after STZ) did not demonstrate the same degree of mechanical sensitivity, as no significant difference was observed between control and gabapentin-treated diabetic rats at 8 weeks (30.6 g ± 5.0, Figure 1(a)). In addition, diabetes-associated thermal hyperalgesia was prevented with gabapentin (untreated diabetic: 7.8 s ± 0.6 versus gabapentin-treated diabetic: 10.1 s ± 1.6, $p < 0.05$; Figure 1(b)). Gabapentin therefore protects against diabetes-associated allodynia and thermal hyperalgesia, without affecting terminal blood glucose levels (control: 8.1 ± 0.4 mmol/L; untreated diabetic: 34.1 ± 4.8 mmol/L ($p < 0.001$); diabetic + gabapentin: 39.2 ± 7.1 mmol/L ($p < 0.001$) compared with blood glucose levels in control rats: 1-way ANOVA with Bonferroni post hoc tests).

3.2. Diabetes-Induced Increase in L-Selectin in the Spinal Cord is Prevented by Gabapentin. An increase in the levels of proinflammatory cytokines has previously been reported in the rat spinal cord at early time points of diabetes (4-5 weeks after STZ: [28, 29]). We therefore conducted a cytokine profiling array using lumbar spinal cord samples (Figures 2(a)–2(c); Table 1) to determine which were altered at the 8-week time point of diabetes. The levels of classical "proinflammatory" cytokines such as TNF-α (Figure 2(d)), IL-1α (Figure 2(e)), and IL-1β (Figure 2(f)) were not significantly different in spinal cord samples from diabetic rats. Indeed, the only significant change (the full panel of results of 29 cytokines is shown in Table 1) was in the expression of L-selectin, which was significantly increased by 55% in spinal cords from untreated diabetic rats compared to control rats; this increase was prevented by gabapentin (Figure 2(g)). This observation was validated using ELISA (untreated diabetic: 0.14 ± 0.13 ρg L-selectin/μg spinal cord protein versus gabapentin-treated diabetic: 0.04 ρg ± 0.02 L-selectin/μg spinal cord protein; $p < 0.05$; Figure 2(h)).

3.3. Glial Cell Profiling in the Dorsal Horn of Rats with 8 Weeks of Experimental Diabetes. A change in spinal microglial number and morphology has previously been reported at early time points of diabetes (2–4 weeks after STZ: [11, 20]). Here, we examined Iba-1-immunopositive microglia at a later time point in the lumbar spinal cord of control, untreated diabetic, and diabetic rats treated with gabapentin (Figure 3). At 8 weeks after STZ, there was a small but significant increase in the numbers of Iba-1-positive cells present in the superficial dorsal horn of untreated diabetic rats (Figures 3(a)–3(d);

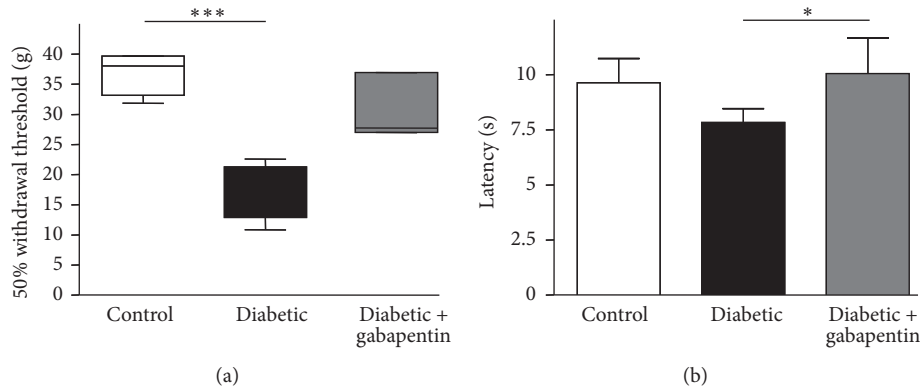


Figure 1: Gabapentin corrects diabetes-induced hypersensitivity. (a) Mechanical allodynia was observed in untreated diabetic rats (8 weeks after STZ), whilst diabetic rats treated with gabapentin showed near-control thresholds. Data are displayed as box and whisker plots, $^{***}p < 0.001$, in a Kruskal-Wallis test with Dunn's post hoc tests. (b) Thermal hyperalgesia was determined using a Hargreaves device; again diabetic rats treated with gabapentin showed near-control thresholds $^{*}p < 0.05$, in a 1-way ANOVA with Bonferroni post hoc tests. Data represent mean latency + SD; $n = 6$.

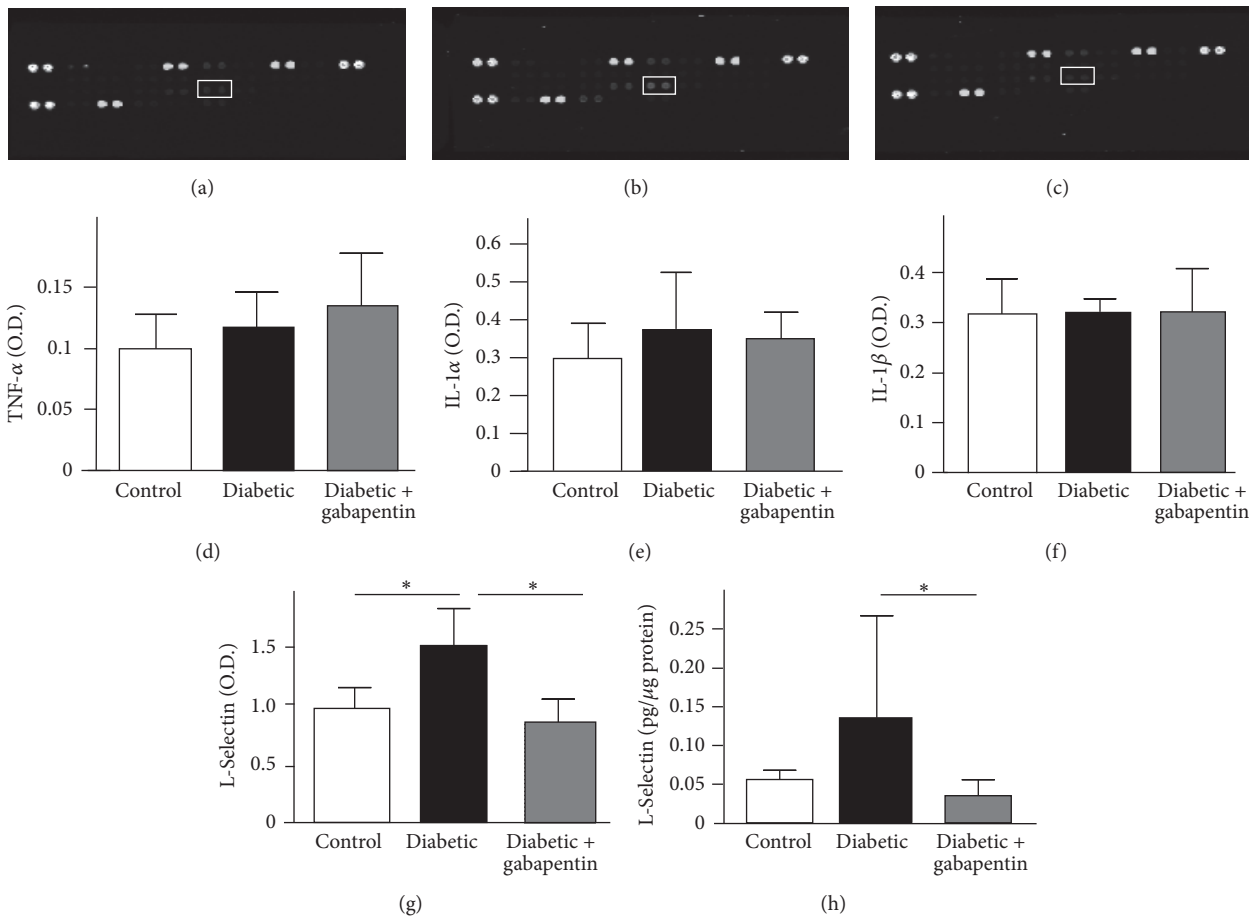


Figure 2: L-Selectin is increased in the spinal cord of diabetic rats. Cytokine proteome arrays were used to assay levels of 29 cytokines in (a) control, (b) untreated diabetic, and (c) gabapentin-treated diabetic rats. There was no change in the spinal levels of classically proinflammatory cytokines such as (d) TNF-α, (e) IL-1α, or (f) IL-1β (g). However, L-selectin (highlighted in box in (a)–(c)) was significantly increased in diabetic rat lumbar spinal cord, and this increase was ameliorated by treatment with gabapentin ($^{*}p < 0.05$ in a 1-way ANOVA with Bonferroni post hoc tests. Mean optical densities + SD are displayed; $n = 4$). (h) This was independently confirmed with an ELISA; levels of L-selectin were reduced when diabetic rats were treated with gabapentin ($^{*}p < 0.05$ in a Kruskal-Wallis test with Dunn's multiple comparison test; $n = 5$-6).

Table 1: Rat spinal cord cytokine proteome array. The levels of 29 cytokines in the lumbar spinal cord of control, diabetic, and gabapentin-treated diabetic rats. Mean values ± SD are displayed. $n = 4$. $^{*}p < 0.05$ in a 1-way ANOVA.

Name	Mean OD ± SD						p value
	Control		Diabetic		Diabetic + gabapentin		
IL-1α	**0.30**	0.09	**0.37**	0.15	**0.35**	0.07	*0.60*
IL-1β	**0.32**	0.07	**0.33**	0.03	**0.32**	0.09	*0.96*
IL-1ra	**0.30**	0.03	**0.37**	0.02	**0.33**	0.05	*0.09*
IL-2	**0.25**	0.03	**0.26**	0.06	**0.36**	0.09	*0.10*
CINC-1	**0.39**	0.21	**0.51**	0.34	**0.56**	0.24	*0.68*
CINC-2	**0.10**	0.02	**0.12**	0.02	**0.13**	0.05	*0.40*
CINC-3	**0.31**	0.04	**0.37**	0.04	**0.33**	0.02	*0.12*
IFN-γ	**0.31**	0.05	**0.29**	0.02	**0.27**	0.02	*0.19*
IL-3	**0.26**	0.09	**0.26**	0.05	**0.28**	0.08	*0.91*
IL-4	**0.23**	0.08	**0.25**	0.06	**0.27**	0.08	*0.74*
IL-6	**0.25**	0.04	**0.24**	0.03	**0.20**	0.04	*0.26*
IL-10	**0.25**	0.08	**0.22**	0.03	**0.24**	0.04	*0.82*
CNTF	**6.52**	1.91	**8.68**	2.50	**9.49**	3.47	*0.32*
sICAM-1	**9.18**	3.28	**13.10**	2.74	**12.80**	2.63	*0.15*
Thymus chemokine	**19.90**	0.36	**23.20**	3.03	**17.10**	5.85	*0.13*
Fractalkine	**1.02**	0.24	**1.00**	0.18	**1.02**	0.27	*0.99*
GM-CSF	**0.37**	0.20	**0.34**	0.06	**0.28**	0.04	*0.55*
RANTES	**0.29**	0.06	**0.40**	0.13	**0.40**	0.09	*0.22*
TIMP-1	**0.46**	0.28	**0.75**	0.43	**0.96**	0.94	*0.54*
TNF-α	**0.10**	0.03	**0.12**	0.03	**0.13**	0.04	*0.38*
VEGF	**0.56**	0.17	**0.41**	0.02	**0.49**	0.03	*0.07*
CXCl5	**0.71**	0.1	**1.1**	0.31	**0.72**	0.15	*0.07*
MIG	**0.36**	0.07	**0.35**	0.04	**0.33**	0.07	*0.81*
IL-13	**0.26**	0.17	**0.18**	0.04	**0.22**	0.05	*0.57*
IL-17	**0.22**	0.04	**0.24**	0.06	**0.31**	0.05	*0.10*
MIP-1α	**0.13**	0.02	**0.14**	0.01	**0.13**	0.03	*0.38*
MIP3α	**0.21**	0.04	**0.21**	0.01	**0.19**	0.02	*0.32*
IP-10	**0.27**	0.04	**0.33**	0.01	**0.30**	0.03	*0.06*
L-Selectin	**0.99**	0.18	**1.53**	0.32	**0.87**	0.20	*0.01**

IL-1α: interleukin-1 alpha; IL-1β: interleukin-1 beta; IL-1ra: interleukin-l receptor antagonist; IL-2: interleukin-2; IL-3: interleukin-3; IL-4: interleukin-4; IL-6: interleukin-6; IL-10: interleukin-10; CNTF: ciliary neurotrophic factor; sICAM: soluble intercellular adhesion molecule; CINC-1: Cytokine-Induced Neutrophil Chemoattractant-1; CINC-2: Cytokine-Induced Neutrophil Chemoattractant-2; CINC-3: Cytokine-Induced Neutrophil Chemoattractant-3; IFN-γ: interferon-gamma; GM-CSF: granulocyte macrophage colony stimulating factor; RANTES: regulated upon activation, normal T-cell expressed and presumably secreted; TIMP-1: tissue inhibitor of metalloproteinases-1; TNF-α: tumour necrosis factor; VEGF: vascular endothelial growth factor; CXCl5: lipopolysaccharide-induced CXC; MIG: monokine induced by gamma interferon; IL-13: interleukin-13; IL-17: interleukin-17; MIP-1α: Macrophage Inflammatory Protein-1 alpha; MIP-3α: Macrophage Inflammatory Protein-3 alpha; IP-10, interferon-gamma-induced protein-10 kDa/CXC motif chemokine 10.

$p < 0.05$ 1-way ANOVA with Bonferroni post hoc tests); however, cell morphology was equivalent in all treatment groups (Figure 3(e); control: 67% ± 4.6: untreated diabetic: 61% ± 8.1; gabapentin-treated diabetic 63% ± 4.8 microglia had "effector" morphology; i.e., processes were less than twice the length of the cell body).

3.4. Increased Numbers of Neutrophils Are Present in the Spinal Cord Parenchyma of Diabetic Rats, Which Is Ameliorated with Gabapentin Treatment. The diabetes-associated upregulation of L-selectin, an adhesion molecule important for neutrophil transmigration [30], suggested possible immune cell recruitment/infiltration into the spinal cord. Therefore, immunohistochemistry was performed to identify whether neutrophils had infiltrated the spinal cord. The spinal cord parenchyma of untreated diabetic rats (arrows, Figures 4(b), 4(e), and 4(h)) contained more neutrophils than control (Figures 4(a), 4(d), and 4(g)) and gabapentin-treated (Figures 4(c), 4(f), and 4(i)) rats. Since rats were not transcardially perfused prior to tissue harvest, only neutrophils present in spinal parenchyma (arrows, Figures 4(k) and 4(l)) were counted and not those contained within blood vessels (asterisks, Figures 4(j) and 4(l)). There was a significant increase in the number of parenchymal neutrophils throughout the spinal cord in untreated diabetic rats compared with control rats (untreated diabetic: 2.60 ± 1.4 versus control: 0.53 ± 0.55; Figure 4(m) $p < 0.01$). Interestingly, this increase was not observed in diabetic animals treated with gabapentin (1.03 ± 0.63, $p < 0.05$ compared with untreated diabetic rats).

4. Discussion

In this study, we demonstrate an increase in the levels of L-selectin, an adhesion molecule important for neutrophil transmigration, in the lumbar spinal cord after 8 weeks of diabetes. In addition, we show an increase in the number of parenchymal neutrophils in the spinal cord of diabetic rats. Together, these data suggest a role for dysregulated L-selectin and spinal vasculature in diabetes that leads to an increase in infiltrating neutrophils in experimental diabetic neuropathy, which is ameliorated by treatment with gabapentin.

The cytokine profiling array did not show diabetes-associated increases in classic proinflammatory cytokines such as IL-1β, TNF-α, fractalkine, and IL-6 cytokines, which are associated with microgliosis and hyperalgesia [11, 20, 28, 29, 31, 32]. Microglial activation has been associated with pain in a number of neuropathy models, notably traumatic nerve-injury models [14] and early time points of STZ-diabetes [11, 31]. However, microgliosis was not associated with viral-induced hypersensitivity [33] and minimal in a nucleoside reverse transcriptase inhibitor- (stavudine-) induced HIV hypersensitivity model [34]. Our finding that there was a small increase in microglial number, but no change in morphology at 8 weeks, perhaps indicates why we did not detect increases in the proinflammatory cytokines in our arrays but does not preclude the possibility that microglial activation may have occurred at an earlier time point. It is also possible that subtle changes in proinflammatory cytokines specifically

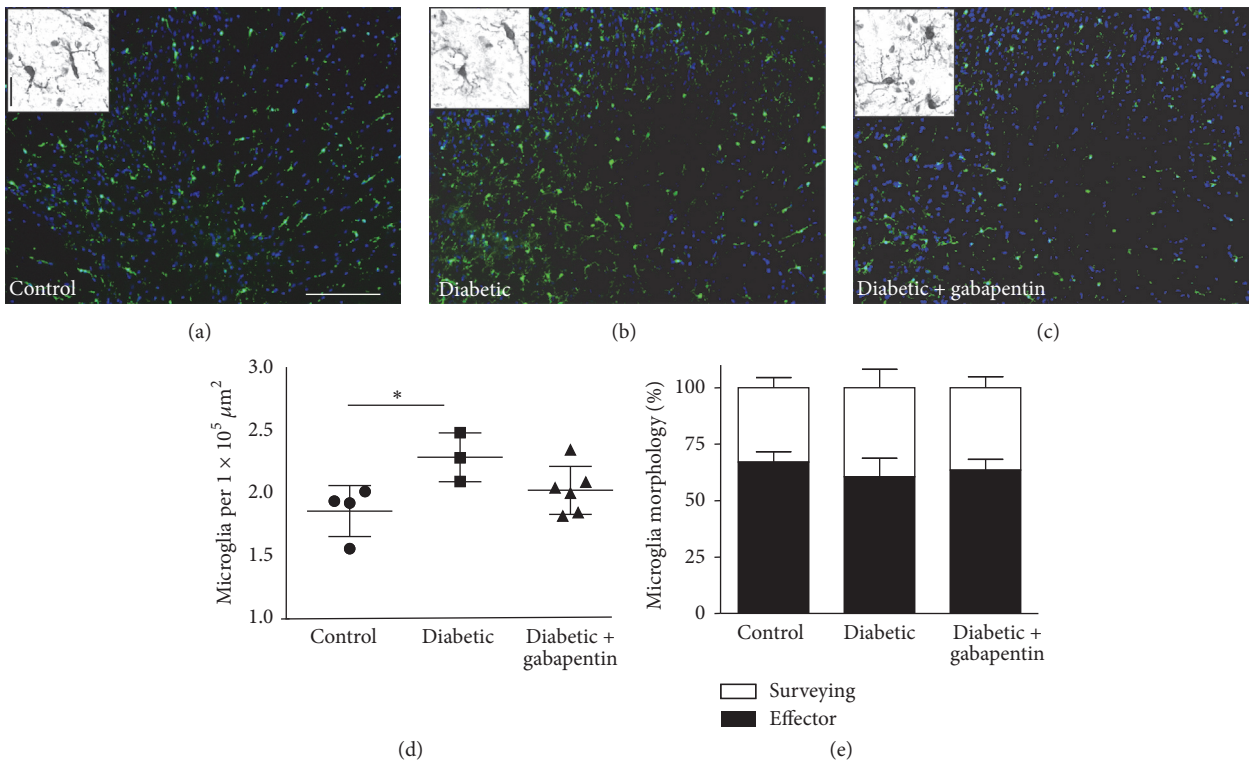


Figure 3: Microglial changes in the spinal cord of control, untreated diabetic, and gabapentin-treated diabetic rats. Representative photomicrographs of the L4/5 dorsal horn of the spinal cord of (a) control, (b) untreated diabetic, and (c) diabetic rats treated with gabapentin (a–c), micrographs (and insets) show examples of typical Iba1-immunoreactivity. Scale bars represent 100 μm (main micrograph, (a)–(c)) and 25 μm (insets (a)–(c)). Microglial numbers are increased in the superficial ((d),* $p < 0.05$ 1-way ANOVA Bonferroni post hoc tests) but not deep (e) dorsal horn of diabetic rats; however, morphology of microglia was not significantly different (e).

within the dorsal horn may have not been detected, as whole lumbar spinal cord lysates were used in our assay.

However, interestingly, we found a significant increase in L-selectin (a type I cell adhesion molecule which is constitutively expressed on the cell surface of most circulating leukocytes [30]) in spinal cords from diabetic rats, which was not evident in spinal cords from diabetic rats treated with gabapentin. L-Selectin mediates the initial tethering and rolling of leukocytes along activated endothelial cells and then the transendothelial migration of leukocytes through the vasculature. L-Selectins are cleaved from the cell surface by the action of ADAM17 (disintegrins and metalloproteinase 17) or TACE (TNF-α converting enzyme), a process known as "shedding," which may dramatically regulate migratory cell behaviour [35].

Neutrophils are one of the first types of peripheral immune cell to attend sites of inflammation following injury or infection [36]. They are important in mounting an initial immune response, able to produce both proinflammatory mediators, as well as present antigen to T-cells [37]. In experimental nerve-injury models, the recruitment of immune cells both peripherally and centrally is an important mechanism underlying establishment and maintenance of neuropathic pain [38]. Neutrophil migration into the CNS is well characterised in spinal cord injury models [39], and reductions in leukocyte infiltration in the CNS have been linked to reductions in neuropathic pain [40]. The reactive glycolytic metabolite methylglyoxal is increased in the plasma of both STZ-diabetic mice and diabetic patients [41]. Interestingly, peripheral injection of methylglyoxal induces leukocyte recruitment to the microvasculature of the injection site and approximately 90% of the recruited cells are neutrophils [42], suggesting that recruitment of this cell type may be particularly important in diabetes. In diabetic retinopathy, higher numbers of $CD45^+$ immune cells have been recorded in the retina of diabetic mice compared with controls [43]. In diabetic rats too, increased numbers of leukocytes are associated with retinal endothelial cells, when compared with retina from control rats [44]. Similarly, an increase in neutrophils in the kidney has been described in mice with diabetic nephropathy [45]. Increased numbers of neutrophils in the parenchyma of spinal cords from diabetic rats compared with controls indicate commonality between the three major diabetic complications.

This increase in spinal neutrophil recruitment in diabetes could be a consequence of altered afferent activity, spinal sensitisation, structural damage, or altered blood-brain barrier function. Interestingly, the reduction in numbers of neutrophils within the spinal cord parenchyma after gabapentin followed the reduction in allodynia and thermal

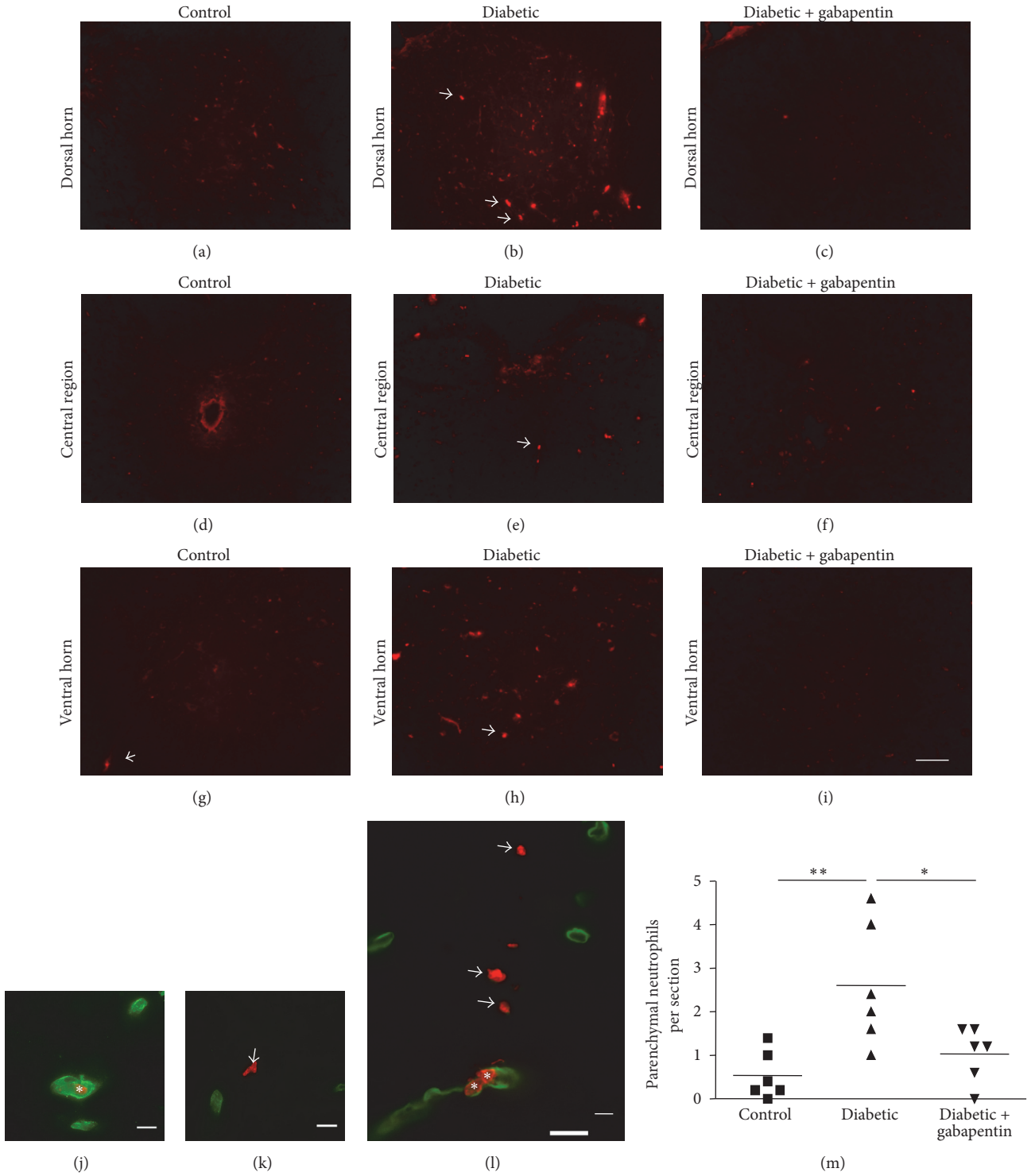


Figure 4: Diabetes is associated with an increase in neutrophil numbers within the spinal cord parenchyma, which is ameliorated with gabapentin treatment. (a–i) There is an increase in antineutrophil immunoreactivity within the dorsal horn (a–c), central region (d–f), and ventral horns (g–i) of untreated diabetic rats (arrows, (b), (e), and (h), scale bar represents 100 μm.). Dual immunofluorescence with antilaminin (green) to label blood vessels reveals the presence of intraluminal (asterisks, (j) and (l)) and parenchymal neutrophils (arrows, (k) and (l)). Only parenchymal neutrophils (i.e., those not associated with a vessel) were counted (m) and revealed an increase in neutrophils in the spinal cord of untreated diabetic rats and revealed that gabapentin treatment reduced the numbers to near-control levels ((m), $^{*}p < 0.05$, $^{**}p < 0.01$ in a 1-way ANOVA with Bonferroni post hoc tests. $n = 6$).

hyperalgesia in diabetic rats. The reduced numbers of spinal neutrophils in diabetic rats treated daily with gabapentin may be a downstream consequence of the transitory analgesic effects of gabapentin ([46]). This may be through inhibition of excitatory postsynaptic currents from dorsal horn neurons or a reduction in microgliosis persisting for a sufficient length of time to prevent neutrophil infiltration into the spinal cord. It will be interesting for future studies to examine whether gabapentin has a direct effect on L-selectin expression. This effect may be via the spinal vasculature, neutrophil migration across the endothelium, and/or release from the bone marrow. Indeed, in an induced paw edema model, gabapentin treatment reduced both leukocyte counts and MPO activity (neutrophil marker) in the foot [47]. Depletion of circulating neutrophils has also previously been shown to attenuate the development of hyperalgesia following a peripheral nerve injury [48]. We suggest that future studies will be important to determine the impact of blocking neutrophil recruitment to the spinal cord on neuropathic pain in diabetes. Using intrathecal L-selectin function-blocking antibodies or inhibitors may provide valuable mechanistic evidence linking spinal L-selectin expression and neutrophil invasion with the pathogenesis of diabetic neuropathy.

In conclusion, this current work highlights the importance of considering not only glial activation, changes in ion channel expression, and altered neuronal activity in painful diabetic neuropathy, but also the effect of immune cell infiltration into the spinal cord; this may open more avenues by which to direct future therapeutic targeting.

Abbreviations

CNS: Central nervous system
DPN: Distal sensory polyneuropathy
GABA: γ-Aminobutyric acid
IBA1: Ionized calcium binding adaptor molecule 1
PBS: Phosphate buffered saline
STZ: Streptozotocin.

Disclosure

The funders had no role in study design, data collection, and analysis or preparation of the manuscript.

Competing Interests

The authors declare that they have no competing interests.

Acknowledgments

This work was supported by a GlaxoSmithKline-BBSRC (UK) Ph.D. studentship (VLN; BB/J014478/1) and the authors thank the Centre for Integrated Mammalian Biology, Manchester, for support. Natalie J. Gardiner was supported by the Juvenile Diabetes Research Foundation (Natalie J. Gardiner: JDRF, USA: 2-2009-226). The authors thank Sumia Ali and Sarah Al-Adham for immunocytochemistry advice and Roger Meadows (University of Manchester Bioimaging Facility) for helping with microscopy.

References

[1] N. Hex, C. Bartlett, D. Wright, M. Taylor, and D. Varley, "Estimating the current and future costs of Type1 and Type2 diabetes in the UK, including direct health costs and indirect societal and productivity costs," *Diabetic Medicine*, vol. 29, no. 7, pp. 855–862, 2012.

[2] A. I. Vinik, T. S. Park, K. B. Stansberry, and G. L. Pittenger, "Diabetic neuropathies," *Diabetologia*, vol. 43, no. 8, pp. 957–973, 2000.

[3] J. Sandkühler, "Models and mechanisms of hyperalgesia and allodynia," *Physiological Reviews*, vol. 89, no. 2, pp. 707–758, 2009.

[4] B. S. Galer, A. Gianas, and M. P. Jensen, "Painful diabetic polyneuropathy: epidemiology, pain description, and quality of life," *Diabetes Research and Clinical Practice*, vol. 47, no. 2, pp. 123–128, 2000.

[5] D. Ziegler, "Painful diabetic neuropathy: treatment and future aspects," *Diabetes/Metabolism Research and Reviews*, vol. 24, S1, pp. S52–S57, 2008.

[6] A. B. O'Connor and R. H. Dworkin, "Treatment of neuropathic pain: an overview of recent guidelines," *The American Journal of Medicine*, vol. 122, no. 10, pp. S22–S32, 2009.

[7] N. Attal, G. Cruccu, R. Baron et al., "EFNS guidelines on the pharmacological treatment of neuropathic pain: 2010 revision," *European Journal of Neurology*, vol. 17, no. 9, pp. 1113–1123, 2010.

[8] M. Bialer, "Why are antiepileptic drugs used for nonepileptic conditions?" *Epilepsia*, vol. 53, pp. 26–33, 2012.

[9] J.-K. Cheng and L.-C. Chiou, "Mechanisms of the antinociceptive action of gabapentin," *Journal of Pharmacological Sciences*, vol. 100, no. 5, pp. 471–486, 2006.

[10] E. M. del Amo, A. Urtti, and M. Yliperttula, "Pharmacokinetic role of L-type amino acid transporters LAT1 and LAT2," *European Journal of Pharmaceutical Sciences*, vol. 35, no. 3, pp. 161–174, 2008.

[11] R. Wodarski, A. K. Clark, J. Grist, F. Marchand, and M. Malcangio, "Gabapentin reverses microglial activation in the spinal cord of streptozotocin-induced diabetic rats," *European Journal of Pain*, vol. 13, no. 8, pp. 807–811, 2009.

[12] C. Morgado, P. P. Terra, and I. Tavares, "Neuronal hyperactivity at the spinal cord and periaqueductal grey during painful diabetic neuropathy: effects of gabapentin," *European Journal of Pain*, vol. 14, no. 7, pp. 693–699, 2010.

[13] M. Costigan, J. Scholz, and C. J. Woolf, "Neuropathic pain: a maladaptive response of the nervous system to damage," *Annual Review of Neuroscience*, vol. 32, pp. 1–32, 2009.

[14] S. B. McMahon and M. Malcangio, "Current challenges in glia-pain biology," *Neuron*, vol. 64, no. 1, pp. 46–54, 2009.

[15] S. Jergová and D. Čížková, "Microglial activation in different models of peripheral nerve injury of the rat," *Journal of Molecular Histology*, vol. 38, no. 3, pp. 245–251, 2007.

[16] S.-X. Jin, Z.-Y. Zhuang, C. J. Woolf, and R.-R. Ji, "p38 mitogen-activated protein kinase is activated after a spinal nerve ligation in spinal cord microglia and dorsal root ganglion neurons and contributes to the generation of neuropathic pain," *The Journal of Neuroscience*, vol. 23, no. 10, pp. 4017–4022, 2003.

[17] Z.-Y. Zhuang, Y.-R. Wen, D.-R. Zhang et al., "A peptide c-Jun N-terminal kinase (JNK) inhibitor blocks mechanical allodynia

after spinal nerve ligation: respective roles of JNK activation in primary sensory neurons and spinal astrocytes for neuropathic pain development and maintenance," *Journal of Neuroscience*, vol. 26, no. 13, pp. 3551–3560, 2006.

[18] C. J. Woolf and Q. Ma, "Nociceptors—noxious stimulus detectors," *Neuron*, vol. 55, no. 3, pp. 353–364, 2007.

[19] L. Daulhac, C. Mallet, C. Courteix et al., "Diabetes-induced mechanical hyperalgesia involves spinal mitogen-activated protein kinase activation in neurons and microglia via N-methyl-D-aspartate- dependent mechanisms," *Molecular Pharmacology*, vol. 70, no. 4, pp. 1246–1254, 2006.

[20] M. Tsuda, H. Ueno, A. Kataoka, H. Tozaki-Saitoh, and K. Inoue, "Activation of dorsal horn microglia contributes to diabetes-induced tactile allodynia via extracellular signal-regulated protein kinase signaling," *GLIA*, vol. 56, no. 4, pp. 378–386, 2008.

[21] K. Mitchell, H.-Y. T. Yang, P. A. Tessier et al., "Localization of S100A8 and S100A9 expressing neutrophils to spinal cord during peripheral tissue inflammation," *Pain*, vol. 134, no. 1-2, pp. 216–231, 2008.

[22] F. C. Barouch, K. Miyamoto, J. R. Allport et al., "Integrin-mediated neutrophil adhesion and retinal leukostasis in diabetes," *Investigative Ophthalmology and Visual Science*, vol. 41, no. 5, pp. 1153–1158, 2000.

[23] E. Galkina and K. Ley, "Leukocyte recruitment and vascular injury in diabetic nephropathy," *Journal of the American Society of Nephrology*, vol. 17, no. 2, pp. 368–377, 2006.

[24] S. R. Chaplan, F. W. Bach, J. W. Pogrel, J. M. Chung, and T. L. Yaksh, "Quantitative assessment of tactile allodynia in the rat paw," *Journal of Neuroscience Methods*, vol. 53, no. 1, pp. 55–63, 1994.

[25] W. J. Dixon, "Efficient analysis of experimental observations," *Annual Review of Pharmacology and Toxicology*, vol. 20, pp. 441–462, 1980.

[26] M. A. Trevethick, A. K. Bahl, N. M. Clayton, P. Strong, S. Sanjar, and I. W. Harman, "Neutrophil infiltration does not contribute to the ulcerogenic effects of indomethacin in the rat gastric antrum," *Agents and Actions*, vol. 43, no. 1-2, pp. 39–43, 1994.

[27] M. Calvo, N. Zhu, J. Grist, Z. Ma, J. A. Loeb, and D. L. H. Bennett, "Following nerve injury neuregulin-1 drives microglial proliferation and neuropathic pain via the MEK/ERK pathway," *GLIA*, vol. 59, no. 4, pp. 554–568, 2011.

[28] M. Liu, K. Liao, C. Yu, X. Li, S. Liu, and S. Yang, "Puerarin alleviates neuropathic pain by inhibiting neuroinflammation in spinal cord," *Mediators of Inflammation*, vol. 2014, Article ID 485927, 9 pages, 2014.

[29] M. Bishnoi, C. A. Bosgraaf, M. Abooj, L. Zhong, and L. S. Premkumar, "Streptozotocin-induced early thermal hyperalgesia is independent of glycemic state of rats: role of transient receptor potential vanilloid 1(TRPV1) and inflammatory mediators," *Molecular Pain*, vol. 7, article 52, 2011.

[30] U. Jung and K. Ley, "Mice lacking two or all three selectins demonstrate overlapping and distinct functions for each selectin," *Journal of Immunology*, vol. 162, no. 11, pp. 6755–6762, 1999.

[31] C. Morgado, P. Pereira-Terra, C. D. Cruz, and I. Tavares, "Minocycline completely reverses mechanical hyperalgesia in diabetic rats through microglia-induced changes in the expression of the potassium chloride co-transporter 2 (KCC2) at the spinal cord," *Diabetes, Obesity and Metabolism*, vol. 13, no. 2, pp. 150–159, 2011.

[32] M. Calvo and D. L. H. Bennett, "The mechanisms of microgliosis and pain following peripheral nerve injury," *Experimental Neurology*, vol. 234, no. 2, pp. 271–282, 2012.

[33] J. Blackbeard, V. C. J. Wallace, K. P. O'Dea et al., "The correlation between pain-related behaviour and spinal microgliosis in four distinct models of peripheral neuropathy," *European Journal of Pain*, vol. 16, no. 10, pp. 1357–1367, 2012.

[34] W. Huang, M. Calvo, K. Karu et al., "A clinically relevant rodent model of the HIV antiretroviral drug stavudine induced painful peripheral neuropathy," *Pain*, vol. 154, no. 4, pp. 560–575, 2013.

[35] K. Rzeniewicz, A. Newe, A. Rey Gallardo et al., "L-selectin shedding is activated specifically within transmigrating pseudopods of monocytes to regulate cell polarity in vitro," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 112, no. 12, pp. E1461–E1470, 2015.

[36] J. A. Smith, "Neutrophils, host defense, and inflammation: a double-edged sword," *Journal of Leukocyte Biology*, vol. 56, no. 6, pp. 672–686, 1994.

[37] H. L. Wright, R. J. Moots, R. C. Bucknall, and S. W. Edwards, "Neutrophil function in inflammation and inflammatory diseases," *Rheumatology*, vol. 49, no. 9, pp. 1618–1631, 2010.

[38] L. R. Watkins and S. F. Maier, "Beyond neurons: evidence that immune and glial cells contribute to pathological pain states," *Physiological Reviews*, vol. 82, no. 4, pp. 981–1011, 2002.

[39] E. D. Means and D. K. Anderson, "Neuronophagia by leukocytes in experimental spinalcord injury," *Journal of Neuropathology & Experimental Neurology*, vol. 42, no. 6, pp. 707–719, 1983.

[40] F. Bao, J. C. Fleming, R. Golshani et al., "A selective phosphodiesterase-4 inhibitor reduces leukocyte infiltration, oxidative processes, and tissue damage after spinal cord injury," *Journal of Neurotrauma*, vol. 28, no. 6, pp. 1035–1049, 2011.

[41] A. Bierhaus, T. Fleming, S. Stoyanov et al., "Methylglyoxal modification of Nav1.8 facilitates nociceptive neuron firing and causes hyperalgesia in diabetic neuropathy," *Nature Medicine*, vol. 18, pp. 926–933, 2012.

[42] Y. Su, X. Lei, L. Wu, and L. Liu, "The role of endothelial cell adhesion molecules P-selectin, E-selectin and intercellular adhesion molecule-1 in leucocyte recruitment induced by exogenous methylglyoxal," *Immunology*, vol. 137, no. 1, pp. 65–79, 2012.

[43] J. Yellowlees Douglas, A. D. Bhatwadekar, S. Li Calzi et al., "Bone marrow-CNS connections: implications in the pathogenesis of diabetic retinopathy," *Progress in Retinal and Eye Research*, vol. 31, no. 5, pp. 481–494, 2012.

[44] A. M. Joussen, T. Murata, A. Tsujikawa, B. Kirchhof, S.-E. Bursell, and A. P. Adamis, "Leukocyte-mediated endothelial cell injury and death in the diabetic retina," *The American Journal of Pathology*, vol. 158, no. 1, pp. 147–152, 2001.

[45] R. Mohamed, C. Jayakumar, P. V. Ranganathan, V. Ganapathy, and G. Ramesh, "Kidney proximal tubular epithelial-specific overexpression of netrin-1 suppresses inflammation and albuminuria through suppression of COX-2-mediated PGE2 production in streptozotocin-induced diabetic mice," *The American Journal of Pathology*, vol. 181, no. 6, pp. 1991–2002, 2012.

[46] W. Xiao, A. Boroujerdi, G. J. Bennett, and Z. D. Luo, "Chemotherapy-evoked painful peripheral neuropathy: analgesic effects of gabapentin and effects on expression of the alpha-2-delta type-1 calcium channel subunit," *Neuroscience*, vol. 144, no. 2, pp. 714–720, 2007.

[47] J. M. Dias, T. V. de Brito, D. de Aguiar Magalhães et al., "Gabapentin, a synthetic analogue of gamma aminobutyric acid, reverses systemic acute inflammation and oxidative stress in mice," *Inflammation*, vol. 37, no. 5, pp. 1826–1836, 2014.

[48] N. M. Perkins and D. J. Tracey, "Hyperalgesia due to nerve injury: role of neutrophils," *Neuroscience*, vol. 101, no. 3, pp. 745–757, 2000.

Hindawi
Journal of Diabetes Research
Volume 2017, Article ID 6943851, 9 pages
https://doi.org/10.1155/2017/6943851

Review Article

Lipids: A Suitable Therapeutic Target in Diabetic Neuropathy?

M. C. Perez-Matos,[1] M. C. Morales-Alvarez,[1] and C. O. Mendivil[1,2]

[1]*School of Medicine, Universidad de Los Andes, Bogotá, Colombia*
[2]*Fundación Santa Fe de Bogotá, Department of Internal Medicine, Section of Endocrinology, Bogotá, Colombia*

Correspondence should be addressed to C. O. Mendivil; carlosolimpo@gmail.com

Received 4 October 2016; Revised 23 November 2016; Accepted 13 December 2016; Published 16 January 2017

Academic Editor: Mitra Tavakoli

Diabetic polyneuropathy (DPN) encompasses multiple syndromes with a common pathogenesis. Glycemic control shows a limited correlation with DPN, arguing in favor of major involvement of other factors, one of which is alterations of lipid and lipoprotein metabolism. Consistent associations have been found between plasma triglycerides/remnant lipoproteins and the risk of DPN. Studies in cultured nerve tissue or in murine models of diabetes have unveiled mechanisms linking lipid metabolism to DPN. Deficient insulin action increases fatty acids flux to nerve cells, inducing mitochondrial dysfunction, anomalous protein kinase C signaling, and perturbations in the physicochemical properties of the plasma membrane. Oxidized low-density lipoproteins bind to cellular receptors and promote generation of reactive oxygen species, worsening mitochondrial function and altering the electrical properties of neurons. Supplementation with specific fatty acids has led to prevention or reversal of different modalities of DPN in animal models. Post hoc and secondary analyses of clinical trials have found benefits of cholesterol reducing (statins and ezetimibe), triglyceride-reducing (fibrates), or lipid antioxidant (thioctic acid) therapies over the progression and severity of DPN. However, these findings are mostly hypothesis-generating. Randomized trials are warranted in which the impact of intensive plasma lipids normalization on DPN outcomes is specifically evaluated.

1. Introduction

Diabetic neuropathy is a frequent and serious complication of both type 1 (DM1) and type 2 (DM2) diabetes. In patients with DM2, the prevalence of diabetic neuropathy has been estimated at 20–40% in different populations [1–3]. Diabetic neuropathy is a progressive, debilitating condition with a major impact on patient morbidity, mortality, and quality of life. There are five types of neurological syndromes related to diabetes mellitus: distal symmetric polyneuropathy (most frequent), autonomic neuropathy, small-fiber neuropathy (earliest), polyradiculopathy, and mononeuropathies [4, 5]. Despite important advances, results from observational studies and clinical trials suggest that other factors besides glycaemia play a large role in this particular complication.

2. Glycemic Control Is Not the Only Determinant of Diabetic Neuropathy

In the Diabetes Control and Complications Trials (DCCT), patients randomized to the intensive control arm achieved an HbA1c 1.8% lower than the conventional treatment arm after a follow-up period of 6.5 years and developed 69% less distal symmetrical polyneuropathy (DSP) (defined as DSP on physical examination plus abnormal nerve conduction in 2 different nerves or unequivocally abnormal autonomic test results) [6]. In the Epidemiology of Diabetes Intervention and Complications (EDIC) study, the original cohort of DCCT was followed observationally for another 8 years. The HbA1c difference between groups had entirely dissipated (8.0% prior intensive group versus 7.9% prior conventional therapy group) [7], yet the difference in diabetic polyneuropathy (DPN) incidence persisted (cumulative incidence 7% in the intensive group versus 3.5% control group). Furthermore, the NeuroEDIC study extended this follow-up for up to 14 years after the DCCT closure, and the between-group difference in the risk for neuropathy not only persisted but widened (25% in the former intensive group versus 35% in the former control group, $p < 0.001$) [8]. So the relevance of glycemic control in the progression of DPN in DM1 is paramount.

The Kumamoto and the Action to Control Cardiovascular Risk in Diabetes (ACCORD) trials found similar results

in patients with DM2. In the Kumamoto study, patients treated with multiple insulin therapy (MIT) (3 or more daily administrations) achieved better glycemic control than those under conventional insulin therapy (HbA1c 7.1% MIT group versus 9.4% conventional therapy, $p < 0.05$). This better glycemic control translated into less nerve damage after 6 years, with a small but significant difference (median nerve conduction velocity [NCV] 53.2 m/s in MIT versus 50.2 m/s in conventional group, $p < 0.05$) [9]. Similarly, in the glycemic component of the ACCORD trial, patients originally randomized to strict glycemic control (HbA1c at glycemic component discontinuation 6.4%) had a slower progression of DPN versus the standard treatment group (HbA1c 7.5%) (hazard ratio [HR] for loss of ankle jerk at study end 0.90, 95% CI: 0.84–0.97, $p = 0.005$) [10].

