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ORIGINAL ARTICLE

Bilirubin is inversely related to diabetic peripheral neuropathy assessed by sural nerve conduction study

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ABSTRACT

Aims/Introduction: Diagnosis of diabetic peripheral neuropathy (DPN) depends on subjective findings, certain investigations for DPN risks have not been performed enough. Bilirubin protects against vascular complications by reducing oxidative stress in diabetes, but is not fully tested for DPN. This study aimed to evaluate sural nerve conduction impairments (SNCI) as an objective DPN marker and the contribution of bilirubin to SNCI.

Materials and methods: Using DPN-Check®, SNCI was defined as a decline of amplitude potential or conduction velocity below the normal limit in 150 inpatients with diabetes. The correlations between SNCI and conventional DPN diagnosis criteria, the incidence of diabetic retinopathy/nephropathy, biomarkers for atherosclerosis, cardiac function by ultrasonic cardiogram, and bilirubin were statistically tested, followed by the comparison of logistic regression models for SNCI to find confounders with bilirubin.

Results: The incidence of SNCI was 72.0%. The sensitivity and specificity of SNCI for DPN prediagnosis by simplified criteria were 54.6 and 90.5%, respectively, and similarly corresponded with diabetic retinopathy and nephropathy (sensitivity 57.4 and 50.0%, respectively). SNCI significantly related to diabetes duration, declined estimated glomerular filtration rate, albuminuria and total bilirubin. SNCI incidence was attenuated in the higher bilirubin tertiles (89.8/65.3/54.8%, $P < 0.001$). Bilirubin was an independent inverse risk factor for SNCI, even after adjustment by known risk factors for DPN and markers for microvascular complications.

Conclusions: SNCI is a comprehensive marker for diabetic complications. We first showed the independent inverse relationship between bilirubin and SNCI through the independent pathway with other complications, provably reducing oxidative stress, as previously reported.

INTRODUCTION

Diabetic neuropathy is one of the most prevalent diabetic complications affecting approximately 50% of patients with type 1 and type 2 diabetes[1,2]. Diabetic peripheral neuropathy (DPN) is known to be a risk factor for not only diabetic foot, but also mortality in diabetes[3]. Early diagnosis of DPN is essential, as early appropriate care can prevent its development, even several years later[4,5]. Generally, DPN is diagnosed by the presence of subjective symptoms and/or objective peripheral nerve dysfunction in patients with diabetes after exclusion of non-diabetic etiologies[2]. However, asymptomatic DPN and inconsistent techniques of neurological examination frustrate the formulation of concrete diagnostic criteria.

Nerve conduction study (NCS), a quantitative electrophysiology detecting sensorimotor dysfunction, has been applied to the evaluation of polyneuropathy in diabetes[6-8]. NCS abnormities

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are closely related to morphological changes in nerve fibers of patients prediagnosed with diabetic neuropathy[9]. Recently, a non-invasive point-of-care nerve conduction device (DPN-Check®; Neurometrix Inc., Waltham, MA, USA) has been developed, which detects sural nerve conduction impairments (SNCI) identified as the diminished amplitude potential (AP) and conduction velocity (CV) in sural NCS. Its diagnostic reliability has been validated by several studies, widely contributing to the accumulation of knowledges about DPN[10-13].

A considerable number of experimental diabetic polyneuropathy models elucidated the hypothesis that oxidative stress was a causative factor of neuronal dysfunction[14-16]. Unfortunately, no human study could directly demonstrate this, probably because of a lack of non-invasive quantitative clinical markers for either oxidative status or DPN grading. Some clinical data found the association between oxidative stress markers and the presence of DPN, which might not establish a direct cause–effect relationship[17-18].

Bilirubin, a potent radical scavenger that possibly reduces oxidative stress in the diabetic state enhanced through the PKC-NAD(P)H oxidase pathway, ameliorates diabetic complications in rodents[19,20]. We previously reported a protective effect of genetic hyperbilirubinemia against diabetic complications[21]. Several following clinical investigations have verified that serum bilirubin levels are inversely associated with the incidence of retinopathy, nephropathy and cardiovascular diseases in individuals with diabetes[22-26]. Kim *et al.* first reported the relationship between bilirubin and DPN, but DPN diagnosis was based on subjective symptoms and physical examination[27].