Nonetheless, not all outcome studies in DM2 have found a significant impact of glycemic control on neuropathy. A very large difference in final HbA1c (8.4% in control group versus 6.9% in intensive group) had no impact on the cumulative incidence of any type of neuropathy in the Veterans Affairs Diabetes Trial (VADT) (43.5% control group, 43.8% intensive group) [11]. The United Kingdom Prospective Diabetes Study (UKPDS) of intensive treatment with sulphonylureas or insulin versus standard therapy in patients with DM2 produced comparable findings. Despite better HbA1c control (7.0% in intensive arm versus 7.9% in standard arm, $p < 0.001$), incidence of DSP measured by absent ankle reflexes did not differ between groups (35% in the intensive treatment group versus 37% in the standard treatment group, $p = 0.60$) [12].

Finally, the Action in Diabetes and Cardiovascular Disease: Preterax and Diamicron Modified Release Controlled Evaluation (ADVANCE) study showed a benefic effect of intensive glycemic control on nephropathy (HR 0.79, IC 95%: 0.66–0.93) but no significant effect on either retinopathy or neuropathy in patients with DM2 [13]. Two recent large cardiovascular outcome trials in patients with DM2 (LEADER with the Glucagon Like Peptide-1 [GLP-1] agonist liraglutide and EMPA-REG with the Sodium-Glucose Cotransporter-2 [SGLT-2] inhibitor empagliflozin) found significant reductions of cardiovascular risk with newer oral antidiabetic therapies but did not report on neuropathy endpoints [14, 15].

So hyperglycemia does not seem to be the sole factor explaining the appearance and progression of DPN, and the effect of glycemic control on the improvement of DPN appears to be variable, particularly among patients with DM2.

Diverse pathophysiological mechanisms have been implicated in the development and progression of DPN. The high oxidative stress characteristic of hyperglycemia exerts injury to nerve cells through lipid peroxidation, direct damage to DNA with pathological activation of repair pathways, depletion of cellular antioxidants, and induction of proinflammatory transcription factors [16]. Another pathway leading from hyperglycemia to DPN entails the activation of the intracellular enzyme aldose reductase, which transforms glucose that has not been oxidized via glycolysis into sorbitol and fructose. This so-called polyol pathway leads to the intracellular accumulation of osmotically active sorbitol, causing cellular edema and loss of important metabolic mediators like taurine, myoinositol, and adenosine. Also, the reaction catalyzed by aldose reductase utilizes NADPH, so this pathway depletes the cell of NADPH, necessary for the regeneration of glutathione, the main defense against oxidative damage [17]. Local alterations of nociceptors and neural growth factors (neurotrophins) also play a role, especially in painful DPN. Chronic and continuous stimulation of the nociceptor transient receptor potential cation channel subfamily V member 1 (TRPV-1) in early DPN leads to local release of various growth factors, importantly NGF (nerve growth factor) and brain-derived neurotrophic factor (BDNF). This creates a feedforward loop in which NGF binds to the trkA receptor, which lowers the threshold for TRPV-1, leading to further sensitization and pain and to further NGF release [18]. The activation of certain isoforms of protein kinase C is characteristic of diabetic complications, and it is presumed to be involved in DPN as well [19]. Protein kinase C is a second messenger kinase that activates nuclear factor kappa-B (NF kappa-B) and other proinflammatory transcription factors.

The hexosamine pathway may also contribute to diabetic neuropathy. When cells have a high glucose influx, some of the fructose-6 phosphate in the glycolytic pathway is diverted by glutamine:fructose-6-phosphate transferase to glucosamine-6 phosphate. This hexosamine is used to produce UDP-N-acetyl glucosamine (UDP-GlcNAc). UDP-GlcNAc is then enzymatically added to the serine and threonine residues of multiple transcription factors, modifying their activity. Involvement of this pathway has been clearly demonstrated for other diabetic complications [20], but its role in DPN is less clear. Yet another plausible mechanism of nerve dysfunction in diabetes involves the nonenzymatic glycation of cellular proteins. Chronic elevation of glucose in the cellular milieu facilitates the formation of advanced glycation end-products (AGEs), which directly hinder the function of multiple essential cellular and extracellular proteins (tubulin, actin, and laminin). AGEs also bind to and activate a specific receptor (the receptor for AGEs or RAGE), inducing a proinflammatory, prooxidative transcriptional program in peripheral nerves [21].

However, despite these well-known pathogenic mechanisms of glucose burden on DPN, the same has not been consistently replicated in various clinical trials as mentioned. This suggests the role of additional factors which might influence the appearance and progression of DPN. Here, we propose that alterations of lipid metabolism (which are very frequent in patients with DM2 and/or the metabolic syndrome) participate in several key pathways of DPN pathogenesis and that normalization of lipid metabolism may constitute an appealing target for the prevention or treatment of DPN.

3. Plasma Lipids Are Associated with Progressive DPN

In several large observational studies, an interesting observation has been the baseline between-group differences in lipid profile in patients with DM2 who go on to develop DPN

and those who do not. In the European Diabetes Prospective Complications (EURODIAB) study of patients with DM1, total cholesterol (TC), LDL cholesterol (LDLc), and TG levels were significantly associated with incident DPN over a 7.3-year follow-up (Odds Ratio [OR] 1.26, $p = 0.001$; OR: 1.22, $p = 0.02$; and OR 1.35, $p < 0.001$, resp.), even after adjustment for baseline HbA1c and diabetes duration [22]. Concordantly, in a 52-week prospective study of patients with DM2, plasma triglycerides (TG) were associated with progressive DPN, defined as a loss of more than 500 fibers/mm^2 in a sural nerve biopsy ($p = 0.04$ for plasma TG difference between progressors and nonprogressors) [23]. Likewise, the Utah Diabetic Neuropathy study found an association between plasma triglycerides ≥150 mg/dl and the risk of DPN at entry in patients with DM2 (relative risk [RR]: 2.3, 95% CI: 1.1–4.7) [24]. Also, LDL particle size as a marker of atherogenic dyslipidemia appears to be an independent risk factor for neuropathy [25], and patients with mixed dyslipidemia have been shown to exhibit prolonged cutaneous silent period latency, a measure of small-fiber neuropathy [26].

4. Mechanisms Linking Lipids to Diabetic Neuropathy

4.1. Peripheral Nerves Are Affected by Insulin Resistance. Even though glucose uptake in the nervous system is largely insulin-independent, there is evidence of insulin signaling in peripheral nerves [27]. Insulin signaling in peripheral neurons proceeds in a way analog to that in other cells, with successive phosphorylation of the insulin receptor itself, then the insulin receptor substrate 2 (IRS-2), phosphoinositide 3-kinase (PI3K), phosphoinositide-dependent kinase-1 (PDK1), and subsequently protein kinase B (PKB/Akt) [28]. Direct insulin administration of insulin at doses insufficient to change plasma glucose was able to prevent and reverse features of diabetic neuropathy (motor conduction velocities and axonal atrophy) in the sural nerves of streptozotocin-induced diabetic mice [29]. Studies in obese diabetic ob/ob mice have demonstrated a lack of PKB/Akt activation in peripheral nerves in response to direct (intrathecal) administration of insulin [30]. Hyperglycemia may directly affect the neural response to insulin. In vitro studies of the impact of insulin on the nerve action potential under normal or high glucose conditions have found that hyperglycemia prolongs the action potential, an effect that is abolished by insulin [31]. However, under normoglycemic conditions the effect of insulin was to reduce the conduction velocity of oxygenated nerves. Furthermore, in vitro studies have shown that continuous exposure to high insulin concentrations abolishes the ability of acute insulin exposure to activate the Akt signaling pathway in dorsal root ganglion neurons [32]. Thus, the hallmarks of molecular resistance to insulin action in other tissues (adipose and liver) are also present in nerve tissue. Human patients with the metabolic syndrome are characterized by insulin resistance and a chronic low-degree inflammation status [33, 34]. In these patients, insulin resistance assessed through the homeostatic model assessment–insulin resistance (HOMA-IR) index has shown a positive and independent association with clinical scores of peripheral neuropathy (Odds Ratio: 1.2 per unit, 95% CI: 1.1–1.4) [35].

4.2. Free Fatty Acids Mediate Insulin Resistance and Dysfunction in Peripheral Nerves. High plasma levels of free fatty acids (FFA) are a hallmark of insulin resistance. Decreased inhibition of adipocyte hormone-sensitive lipase due to insulin resistance leads to a continuous release of FFA [36]. FFA in turn perpetuate and worsen insulin resistance in adipose and other tissues by inducing intracellular formation of diacylglycerol and ceramides that activate protein kinase C-theta and delta isoforms (PKC-theta and PKC-delta) and serine-threonine kinases that phosphorylate IRS and reduce their signaling capacity [37]. On the other hand, the phospholipid bilayer of cells from healthy patients is characterized by a high concentration of polyunsaturated fatty acids (PUFA), a composition that facilitates insertion of membrane receptors and transporters and uptake of external substrates. In DM2, increased FFA lead to high cytoplasmic saturated fatty acyl-CoA, which allosterically inhibits fatty acid desaturases and reduces synthesis of PUFA [38]. Under these circumstances, membrane flexibility decreases and multiple functions associated with electrical conduction and signal transduction may become affected [39]. A rigid membrane increases oxidative stress and further induces insulin resistance by its limited capability glucose transporter (GLUT) expression. High intracellular saturated FFA levels also activate nuclear factor kappa-B (NF-kB) signaling by directly stimulating expression of the p65 subunit of NF-kB [40]. This pathway raises production of reactive oxygen species (ROS) and promotes oxidative stress, which is a central factor in the appearance and progression of DPN [41].

In streptozotocin diabetic rats, 5 weeks of supplementation with PUFAs gamma-linolenic (omega 6) and eicosapentaenoic (omega 3) acids led to a significant decrease in the progression of DPN measured as sensitive and motor NCV [42]. A multicenter clinical trial revealed a significant improvement of 13 DPN parameters (including conduction velocities, thermal sensitivity, and tendon reflexes) in DM2 patients supplemented with gamma-linolenic acid for 1 year [43]. A recent study focused on the causality of the association between FFA and DPN. Patients with DM2 received an intralipid and heparin infusion to intentionally raise FFA levels and had their heart rate variability measured by spectral analysis for 3 hours. Plasma FFA correlated positively with the low frequency/high frequency variability ratio (higher values indicate lower heart rate variability) ($r = 0.57$, $p < 0.02$). After three months of good glycemic control, when circulating FFA had dropped to normal levels, heart rate variability measures also returned to normal [44].

4.3. Imbalance of Mitochondrial Bioenergetics Further Mediates Neuropathy. Cellular energy metabolism is centered at the mitochondria, which is consequently the main site of reactive oxygen species (ROS) generation. In neurons and glial cells, a dysregulation of mitochondrial bioenergetics as seen in DM2 has been associated with abnormal increases in mitochondrial fission and biogenesis [45]. Mitochondria

shift their balance from fatty acid biosynthesis towards continued oxidation, using for this purpose most available acyl-carnitines and depleting a key substrate for myelin lipid biosynthesis [46]. Derangement of substrate utilization may lead to increased production of mitochondrial ROS, release of cytochrome C, and activation of proapoptotic pathways leading to neuronal damage [46, 47].

Transcriptional, proteomic, and functional changes indicative of altered mitochondrial substrate utilization associated with greater ROS generation and less respiratory capacity in the context of insulin-resistant diabetes have been detected in heart [48], skeletal muscle [49, 50], and sensory neurons [51].

FFA have the ability to directly inhibit the respiratory chain [52–54], a property that has been demonstrated in Schwann cells in vitro [55]. A study in streptozotocin diabetic rats found that insulin doses insufficient to induce changes in plasma glucose were still able to normalize rates of mitochondrial coupled respiration in cells from dorsal root ganglia [56]. Murine models of DM2 display reduced glycolytic intermediaries in peripheral nerves and dorsal root ganglia, in association with increased oxidative damage of proteins and lipids [57]. These changes appear to affect first neurons from longer peripheral nerves, like the sciatic nerve [58]. Mechanistically, AMP-activated protein kinase (AMPK) and peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1 alpha) are "central hubs" of energy metabolism [59, 60] that appear to be involved in the pathway from fatty acids to mitochondrial dysfunction and DPN. A high-fat diet increases mitochondrial concentrations of fatty acid oxidation intermediaries and decreases PGC-1 alpha expression in skeletal muscle [61]. A cross-sectional study comparing gene expression patterns in skeletal muscle biopsies from patients with insulin resistance, patients with DM2, and controls found a significant downregulation of PGC-1-responsive genes involved in mitochondrial ATP production in the first two groups [62]. The expression of PGC-1 and nuclear respiratory factor-1 (NRF-1) responsive oxidative metabolism genes is reduced in muscle tissue of patients with DM2 and in normoglycemic relatives of patients with diabetes [50]. Interestingly, stimulation of AMPK signaling has improved neuropathic manifestations like thermal hypoalgesia in a rodent model of diabetic neuropathy [63]. Administration of troglitazone (a PPAR-gamma agonist) to diabetic obese rats improved NCV [64].

4.4. Oxidized Lipids May Promote DPN. Increased LDL cholesterol and TG levels have been shown to be associated with a faster progression to end-stage renal disease, blindness and peripheral neuropathy in patients with DM2 [65]. The mechanism behind the LDL-DPN relationship is thought to reside in increased oxidative environment, as explained above. In fact, oxidation of LDL cholesterol is increased in patients with diabetes compared to healthy controls [66], resulting in a proinflammatory state. Dorsal root ganglia express the lectin-like oxLDL receptor (LOX-1). When oxidized LDL (oxLDL) bind to this receptor, a signaling pathway is activated that increases ROS and oxidative stress. The same process occurs in the nerve roots of patients with DPN, particularly via activation of NADPH oxidase, before a significant impairment of glycemia becomes evident [67].

4.5. Atypical Sphingolipids, Another Metabolic and Neurotoxic Link? Sphingolipids are a class of naturally occurring lipids made by subsequent modifications of a sphingoid base, mostly sphingosine [68]. The rate-limiting step in their synthesis is the condensation of L-serine and palmitoyl-CoA, catalyzed by the enzyme serine-palmitoyl transferase (SPT) [69]. Complex lipids from this group such as ceramide and sphingomyelin are involved in cell structure and signaling [68]. Deoxy-sphingolipids (DOSL) are atypical sphingolipids characterized by the lack of an OH group in C1. Several DOSL display neurotoxic activity [70]. DOSL are produced when SPT activity is altered and it uses L-alanine or glycine instead of serine as amino acid substrate [68]. As serine and alanine are involved in carbohydrate metabolism, it is believed that DOSL synthesis is a metabolic intersection between lipid, carbohydrate pathways, and oxidative stress [71], especially in patients with DM2 [72].

Observational studies have demonstrated that DOSL levels are increased in patients with metabolic syndrome and/or DM2. A study comparing the sphingolipid profile of patients with DM1, DM2, and controls found increased levels of DOSL in patients with DM2 (0.05, 0.09, and 0.05 arbitrary units, resp.) [71]. In a case-control study, patients with DM2 also had higher DSOL plasma levels compared to controls (0.19 microM and 0.12 microM, resp., $p = 0.005$) [72]. Plasma sphingolipid profiling of patients with DPN compared to other types of neuropathy and patients without neuropathy reveals increased atypical sphingolipids (0.11 microM DPN versus 0.06 microM controls, $p < 0.001$) [73]. In a subgroup study from EDIC, patients who reported neuropathy at any point of follow-up exhibited higher deoxy-ceramide levels than those without neuropathy (12.3 versus 10.6, $p = 0.049$ units/curve area) [74]. A pilot model with diabetic rats demonstrated that intentionally lowering plasma DOSL may improve neuropathy measures like mechanical sensitivity and NCV [75]. In a trial comparing treatment with fenofibrate versus niacin for 6 weeks in patients with primary hypercholesterolemia or mixed dyslipidemia, fenofibrate effectively lowered atypical sphingolipids (0.13 microM before, 0.09 microM after treatment, $p \leq 0.001$) [76], which suggests that PPAR-alpha agonists may provide a positive impact on DPN (see below). The mechanism of DOSL-induced neurotoxicity remains to be elucidated.

The mechanisms linking deranged lipid metabolism to DPN are summarized in Figure 1.

5. Treatment of Dyslipidemia: Its Impact on DPN

5.1. Triglyceride-Reducing Therapy. Fibrates are a class of lipid-lowering therapies with demonstrated efficacy at reducing TG and increasing HDLc in patients with DM2. Recent evidence suggests a positive effect of fibrates on DPN progression. In a report from the Fremantle Diabetes Study

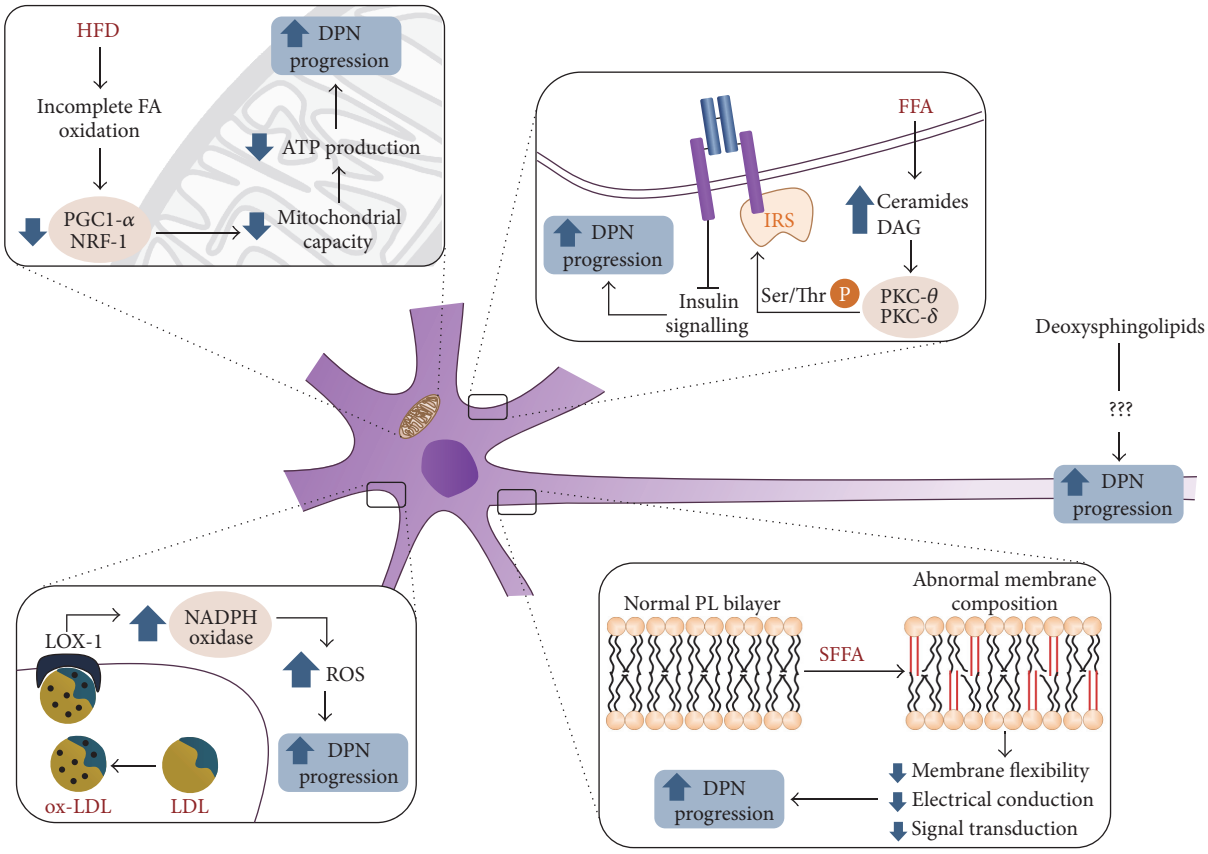


Figure 1: Pathogenic mechanisms linking abnormal lipid metabolism to progression of diabetic neuropathy. HFD: high-fat diet, FA: fatty acids, PGC-1alpha: PPAR-gamma coactivator 1-alpha, NRF-1: nuclear respiratory factor-1, DPN: diabetic polyneuropathy, FFA: free fatty acids, IRS: insulin receptor substrates, PKC-theta: protein kinase C, theta isoform, PKC-delta: protein kinase C, delta isoform, Ser/ThrP: phosphorylation in serine or threonine, ox-LDL: oxidized LDL, LOX-1: lectin-like oxidized LDL receptor, NADPH oxidase: reduced nicotinamide-adenine dinucleotide phosphate oxidase, PL: phospholipid, and SFFA: saturated free fatty acids. Insulin resistance or a high-fat diet increase the cellular supply of FFA, leading to decreased expression of PGC-1alpha and NRF1-alpha-responsive genes and subsequently to impaired mitochondrial capacity and nerve dysfunction. Increased supply of FFA also causes uncontrolled formation of DAG and ceramides, which activate atypical PKC isoforms and promote serine/threonine phosphorylation of IRS, decreased insulin signaling, and defective nerve growth and repair. The augmented availability of SFFA in insulin resistance leads to changes in the fatty acid composition of plasma membrane phospholipids. Membranes richer in saturated FA are more rigid and exhibit disturbances of electrical conduction and a reduced capacity for receptor expression and signal transduction, all of which worsen DPN. Accelerated ROS production in diabetes generates oxLDL that bind to the LOX-1 receptor and activate NADPH oxidase, worsening ROS production even further and hastening the progression of DPN. Finally, oxidized deoxysphingolipids are neurotoxic lipids associated with DPN, but their mechanism of action is still unknown.

that included 531 patients with DM2 followed for 5 years using either statins or fibrates as lipid-lowering therapy, treatment with fenofibrate was associated with a significant decrease in the appearance of neuropathy (measured by the Michigan Neuropathy Scoring Instrument (MNSI) [HR 0.52, $p = 0.042$]) [77]. In the Fenofibrate and Event-Lowering in Diabetes (FIELD) study of 9795 patients with DM2 who were randomized to fenofibrate or placebo, the fenofibrate group had a significantly lower rate of nontraumatic amputations (HR 0.62, $p = 0.011$) [78]. Mechanistic studies in obese db/db mice have found that fenofibrate markedly activates the above-mentioned PPARalpha-AMPK-PGC1 pathway in the sciatic nerve, while improving the animals' tactile threshold [79].

Omega-3 fatty acids are essential polyunsaturated fatty acids, a group that includes eicosapentaenoic and docosahexaenoic acids (DHA). In patients with DM2, plasma levels of omega-3 acids correlate negatively with insulin resistance and dyslipidemia [80]. Experimentally, an increased production of omega-3 in a diabetic mice model confers resistance to diet induced obesity and diabetes [81]. Supplementation with fish oil containing DHA completely prevented the development of neuropathy in streptozotocin-induced diabetic mice [82] and led to preservation of NCV and Na+/K+ ATPase activity in sural nerve of streptozotocin-induced diabetic rats [83]. The polyunsaturated and anti-inflammatory nature of omega-3 may be key to these effects against diabetes-induced nerve dysfunction.

5.2. Cholesterol-Lowering Therapy. Statins are the cornerstone of hypercholesterolemia management. By inhibiting the rate-limiting enzyme in the cholesterol biosynthesis pathway (conversion of hydroxymethylglutaryl CoA to mevalonate), they also prevent the formation of isoprenoid intermediaries like isopentenyl-pyrophosphate, dimethylallyl-pyrophosphate, geranyl-pyrophosphate, and farnesyl-pyrophosphate. Isoprenoids play an important role in the posttranslational modification and membrane attachment of multiple signaling molecules, among them GTP-binding proteins of the Ras and Rho family. Therefore changes in the availability of farnesyl-PP (associated with Ras proteins) or geranyl-PP (associated with Rho proteins) affect a great number of cellular processes beyond cholesterol production [84]. Streptozotocin-induced diabetic mice showed normalization of their NCV of the saphenous and sciatic nerves after 2 weeks of treatment with 0.3–20 mg/kg of rosuvastatin and a normalization of thermal hyperalgesia with the 20 mg/kg dose. These results indicated improvement in both large and small nerve fibers. The complete reversal of these effects with mevalonate supplementation implies that they were mediated by reduced production of isoprenoid precursors [85].

There is also evidence of DPN improvement with cholesterol-lowering therapies such as statins or ezetimibe in clinical studies. In the Fremantle Diabetes Study, patients with DM2 treated with statins evidenced a 35% reduction in the incidence of DPN [77]. A recent study demonstrated that patients with DM2 treated with simvastatin + ezetimibe or rosuvastatin had lower lipid peroxidation (LPO) markers versus placebo and a significant reduction in the Neuropathy Symptoms Score from baseline [86], lending further support for this pathway as a pharmacological target in DPN.

5.3. Lipoic Acid as a DPN Therapy. In the context previously described, current approaches for DPN therapy include molecules with antioxidant properties [87]. Lipoic acid or thioctic acid (LA) is an octanoic acid derivative that has been used for symptomatic relief in diabetic polyneuropathy with positive results. Three pathways may explain its effect: (1) LA has the capacity to directly scavenge reactive oxygen species; (2) LA regenerates endogenous antioxidants (glutathione, vitamin E, vitamin C, and coenzyme Q); and (3) LA has metal chelating activity over iron and copper. Several clinical trials have provided evidence of the efficacy of LA against neuropathy in patients with DM1 and DM2. A recent meta-analysis of 15 randomized controlled trials evaluating the efficacy of LA administration on improvement of objective DSP measures found a positive effect on peripheral NCVs with the 300–600 mg i.v. dose for at least 3 weeks (OR 4.03, 95% IC 2.73–5.94), with no significant adverse effects [88].

6. Summary/Conclusion

DPN is a frequent, serious, and debilitating chronic complication of diabetes mellitus. Despite its relevance, very little is known about the details of its molecular pathogenesis and consequently the availability of targeted, efficacious therapies is limited. Alterations in the metabolism of lipids including triglycerides, cholesterol, fatty acids, and sphingolipids have been implicated in the pathogenesis of DPN and constitute an interesting molecular target for the treatment of clinical DPN. However, most of the available evidence in this respect is mechanistic (i.e., animal on in vitro studies) or from observational human studies.

The evidence from secondary or post hoc analysis of randomized trials is limited by patient heterogeneity, variations in dose and follow-up duration, and particularly the methodology used to define DPN. Most studies have used sign-driven scales (like the Michigan Neuropathy Score), symptom-driven scales (like the Total Symptom Score), or vibration perception thresholds in an attempt to make DPN a measurable, comparable variable, but only a few have measured NCV, a truly objective measure of nerve functionality. Furthermore, it is known that small-fiber neuropathy, the earliest manifestation of DPN, can be missed by all these methodologies. For that reason a group of new techniques for DPN diagnosis have come into place, including corneal confocal microscopy, laser Doppler image flare, sudomotor reflex assessment, quantitative sensory testing, and skin biopsy [89]. These small-fiber neuropathy detection tools should be incorporated into endpoint ascertainment in future studies of lipids and DPN.

In summary, DPN is a complex and multifactorial entity in which various factors besides hyperglycemia play an important role. There is a host of indirect evidence showing that deranged lipid metabolism at the cellular and whole-organism level aggravates or perpetuates DPN, and mitigation of such alterations improves DPN in animal models of diabetes.

In consonance with these observations, clinical trials in which lipid-modifying therapies have been assessed for their impact on cardiovascular morbidity and mortality have shown as descriptive findings positive effects on DPN, but the available evidence is insufficient to solidly implicate lipids as a pharmacological target in DPN.

Future research should concentrate on targeting lipids with one or more aggressive interventions specifically in patients with DM2 whose DPN is detectable but whose progression can still be largely prevented. Such studies could have selection criteria focused on the presence and severity of DPN instead of plasma lipid concentrations. Until then, careful control and follow-up of plasma lipids in patients with diabetes can only be considered an adjunct strategy against DPN.

Competing Interests

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

References

[1] M. Davies, S. Brophy, R. Williams, and A. Taylor, "The prevalence, severity, and impact of painful diabetic peripheral neuropathy in type 2 diabetes," *Diabetes Care*, vol. 29, no. 7, pp. 1518–1522, 2006.

[2] C. A. Abbott, R. A. Malik, E. R. E. Van Ross, J. Kulkarni, and A. J. M. Boulton, "Prevalence and characteristics of painful diabetic neuropathy in a large community-based diabetic population in the U.K.," *Diabetes Care*, vol. 34, no. 10, pp. 2220–2224, 2011.

[3] S. Tesfaye, A. J. M. Boulton, and A. H. Dickenson, "Mechanisms and management of diabetic painful distal symmetrical polyneuropathy," *Diabetes Care*, vol. 36, no. 9, pp. 2456–2465, 2013.

[4] S. Tesfaye, A. J. M. Boulton, P. J. Dyck et al., "Diabetic neuropathies: update on definitions, diagnostic criteria, estimation of severity, and treatments," *Diabetes Care*, vol. 33, no. 10, pp. 2285–2293, 2010.

[5] A. C. Themistocleous, J. D. Ramirez, J. Serra, and D. L. H. Bennett, "The clinical approach to small fiber neuropathy and painful channelopathy," *Practical Neurology*, vol. 14, no. 6, pp. 368–379, 2014.

[6] The Diabetes Control and Complications Trial Research Group, "The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus," *New England Journal of Medicine*, vol. 329, no. 14, pp. 977–986, 1993.

[7] C. L. Martin, J. Albers, W. H. Herman et al., "Neuropathy among the diabetes control and complications trial cohort 8 years after trial completion," *Diabetes Care*, vol. 29, no. 2, pp. 340–344, 2006.

[8] J. W. Albers, W. H. Herman, R. Pop-Busui et al., "Effect of prior intensive insulin treatment during the Diabetes Control and Complications Trial (DCCT) on peripheral neuropathy in type 1 diabetes during the Epidemiology of Diabetes Interventions and Complications (EDIC) study," *Diabetes Care*, vol. 33, no. 5, pp. 1090–1096, 2010.

[9] Y. Ohkubo, H. Kishikawa, E. Araki et al., "Intensive insulin therapy prevents the progression of diabetic microvascular complications in Japanese patients with non-insulin-dependent diabetes mellitus: a randomized prospective 6-year study," *Diabetes Research and Clinical Practice*, vol. 28, no. 2, pp. 103–117, 1995.

[10] F. Ismail-Beigi, T. Craven, M. A. Banerji et al., "Effect of intensive treatment of hyperglycaemia on microvascular outcomes in type 2 diabetes: an analysis of the ACCORD randomised trial," *The Lancet*, vol. 376, no. 9739, pp. 419–430, 2010.

[11] W. Duckworth, C. Abraira, T. Moritz et al., "Glucose control and vascular complications in veterans with type 2 diabetes," *New England Journal of Medicine*, vol. 360, no. 2, pp. 129–139, 2009.

[12] R. Turner, "Intensive blood-glucose control with sulphonylureas or insulin compared with conventional treatment and risk of complications in patients with type 2 diabetes (UKPDS 33)," *The Lancet*, vol. 352, no. 9131, pp. 837–853, 1998.

[13] ADVANCE Collaborative Group, A. Patel, S. MacMahon et al., "Intensive blood glucose control and vascular outcomes in patients with type 2 diabetes," *The New England Journal of Medicine*, vol. 358, pp. 2560–2572, 2008.

[14] S. P. Marso, G. H. Daniels, K. B. Frandsen et al., "Liraglutide and cardiovascular outcomes in type 2 diabetes," *New England Journal of Medicine*, vol. 375, no. 4, pp. 311–322, 2016.

[15] B. Zinman, C. Wanner, J. M. Lachin et al., "Empagliflozin, cardiovascular outcomes, and mortality in type 2 diabetes," *The New England Journal of Medicine*, vol. 373, no. 22, pp. 2117–2128, 2015.

[16] G. Negi, A. Kumar, R. P. Joshi, and S. S. Sharma, "Oxidative stress and Nrf2 in the pathophysiology of diabetic neuropathy: old perspective with a new angle," *Biochemical and Biophysical Research Communications*, vol. 408, no. 1, pp. 1–5, 2011.

[17] A. B. Oyenihi, A. O. Ayeleso, E. Mukwevho, and B. Masola, "Antioxidant strategies in the management of diabetic neuropathy," *BioMed Research International*, vol. 2015, Article ID 515042, 15 pages, 2015.

[18] M. Greig, S. Tesfaye, D. Selvarajah, and I. D. Wilkinson, "Insights into the pathogenesis and treatment of painful diabetic neuropathy," *Handbook of Clinical Neurology*, vol. 126, pp. 559–578, 2014.

[19] N. Das Evcimen and G. L. King, "The role of protein kinase C activation and the vascular complications of diabetes," *Pharmacological Research*, vol. 55, no. 6, pp. 498–510, 2007.

[20] X.-L. Du, D. Edelstein, L. Rossetti et al., "Hyperglycemia-induced mitochondrial superoxide overproduction activates the hexosamine pathway and induces plasminogen activator inhibitor-1 expression by increasing Sp1 glycosylation," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 97, no. 22, pp. 12222–12226, 2000.

[21] K. Sugimoto, M. Yasujima, and S. Yagihashi, "Role of advanced glycation end products in diabetic neuropathy," *Current Pharmaceutical Design*, vol. 14, no. 10, pp. 953–961, 2008.

[22] S. Tesfaye, N. Chaturvedi, S. E. M. Eaton et al., "Vascular risk factors and diabetic neuropathy," *The New England Journal of Medicine*, vol. 352, no. 4, pp. 341–450, 2005.

[23] T. D. Wiggin, K. A. Sullivan, R. Pop-Busui, A. Amato, A. A. F. Sima, and E. L. Feldman, "Elevated triglycerides correlate with progression of diabetic neuropathy," *Diabetes*, vol. 58, no. 7, pp. 1634–1640, 2009.

[24] A. G. Smith and J. R. Singleton, "Obesity and hyperlipidemia are risk factors for early diabetic neuropathy," *Journal of Diabetes and its Complications*, vol. 27, no. 5, pp. 436–442, 2013.

[25] B. Isomaa, M. Henricsson, P. Almgren, T. Tuomi, M.-R. Taskinen, and L. Groop, "The metabolic syndrome influences the risk of chronic complications in patients with Type II diabetes," *Diabetologia*, vol. 44, no. 9, pp. 1148–1154, 2001.

[26] A. Leventoglu and G. Morkavuk, "P225: small fiber neuropathy associated with hyperlipidemia: utility of cutaneous silent periods and autonomic tests," *Clinical Neurophysiology*, vol. 125, supplement 1, p. S108, 2014.

[27] B. Kim and E. L. Feldman, "Insulin resistance in the nervous system," *Trends in Endocrinology and Metabolism*, vol. 23, no. 3, pp. 133–141, 2012.

[28] C. W. Grote, J. K. Morris, J. M. Ryals, P. C. Geiger, and D. E. Wright, "Insulin receptor substrate 2 expression and involvement in neuronal insulin resistance in diabetic neuropathy," *Experimental Diabetes Research*, vol. 2011, Article ID 212571, 12 pages, 2011.

[29] V. Brussee, F. A. Cunningham, and D. W. Zochodne, "Direct insulin signalling of neurons reverses diabetic neuropathy," *Diabetes*, vol. 53, no. 7, pp. 1824–1830, 2004.

[30] C. W. Grote, A. L. Groover, J. M. Ryals, P. C. Geiger, E. L. Feldman, and D. E. Wright, "Peripheral nervous system insulin resistance in ob/ob mice," *Acta Neuropathologica Communications*, vol. 1, no. 1, article 15, 2013.

[31] M. Stecker and M. Stevenson, "Effects of insulin on peripheral nerves," *Journal of Diabetes and its Complications*, vol. 30, no. 5, pp. 770–777, 2016.

[32] B. Kim, L. L. McLean, S. S. Philip, and E. L. Feldman, "Hyperinsulinemia induces insulin resistance in dorsal root ganglion neurons," *Endocrinology*, vol. 152, no. 10, pp. 3638–3647, 2011.

[33] B. Draznin, "Molecular mechanisms of insulin resistance: serine phosphorylation of insulin receptor substrate-1 and increased expression of p85α: the two sides of a coin," *Diabetes*, vol. 55, no. 8, pp. 2392–2397, 2006.

[34] B. Sears and M. Perry, "The role of fatty acids in insulin resistance," *Lipids in Health and Disease*, vol. 14, no. 1, article 121, 2015.

[35] L. Han, L. Ji, J. Chang et al., "Peripheral neuropathy is associated with insulin resistance independent of metabolic syndrome," *Diabetology and Metabolic Syndrome*, vol. 7, no. 1, article no. 14, 2015.

[36] A. J. Garber, "The metabolic syndrome," *Medical Clinics of North America*, vol. 88, no. 4, pp. 837–846, 2004.

[37] D. Dey, D. Basu, S. S. Roy, A. Bandyopadhyay, and S. Bhattacharya, "Involvement of novel PKC isoforms in FFA induced defects in insulin signaling," *Molecular and Cellular Endocrinology*, vol. 246, no. 1-2, pp. 60–64, 2006.

[38] T. C. Coste, A. Gerbi, P. Vague, J. M. Maixent, G. Pieroni, and D. Raccah, "Peripheral diabetic neuropathy and polyunsaturated fatty acid supplementations: natural sources or biotechnological needs?" *Cellular and Molecular Biology*, vol. 50, no. 7, pp. 845–853, 2004.

[39] R. N. M. Weijers, "Membrane flexibility, free fatty acids, and the onset of vascular and neurological lesions in type 2 diabetes," *Journal of Diabetes and Metabolic Disorders*, vol. 15, article 13, 2016.

[40] M. K. Piya, P. G. McTernan, and S. Kumar, "Adipokine inflammation and insulin resistance: the role of glucose, lipids and endotoxin," *Journal of Endocrinology*, vol. 216, no. 1, pp. T1–T15, 2013.

[41] M. Roustit, J. Loader, C. Deusenbery, D. Baltzis, and A. Veves, "Endothelial dysfunction as a link between cardiovascular risk factors and peripheral neuropathy in diabetes," *The Journal of Clinical Endocrinology & Metabolism*, vol. 101, no. 9, pp. 3401–3408, 2016.

[42] P. O. O. Julu, "Essential fatty acids prevent slowed nerve conduction in streptozotocin diabetic rats," *Journal of Diabetic Complications*, vol. 2, no. 4, pp. 185–188, 1988.

[43] H. Keen, J. Payan, J. Allawi et al., "Treatment of diabetic neuropathy with γ-linolenic acid," *Diabetes Care*, vol. 16, no. 1, pp. 8–15, 1993.

[44] D. Manzella, M. Barbieri, M. R. Rizzo et al., "Role of free fatty acids on cardiac autonomic nervous system in noninsulin-dependent diabetic patients: effects of metabolic control," *Journal of Clinical Endocrinology and Metabolism*, vol. 86, no. 6, pp. 2769–2774, 2001.

[45] A. M. Vincent, J. L. Edwards, L. L. McLean et al., "Mitochondrial biogenesis and fission in axons in cell culture and animal models of diabetic neuropathy," *Acta Neuropathologica*, vol. 120, no. 4, pp. 477–489, 2010.

[46] A. Viader, Y. Sasaki, S. Kim et al., "Aberrant schwann cell lipid metabolism linked to mitochondrial deficits leads to axon degeneration and neuropathy," *Neuron*, vol. 77, no. 5, pp. 886–898, 2013.

[47] M. J. McManus, M. P. Murphy, and J. L. Franklin, "Mitochondria-derived reactive oxygen species mediate caspase-dependent and -independent neuronal deaths," *Molecular and Cellular Neuroscience*, vol. 63, pp. 13–23, 2014.

[48] S. Boudina, S. Sena, H. Theobald et al., "Mitochondrial energetics in the heart in obesity-related diabetes: direct evidence for increased uncoupled respiration and activation of uncoupling proteins," *Diabetes*, vol. 56, no. 10, pp. 2457–2466, 2007.

[49] D. E. Kelley, J. He, E. V. Menshikova, and V. B. Ritov, "Dysfunction of mitochondria in human skeletal muscle in type 2 diabetes," *Diabetes*, vol. 51, no. 10, pp. 2944–2950, 2002.

[50] M. E. Patti, A. J. Butte, S. Crunkhorn et al., "Coordinated reduction of genes of oxidative metabolism in humans with insulin resistance and diabetes: potential role of PGC1 and NRF1," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 100, no. 14, pp. 8466–8471, 2003.

[51] E. Akude, E. Zherebitskaya, S. K. R. Chowdhury, D. R. Smith, R. T. Dobrowsky, and P. Fernyhough, "Diminished superoxide generation is associated with respiratory chain dysfunction and changes in the mitochondrial proteome of sensory neurons from diabetic rats," *Diabetes*, vol. 60, no. 1, pp. 288–297, 2011.

[52] D. Penzo, C. Tagliapietra, R. Colonna, V. Petronilli, and P. Bernardi, "Effects of fatty acids on mitochondria: implications for cell death," *Biochimica et Biophysica Acta—Bioenergetics*, vol. 1555, no. 1-3, pp. 160–165, 2002.

[53] E. Rial, L. Rodríguez-Sánchez, E. Gallardo-Vara, P. Zaragoza, E. Moyano, and M. M. González-Barroso, "Lipotoxicity, fatty acid uncoupling and mitochondrial carrier function," *Biochimica et Biophysica Acta—Bioenergetics*, vol. 1797, no. 6-7, pp. 800–806, 2010.

[54] L. Wojtczak and M. R. Wieckowski, "The mechanisms of fatty acid-induced proton permeability of the inner mitochondrial membrane," *Journal of Bioenergetics and Biomembranes*, vol. 31, no. 5, pp. 447–455, 1999.

[55] L. M. Hinder, C. Figueroa-Romero, C. Pacut et al., "Long-chain acyl coenzyme a synthetase 1 overexpression in primary cultured schwann cells prevents long chain fatty acid-induced oxidative stress and mitochondrial dysfunction," *Antioxidants and Redox Signaling*, vol. 21, no. 4, pp. 588–600, 2014.

[56] S. K. Roy Chowdhury, E. Zherebitskaya, D. R. Smith et al., "Mitochondrial respiratory chain dysfunction in dorsal root ganglia of streptozotocin-induced diabetic rats and its correction by insulin treatment," *Diabetes*, vol. 59, no. 4, pp. 1082–1091, 2010.

[57] L. M. Hinder, A. Vivekanandan-Giri, L. L. McLean, S. Pennathur, and E. L. Feldman, "Decreased glycolytic and tricarboxylic acid cycle intermediates coincide with peripheral nervous system oxidative stress in a murine model of type 2 diabetes," *Journal of Endocrinology*, vol. 216, no. 1, pp. 1–11, 2013.

[58] O. J. Freeman, R. D. Unwin, A. W. Dowsey et al., "Metabolic dysfunction is restricted to the sciatic nerve in experimental diabetic neuropathy," *Diabetes*, vol. 65, no. 1, pp. 228–238, 2016.

[59] J. N. Feige and J. Auwerx, "Transcriptional coregulators in the control of energy homeostasis," *Trends in Cell Biology*, vol. 17, no. 6, pp. 292–301, 2007.

[60] Z. Wu, P. Puigserver, U. Andersson et al., "Mechanisms controlling mitochondrial biogenesis and respiration through the thermogenic coactivator PGC-1," *Cell*, vol. 98, no. 1, pp. 115–124, 1999.

[61] T. R. Koves, P. Li, J. An et al., "Peroxisome proliferator-activated receptor-γ co-activator 1α-mediated metabolic remodeling of skeletal myocytes mimics exercise training and reverses lipid-induced mitochondrial inefficiency," *Journal of Biological Chemistry*, vol. 280, no. 39, pp. 33588–33598, 2005.

[62] V. K. Mootha, C. M. Lindgren, K.-F. Eriksson et al., "PGC-1α-responsive genes involved in oxidative phosphorylation are coordinately downregulated in human diabetes," *Nature Genetics*, vol. 34, no. 3, pp. 267–273, 2003.

[63] S. K. Roy Chowdhury, D. R. Smith, A. Saleh et al., "Impaired adenosine monophosphate-activated protein kinase signalling

in dorsal root ganglia neurons is linked to mitochondrial dysfunction and peripheral neuropathy in diabetes," *Brain*, vol. 135, no. 6, pp. 1751–1766, 2012.

[64] T. Shibata, S. Takeuchi, S. Yokota, K. Kakimoto, F. Yonemori, and K. Wakitani, "Effects of peroxisome proliferator-activated receptor-α and -γ agonist, JTT-501, on diabetic complications in Zucker diabetic fatty rats," *British Journal of Pharmacology*, vol. 130, no. 3, pp. 495–504, 2000.

[65] I. G. Obrosova, O. Ilnytska, V. V. Lyzogubov et al., "High-fat diet-induced neuropathy of pre-diabetes and obesity: effects of "healthy" diet and aldose reductase inhibition," *Diabetes*, vol. 56, no. 10, pp. 2598–2608, 2007.

[66] O. T. Njajou, A. M. Kanaya, P. Holvoet et al., "Association between oxidized LDL, obesity and type 2 diabetes in a population-based cohort, the health, aging and body composition study," *Diabetes/Metabolism Research and Reviews*, vol. 25, no. 8, pp. 733–739, 2009.

[67] A. M. Vincent, J. M. Hayes, L. L. McLean, A. Vivekanandan-Giri, S. Pennathur, and E. L. Feldman, "Dyslipidemia-induced neuropathy in mice: the role of oxLDL/LOX-1," *Diabetes*, vol. 58, no. 10, pp. 2376–2385, 2009.

[68] J. Duan and A. H. Merrill, "1-deoxysphingolipids encountered exogenously and made de novo: dangerous mysteries inside an enigma," *Journal of Biological Chemistry*, vol. 290, no. 25, pp. 15380–15389, 2015.

[69] R. Pralhada Rao, N. Vaidyanathan, M. Rengasamy, A. Mammen Oommen, N. Somaiya, and M. R. Jagannath, "Sphingolipid metabolic pathway: an overview of major roles played in human diseases," *Journal of Lipids*, vol. 2013, Article ID 178910, 12 pages, 2013.

[70] R. Kramer, J. Bielawski, E. Kistner-Griffin et al., "Neurotoxic 1-deoxysphingolipids and paclitaxel-induced peripheral neuropathy," *FASEB Journal*, vol. 29, no. 11, pp. 4461–4472, 2015.

[71] N. Wei, J. Pan, R. Pop-Busui et al., "Altered sphingoid base profiles in type 1 compared to type 2 diabetes," *Lipids in Health and Disease*, vol. 13, no. 1, article no. 161, 2014.

[72] M. Bertea, M. F. Rütti, A. Othman et al., "Deoxysphingoid bases as plasma markers in diabetes mellitus," *Lipids in Health and Disease*, vol. 9, article no. 84, 2010.

[73] M. F. Dohrn, A. Othman, S. K. Hirshman et al., "Elevation of plasma 1-deoxy-sphingolipids in type 2 diabetes mellitus: a susceptibility to neuropathy?" *European Journal of Neurology*, vol. 22, no. 5, pp. 806–e55, 2015.

[74] S. M. Hammad, N. L. Baker, J. M. El Abiad et al., "Increased plasma levels of select deoxy-ceramide and ceramide species are associated with increased odds of diabetic neuropathy in type 1 diabetes: a pilot study," *NeuroMolecular Medicine*, 2016.

[75] A. Othman, R. Bianchi, I. Alecu et al., "Lowering plasma 1-deoxysphingolipids improves neuropathy in diabetic rats," *Diabetes*, vol. 64, no. 3, pp. 1035–1045, 2015.

[76] A. Othman, R. Benghozi, I. Alecu et al., "Fenofibrate lowers atypical sphingolipids in plasma of dyslipidemic patients: a novel approach for treating diabetic neuropathy?" *Journal of Clinical Lipidology*, vol. 9, no. 4, pp. 568–575, 2015.

[77] T. M. E. Davis, B. B. Yeap, W. A. Davis, and D. G. Bruce, "Lipid-lowering therapy and peripheral sensory neuropathy in type 2 diabetes: The Fremantle Diabetes Study," *Diabetologia*, vol. 51, no. 4, pp. 562–566, 2008.

[78] K. Rajamani, P. G. Colman, L. P. Li et al., "Effect of fenofibrate on amputation events in people with type 2 diabetes mellitus (FIELD study): a prespecified analysis of a randomised controlled trial," *The Lancet*, vol. 373, no. 9677, pp. 1780–1788, 2009.

[79] Y. R. Cho, J. H. Lim, M. Y. Kim et al., "Therapeutic effects of fenofibrate on diabetic peripheral neuropathy by improving endothelial and neural survival in db/db mice," *PLoS ONE*, vol. 9, no. 1, Article ID 0083204, 2014.

[80] D.-J. Lou, Q.-Q. Zhu, X.-W. Si et al., "Serum phospholipid omega-3 polyunsaturated fatty acids and insulin resistance in type 2 diabetes mellitus and non-alcoholic fatty liver disease," *Journal of Diabetes and Its Complications*, vol. 28, no. 5, pp. 711–714, 2014.

[81] J. Li, F. R. Li, D. Wei et al., "Endogenous ω-3 polyunsaturated fatty acid production confers resistance to obesity, dyslipidemia, and diabetes in mice," *Molecular Endocrinology*, vol. 28, no. 8, pp. 1316–1328, 2014.

[82] T. C. Coste, A. Gerbi, P. Vague, G. Pieroni, and D. Raccah, "Neuroprotective effect of docosahexaenoic acid-enriched phospholipids in experimental diabetic neuropathy," *Diabetes*, vol. 52, no. 10, pp. 2578–2585, 2003.

[83] A. Gerbi, J.-M. Maixent, J.-L. Ansaldi et al., "Fish oil supplementation prevents diabetes-induced nerve conduction velocity and neuroanatomical changes in rats," *Journal of Nutrition*, vol. 129, no. 1, pp. 207–213, 1999.

[84] F. R. Danesh, R. L. Anel, L. Zeng, J. Lomasney, A. Sahai, and Y. S. Kanwar, "Immunomodulatory effects of HMG-CoA reductase inhibitors," *Archivum Immunologiae et Therapiae Experimentalis*, vol. 51, no. 3, pp. 139–148, 2003.

[85] N. Cameron, M. Cotter, M. Inkster, and M. Nangle, "Looking to the future: diabetic neuropathy and effects of rosuvastatin on neurovascular function in diabetes models," *Diabetes Research and Clinical Practice*, vol. 61, supplement 1, pp. S35–S39, 2003.

[86] G. Villegas-Rivera, L. M. Román-Pintos, E. G. Cardona-Muñoz et al., "Effects of ezetimibe/simvastatin and rosuvastatin on oxidative stress in diabetic neuropathy: a randomized, double-blind, placebo-controlled clinical trial," *Oxidative Medicine and Cellular Longevity*, vol. 2015, Article ID 756294, 10 pages, 2015.

[87] L. Packer, K. Kraemer, and G. Rimbach, "Molecular aspects of lipoic acid in the prevention of diabetes complications," *Nutrition*, vol. 17, no. 10, pp. 888–895, 2001.

[88] T. Han, J. Bai, W. Liu, and Y. Hu, "A systematic review and meta-analysis of α-lipoic acid in the treatment of diabetic peripheral neuropathy," *European Journal of Endocrinology*, vol. 167, no. 4, pp. 465–471, 2012.

[89] A. Hovaguimian and C. H. Gibbons, "Diagnosis and treatment of pain in small-fiber neuropathy," *Current Pain and Headache Reports*, vol. 15, no. 3, pp. 193–200, 2011.

Hindawi Publishing Corporation
Journal of Diabetes Research
Volume 2016, Article ID 3425617, 16 pages
http://dx.doi.org/10.1155/2016/3425617

Review Article

Diabetic Polyneuropathy in Type 2 Diabetes Mellitus: Inflammation, Oxidative Stress, and Mitochondrial Function

Luis Miguel Román-Pintos,[1] Geannyne Villegas-Rivera,[1,2] Adolfo Daniel Rodríguez-Carrizalez,[3] Alejandra Guillermina Miranda-Díaz,[3] and Ernesto Germán Cardona-Muñoz[3]

[1]*Departamento de Ciencias de la Salud-Enfermedad, Centro Universitario de Tonalá, Universidad de Guadalajara, Guadalajara, JAL, Mexico*
[2]*Unidad de Investigación Médica, Instituto de Investigación Clínica de Occidente, Guadalajara, JAL, Mexico*
[3]*Instituto de Terapéutica Experimental y Clínica, Departamento de Fisiología, Centro Universitario de Ciencias de la Salud, Universidad de Guadalajara, Guadalajara, JAL, Mexico*

Correspondence should be addressed to Alejandra Guillermina Miranda-Díaz; kindalex1@outlook.com

Received 8 September 2016; Accepted 7 November 2016

Academic Editor: Abd Tahrani

Diabetic polyneuropathy (DPN) is defined as peripheral nerve dysfunction. There are three main alterations involved in the pathologic changes of DPN: inflammation, oxidative stress, and mitochondrial dysfunction. Inflammation induces activation of nuclear factor kappa B, activator protein 1, and mitogen-activated protein kinases. Oxidative stress induced by hyperglycemia is mediated by several identified pathways: polyol, hexosamine, protein kinase C, advanced glycosylation end-products, and glycolysis. In addition, mitochondrial dysfunction accounts for most of the production of reactive oxygen and nitrosative species. These free radicals cause lipid peroxidation, protein modification, and nucleic acid damage, to finally induce axonal degeneration and segmental demyelination. The prevalence of DPN ranges from 2.4% to 78.8% worldwide, depending on the diagnostic method and the population assessed (hospital-based or outpatients). Risk factors include age, male gender, duration of diabetes, uncontrolled glycaemia, height, overweight and obesity, and insulin treatment. Several diagnostic methods have been developed, and composite scores combined with nerve conduction studies are the most reliable to identify early DPN. Treatment should be directed to improve etiologic factors besides reducing symptoms; several approaches have been evaluated to reduce neuropathic impairments and improve nerve conduction, such as oral antidiabetics, statins, and antioxidants (alpha-lipoic acid, ubiquinone, and flavonoids).

1. Introduction

Diabetes mellitus (DM) leads to important morbidity and mortality, consequence of macro- and microvessels complications [1]. Type 2 DM is characterized by insulin resistance, with or without insulin deficiency that induces organ dysfunction [2]. Persistent hyperglycemia in DM generates reactive oxygen species (ROS) and nitrosative species (RNS); both are considered an essential factor for DM macro- and microvessels complications [3]. Along with overproduction of ROS and RNS, a reduction of the activity of antioxidant enzymes is known to cause endothelial dysfunction, insulin resistance, and DM complications [4]. Furthermore, oxidative stress inhibits insulin secretion in pancreatic β-cells by activation of uncoupling protein 2 (UCP-2), which, in turn, reduces the adenosine triphosphate (ATP)/adenosine diphosphate (ADP) ratio, and thus reduces the insulin-secretory response [5]. This approach explains the pancreatic dysfunction induced by glucose toxicity, as part of the pathophysiology of DM.

ROS and RNS are responsible for structural derangement of carbohydrates, proteins, lipids, and nucleic acids [6]. These free radicals activate different signaling pathways, which leads to transcriptional genes related to diabetic complications; activation of nuclear factor kappa B results in induction of proinflammatory proteins, also observed in

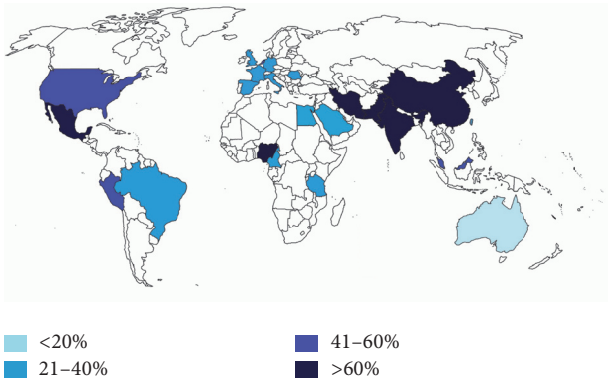


Figure 1: Prevalence of DPN by country. Colors represent the percentiles 25, 50, and 75 of epidemiology studies.

diabetic polyneuropathy (DPN) [7]. In this review, we aimed to describe the behavior of inflammatory response, oxidative stress, and mitochondrial function in DPN of type 2 DM.

2. Diabetic Polyneuropathy

Diabetic neuropathies are a heterogeneous group of pathological manifestations with the potential to affect every organ, with clinical implications such as organ dysfunction, which leads to low quality life and increased morbidity [8]. DPN is defined as peripheral nerve dysfunction with positive and negative symptoms [9]. Some authors describe it as the most frequent microvessel diabetic complication, and it is present in approximately 10% of recently diagnosed diabetic patients [10, 11].

2.1. Epidemiology. For the analysis of prevalence of DPN we included some of the most representative studies by country. Noteworthy, we only report the prevalence of type 2 DM patients with the information contained in each article. The distinction of the population where the prevalence is reported is important due to the particular characteristics of the evaluated patients. There are several biases to take into consideration in particular with each study: intra- or interobserver variability, examiners ability to perform the tests, different detection methods, presence/absence of arteriopathy, and comorbidities, among others. Ziegler et al. minutely described the prevalence of DPN from 1986 to 2009 and separated the known information in type 1 and 2 DM [12].

The prevalence of DPN ranged from 2.4 to 78.8%, both in Chinese population. The median prevalence of evaluated studies was 59%. We established percentiles around the minimum and maximum prevalence reported and divided those reported by country into the following: above percentile 75 (>60% of DPN), higher than percentile 50 (41–60%), between percentile 25 and 50 (21–40%), and below percentile 25 (<20%). Figure 1 resumes the highest prevalence reported by country [13–44].

There was one study performed in Spain where the prevalence was reported as low as 1.33%; however, the method of detection was not reported, and apparently the patients were classified using their hospital discharge records; thus, we did not include this study in the analysis because we believe it lacks essential information [45]. In another study from Germany and UK type 2 DM patients who were recruited from general practice nationwide, the authors used the 10th revision of the International Statistical Classification of Diseases and Related Health Problems (ICD-10) to establish the prevalence of DPN in their communities, but then again they do not report the diagnostic method employed by the physicians: in Germany it was 5.7% and in the UK 2.4% [46]. One study that explores the presence of painful neuropathy demonstrated a prevalence of mild symptoms of 14%, moderate 18%, and severe 16%: the overall presence of painful neuropathy was 34% [47]. Davies et al. also reported the prevalence of painful DPN in 269 patients with type 2 DM of a population-based sample recruited in an urban community in South Wales, UK; the results were an overall prevalence of 26.4% patients with DPN: neuropathic pain 19%, nonneuropathic pain 36.8%, mixed pain 7.4%, and no pain 36.8% [18]. However, some authors report a prevalence up to 77.4% in some series [33].

2.2. Risk Factors. The Rochester Diabetic Neuropathy Study (RDNS) is a classical cohort in the 90s that gives us plenty of information regarding demographic and clinical outcomes in this area. The classical variables which influence neuropathic endpoints are age, gender, height, weight, body surface area, and body mass index (BMI) [48]. The temperature also influences the measures, as shown in nerve conduction studies of normal subjects, and in consequence the protocols for nerve conduction studies include having a standardized normal temperature room [49, 50]. *Age* is the most evaluated risk factor in the majority of epidemiological studies, with those ≥70 years old considered the most prevalent group for DPN (odds ratio [OR] 1.073 [95% CI 1.051–1.097], $p < 0.001$); it is considered a risk factor for symptoms, deficits, and sensation perception deficits, so much for presence and severity of DPN [17, 19, 39, 42, 43]. Disparity results are found regarding *gender*, with some authors reporting male sex as a severity risk factor (OR 2.01, $p = 0.02$) [19, 51]. A more recent report found that male gender in type 2 DM patients from a survey conducted in a tertiary hospital showed an OR of 2.7 (95% CI 1.4–5.1, $p = 0.001$). However, in patients with established DPN, apparently female gender is associated with more symptomatic disease and severity of pain [42]. Furthermore, one study demonstrated that, after adjustments for age, diabetes duration, and differences in clinical neuropathy, women still had a 50% increased risk of painful symptoms compared with men (OR 1.5 [95% CI 1.4–1.6], $p = 0.0001$) [47, 52]. One study demonstrated the correlation between *height* and nerve conduction studies in normal subjects from 17 to 77 years old. A strong inverse correlation was found between height and sural ($r = -0.7104$), peroneal ($r = -0.6842$), and tibiae ($r = -0.5044$) conduction velocities and is also correlated to nerve latencies (sural $r = 0.6518$, peroneal $r = 0.4583$, tibiae $r = 0.7217$, and median $r = 0.5440$) [49]. *Overweight and obesity* are considered as risk factors for the presence of DPN with OR 1.036 (95% CI 1.005–1.068, $p = 0.022$) [14]. Furthermore, *weight* by itself is also a risk factor with OR 1.01 (95% CI 1.00–1.03, $p = 0.044$) [28].

A study performed from 2010 to 2012 in 16 diabetes outpatient clinics in Japan, where 298 patients were included, reported that overweight and obesity are risk factors for pain and numbness in patients with DPN [52]. In type 2 DM patients with BMI ≥ 25, age (OR 1.016 [95% CI 1.008–1.024]), duration of DM (OR 1.072 [95% CI 1.056–1.087]), and HbA1c (OR 1.053 [95% CI 1.013–1.095]) are considered risk factors for the presence of DPN [32]. Dyck et al. established the risk factors for DPN in 264 diabetic patients of both type 1 and 2 DM and found that more than 20 covariates were statistically associated with DPN severity. They divided the risk factors for both type 1 and type 2, type 1 DM only, and type 2 DM only; after inclusion of the variables in a multivariate analysis and excluding markers of microvessel and macrovessel disease, for type 2 DM, an altered *glycated hemoglobin* (HbA1c) was the most significant independent risk factor associated with severity [53]. Moreover, a quantitative assessment of nerve conduction studies in patients with type 2 DM found that increased HbA1c is a risk factor for severity of DPN, resulting in an OR of 5.233 (95% CI 1.700–16.103), $p = 0.004$) [54]. These findings have been constantly reported, with a more recent study associating the presence of an altered HbA1c with DPN (OR 1.139 [95% CI 1.021–1.271]) [39] and >50% of patients with DPN having HbA1c ≥ 7% [19, 43]. Another risk factor is longer *diabetes duration*, with adjusted OR 1.05 (95% CI 1.02–1.08) [19, 28, 39]. *Ethnicity* also influences severity of DPN, being the black non-Hispanic, mixed, or Asian patients more affected than Caucasians [19, 42]. A higher *education* level is a protective factor for DPN in type 2 DM (OR 0.72 [95% CI 0.58–0.88, $p < 0.05$]). A history of *hypertension* is more frequently found in patients with type 2 DM with DPN and coronary artery disease (CAD) in the BARI2 cohort, although no regression analysis was made [19].

Some authors have also considered pharmacological treatments for DM, such as *insulin*, as a risk factor for the presence of DPN (OR 1.57 [95% CI 1.15–2.13]), and related with greater risk of numbness (OR 3.21 [95% CI 1.52–6.97]), after adjustment for duration of diabetes, HbA1c levels, and age [19, 52]. Painful symptoms also seem to be more prevalent when patients are treated with insulin, compared with those on oral hypoglycemic agents and diet-only (54.7, 50.6, and 42.1%, respectively; $p = 0.0001$) [47].

DPN is strongly associated with diabetic retinopathy (OR 1.10, $p < 0.01$), not considered as a risk factor, but part of the same physiopathological cause for microvessel disease in DM [51, 53]. DPN is also more frequent in patients with nephropathy compared with those without overt nephropathy (62% versus 32%), although not statistically different when evaluated in 156 type 2 DM patients. Macrovessel disease is also associated with DPN; peripheral vascular disease is three times more common in patients with DPN (OR 2.31, $p = 0.007$) [51].

Modern risk factors have been explored lately, with interesting results addressing more complex determinations. Ankle-brachial index as a marker for peripheral arterial disease constitutes an independent predictor factor for neuropathy (OR 2.260 (95% CI 1.324–3.858, $p = 0.003$) [39]. In a hospital-based report in type 2 DM patients from Sub-Saharan Africa, the determinants of polyneuropathy were urban residence (OR 7.9 [95% CI 1.4–44.9], $p = 0.02$), infection with hepatitis C virus (OR 4.8 [95% CI 1.1–21.4], $p = 0.002$), infection with HIV (OR 3.4 [1.1–10.5], $p = 0.012$), and presence of albuminuria (OR 20.4 [95% CI 6.5–63.9], $p = 0.0001$) [33]. The latter was also reported previously in patients with type 2 DM and coronary artery disease as more prevalent in patients with DPN [19].

An association between cardiovascular disease and DPN has been mentioned previously in between lines; however, there were no adapted studies that addressed this particular issue until 2014, where Ybarra-Muñoz et al. demonstrated that patients with type 2 DM and cardiovascular disease presented an increased risk of developing DPN at 10 years of follow-up (OR 2.32 [95% CI 1.03–5.22], $p = 0.04$). It was performed in a primary care setting from 2002 to 2012, and the selection of patients was according to the presence of previous history of myocardial infarction, angina, coronary artery disease, bypass-grafting, stroke, peripheral vascular disease, or ischemic changes detected on a 12-lead electrocardiogram [55].

2.3. Pathophysiology. Three main characteristics are involved in the pathologic changes of DPN, namely, inflammation, oxidative stress, and mitochondrial dysfunction (Figure 2). They account for most of the pathologic processes that affect microvessels and nerve fibers. It is now known that there is a structural abnormality of nerve capillaries in DM and an association between pathologic abnormality of vessels and pathologic abnormality of nerve fibers [56].

Biopsy findings reflect a loss of multifocal and focal proximal nerve fibers, but a more severe damage in distal fibers; the number of myelinated fibers diminishes from proximal to distal nerves [57]. Axonal degeneration and segmental demyelination are the main pathological characteristics of neuropathic damage induced by hyperglycemia. It has been established that the first pathologic changes in DPN are axonal degeneration with subsequent regeneration, but insufficient to reestablish the structural abnormalities due to chronic hyperglycemia. Onion bulbs are shown in nerve biopsies, characteristic of hypertrophic neuropathy, representing the demyelination and remyelination of the nerve fibers [57].

The number of capillaries per mm^2 and the minimum intercapillary distance are not affected in DM patients; however, the percentage of capillaries closed is higher in subjects with DPN compared with healthy controls and also related to the severity of the neuropathy [58]. The walls of microvessels are thickened, due mainly to excessive numbers of basement membranes, and undergo degenerative changes, which cannot be explained by age. Degeneration of both endothelial and periendothelial cells is considered characteristic changes in DPN; however, some authors have shown that transformation of the microvascular wall rather than a process of microvascular de- and regeneration is the main pathological change [59]. In streptozotocin-diabetic rats, axonal outgrowth of dorsal root ganglion neurons exhibited a twofold elevation of ROS in axons after 24 hours of 25 mmol/L of glucose exposure compared to controls, predisposing the axon to degeneration and dissolution [60].

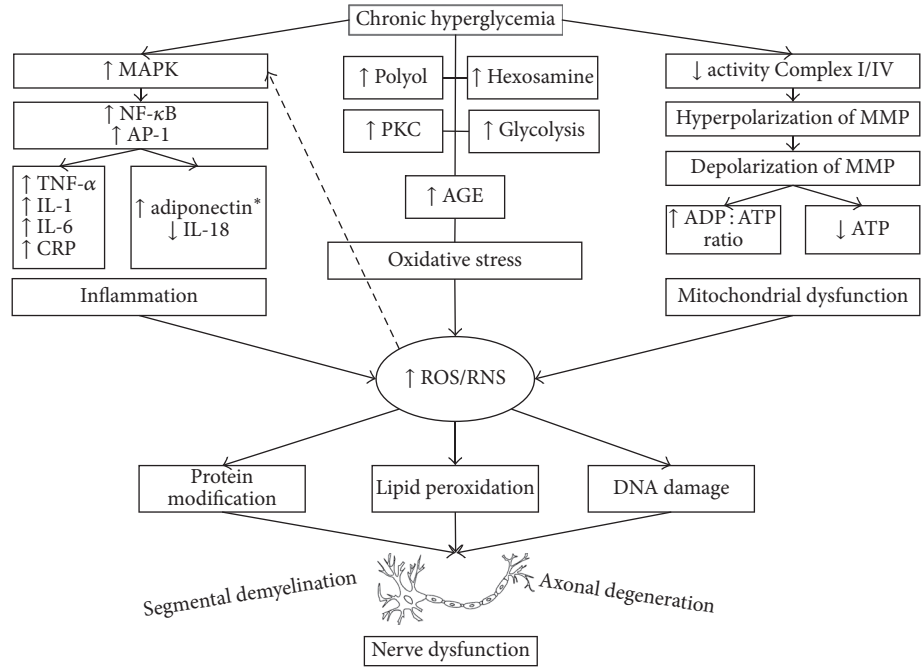


Figure 2: Mechanisms of nerve dysfunction induced by hyperglycemia. The description of how inflammation, oxidative stress, and mitochondrial dysfunction contributes to ROS/RNS formation and nerve damage. *Paradoxical increase of adiponectin in DPN.

2.3.1. Inflammation. The ROS-mediated inflammation induces activation of nuclear factor kappa B (NF-κB), activator protein 1 (AP-1), and mitogen-activated protein kinases (MAPK). NF-κB at the same time facilitates the production of inflammatory cytokines: tumoral necrosis factor alpha (TNF-α), interleukin- (IL-) 6, cyclooxygenase 2 (COX-2), and inducible nitric oxide synthase (iNOS). The MAPK is promoted by hyperglycemia through apoptosis signaling kinase 1 or directly by ROS and causes activation of cytosolic NF-κB [61]. It is known that Nrf2 consist in the counterpart of NF-κB maintaining a cellular homeostasis, but ROS overproduction can generate an imbalance between them and associates with nerve damage [62, 63].

Adipocytokines are inflammatory substances secreted by fat tissue, which includes TNF-α, adiponectin, and leptin [64]. In overweight and obese patients a low insulin sensitivity and adiponectin levels have been found, and they had a positive correlation. Inversely, TNF-α receptors had a negative correlation with insulin sensitivity, which leads to the conclusion that TNF-α might be associated with insulin resistance through its relationship with substrate oxidation in hyperinsulinemic condition and nonoxidative glucose metabolism [65]. Furthermore, hypoadiponectinemia is related to higher fasting plasma glucose and triglycerides, lower HDL cholesterol, and visceral obesity and also to higher levels of inflammatory cytokines (IL-6 and IL-1) and C reactive protein (CRP) in type 2 DM subjects [66].

From 2002 and 2004 a total of 105 type 2 DM subjects were evaluated to find the role of adipocytokines in DPN. Matsuda et al. found a relationship between leptin and TNF-α with sural conduction velocity in patients with DPN; however, the study lacked plausibility criteria for it was a cross-sectional study, and they had difficulty explaining these correlations [67]. Years later, in 2009, the clinical implications of inflammation in DPN were addressed again, with enough findings to establish the association of inflammation markers with DPN. Herder et al. studied a total of 10 inflammation biomarkers in 227 type 2 DM patients selected from a population-based survey in Germany and found that higher levels of CRP and IL-6 were most consistently associated with DPN compared to type 2 DM patients without neuropathy. These two markers were directly associated with presence and severity of DPN, while IL-18 was inversely related to neuropathy [68].

In type 2 DM patients of recent diagnosis, an increase of IL-6, total adiponectin, and high molecular weight adiponectin were found in subjects with DPN. These results seem to be counterintuitive, since adiponectin is considered an anti-inflammatory cytokine; however, it has been suggested that adiponectin could be upregulated by metabolic and/or inflammatory insults. A positive association of IL-6 and adiponectin with the presence of DPN was found, after adjusting for age, sex, time since diagnosis of diabetes, HbA1c, waist circumference, height, total cholesterol, hypertension, current smoking, physical activity, use of lipid-lowering medication, use of NSAIDs, history of myocardial infarction, and/or stroke; therefore, it can be used as an independent marker for the presence of DPN [69]. Also, in type 2 DM patients with DPN, an inverse association between TNF-α and nerve conduction parameters has been proven, mainly for sural, median, and ulnar nerve conduction velocity. This inflammatory marker was tested in patients without DPN or with less than 8 years and >8 years from the diagnosis of DPN. The serum levels of TNF-α were markedly

raised in neuropathy patients and the trend continued when duration of disease increased [70]. Another study included patients aged from 61 to 82 years with DM and DPN from the population-based Cooperative Health Research in the Region of Augsburg (KORA) F4 study and found that serum concentrations of IL-1 receptor antagonist (IL-1RA) were positively associated with the presence of DPN and higher Michigan Neuropathy Screening Instrument (MNSI) scores in age-adjusted and sex-adjusted analyses, reassuring the association of inflammatory cytokines in the older population [44]. A restriction of glucotoxicity from the diet has demonstrated that inflammatory changes are diminished in diabetic patients, an improvement of insulin resistance has been observed, and a reduction of leptin and an increase of adiponectin levels have been reported [71, 72].

2.3.2. Oxidative Stress. There are several pathways for ROS production, such as glycolysis, hexosamine pathway, protein kinase C (PKC) pathway, polyol pathway, and autoxidation [73]. High extracellular glucose enhances glucose activation and the *glycolytic pathway*, resulting in an enhanced pyruvate formation. Pyruvate oxidation in the mitochondria is associated with an increase in the mitochondrial membrane potential (MMP), and high MMP is responsible for an overproduction of ROS, which in turn inhibits glycolysis by a negative feedback. Hence, the flux of carbon is then rerouted towards the glucosamine pathway, which is responsible for the transcriptional consequence of high extracellular glucose [74]. Moreover, an inhibition of glucose-6-phosphate dehydrogenase leads to an increase of free radicals through the production of nicotinamide adenine dinucleotide phosphate (NADPH), induced by hyperglycemia [75]. In *hexosamine pathway*, fructose-6-phosphate is diverted from glycolysis to produce glucosamine-6-phosphate, which in turn converts into uridine diphosphate- (UDP-) N-acetyl glucosamine (UDP-GlcNAc), a substrate for the formation of proteoglycans and other glycoproteins [76]. The UDP-GlcNAc can inhibit the endothelial nitric oxide synthase (eNOS) activity and induce an increased expression of transforming growth factor beta (TGF-β) [77–79]. Intracellular hyperglycemia also induces the formation of diacylglycerol (DAG) from the glycolytic intermediate dihydroxyacetone phosphate, through reduction of the latter to glycerol-3-phosphate. Increased DAG activates *protein kinase C*, but the polyol pathway can also activate some PKC isoforms, particularly relevant to explain microvascular complications of DM [80]. The excess of glucose can be diverted to the *polyol pathway* by the enzyme aldose reductase to produce sorbitol, which is then accumulated in nerve fibers, and reduce the levels of myoinositol [81]. In consequence, there is a reduction in the axon capacity to propagate the membrane action potential and diminished capacity of nerve regeneration [82, 83]. In rat model, an association between exhaustion of myoinositol and a reduction of the ATPase induces a nerve conduction deficit [84]. *Autoxidation* is considered a mechanism where glucose itself can be toxic as a result of a nonenzymatic glucose binding to proteins, resulting in advanced glycation end stage (AGE) products [85]. An increase of AGE in axons and Schwann cells have been reported in peripheral nerves of patients and animal model of DM [86]. It is a fact that AGE are related to the progression of type 2 DM complications, including DPN, diabetic nephropathy, myocardiopathy, peripheral artery disease, and retinopathy [87]. Experimental studies in rats showed that, after a high AGE diet during a long period of time, insulin resistance appears, and type 2 DM [88].

Oxidative Stress Biomarkers. The ROS and RNS are intended to induce apoptosis of dysfunctional cells and recycling some of their components; however, imbalance occurs when the antioxidant capacity of patients with DM is exceeded with the production of free radicals. The ROS include free radicals, such as superoxide ($O^{2\bullet-}$), hydroxyl ($HO^{\bullet}$), peroxyl ($RO^{2-\bullet}$), and hydroperoxyl ($^{\bullet}HRO^{2-}$), and nonradical species, such as hydrogen peroxide (H_2O_2) and hydrochloric acid (HOCl). Among RNS we can find free radicals as nitric oxide ($NO^{\bullet}$) and nitrogen dioxide ($NO^{2\bullet}$) and nonradical peroxynitrite ($ONOO^{-}$), nitrous oxide (HNO^2), and alkyl peroxynitrates (RONOO) [89].

Oxidative and nitrosative stress markers have been extensively studied in DPN, and the relationship between ROS and neuropathy in DM has been assessed in vitro and in vivo. A loss of 53% of large myelinated fibers due to ROS overproduction in chronically diabetic animals has been proven: variations in basal glucose as small as 10 mM induce neuronal injury [90]. In vitro and in vivo studies have shown an increase in oxidative stress biomarkers in lipids (thiobarbituric acid-reactive substances [TBARS], malondialdehyde [MDA], and isoprostanes), proteins (protein carbonylation and nitrosylation), carbohydrates (AGE products), and DNA (8-hydroxy-deoxyguanin), along with inhibition of endogenous antioxidant synthesis [91]. Ziegler et al. found significant elevations in three reliable oxidative stress markers, plasma 8-iso-prostaglandin $F_{2\alpha}$ (8-iso-$PGF_{2\alpha}$), $O^{2\bullet-}$ and $ONOO^{-}$ of patients with DM and DPN, compared to healthy controls and diabetic patients without DPN. In multivariate models, $O^{2\bullet-}$ and $ONOO^{-}$ were independently associated with neuropathic deficits and related to the presence and severity of DPN [92].

Free radicals have the capacity to attach to membranes and induce cell damage; when they affect structural lipids of cells is called lipid peroxidation (LPO), and MDA is considered an index of endogenous LPO [93, 94]. A significant elevation in LPO markers like MDA and TBARS of patients with type 2 DM are a consistent finding in several publications [95–98]. Moreover, when type 2 DM patients exhibit DPN, MDA levels are ~40% higher than diabetics without neuropathy and almost three times higher than healthy controls, with similar increases in total antioxidant capacity [99]. The TBARS are also increased in patients with DPN compared to type 2 DM subjects without neuropathy [100] suggesting that oxidative stress is an important if not essential pathophysiologic process for DPN. The increase in ROS and impaired regulation of oxidative stressors result in programmed cell death of neurons and provide a mechanism to explain how impaired regulation of peak glucose levels leads to ROS-induced injury in diabetic neuropathy [90].

Antioxidant Status. Antioxidants participate in mechanisms to reduce the deleterious effects of free radicals by preventing their production and/or inactivating them through enzymatic defense systems. The most studied endogenous antioxidants are superoxide dismutase (SOD), catalase (CAT), glutathione S-transferase (GST), and glutathione peroxidase (GPx) and nonenzymatic antioxidants as uric acid, carotenoids, flavonoids, and lipoic acid vitamins A, C, and E. Aydin et al. selected type 2 DM patients from an outpatient clinic of an endocrinology department and evaluated their levels of copper zinc SOD, erythrocyte and plasma selenium dependent GPx, and erythrocyte CAT activities, before and after treatment with oral antidiabetics (OA) comparing them with healthy controls. The results were an increase in levels of SOD but normal activity of GPx and CAT. All antioxidants reduced after 3 months of treatment with OA compared to controls, probably by ameliorating the hyperglycemic state and/or the powerful O^{2-} radical scavenging activity of oral antidiabetics [97]. Furthermore, vitamin E-to-lipid ratio and vitamin C, two potent endogenous antioxidants with well-identified plasma scavenging features, are decreased in patients with type 2 DM and DPN [92]. Similar reports have been published regarding the antioxidant status of type 2 DM patients, where an increased activity is found in hyperglycemic conditions [92, 98], and higher elevations are shown in patients with DPN [100].

2.3.3. Mitochondrial Dysfunction. Mitochondria are intrinsically associated with ROS production; its normal function is altered by hyperglycemia [101]. More than 90% of ROS are generated in the mitochondria [102]. The damage to this organelle can lead to cellular apoptosis and/or reduce the capacity to generate ATP, finally altering the axon through its degeneration. A lower activity of complex I and complex IV secondary to reduced protein expression of certain complex components has been shown in animal models, and an impaired electron transport chain function also causes an increase in ROS generation [103, 104]. The electron chain transport induces the production of $O^{2\bullet-}$ as an end-product from electron uncoupling, followed by a reduction in oxygen to form this free radical. Moreover, mitochondria can also generate $HO^{\bullet}$, H_2O_2, and $NO^{\bullet}$, capable of causing deleterious effects to other proteins or the DNA [62]. The nervous system seems to be particularly vulnerable to oxidative stress damage due to a high energetic demand and elevated lipids content [105]. When hyperglycemia is controlled, the mitochondria experience a reduction of $O^{2\bullet-}$ inhibition, along with improvement of mitochondrial function and DNA [106].

It has been proved that hyperglycemia induces a dose-dependent effect on cleavage of caspases through ATP depletion. Hyperglycemia generates ROS coupled with hyperpolarization of the mitochondrial membrane potential (MMP), followed by mitochondrial membrane depolarization, which is temporally related to an increase in ADP : ATP ratio and an absolute decrease in ATP levels. This in turn is coupled with cytochrome *c* release from the intermitochondrial membrane space and cleavage of caspases, resulting in dorsal root ganglion apoptosis [90].

In diabetic dorsal root ganglion neurons exposed to increase concentrations of glucose both in vivo and in vitro, there is a loss of electrons from the mitochondrial electron transfer chain, coupled with initial hyperpolarization of the MMP, and it results in generation of excess ROS in the mitochondria; in turn, there is increased mitochondrial injury, mitochondrial membrane depolarization, and swelling, with release of apoptosis-inducing factors from the mitochondria into the cytosol, leading to formation of an apoptosome [107]. The relation between ROS overproduction and mitochondrial dysfunction relies in these findings: high glucose increases ROS, destabilizes the MMP, and induces mitochondrial apoptosis [90, 107]. However, there is more than one pathway for ROS increase, as demonstrated by Akude et al., who analyzed the proteins associated with mitochondrial dysfunction, oxidative phosphorylation, ubiquinone biosynthesis, and the citric acid cycle and found that these were downregulated in diabetic samples. Respiration and mitochondrial complex activity was significantly decreased by 15 to 32% compared with control, which leads to reduced levels of intramitochondrial $O^{2\bullet-}$. Even so, the axons of diabetic neurons exhibited oxidative stress and depolarized mitochondria, concluding that alternative pathways appear to contribute to raised ROS in axons of diabetic neurons under high glucose concentration [108].

2.4. Diagnosis. There is no gold standard for the diagnosis of DPN. The expert panel of San Antonio conference recommends that it should be made on the basis of neuropathic symptoms, signs, and nerve conduction studies (NCS) [109]. In 2005 a report of the American Academy of Neurology, the American Association of Electrodiagnostic Medicine, and the American Academy of Physical Medicine and Rehabilitation developed a definition of distal symmetric polyneuropathy (DSPN) that served as a basis for actual research studies. It was established that electrodiagnostic studies are not required for field or epidemiologic studies; therefore, many studies lack nerve conduction studies as part of the evaluation [50]. However, a combination of symptoms and sings is required to increase the specificity of the tests, and therefore, more consideration on the results should be made while interpreting the prevalence in type 2 DM.

A classification for DPN has been proposed for clinical and research purposes by the Toronto Diabetic Neuropathy Expert Group. The following definitions are included: *possible*, when symptoms or signs are present; *probable*, diagnosed with a combination of symptoms and signs of neuropathy; *confirmed*, when NCS are abnormal and symptoms/signs are present. A fourth classification (subclinical) is appointed to those patients without clinical findings but abnormal NCS or a validated measure of small fiber neuropathy may be used. Among these, nerve biopsy, skin biopsy (morphometric quantification of intraepidermal nerve fibers), corneal confocal microscopy, and nerve axon reflex/flare response are considered validated tools. However, subclinical tests for DPN are only recommended for research studies [110].

To assess the severity of DSPN, several approaches can be recommended: the graded approach outlined above, various continuous measures of sum scores of neurologic signs,

symptoms or nerve test scores, and scores of function of acts of daily living or of predetermined tasks or of disability. Irrespective of which approach is used, it is necessary to ensure good performance of evaluations with monitoring proficiency.

The first alterations observed in DPN patients are alteration in vibration perception threshold and reduction of ankle jerks [111]. The American Diabetes Association (ADA) recommended five simple examinations for DPN screening based on clinical signs: ankle reflex, pinprick sensation, temperature sensation, vibration perception thresholds, and pressure sensation [9]. NCS are the most precise tool for the detection of DN; along with symptoms and signs they give an accurate diagnosis. In 2010, the ADA suggested that NCSs should be considered as the gold standard for the diagnosis of DSPN [112].

One of the most employed items to detect the presence of DPN in clinical practice consists in the MNSI that includes the sum of scores varying from 0 to 1 for each abnormality revealed in foot appearance, Achilles reflexes presence, and vibratory threshold (VPT) by tuning fork (maximum score = 8). MNSI by using 2.5 score as cut-off may be considered a rapid, simple, reproducible, and reliable test for rapid ambulatory screening of PDN from the diabetologists [113]. The positive likelihood ratio of MNSI is 5.56 for those patients with ≥2.5 points of the composite score and raises up to 5.83 with >3 points [114].

Another tool for detection of DPN was validated in 2002, known as the Toronto Clinical Neuropathy Scoring System (TCSS), and evaluates the presence of symptomatic DPN (symptom, reflex, and sensory tests scores). It showed a significant negative correlation with sural nerve fiber density, and it was lower in those with better glycemic control. TCSS correlates well with the underlying structural damage in peripheral nerve as shown by the loss of myelinated nerve fibers [115]. In Table 1 we report a compilation of diagnostic methods for the diagnosis of DPN, with their respective sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV).

2.5. Treatment. We focused on etiology-based treatment, leaving symptomatic treatment beyond our review. The basis of treatment for DPN and other microvascular complications of DM is glycemic control due to the basic mechanisms explained previously where excess of glucose is responsible for nerve damage and lack of regeneration. Once glucose levels normalize, nerve dysfunction can be stopped and the nerve fibers can improve [121].

2.5.1. Metformin. The most worldwide used OA drug is still Metformin, a dimethylbiguanide, and the first line treatment for almost any kind of DM, but essential for type 2 DM. It has many advantages when compared to other OAs, such as lack of weight gain, low risk of hypoglycemia, and favorable effects on the lipid profile [122]. Beneficial effects of Metformin have also been observed in adipose tissue by reducing fatty acid oxidation, activation of adenosine monophosphate (AMP) kinase to increase glucose transporter (GLUT4) translocation, and reduction of gluconeogenesis in liver [123]. However, the reason why we included this particular OA is because it has the capacity to prevent mitochondrial permeability transition, reduces the risk of cell death, and has a mild inhibitory effect on complex I of the respiratory chain reaction of mitochondria, which traduce in cell protection from apoptosis induced by mitochondria-related toxicity of hyperglycemia [124]. Furthermore, another investigation of Metformin effects on mitochondria revealed that elevated glucose concentration leads to an oxidative stress that favors the mitochondrial permeability transition pore (PTP) opening and subsequent cell death in several endothelial cell types, and Metformin prevents this PTP opening-related cell death [125]. Kooy et al. studied the effect of Metformin added to insulin therapy in 390 patients with type 2 DM and macro- and microvascular complications; they discovered that Metformin reduced macrovascular complications at 4.3 years of follow-up, although no beneficial effects on DPN were observed [126]. Recently, Hasanvand et al. demonstrated that the activation of AMP kinase signaling pathway in diabetic neuropathy might be associated with the anti-inflammatory response, and Metformin reduced the levels of inflammatory cytokines in diabetic rats; it also improved motor nerve conduction velocities of the sciatic nerves [127].

Recent studies have addressed the importance of vitamin B12 deficiency among long-term users of Metformin as OA therapy. The prevalence varies depending on the cut-off point from 8.6 to 28.1%, and other known risk factors are DM duration ≥10 years, and concomitant use of proton pump inhibitors (PPI) or histamine H2 antagonists (H2A), but the clinical relevance of the deficiency is yet unclear [128, 129]. Ahmed et al. in 2016 published a cross-sectional study where vitamin B12 was measured in 121 type 2 DM patients and the association with DPN was evaluated. Forty-three (35.54%) patients had DPN and vitamin B12 deficiency was defined as levels <150 pmol/L. The prevalence of vitamin B12 deficiency was 28.1%; however, there was no association between vitamin B12 deficiency and DPN, and Metformin dose did not confer an increase risk on DPN presence [129]. Similar results were reported by other authors, with controversial results, but without strong evidence that vitamin B12 deficiency influences the presence or severity of peripheral neuropathy [130–132]. We recommend supplementation with vitamin B12 in those patients with long-term use of Metformin (≥10 years) or concomitant use of PPI/H2A, and evidence of clinical DPN. However, consider suspending supplementation if there is no evidence of improvement.

2.5.2. Statins. The hydroxy methyl glutaryl-CoA reductases, also known as statins, have potent antioxidant properties evaluated in multiple clinical trials. Possible mechanisms reported are inhibition of NADPH oxidase, thus, reducing intracellular production of ROS and acting as free radical scavengers [133]. A subgroup of 136 patients with type 2 DM and diagnosis of DPN established by means of MNSI in the Fremantle Diabetes Study (FDS) were evaluated to observe the benefits of fibrates and statins. The results of a 5-year follow-up demonstrated that statins have a beneficial effect on the incidence of neuropathy, with a hazard ratio (HR) of 0.65 (CI 95% 0.46–0.93) [134]. Ezetimibe/Simvastatin and

Table 1: Diagnostic methods for DPN.

Author(s)	Diagnostic method	Sensitivity	Specificity	PPV	NPV
	Single tests				
Dyck et al. [116]	Abnormal ankle reflex	60.3	90.5		
	Abnormal VPT	17.2	96.4		
Al-Geffari [117]	Abnormal ankle reflex	51.4	97.7	94.9	71.0
	10 g SW Monofilament	69.7	87.9	82.6	78.0
	128-Hz tuning fork	72.5	88.7	84.0	79.7
	Composite scores				
Dyck et al. [116]	NIS (LL) + 7 tests	100	100		
	NIS (LL)	69	86.9		
Feldman et al. [118]	MNSI > 2	80	90	97	74
Moghtaderi et al. [114]	MNSI > 1.5	79	65	59	83
	MNSI > 2.0	65	83	71	79
	MNSI > 2.5	50	91	77	74
	MNSI > 3.0	35	94	80	70
Xiong et al. [119]	NSC	85.96	77.03	74.24	87.69
	NIS	59.65	98.65	97.14	76.04
	MNSI > 1.0	70.18	81.08	74.07	77.92
	MNSI > 1.5	57.89	97.30	94.29	75.00
	MNSI > 2.0	49.12	97.30	93.33	71.29
	MNSI > 2.5	36.84	98.65	95.45	66.97
Al-Geffari [117]	Combined tuning fork & SW Monofilament	89.5	84.9	92.8	89.5
Liu et al. [120]	TCSS	77.2	75.6		
	Nerve conduction studies				
Dyck et al. [116]	≥1 nerve w/abnormal NCS	93.1	57.7		
	≥2 nerves w/abnormal NCS	81	91.2		
	≥3 nerves w/abnormal NCS	51.7	97.8		

DM, diabetes mellitus; IDDM, insulin-dependent diabetes mellitus; NIDDM, non-insulin-dependent diabetes mellitus; IGT, impaired glucose tolerance; MNSI, Michigan Neuropathy Screening.
Instrument; NCS, Nerve Conduction Study; NIS (LL), neuropathic impairment (disability) score of lower limbs; NSC, nerve symptomatic change score; SW, Semmes-Weinstein.