The purpose of the present study was to evaluate the relationship of SNCI assessed by DPN-Check® to conventional diagnosis for DPN and clinical features. We also aimed to elucidate whether serum bilirubin could be a protective factor against DPN, using SNCI as an objective biomarker for DPN.

MATERIALS AND METHODS

Ethics statement

The present single-center study was carried out at the department of Endocrine & Metabolic Diseases/Diabetes Mellitus, Kyushu University Hospital, Fukuoka, Japan. A total of 150 inpatients (≥20 years) with any overt diabetes were included in the cross-sectional registry during October 2014 to July 2015. Patients prediagnosed with severe systemic illness, alcoholism, orthopedic/neuromuscular diseases, critical limb ischemia, diabetic foot or any wounds disturbing electrophysiology; or treated with implanted electronic devices (e.g., defibrillators or pacemakers) were excluded. The study was approved by the ethics committee at the Kyushu University and fulfilled the Declaration of Helsinki. (Approval number 27-192). Written and oral informed consent was obtained from all participants.

Study procedures

Sex, age, height, weight, body mass index (weight in kilograms divided by height in meters squared), habitual alcohol drinking (moderate drinking; up to 1 drink per day for women and up to 2 drinks per day for men), smoking history (current and ever-smoker), clinical blood pressure (systolic and diastolic), and type and duration of diabetes mellitus were obtained from medical records.

Diagnosis of DPN was carried out by certified diabetologists according to 'simplified diagnostic criteria of diabetic polyneuropathy and suggested staging' proposed from the consensus of the Japanese study group of diabetic neuropathy[28]. Briefly, DPN was determined as positive for at least two of the three criteria: (i) subjective symptoms (numbness, pain or dysesthesia in bilateral lower extremities); (ii) diminished bilateral Achilles tendon reflexes (ATR), which include decreased and absent ATR; or (iii) diminished bilateral vibratory sensation at the malleolus medialis (<10 s using a tuning fork at 128 Hz). Sural nerve conduction velocities were measured using DPN-Check® (NeuroMetrix Inc.; Woburn, MA, USA) by trained technicians in compliance with the reference method[10]. Both AP and CV were respectively calculated as averages of bilateral results. Undetectable nerve conduction was treated as AP 0 μV and CV 0 m/s for SNCI diagnosis. Grading for SNCI severity was automatically reported by DPN-Check® based on AP/CV values: (i) normal (AP ≥4 μV, CV ≥40 m/s); (ii) mild (AP ≥4 μV, CV <40 m/s); (iii) moderate (AP ≥1, <4 μV); and (iv) severe (AP <1 μV). However, the preset thresholds were not based on published evidence nor validated in the Asian population. According to the previous study, which provided the regression formulas representing normal limits optimized in Japanese individuals as follows: AP limit = 12.62 − 0.103 × age (years), CV limit = 94.88 − 0.148 × age (years) − 0.231 × height (cm), [29] we determined a 'modified SNCI' as either AP or CV below the normal limits. Coefficient of variation of RR intervals (CVRR) in the resting state and during deep breathing were also measured by technicians to assess autonomic nervous function.

Diabetic retinopathy (DR) was defined as the presence of disease greater than simple DR or the presence of diabetic macular edema. DR grade for each patient with fundus examination was informed by certified ophthalmologists collaborating at Kyushu University Hospital. Diabetic nephropathy (DN) was defined as urinary albumin/creatinine ratio (UACR) of ≥30 mg/gCr or estimated glomerular filtration rate (eGFR) of <30 mL/min per 1.73 m^2 after exclusion of renal dysfunction originating from other overt etiology (e.g., autoimmune glomerulonephritis or drug-induced nephrotoxicity).

The ankle-brachial pressure index (ABI) and brachial-ankle pulse wave velocity (baPWV) were measured by trained technicians using an oscillometric device (Form PWV/ABI; Omron Colin Co., Ltd., Komaki, Japan) as markers for atherosclerosis and arteriosclerosis, respectively. The lower value for ABI and mean for baPWV were used to round bilateral values up for the following analyses. Left ventricular ejection fraction and the ratio of early transmitral flow velocity to early diastolic velocity of the mitral annulus (E/e′)

were measured using an ultrasonic cardiogram by trained technicians or cardiologists.