Rosuvastatin have been shown to reduce LPO after 16 weeks of treatment in type 2 DM patients with DPN, although no clinical outcomes were drastically changed compared to placebo [135]. However, a study performed in coronary artery disease patients where Ezetimibe/Simvastatin 10/20 mg was compared to Simvastatin 80 mg as monotherapy demonstrated that inflammation biomarkers (CRP, IL-6, monocyte chemoattractant protein-1, and soluble CD40) were unaltered after 6 weeks of treatment, probably explained by the theory that the target of statins resides on oxidative stress rather than inflammatory response [136]. A study performed in healthy male subjects treated with Simvastatin monotherapy showed no changes in oxidative stress to nucleic acids, LPO, and plasma antioxidants [137], probably due to the lack of free radicals increase, since Ezetimibe alone has been shown to reduce 8-isoprostanes and reactive oxygen metabolites levels only in hypercholesterolemic patients with high oxidative stress at baseline, but not in those with near-normal oxidative status [138]. Rosuvastatin 20 mg was assessed in 17 patients with type 2 DM and DPN, and a reduction of Neuropathic Symptoms Score (NSS) and NCS were observed after 12 weeks of treatment, along with a significant reduction in LPO but no changes in nerve growth factor beta [139]. Finally, both Atorvastatin and Rosuvastatin have proven an increase of total antioxidant capacity when given to type 2 DM patients with high low-density lipoprotein levels after 3 months of treatment, confirming the fact that statins increase the antioxidant status in patients with high levels of oxidative stress [140].

2.5.3. Fenofibrate. A fibric acid derivative, Fenofibrate, is a peroxisome proliferator activated-receptor alpha (PPARα) agonist recently approved for the management of diabetic retinopathy (DR). A subgroup of 1012 patients with DR aged 50–75 years who had type 2 diabetes in the Fenofibrate Intervention and Event Lowering in Diabetes (FIELD) study were assessed regarding the need for laser therapy, and it was found that Fenofibrate has an important effect reducing courses of laser treatment in patients with maculopathy or proliferative retinopathy [141]. The preliminary observational findings in the FDS indicate that fibrates have protective properties against neuropathy, with HR even lower than

statins of 0.52 (CI 95% 0.27–0.98) [134]. An experimental study in *db/db* mice model of diabetic peripheral neuropathy published in 2014 demonstrated that Fenofibrate treatment ameliorated neural and endothelial damage by activating the PPARα-adenosine monophosphate kinase- (AMPK-) PPARγ coactivator- (PGC-) 1a-endothelial nitric oxide (eNOS) pathway [142]. Fenofibrate possesses anti-inflammatory, antioxidant, and anti-ischemic properties, and it could have beneficial effects on DPN, but large randomized clinical studies are needed to consider Fenofibrate an adequate treatment for DPN in type 2 DM patients [143].

2.5.4. Ubiquinone (Coenzyme Q10). Ubiquinone is a vitamin-like substance that contributes to adenosine triphosphate synthesis in mitochondrial electron transport chain. It is reduced to ubiquinol and redistributed into lipoproteins, possibly to protect them from oxidation [144]. In a study on diabetes-induced rat model where Coenzyme Q10 (CoQ10) was compared with α lipoic acid (ALA), the CoQ10 proved to stop the shift of actively contributing nerve fibers toward slower conduction velocities and tended to restore velocities of sciatic nerves toward those of the age-matched control group, whereas ALA did not produce statistically significant effects [145]. Clinical observation of endogenous CoQ10 has shown that, in type 2 DM patients, plasma and platelet MDA, as a marker of oxidative stress, were significantly higher, and the level of CoQ10, as antioxidant capacity, was significantly lower compared to controls, with a negative correlation between plasma CoQ10 and HbA1c [146]. When patients with type 2 DM and DPN were supplemented with 400 mg/day of CoQ10, NSS, Neuropathic Disability Score (NDS), and NCS were improved compared to placebo and associated with a reduction in LPO after 12 weeks of treatment [147]. Furthermore, an increase on total antioxidant capacity, anti-inflammatory effects, shown by reduction of CRP, and probably a protective effect on insulin resistance have been proven when supplementing with 200 mg/day of CoQ10 for 12 weeks, although, at this doses, no beneficial effects on clinical and nerve conduction were observed [148].

2.5.5. Flavonoids. Polyphenols are useful nutraceuticals for type 2 DM patients. Some of their established effects are improving glycemic control, lipid profile, and insulin sensitivity. Furthermore, by regulating adipose metabolism, flavonoids can attenuate oxidative stress and modulate signaling pathways induced by ROS and inflammation [149]. In rat experimental models, an improvement of DPN was found with early intervention based on proanthocyanidins of the grape seed. They can also maintain the normal morphology of nerve tissue by reducing hyperglycemia and calcium overload in sciatic nerves [150]. Grape seed proanthocyanidins reduced low-density lipoproteins and enhanced nerve conduction velocity in Sprague-Dawley with induced type 2 DM [151]. One randomized, double-blinded, placebo-controlled clinical trial used QR-333, a topic drug with quercetin—a flavonoid contained in red wine—three times daily during 4 weeks for symptomatic DPN, and demonstrated a significant improvement in quality of life, neuropathic symptoms, with a good security profile. QR-333 reduced the severity of numbness, irritation, and pain when compared to placebo [152]. Another study evaluated the effect isoflavones-enriched bean sprouts on diabetic gastroparesis (autonomic neuropathy) in patients with type 2 DM with an improvement on gastric emptying compared to placebo [153]. Moreover, puerarin, an important isoflavonoid extracted from a Chinese herb *Puerariae radix,* has come into research attention, since a meta-analysis, where 22 clinical trials with 1664 subjects have investigated the efficacy of intravenous puerarin for DPN, concluded that combined with western medication it was more effective than conventional therapy for DPN in terms of total effective rate, nerve conduction velocity, and hemorheology index [154]. Naringenin is a flavone contained in citric fruits such as grapefruit and orange; it neutralizes oxidative stress and alterations in nerve growth factor in experimental DPN models [155, 156]. Hasanein and Fazeli demonstrated that long-term naringenin exposure has the capacity to exert significant analgesic and glucose lowering dose-dependent effects in a rat model with DPN [157]. In the same way, baicalein, a flavonoid originally isolated from the root *Scutellaria baicalensis,* has been used for many centuries in traditional herbal Chinese medicine for its antibacterial and antiviral properties [158]; it is a potent anti-inflammatory and antitumor agent, a free radical scavenger, and xanthine oxidase inhibitor, thus improving endothelial function and conferring cardiovascular protective actions against oxidative stress-induced cell injury. Its effects on DPN have not been proven yet, but because of the abovementioned properties it could exert beneficial effects on pathological nerve changes discussed here before. Finally, curcumin, a natural extract from *Curcuma longa* roots, exhibits antioxidant, antitumoral, and anti-inflammatory effects in experimental models [159]. It also promotes nerve regeneration and functional recovery after sciatic nerve injury in diabetic rats [160].

2.5.6. Alpha-Lipoic Acid. Also known as thioctic acid, alpha-lipoic acid is a natural compound that acts as a cofactor for major complexes in mitochondrial enzymes. It contains two thiol groups capable of being oxidized or reduced. Its reduced form is named dihydrolipoic acid and its oxidized form as ALA. It can cross the hematoencephalic barrier and regenerate other antioxidants, such as vitamin C, vitamin E, and glutathione [161, 162]. The first series of studies addressing the capacity of ALA to exert beneficial effects on DPN were called ALADIN (Alpha-Lipoic Acid in Diabetic Neuropathy). In 1995, ALADIN I 328 non-insulin-dependent diabetic patients with symptomatic peripheral neuropathy were included and randomly allocated to one of three arms of treatment with 1200, 600, or 100 mg of intravenous ALA. The results were positive with significant reduction of symptoms score when compared to placebo after 19 days of treatment and a good security profile with the dose of 600 mg/day [163]. In 1999, ALADIN II was published with results after 24 months of treatment, initially with intravenous ALA for 5 days and then orally; even when a short number of patients were evaluated (27 in group 600 mg and 18 with 1200 mg/day), they evaluated the results with NCS and reported an improvement

of sural sensory nerve conduction velocity and sural sensory nerve action potential for both arms. Tibiae motor nerve conduction velocity was only modified with 1200 mg/day [164]. Finally, in ALADIN III, 509 outpatients were tested for 6 months with 600 mg/day of ALA, with a reduction on neuropathic impairment score (NIS) but no changes in symptoms. The authors adjudged this unfavorable result possibly due to increasing intercenter variability in symptom scoring during the study [165]. ALA was included as the only etiologic treatment for DPN in the international guidelines, and some other studies have been conducted afterwards with favorable results even after 4 years of treatment [166–168].

2.5.7. Aldose Reductase Inhibitors (ARI). This group of drugs are intended to reduce the extent of polyol pathway deleterious events, by decreasing the accumulation of sorbitol in nerve fibers; however, as explained before, it is not the only pathway involved in the pathological findings of DPN [169, 170]. In experimental models a sorbitol dehydrogenase inhibitor did not have the expected results, and the effects on oxidative stress were counterproductive, by increasing MDA and 4-hydroxyalkenals, with reduced glutathione concentration [171]. Ranirestat is the most studied ARI in clinical trials. At first, in patients with mild to moderate DPN, great improvements in nerve conduction velocities above 1 m/s and vibration perception thresholds were observed, with promising results even in long-term evaluations [172, 173]; however, recent studies have been controversial, with no effect on efficacy endpoints and a mild improvement of less than 1.2 m/s in peroneal motor nerve conduction velocity with ranirestat [174] and no difference in clinical assessments compared to placebo [175].

2.5.8. Miscellaneous. L-acetyl-carnitine (ALCAR) is a derivative from the amino acid carnitine that acts as a cofactor for lipid utilization as energy, mainly in the mitochondrial electron transport chain. ALCAR promotes regeneration of peripheral nerves in DPN in experimental studies [176].

3. Conclusions

There is still a lot of research to be done to fully understand the complex pathways in which hyperglycemia alters nerve function and even more regarding therapeutic approaches to reduce inflammatory, oxidative stress, and mitochondrial dysfunction, as part of the etiologic treatment for DPN. The lack of evidence with most of the treatments for DPN is associated probably with the selection of patients, since glucose control must be achieved in order to modify the overproduction of ROS/RNS; however, out of the clinical trials, the main problem of the diabetic population is the lack of prolonged glycemic control and specially the first years after the diagnosis of type 2 DM. If a prompt euglycemia is not accomplished, little or no effect of the abovementioned therapeutic approaches will continue to appear. The following clinical trials should be performed in early diagnosed DPN, and longer periods of time are needed to make further conclusions in terms of improvements. As an opinion of the authors in this review, for clinical trials, NCS should be assessed as an objective parameter for the evaluation of the therapies.

Competing Interests

The authors declare that there are no competing interests.

References

[1] American Diabetes Association, "Microvascular complications and foot care," *Diabetes Care*, vol. 39, no. 1, pp. S72–S80, 2016.

[2] W.-T. Wang, P. Lee, H.-W. Yeh, I. V. Smirnova, and I.-Y. Choi, "Effects of acute and chronic hyperglycemia on the neurochemical profiles in the rat brain with streptozotocin-induced diabetes detected using in vivo ^{1}H MR spectroscopy at 9.4 T," *Journal of Neurochemistry*, vol. 121, no. 3, pp. 407–417, 2012.

[3] G. Negi, A. Kumar, R. P. Joshi, P. K. Ruby, and S. S. Sharma, "Oxidative stress and diabetic neuropathy: current status of antioxidants," *Institute of Integrative Omics and Applied Biotechnology Journal*, vol. 2, no. 6, pp. 71–78, 2011.

[4] S. D. M. Bandeira, L. J. S. da Fonseca, G. D. S. Guedes, L. A. Rabelo, M. O. F. Goulart, and S. M. L. Vasconcelos, "Oxidative stress as an underlying contributor in the development of chronic complications in diabetes mellitus," *International Journal of Molecular Sciences*, vol. 14, no. 2, pp. 3265–3284, 2013.

[5] M. Brownlee, "A radical explanation for glucose-induced β cell dysfunction," *Journal of Clinical Investigation*, vol. 112, no. 12, pp. 1788–1790, 2003.

[6] A. Negre-Salvayre, C. Coatrieux, C. Ingueneau, and R. Salvayre, "Advanced lipid peroxidation end products in oxidative damage to proteins. Potential role in diseases and therapeutic prospects for the inhibitors," *British Journal of Pharmacology*, vol. 153, no. 1, pp. 6–20, 2008.

[7] O. R. Ayepola, N. N. Chegou, N. L. Brooks, and O. O. Oguntibeju, "Kolaviron, a *Garcinia* biflavonoid complex ameliorates hyperglycemia-mediated hepatic injury in rats via suppression of inflammatory responses," *BMC Complementary and Alternative Medicine*, vol. 13, article 363, 2013.

[8] P. M. Rijken, J. Dekker, E. Dekker et al., "Clinical and functional correlates of foot pain in diabetic patients," *Disability and Rehabilitation*, vol. 20, no. 9, pp. 330–336, 1998.

[9] A. J. M. Boulton, A. I. Vinik, J. C. Arezzo et al., "Diabetic neuropathies: a statement by the American Diabetes Association," *Diabetes Care*, vol. 28, no. 4, pp. 956–962, 2005.

[10] A. J. M. Boulton, R. A. Malik, J. C. Arezzo, and J. M. Sosenko, "Diabetic somatic neuropathies," *Diabetes Care*, vol. 27, no. 6, pp. 1458–1486, 2004.

[11] R. Raman, A. Gupta, S. Krishna, V. Kulothungan, and T. Sharma, "Prevalence and risk factors for diabetic microvascular complications in newly diagnosed type II diabetes mellitus. Sankara Nethralaya Diabetic Retinopathy Epidemiology and Molecular Genetic Study (SN-DREAMS, report 27)," *Journal of Diabetes and Its Complications*, vol. 26, no. 2, pp. 123–128, 2012.

[12] D. Ziegler, N. Papanas, A. I. Vinik, and J. E. Shaw, "Epidemiology of polyneuropathy in diabetes and prediabetes," *Handbook of Clinical Neurology*, vol. 126, pp. 3–22, 2014.

[13] S. Soheilykhah, M. Rashidi, and F. Dehghan, "Prevalence of peripheral neuropathy in diabetic patients," *Iranian Journal of Diabetes and Obesity*, vol. 5, no. 3, pp. 107–113, 2013.

[14] M. W. Knuiman, T. A. Welborn, V. J. McCann, K. G. Stanton, and I. J. Constable, "Prevalence of diabetic complications in

relation to risk factors," *Diabetes*, vol. 35, no. 12, pp. 1332–1339, 1986.

[15] G. M. Franklin, L. B. Kahn, J. Baxter, J. A. Marshall, and R. F. Hamman, "Sensory neuropathy in non-insulin-dependent diabetes mellitus: the san luis valley diabetes study," *American Journal of Epidemiology*, vol. 131, no. 4, pp. 633–643, 1990.

[16] D. P. Walters, W. Gatling, M. A. Mullee, and R. D. Hill, "The prevalence of diabetic distal sensory neuropathy in an English community," *Diabetic Medicine*, vol. 9, no. 4, pp. 349–353, 1992.

[17] P. J. Dyck, K. M. Kratz, J. L. Karnes et al., "The prevalence by staged severity of various types of diabetic neuropathy, retinopathy, and nephropathy in a population-based cohort: the Rochester diabetic neuropathy study," *Neurology*, vol. 43, no. 4, pp. 817–824, 1993.

[18] M. Davies, S. Brophy, R. Williams, and A. Taylor, "The prevalence, severity, and impact of painful diabetic peripheral neuropathy in type 2 diabetes," *Diabetes Care*, vol. 29, no. 7, pp. 1518–1522, 2006.

[19] R. Pop-Busui, J. Lu, N. Lopes, and T. L. Z. Jones, "Prevalence of diabetic peripheral neuropathy and relation to glycemic control therapies at baseline in the BARI 2D cohort," *Journal of the Peripheral Nervous System*, vol. 14, no. 1, pp. 1–13, 2009.

[20] J. Camacho-López, "Prevalencia de neuropatía periférica en pacientes con diabetes mellitus tipo 2, atendidos en la Clínica Hospital del ISSSTE en Mazatlán, Sinaloa," *Revista de Especialidades Médico Quirúrgicas*, vol. 16, no. 2, pp. 71–74, 2011.

[21] O. Tabatabaei-Malazy, M. R. Mohajeri-Tehrani, S. P. Madani, R. Heshmat, and B. Larijani, "The prevalence of diabetic peripheral neuropathy and related factors," *Iranian Journal of Public Health*, vol. 40, no. 3, pp. 55–62, 2011.

[22] R. C. T. Ibarra, L. J. J. Rocha, O. R. Hernández, R. R. E. Nieves, and J. R. Leyva, "Prevalence of peripheral neuropathy among primary care type 2 diabetic patients," *Revista Médica de Chile*, vol. 140, no. 9, pp. 1126–1131, 2012.

[23] H. K. V. S. Kumar, S. K. Kota, A. Basile et al., "Profile of microvascular disease in type 2 diabetes in a tertiary health care hospital in india," *Annals of Medical and Health Sciences Research*, vol. 2, no. 2, pp. 103–108, 2012.

[24] A. K. Das, V. Seshiah, B. K. Sahay et al., "Improving management practices and clinical outcomes in type 2 diabetes study: prevalence of complications in people with type 2 diabetes in India," *Indian Journal of Endocrinology and Metabolism*, vol. 16, no. 2, pp. S430–S431, 2012.

[25] B. Lu, J. Hu, J. Wen et al., "Determination of peripheral neuropathy prevalence and associated factors in Chinese subjects with diabetes and pre-diabetes—ShangHai diabetic neuRopathy epidemiology and molecular genetics study (SH-DREAMS)," *PLoS ONE*, vol. 8, no. 4, Article ID e61053, 2013.

[26] M. Jaiswal, A. Lauer, C. L. Martin et al., "Peripheral neuropathy in adolescents and young adults with type 1 and type 2 diabetes from the SEARCH for diabetes in youth follow-up cohort: a pilot study," *Diabetes Care*, vol. 36, no. 12, pp. 3903–3908, 2013.

[27] A. Ali, F. Iqbal, A. Taj, Z. Iqbal, M. J. Amin, and Q. Z. Iqbal, "Prevalence of microvascular complications in newly diagnosed patients with type 2 diabetes," *Pakistan Journal of Medical Sciences*, vol. 29, no. 4, pp. 899–902, 2013.

[28] J. Kiani, A. Moghimbeigi, H. Azizkhani, and S. Kosarifard, "The prevalence and associated risk factors of peripheral diabetic neuropathy in Hamedan, Iran," *Archives of Iranian Medicine*, vol. 16, no. 1, pp. 17–19, 2013.

[29] D. Bansal, K. Gudala, H. Muthyala, H. P. Esam, R. Nayakallu, and A. Bhansali, "Prevalence and risk factors of development of peripheral diabetic neuropathy in type 2 diabetes mellitus in a tertiary care setting," *Journal of Diabetes Investigation*, vol. 5, no. 6, pp. 714–721, 2014.

[30] K. Kung, K. M. Chow, E. M. Hui et al., "Prevalence of complications among Chinese diabetic patients in urban primary care clinics: a cross-sectional study," *BMC Family Practice*, vol. 15, article 8, 2014.

[31] S. H. Assaad-Khalil, A. Zaki, A. A. Rehim et al., "Prevalence of diabetic foot disorders and related risk factors among Egyptian subjects with diabetes," *Primary Care Diabetes*, vol. 9, no. 4, pp. 297–303, 2015.

[32] L. Li, J. Chen, J. Wang, and D. Cai, "Prevalence and risk factors of diabetic peripheral neuropathy in type 2 diabetes mellitus patients with overweight/obese in Guangdong province, China," *Primary Care Diabetes*, vol. 9, no. 3, pp. 191–195, 2015.

[33] C. Kuate-Tegueu, E. Temfack, S. Ngankou, J. Doumbe, V. P. Djientcheu, and A. P. Kengne, "Prevalence and determinants of diabetic polyneuropathy in a sub-Saharan African referral hospital," *Journal of the Neurological Sciences*, vol. 355, no. 1-2, pp. 108–112, 2015.

[34] M. Olamoyegun, W. Ibraheem, S. Iwuala, M. Audu, and B. Kolawole, "Burden and pattern of micro vascular complications in type 2 diabetes in a tertiary health institution in Nigeria," *African Health Sciences*, vol. 15, no. 4, pp. 1136–1141, 2015.

[35] M. De Los Angeles Lazo, A. Bernabé-Ortiz, M. E. Pinto et al., "Diabetic peripheral neuropathy in ambulatory patients with type 2 diabetes in a general hospital in a middle income country: a cross-sectional study," *PLoS ONE*, vol. 9, no. 5, Article ID e95403, 2014.

[36] Y. Jiang, X. Ran, L. Jia et al., "Epidemiology of type 2 diabetic foot problems and predictive factors for amputation in China," *The International Journal of Lower Extremity Wounds*, vol. 14, no. 1, pp. 19–27, 2015.

[37] M. Kulshrestha, S. Seth, A. Tripathi, A. Seth, and A. Kumar, "Prevalence of complications and clinical audit of management of type 2 diabetes mellitus: a prospective hospital based study," *Journal of Clinical and Diagnostic Research*, vol. 9, no. 11, pp. OC25–OC28, 2015.

[38] D. Wu, Y. Xuan, Y. Ruan et al., "Prevalence of macro- and microvascular complications in patients with type 2 diabetes and kidney disease with or without albuminuria in a single Chinese Diabetes Centre," *Diabetes & Vascular Disease Research*, vol. 13, no. 1, pp. 21–30, 2016.

[39] L. Salvotelli, V. Stoico, F. Perrone et al., "Prevalence of neuropathy in type 2 diabetic patients and its association with other diabetes complications: the Verona Diabetic Foot Screening Program," *Journal of Diabetes and Its Complications*, vol. 29, no. 8, pp. 1066–1070, 2015.

[40] M. D'Souza, V. Kulkarni, U. Bhaskaran et al., "Diabetic peripheral neuropathy and its determinants among patients attending a tertiary health care centre in Mangalore, India," *Journal of Public Health Research*, vol. 4, no. 2, p. 450, 2015.

[41] E. M. Khedr, G. Fawi, M. A. Allah Abbas et al., "Prevalence of diabetes and diabetic neuropathy in Qena Governorate: population-based survey," *Neuroepidemiology*, vol. 46, no. 3, pp. 173–181, 2016.

[42] M. C. R. Parisi, N. A. Moura, and F. H. Menezes, "Baseline characteristics and risk factors for ulcer, amputation and severe neuropathy in diabetic foot at risk: the BRAZUPA study," *Diabetology & Metabolic Syndrome*, vol. 25, article 8, 2016.

[43] S. Popescu, B. Timar, F. Baderca et al., "Age as an independent factor for the development of neuropathy in diabetic patients," *Clinical Interventions in Aging*, pp. 313–318, 2016.

[44] C. Herder, B. W. C. Bongaerts, W. Rathmann et al., "Association of subclinical inflammation with polyneuropathy in the older population: KORA F4 Study," *Diabetes Care*, vol. 36, no. 11, pp. 3663–3670, 2013.

[45] E. Alonso-Morán, J. F. Orueta, J. I. F. Esteban et al., "The prevalence of diabetes-related complications and multimorbidity in the population with type 2 diabetes mellitus in the Basque Country," *BMC Public Health*, vol. 14, article 1059, 2014.

[46] K. Kostev, A. Jockwig, A. Hallwachs, and W. Rathmann, "Prevalence and risk factors of neuropathy in newly diagnosed type 2 diabetes in primary care practices: a retrospective database analysis in Germany and UK," *Primary Care Diabetes*, vol. 8, no. 3, pp. 250–255, 2014.

[47] C. A. Abbott, R. A. Malik, E. R. E. Van Ross, J. Kulkarni, and A. J. M. Boulton, "Prevalence and characteristics of painful diabetic neuropathy in a large community-based diabetic population in the U.K," *Diabetes Care*, vol. 34, no. 10, pp. 2220–2224, 2011.

[48] P. J. Dyck, W. J. Litchy, K. A. Lehman, J. L. Hokanson, P. A. Low, and P. C. O'Brien, "Variables influencing neuropathic endpoints: the Rochester Diabetic Neuropathy Study of Healthy Subjects," *Neurology*, vol. 45, no. 6, pp. 1115–1121, 1995.

[49] M. H. Rivner, T. R. Swift, B. O. Crout, and K. P. Rhodes, "Toward more rational nerve conduction interpretations: the effect of height," *Muscle and Nerve*, vol. 13, no. 3, pp. 232–239, 1990.

[50] J. D. England, G. S. Gronseth, G. Franklin et al., "Distal symmetric polyneuropathy: a definition for clinical research—report of the American Academy of Neurology, the American Association of Electrodiagnostic Medicine, and the American Academy of Physical Medicine and Rehabilitation," *Neurology*, vol. 64, no. 2, pp. 199–207, 2005.

[51] L. Kärvestedt, E. Mårtensson, V. Grill et al., "Peripheral sensory neuropathy associates with micro- or microangiopathy: results from a population-based study of type 2 diabetic patients in Sweden," *Diabetes Care*, vol. 32, no. 2, pp. 317–322, 2009.

[52] R. Inoue, M. Sumitani, T. Yasuda et al., "Independent risk factors for positive and negative symptoms in patients with diabetic polyneuropathy," *Journal of Pain & Palliative Care Pharmacotherapy*, vol. 30, no. 3, pp. 178–183, 2016.

[53] P. J. Dyck, J. L. Davies, D. M. Wilson, F. J. Service, L. J. Melton III, and P. C. O'Brien, "Risk factors for severity of diabetic polyneuropathy: intensive longitudinal assessment of the Rochester Diabetic Neuropathy Study cohort," *Diabetes Care*, vol. 22, no. 9, pp. 1479–1486, 1999.

[54] W. J. Lee, S. Jang, S. H. Lee, and H. Lee, "Correlation between the severity of diabetic peripheral polyneuropathy and glycosylated hemoglobin levels: a quantitative study," *Annals of Rehabilitation Medicine*, vol. 40, no. 2, pp. 263–270, 2016.

[55] J. Ybarra-Muñoz, J. Jurado-Campos, M. Garcia-Gil et al., "Cardiovascular disease predicts diabetic peripheral polyneuropathy in subjects with type 2 diabetes: A 10-year Prospective Study," *European Journal of Cardiovascular Nursing*, vol. 15, no. 4, pp. 248–254, 2016.

[56] P. J. Dyck, A. Lais, J. L. Karnes et al., "Fiber loss is a primary and multifocal in sural nerves in diabetic polyneuropathy," *Annals of Neurology*, vol. 10, pp. 13–18, 1973.

[57] P. J. Dyck and C. Giannini, "Pathologic alterations in the diabetic neuropathies of humans: a review," *Journal of Neuropathology & Experimental Neurology*, vol. 55, no. 12, pp. 1181–1193, 1996.

[58] P. J. Dyck, S. Hansen, J. Karnes et al., "Capillary number and percentage closed in human diabetic sural nerve," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 82, no. 8, pp. 2513–2517, 1985.

[59] C. Giannini and P. J. Dyck, "Ultrastructural morphometric abnormalities of sural nerve endoneurial microvessels in diabetes mellitus," *Annals of Neurology*, vol. 36, no. 3, pp. 408–415, 1994.

[60] E. Zherebitskaya, E. Akude, D. R. Smith, and P. Fernyhough, "Development of selective axonopathy in adult sensory neurons isolated from diabetic rats: role of glucose-induced oxidative stress," *Diabetes*, vol. 58, no. 6, pp. 1356–1364, 2009.

[61] M. Karin, "The regulation of AP-1 activity by mitogen-activated protein kinases," *Journal of Biological Chemistry*, vol. 270, no. 28, pp. 16483–16486, 1995.

[62] G.-H. Liu, J. Qu, and X. Shen, "NF-κB/p65 antagonizes Nrf2-ARE pathway by depriving CBP from Nrf2 and facilitating recruitment of HDAC3 to MafK," *Biochimica et Biophysica Acta—Molecular Cell Research*, vol. 1783, no. 5, pp. 713–727, 2008.

[63] V. Ganesh Yerra, G. Negi, S. S. Sharma, and A. Kumar, "Potential therapeutic effects of the simultaneous targeting of the Nrf2 and NF-κB pathways in diabetic neuropathy," *Redox Biology*, vol. 1, no. 1, pp. 394–397, 2013.

[64] I. Shimomura, T. Funahashi, and Y. Matsuzawa, "Significance of adipocytokine, fat-derived hormones, in metabolic syndrome," *Tanpakushitsu kakusan koso. Protein, nucleic acid, enzyme*, vol. 47, no. 14, pp. 1896–1903, 2002.

[65] A. Adamska, A. Nikołajuk, M. Karczewska-Kupczewska et al., "Relationships between serum adiponectin and soluble TNF-α receptors and glucose and lipid oxidation in lean and obese subjects," *Acta Diabetologica*, vol. 49, no. 1, pp. 17–24, 2012.

[66] U. Salmenniemi, J. Zacharova, E. Ruotsalainen et al., "Association of adiponectin level and variants in the adiponectin gene with glucose metabolism, energy expenditure, and cytokines in offspring of type 2 diabetic patients," *Journal of Clinical Endocrinology and Metabolism*, vol. 90, no. 7, pp. 4216–4223, 2005.

[67] M. Matsuda, F. Kawasaki, H. Inoue et al., "Possible contribution of adipocytokines on diabetic neuropathy," *Diabetes Research and Clinical Practice*, vol. 66, supplement, pp. S121–S123, 2004.

[68] C. Herder, M. Lankisch, D. Ziegler et al., "Subclinical inflammation and diabetic polyneuropathy," *Diabetes Care*, vol. 32, no. 4, pp. 680–682, 2009.

[69] I. Schamarek, C. Herder, B. Nowotny et al., "Adiponectin, markers of subclinical inflammation and nerve conduction in individuals with recently diagnosed type 1 and type 2 diabetes," *European Journal of Endocrinology*, vol. 174, no. 4, pp. 433–443, 2016.

[70] G. Hussain, S. A. A. Rizvi, S. Singhal, M. Zubair, and J. Ahmad, "Serum levels of TNF-α in peripheral neuropathy patients and its correlation with nerve conduction velocity in type 2 diabetes mellitus," *Diabetes and Metabolic Syndrome: Clinical Research and Reviews*, vol. 7, no. 4, pp. 238–242, 2013.

[71] H. Vlassara, W. Cai, J. Crandall et al., "Inflammatory mediators are induced by dietary glycotoxins, a major risk factor for diabetic angiopathy," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 99, no. 24, pp. 15596–15601, 2002.

[72] H. Vlassara and G. E. Striker, "Advanced Glycation Endproducts in Diabetes and Diabetic Complications," *Endocrinology and*

Metabolism Clinics of North America, vol. 42, no. 4, pp. 697–719, 2013.

[73] M. Brownlee, "Biochemistry and molecular cell biology of diabetic complications," *Nature*, vol. 414, no. 6865, pp. 813–820, 2001.

[74] X. M. Leverve, B. Guigas, D. Detaille et al., "Mitochondrial metabolism and type-2 diabetes: a specific target of metformin," *Diabetes and Metabolism*, vol. 29, no. 4, pp. 6588–6594, 2003.

[75] Z. Zhang, C. W. Liew, D. E. Handy et al., "High glucose inhibits glucose-6-phosphate dehydrogenase, leading to increased oxidative stress and β-cell apoptosis," *FASEB Journal*, vol. 24, no. 5, pp. 1497–1505, 2010.

[76] M. G. Buse, "Hexosamines, insulin resistance, and the complications of diabetes: current status," *American Journal of Physiology—Endocrinology and Metabolism*, vol. 290, no. 1, pp. E1–E8, 2006.

[77] F. I. Comer and G. W. Hart, "Reciprocity between O-GlcNAc and O-phosphate on the carboxyl terminal domain of RNA polymerase II," *Biochemistry*, vol. 40, no. 26, pp. 7845–7852, 2001.

[78] M. Federici, R. Menghini, A. Mauriello et al., "Insulin-dependent activation of endothelial nitric oxide synthase is impaired by O-linked glycosylation modification of signaling proteins in human coronary endothelial cells," *Circulation*, vol. 106, no. 4, pp. 466–472, 2002.

[79] X.-L. Du, D. Edelstein, L. Rossetti et al., "Hyperglycemia-induced mitochondrial superoxide overproduction activates the hexosamine pathway and induces plasminogen activator inhibitor-1 expression by increasing Sp1 glycosylation," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 97, no. 22, pp. 12222–12226, 2000.

[80] K. V. Ramana, B. Friedrich, R. Tammali, M. B. West, A. Bhatnagar, and S. K. Srivastava, "Requirement of aldose reductase for the hyperglycemic activation of protein kinase C and formation of diacylglycerol in vascular smooth muscle cells," *Diabetes*, vol. 54, no. 3, pp. 818–829, 2005.

[81] K. H. Gabbay, L. O. Merola, and R. A. Field, "Sorbitol pathway: presence in nerve and cord with substrate accumulation in diabetes," *Science*, vol. 151, no. 3707, pp. 209–210, 1966.

[82] M. A. Yorek, J. A. Dunlap, and B. H. Ginsberg, "myo-Inositol metabolism in 41A3 neuroblastoma cells: Effects of high glucose and sorbitol levels," *Journal of Neurochemistry*, vol. 48, no. 1, pp. 53–61, 1987.

[83] G. Majno and M. L. Karnovsky, "A biochemical and morphologic study of myelination and demyelination: II. Lipogenesis in vitro by rat nerves following transection," *Journal of Experimental Medicine*, vol. 108, no. 2, pp. 197–214, 1958.

[84] D. A. Greene, S. Yagihashi, S. A. Lattimer, and A. A. Sima, "Nerve Na+-K+-ATPase, conduction, and myo-inositol in the insulin-deficient BB rat," *The American Journal of Physiology*, vol. 247, no. 4, pp. E534–E539, 1984.

[85] M. Brownlee, "Advanced protein glycosylation in diabetes and aging," *Annual Review of Medicine*, vol. 46, pp. 223–234, 1995.

[86] B. Duran-Jimenez, D. Dobler, S. Moffatt et al., "Advanced glycation end products in extracellular matrix proteins contribute to the failure of sensory nerve regeneration in diabetes," *Diabetes*, vol. 58, no. 12, pp. 2893–2903, 2009.

[87] S.-Y. Goh and M. E. Cooper, "The role of advanced glycation end products in progression and complications of diabetes," *Journal of Clinical Endocrinology and Metabolism*, vol. 93, no. 4, pp. 1143–1152, 2008.

[88] W. Cai, M. Ramdas, L. Zhu, X. Chen, G. E. Striker, and H. Vlassara, "Oral advanced glycation endproducts (AGEs) promote insulin resistance and diabetes by depleting the antioxidant defenses AGE receptor-1 and sirtuin 1," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 109, no. 39, pp. 15888–15893, 2012.

[89] M. Valko, D. Leibfritz, J. Moncol, M. T. D. Cronin, M. Mazur, and J. Telser, "Free radicals and antioxidants in normal physiological functions and human disease," *International Journal of Biochemistry and Cell Biology*, vol. 39, no. 1, pp. 44–84, 2007.

[90] J. W. Russell, D. Golovoy, A. M. Vincent et al., "High glucose-induced oxidative stress and mitochondrial dysfunction in neurons," *FASEB Journal*, vol. 16, no. 13, pp. 1738–1748, 2002.

[91] J. Cederberg, S. Basu, and U. J. Eriksson, "Increased rate of lipid peroxidation and protein carbonylation in experimental diabetic pregnancy," *Diabetologia*, vol. 44, no. 6, pp. 766–774, 2001.

[92] D. Ziegler, C. G. H. Sohr, and J. Nourooz-Zadeh, "Oxidative stress and antioxidant defense in relation to the severity of diabetic polyneuropathy and cardiovascular autonomic neuropathy," *Diabetes Care*, vol. 27, no. 9, pp. 2178–2183, 2004.

[93] E. Niki, Y. Yoshida, Y. Saito, and N. Noguchi, "Lipid peroxidation: mechanisms, inhibition, and biological effects," *Biochemical and Biophysical Research Communications*, vol. 338, no. 1, pp. 668–676, 2005.

[94] M. A. Carbonneau, E. Penchant, D. Sess, P. Canioni, and M. Clerc, "Free and bound malondialdehyde measured as thiobarbituric acid adduct by HPLC in serum and plasma," *Clinical Chemistry*, vol. 37, no. 8, pp. 1423–1429, 1991.

[95] F. N. Ahmed, F. N. Naqvi, and F. Shafiq, "Lipid peroxidation and serum antioxidant enzymes in patients with type 2 diabetes mellitus," *Annals of the New York Academy of Sciences*, vol. 1084, pp. 481–489, 2006.

[96] K. N. Kalaivanam, M. Dharmalingam, and S. R. Marcus, "Lipid peroxidation in type 2 diabetes mellitus," *International Journal of Diabetes in Developing Countries*, vol. 26, no. 1, pp. 30–32, 2006.

[97] A. Aydin, H. Orhan, A. Sayal, M. Özata, G. Şahin, and A. Işımer, "Oxidative stress and nitric oxide related parameters in type II diabetes mellitus: effects of glycemic control," *Clinical Biochemistry*, vol. 34, no. 1, pp. 65–70, 2001.

[98] M. Mahboob, M. F. Rahman, and P. Grover, "Serum lipid peroxidation and antioxidant enzyme levels in male and female diabetic patients," *Singapore Medical Journal*, vol. 46, no. 7, pp. 322–324, 2005.

[99] N. A. El Boghdady and G. A. Badr, "Evaluation of oxidative stress markers and vascular risk factors in patients with diabetic peripheral neuropathy," *Cell Biochemistry and Function*, vol. 30, no. 4, pp. 328–334, 2012.

[100] R. Srivatsan, S. Das, R. Gadde et al., "Antioxidants and lipid peroxidation status in diabetic patients with and without complications," *Archives of Iranian Medicine*, vol. 12, no. 2, pp. 121–127, 2009.

[101] M. P. Murphy, "How mitochondria produce reactive oxygen species," *Biochemical Journal*, vol. 417, no. 1, pp. 1–13, 2009.

[102] R. S. Balaban, S. Nemoto, and T. Finkel, "Mitochondria, oxidants, and aging," *Cell*, vol. 120, no. 4, pp. 483–495, 2005.

[103] A. Naudi, M. Jove, V. Ayala et al., "Cellular dysfunction in diabetes as maladaptive response to mitochondrial oxidative stress," *Experimental Diabetes Research*, vol. 2012, Article ID 696215, 14 pages, 2012.

[104] C. R. Cashman and A. Höke, "Mechanisms of distal axonal degeneration in peripheral neuropathies," *Neuroscience Letters*, vol. 596, pp. 33–50, 2015.

[105] D. Metodiewa and C. Kośka, "Reactive oxygen species and reactive nitrogen species: relevance to cyto(neuro)toxic events and neurologic disorders. An overview," *Neurotoxicity Research*, vol. 1, no. 3, pp. 197–233, 1999.

[106] S. Tewari, J. M. Santos, and R. A. Kowluru, "Damaged mitochondrial DNA replication system and the development of diabetic retinopathy," *Antioxidants and Redox Signaling*, vol. 17, no. 3, pp. 492–504, 2012.

[107] A. M. Vincent, M. Brownlee, and J. W. Russell, "Oxidative stress and programmed cell death in diabetic neuropathy," *Annals of the New York Academy of Sciences*, vol. 959, pp. 368–383, 2002.

[108] E. Akude, E. Zherebitskaya, S. K. R. Chowdhury, D. R. Smith, R. T. Dobrowsky, and P. Fernyhough, "Diminished superoxide generation is associated with respiratory chain dysfunction and changes in the mitochondrial proteome of sensory neurons from diabetic rats," *Diabetes*, vol. 60, no. 1, pp. 288–297, 2011.

[109] J.-W. G. Meijer, E. Bosma, J. D. Lefrandt et al., "Clinical diagnosis of diabetic polyneuropathy with the diabetic neuropathy symptom and diabetic neuropathy examination scores," *Diabetes Care*, vol. 26, no. 3, pp. 697–701, 2003.

[110] S. Tesfaye, A. J. M. Boulton, P. J. Dyck et al., "Diabetic neuropathies: update on definitions, diagnostic criteria, estimation of severity, and treatments," *Diabetes Care*, vol. 33, no. 12, p. 2725, 2010.

[111] P. J. Dyck, "Detection, characterization, and staging of polyneuropathy: assessed in diabetics," *Muscle and Nerve*, vol. 11, no. 1, pp. 21–32, 1988.

[112] S. Tesfaye, A. J. Boulton, P. J. Dyck et al., "Diabetic neuropathies: update on definitions, diagnostic criteria, estimation of severity, and treatments," *Diabetes Care*, vol. 33, no. 10, pp. 2285–2293, 2010.

[113] M. Lunetta, R. Le Moli, G. Grasso, and L. Sangiorgio, "A simplified diagnostic test for ambulatory screening of peripheral diabetic neuropathy," *Diabetes Research and Clinical Practice*, vol. 39, no. 3, pp. 165–172, 1998.

[114] A. Moghtaderi, A. Bakhshipour, and H. Rashidi, "Validation of Michigan neuropathy screening instrument for diabetic peripheral neuropathy," *Clinical Neurology and Neurosurgery*, vol. 108, no. 5, pp. 477–481, 2006.

[115] V. Bril and B. A. Perkins, "Validation of the Toronto clinical scoring system for diabetic polyneuropathy," *Diabetes Care*, vol. 25, no. 11, pp. 2048–2052, 2002.

[116] P. J. Dyck, J. L. Davies, W. J. Litchy, and P. C. O'Brien, "Longitudinal assessment of diabetic polyneuropathy using a composite score in the Rochester Diabetic Neuropathy Study cohort," *Neurology*, vol. 49, no. 1, pp. 229–239, 1997.

[117] M. Al-Geffari, "Comparison of different screening tests for diagnosis of diabetic peripheral neuropathy in Primary Health Care setting," *International Journal of Health Sciences*, vol. 6, no. 2, pp. 127–134, 2012.

[118] E. L. Feldman, M. J. Stevens, P. K. Thomas, M. B. Brown, N. Canal, and D. A. Greene, "A practical two-step quantitative clinical and electrophysiological assessment for the diagnosis and staging of diabetic neuropathy," *Diabetes Care*, vol. 17, no. 11, pp. 1281–1289, 1994.

[119] Q. Xiong, B. Lu, H. Ye, X. Wu, T. Zhang, and Y. Li, "The diagnostic value of neuropathy symptom and change score, neuropathy impairment score and Michigan neuropathy screening instrument for diabetic peripheral neuropathy," *European Neurology*, vol. 74, no. 5-6, pp. 323–327, 2015.

[120] F. Liu, J. P. Mao, and X. Yan, "Toronto clinical scoring system in diabetic peripheral neuropathy," *Zhong Nan Da Xue Xue Bao Yi Xue Ban*, vol. 33, no. 12, pp. 1137–1141, 2008.

[121] J. Shakher and M. J. Stevens, "Update on the management of diabetic polyneuropathies," *Diabetes, Metabolic Syndrome and Obesity: Targets and Therapy*, vol. 4, pp. 289–305, 2011.

[122] J. H. B. Scarpello and H. C. S. Howlett, "Metformin therapy and clinical uses," *Diabetes and Vascular Disease Research*, vol. 5, no. 3, pp. 157–167, 2008.

[123] C. J. Dunn and D. H. Peters, "Metformin. A review of its pharmacological properties and therapeutic use in non-insulin-dependent diabetes mellitus," *Drugs*, vol. 49, no. 5, pp. 721–749, 1995.

[124] B. Guigas, D. Detaille, C. Chauvin et al., "Metformin inhibits mitochondrial permeability transition and cell death: a pharmacological in vitro study," *Biochemical Journal*, vol. 382, no. 3, pp. 877–884, 2004.

[125] D. Detaille, B. Guigas, C. Chauvin et al., "Metformin prevents high-glucose-induced endothelial cell death through a mitochondrial permeability transition-dependent process," *Diabetes*, vol. 54, no. 7, pp. 2179–2187, 2005.

[126] A. Kooy, J. De Jager, P. Lehert et al., "Long-term effects of metformin on metabolism and microvascular and macrovascular disease in patients with type 2 diabetes mellitus," *Archives of Internal Medicine*, vol. 169, no. 6, pp. 616–625, 2009.

[127] A. Hasanvand, H. Amini-Khoei, M. R. Hadian et al., "Anti-inflammatory effect of AMPK signaling pathway in rat model of diabetic neuropathy," *Inflammopharmacology*, vol. 24, no. 5, pp. 207–219, 2016.

[128] K. S. Akinlade, S. O. Agbebaku, S. K. Rahamon, and W. O. Balogun, "Vitamin B_{12} levels in patients with type 2 diabetes mellitus on metformin," *Annals of Ibadan Postgraduate Medicine*, vol. 13, no. 2, pp. 79–83, 2015.

[129] M. A. Ahmed, G. Muntingh, and P. Rheeder, "Vitamin B12 deficiency in metformin-treated type-2 diabetes patients, prevalence and association with peripheral neuropathy," *BMC Pharmacology and Toxicology*, vol. 17, article 44, 2016.

[130] G. T. Russo, A. Giandalia, E. L. Romeo et al., "Diabetic neuropathy is not associated with homocysteine, folate, vitamin B12 levels, and MTHFR C677T mutation in type 2 diabetic outpatients taking metformin," *Journal of Endocrinological Investigation*, vol. 39, no. 3, pp. 305–314, 2016.

[131] D. M. de Groot-Kamphuis, P. R. van Dijk, K. H. Groenier, S. T. Houweling, H. J. G. Bilo, and N. Kleefstra, "Vitamin B12 deficiency and the lack of its consequences in type 2 diabetes patients using metformin," *Netherlands Journal of Medicine*, vol. 71, no. 7, pp. 386–390, 2013.

[132] R. P. Roy, K. Ghosh, M. Ghosh et al., "Study of Vitamin B_{12} deficiency and peripheral neuropathy in metformin-treated early Type 2 diabetes mellitus," *Indian Journal of Endocrinology and Metabolism*, vol. 20, no. 5, pp. 631–637, 2016.

[133] S. Mennickent, M. Bravo, C. Calvo, and M. Avello, "Pleiotropic effects of statins," *Revista Medica de Chile*, vol. 136, no. 6, pp. 775–782, 2008.

[134] T. M. E. Davis, B. B. Yeap, W. A. Davis, and D. G. Bruce, "Lipid-lowering therapy and peripheral sensory neuropathy in type 2 diabetes: The Fremantle Diabetes Study," *Diabetologia*, vol. 51, no. 4, pp. 562–566, 2008.

[135] G. Villegas-Rivera, L. M. Román-Pintos, E. G. Cardona-Muñoz et al., "Effects of ezetimibe/simvastatin and rosuvastatin on oxidative stress in diabetic neuropathy: a randomized, double-blind, placebo-controlled clinical trial," *Oxidative Medicine and Cellular Longevity*, vol. 2015, Article ID 756294, 10 pages, 2015.

[136] A. E. P. Pesaro, C. V. Serrano Jr., J. L. Fernandes et al., "Pleiotropic effects of ezetimibe/simvastatin vs. high dose simvastatin," *International Journal of Cardiology*, vol. 158, no. 3, pp. 400–404, 2012.

[137] S. T. Rasmussen, J. T. Andersen, T. K. Nielsen et al., "Simvastatin and oxidative stress in humans: a randomized, double-blinded, placebo-controlled clinical trial," *Redox Biology*, vol. 9, pp. 32–38, 2016.

[138] M. S. Kostapanos, A. T. Spyrou, C. C. Tellis et al., "Ezetimibe treatment lowers indicators of oxidative stress in hypercholesterolemic subjects with high oxidative stress," *Lipids*, vol. 46, no. 4, pp. 341–348, 2011.

[139] J. Hernández-Ojeda, L. M. Román-Pintos, A. D. Rodríguez-Carrízalez et al., "Effect of rosuvastatin on diabetic polyneuropathy: A Randomized, Double-Blind, Placebo-Controlled Phase IIa Study," *Diabetes, Metabolic Syndrome & Obesity: Targets and Therapy*, vol. 7, pp. 401–407, 2014.

[140] M. Koksal, M. A. Eren, M. N. Turan, and T. Sabuncu, "The effects of atorvastatin and rosuvastatin on oxidative stress in diabetic patients," *European Journal of Internal Medicine*, vol. 22, no. 3, pp. 249–253, 2011.

[141] N. Sharma, J.-L. Ooi, J. Ong, and D. Newman, "The use of fenofibrate in the management of patients with diabetic retinopathy: an evidence-based review," *Australian Family Physician*, vol. 44, no. 6, pp. 367–370, 2015.

[142] Y. R. Cho, J. H. Lim, M. Y. Kim et al., "Therapeutic effects of fenofibrate on diabetic peripheral neuropathy by improving endothelial and neural survival in *db/db* mice," *PLoS ONE*, vol. 9, no. 1, Article ID 0083204, pp. 1–12, 2014.

[143] R. Simó, O. Simó-Servat, and C. Hernández, "Is Fenofibrate a Reasonable Treatment for Diabetic Microvascular Disease?" *Current Diabetes Reports*, vol. 15, no. 24, 2015.

[144] G. Lenaz, R. Fato, G. Formiggini, and M. L. Genova, "The role of Coenzyme Q in mitochondrial electron transport," *Mitochondrion*, vol. 7, pp. S8–S33, 2007.

[145] M. Ayaz, S. Tuncer, N. Okudan, and H. Gökbel, "Coenzyme Q10 and α-lipoic acid supplementation in diabetic rats: conduction velocity distributions," *Methods and Findings in Experimental and Clinical Pharmacology*, vol. 30, no. 5, pp. 367–374, 2008.

[146] E. A. El-ghoroury, H. M. Raslan, E. A. Badawy et al., "Malondialdehyde and coenzyme Q10 in platelets and serum in type 2 diabetes mellitus: correlation with glycemic control," *Blood Coagulation & Fibrinolysis*, vol. 20, no. 4, pp. 248–251, 2009.

[147] J. Hernández-Ojeda, E. G. Cardona-Muñoz, L. M. Román-Pintos et al., "The effect of ubiquinone in diabetic polyneuropathy: a randomized double-blind placebo-controlled study," *Journal of Diabetes and Its Complications*, vol. 26, no. 4, pp. 352–358, 2012.

[148] M. Akbari Fakhrabadi, A. Zeinali Ghotrom, H. Mozaffari-Khosravi, H. H. Nodoushan, and A. Nadjarzadeh, "Effect of Coenzyme Q10 on oxidative stress, glycemic control and inflammation in diabetic neuropathy: a double blind randomized clinical Trial," *International Journal for Vitamin and Nutrition Research*, vol. 84, no. 5-6, pp. 252–260, 2014.

[149] Z. Bahadoran, P. Mirmiran, and F. Azizi, "Dietary polyphenols as potential nutraceuticals in management of diabetes: a review," *Journal of Diabetes and Metabolic Disorders*, vol. 12, no. 1, article no. 43, 2013.

[150] Y. Ding, X. Dai, Y. Jiang, Z. Zhang, and Y. Li, "Functional and morphological effects of grape seed proanthocyanidins on peripheral neuropathy in rats with type 2 diabetes mellitus," *Phytotherapy Research*, vol. 28, no. 7, pp. 1082–1087, 2014.

[151] Y. Ding, X. Dai, Z. Zhang et al., "Proanthocyanidins protect against early diabetic peripheral neuropathy by modulating endoplasmic reticulum stress," *Journal of Nutritional Biochemistry*, vol. 25, no. 7, pp. 765–772, 2014.

[152] P. Valensi, C. Le Devehat, J.-L. Richard et al., "A multicenter, double-blind, safety study of QR-333 for the treatment of symptomatic diabetic peripheral neuropathy. A preliminary report," *Journal of Diabetes and Its Complications*, vol. 19, no. 5, pp. 247–253, 2005.

[153] K. D. R. Setchell, E. Nardi, P.-M. Battezzati et al., "Novel soy germ pasta enriched in isoflavones ameliorates gastroparesis in type 2 diabetes: a pilot study," *Diabetes Care*, vol. 36, no. 11, pp. 3495–3497, 2013.

[154] J. Wu, X. Zhang, and B. Zhang, "Efficacy and safety of puerarin injection in treatment of diabetic peripheral neuropathy: a systematic review and Meta-analysis of randomized controlled trials," *Journal of Traditional Chinese Medicine*, vol. 34, no. 4, pp. 401–410, 2014.

[155] C.-H. Lee, T.-S. Jeong, Y.-K. Choi et al., "Anti-atherogenic effect of citrus flavonoids, naringin and naringenin, associated with hepatic ACAT and aortic VCAM-1 and MCP-1 in high cholesterol-fed rabbits," *Biochemical and Biophysical Research Communications*, vol. 284, no. 3, pp. 681–688, 2001.

[156] S. S. Al-Rejaie, A. M. Aleisa, H. M. Abuohashish et al., "Naringenin neutralises oxidative stress and nerve growth factor discrepancy in experimental diabetic neuropathy," *Neurological Research*, vol. 37, no. 10, pp. 924–933, 2015.

[157] P. Hasanein and F. Fazeli, "Role of naringenin in protection against diabetic hyperalgesia and tactile allodynia in male Wistar rats," *Journal of Physiology and Biochemistry*, vol. 70, no. 4, pp. 997–1006, 2014.

[158] Y. Huang, S.-Y. Tsang, X. Yao, and Z.-Y. Chen, "Biological properties of baicalein in cardiovascular system," *Current Drug Targets—Cardiovascular and Haematological Disorders*, vol. 5, no. 2, pp. 177–184, 2005.

[159] H. Zhou, C. S. Beevers, and S. Huang, "The targets of curcumin," *Current Drug Targets*, vol. 12, no. 3, pp. 332–347, 2011.

[160] J. Ma, H. Yu, J. Liu, Y. Chen, Q. Wang, and L. Xiang, "Curcumin promotes nerve regeneration and functional recovery after sciatic nerve crush injury in diabetic rats," *Neuroscience Letters*, vol. 610, pp. 139–143, 2016.

[161] R. S. Sebastian, L. E. Cleveland, J. D. Goldman, and A. J. Moshfegh, "Older adults who use vitamin/mineral supplements differ from nonusers in nutrient intake adequacy and dietary attitudes," *Journal of the American Dietetic Association*, vol. 107, no. 8, pp. 1322–1332, 2007.

[162] A. R. Smith, S. V. Shenvi, M. Widlansky, J. H. Suh, and T. M. Hagen, "Lipoic acid as a potential therapy for chronic diseases associated with oxidative stress," *Current Medicinal Chemistry*, vol. 11, no. 9, pp. 1135–1146, 2004.

[163] D. Ziegler, M. Hanefeld, K. J. Ruhnau et al., "Treatment of symptomatic diabetic peripheral neuropathy with the antioxidant α-lipoic acid - A 3-week multicentre randomized controlled trial (ALADIN Study)," *Diabetologia*, vol. 38, no. 12, pp. 1425–1433, 1995.

[164] M. Reljanovic, G. Reichel, K. Rett et al., "Treatment of diabetic polyneuropathy with the antioxidant thioctic acid (α-lipoic acid): a two year multicenter randomized double-blind placebo-controlled trial (ALADIN II)," *Free Radical Research*, vol. 31, no. 3, pp. 171–179, 1999.

[165] D. Ziegler, M. Hanefeld, K.-J. Ruhnau et al., "Treatment of symptomatic diabetic polyneuropathy with the antioxidant α-lipoic acid: a 7-month multicenter randomized controlled trial (ALADIN III study)," *Diabetes Care*, vol. 22, no. 8, pp. 1296–1301, 1999.

[166] A. S. Ametov, A. Barinov, P. J. Dyck et al., "The sensory symptoms of diabetic polyneuropathy are improved with α-lipoic acid. The SYDNEY Trial," *Diabetes Care*, vol. 26, no. 3, pp. 770–776, 2003.

[167] D. Ziegler, A. Ametov, A. Barinov et al., "Oral treatment with α-lipoic acid improves symptomatic diabetic polyneuropathy," *Diabetes Care*, vol. 29, no. 11, pp. 2365–2370, 2006.

[168] D. Ziegler, P. A. Low, W. J. Litchy et al., "Efficacy and safety of antioxidant treatment with α-lipoic acid over 4 years in diabetic polyneuropathy: the NATHAN 1 trial," *Diabetes Care*, vol. 34, no. 9, pp. 2054–2060, 2011.

[169] D. A. Greene and A. A. F. Sima, "Effects of aldose reductase inhibitors on the progression of nerve damage," *Diabetic Medicine*, vol. 10, no. 2, pp. 31S–32S, 1993.

[170] D. R. Tomlinson, A. L. Carrington, L. T. Diemel, C. B. Ettlinger, W. J. Smith, and P. Fernyhough, "Limitations of the polyol hypothesis in the pathobiology of experimental diabetic neuropathy," *Diabetic Medicine*, vol. 10, supplement 2, 1993.

[171] I. G. Obrosova, L. Fathallah, H. J. Lang, and D. A. Greene, "Evaluation of a sorbitol dehydrogenase inhibitor on diabetic peripheral nerve metabolism: a prevention study," *Diabetologia*, vol. 42, no. 10, pp. 1187–1194, 1999.

[172] V. Bril, R. A. Buchanan, and The AS-3201 Study Group, "Aldose reductase inhibition by AS-3201 in sural nerve from patients with diabetic sensorimotor polyneuropathy," *Diabetes Care*, vol. 27, no. 10, pp. 2369–2375, 2004.

[173] V. Bril, R. A. Buchanan, and The Ranirestat Study Group, "Long-Term Effects of Ranirestat (AS-3201) on peripheral nerve function in patients with diabetic sensorimotor polyneuropathy," *Diabetes Care*, vol. 29, no. 1, pp. 68–72, 2006.

[174] M. Polydefkis, J. Arezzo, M. Nash et al., "Safety and efficacy of ranirestat in patients with mild-to-moderate diabetic sensorimotor polyneuropathy," *Journal of the Peripheral Nervous System*, vol. 20, no. 4, pp. 363–371, 2015.

[175] V. Bril, T. Hirose, S. Tomioka, and R. Buchanan, "Ranirestat for the management of diabetic sensorimotor polyneuropathy," *Diabetes Care*, vol. 32, no. 7, pp. 1256–1260, 2009.

[176] S. Chiechio, A. Copani, R. W. Gereau IV, and F. Nicoletti, "Acetyl-L-carnitine in neuropathic pain: experimental data," *CNS Drugs*, vol. 21, supplement 1, pp. 31–38, 2007.

Hindawi Publishing Corporation
Journal of Diabetes Research
Volume 2016, Article ID 3157123, 10 pages
http://dx.doi.org/10.1155/2016/3157123

Research Article

Shear Stress-Normal Stress (Pressure) Ratio Decides Forming Callus in Patients with Diabetic Neuropathy

Ayumi Amemiya,[1,2] Hiroshi Noguchi,[3] Makoto Oe,[4] Kimie Takehara,[5] Yumiko Ohashi,[6] Ryo Suzuki,[7] Toshimasa Yamauchi,[7] Takashi Kadowaki,[7] Hiromi Sanada,[1] and Taketoshi Mori[3]

[1]*Department of Wound Care Management, Graduate School of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo, Japan*
[2]*Department of Nursing Physiology, Graduate School of Nursing, Chiba University, 1-8-1 Inohana, Chuo-ku, Chiba-shi, Chiba, Japan*
[3]*Department of Life Support Technology (Molten), Graduate School of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo, Japan*
[4]*Department of Advanced Nursing Technology, Graduate School of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo, Japan*
[5]*Department of Nursing Administration, Graduate School of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo, Japan*
[6]*Department of Nursing, The University of Tokyo Hospital, 7-3-1 Hongo, Bunkyo-ku, Tokyo, Japan*
[7]*Department of Diabetes and Metabolic Diseases, Graduate School of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo, Japan*

Correspondence should be addressed to Taketoshi Mori; tmoriics-tky@umin.ac.jp

Received 29 August 2016; Revised 26 October 2016; Accepted 9 November 2016

Academic Editor: Frank Bowling

Aim. Callus is a risk factor, leading to severe diabetic foot ulcer; thus, prevention of callus formation is important. However, normal stress (pressure) and shear stress associated with callus have not been clarified. Additionally, as new valuables, a shear stress-normal stress (pressure) ratio (SPR) was examined. The purpose was to clarify the external force associated with callus formation in patients with diabetic neuropathy. *Methods*. The external force of the 1st, 2nd, and 5th metatarsal head (MTH) as callus predilection regions was measured. The SPR was calculated by dividing shear stress by normal stress (pressure), concretely, peak values (SPR-p) and time integral values (SPR-i). The optimal cut-off point was determined. *Results*. Callus formation region of the 1st and 2nd MTH had high SPR-i rather than noncallus formation region. The cut-off value of the 1st MTH was 0.60 and the 2nd MTH was 0.50. For the 5th MTH, variables pertaining to the external forces could not be determined to be indicators of callus formation because of low accuracy. *Conclusions*. The callus formation cut-off values of the 1st and 2nd MTH were clarified. In the future, it will be necessary to confirm the effect of using appropriate footwear and gait training on lowering SPR-i.

1. Introduction

Diabetic neuropathy is the most common complication of diabetes; approximately half of patients with diabetes have symptoms of diabetic neuropathy [1]. Loss of sensation is particularly important because it can allow the injury to go unnoticed, leading to foot ulcers. Diabetic foot ulcer is defined as cutaneous erosions characterized by a loss of epithelium that extends into or through the dermis to deeper tissues [2]; lifetime prevalence of diabetic foot ulcer is 15%–25% in the population with diabetes [3]. These foot ulcers seriously affect quality of life (QOL), reducing physical activity and increasing psychological stress [4]. Furthermore, a 2004 study estimated that diabetic ulcer-related costs averaged over $13,000 per episode, not including costs associated with psychosocial issues, decrease in QOL, and lost productivity [5]. In addition, even when an ulcer is successfully healed, the risk of recurrence is high, with

reported rates ranging between 30% and 40% within one year [6, 7]. Therefore, prevention of foot ulcers is of paramount importance and has long been recognized as a priority by the International Working Group on the Diabetic Foot [8].

The pathogenesis of foot ulceration is a complex process in which many factors are involved. The most important factor appears to be peripheral neuropathy with a loss of sensation. However, neuropathy alone may not cause plantar ulceration [9]. Other risk factors are associated with developing diabetic foot ulcers, one of which is said to be the external force on the plantar [8]. Repeated normal stress (pressure) and shear stress during walking contribute to callus formation in the plantar region [10, 11]. Callus formation may lead to the development of foot ulcers and involves hyperkeratosis caused by excessive mechanical loading [2, 8, 12, 13]. The callus formation precedes ulcer formation in over 82% of patients with diabetic foot ulcers [14]. The relative risk for ulceration in a callused area was 11.0 compared with that of an area without callus [15, 16]. Once the foot ulcer occurs, its treatment will be difficult and take a long time. Therefore, prevention of the callus formation is important.

Patients with calluses are known to have significantly higher peak normal stress (pressure) during walking than patients without callus [17, 18]. Assuming that an average person walks approximately 10,000 steps a day, a callus may cause 18,600 kg of excess plantar normal stress (pressure) per day [19], highlighting the deleterious impact of calluses.

On the other hand, studies on shear stress are limited because the measurement of shear stress is technically difficult. Shear sensor technology has been still far from miniaturization to the point where it could accurately map shear load distribution. The shear stress has been commonly measured as ground reaction forces typically along, with a force platform providing resultant force acting on the outsole or barefoot because of technical difficulties. This fact appears to have acted as an almost complete barrier to practical, useful research relating to friction loads [20]. However, it is important to measure the in-shoe shear stress for considering the callus related factors. The shear stress of the callus formation area is hardly ever measured in patients with diabetes, and therefore, there are few studies on shear stress in patients with diabetes. As one of the few studies, patients with diabetes had higher shear stress under the first/second metatarsal head and lower shear stress under the third/fourth metatarsal head compared with healthy subjects [21, 22]. However, these studies used special type of shoes for measurement that could accommodate the thick sensor. It has been revealed that the shear stress of each metatarsal head differs in terms of direction and magnitude, depending on the difference of heel height using special type of shoes for measurement [23]. Therefore, it is important to perform measurements on the patient, while the patient is wearing his/her own ordinary footwear to identify causes associated with callus formation.

In the clinical setting, many patients redevelop calluses, despite wearing tailor-made footwear developed with a view for preventing callus. Tailor-made footwear that is declared for the callus prevention is available, the effectiveness of which has already been demonstrated [24, 25]; however, Scirè et al. also showed that the callus recurred in 41% patients, even if they wore therapeutic footwear [26]. The callus recurred in 24 of 31 patients, even if they received foot care and wore recommended footwear, according to the diabetic foot care program [27]. From these results, it is considered that the causes of callus have not been completely excluded. It is assumed that the callus has been intervened without considering shear stress during walking. Although repeated normal stress (pressure) and shear stress during walking contribute to callus formation, many studies on intervention were focusing only on normal stress (pressure). Shear stress is another essential factor to consider for developing more effective care.

It is also unclear how much the external force on plantar should be decreased, despite many studies being available on normal stress (pressure) analysis. Identification of a cut-off value of the normal stress (pressure) has been attempted for foot ulcer prevention, but the cut-off value has less accuracy [16, 28]. Moreover, a study of a cut-off value of the shear stress is hardly available. The cut-off value of the normal stress (pressure) and shear stress on callus formation prevention has not been studied. As for the future clinical image, in the next procedure of intervention, the external forces will be adjusted referring to the cut-off value. If the callus recurs, the external forces will be readjusted to the smaller value of the current value.

The purpose of this study was to clarify external forces associated with callus formation in patients with diabetic neuropathy and to identify the cut-off value of the forces. First, differences in normal stress (pressure) and shear stress in callus formation region and noncallus formation region will be clarified in patients with diabetic neuropathy. Second, the external force cut-off value for callus formation will be identified in patients with diabetic neuropathy.

2. Research Design and Methods

2.1. Research Design. The walking measurement was cross-sectional study, and observation of callus formation was carried out as longitudinal study.

2.2. Participants. This survey was conducted at the Diabetic Foot Outpatient Clinic at the University of Tokyo Hospital from April to October 2015. Sixty-four patients with diabetes who visited this outpatient clinic were recruited. Inclusion criteria were patients with diabetic neuropathy ≥20 years old who could walk without aid. Participants who had a current diabetic foot ulcer and a history of more proximal lower limb of metatarsophalangeal (MTP) joint amputation were excluded. The survey protocol was approved by the Ethical Committee of the Graduate School of Medicine, The University of Tokyo (#10797).

Aim of this outpatient clinic is to prevent diabetic foot ulcers, and therefore, most patients experience symptoms of the foot or are interested in the prevention of complications. After general examination in the diabetic foot outpatient clinic including regular callus removal, written informed consent was obtained from the participant. Sensors were attached and walking measurement was executed. The patient walked approximately 50 m as a practice to confirm that there was no

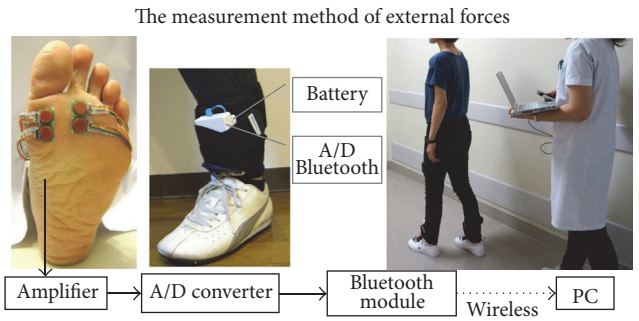


Figure 1: The state of attaching the sensors at the 1st and 2nd metatarsal head.

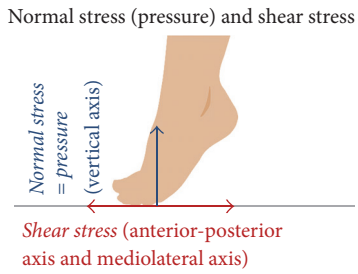


Figure 2: Normal stress (pressure) is vertical axis. Shear stress is the resultant force of anterior-posterior axis and mediolateral axis.

pain and no interfering in walking by the sensor attachment. The patient then walked about 15 m twice as the measurement walk, and the researcher recorded all sensor data ensuring that sensors were operating during the measurement. An assistant measured 15 m walking time using a stopwatch and walked diagonally behind the patient to prevent them from falling or other adverse events (Figure 1).

The participants were asked to wear "usual shoes" when they visited the Diabetic Foot Outpatient Clinic. It was assumed that those shoes were worn for the longest time, such as during exercise therapy. All participants were provided with standardized socks to use during the measurement. When the pretest participants had come with wearing tight or long socks, attached sensors had been often displaced in the pretest. In these situations, remeasurement and confirmation of the position of attaching sensor had been repeated. Thus, the patients' own socks could not be used to shorten the investigation time and to reduce the burden of the patients.

The presence or absence of callus was checked after one month again, which means the formation of callus check; this is also the definition of callus formation region in this study.

2.3. Data Collection

2.3.1. Callus. The definition of callus is not clear universally; therefore the presence/absence of callus is determined in the clinical setting using expert opinion in this study. That is, in this study, a callus was defined by plate-shaped hyperkeratosis observed by two expert nurses in foot care. All calluses were removed in the diabetic foot clinic using a corn cutter, and walking was measured afterward.

The presence or absence of callus formation was checked again after one month in all patients, because, generally, a callus would have recurred within one month. The region of such callus was classified into the callus formation group if callus recurred. If the callus does not recur in the region, it will be excluded from the analysis. The region of noncallus was classified into the noncallus formation group if a callus did not develop after one month. If a callus developed in the noncallus region during the follow-up for 1 month, it was also excluded from the analysis, since it could not be determined that the measured gait and foot condition were related to callus formation.

2.3.2. External Forces. In this study, in-shoe normal stress (pressure) and shear stress of the 1st, 2nd, and 5th MTH were measured; these are the predilection regions of callus and foot ulcers. Reliability and validity of the system for measuring in-shoe plantar normal stress (pressure) and shear stress have already been verified [29]. These in-shoe normal stress (pressure) and two axes' shear stress were measured by ShokacChip™, which was newly developed by Touchence Inc. and released in 2013. ShokacChip is a tactile sensor with small-high sensitivity based on MEMS (Micro Electro Mechanical Systems) technology. High sensitivity is realized for three-dimensional axes by processing three piezoelectric elements and locating them at three-dimensional axes on the 2 mm^2 chip. Sensor size is φ10.0 mm × 1.3 mm (t); it is very small and thin [30]. Thus, it can measure the in-shoe external forces of each MTH. Calibration using a load-compensating device has already been done before shipment of the sensors. It has been guaranteed that this sensor is not necessary to calibrate in each time of measurement on the characteristic of the sensor. However, calibration was done by rising a foot to each foot before the measurement in a state of wearing shoes, because the sensor caught some stresses when patients wore shoes. Subject's plantar was fixed perpendicular to the ground and the sensor was attached to be parallel to the ground at the region of ranging from the MTH top to the base of toe by double sided tape. The fixed method of the sensors was considered before pretest. Additionally, location of the sensor was checked visually after each measurement. Shear stress is the resultant force of anterior-posterior axis and mediolateral axis on the assumption of some sensor shift (Figure 2). From results of pretests, measurements should use two sensors in each region. Only two regions could be measured per foot in one measurement, because four sensors, at a maximum, could be used per foot by the sensor circuit system hardware restriction. The callus regions were preferentially selected for measurement, and the noncallus regions were selected at random in these three regions. If a noncallus patient could be matched by sex and age (±3 years), the same regions were measured in the callus patient. If a foot had three calluses, measurements were conducted twice by changing the attachment of the sensor because the number of sensors was limited. The noncallus region will be excluded when one foot has callus region and noncallus region, because noncallus region might be also affected by callus. It is considered that the cause of callus formation

of toe is the contact with shoes. These calluses are clearly different cause to callus of metatarsal heads. Therefore, feet with calluses of toe are not excluded.

Peak plantar normal stress (pressure) (PP), normal stress (pressure) time integral (PI), peak shear stress (PSS), and shear stress integral (SSI) of each gait cycle were calculated from the recorded force profile; these variables have been investigated in many previous studies of external force [31–33]. In addition, during measurements, we newly noticed that some callus patients had a high shear stress in spite of the not so high normal stress (pressure). One previous study [34] examined the combinations of normal stress (pressure) and shear stress; they concluded that skin breakdown occurred at higher shear load levels in animal experiment. However, it was indicated in the results that loading pad was slipped and skin breakdown did not occur in the combination of some shear stress and low normal stress (pressure) loading. From these results, we considered that the balance of normal stress (pressure) and shear stress is important. Thus, shear stress-normal stress (pressure) ratios (SPR) were calculated by dividing shear stress by normal stress (pressure), concretely, peak values (SPR-p) and time integral values (SPR-i).

2.3.3. Patient Characteristics. Data regarding age, sex, height, weight, foot length, and width (standing position) and usual daily activities were obtained from medical records or by interview. Hallux valgus, bunionette, claw toe, and hammer toe were regarded as foot deformities and identified by the visual inspection of well-trained nurse.

Patients were diagnosed with diabetic neuropathy if two of the following three items were fulfilled: (1) sensory symptoms considered to be due to diabetic neuropathy, (2) bilaterally decreased or absent ankle reflex, and (3) decreased vibratory sensation in bilateral medial malleoli [35]. The sensory symptoms considered to be due to diabetic neuropathy were clarified during the interview, whereas the bilaterally decreased or absent ankle reflex was examined with the patient in a kneeling position. Decreased vibratory sensation in bilateral medial malleoli was examined using an AC128 tuning fork for evaluation of vibration sense. The total time span in which the patient felt vibratory sensation was evaluated, and a duration of <10 s was considered as decreased sensation [35]. In addition, the results of the monofilament test were confirmed using medical records; this test was performed on the basis of the Practical Guidelines of International Working Group on the Diabetic Foot using 5.07 Semmes-Weinstein monofilament (ARKRAY Inc., Tokyo, Japan). Data regarding duration of diabetes, hemoglobin A1c (HbA1c), foot ulcer history, and foot amputation history were obtained from medical records or by interview. Angiopathy data were collected from medical records, and an ankle-brachial index (ABI) <0.9 was regarded as angiopathy [36].

2.4. Data Analysis. The variables of the external forces were obtained by averaging a total 15 steps after removing the initial three steps and the final three steps. Data processing was performed using MATLAB R2012a (The Math Works, Inc., MA, USA).

Descriptive data were expressed as means ± standard deviations for continuous variables and n (%) for categorical variables. Statistical analyses were performed using IBM SPSS Statistics ver. 23.0 (Chicago, IL, USA). Statistical significance was set at $p = 0.05$. Patient characteristics were compared between the callus formation group and noncallus formation group using Student's t-test and Fisher's exact test. For comparison of the external forces in callus formation regions and noncallus formation regions, Student's t-test was used.

Receiver operating characteristic (ROC) curves were drawn for all external force variables, and the area under curve (AUC) was calculated. The optimal cut-off point was determined with the specificity being approximately 0.8. Positive predictive values (PPVs) and negative predictive values (NPVs) were also calculated. The reason why the specificity has priority is that the callus can be removed unlike a foot ulcer even if it occurs once. On the other hand, intervention to improve the external force (e.g., the introduction of custom-made footwear) has a large burden on medical staff and patients. Therefore, the cut-off value was examined to avoid excessive intervention.

3. Results

Sixty-four patients with diabetes were attended to at the Diabetic Foot Outpatient Clinic during the observation period, and fifty-nine patients with diabetic neuropathy participated in the survey. Five patients were excluded because the patient (1) did not have neuropathy ($n = 1$), (2) used a wheelchair ($n = 1$), (3) had a current diabetic foot ulcer ($n = 1$), (4) had a history of bilateral knee amputation ($n = 1$), and (5) was unable to provide consent for participation ($n = 1$).

Twenty patients (33.9%) had more than one callus in the target region, whereas the number of patients without callus was 39 (66.1%). Measurement regions were 244 regions; four patients had 3 calluses in one foot. Thirty-eight regions (15.6%) were excluded from the analysis because other regions had a callus in one foot. As for the 1st MTH, the number of callus formation feet was 13 (23.6%), whereas the number of noncallus formation feet was 42 (76.4%). As for the 2nd MTH, the number of callus formation feet was 16 (22.2%), whereas the number of noncallus formation feet was 56 (77.8%). As for the 5th MTH, the number of callus formation feet was 21 (26.6%), and the number of noncallus formation feet was 58 (73.4%).

All calluses had recurred by the time of the next follow-up (1 month later), whereas no calluses were seen in any of the noncallus regions at the follow-up. Therefore, all regions of callus removal were assigned to regions of "callus formation," and all regions with no calluses were assigned to regions of "noncallus formation" in this study.

Measurement survey took about 15 min; approximately five minutes was required for follow-up survey. No adverse events were observed during the survey.

The patient characteristics for each MTH are shown in Table 1.

In the 1st MTH results, the foot deformity was significantly higher in the callus formation group. In the 2nd MTH results, there were significantly more female patients, light

Table 1: Patient characteristics for each metatarsal head observation.

	1st metatarsal head			2nd metatarsal head			5th metatarsal head		
	Noncallus formation group	Callus formation group	p value	Noncallus formation group	Callus formation group	p value	Noncallus formation group	Callus formation group	p value
Number of feet	42	13		56	16		58	21	
Patient characteristics									
Age (y)	66.0 ± 11.2	63.7 ± 8.6	0.450[1]	66.7 ± 10.8	65.1 ± 7.8	0.517[1]	69.3 ± 10.2	67.0 ± 13.4	0.471[1]
Sex			0.053[2]			0.004[2*]			0.599[2]
Male	20 (47.6)	2 (15.4)		30 (53.6)	2 (12.5)		38 (65.5)	12 (57.1)	
Female	22 (52.4)	11 (84.6)		26 (46.4)	14 (87.5)		20 (34.5)	9 (42.9)	
Height (m)	1.61 ± 0.1	1.56 ± 0.1	0.133[1]	1.61 ± 0.09	1.57 ± 0.13	0.289[1]	1.62 ± 0.09	1.64 ± 0.13	0.504[1]
Weight (kg)	66.8 ± 17.5	66.9 ± 23.9	0.999[1]	68.0 ± 17.7	58.3 ± 9.3	0.005[1*]	63.1 ± 14.6	65.1 ± 14.8	0.595[1]
BMI	25.5 ± 5.4	26.8 ± 6.2	0.495[1]	25.9 ± 5.2	23.7 ± 3.9	0.068[1]	24.0 ± 4.4	24.2 ± 4.0	0.856[1]
HbA1c: NGSP (%)	6.9 ± 1.3	6.7 ± 0.7	0.134[1]	7.1 ± 1.0	6.3 ± 0.5	<0.001[1*]	6.7 ± 0.8	6.9 ± 1.2	0.507[1]
HbA1c: IFCC (mmol/mol)	52 ± 10	50 ± 5		54 ± 8	45 ± 4		50 ± 6	52 ± 9	
Diabetes duration (y)	15.6 ± 10.3	13.5 ± 7.5	0.683[1]	17.9 ± 9.9	13.4 ± 10.7	0.143[1]	15.7 ± 11.3	15.8 ± 7.1	0.986[1]
Monofilament test (abnormal)	5 (11.9)	1 (7.7)	1.000[2]	4 (7.1)	0 (0.0)	1.000[2]	5 (8.6)	4 (19.0)	0.236[2]
History of diabetic Foot ulcer	2 (4.8)	0 (0.0)	1.000[2]	0 (0.0)	0 (0.0)		2 (3.4)	0 (0.0)	1.000[2]
Deformity	9 (21.4)	10 (76.9)	<0.001[2*]	7 (12.5)	7 (43.8)	0.010[2*]	8 (13.8)	5 (23.8)	0.314[2]
Angiopathy	2 (4.8)	1 (7.7)	0.562[2]	2 (3.6)	1 (6.3)	0.535[2]	4 (6.9)	1 (4.8)	1.000[2]

Mean ± SD, n (%) $^{*}p < 0.05$ [1]t-test, [2]Fisher's exact test.

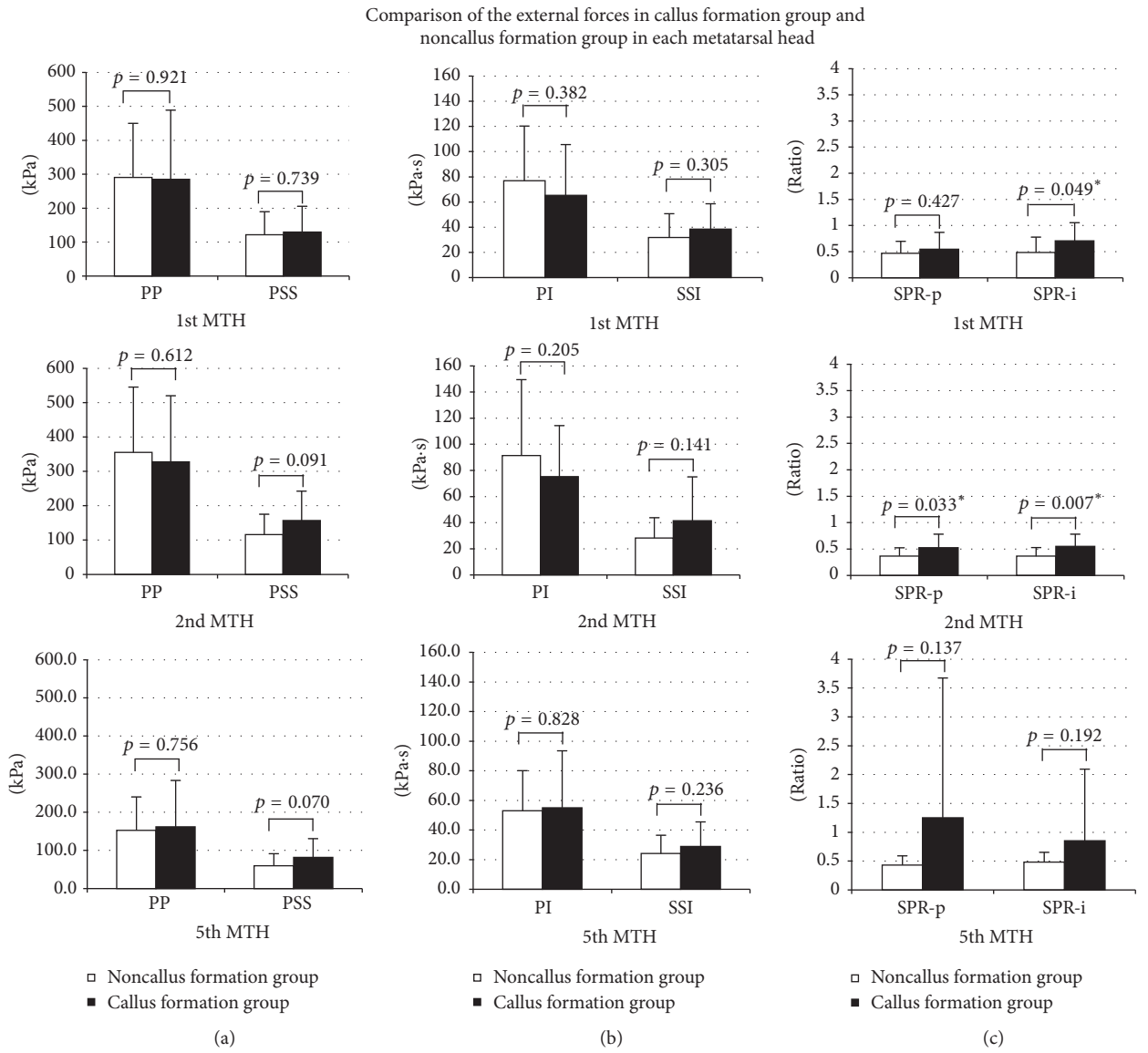


Figure 3: MTH: metatarsal head. (a) Peak normal stress (pressure) (PP) and peak shear stress (PSS), (b) normal stress (pressure) time integral (PI) and shear stress time integral (SSI), and (c) shear stress/normal stress (pressure) ratio of peak value (SPR-p) and shear stress/normal stress (pressure) ratio of time integral value (SPR-i).

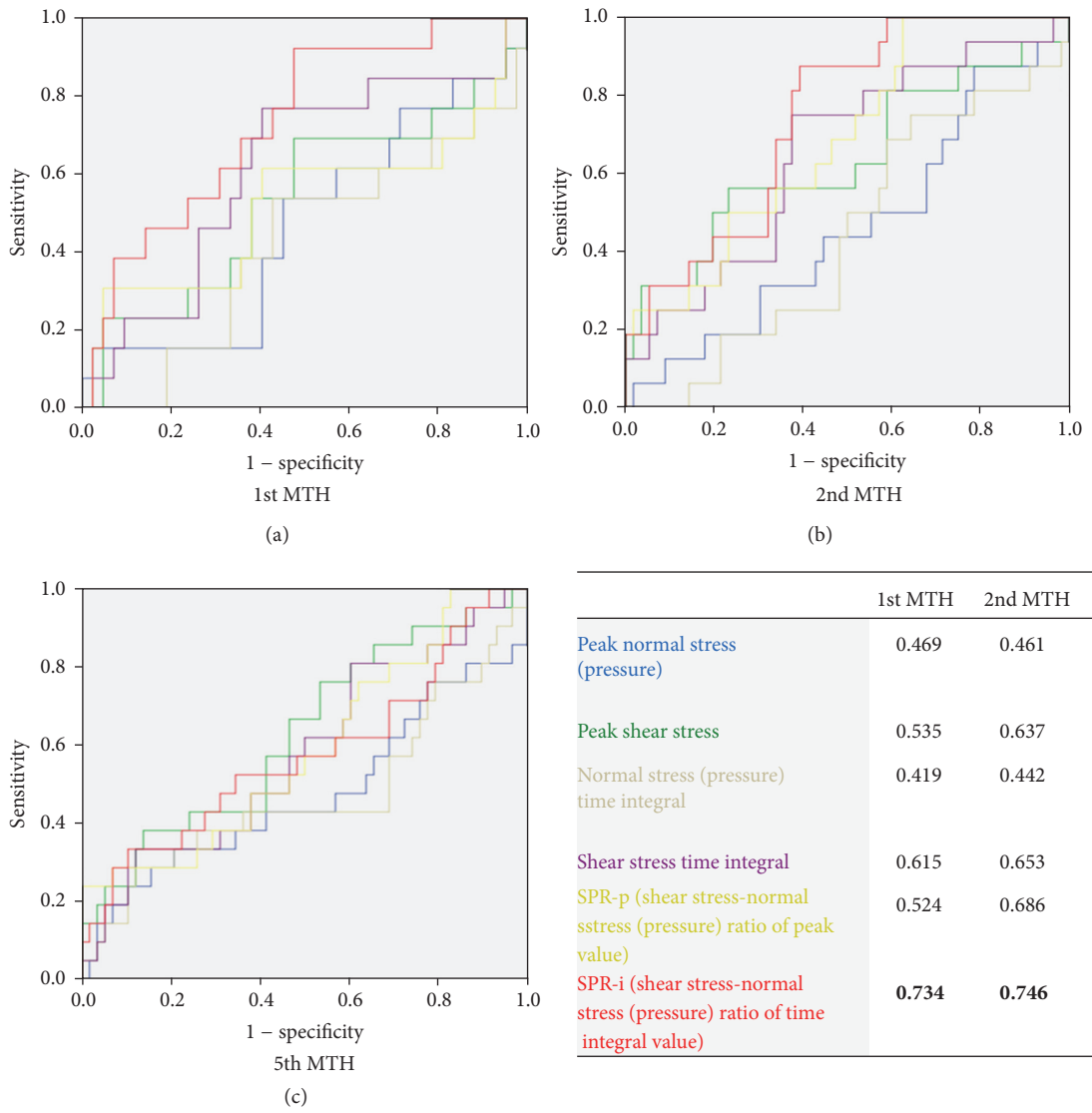
weight patients, patients with low HbA1c, and patients with foot deformity in the callus formation group. In the 5th MTH results, the two groups had no significant difference.

The results for the 1st MTH are shown in Figure 3. Single variables of normal stress (pressure) and shear stress did not differ significantly; only SPR-i was significantly higher in the callus formation group. The results for the 2nd MTH are shown in 2nd row of Figure 3. Single variables of normal stress (pressure) and shear stress did not differ significantly; only SPR-p and SPR-i were significantly higher in the callus formation group. The results for the 5th MTH are shown in 3rd row of Figure 3. No variables differed significantly; however, PSS had a tendency toward being high in the callus formation group ($p = 0.070$).

ROC curves for the 1st MTH are shown in Figure 4(a). The cut-off value of SPR-i was 0.60 (sensitivity, 0.54; specificity, 0.76) for the 1st MTH. The ROC curves for the 2nd MTH are shown in Figure 4(b). The cut-off value of SPR-i was 0.50 (sensitivity, 0.44; specificity, 0.80) in the 2nd MTH. ROC curves for the 5th MTH are shown in Figure 4(c). For the 5th MTH, variables pertaining to the external forces could not be determined to be indicators of callus formation, because the AUC of all variables was <0.7, although PSS in AUC was 0.63.

4. Conclusions

This is the first study that measures the in-shoe external force of the 1st, 2nd, and 5th metatarsal heads of the foot. Since this



	1st MTH	2nd MTH	5th MTH
Peak normal stress (pressure)	0.469	0.461	0.475
Peak shear stress	0.535	0.637	**0.630**
Normal stress (pressure) time integral	0.419	0.442	0.472
Shear stress time integral	0.615	0.653	0.580
SPR-p (shear stress-normal sstress (pressure) ratio of peak value)	0.524	0.686	0.589
SPR-i (shear stress-normal stress (pressure) ratio of time integral value)	**0.734**	**0.746**	0.570

Figure 4: Receiver operating characteristics (ROC) curve of all external force variables in each metatarsal head. (a) 1st MTH, (b) 2nd MTH, and (c) 5th MTH.

measurement had been enabled, it was clarified that callus formation of the 1st and 2nd metatarsal head were related to high shear stress time integral/normal stress (pressure) time integral (SPR-i) rather than the single variables of normal stress (pressure) and shear stress. Additionally, the cut-off values for each region were found to be different.

The measurement of the actual in-shoe normal stress (pressure) and shear stress on the plantar has been associated with technical difficulties. In this study, the in-shoe external forces were measured in the clinic setting, since it became possible to conduct measurements in any shoe utilizing the thin and small sensor that was recently developed. In addition, all patients with diabetic neuropathy could participate in this survey without adverse events. Furthermore, the insole type sensor systems used in the previous studies were unclear whether the systems measured external force applied to the callus region since it had been difficult to confirm the sensor attached to the callus region [23, 37]. In this study, this problem was solved by directly attaching the sensor on the target area on the plantar. Hence, this study is highly important because normal stress (pressure) and shear stress of the callus regions could be accurately measured simultaneously.

It was revealed that SPR-i is the new indicator of callus formation; that is, dividing shear stress with normal stress (pressure) indicated the balance between shear stress and normal stress (pressure). The balance of the external forces had not been previously investigated. With higher shear stress-normal stress (pressure) ratio, as the high shear stress applies with a certain degree of normal stress (pressure), mechanical stress on the skin will become increased, and the body attempts to protect irritated skin by forming a hyperkeratotic lesion, such as a corn or a callus [11]. With lower shear stress-normal stress (pressure) ratio, when high normal stress (pressure) applies with small shear stress, it is considered that the callus, which is the normal physiologic response of the skin, will be hardly formed, since the external

forces are applied to the subcutaneous tissue rather than being exerted on the skin surface. Calluses form as a result of hyperproliferation and incomplete differentiation of epidermal keratinocytes and increased expression of adhesion molecules in the epidermis [38]. Forces and time integration were associated with the callus formation rather than the maximum forces, because the time integral ratio had a high accuracy than the peak ratio.

Two reasons could be considered as SPR-i of the 5th metatarsal head did not have significant difference between callus formation group and noncallus formation group. First, sample size of the 5th metatarsal head was limited. If enough patients with the 5th metatarsal head callus will participate, SPR of the 5th metatarsal head might also become the indicator. More patients were needed for the 5th metatarsal head, because external force of the 5th metatarsal head had large variability and small value of difference compared with other regions. Second, peak shear stress affected the callus formation of 5th metatarsal head rather than SPR-i. Mechanical stress might not be absorbed and off-loaded in the subcutaneous tissue, because the 5th metatarsal head has thin subcutaneous tissue comparing other two regions. Therefore, peak shear stress might affect the callus formation of 5th metatarsal head rather than the balance of normal stress (pressure) and shear stress (SPR-i).