All laboratory tests were carried out at the single unit in Kyushu University Hospital approved by the laboratory quality management system (ISO 15189) after 10 h of fasting. Most recent hemoglobin A1c (HbA1c; Japan Diabetes Society) levels converted to National Glycohemoglobin Standardization Program units and International Federation of Clinical Chemistry units were used for analyses.

Statistical analysis

We compared the clinical features of individuals with or without SNCI by Welch's *t*-tests for continuous variables and Pearson's χ^2-tests (or Fisher's exact test) for categorical variables. Diagnostic accuracy was presented as positive percent agreement (PPA) and negative percent agreement (NPA). PPA (sensitivity) is the proportion of comparative/reference method positive results in which the test method result is positive. NPA (specificity) is the proportion of comparative/reference method negative results in which the test method result is negative. Total bilirubin was divided into tertiles: T1 (0–0.6 mg/dL, n = 59), T2 (0.7–0.8 mg/dL, n = 49) and T3 (0.9- mg/dL, n = 42) mg/dL. Welch's ANOVA was used to test the equality of AP/CV means among tertiles. Logistic regression models were used to compare the contribution of risk factors for SNCI (age, sex, BMI, smoking, alcohol, HbA1c, systolic/diastolic blood pressures, lipid profile, eGFR, UACR, ABI, baPWV and total bilirubin) to any SNCI. All statistical analyses were carried out using JMP Version 15 statistical software (SAS institute Inc., Cary, NC, USA). *P*-values of <0.05 were considered statistically significant.

RESULTS

Clinical characteristics and SNCI prevalence

Clinical characteristics including DPN-Check® data of all participants are shown in Table 1. All participants with diabetes enrolled in the current study were automatically classified into 103 (68.7%) no SNCI, seven (4.7%) mild, 35 (23.3%) moderate and five (3.3%) severe SNCI according to the manufacturer's criteria based on AP/CV values. The prevalence of DPN prediagnosis by simplified criteria was 42.0%. Unfortunately, the deviated distribution of SNCI severity disabled a proper comparison of clinical features as to SNCI severity trends.

Clinical features of SNCI

Thus, clinical features of 108 (72.0%) participants with modified SNCI were statistically compared with those with no SNCI, as shown in Table 2. Diabetes duration was remarkably longer in participants with any SNCI, whereas age, sex, body mass index, smoking and habitual alcohol drinking were not. There was no significant change in the prevalence of modified SNCI between participants with type 1 and 2 diabetes. HbA1c and lipid profiles did not present any relationships with SNCI. UACR of seven participants were not available because of hemodialysis, menstruation or urinary tract infection. Significantly lower eGFR and higher UACR, representing the presence of DN or its severities, were found in SNCI. ABI (n = 145) and baPWV (n = 143) were available after exclusion of participants with known peripheral artery disease or uncertainty of measurement because of incompressible arteries. CVRR (n = 141) and ultrasonic cardiogram (n = 138, of which 5 E/e′ were not available) were obtained because of the limited duration of hospitalization. Participants with modified SNCI showed no correlation to markers for macrovascular complications to SNCI, contrary to those for microvascular complications. Lower serum bilirubin, which indicates the risk for micro- and macrovascular complications through enhanced oxidative stress status in diabetes, was also significantly associated with modified SNCI.