According to these results, there was no significant difference in the normal stress (pressure) and shear stress variables between the callus formation group and noncallus formation group in all regions. It was only observed that peak shear stress tended toward being high in the 2nd and 5th metatarsal head. In some previous studies, where the calluses were measured without prior removal, the normal stress (pressure) and shear stress were significantly higher in the callus group. Some studies had shown that the normal stress (pressure) decreased after removing the callus [19, 39]. Therefore, it is natural that single variables of normal stress (pressure) and shear stress had no significant differences in the absence of hyperkeratosis.

The cut-off values of external force on plantar associated with callus formation were found; SPR-i of the 1st metatarsal head was 0.60 and SPR-i of the 2nd metatarsal head was 0.50. The cut-off value of SPR-i for the 2nd metatarsal head was lower than that for the 1st metatarsal head. This may be the reason why the 2nd metatarsal head was associated with a higher normal stress (pressure) than that associated with the 1st metatarsal head. In general, the center of normal stress (pressure) is applied to the 2nd metatarsal head during walking, and the center of normal stress (pressure) during the push-off phase, which is higher, often applies to this region in patients with diabetes [40]. Thus, the normal stress (pressure) in the 2nd metatarsal head was higher than the normal stress (pressure) of the 1st metatarsal head.

In the 5th metatarsal head, individual differences were more pronounced than for the other regions. The reason might be that the moving of the center of gravity from the heel to the 5th metatarsal head during walking hardly connects to the propulsion. Therefore, the external force could not be determined to be the sole indicator of callus formation in the 5th metatarsal head.

In various previous studies, normal stress (pressure) had been associated with foot ulcer development [16, 38, 41], and therefore, the following process was considered. Calluses were developed under the influence of high SPR-i. High normal stress (pressure) was applied to the callus region by hyperkeratosis [19], and foot ulcer subsequently developed causing subcutaneous tissue damage due to high normal stress (pressure).

The patients of this survey were limited to mild neuropathy and mild foot deformity patients. Only one patient had a history of amputation. Therefore, the results of this study might not be applicable to patients with a progressed neuropathy, severe foot deformity, and amputation history. It will be necessary to confirm it in the future whether this result is applicable to such a high risk subject.

It is limitation that the walking measurements were taken on one occasion only. It was necessary to check that the gait features were not changed, when observing callus formation after one month. However, it was difficult because this study was carried out during medical practice. This study was conducted on the assumption that the gait features were not changed during this one month.

For the first time, each region of the normal stress (pressure) and shear stress wearing the patient's own shoes was measured in a clinical setting. As a result, the following two points were revealed. First, callus formation in the 1st and 2nd metatarsal head is related to high shear stress time integral/normal stress (pressure) time integral (SPR-i) rather than the single variables of normal stress (pressure) and shear stress. Peak shear stress had a tendency towards being high in the callus formation group in the 5th metatarsal head. Second, the external force cut-off values were found to differ in each site, SPR-i of the 1st metatarsal head being 0.60 and SPR-i of the 2nd metatarsal head being 0.50. External force could not be determined to be a sole indicator of callus formation in the 5th metatarsal head. Considering the results presented here, intervention based on cut-off values of SPR-i of each metatarsal head will be effective. In future, it will be necessary to confirm the effect of using appropriate footwear and gait training on lowering external forces associated with callus formation.

Competing Interests

The authors report no relevant conflict of interests.

Acknowledgments

This work was supported by the Grant-in-Aid for Fellows of Japan Society for the Promotion of Science [Grant no. 26-5726]. The authors would like to express their appreciation to all participants of this survey.

References

[1] A. J. M. Boulton, A. I. Vinik, J. C. Arezzo et al., "Diabetic neuropathies: a statement by the American Diabetes Association," *Diabetes Care*, vol. 28, no. 4, pp. 956–962, 2005.

[2] G. E. Reiber, L. Vileikyte, E. J. Boyko et al., "Causal pathways for incident lower-extremity ulcers in patients with diabetes

from two settings," *Diabetes Care*, vol. 22, no. 1, pp. 157–162, 1999.

[3] N. Singh, D. G. Armstrong, and B. A. Lipsky, "Preventing foot ulcers in patients with diabetes," *Journal of the American Medical Association*, vol. 293, no. 2, pp. 217–228, 2005.

[4] M. Brod, "Quality of life issues in patients with diabetes and lower extremity ulcers: patients and care givers," *Quality of Life Research*, vol. 7, no. 4, pp. 365–372, 1998.

[5] K. Stockl, A. Vanderplas, E. Tafesse, and E. Chang, "Costs of lower-extremity ulcers among patients with diabetes," *Diabetes Care*, vol. 27, no. 9, pp. 2129–2134, 2004.

[6] N. Pound, S. Chipchase, K. Treece, F. Game, and W. Jeffcoate, "Ulcer-free survival following management of foot ulcers in diabetes," *Diabetic Medicine*, vol. 22, no. 10, pp. 1306–1309, 2005.

[7] S. A. Bus, R. Waaijman, M. Arts et al., "Effect of custom-made footwear on foot ulcer recurrence in diabetes: a multicenter randomized controlled trial," *Diabetes Care*, vol. 36, no. 12, pp. 4109–4116, 2013.

[8] International Working Group on the Diabetic Foot, *International Consensus on the Diabetic Foot and Practical Guidelines on the Management and the Prevention of the Diabetic Foot*, International Working Group on the Diabetic Foot, Amsterdam, The Netherlands, on DVD, 2011.

[9] J. E. Shaw and A. J. M. Boulton, "The pathogenesis of diabetic foot problems: an overview," *Diabetes*, vol. 46, no. 2, pp. S58–S61, 1997.

[10] D. Singh, G. Bentley, and S. G. Trevino, "Callosities, corns, and calluses," *British Medical Journal*, vol. 312, no. 7043, pp. 1403–1406, 1996.

[11] D. B. Freeman, "Corns and calluses resulting from mechanical hyperkeratosis," *American Family Physician*, vol. 65, no. 11, pp. 2277–2280, 2002.

[12] O.-Y. Kwon, S. D. Minor, K. S. Maluf, and M. J. Mueller, "Comparison of muscle activity during walking in subjects with and without diabetic neuropathy," *Gait and Posture*, vol. 18, no. 1, pp. 105–113, 2003.

[13] L. A. Lavery, E. J. G. Peters, and D. G. Armstrong, "What are the most effective interventions in preventing diabetic foot ulcers?" *International Wound Journal*, vol. 5, no. 3, pp. 425–433, 2008.

[14] R. A. Sage, J. K. Webster, and S. G. Fisher, "Outpatient care and morbidity reduction in diabetic foot ulcers associated with chronic pressure callus," *Journal of the American Podiatric Medical Association*, vol. 91, no. 6, pp. 275–279, 2001.

[15] H. J. Murray, M. J. Young, S. Hollis, and A. J. M. Boulton, "The association between callus formation, high pressures and neuropathy in diabetic foot ulceration," *Diabetic Medicine*, vol. 13, no. 11, pp. 979–982, 1996.

[16] R. Waaijman, M. de Haart, M. L. J. Arts et al., "Risk factors for plantar foot ulcer recurrence in neuropathic diabetic patients," *Diabetes Care*, vol. 37, no. 6, pp. 1697–1705, 2014.

[17] A. C. Duffin, R. Kidd, A. Chan, and K. C. Donaghue, "High plantar pressure and callus in diabetic adolescents. Incidence and treatment," *Journal of the American Podiatric Medical Association*, vol. 93, no. 3, pp. 214–220, 2003.

[18] H. B. Menz, G. V. Zammit, and S. E. Munteanu, "Plantar pressures are higher under callused regions of the foot in older people," *Clinical and Experimental Dermatology*, vol. 32, no. 4, pp. 375–380, 2007.

[19] Z. Pataky, A. Golay, L. Faravel et al., "The impact of callosities on the magnitude and duration of plantar pressure in patients with diabetes mellitus. A callus may cause 18,600 kilograms of excess plantar pressure per day," *Diabetes and Metabolism*, vol. 28, no. 5, pp. 356–361, 2002.

[20] J. M. Carlson, "Functional limitations from pain caused by repetitive loading on the skin: a review and discussion for practitioners, with new data for limiting friction loads," *Journal of Prosthetics and Orthotics*, vol. 18, no. 4, pp. 93–103, 2006.

[21] M. Lord and R. Hosein, "A study of in-shoe plantar shear in patients with diabetic neuropathy," *Clinical Biomechanics*, vol. 15, no. 4, pp. 278–283, 2000.

[22] R. Hosein and M. Lord, "A study of in-shoe plantar shear in normals," *Clinical Biomechanics*, vol. 15, no. 1, pp. 46–53, 2000.

[23] Y. Cong, J. T.-M. Cheung, A. K. Leung, and M. Zhang, "Effect of heel height on in-shoe localized triaxial stresses," *Journal of Biomechanics*, vol. 44, no. 12, pp. 2267–2272, 2011.

[24] J. A. Mayfield, G. E. Reiber, L. J. Sanders, D. Janisse, and L. M. Pogach, "Preventive foot care in people with diabetes," *Diabetes Care*, vol. 26, pp. S78–S79, 2003.

[25] D. Janisse and E. Janisse, "Pedorthic management of the diabetic foot," *Prosthetics and Orthotics International*, vol. 39, no. 1, pp. 40–47, 2015.

[26] V. Scirè, E. Leporati, L. Teobaldi, L. A. Nobili, L. Rizzo, and A. Piaggesi, "Effectiveness and safety of using podikon digital silicone padding in the primary prevention of neuropathic lesions in the forefoot of diabetic patients," *Journal of the American Podiatric Medical Association*, vol. 99, no. 1, pp. 28–34, 2009.

[27] Y. Fujiwara, K. Kishida, M. Terao et al., "Beneficial effects of foot care nursing for people with diabetes mellitus: an uncontrolled before and after intervention study," *Journal of Advanced Nursing*, vol. 67, no. 9, pp. 1952–1962, 2011.

[28] T. M. Owings, J. Apelqvist, A. Stenström et al., "Plantar pressures in diabetic patients with foot ulcers which have remained healed," *Diabetic Medicine*, vol. 26, no. 11, pp. 1141–1146, 2009.

[29] A. Amemiya, H. Noguchi, M. Oe, H. Sanada, and T. Mori, "Establishment of a measurement method for in-shoe pressure and shear stress in specific regions for diabetic ulcer prevention," in *2016 38th Annual International Conference of the IEEE Engineering in Medicine and Biology Society (EMBC)*, pp. 2291–2294, Orlando, FL, USA, August 2016.

[30] ShokacChip™ Product outline, Touchence Inc., http://www.touchence.jp/en/chip/index.html.

[31] M. Yavuz, A. Tajaddini, G. Botek, and B. L. Davis, "Temporal characteristics of plantar shear distribution: relevance to diabetic patients," *Journal of Biomechanics*, vol. 41, no. 3, pp. 556–559, 2008.

[32] M. Yavuz, "American Society of Biomechanics Clinical Biomechanics Award 2012: plantar shear stress distributions in diabetic patients with and without neuropathy," *Clinical Biomechanics*, vol. 29, no. 2, pp. 223–229, 2014.

[33] I. C. N. Sacco, A. N. Hamamoto, L. M. G. Tonicelli, R. Watari, N. R. S. Ortega, and C. D. Sartor, "Abnormalities of plantar pressure distribution in early, intermediate, and late stages of diabetic neuropathy," *Gait and Posture*, vol. 40, no. 4, pp. 570–574, 2014.

[34] B. Goldstein and J. Sanders, "Skin response to repetitive mechanical stress: a new experimental model in pig," *Archives of Physical Medicine and Rehabilitation*, vol. 79, no. 3, pp. 265–272, 1998.

[35] H. Yasuda, M. Sanada, K. Kitada et al., "Rationale and usefulness of newly devised abbreviated diagnostic criteria and staging for diabetic polyneuropathy," *Diabetes Research and Clinical Practice*, vol. 77, no. 3, pp. S178–S183, 2007.

[36] American Diabetes Association, "Peripheral arterial disease in people with diabetes," *Diabetes Care*, vol. 26, no. 12, pp. 3333–3341, 2003.

[37] H. Noguchi, M. Oe, K. Takehara, T. Mori, and H. Sanada, "Reliability and validity of an on-site measurement and visualization system to measure plantar pressure and shear force in footwear for the education of diabetic patients," *Journal of Japanese Society of Wound, Ostomy, and Continence Management*, vol. 19, no. 3, pp. 327–335, 2015.

[38] S. H. Kim, S. Kim, H. I. Choi et al., "Callus formation is associated with hyperproliferation and incomplete differentiation of keratinocytes, and increased expression of adhesion molecules," *British Journal of Dermatology*, vol. 163, no. 3, pp. 495–501, 2010.

[39] R. A. Slater, I. Hershkowitz, Y. Ramot, A. Buchs, and M. J. Rapoport, "Reduction of digital plantar pressure by debridement and silicone orthosis," *Diabetes Research and Clinical Practice*, vol. 74, no. 3, pp. 263–266, 2006.

[40] M. Ko, L. Hughes, and H. Lewis, "Walking speed and peak plantar pressure distribution during barefoot walking in persons with diabetes," *Physiotherapy Research International*, vol. 17, no. 1, pp. 29–35, 2012.

[41] W. R. Ledoux, J. B. Shofer, M. S. Cowley, J. H. Ahroni, V. Cohen, and E. J. Boyko, "Diabetic foot ulcer incidence in relation to plantar pressure magnitude and measurement location," *Journal of Diabetes and Its Complications*, vol. 27, no. 6, pp. 621–626, 2013.

Hindawi Publishing Corporation
Journal of Diabetes Research
Volume 2016, Article ID 1567405, 9 pages
http://dx.doi.org/10.1155/2016/1567405

Research Article

Delay between Onset of Symptoms and Seeking Physician Intervention Increases Risk of Diabetic Foot Complications: Results of a Cross-Sectional Population-Based Survey

Norina A. Gavan,[1] Ioan A. Veresiu,[2] Etta J. Vinik,[3] Aaron I. Vinik,[4] Bogdan Florea,[5] and Cosmina I. Bondor[6]

[1]*Society of Diabetic Neuropathy, Worwag Pharma GmbH&Co.KG, Romanian Representative Office, 11 Fagului Street, 400483 Cluj-Napoca, Romania*
[2]*Department of Diabetes, Nutrition and Metabolic Diseases, Iuliu Hatieganu University of Medicine and Pharmacy Cluj-Napoca, 4-6 Clinicilor Street, 400006 Cluj-Napoca, Romania*
[3]*Eastern Virginia Medical School, Strelitz Diabetes Center, 855 West Brambleton Avenue, Norfolk, VA 23510, USA*
[4]*Research & Neuroendocrine Unit, Eastern Virginia Medical School, 855 West Brambleton Avenue, Norfolk, VA 23510, USA*
[5]*IMOGEN Research Center, Iuliu Hatieganu University of Medicine and Pharmacy Cluj-Napoca, 8 Victor Babeş Street, 400012 Cluj-Napoca, Romania*
[6]*Department of Medical Informatics and Biostatistics, Iuliu Hatieganu University of Medicine and Pharmacy Cluj-Napoca, 6 Pasteur Street, 400349 Cluj-Napoca, Romania*

Correspondence should be addressed to Cosmina I. Bondor; cosmina_ioana@yahoo.com

Received 7 September 2016; Accepted 16 November 2016

Academic Editor: Frank Bowling

We present a post hoc analysis of 17,530 questionnaires collected as part of the 2012 screening for neuropathy using Norfolk Quality of Life tool in patients with diabetes in Romania, to assess the impact on foot complications of time between the onset of symptoms of diabetes/its complications and the physician visit. Odds ratios (ORs) for self-reporting neuropathy increased from 1.16 (95% CI: 1.07–1.25) in those who sought medical care in 1–6 months from symptoms of diabetes/its complications onset to 2.27 in those who sought medical care >2 years after symptoms onset. The ORs for having a history of foot ulcers were 1.43 (95% CI: 1.26–1.63) in those who sought medical care in 1–6 months and increased to 3.08 (95% CI: 2.59–3.66) in those who sought medical care after >2 years from symptoms of diabetes/its complications onset. The highest ORs for a history of gangrene (2.49 [95% CI: 1.90–3.26]) and amputations (2.18 [95% CI: 1.60–2.97]) were observed in those who sought medical care after >2 years following symptoms onset. In conclusion, we showed that waiting for >1 month after symptoms onset dramatically increases the risk of diabetic foot complications. These results show the need for accessible educational programs on diabetes and its chronic complications and the need to avoid delays in reporting.

1. Introduction

Diabetes represents a major worldwide epidemic that poses a great social, economic, and medical burden on both developed and underdeveloped countries [1]. An analysis of health examination surveys and epidemiological data involving 2.7 million participants performed in 2011 by Danaei et al. [2] showed that the number of people with diabetes doubled between 1980 and 2008, increasing from 153 million to 347 million. According to the International Diabetes Federation these figures further increased to 415 million in 2015 and are estimated to reach 642 million in 2040 [1]. Furthermore, it is estimated that 192.8 million people with diabetes have not been diagnosed and have an increased risk of developing complications, thus posing an additional burden on society [1]. The financial cost of diabetes is high. The estimated

global health spending for treatment of diabetes and its complications for 2015 ranged between 673 and 1,197 billion USD [1]. An increase in the number of patients with diabetes has been also reported for Romania; the number of patients increased from 482,250 in 2005 to 803,489 in 2011 [3, 4]. The most recent epidemiological study performed in Romania between December 2012 and February 2014 showed that in adults 20 to 79 years of age the overall prevalence of diabetes adjusted for age and gender was 11.6% [5]. However, except for the analysis of the trends in the diabetes-related lower extremities [6], little information is available on the prevalence of its chronic complications or patient attitudes toward diabetes in Romania.

The Quality of Life in Patients with Diabetic Neuropathy in Romania was a cross-sectional, noninterventional, multicenter survey performed with the involvement of 181 healthcare professionals and aimed to capture undiagnosed neuropathy in patients with self-reported diabetes in Romania by using the Norfolk Quality of Life (Norfolk-QOL-DN) questionnaire as a screening tool [7]. This survey revealed a high prevalence of undisclosed neuropathy (50%) [7], as well as a high prevalence of foot ulcers (14.85%) and amputations (3.60%) in this population [8].

Here we present a post hoc analysis of data collected in this survey aiming to assess diabetes chronic complications (i.e., neuropathy, foot ulcers, gangrene, and amputations) as a function of time between the onset of symptoms of diabetes or its complications and the physician visit for those symptoms. At this time, no studies in Romania have assessed this association in patients with neuropathic symptoms. These results may help to fill the knowledge gap on patients' health beliefs and provide invaluable support for developing future educational programs aimed at preventing diabetes complications.

2. Materials and Methods

The methodology of this cross-sectional survey performed between January and December 2012 was previously described elsewhere [7]. Briefly, self-administered questionnaires were distributed by physician specialists in diabetes, neurologists, general practitioners, and nurses from all regions in Romania to their patients with diabetes. Data were collected using the Romanian version of the Norfolk QOL-DN questionnaire [7]. Of the 25,000 questionnaires distributed, 23,543 were returned and fully completed ones were entered in the database.

The questionnaire used comprises 35 scored items reflecting patients' health perception and used to calculate the total Norfolk QOL-DN score and 5 subdomain scores for symptoms, activities of daily living (ADLs), autonomic neuropathy, physical functioning/large fiber neuropathy, and small fiber neuropathy [5]. Additionally, the questionnaire has items which are not scored and were used to collect demographic (age, gender) and medical history information [7].

The analysis presented here contains the responses to the following medical history questions. "Do you have diabetes?" "Do you have neuropathy?" "Have you ever had an ulcer on your feet?" "Have you ever had gangrene?" "Have you had any toes or fingers amputated?" "How soon after the onset of the first symptoms of diabetes/its complications did you make an appointment for a physician visit and see the physician?" The patients were asked to respond with "Yes" or "No" to all these questions, except for the last one in which the patients were asked to choose among the 6 possible responses:

(i) less than 1 month,

(ii) between 1 and 6 months,

(iii) between 6 and 12 months,

(iv) between 1 and 2 years,

(v) over 2 years,

(vi) I do not know/I do not remember.

Before completion of questionnaires, the patients were informed that their personal data would be collected as part of this survey and consented for their data to be analyzed. The survey was approved by The National Supervisory Authority for Personal Data Processing under the number 0006753.22-03-2012.

2.1. Main Objective. The main objective of the analysis presented here was to assess the association between the presence of self-reported neuropathy, foot ulcers, gangrene and amputations and the time interval between the onset of symptoms of diabetes or its complications and the physician visit for those symptoms.

2.2. Statistical Analysis. For the analysis presented here we included only those questionnaires which provided "Yes" as an answer to the question "do you have diabetes?" Of these, only those with an answer other than "I do not know/I do not remember" to the question "how soon after the onset of the first symptoms of diabetes/its complications did you make an appointment for a physician visit and see the physician?" were included in the analysis.

Qualitative variables were summarized with frequency tables. Descriptive statistics (mean, standard deviation, and standard error, minimum, and maximum) were calculated for continuous variables. The age and total Norfolk QOL-DN and subdomain scores were compared between categories of responses to the questions "how soon after the onset of the first symptoms of diabetes/its complications did you make an appointment for a physician visit and see the physician?" by student t-test and Kruskal Wallis test. Gender, frequency of self-reported neuropathy, foot ulcers, gangrene, and amputations were compared by Chi-square test.

The association of the time interval between patient symptoms onset and physician visit for those symptoms with self-declaring the presence of neuropathy and the probability a history of foot ulcers, gangrene, or amputations was tested by logistic regression while controlling for age and sex. Additionally, the models with the history of foot ulcers, gangrene, or amputations as dependent variables were adjusted for the presence of neuropathy.

Two-way bifactorial ANOVA with age and gender as covariates was used to test the influence of time interval

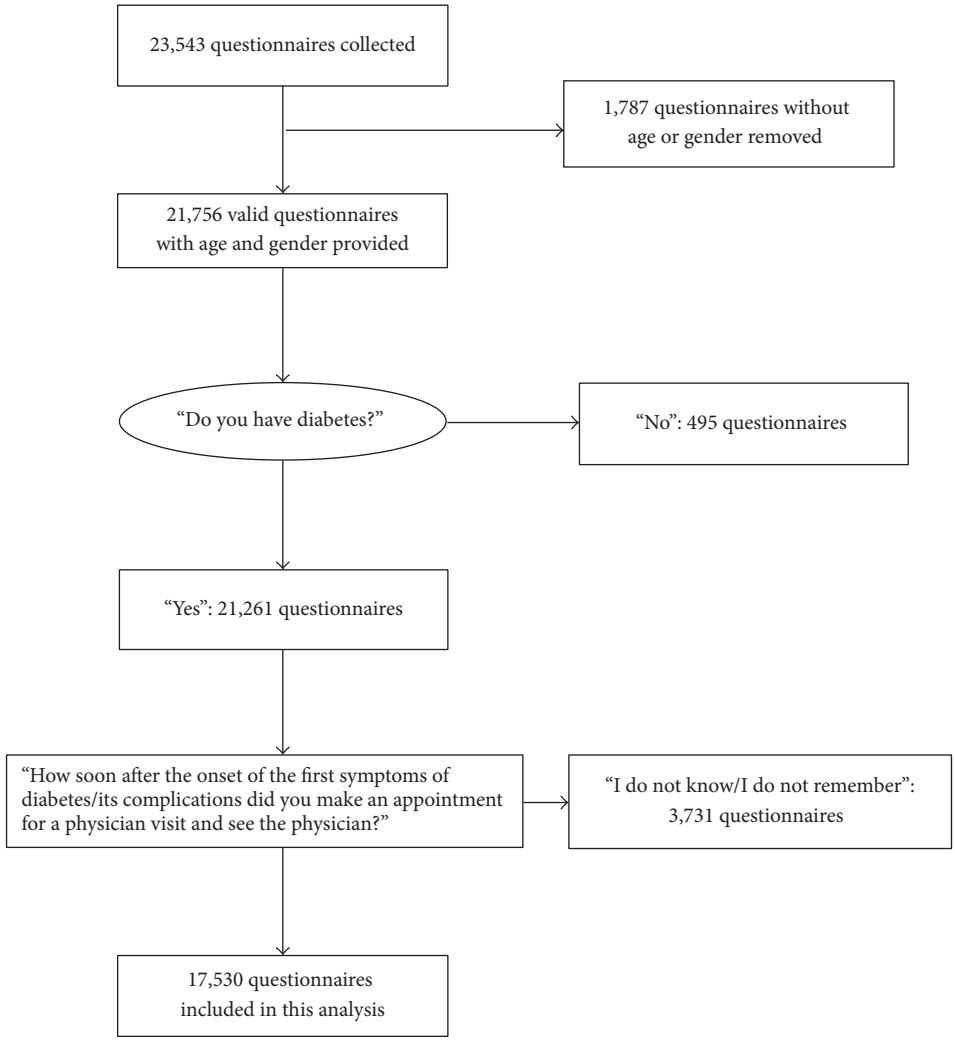


Figure 1: Participant flow diagram.

between symptoms onset and physician visit on the total Norfolk QOL-DN and the presence of self-reported neuropathy. The age and sex were used as covariates due to significant differences between groups in terms of age observed in the current analysis and the differences in the total Norfolk QOL-DN score in men and women previously observed in our sample [7]. The estimated marginal means of the Norfolk QOL-DN total score adjusted for age and sex calculated with this model were further compared with the cut-off previously used as suggestive for the presence of diabetic neuropathy [7].

All statistical analyses were performed using IBM SPSS Statistics for Windows, Version 15.0 (Armonk, NY: IBM Corp). A p value < 0.05 was considered statistically significant.

3. Results and Discussions

3.1. Results. As previously described [7], 23,543 completed questionnaires were collected. Of these, 21,756 had age and gender provided and were considered valid. After removing those with a missing answer or "No" as an answer to the question "do you have diabetes?" (495 questionnaires; 2.3% of the valid questionnaires) and those with a missing answer or "I do not know/I do not remember" as an answer to the question "how soon after the onset of the first symptoms of diabetes/its complications did you make an appointment for a physician visit and see the physician?" (3,731 questionnaires; 17.1%), 17,530 questionnaires were included in the analysis presented here (Figure 1).

The majority of patients reported having sought medical care within 6 months after the onset of symptoms of diabetes/its complications: 4,401 of 17,530 (25.1%) reported having sought medical care within 1 month after the symptoms of diabetes/its complications onset and 7,023 of 17,530 (40.1%) between 1 and 6 months after the onset of symptoms. However, 1,558 of the 17,530 included in the analysis (8.9%) and 1,239 of the 17,530 included in the analysis (7.1%) reported having sought medical care only after 1 to 2 years and more than 2 years after the symptoms of diabetes/its complications onset, respectively (Figure 2).

Table 1: Demographic characteristics and history of self-reported neuropathy, foot ulcers, gangrene, and amputations according to the time interval between symptoms of diabetes/its complication onset and physician visit for those symptoms.

	N'	Time between symptom onset and physician visit for those symptoms					Total $N = 17{,}530$	p
		<1 month $N = 4{,}401$	1–6 months $N = 7{,}023$	6–12 months $N = 3{,}309$	1-2 years $N = 1{,}558$	>2 years $N = 1{,}239$		
Women, n (%)	17,490	2,274 (51.8)	3,700 (52.8)	1,789 (54.2)	833 (53.6)	678 (54.8)	9,274 (53.0)	0.18
Age, years Mean ± SD	17,530	58.7 ± 12.3	60.3 ± 11.2	61.6 ± 10.7	62.2 ± 10.5	62.1 ± 10.1	60.5 ± 10.1	<0.001
Diabetes with self-reported neuropathy, n (%)	16,928	2,568 (60.7)	4,370 (64.7)	2,360 (72.9)	1,202 (78.9)	934 (78.8)	11,434 (67.5)	<0.001
Diabetes with history of foot ulcers, n (%)	17,242	382 (8.8)	853 (12.4)	526 (16.0)	308 (20.0)	328 (27.0)	2397 (13.9)	<0.001
Diabetes with history of gangrene, n (%)	17,240	135 (3.1)	266 (3.9)	148 (4.5)	93 (6.1)	105 (8.6)	747 (4.3)	<0.001
Diabetes with history of amputations, n (%)	1,7251	110 (2.5)	199 (2.9)	120 (3.7)	70 (4.6)	74 (6.1)	573 (3.3)	<0.001

N = number of patients in given category; N' = number of patients with available responses to a given question; n (%) = number (percentage); SD = standard deviation.

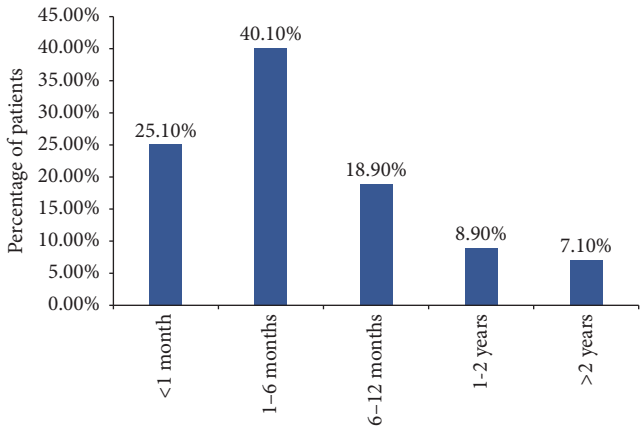


Figure 2: The time interval between symptoms of diabetes/its complication onset and physician visit for those symptoms.

No difference was observed in the time interval between symptoms of diabetes/its complications onset and physician visit in terms of gender (Table 1). Persons who sought medical care after more than 1 year following symptom onset were significantly older than those who sought medical care less than 1 month from symptom onset ($p < 0.001$).

The percentage of patients with a history of foot ulcers, gangrene, and amputations increased with the time interval between the symptoms of diabetes/its complications onset and the physician visit for those symptoms. The percentage of patients with a history of foot ulcers increased from 8.8% in the groups who sought medical care <1 month from symptom onset to 12.4% in those who sought medical care between 1 to 6 months, 16.0% in those who sought medical care between 6 and 12 months from symptom onset, 20% in those who sought medical care between 1 and 2 years, and 27.0% in those sought medical care after 2 years. For gangrene and amputations, the percentage increased from 3.1% and 2.5%, respectively, in those who sought medical care within 1 month to 8.6% and 6.1%, respectively, in those who sought medical care after more than 2 years. A similar trend was also observed for self-reported neuropathy on the prevalence of foot complications (Table 1).

The odds of self-reporting the presence of neuropathy were significantly higher in those who sought medical care later than 1 month from symptoms of diabetes/its complications onset as compared to those who sought medical care within 1 month from symptoms onset. Odds ratios (ORs) for reporting neuropathy increased from 1.16 (95% confidence interval [CI]: 1.07–1.25) in those who sought medical care between 1 and 6 months from symptoms of diabetes/its complications onset to 2.28 and 2.27 in those who sought medical care in 1 to 2 years or in more than 2 years after symptoms onset. The ORs for having a history of foot ulcers were also significantly higher in those who sought medical care after 1 month from symptoms of diabetes/its complications onset: 1.43 (95% CI: 1.26–1.63) in those who sought medical care in 1 to 6 months, 1.78 (95% CI: 1.54–2.06) in those who sought medical care in 6 to 12 months, 2.18 (95% CI: 1.84–2.58) in those who sought medical care in 1 to 2 years, and 3.08 (95% CI: 2.59–3.66) in those who sought medical care after more than 2 years from symptom onset. The ORs for having a history of gangrene were significantly higher as compared to the reference category (those who sought medical care in less than 1 month from symptom of diabetes/its complications onset) only in patients who sought medical care more than 6 months from symptom onset. The ORs for having a history of amputations were significantly higher as compared to the reference category only in patients who sought medical care more than 1 year from symptom of diabetes/its complications onset. The highest ORs for a history of gangrene and amputations were observed in those who sought medical care after more than 2 years following symptom onset: 2.49 (95% CI: 1.90–3.26) for gangrene and 2.18 (95% CI: 1.60–2.97) for amputations (Figure 3).

The mean scores for total Norfolk QOL-DN and all subdomain scores increased significantly and in parallel with

Dependent variables	Time interval between symptoms onset and physician visit	OR (95% CI)	p
Self-reported neuropathy[a]	1–6 months	1.16 (1.07–1.25)	<0.001
	6–12 months	1.66 (1.50–1.84)	<0.001
	1-2 years	2.28 (1.98–2.62)	<0.001
	>2 years	2.27 (1.95–2.65)	<0.001
Self-reported history of foot ulcers[b]	1–6 months	1.43 (1.26–1.63)	<0.001
	6–12 months	1.78 (1.54–2.06)	<0.001
	1-2 years	2.18 (1.84–2.58)	<0.001
	>2 years	3.08 (2.59–3.66)	<0.001
Self-reported history of gangrene[b]	1–6 months	1.20 (0.97–1.49)	0.10
	6–12 months	1.28 (1.01–1.64)	0.04
	1-2 years	1.62 (1.23–2.14)	0.0007
	>2 years	2.49 (1.90–3.26)	<0.001
Self-reported history of amputations[b]	1–6 months	1.09 (0.86–1.39)	0.463
	6–12 months	1.29 (0.99–1.69)	0.061
	1-2 years	1.50 (1.09–2.05)	0.012
	>2 years	2.18 (1.60–2.97)	<0.001

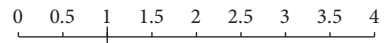


Figure 3: Forest plot for the probability of declaring a history of neuropathy, foot ulcers, gangrene, and amputations according to the time interval between onset of symptoms of diabetes/complications and physician visit. Category <1 month was considered as reference in the model. [a]Regression model adjusted for age and gender. [b]Regression model adjusted for the presence of self-reported neuropathy, age, and gender. OR: odds ratio; CI: confidence interval.

the time interval between the symptoms onset and the physician visit for those symptoms (Figure 4, $p < 0.001$ for all). For all these comparisons, the lower scores, which represent better QOL, were observed in those who sought medical care within 1 month from symptoms of diabetes/its complications onset: 22.72 for total Norfolk QOL-DN score; 12.53 for physical functioning/large fiber neuropathy; 5.27 for symptoms; 2.05 for ADLs; 1.34 for autonomic neuropathy; and 1.53 for small fiber subdomain. The maximum score, indicating poorer QOL, was observed in those who sought medical care more than 2 years after the symptom onset: 40.96 for Norfolk QOL-DN score; 21.72 for physical functioning/large fiber neuropathy; 8.31 for symptoms; 4.59 for ADLs; 2.59 for autonomic neuropathy; and 3.75 for small fiber subdomain.

The ANOVA analysis confirmed that the time between symptoms of diabetes/its complications onset and physician visit for those symptoms had a significant impact on the total Norfolk QOL-DN score and that this association was independent of age and gender. A significant interaction was also observed in this model between the Norfolk QOL-DN and self-reported neuropathy (Figure 5). The estimated marginal means of Norfolk QOL-DN, adjusted for age and

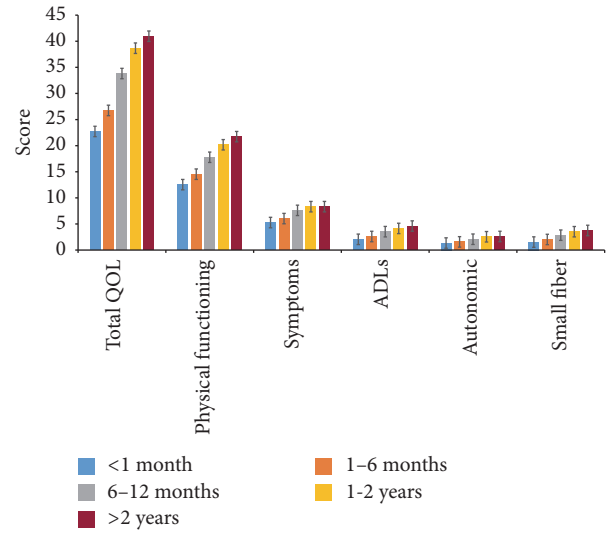


Figure 4: Norfolk QOL-DN total and subscale scores in Romanian patients with self-reported diabetes mellitus according to the time interval between onset of symptoms of diabetes/complications and physician visit for those symptoms. QOL: quality of life; ADLs: activities of daily living. $p < 0.001$ for trend for Norfolk QOL-DN total and subscale scores.

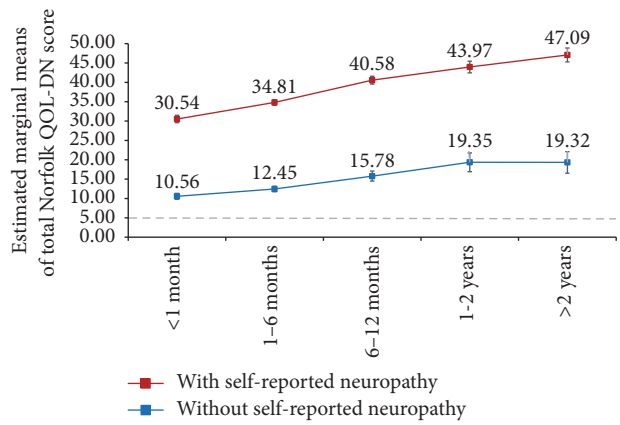


Figure 5: Estimated marginal means of Norfolk QOL-DN total score in Romanian patients with self-reported diabetes mellitus with and without neuropathy according to the time interval between onset of symptoms of diabetes/complications and physician visit for those symptoms after controlling for age and gender. Error bars represent 95% confidence interval of the estimated marginal means of the total Norfolk-QOL score. Dashed line represents the total Norfolk-QOL score cut-off value suggestive for the presence of neuropathy [7].

sex, in those with self-reported neuropathy were 30.54 for those who sought medical care within 1 month after symptoms of diabetes/its complications onset and increased to 47.09 in those who sought medical care after more than 2 years from symptoms onset. A similar trend was observed in patients without self-reported neuropathy. The estimated marginal means of Norfolk QOL-DN, adjusted for age and sex, increased in parallel with time interval between symptoms of diabetes/its complications onset and a physician visit (ranging from 10.56 in those who sought medical care in less than 1 month after symptoms onset and 19.35 in those who sought medical care between 1 and 2 years after the symptoms onset). All estimated marginal means of Norfolk QOL-DN were higher than the cut-off previously used suggestive for the presence of neuropathy [7].

3.2. Discussion. The results of this analysis of the data collected in the screening for neuropathy survey using the QOL-DN Norfolk tool in patients with diabetes in Romania showed that the majority of patients sought medical care after only 1 month from the onset of symptoms of diabetes or its complications, while 33% waited for more than 6 months before addressing a physician and 16% sought medical care only after 1 year.

We found a significant association between the time between the occurrence of symptoms of diabetes/its complications and the consult with a physician and the occurrence of self-reported neuropathy, foot ulcers, gangrene, and amputations, confirming previously observed results. The odds of self-reported neuropathy and foot ulcers were significantly higher in those who delayed seeking medical care for more than 1 month after the onset of symptoms of diabetes or its complications as compared to those presenting within 1 month from symptoms onset and increased in parallel with the time between the symptoms onset and the physician visit. Notably, the risk of more serious complications, such as gangrene and amputations, became significantly higher in those who sought medical care after 1 year from symptoms onset and more than doubled in those who sought medical care after 2 years from symptoms onset.

Previously we found a high prevalence (52.5%) of undisclosed diabetic neuropathy in this population [7]. The results of the analysis presented here show that the high prevalence of the undisclosed neuropathy is partially attributable to patients who do not report their symptoms to the healthcare providers or report them late after the symptoms onset, thus showing the limited efficacy of existing educational programs in diabetes in Romania. This is in line with results of previous studies and surveys which showed that diabetes is often perceived by patients as a nonserious disease until complications occur [9–11]. According to the Health Belief Model, a person will probably take action toward a disease if he/she perceives themselves to be at risk for the disease, is aware of the severity of a condition and of the benefits of certain actions, and is given cues for actions [12]. Additionally, identifying barriers and practical ways to overcome them is also important [13]. Previous studies in patients with diabetes showed that a patient-centered multidisciplinary educational approach adapted to their level of understanding is effective in increasing the compliance and adherence to diabetes management and preventive care of chronic complications [14–17] and has a positive impact on reducing the diabetes-associated complications and costs [18–24]. Here we show that the answer to a single question on a positive response to the development of symptoms of diabetes/its complications should alert the individual to seek care forthwith. Our results clearly demonstrate that delaying more than one month can have a disastrous outcome on the foot complications of diabetes and education programs should impart this simple message.

The type of symptoms experienced by patients may be another cause of delay between onset of symptoms and seeking physician intervention. In a previous analysis of this survey (Gavan et al., Symptom Characteristics That Alert Patients to the Diagnosis of Diabetic Neuropathy, 2015, submitted), we identified a group of patients who self-reported neuropathy without being diagnosed by a physician. This group reported more frequently symptoms in hands and arms than the group with a previous diagnosis of neuropathy who reported more frequently symptoms in legs and feet. A higher likelihood of the patients to self-report neuropathy although they had not been told they had this diagnosis was also associated with autonomic neuropathy, large fiber neuropathy, and ADLs subdomain scores. The presence of symptoms in feet and legs usually alerts patient to their neuropathy and probably these patients sought for medical care soon after the symptoms onset. The group of patients with symptoms in hands and arms, autonomic neuropathy, or symptoms attributed to small fiber neuropathy may have failed to recognize the defined symptoms or, if recognized, did not report them to their physician.

The mean total Norfolk QOL-DN score in those who sought medical care within 1 month from symptoms of diabetes/its complications onset was similar to those previously

reported in patients with neuropathy without symptoms (22.72) and the mean total Norfolk QOL-DN score in those who sought medical care after more than 2 years after symptoms onset (40.96) was similar to the ones of a group with symptomatic neuropathy in a German population [25]. These results confirmed once gain the deleterious impact that delays in diagnosis and treatment have on the progression of diabetic neuropathy.

Another cause for the delayed appointment to a physician after the symptoms of diabetes/its complications onset may be the limited access to foot examination in the diabetic population [7]. The foot examination in primary care is limited in both Romania and elsewhere [26–28]. Although as per Romanian medical system organization each patient with diabetes should be seen by their primary care physician or a physician specialist in diabetes every 3 months, we have reasons to believe that the majority of them do not have a foot examination performed at least once a year. This may also have a negative impact on the patient's perception on the importance of seeking medical care when symptoms of diabetes or its complications occur. A study performed as part of the Quality of Care and Outcomes in Type 2 Diabetes project aiming to assess the physician's attitude towards foot care education and foot exam in patients with type 2 diabetes showed that foot examination was performed less frequently by general practitioners as compared to physician specialists in diabetes and more frequently in those with foot complications (not diabetic neuropathy) [27]. Additionally, it was shown that patients who had a foot exam were more likely to check their feet regularly [27]. Educational programs targeting physicians have been shown to increase the performance of screening activities [26] and thus may have a positive impact on reducing the time between the onset of symptoms of diabetes or its complications and medical care.

Notwithstanding that our survey has strengths derived from the large number of patients included and which makes its results representative for Romanian patients with diabetes, it also has limitations. Although it would have provided more detailed information, we did not ask for separate answers for the time between the diabetes symptoms onset and the physician visit for those symptoms and between the time of symptoms of complications onset and the physician visit for those symptoms. We have not asked either specific question to assess which diabetes complication the patients referred to when answering the following question: "how soon after the onset of the first symptoms of diabetes/its complications did you make an appointment for a physician visit and see the physician?" We chose this approach to assess patients' beliefs, education, and knowledge and the overall attitude toward the health status and the primary and secondary prevention of diabetes and its complications. Therefore, we do not know if the patient answers referred to the time between the onset of symptoms of diabetes alone or its complications and the moment of seeking medical care for these symptoms. As we previously mentioned the Norfolk QOL-DN questionnaire was specifically designed for the evaluation of the impact of diabetic neuropathy on QOL and showed good sensitivity, specificity, and positive and negative predictive values for the severity of neuropathy [25, 29, 30]. In addition, this tool has been used in Romania to screen for diabetic neuropathy [7]. It has also been used to monitor QOL in patients with amyloid neuropathy [31]. Here we used an additional question to define the onset of symptoms of diabetes/complications which did not correspond with the items of the Norfolk QOL-DN. It remains to be ascertained more specifically what the content of the question on diabetes and its complications would be the most useful in proposing an education program to reduce the latency of patients' decision to visit a physician. However, we based our analysis on the questions asked of patients and their self-reported responses and not on reviewing medical charts or objective measures of neuropathy and its complications recall bias cannot be excluded and more specifically must depend on the nature of the question itself.

4. Conclusions

In conclusion, we showed that waiting for more than 1 month after symptom onset of diabetes/neuropathy dramatically increases the risk of neuropathy, foot ulcers, gangrene, and amputations. Seventy-five percent of the patients who completed the questionnaires waited for more than 1 month after the symptom onset to seek medical attention, and 16% sought medical attention after 1 year following the symptom onset. These results are alarming and support the need to implement easily accessible educational programs on diabetes and its chronic complications.

Competing Interests

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

Acknowledgments

The authors wish to thank Adriana Rusu (on behalf of Worwag Pharma GmbH&Co.KG, Romanian Rep. Office) for medical writing support. The authors would also like to thank to the members of the Diabetic Neuropathy Society (https://www.neurodiab.org/) for their kind support during this survey. This work was supported by Worwag Pharma GmbH&Co.KG, Romanian Rep. Office. Worwag Pharma GmbH&Co.KG, Romanian Rep. Office, was involved in all stages of the survey conduct and analysis. Worwag Pharma GmbH&Co.KG, Romanian Rep. Office, also took in charge all costs associated with the development and the publishing of the present manuscript. All authors had full access to the data.

References

[1] International Diabetes Federation, *IDF Diabetes Atlas*, International Diabetes Federation, Brussels, Belgium, 7th edition, 2015.

[2] G. Danaei, M. M. Finucane, Y. Lu et al., "National, regional, and global trends in fasting plasma glucose and diabetes prevalence since 1980: systematic analysis of health examination surveys and epidemiological studies with 370 country-years and 2·7 million participants," *The Lancet*, vol. 378, no. 9785, pp. 31–40, 2011.

[3] Ministerul Sanatatii, 2011 http://www.ms.ro/?pag=62&id=9226.

[4] M. Mota and I.-R. Dinu, "The analysis of prevalence and incidence of diabetes mellitus in Romania," *Romanian Journal of Diabetes, Nutrition and Metabolic Diseases*, vol. 20, no. 2, pp. 135–139, 2013.

[5] M. Mota, S. G. Popa, E. Mota et al., "Prevalence of diabetes mellitus and prediabetes in the adult Romanian population: PREDATORR study," *Journal of Diabetes*, vol. 8, no. 3, pp. 336–344, 2016.

[6] I. A. Veresiu, S. S. Iancu, and C. Bondor, "Trends in diabetes-related lower extremities amputations in Romania—A five year nationwide evaluation," *Diabetes Research and Clinical Practice*, vol. 109, no. 2, pp. 293–298, 2015.

[7] A. I. Veresiu, C. I. Bondor, B. Florea, E. J. Vinik, A. I. Vinik, and N. A. Gâvan, "Detection of undisclosed neuropathy and assessment of its impact on quality of life: a survey in 25,000 Romanian patients with diabetes," *Journal of Diabetes and its Complications*, vol. 29, no. 5, pp. 644–649, 2015.

[8] C. I. Bondor, I. A. Veresiu, B. Florea, E. J. Vinik, A. I. Vinik, and N. A. Gavan, "Epidemiology of diabetic foot ulcers and amputations in Romania: results of a cross-sectional quality of life questionnaire based survey," *Journal of Diabetes Research*, vol. 2016, Article ID 5439521, 7 pages, 2016.

[9] M. Johnson, P. Newton, and E. Goyder, "Patient and professional perspectives on prescribed therapeutic footwear for people with diabetes: A Vignette Study," *Patient Education & Counseling*, vol. 64, no. 1–3, pp. 167–172, 2006.

[10] M. Johnson, P. Newton, M. Jiwa, and E. Goyder, "Meeting the educational needs of people at risk of diabetes-related amputation: a vignette study with patients and professionals," *Health Expectations*, vol. 8, no. 4, pp. 324–333, 2005.

[11] J. J. Gagliardino, C. González, J. E. Caporale et al., "The diabetes-related attitudes of health care professionals and persons with diabetes in Argentina," *Revista Panamericana de Salud Pública*, vol. 22, no. 5, pp. 304–307, 2007.

[12] N. K. Janz and M. H. Becker, "The health belief model: a decade later," *Health Education Quarterly*, vol. 11, no. 1, pp. 1–47, 1984.

[13] K. M. Chapman, J. O. Ham, P. Liesen, and L. Winter, "Applying behavioral models to dietary education of elderly diabetic patients," *Journal of Nutrition Education*, vol. 27, no. 2, pp. 75–79, 1995.

[14] M. I. Anselmo, M. Nery, and M. C. R. Parisi, "The effectiveness of educational practice in diabetic foot: a view from Brazil," *Diabetology & Metabolic Syndrome*, vol. 2, no. 1, article no. 45, 2010.

[15] F. Sharaf, "Impact of health education on compliance among patients of chronic diseases in Al Qassim, Saudi Arabia," *International Journal Health Sciences*, vol. 4, no. 2, pp. 139–148, 2010.

[16] S. L. Norris, M. M. Engelgau, and K. M. Venkat Narayan, "Effectiveness of self-management training in type 2 diabetes," *Diabetes Care*, vol. 24, no. 3, pp. 561–587, 2001.

[17] L. Steed, D. Cooke, and S. Newman, "A systematic review of psychosocial outcomes following education, self-management and psychological interventions in diabetes mellitus," *Patient Education and Counseling*, vol. 51, no. 1, pp. 5–15, 2003.

[18] I. Duncan, C. Birkmeyer, S. Coughlin, Q. Li, D. Sherr, and S. Boren, "Assessing the value of diabetes education," *Diabetes Educator*, vol. 35, no. 5, pp. 752–760, 2009.

[19] S. A. Boren, K. A. Fitzner, P. S. Panhalkar, and J. E. Specker, "Costs and benefits associated with diabetes education a review of the literature," *Diabetes Educator*, vol. 35, no. 1, pp. 72–96, 2009.

[20] M. R. Dalal, S. B. Robinson, and S. D. Sullivan, "Real-world evaluation of the effects of counseling and education in diabetes management," *Diabetes Spectrum*, vol. 27, no. 4, pp. 235–243, 2014.

[21] J.-P. Assal, A. Albeanu, B. Peter-Riesch, and J. Vaucher, "Cost of training a diabetes mellitus patient. Effects on the prevention of amputation," *Diabete & Metabolisme*, vol. 19, supplement 5, pp. 491–495, 1993.

[22] J. M. Malone, M. Snyder, G. Anderson, V. M. Bernhard, G. A. Holloway Jr., and T. J. Bunt, "Prevention of amputation by diabetic education," *The American Journal of Surgery*, vol. 158, no. 6, pp. 520–524, 1989.

[23] G. R. Tennvall and J. Apelqvist, "Prevention of diabetes-related foot ulcers and amputations: a cost-utility analysis based on Markov model simulations," *Diabetologia*, vol. 44, no. 11, pp. 2077–2087, 2001.

[24] L. A. Lavery, R. P. Wunderlich, and J. L. Tredwell, "Disease management for the diabetic foot: effectiveness of a diabetic foot prevention program to reduce amputations and hospitalizations," *Diabetes Research and Clinical Practice*, vol. 70, no. 1, pp. 31–37, 2005.

[25] E. J. Vinik, J. F. Paulson, S. L. Ford-Molvik, and A. I. Vinik, "German-translated Norfolk quality of life (QOL-DN) identifies the same factors as the english version of the tool and discriminates different levels of neuropathy severity," *Journal of Diabetes Science and Technology*, vol. 2, no. 6, pp. 1075–1086, 2008.

[26] K. E. O'Brien, V. Chandramohan, D. A. Nelson, J. R. Fischer Jr., G. Stevens, and J. A. Poremba, "Effect of a physician-directed educational campaign on performance of proper diabetic foot exams in an outpatient setting," *Journal of General Internal Medicine*, vol. 18, no. 4, pp. 258–265, 2003.

[27] G. De Berardis, F. Pellegrini, M. Franciosi et al., "Physician attitudes toward foot care education and foot examination and their correlation with patient practice," *Diabetes Care*, vol. 27, no. 1, pp. 286–287, 2004.

[28] A. E. Pinilla, A. L. Sánchez, A. Mejía, and M. P. Barrera, "Primary-care prevention activities in outpatients suffering from diabetic foot care," *Revista de Salud Pública*, vol. 13, no. 2, pp. 262–273, 2011.

[29] E. J. Vinik, R. P. Hayes, A. Oglesby et al., "The development and validation of the Norfolk QOL-DN, a new measure of patients' perception of the effects of diabetes and diabetic neuropathy," *Diabetes Technology and Therapeutics*, vol. 7, no. 3, pp. 497–508, 2005.

[30] A. Boyd, C. Casselini, E. Vinik, and A. Vinik, "Quality of life and objective measures of diabetic neuropathy in a prospective placebo-controlled trial of ruboxistaurin and topiramate," *Journal of Diabetes Science and Technology*, vol. 5, no. 3, pp. 714–722, 2011.

[31] E. J. Vinik, A. I. Vinik, J. F. Paulson et al., "Norfolk QOL-DN: validation of a patient reported outcome measure in transthyretin familial amyloid polyneuropathy," *Journal of the Peripheral Nervous System*, vol. 19, no. 2, pp. 104–114, 2014.

Hindawi Publishing Corporation
Journal of Diabetes Research
Volume 2016, Article ID 9027484, 8 pages
http://dx.doi.org/10.1155/2016/9027484

Research Article

Mean Daily Dosage of Aspirin and the Risk of Incident Alzheimer's Dementia in Patients with Type 2 Diabetes Mellitus: A Nationwide Retrospective Cohort Study in Taiwan

Cheng-Wei Chang,[1,2] Jorng-Tzong Horng,[3,4] Chi-Chang Hsu,[4] and Jui-Ming Chen[1,3]

[1]*Department of Endocrinology and Metabolism, Tungs' Taichung MetroHarbor Hospital, Taichung 435, Taiwan*
[2]*Department of Information Management, Hsing Wu University, New Taipei City, Taiwan*
[3]*Department of Biomedical Informatics, Asia University, Taichung 413, Taiwan*
[4]*Department of Computer Science and Information Engineering, National Central University, Chungli, Taiwan*

Correspondence should be addressed to Jui-Ming Chen; crm4545@seed.net.tw

Received 11 July 2016; Accepted 29 September 2016

Academic Editor: Dilek Yavuz

Background. Type 2 diabetes mellitus patients are known to have higher risk of developing dementia while aspirin use has been shown to prevent incident dementia. This study was conducted to evaluate the potential benefits of aspirin use on dementia in patients with type 2 diabetes mellitus and identify the appropriate dosage of aspirin that provides the most benefit. *Method*. A Taiwan nationwide, population-based retrospective 8-year study was employed to analyze the association between the use of aspirin and incidence of dementia including Alzheimer's disease and non-Alzheimer's dementia using multivariate Cox-proportional hazards regression model and adjusting for several potential confounders. *Results*. Regular aspirin use in mean daily dosage of within 40 mg was associated with a decreased risk of developing incident Alzheimer's dementia in patients with type 2 diabetes mellitus (adjusted HR of 0.51 with 95% CI of 0.27–0.97, p value 0.041). *Conclusion*. A mean daily dosage of aspirin use within 40 mg might decrease the risk of developing Alzheimer's disease in patients with type 2 diabetes mellitus.

1. Introduction

Diabetic patients have been reported to have a greater decline in cognitive function and a higher risk of developing dementia, although some studies announced that the relation between diabetes and dementia remains contestable [1–6].

Many previous studies have indicated that patients taking NSAIDs or aspirin had a decreased risk of Alzheimer's disease [7–15], including case control, prospective, and meta-analytical studies. However, there are also other investigations that failed to confirm such protective effect of NSAIDs or aspirin [16–18]. One study indicated that high but not low dose aspirin can prevent Alzheimer's disease [15], while another case control study reported that such a dosage effect did not exist [9]. These controversial findings indicated the need for further research on whether the use of aspirin might benefit certain group of elderly subjects and the appropriate dose to be prescribed.

Although many previous studies revealed a positive association between aspirin use and dementia, few of them focused on type 2 diabetes mellitus patients and did not indicate the appropriate dose needed to prevent Alzheimer's disease. So, we conducted this study using the National Health Insurance Research Database (NHIRD) in Taiwan [19] to evaluate the effect of aspirin use on decreasing the risk of dementia in type 2 diabetes mellitus cohort and the appropriate dose of aspirin.

2. Materials and Method

2.1. Data Sources. This study utilized claims data from the National Health Insurance Research Dataset (NHIRD), maintained by the National Health Research Institute (NHRI) in Taiwan, which includes statistical information of more than 96% of the Taiwan population [20]. The National Health

Insurance Research Database (NHIRD), which is provided to researchers for academic research purpose in Taiwan, contains a number of large computerized databases including original files and registration data. These data were obtained from the insurance system constructed by the Bureau of National Health Insurance (NHI). These files are deidentified by scrambling the identification codes of individuals and medical facilities; then the patients' information is sent to the National Health Research Institutes and forms the original files of the NHIRD [21].

This study used a 1,000,000-individual randomly selected sample from the Taiwan NHIRD. The enrolled patients were followed up from 1997 to 2008.

In this article, we applied the databases for patients' information including encoded identification number, gender, birth and death date, diagnostic data, and procedures and discharge status (diagnosed cancers before, died or transferred out, less than three diagnoses). The diagnostic data included initial diagnosis date, specific treatment items, and the relevant International Classification of Diseases, Revision Ninth, Clinical Modification (ICD-9-CM) diagnosis codes.

2.2. Study Sample. This study was designed as a population-based retrospective cohort study. Cohort study is an association research study that tracks the same people over a period of time for identifying the incidence of dementia occurrence with or without exposure to the use of aspirin. The study sample was drawn from 1997–2008 Taiwan NHIRD [19]. As the data files consisted of unidentified secondary data (National Health Insurance Research Database), the study was exempted from a full review by the Institutional Review Board of Tungs' Taichung MetroHarbor Hospital. Obtaining informed consent from the study population was not required due to the deidentified data files, the large size of the population (1 million), and the deceased status of some of the population by the time of the study.

We identified 28,321 patients with diagnosis of type 2 diabetes mellitus who were above 50 years old with no history of dementia on January 1, 2000, and who presented in ambulatory visit file for at least 2 times within any one year between 2000 and 2008. NHIRD employed A-code until December 31, 1999. Since A-code cannot precisely identify subjects with Alzheimer's disease and non-Alzheimer dementia, we excluded type 2 diabetes mellitus patients with dementia ($n = 612$) (A-codes A210 and A222) before 2000 from the study samples. The study then separated the type 2 diabetes mellitus samples into two groups: those who had never used aspirin ($n = 10{,}720$) and those who used aspirin regularly ($n = 2{,}876$). The detailed study flow was presented in Figure 1.

We defined regular aspirin users as the regular use of aspirin for more than a year and the interval between successive prescription drug records cannot exceed 120 days. A total of 13,596 patients in this study were individually traced until December 31, 2008, after index prescription of aspirin to identify patients who had been diagnosed with Alzheimer's disease (ICD-9-CM codes 290.0, 290.10–290.13, 290.20, 290.21, 290.3, 294.1, and 331.0) or non-Alzheimer dementia (ICD-9-CM codes 046.1, 290.1, 290.2, 290.4, 290.40–290.43, 294.11, 331.1, 331.11, 331.19, 331.2, and 331.7–331.9) at least twice in any one year during the follow-up period. In this study, we further classified regular aspirin users into three subgroups based on their average daily dose.

The index date of follow-up period for the group that never used aspirin was assigned to the date of type 2 diabetes mellitus diagnosis, whereas the index date of follow-up period for the group that used aspirin regularly was assigned to the first prescription date of aspirin. The end date of follow-up period for both nonaspirin users and regular aspirin users was assigned to the date of dementia, the date of death, or 31 of December, 2008, whichever came first. The dose of aspirin used was a mean daily dose which was calculated by cumulative doses divided by cumulative observation days. It is not the real daily dose prescribed to the patients.

The comorbid medical conditions for each individual were evaluated by using the established Charlson-Deyo comorbidity index (CCI). Chronic concomitant diseases of study samples related to arthropathy, cardiovascular, gastrointestinal, hepatic, neoplastic, neurologic, pulmonary, and renal diseases were categorized from the CCI index and have been described in detail in Table 1.

2.3. Statistical Analysis. The SAS statistical software (version 9.2) was used to perform all programming and statistical analyses in this study. The t-test and Pearson $\chi 2$ test were used to examine the differences in demographic characteristics of T2DM patients with and without regular use of aspirin. Demographic characteristics included categorical variables such as age group, gender, types of stroke, statins, antihypertensive drugs, CCI group, follow-up group, antidiabetic drug types, and underlying chronic diseases.

The risk for dementia associated with type 2 diabetes mellitus and exposure to regular use of aspirin was estimated by the cox proportional hazards models. Cox proportional hazards models provided both unadjusted and adjusted hazard ratios with 95% confidence interval. The adjusted hazard ratios included age group, gender, CCI group, stroke types, and antidiabetic drugs as potential covariates. The Kaplan-Meier method was used to compare the cumulative Alzheimer's disease and non-Alzheimer dementia events-free unjustified survival probabilities from 1997 to 2008 with different dosage of aspirin used in patients with type 2 diabetes mellitus. Survival curves among type 2 diabetes mellitus patients were created individually based on their aspirin medication status. The log-rank test was used to determine the significance of inequality with respect to aspirin medication status curves.

3. Results

3.1. Demographic Characteristics. A total of 28,321 patients diagnosed with type 2 diabetes mellitus whose age was above 50 and dementia-free before 1 January 2000 were selected. Among these, 10,720 were patients who never used aspirin and 2,876 were defined as patients who regularly used aspirin.

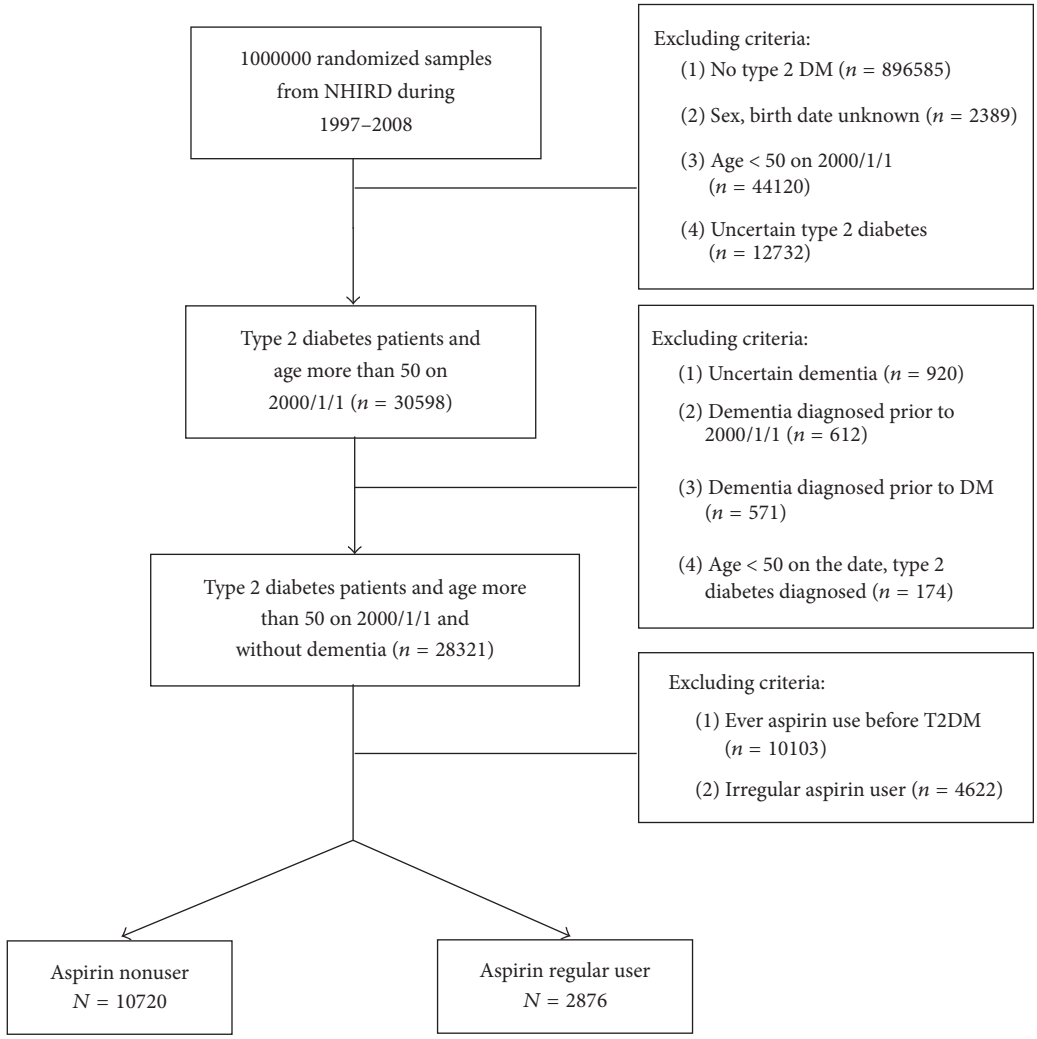


Figure 1: Study flowchart.

Table 1 compared the baseline characteristics between the aspirin users group and nonaspirin users group. Aspirin users with type 2 diabetes mellitus were older and more often male, more often had history of stroke, used more types of antidiabetes drugs, used antihypertensive drugs and statins more often, and had more chronic comorbidities. The mean duration of follow-up for the aspirin users group was 4.5 years and 5.0 years in nonaspirin users group.

3.1.1. Kaplan-Meier Dementia-Free Survival Curves. The log-rank test indicated that the nonaspirin users had significantly higher Alzheimer's disease and non-Alzheimer dementia events-free unjustified survival probabilities than aspirin users in Supplemental Figures 1 and 2 (see Supplementary Material available online at http://dx.doi.org/10.1155/2016/9027484). It is interesting to note that aspirin users had lower Alzheimer's disease and non-Alzheimer dementia events-free survival probabilities not until 2.5 years of follow-up and 1.5 years of follow-up, respectively. The regular aspirin users were then further classified into three subgroups based on their average daily dose. In Figure 2 and Supplemental Figures 3 and 4, regular aspirin users with low mean daily dose of aspirin (<40 mg) had higher Alzheimer's disease and non-Alzheimer's dementia events-free unjustified survival probabilities than the other subgroups and nonaspirin users.

3.1.2. Risk of Alzheimer's Disease and Non-Alzheimer's Dementia. Table 2 presents the results of the cox proportional hazard model for different status of aspirin usage during the follow-up period. Type 2 diabetes mellitus patients using aspirin had a higher risk of Alzheimer's disease (unadjusted HR: 1.33; 95% CI 1.06–1.68, $p = 0.016$) and non-Alzheimer's dementia (unadjusted HR: 1.94; 95% CI 1.19–3.16, $p = 0.008$) than type 2 diabetes mellitus patients without using aspirin in the unadjusted analyses. After adjusting for age, gender, history of stroke, types of antidiabetic drugs, statins, antihypertensive drugs, and CCI score, the adjusted results revealed no significant differences in Alzheimer's disease (adjusted HR: 1.37; 95% CI 1.05–1.78, $p = 0.019$) and

Table 1: Demographics of study subjects by aspirin using conditions between 1997 and 2008 in Taiwan.

Descriptor	Nonaspirin users among T2DM patients[a]		Aspirin users among T2DM patients[b]		p value[c]
	10720	(%)	2876	(%)	
Age group\mean ± SD	64.8 ± 8.2		66.9 ± 7.9		<0.001
50–59	3507	32.7	618	21.5	<0.001
60–69	4469	41.7	1265	44.0	
70–79	2182	20.4	810	28.2	
≥80	562	5.2	183	6.4	
Gender					
Male	5194	48.5	1479	51.4	<0.005
Female	5526	51.5	1397	48.6	
Stroke[d]	385	3.6	435	15.1	<0.001
No stroke	10335	96.4	2441	84.9	<0.001
Haemorrhagic stroke	123	1.1	49	1.7	<0.018
Ischemic stroke	154	1.4	262	9.1	<0.001
Transient ischemic stroke	69	0.6	87	3.0	<0.001
Unclassified	93	0.9	113	3.9	<0.001
Antidiabetes drug type[e]					
Never use	7026	65.5	880	30.6	<0.001
Acarbose	357	3.3	292	10.2	<0.001
Metformin	2291	21.4	1308	45.5	<0.001
Thiazolidinedione (TZD)	196	1.8	141	4.9	<0.001
Sulfonylureas	2563	23.9	1481	51.5	<0.001
Meglitinide	246	2.3	193	6.7	<0.001
Insulin	101	0.9	111	3.9	<0.001
Statin type[e]					
Never use	9811	91.5	2193	76.3	<0.001
Atorvastatin	352	3.3	296	10.3	<0.001
Fluvastatin	104	1.0	93	3.2	<0.001
Lovastatin	209	1.9	98	3.4	<0.001
Pravastatin	108	1.0	67	2.3	<0.001
Rosuvastatin	102	1.0	114	4.0	<0.001
Simvastatin	172	1.6	133	4.6	<0.001
Hypertensive drug type[e]					
Never use	6418	59.9	509	17.7	<0.001
Alpha blocker	545	5.1	294	10.2	<0.001
ARB	1415	13.2	1116	38.8	<0.001
ACEI	1073	10.0	755	26.3	<0.001
Beta blocker	1649	15.4	1087	37.8	<0.001
CCB	2560	23.9	1567	54.5	<0.001
Diuretics	785	7.3	498	17.3	<0.001
CCI score[f]\ mean ± SD	1.1 ± 1.3		1.6 ± 1.5		<0.001
CCI score of 0	4375	40.8	782	27.2	<0.001
CCI score of 1, 2	4813	44.9	1465	50.9	
CCI score of 3, 4	1251	11.7	500	17.4	
CCI score of ≥5	281	2.6	129	4.5	

Table 1: Continued.

Descriptor	Nonaspirin users among T2DM patients[a]		Aspirin users among T2DM patients[b]		p value[c]
	10720	(%)	2876	(%)	
Chronic diseases[d]					
No chronic disease	4046	37.7	744	25.9	<0.001
Arthropathy	414	3.9	132	4.6	0.078
Cardiovascular	1480	13.8	750	26.1	<0.001
Gastrointestinal	1600	14.9	428	14.9	0.954
Hepatic	2358	22.0	873	30.4	<0.001
Neoplasm	313	2.9	97	3.4	0.208
Neurologic	46	0.4	22	0.8	0.024
Pulmonary	3820	35.6	1224	42.6	<0.001
Renal	1065	9.9	439	15.3	<0.001

[a]Diagnosed T2DM patients who did not use aspirin before the end of follow-up date.

[b]The aspirin regular user is the patient who uses aspirin continuously at least over one year in the follow-up time that nearly 2 aspirin prescriptions cannot exceed 120 days. This group of diagnosed T2DM patients is aspirin regular user group.

[c]Wilcoxon rank sum test and Pearson's Chi-square test.

[d]The case number is calculated before patient's index date. The index date of patient who never uses aspirin before the end of follow-up date is the T2DM diagnosed date. The index date of patient who is aspirin regular user is the date of starting to take aspirin regularly.

[e]The case number is the regular use of the specific drugs before patient's end date of observation.

[f]Charlson comorbidity index (CCI). The diagnoses recorded in the National Health Insurance Research Database before the index date are used to calculate CCI score. We exclude the diagnosis of diabetes mellitus and stroke from CCI score calculation, because these two disease entities are considered separately.

SD: standard deviation, T2DM: type 2 diabetes mellitus, ARB: angiotensin II receptor blockers, ACEI: angiotensin converting enzyme inhibitors, and CCB: calcium channel blocker.

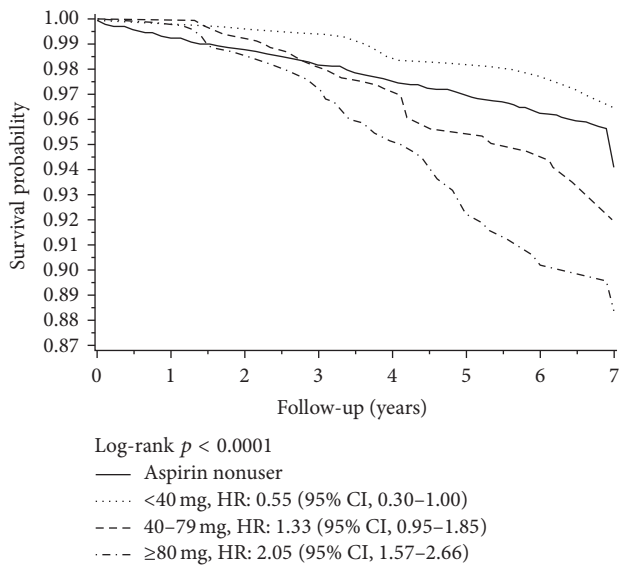


Figure 2: All-cause dementia-free survival curves by mean daily dosage of aspirin.

non-Alzheimer's dementia (adjusted HR: 1.91; 95% CI 1.09–3.36, $p = 0.025$) between type 2 diabetes mellitus patients with or without use of aspirin.

For the subgroup prescribed with a mean daily dose of less than 40 mg of aspirin, there was a trend towards a reduced risk of Alzheimer's disease (adjusted HR: 0.51, 95% CI 0.27–0.97, p value 0.041) as compared with nonaspirin users. However, no trend was detected in the risk of non-Alzheimer's dementia (adjusted HR: 0.28; 95% CI 0.04–2.05, $p = 0.209$) for patients prescribed with a mean daily dose of less than 40 mg of aspirin. In the subgroup prescribed with a mean daily dose between 40 mg and 80 mg of aspirin, the adjusted ratios were 1.27 (95% CI 0.84–1.91) and 2.54 (95% CI 1.23–5.24) for the risk of Alzheimer's disease and non-Alzheimer's dementia, respectively. For the subgroup prescribed with a mean daily dose of more than 80 mg of aspirin, an increased risk of developing Alzheimer's disease (adjusted HR: 2.26, 95% CI 1.64–3.12, $p < 0.001$) and non-Alzheimer's dementia (adjusted HR: 2.95, 95% CI 1.44–6.05, $p = 0.003$) was found in this study. As can be seen from Table 2, the risk of non-Alzheimer's dementia in type 2 diabetes mellitus patients increased with the increasing amount of aspirin usage.

4. Discussion

The main finding of this study showed that regular use of aspirin in a mean daily dose of within 40 mg might decrease the risk of developing Alzheimer's disease in patients with type 2 diabetes mellitus while no benefit was observed in non-Alzheimer's dementia. But once the mean daily dose of aspirin was higher than 80 mg per day, both the risks of incident Alzheimer's dementia and non-Alzheimer's dementia increased in patients with type 2 diabetes mellitus.

Our data confirmed the association between aspirin use and risk reduction of Alzheimer's dementia as seen in previous reports [7, 9, 10, 14, 15, 22–24]. In 2000, Broe et al. reported an inverse association between aspirin and

Table 2: Risk of Alzheimer's disease, non-Alzheimer dementia, and all-cause dementia among T2DM patients who regularly use aspirin.

Study subjects			All-cause dementia			
Aspirin status	Mean daily dose (mg)[a]	Dementia cases/total	Unadjusted HR (95% CI)	p value	Adjusted HR (95% CI)[b]	p value
No aspirin		360/10720	Reference (N/A)		Reference (N/A)	
Regular user		117/2876	1.42 (1.15–1.75)	0.001	1.45 (1.15–1.84)	0.002
Cohort 1	<40	11/522	0.55 (0.30–1.00)	0.050	0.48 (0.26–0.89)	0.019
Cohort 2	40–79	39/1003	1.33 (0.95–1.85)	0.096	1.47 (1.03–2.09)	0.033
Cohort 3	≥80	67/1351	2.05 (1.57–2.66)	<0.001	2.35 (1.75–3.15)	<0.001
Study subjects			Alzheimer's disease			
Aspirin status	Mean daily dose (mg)[a]	Dementia cases/total	Unadjusted HR (95% CI)	p value	Adjusted HR (95% CI)[b]	p value
No aspirin		308/10668	Reference (N/A)		Reference (N/A)	
Regular user		93/2852	1.33 (1.06–1.68)	0.016	1.37 (1.05–1.78)	0.019
Cohort 1	<40	10/521	0.58 (0.31–1.10)	0.094	0.51 (0.27–0.97)	0.041
Cohort 2	40–79	28/992	1.13 (0.77–1.66)	0.545	1.27 (0.84–1.91)	0.257
Cohort 3	≥80	55/1339	1.99 (1.49–2.66)	<0.001	2.26 (1.64–3.12)	<0.001
Study subjects			Non-Alzheimer dementia			
Aspirin status	Mean daily dose (mg)[a]	Dementia cases/total	Unadjusted HR (95% CI)	p value	Adjusted HR (95% CI)[b]	p value
No aspirin		52/10412	Reference (N/A)		Reference (N/A)	
Regular user		24/2783	1.94 (1.19–3.16)	0.008	1.91 (1.09–3.36)	0.025
Cohort 1	<40	1/512	0.34 (0.05–2.46)	0.286	0.28 (0.04–2.05)	0.209
Cohort 2	40–79	11/975	2.49 (1.29–4.78)	0.006	2.54 (1.23–5.24)	0.012
Cohort 3	≥80	12/1296	2.42 (1.29–4.57)	0.006	2.95 (1.44–6.05)	0.003

[a]Mean daily dose (mg) = cumulative doses starting from the regular taking drug date to the end of observation date/days between the start regular taking drug date and the end of observation date.
[b]Adjust age group, gender, CCI group, stroke types, antidiabetic drugs, statins, and hypertensive drugs.
HR: hazard ratio; CI: confidence interval; T2DM: type 2 diabetes mellitus.

Alzheimer's dementia, but such association was not observed with vascular dementia [9]. Another population study of Alzheimer's dementia in Cache County reported that use of aspirin was also specifically associated with reduced occurrence of Alzheimer's dementia [11]. But the above studies did not specifically target type 2 diabetes mellitus patients and their patient numbers were also relatively smaller compared to our study. Besides, most of them also did not indicate the appropriate dose of aspirin used to prevent Alzheimer dementia. Their analysis also did not distinguish Alzheimer dementia and non-Alzheimer dementia.

There are also some studies with opposing results. An investigation of up to 2,300 participants from the Baltimore Longitudinal Study of Aging concluded that aspirin use was associated with greater prospective cognitive decline on select measures [16]. Another 12-year longitudinal clinical-pathologic study of 1,019 older Catholic clergy did not support a strong relation between aspirin and Alzheimer disease (HR 0.84, 95% CI 0.63–1.11) [17]. Results from a population-based cross-sectional study with 2,708 patients enrolled revealed that long-term NSAIDs use has a protective effect against Alzheimer's dementia, but this association was statistically significant only for nonaspirin NSAIDs use [25]. Another two-wave longitudinal study over 3.6 years also was not able to conclude the protective effect of aspirin on Alzheimer dementia [18].

In our present study, we did not show a risk reduction of non-Alzheimer dementia in response to regular aspirin use. The lack of statistical significance of the protecting effects of low dose aspirin in developing non-Alzheimer dementia was likely due to limited sample size since the punctual estimate was quite low (HR = 0.28). In fact, vascular dementia accounts for about 20–30% of all the dementia and comprises the majority of non-Alzheimer disease dementia. In support of our findings, Broe et al. reported an inverse association between aspirin and Alzheimer's dementia, but such association was not observed with vascular dementia [9].

In our study, we also found that a mean daily dose of aspirin of within 40 mg is statistically significant in preventing Alzheimer's dementia (adjusted HR: 0.48, 95% CI 0.25–0.90, p value 0.022). Although there were many studies about the use of aspirin in preventing Alzheimer's dementia, only a few of them indicated the appropriate dose. A Swedish population-based study on individuals 80 years old or more revealed users of high-dose (>500 mg/day) aspirin had significantly lower prevalence of Alzheimer's dementia whereas users of 75 mg daily dose had only numerical but

insignificant reduction of Alzheimer's dementia, even after correction of stroke, transient ischemic attack, myocardial infarction, angina pectoris, and congestive heart failure [15]. In some studies examining dosage effects, elderly persons who took high-dose NSAIDs got poorer memory and decline faster than those taking low doses [26]. Another case control study involving subjects with average age of 81 disclosed that not only a high-dose (>1000 mg/day) anti-inflammatory action of aspirin but also low-dose antiplatelet action (<500 mg/day) is protective against Alzheimer's dementia [9]. Their results suggested that the anti-inflammatory drug hypothesis of Alzheimer's dementia prevention should be reviewed. In addition, alternate mechanisms of low-dose NSAID and/or aspirin drug action should be considered. They proposed that such low doses of NSAID and/or aspirin can protect against Alzheimer's dementia by ameliorating platelet and endothelium dysfunction [9].

As with most retrospective studies, there are strengths and limitations in our study. The greatest strength of our study lies in its large-scale population-based data and relative longer follow-up period (8 years). But there are some pertinent limitations in our study. The dose of aspirin used was a mean daily dose which was calculated by cumulative dose divided by cumulative observation days. It was not the real daily dose prescribed to the patients.

Besides, we only identified 11 patients with all-cause dementia whose mean daily dose of aspirin was less than 40 mg. Therefore, if we further divided those 11 patients into 4 different age groups, a relative low sample size has a weak power to detect the difference in significance which is listed in Table 2. Also, ICD-9 codes may not accurately reflect the patients' diagnoses due to some artificial errors. Besides, the clinical status of patients' glycemic and blood pressure control, other potential risk factors for dementia such as education, diet, smoking, and alcohol use and apolipoprotein E4 genotype were not provided by the administrative claims dataset [6]. Another limitation is that our study cohort is all Taiwanese people; the results of our study may not apply to type 2 diabetic patients with different ethnicity.

5. Conclusion

Mean daily dose of aspirin use within 40 mg might decrease the risk of developing Alzheimer's disease in patients with type 2 diabetes mellitus while no benefit was observed in non-Alzheimer's dementia. But once the mean daily dose of aspirin was higher than 80 mg per day, both the risks of incident Alzheimer's dementia and non-Alzheimer's dementia increased in patients with type 2 diabetes mellitus. The exact mechanism of these effects needs further elucidation and investigations.

Competing Interests

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

Authors' Contributions

Cheng-Wei Chang and Jorng-Tzong Horng contributed equally to this work. Jui-Ming Chen and Cheng-Wei Chan conceived and designed the experiments. Jui-Ming Chen, Cheng-Wei Chan, and Chi-Chang Hsu performed the experiments. Jui-Ming Chen, Cheng-Wei Chan, and Chi-Chang Hsu analyzed the data. Jorng-Tzong Horng contributed reagents/materials/analysis tools. Jui-Ming Chen and Cheng-Wei Chan wrote the manuscript.

References

[1] T. Cukierman, H. C. Gerstein, and J. D. Williamson, "Cognitive decline and dementia in diabetes—systematic overview of prospective observational studies," *Diabetologia*, vol. 48, no. 12, pp. 2460–2469, 2005.

[2] K.-C. Wang, L.-C. Woung, M.-T. Tsai, C.-C. Liu, Y.-H. Su, and C.-Y. Li, "Risk of Alzheimer's disease in relation to diabetes: a population-based cohort study," *Neuroepidemiology*, vol. 38, no. 4, pp. 237–244, 2012.

[3] S. Ahtiluoto, T. Polvikoski, M. Peltonen et al., "Diabetes, Alzheimer disease, and vascular dementia: a population-based neuropathologic study," *Neurology*, vol. 75, no. 13, pp. 1195–1202, 2010.

[4] Z. Arvanitakis, R. S. Wilson, J. L. Bienias, D. A. Evans, and D. A. Bennett, "Diabetes mellitus and risk of Alzheimer disease and decline in cognitive function," *Archives of Neurology*, vol. 61, no. 5, pp. 661–666, 2004.

[5] J. Janson, T. Laedtke, J. E. Parisi, P. O'Brien, R. C. Petersen, and P. C. Butler, "Increased risk of type 2 diabetes in Alzheimer disease," *Diabetes*, vol. 53, no. 2, pp. 474–481, 2004.

[6] J. M. Chen, C. W. Chang, T. H. Chang, C. C. Hsu, J. T. Horng, and W. H.-H. Sheu, "Effects of statins on incident dementia in patients with type 2 DM: a population-based retrospective cohort study in Taiwan," *PLoS ONE*, vol. 9, Article ID e88434, 2014.

[7] J. C. S. Breitner, B. A. Gau, K. A. Welsh et al., "Inverse association of anti-inflammatory treatments and Alzheimer's disease: initial results of a co-twin control study," *Neurology*, vol. 44, no. 2, pp. 227–232, 1994.

[8] J. B. Rich, D. X. Rasmusson, M. F. Folstein, K. A. Carson, C. Kawas, and J. Brandt, "Nonsteroidal anti-inflammatory drugs in Alzheimer's disease," *Neurology*, vol. 45, no. 1, pp. 51–55, 1995.

[9] G. A. Broe, D. A. Grayson, H. M. Creasey et al., "Anti-inflammatory drugs protect against Alzheimer disease at low doses," *Archives of Neurology*, vol. 57, no. 11, pp. 1586–1591, 2000.

[10] J. C. Anthony, J. C. S. Breitner, P. P. Zandi et al., "Reduced prevalence of AD in users of NSAIDS and H2 receptor antagonists: the Cache County study," *Neurology*, vol. 54, no. 11, pp. 2066–2071, 2000.

[11] P. P. Zandi, J. C. Anthony, K. M. Hayden, K. Mehta, L. Mayer, and J. C. S. Breitner, "Reduced incidence of AD with NSAID but not H_2 receptor antagonists: the Cache County study," *Neurology*, vol. 59, no. 6, pp. 880–886, 2002.

[12] C. Cornelius, J. Fastbom, B. Winblad, and M. Viitanen, "Aspirin, NSAIDs, risk of dementia, and influence of the apolipoprotein E epsilon 4 allele in an elderly population," *Neuroepidemiology*, vol. 23, no. 3, pp. 135–143, 2004.

[13] C. A. Szekely, J. C. S. Breitner, A. L. Fitzpatrick et al., "NSAID use and dementia risk in the Cardiovascular Health Study: role

of APOE and NSAID type," *Neurology*, vol. 70, no. 1, pp. 17–24, 2008.

[14] C. A. Szekely, R. C. Green, J. C. S. Breitner et al., "No advantage of Aβ_{42}-lowering NSAIDs for prevention of Alzheimer dementia in six pooled cohort studies," *Neurology*, vol. 70, no. 24, pp. 2291–2298, 2008.

[15] S. E. Nilsson, B. Johansson, S. Takkinen et al., "Does aspirin protect against Alzheimer's dementia? A study in a Swedish population-based sample aged > or =80 years," *European Journal of Clinical Pharmacology*, vol. 59, no. 4, pp. 313–319, 2003.

[16] S. R. Waldstein, C. R. Wendell, S. L. Seliger, L. Ferrucci, E. J. Metter, and A. B. Zonderman, "Nonsteroidal anti-inflammatory drugs, aspirin, and cognitive function in the Baltimore longitudinal study of aging," *Journal of the American Geriatrics Society*, vol. 58, no. 1, pp. 38–43, 2010.

[17] Z. Arvanitakis, F. Grodstein, J. L. Bienias et al., "Relation of NSAIDs to incident AD, change in cognitive function, and AD pathology," *Neurology*, vol. 70, no. 23, pp. 2219–2225, 2008.

[18] A. S. Henderson, A. F. Jorm, H. Christensen, P. A. Jacomb, and A. E. Korten, "Aspirin, anti-inflammatory drugs and risk of dementia," *International Journal of Geriatric Psychiatry*, vol. 12, no. 9, pp. 926–930, 1997.

[19] NHIRD Introduction to the National Health Insurance Research Database (NHIRD), Taiwan.

[20] I.-C. Chien, Y.-J. Chou, C.-H. Lin, S.-H. Bih, P. Chou, and H.-J. Chang, "Prevalence and incidence of schizophrenia among national health insurance enrollees in Taiwan, 1996–2001," *Psychiatry and Clinical Neurosciences*, vol. 58, no. 6, pp. 611–618, 2004.

[21] C.-W. Chang, V. C. Kok, T.-C. Tseng, J.-T. Horng, and C.-E. Liu, "Diabetic patients with severe sepsis admitted to intensive care unit do not fare worse than non-diabetic patients: a nationwide population-based cohort study," *PLoS ONE*, vol. 7, no. 12, Article ID e50729, 2012.

[22] C. M. Beard, S. C. Waring, P. C. O'Brien, L. T. Kurland, and E. Kokmen, "Nonsteroidal anti-inflammatory drug use and Alzheimer's disease: a case- control study in Rochester, Minnesota, 1980 through 1984," *Mayo Clinic Proceedings*, vol. 73, no. 10, pp. 951–955, 1998.

[23] W. F. Stewart, C. Kawas, M. Corrada, and E. J. Metter, "Risk of Alzheimer's disease and duration of NSAID use," *Neurology*, vol. 48, no. 3, pp. 626–632, 1997.

[24] P. M. Doraiswamy, A. Krishen, F. Stallone et al., "NSAIDs and cognition in Alzheimer's disease," *Neurology*, vol. 46, no. 4, p. 1194, 1996.

[25] F. Landi, M. Cesari, G. Onder, A. Russo, S. Torre, and R. Bernabei, "Non-steroidal anti-inflammatory drug (NSAID) use and Alzheimer disease in community-dwelling elderly patients," *American Journal of Geriatric Psychiatry*, vol. 11, no. 2, pp. 179–185, 2003.

[26] T. M. Karplus and K. G. Saag, "Nonsteroidal anti-inflammatory drugs and cognitive function: do they have a beneficial or deleterious effect?" *Drug Safety*, vol. 19, no. 6, pp. 427–433, 1998.

Hindawi Publishing Corporation
Journal of Diabetes Research
Volume 2016, Article ID 3207043, 10 pages
http://dx.doi.org/10.1155/2016/3207043

Review Article

Charcot Neuropathic Arthropathy of the Foot: A Literature Review and Single-Center Experience

Tomas Kucera,[1,2] Haroun Hassan Shaikh,[1] and Pavel Sponer[1,2]

[1] *Department of Orthopaedic Surgery, University Hospital Hradec Kralove, Hradec Kralove, Czech Republic*
[2] *Faculty of Medicine in Hradec Kralove, Charles University in Prague, Prague, Czech Republic*

Correspondence should be addressed to Tomas Kucera; kucerat@tiscali.cz

Received 29 May 2016; Accepted 2 August 2016

Academic Editor: Frank Bowling

Charcot neuropathic osteoarthropathy of the foot is a relatively common complication of diabetic neuropathy. Incorrect diagnosis and improper treatment often result in the extremity having to be amputated. This paper summarises the current view on the etiology, diagnostics, and treatment of diabetic Charcot neuropathic osteoarthropathy, with particular focus on preserving the extremity through surgical intervention from our own experiences.

1. Introduction

Charcot neuropathic osteoarthropathy (CN) is a chronic, progressive condition of bones, joints, and soft tissues, most commonly occurring in the area of the foot and ankle as a result of peripheral neuropathy. It is characterized by a local inflammatory process in the early stages and gradual development of bone loss, joint dislocation, and fixed deformities. These deformities can secondary lead to infected ulcerations and eventually to osteomyelitis. In general, any part of skeleton can be affected.

Diabetes mellitus, together with neuropathy, is currently considered the main cause of CN. Data indicating the prevalence and incidence of the condition suggest that it often goes undiagnosed among sufferers of diabetes, with figures ranging from 0.4 to 13% among diabetics [1]. However, changes diagnosed by X-ray and corresponding with CN are detected in up to 29% of diabetics. Bilateral disability has been observed in numbers ranging from 9 to 39% of patients. When MRI is used as a diagnostic method, the detection rate rises to 75% of documented cases [2, 3]. Sohn et al. [4] state that the mortality rate is 28.3% within five years in patients with CN.

The common issue is an early diagnosis and an appropriate treatment, in case of an acute phase where it is difficult to differentiate an acute osteomyelitis. Even though the treatment of CN is mostly conservative, the surgical options might be beneficial for the patients. However, the crucial question is when, where, and how a surgical therapy has to be used.

2. Materials and Methods

A PubMed search was done with the key word "Charcot foot, neuropathic arthropathy, Charcot arthropathy." We could trace about 400 up-to-date papers on the subject. Electronic database was systematically searched for literature discussing the history, pathophysiology, assessment, imaging methods, diagnosis including osteomyelitis, classification, and management of CN. We applied no restrictions on publication date. Article eligibility was assessed independently by all authors. Reasons for exclusion of articles based on title or abstract were (1) nonoriginal data (e.g., editorials, guidelines, and comments), (2) nonclinical articles (e.g., technical or animal studies), (3) case reports, and (4) articles not written in English language. All authors independently chose the most up-to-date papers with regard to target topics resulting in the identification of 59 "most pertinent" articles. Together we discussed and compared the relevant information from all these sources with our clinical practice and included them in this review.

2.1. History. Musgrave first described neuropathic osteoarthropathy in 1703 as an arthralgia caused by venereal disease [6]. Later, Mitchell [7] supposed the relation between spinal lesion and rheumatism of lower extremities in 1831. Charcot described the neuropathic aspect of the condition in detail in 1868 and detected spinal damage resulting from tabes dorsalis as a cause [8] and his brilliant presentation, *Demonstration of Arthropathic Affections of Locomotor Ataxy*, at the 7th International Medical Congress (1881), established this disease as a distinct pathological entity. Much later, in 1936, Jordan [9] revealed diabetes mellitus to be a possible cause of neuropathic osteoarthropathy. Nevertheless, the etiology, diagnostics, and treatment of this condition have to this day yet to be fully addressed.

2.2. Pathophysiology. Numerous factors contribute to the development of CN. Two main theories concerning the origin of the condition have been discussed in the past. The neurotraumatic theory is based upon damage to sensory feedback resulting from progressive destruction of bones and joints brought about by repeated trauma. The neurovascular theory highlights the changes in blood supply caused by neuropathy, most of all lesions in the sympathetic nerves which affect bone resorption [10]. The current accepted theory of CN origin states that, in susceptible individuals with peripheral neuropathy, an unregulated inflammatory process is triggered which leads to an increased expression of the polypeptide receptor activator of nuclear factor kappa-B ligand (RANKL). RANKL triggers the synthesis of the nuclear transcription factor, nuclear factor-$\kappa\beta$ (NF-$\kappa\beta$), and this in turn stimulates the maturation of osteoclasts from osteoclast precursor cells. At the same time, NF-$\kappa\beta$ stimulates the production of the glycopeptide osteoprotegerin (OPG) from osteoblasts. A repetitive trauma with the loss of pain sensation results in continual production of proinflammatory cytokines, RANKL, NF-$\kappa\beta$, and osteoclasts, which in turn leads to continuing local osteolysis. Another possible cause is decreased secretion of the calcitonin gene related peptide (CGRP) from damaged nervous endings. Under physiological circumstances this peptide works as an antagonist of the RANKL synthesis and at the same time is responsible for the normal integrity of the joint capsule [11]. The powerful bone anabolic Wnt/β-catenin pathway plays a critical role in remodeling as well as preservation of the foot skeleton in the acute and chronic stages of the disease [12]. In general, diabetes may predispose to CN occurrence through a number of mechanisms. Apart from the presence of neuropathy and possible osteopenia, these include the effects of advanced glycation end products, reactive oxygen species, and oxidized lipids, which may all enhance the expression of RANKL in diabetes [13].

2.3. Assessment. The diagnosis is based on patient's history, clinical examination, and imaging methods. Patients are quite often not aware of any injury as a result of their lowered perception of pain. Another triggering factor can be previous surgery of the foot [14]. Diabetic nephropathy is also associated with a higher incidence of CN [15]. Trigger factors of development of the CN can also be infections (osteomyelitis precedes CN) [16] and revascularization [17]. A necessary condition for the emergence of CN is the presence of peripheral neuropathy, in particular, diabetic distal sensitive polyneuropathy. Several methods can be used to test neuropathy. The most common is the Semmes-Weinstein 5.07/10 g monofilament. Next is the pinprick test or even the more sensitive neurometer test [18]. A small fiber-predominant neuropathy is an early manifestation of diabetic neuropathy and it can progress to distal symmetric polyneuropathy. It can be difficult to diagnose because the examination (decreased reflexes, impaired vibration, and weakness) and electrodiagnostic testing can be normal. A skin biopsy is used for this purpose. Autonomic neuropathy (a type of small fiber neuropathy) is also common and plays a key role in the development of the CN. Symptoms include gastroparesis, constipation, urinary retention, erectile dysfunction, and cardiac arrhythmias [19]. Signs of inflammation are an important sign since inflammation plays a key role in the pathophysiology of CN. Oedema, erythema, warmth, and more than a 2°C difference in local temperature in comparison to the contralateral extremity are typical symptoms of active CN and it could be difficult to differentiate them from phlegmon with osteomyelitis or from an acute attack of gout [20], especially since pain occurs in only 50% of neuropathy cases [21]. In this stage the vascular supply to the foot is still maintained though it could be difficult to palpate an arterial pulse due to prominent swelling. The eventual fracture and joint dislocation can lead to deformities, typically to rocker bottom foot with possible ulceration. During this time, critical ischaemia of the extremity is much more frequent [11]. Skin temperature measurement is the most widely used method in the assessment of the activities of the CN. With the use of a surface-sensing temperature device (infrared thermometer), temperatures are recorded in the most affected area of the foot and compared with the same areas of the contralateral foot [22].

For further prognosis and therapy it is necessary to examine the foot's stability. Instability of the forefoot can be assessed according to Assal and Stern: the pressure on the foot in the sagittal plane dorsally when the ankle joint is locked in dorsiflexion [23]. Relatively common in CN is the contraction of the triceps surae muscle which is involved in plantar inclination of the calcaneus (Figure 1).

2.4. Imaging Methods. Primarily it must be emphasized that the changes on the X-ray are typically delayed and have low sensitivity [24].

A basic examination is an X-ray of the talus and the weight bearing foot in the anteroposterior and dorsoplantar lateral projection. During the initial stage the X-ray finding can be negative or only minor bone infractions and joint incongruence are present. In a developed stage fractures and subluxations or luxations are clearly observed. The X-ray finding depends on the specific type of CN. In a typical rocker bottom deformity a plantar dislocation of the navicular and cuboid bone is visible. A lateral projection defines inclination of the calcaneus. In CN we often find a negative inclination with a plantar tilt of the calcaneus. This deformity arises

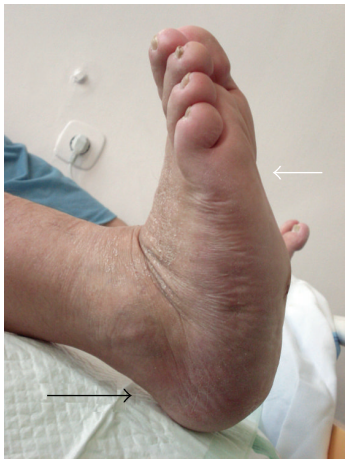


Figure 1: Clinical image of the right foot (CN) with contraction of m. triceps surae and plantar inclination of calcaneus (black arrow), instability of the foot, and dorsal collapse of the forefoot (white arrow).

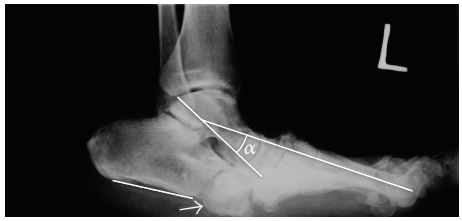


Figure 2: Lateral X-ray image of the weight bearing left foot, visible negative inclination of calcaneus (white line), and collapse of middle part of tarsus (angle α) with plantar prominence of cuboid bone (arrow).

due to deformed midtarsal bones and the shortening of the Achilles tendon, which loses its elasticity during glycosylation [25]. Another finding on the lateral projection due to deformity of the middle part of the tarsus is a negative angle between the axis of talus and I metatarsus (Figure 2). The dorsoplantar projection shows changes of the position in the Lisfranc as well as Chopart joint, with resulting abduction or adduction deformity of the foot.

Examination using magnetic resonance imaging is a very valuable method for the early stages of the illness when X-ray imaging alone results in practically normal findings. An important finding is oedema of the bone marrow of two or more bones, oedema of the adjacent soft tissues, and fluid in several joints or cortical fractures. If conservative treatment is begun during this phase the condition is reversible [26].

Certain methods of nuclear medicine can be helpful not only as an alternative diagnostic method, for example, in revealing the presence of osteomyelitis, but also for monitoring the progress of the treatment. These methods, however, introduce certain difficulties. Three- or four-phase bone scintigraphy (^{99m}Tc-MDP) is highly sensitive but with a low specificity. Scintigraphy with labelled leucocytes (^{99m}Tc-WBC nebo ^{111}In-WBC) is highly sensitive and very specific for diagnosing the infection but it is difficult to differentiate soft tissue from bone. That is why either a combination of both the abovementioned methods or FDG-PET/CT is recommended [27].

2.5. Diagnosis of Osteomyelitis in CN. The diagnostics of osteomyelitis in CN is difficult primarily in the active stage of the disease when clinical symptoms are practically the same both in osteomyelitis and in CN. On the contrary, in the chronic stage symptoms in osteomyelitis due to ischaemia and immunodeficiency may be masked. Diagnosing osteomyelitis is not possible on the basis of one examination alone. In general, for the differential diagnosis of osteomyelitis and CN, an important role plays a complex view of inflammatory markers and clinical manifestations of infection. The origin of osteomyelitis is in most cases caused by spread of infection from the soft tissues. History of ulceration or presence of ulceration and/or previous amputation in the area of the foot are possible factors to weigh when suspecting osteomyelitis. Osteomyelitis in CN without ulceration has a very small probability. On the contrary, a high predictive value for the presence of osteomyelitis is ulcerations bigger than two cm^2 and deeper than three mm [28]. Another supplementary examination is probe-to-bone test (PTB). The basis of the test is whether a fine blunt steel probe can penetrate the ulceration to the bone. Sensitivity of the test is from 38 to 94% and specificity 85 to 98% [28]. Laboratory readings in CN do not show higher markers of inflammation (practically normal numbers of leucocytes, CRP, procalcitonin, and FW). Due to the chronicity of infection in osteomyelitis we can find particularly higher FW (most commonly >70 mm/hour). A basic imaging method is X-ray. Osteomyelitis shows larger osteolytic lesions and periosteal reaction when compared to plain CN. These changes are visible within two to three weeks of the onset of the infection. The abovementioned methods of nuclear medicine (four-phase bone scintigraphy, marked leucocytes, ^{18}F FDG-PET, or ^{67}Ga SPECT/CT) can also be used to diagnose osteomyelitis, and the range of osteomyelitic changes is also highly visible on the MRI [28]. Also ^{99m}Tc-WBC SPECT/CT hybrid image is a useful tool in addition to MRI [29]. The most reliable examination is a bone biopsy, either preoperative percutaneous or peroperative, made with a biopsy needle after disinfection of the ulceration. A tissue sample is sent for microbiological and histological examination. In our clinical practice we do not use swabs to diagnose pathogens but rather send tissue samples directly. Swabs are often contaminated with normal skin flora or colonizers, and their use may result in failure to identify deep tissue pathogens [30]. For identification of pathogens, the International Working Group on Diabetic Foot (IWGDF) has proposed cultures from tissue specimens rather than from swabs [31]. However, swab cultures are less invasive than tissue biopsy or curetted tissue and swab culturing (including a vacuum transport container) may be reliable for identification of pathogens in superficial diabetic foot wounds [32].

2.6. Examination of Vascular Supply. Typical ischaemic symptoms like claudication and pains at rest, which would

Table 1: Eichenholtz classification.

Stage	Radiographic finding	Clinical finding
I development	Osteopenia, osseous fragmentation, joint subluxation or dislocation	Swelling, erythema, warmth, ligamentous laxity
II coalescence	Absorption of debris, sclerosis, fusion of larger fragments	Decreased warmth, decreased swelling, decreased erythema
III reconstruction	Consolidation of deformity, fibrous ankylosis, rounding and smoothing of bone fragments	Absence of warmth, absence of swelling, absence of erythema, fixed deformity

Table 2: Classification of CN based upon MR imaging. Source: [5].

Stage	Severity grade	
	Low severity: grade 0 (without cortical fracture)	High severity: grade 1 (with cortical fracture)
Active arthropathy (acute stage)	Mild inflammation/soft tissue oedema	Severe inflammation/soft tissue oedema
	No skeletal deformity	Severe skeletal deformity
	X-ray: normal	X-ray: abnormal
	MRI: abnormal (bone marrow oedema, microfractures, bone bruise)	MRI: abnormal (bone marrow oedema, macrofractures, bone bruise)
Inactive arthropathy (becalmed stage)	No inflammation	No inflammation
	No skeletal deformity	Severe skeletal deformity
	X-ray: normal	X-ray: abnormal (past macrofractures)
	MRI: no significant bone marrow oedema	MRI: no significant bone marrow oedema

normally appear in the history, might be unrecognised because of the presence of neuropathy. During a physical examination pulsation can be impalpable and trophic changes are often found. An example of a noninvasive diagnostic method that can be also used would be measuring blood pressure in the ankle using a Doppler probe (it carries a higher risk of artificially higher pressures in the case of mediocalcinosis). In our department we use measure pressure on the big toes or transcutaneous oxygen tension (this method carries a risk of artificially lower pressures in the case of oedema) [33]. If a pathologic finding appears, we perform angiography with the possibility of revascularization. If angiography is performed postoperatively, there could be a risk of activating CN after prospective revascularization. Examination of the vascular supply must be repetitive even after surgical reconstruction of CN when a higher risk of ischaemia exists (oedema, thrombus formation of vessels). Based upon our experience, we evaluate this risk as the most significant from the point of view of possible postoperative complications.

2.7. Classification. The most commonly used classification according to Eichenholtz was published in 1966 (Table 1) [34].

Currently, in spite of the quite widespread usage of this classification, it is necessary to consider and search for new alternative methods of imaging. Eichenholtz evaluated X-ray images in 68 patients (altogether 94 joints) of whom only 12 were diabetic [34]. The development of examination methods of nuclear medicine and magnetic resonance have shown that there are already noticeable and recordable changes of CN even when X-ray images are negative, and starting treatment at such an early stage can prevent deformities. This stage has been labelled as stage 0 and has been added to the original classification. Alternatively, stage 1 has been divided into 1a and 1b [5]. With regard to the fact that the initial change in CN is an inflammatory reaction, which corresponds with oedema of bone marrow, a classification of CN based upon MR imaging has been suggested. It recognises two stages of the disease—active and inactive—according to the presence or absence of oedema of the bone marrow and distinguishes two grades—0 and 1—according to the presence or absence of cortical fractures. These fractures represent a worse prognosis from the point of view of developing deformities (Table 2) [5].

2.8. Conservative Treatment. The treatment of CN is mostly conservative. This method is based on immobilisation and the complete absence of weight bearing for the affected extremity in the active stage. There are various opinions concerning the type of immobilisation and the period of nonweight bearing for the foot. The most common immobilisation used is a total contact cast (TCC) changed three days after the initial application and then every week. Alternatively, it is possible to use Charcot Restraint Orthotic Walker (CROW) prefab orthoses. The period of fixation depends on the reduction in oedema and a drop in skin temperature below 2°C compared to the contralateral extremity [35]. The recommended length of fixation varies from six weeks to three months followed by a change of orthosis. Similarly, the recommended period without any weight bearing varies—starting from weight bearing during application of TCC to the usage of a wheelchair as a preventive means against overloading the other extremity [36]. Koller et al. [37] recommend six to eight weeks of TCC and a wheelchair with subsequent change for individual orthosis fixing the affected segment and at

the same time preventing tibial rotation, thus enabling only axial weight bearing (a so-called frame orthosis). In the chronic stage of the condition, a deformed foot in plantigrade position capable of weight bearing in shoes or an orthosis without increasing deformity is suitable for conservative treatment. The type of the prosthetic equipment depends on the gravity of the deformity and on the eventual presence of ulceration. It is possible to use various types of walkers, ankle-foot orthoses, orthotic shoes, or adjusted regular shoes [38]. In general we prefer conservative treatment in both stages by a multidisciplinary team. Only after the failure of conservative management does a patient become a candidate for the surgical treatment. This treatment is beneficial in CN refractory to off-loading and immobilisation or in the case of recalcitrant ulcers [11]. A separate question is the problem of weight bearing in the case of conservatively treated or operated CN. Gait dysfunction has been proven in patients with diabetes [39]. We have the same experience as Koller et al., most of the patients suffering from peripheral neuropathy are not able to reduce weight loading of the foot in a controlled manner with the help of crutches, and there is a risk of overloading the contralateral limb and a risk of fall. Therefore we recommend full weight bearing wherein we gradually prolong the time and speed of the walk [37].

To support healing some medicaments have been used, bisphosphonates, which inhibit osteoplastic bone resorption, and intranasal calcitonin, which has had fewer complications [40]. Nevertheless, beneficial effect of pharmacological treatment (improvement of markers of resorption versus an absence of clinical marks of healing and side effects of the therapy) as well as physical stimulation of the bone growth is yet to be fully demonstrated.

2.9. Indication for Surgical Treatment. Besides conservative treatment, the possibilities of surgical treatment have also been looked into and the benefits and risks of such treatment have been considered. Saltzman et al. [41] evaluated retrospectively conservative treatment of 127 extremities in 115 patients over a period of 20 years. The study found that the annual rate of amputations was 2.7%, 47% of patients used an orthosis for a period longer than 18 months, and the risk of ulceration appeared in 40% of patients. Ulceration is often accompanied by a high risk of amputation. At present, specific methods for the surgical treatment of CN to save the extremity or delay major amputation are still being developed. Foot reconstruction, resection of bony prominences, and major amputations are considered for the surgical treatment (Table 3).

Major amputations in CN (generally we prefer the below-the-knee amputation) are still the current solution. If carried out properly, if the healing is complete, and if the patient is equipped in the prosthetic and rehabilitation department with a suitable prosthesis and has an adequate walking regime as part of the rehabilitation, then we know from experience that these patients, though initially perhaps unwilling to undergo surgery, are more satisfied compared to those who use orthosis for a long time, who required constant dressings of ulcerations and repeated visits to hospital. Based on our personal experiences, we use transcutaneous oxygen tension more than 35 mmHg as a predictive factor for successful healing of below-knee amputation. In dialysis patients we deal with problem of a suitable prosthesis after major amputation due to changes of extremity volume between dialyses.

Table 3: Indications of the surgical treatment.

Surgical treatment	Indications
Reconstruction	Stable, nonplantigrade foot Unstable foot
Resection of bony prominences	Isolated bony prominences in a stable plantigrade foot
Major amputations	Severe peripheral vascular disease Severe bone destruction including osteomyelitis Failed previous surgery

Bone resections are done as a separate intervention in isolated bone prominences, mostly in cases of a high bony pressure that cannot be accommodated with orthotic and prosthetic means and in stable plantigrade foot [11]. In some cases, a Strayer procedure or Achilles tendon prolongation is necessary because of frequent cases of pes equinus in the diabetic foot. Such intervention carries a risk of instable foot in the case of larger bone resection. A bone resection is also done as preparation for reconstruction of the foot in case an infected ulceration is present or if there is suspicion of osteomyelitis.

With regard to poor bone quality and the presence of neuropathy in long-term healing, the so-called superconstruction principles for reconstruction operations have been set up: (a) extending arthrodesis beyond the affected area on neighbouring joints, (b) resection of the bone for mild shortening of the foot enabling adequate repositioning of the deformity without excessive tension of soft tissues, thus helping prevent secondary ischaemisation, (c) usage of the strongest possible implant which can be tolerated, and (d) introduction of an implant that can maximally increase mechanic stability, which is the main goal [42].

2.10. Types of Implants. In general we can use different types of external fixators or internal fixators according to the type of deformity and preference of the surgeon. In the case of external fixators, the most suitable enabling gradual correction seems to be ankle-foot fixators of the Ilizarov type or the Taylor Spatial Frame. Their disadvantage is the relatively high purchase cost. Mostly, a three-plane fixation that combines common types of external fixators is used. An advantage of this method is the absence of internal implant that may increase the risk of infection and the possibility of earlier weight bearing on the foot. As for the internal fixations, plates are recommended if the implantation is from the plantar side, although nowadays angle stable plates are used more often. They enable good stability in an osteoporotic bone and greater variability from the point of view of plate placement. The disadvantage is the need for a wider surgical approach and problematic healing with the exposed implant [42]. In our department we use, besides external

Table 4: Sanders and Frykberg classification.

Type	Localization
I	Metatarsophalangeal and interphalangeal joints
II	Tarsometatarsal articulations (Lisfranc)
III	Midtarsal joint line (Chopart)
IV	Ankle joint and subtalar joint
V	Calcaneus

fixators, a technique of reconstruction by axial screws—Midfoot Fusion Bolt 6.5 mm (DePuy/Synthes). When the resection is done and the reposition is finished we use them to fix intramedullary both the medial and lateral columns and subsequently apply plaster cast fixation. By using such a technique we eliminate the disadvantages of using the plates as mentioned above and we did not observe any osseous healing failure reporting by some authors [43, 44].

2.11. Timing of the Surgery according to the Stage of the Disease. Most of the earlier operations have been carried out only in the chronic, inactive stage. In the active stage, an inflammatory reaction with oedema and osteoporosis are present, thus increasing the risk of complicated healing. On the other hand, this stage enables easier corrections than in the fixed deformity as it is possible to use the remodeling capacity of the bone. Indications for surgery in the active stage are heavy instability, progression of the deformity, prevention of the dislocation of fragments by muscle contraction, and the general failure of conservative treatment. An external fixator is used exclusively. Usually within three to six weeks the position of the foot is gradually corrected by this external fixator into the correct plantigrade position, then an arthrodesis of joints is carried out, and the fixator is left in place for at least three more months [45]. Nevertheless, to date no sufficient relevant studies have been published demonstrating the success rate of the surgeries carried out during this active stage. In our department we carry out surgeries only in the chronic stage.

3. Surgical Treatment in Individual Localizations according to Sanders and Frykberg

Sanders and Frykberg classified individual localizations of CN on the foot (Table 4) [46].

3.1. Sanders I. In this case prevailing resorptive changes were creating deformities of the metatarsal bones of the so-called candy bar type (Figure 3) [37]. This type of CN is relatively often diagnosed as osteomyelitis. Usually it is treated conservatively. In the case of dislocation in the I MTP joint we prefer arthrodesis in a revised position. Popelka recommends a fixation with the use of two screws as sufficient for this surgery [47]. On the basis of our experience we recommend using plates in CN in neuropathic terrain. In the case of heavy deformities or superimposed infection we choose bone resection. It is necessary to distinguish CN and osteomyelitis as mistaking CN for osteomyelitis may often lead to transmetatarsal amputation.

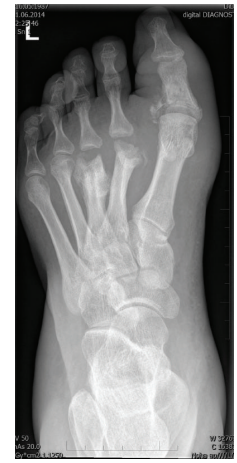


Figure 3: Dorsoplantar X-ray image of the left foot. Resorptive changes I–III MTP of the joints.

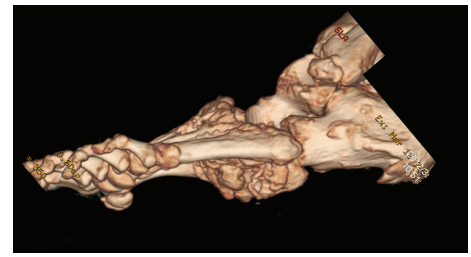


Figure 4: 3D CT of the left foot: plantar prominence of tarsal bones and in this case plantar prominence of cuboid bone.

3.2. Sanders II. Translation of metatarsi medially or laterally is usually associated with lowering the medial column and valgus heel. A frequent consequence is abduction of the forefoot, which includes a perinavicular affection with the talus and navicular bone in plantar flection while the cuneiform bone is dislocated dorsally with the I metatarsus. The contraction of the tibialis anterior muscle worsens the deformity and practically excludes successful conservative treatment. This type of CN is very often combined with the following type III, which is why surgical treatment of both these types will be described together. Moreover, a normal position of the hindfoot is a prerequisite for the correction of the forefoot.

3.3. Sanders III. This is a typical rocker bottom foot with the cuboid bone in plantar prominence. This plantar bone prominence causes chronic ulcerations which do not respond to conservative therapy (Figure 4).

It is necessary to do reconstruction and stabilisation of the medial and lateral column and, in case of persistent instability, subtalar arthrodesis as well.

3.4. Reconstruction of the Foot. Reconstruction of the foot consists of several phases. One advantage is regional anaesthesia and minimal usage of a tourniquet. In the case of

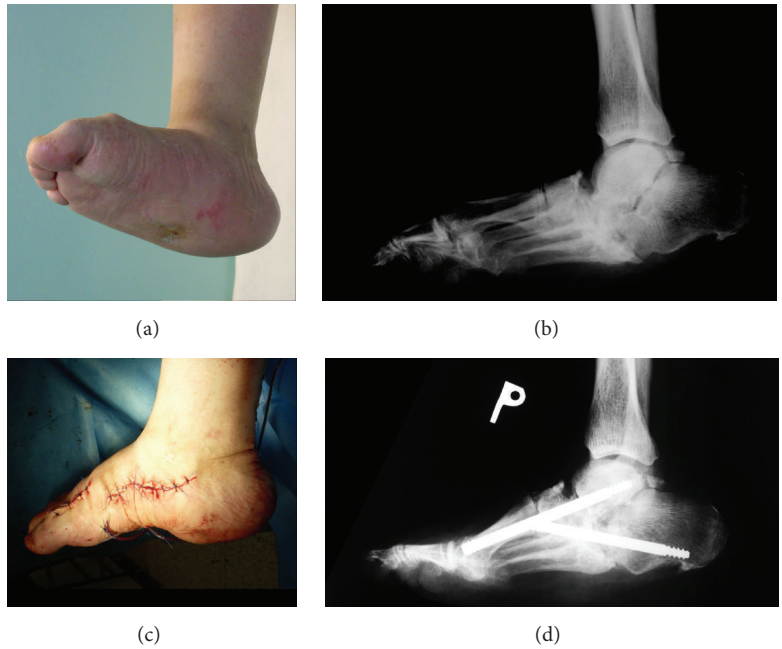


Figure 5: (a) Clinical image of CN with collapsed middle part of tarsus and plantar sinus (right foot). (b) Preoperative X-ray image of collapsed arch in the middle part of tarsus. (c) Clinical image immediately after reconstruction with reconstructed longitudinal arch. (d) Lateral X-ray image demonstrates reconstruction of both columns two months after surgery.

pes equinus, the first phase, according to the preoperative assessment, involves either the Strayer procedure or, more frequently, prolongation of the Achilles tendon, which can be carried out using either a Z-plasty or a technique of three mini-incisions three cm apart from each other and up to one-half of the tendon diameter (most frequently a lateral-medial-lateral incision). The position of the sole is corrected up to 90 degrees in respect to the long axis of the fibula, with the knee in full extension. This procedure restores the positive inclination of the calcaneus and facilitates the reconstruction of the medial part of the tarsus. Care must be taken to avoid pes calcaneus by overextended prolongation; this position causes heel ulcerations that do not heal, leading to the necessity for below-knee amputation. We temporarily fix the corrected position of the hindfoot with the help of the Kirschner wire from the calcaneus to the tibia.

In the next phase we make a slightly S-shaped incision on the medial side of the foot from the talus to the base of the I metatarsus, where we identify individual joint dislocations. Earlier we used the approach to the lateral column according to Ollier which led to a higher percent of secondary healing. That is why, in a deformity where the cuboid bone is the lowest bone, the callus is excised from a plantar approach. Reconstruction of the Lisfranc joint follows, and we correct abduction or adduction deformity performing osteotomy using the previously introduced Kirschner wires. We temporarily fix the corrected position with the help of Kirschner wire.

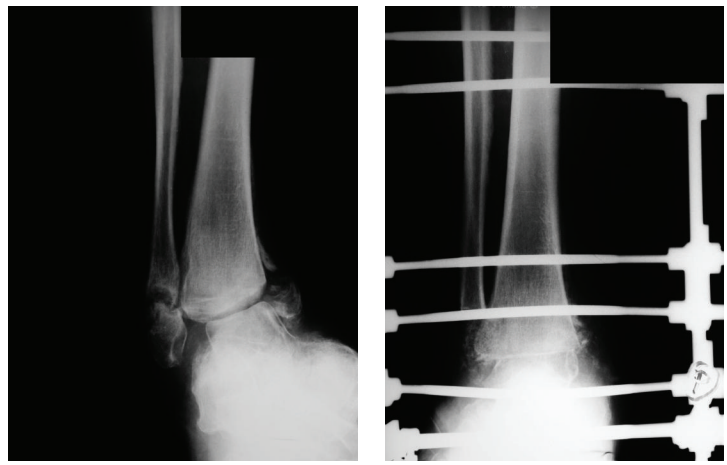
In the third phase we reconstruct the middle part of the tarsus. The navicular and cuboid bones are usually plantarly dislocated, with the goal being resectional talonavicular, naviculocuneiform, and calcaneocuboid arthrodesis in the corrected position, which is again temporarily fixed by the Kirschner wires.

A final fixation with a Midfoot Fusion Bolt of 6.5 mm (DePuy/Synthes) is the last step. We introduce the implant medially over the head of the I metatarsus according to the deformity of the hallux from a dorsal or plantar approach up to the talus bone. Laterally we introduce the implant from the mini-incision from the area of base of the IV metatarsus through the cuboid bone to the calcaneus. To date we did not have to address residual instability between the talus and calcaneus, for which subtalar arthrodesis with the same implant is recommended [45]. We use resected bones as local autografts, reinsert tendon attachments of the medial column, and insert Redon drains and the wounds are sutured. Finally, we apply a padded plaster cast according to the type of intervention (Figure 5).

3.5. Sanders IV. In this type the ankle and frequently a subtalar joint are most affected. Because of the instability the deformity progresses and calluses and ulcerations emerge. We indicate arthrodesis of the ankle and the subtalar joint along with an external fixation (Figure 6). Healing complications have been observed by us in these cases.

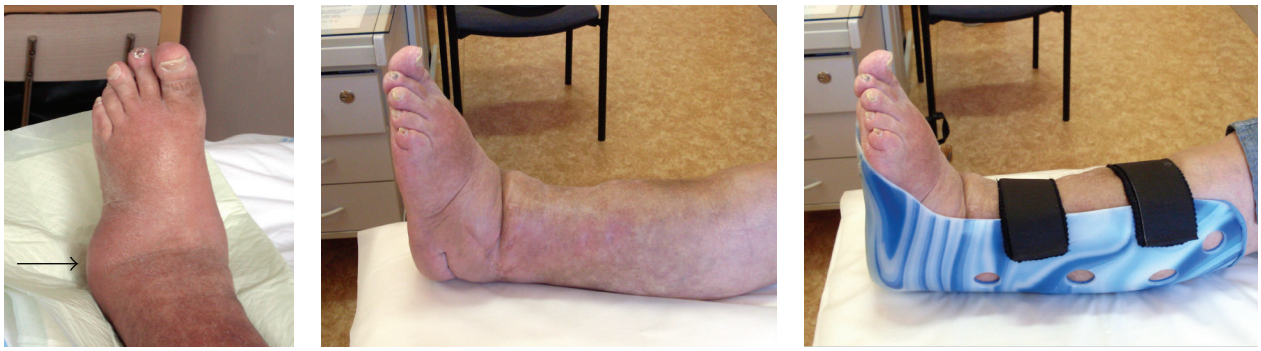
In the case of severe deformities we prefer astragalectomy before performing prospective transtibial amputation. After the tibiocalcaneal arthrodesis is healed it is necessary to use an orthosis for several months (Figure 7).

3.6. Sanders V. This is the least frequent type affecting the calcaneus (Figure 8). With regard to the poor quality of bone and thus to the retention of osteosynthetic material and the



(a) (b)

Figure 6: (a) AP X-ray image of the right ankle: inveterate neuropathic fractures of both malleoli. (b) A primary arthrodesis using an external fixation carried out.



(a) (b) (c)

Figure 7: (a) A clinical image of a left foot deformity with complete dislocation of talus plantolaterally (arrow). (b) A clinical image after astragalectomy and completed healing of tibiocalcaneal fusion. (c) Individual plastic orthosis into shoes.

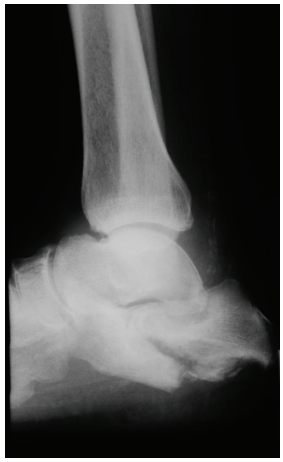


Figure 8: A lateral X-ray image of calcaneus: the pull of the Achilles tendon causes the fragment to be dislocated, with incongruence in the subtalar joint.

risk of infection, we opt for conservative treatment (orthosis). Surgical treatment is considered in the case of progressing deformity when a dislocation of fragments occurs as a result of contracting Achilles tendon. The primary goal is the stability of the hindfoot and preventing the formation of ulcerations. Part of the operative procedure involves subtalar arthrodesis.

4. Postoperative Care

Postoperative care depends on the type of corrected deformity, the implant used (internal or external fixation), the course of healing, whether or not the contralateral extremity is affected, and the ability of nonweight bearing on the operated extremity. In general a great deal of attention must be paid to the appropriate off-loading in the early and subacute postoperative stage and in the case of chronic CN in terms of localization. The advantage of external fixation

is the possibility of earlier weight bearing; internal fixation is supplemented by a plaster cast usually for three to four months. After the external fixator or plaster cast fixation is removed, individual orthoses are applied, and weight bearing is gradually increased by reduction in limitation of walking time and speed. A suitable time for using the orthosis is up to one year. After this period individual orthopaedic shoes are usually made. A lifelong follow-up including diabetes, nutrition, and infection control by antibiotic treatment if necessary is essential.

5. Conclusion

The treatment of CN is mostly conservative. Thanks to new findings from the aetiopathogenesis of the condition and its biomechanics it is possible, in indicated cases, to supplement CN treatment with reconstructive procedures along with suitable implants, thus avoiding major amputation. Nevertheless, to evaluate the benefits and risks of these procedures further evidence-based studies will be necessary.

Competing Interests

The authors declare that there are no competing interests.

Acknowledgments

This work was supported by the programme PRVOUK P 37/04.

References

[1] R. G. Frykberg and R. Belczyk, "Epidemiology of the Charcot foot," *Clinics in Podiatric Medicine and Surgery*, vol. 25, no. 1, pp. 17–28, 2008.

[2] M. E. Munson, J. S. Wrobel, C. M. Holmes, and D. A. Hanauer, "Data mining for identifying novel associations and temporal relationships with Charcot foot," *Journal of Diabetes Research*, vol. 2014, Article ID 214353, 13 pages, 2014.

[3] S. Rajbhandari, R. Jenkins, C. Davies, and S. Tesfaye, "Charcot neuroarthropathy in diabetes mellitus," *Diabetologia*, vol. 45, no. 8, pp. 1085–1096, 2002.

[4] M.-W. Sohn, T. A. Lee, R. M. Stuck, R. G. Frykberg, and E. Budiman-Mak, "Mortality risk of charcot arthropathy compared with that of diabetic foot ulcer and diabetes alone," *Diabetes Care*, vol. 32, no. 5, pp. 816–821, 2009.

[5] E. A. Chantelau and G. Grützner, "Is the Eichenholtz classification still valid for the diabetic Charcot foot?" *Swiss Medical Weekly*, vol. 144, Article ID w13948, 2014.

[6] R. Gupta, "A short history of neuropathic arthropathy," *Clinical Orthopaedics and Related Research*, no. 296, pp. 43–49, 1993.

[7] J. K. Mitchell, "On a new practice in acute and chronic rheumatism," *The American Journal of the Medical Sciences*, vol. 8, pp. 55–64, 1831.

[8] J. M. Charcot, "Sur quelques arthropathies qui paraissent dependre d'une lesion du cerveau ou de la moelle epimere," *Archives de Physiologie Normale et Pathologique*, vol. 1, article 161, 1868.

[9] W. R. Jordan, "Neuritic manifestations in diabetes mellitus," *Archives of Internal Medicine*, vol. 57, no. 2, pp. 307–366, 1936.

[10] E. Gouveri and N. Papanas, "Charcot osteoarthropathy in diabetes: a brief review with an emphasis on clinical practice," *World Journal of Diabetes*, vol. 2, no. 5, pp. 59–65, 2011.

[11] L. C. Rogers, R. G. Frykberg, D. G. Armstrong et al., "The Charcot foot in diabetes," *Diabetes Care*, vol. 34, no. 9, pp. 2123–2129, 2011.

[12] A. Folestad, M. Ålund, S. Asteberg et al., "Role of Wnt/β-catenin and RANKL/OPG in bone healing of diabetic Charcot arthropathy patients," *Acta Orthopaedica*, vol. 86, no. 4, pp. 415–425, 2015.

[13] W. J. Jeffcoate and F. L. Game, "New theories on the cause of the Charcot foot in diabetes," in *The Diabetic Charcot Foot: Principles and Management*, R. G. Frykberg, Ed., pp. 29–44, Data Trace Publishing Company, Brooklandville, Md, USA, 2010.

[14] J. Aragón-Sánchez, J. L. Lázaro-Martínez, and M. J. Hernández-Herrero, "Triggering mechanisms of neuroarthropathy following conservative surgery for osteomyelitis," *Diabetic Medicine*, vol. 27, no. 7, pp. 844–847, 2010.

[15] A. Sämann, S. Pofahl, T. Lehmann et al., "Diabetic nephropathy but not HbA1c is predictive for frequent complications of charcot feet—long-term follow-up of 164 consecutive patients with 195 acute charcot feet," *Experimental and Clinical Endocrinology & Diabetes*, vol. 120, no. 6, pp. 335–339, 2012.

[16] A. A. Fauzi, T. Y. Chung, and L. A. Latif, "Risk factors of diabetic foot Charcot arthropathy: a case-control study at a Malaysian tertiary care centre," *Singapore Medical Journal*, vol. 57, no. 4, pp. 198–203, 2016.

[17] S. V. Edelman, E. M. Kosofsky, R. A. Paul, and G. P. Kozak, "Neuro-osteoarthropathy (Charcot's joint) in diabetes mellitus following revascularization surgery: three case reports and a review of the literature," *Archives of Internal Medicine*, vol. 147, no. 8, pp. 1504–1508, 1987.

[18] A. Nather, W. K. Lin, Z. Aziz, C. H. J. Ong, B. Feng, and C. B. Lin, "Assessment of sensory neuropathy in patients with diabetic foot problems," *Diabetic Foot & Ankle*, vol. 2, article 6367, 2011.

[19] B. C. Callaghan, H. T. Cheng, C. L. Stables, A. L. Smith, and E. L. Feldman, "Diabetic neuropathy: clinical manifestations and current treatments," *The Lancet Neurology*, vol. 11, no. 6, pp. 521–534, 2012.

[20] M. Wünschel, N. Wülker, and M. Gesicki, "Charcot arthropathy of the first metatarsophalangeal joint," *Journal of the American Podiatric Medical Association*, vol. 102, no. 2, pp. 161–164, 2012.

[21] G. Botek, M. A. Anderson, and R. Taylor, "Charcot neuroarthropathy: an often overlooked complication of diabetes," *Cleveland Clinic Journal of Medicine*, vol. 77, no. 9, pp. 593–599, 2010.

[22] D. G. Armstrong, W. F. Tood, L. A. Lavery et al., "The natural history of acute Charcot's arthropathy in a diabetic foot speciality clinic," *Diabetic Medicine*, vol. 14, no. 5, pp. 357–363, 1997.

[23] M. Assal and R. Stern, "Realignment and extended fusion with use of a medial column screw for midfoot deformities secondary to diabetic neuropathy," *The Journal of Bone & Joint Surgery—American Volume*, vol. 91, no. 4, pp. 812–820, 2009.

[24] W. B. Morrison and H. P. Ledermann, "Work-up of the diabetic foot," *Radiologic Clinics of North America*, vol. 40, no. 5, pp. 1171–1192, 2002.

[25] G. K. Reddy, "Glucose-mediated in vitro glycation modulates biomechanical integrity of the soft tissues but not hard tissues," *Journal of Orthopaedic Research*, vol. 21, no. 4, pp. 738–743, 2003.

[26] K. T. A. Low and W. C. G. Peh, "Magnetic resonance imaging of diabetic foot complications," *Singapore Medical Journal*, vol. 56, no. 1, pp. 23–34, 2015.

[27] S. E. Sanverdi, F. B. Ergen, and A. Oznur, "Current challenges in imaging of the diabetic foot," *Diabetic Foot and Ankle*, vol. 3, 2012.

[28] B. M. Ertugrul, B. A. Lipsky, and O. Savk, "Osteomyelitis or charcot neuroosteoarthropathy? Differentiating these disorders in diabetic patients with a foot problem," *Diabetic Foot and Ankle*, vol. 4, Article ID 21855, 2013.

[29] W. A. Erdman, J. Buethe, R. Bhore et al., "Indexing severity of diabetic foot infection with ^{99m}Tc-WBC SPECT/CT hybrid imaging," *Diabetes Care*, vol. 35, no. 9, pp. 1826–1831, 2012.

[30] A. Sotto, J.-L. Richard, N. Jourdan, C. Combescure, N. Bouziges, and J.-P. Lavigne, "Miniaturized oligonucleotide arrays: a new tool for discriminating colonization from infection due to *Staphylococcus aureus* in diabetic foot ulcers," *Diabetes Care*, vol. 30, no. 8, pp. 2051–2056, 2007.

[31] B. A. Lipsky, J. Aragón-Sánchez, M. Diggle et al., "IWGDF guidance on the diagnosis and management of foot infections in persons with diabetes," *Diabetes/Metabolism Research and Reviews*, vol. 32, supplement 1, pp. 45–74, 2016.

[32] Y. Huang, Y. Cao, M. Zou et al., "A comparison of tissue versus swab culturing of infected diabetic foot wounds," *International Journal of Endocrinology*, vol. 2016, Article ID 8198714, 6 pages, 2016.

[33] A. Broulikova, "Syndrom diabetické nohy-diagnostika a léčba," *Interní Medicína pro Praxi*, vol. 15, no. 2, pp. 84–84, 2013.

[34] S. N. Eichenholtz, *Charcot Joints*, Charles C. Thomas, Springfield, Ill, USA, 1st edition, 1966.

[35] R. Bramham, P. Wraight, and K. May, "Management of Charcot neuroarthropathy," *The Diabetic Foot Journal*, vol. 14, pp. 163–170, 2011.

[36] C. L. Ramanujam and Z. Facaros, "An overview of conservative treatment options for diabetic Charcot foot neuroarthropathy," *Diabetic Foot & Ankle*, vol. 2, article 6418, 2011.

[37] A. Koller, R. Springfield, G. Engels et al., "German-Austrian consensus on operative treatment of Charcot neuroarthropathy: a perspective by the Charcot task force of the German association for foot surgery," *Diabetic Foot & Ankle*, vol. 2, 2011.

[38] T. E. Milne, J. R. Rogers, E. M. Kinnear et al., "Developing an evidence-based clinical pathway for the assessment, diagnosis and management of acute Charcot Neuro-Arthropathy: a systematic review," *Journal of Foot and Ankle Research*, vol. 6, no. 1, article 30, 2013.

[39] J. S. Wrobel and B. Najafi, "Diabetic foot biomechanics and gait dysfunction," *Journal of Diabetes Science and Technology*, vol. 4, no. 4, pp. 833–845, 2010.

[40] R. Bem, A. Jirkovská, V. Fejfarová, J. Skibová, and E. B. Jude, "Intranasal calcitonin in the treatment of acute Charcot neuroosteoarthropathy: a randomized controlled trial," *Diabetes Care*, vol. 29, no. 6, pp. 1392–1394, 2006.

[41] C. L. Saltzman, M. L. Hagy, B. Zimmerman, M. Estin, and R. Cooper, "How effective is intensive nonoperative initial treatment of patients with diabetes and Charcot arthropathy of the feet?" *Clinical Orthopaedics and Related Research*, no. 435, pp. 185–190, 2005.

[42] V. J. Sammarco, "Superconstructs in the treatment of charcot foot deformity: plantar plating, locked plating, and axial screw fixation," *Foot and Ankle Clinics*, vol. 14, no. 3, pp. 393–407, 2009.

[43] A. Eschler, A. Wussow, B. Ulmar, T. Mittlmeier, and G. Gradl, "Intramedullary medial column support with the Midfoot Fusion Bolt (MFB) is not sufficient for osseous healing of arthrodesis in neuroosteoarthropathic feet," *Injury*, vol. 45, supplement 1, pp. S38–S43, 2014.

[44] D. A. Butt, T. Hester, A. Bilal, M. Edmonds, and V. Kavarthapu, "The medial column Synthes Midfoot Fusion Bolt is associated with unacceptable rates of failure in corrective fusion for Charcot deformity: results from a consecutive case series," *Bone and Joint Journal*, vol. 97, no. 6, pp. 809–813, 2015.

[45] T. Zgonis, *Surgical Reconstruction of the Diabetic Foot and Ankle*, Lippincott Williams & Wilkins, Philadelphia, Pa, USA, 2009.

[46] L. J. Sanders and R. G. Frykberg, "Diabetic neuropathic osteoarthropathy: the Charcot foot," in *The High Risk Foot in Diabetes Mellitus*, R. G. Frykberg, Ed., pp. 297–336, Churchill Livingstone, New York, NY, USA, 1993.

[47] S. Popelka, *Chirurgie Nohy a Hlezna*, Mladá Fronta, Praha, Czech Republic, 2014.