Agreement ratios of SNCI versus DPN Prediagnosis, DR and DN

Table 3 shows the agreement ratios between modified SNCI and the diagnoses of diabetic complications. PPA (sensitivity) and NPA (specificity) of modified SNCI versus DPN prediagnosis by simplified criteria were 54.6 and 90.5%, respectively. Among three minor criteria of simplified diagnostic criteria for DPN, 'diminished ATR' achieved the best PPA versus modified

Table 1 | Characteristics of the participants

Age (years)	65.0 (51.0–71.0)
Female sex	67 (44.7)
BMI (kg/m^2)	24.1 (21.7–28.4)
Height (m)	1.62 (1.54–1.67)
Diabetes duration (years)	10.0 (4.0–18.3)
T2D/T1D/other diabetes (*n*)	126/12/12
HbA1c (mmol/mol)	70.5(58.5–88.0)
HbA1c (%)	8.6 (7.5–10.2)
Hypertension	91 (60.6)
Dyslipidemia	118 (78.7)
Smoking (current and ever-smoker)	77 (51.3)
Habitual alcohol drinking	55 (36.7)
Diabetic retinopathy	72 (48.0)
Diabetic nephropathy	61 (40.7)
DPN prediagnosis by simplified diagnostic criteria	63 (42.0)
1) Subjective symptoms	55 (36.7)
2) Diminished ATR	83 (55.3)
3) Diminished vibratory sensation	54 (36.0)
Sural nerve conduction study	
Sural nerve AP (μV)	5.0 (4.0–6.0)
Sural nerve CV (m/s)	48 (46.8–50)
SNCI severity (mild/moderate/severe), *n* [undetectable SNCI]	7[0]/35[4]/5[5]
Modified SNCI	108 (72.0)

Total n = 150. Data are presented as the median (Q1, Q3) or n (%). AP, amplitude potential; ATR, Achilles tendon reflexes; CV, conduction velocity; SNCI, sural nerve conduction impairments; T1D, type 1 diabetes; T2D, type 2 diabetes.

SNCI (67.6%) when 'subjective symptoms' did the best NPA versus modified SNCI (90.5%). Sural nerve AP was well correlated with all the DPN criteria, whereas CV was similar, except for the irrelevance with 'diminished vibratory sensation' (Table S1). Meanwhile, both DR and DN incidences were consistently associated with modified SNCI, accompanied by high PPA (57.4 and 50.0%, respectively) and NPA (76.2 and 83.3%, respectively; Table 3). After the adjustment for the other risk factors in the logistic regression models, SNCI was still at risk for DR and DN with significance (odds ratios (ORs) 3.56 and 4.62, respectively; Table S2).

Relationship between bilirubin and SNCI

Total bilirubin was divided into T1 (0–0.6 mg/dL, $n = 59$), T2 (0.7–0.8 mg/dL, $n = 49$) and T3 (≥0.9 mg/dL, $n = 42$). The prevalence of SNCI had a significant difference among tertiles, notably, there was no patient with severe SNCI in the highest tertile (T3; Figure 1a). The higher tertile of total bilirubin, an innate anti-oxidant, had the less modified SNCI prevalence (T1/T2/T3: 89.8/65.3/54.8%) with significance (Figure 1b).

To investigate how bilirubin is involved in the etiology of DPN, we compared three logistic regression models to explain the relationships between risk factors and any SNCI (Table 4). Model 1 showed that bilirubin was a remarkable protective factor against SNCI (OR 0.10 per 1-mg/dL increase), even after the adjustment for other confounding factors (age, sex, body mass index, height, diabetes duration, HbA1c, smoking history, habitual alcohol drinking, blood pressure, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol and triglyceride); of which diabetes duration (OR 1.07, per 1-year increase), systolic blood pressure (OR 1.04, per 1-mmHg increase), smoking history (OR 2.97) and triglyceride (OR 1.00, per 1-mg/dL increase) remained as independent explanatory variables as well. In model 3, designed as a microvascular complication model including bilirubin, the significance of bilirubin was sustained even after the adjustment by DR, eGFR and UACR added in model 2.

DISCUSSION

The present study verified the potential prevalence of peripheral nerve dysfunction in diabetes and the relevance ratio to the

Table 2 | Comparison of the subject with or without modified sural nerve conduction impairments

	No SNCI	*n*	Modified SNCI	*n*	*P*-value
Age (years)	57.7 ± 18.3	42	62.0 ± 14.1	108	0.174
Female sex	20 (47.6)	42	63 (58.3)	108	0.236
BMI (kg/m^2)	25.8 ± 6.0	42	25.1 ± 5.1	108	0.491
Height (m)	1.61 ± 0.10	42	1.60 ± 0.09	108	0.593
Diabetes duration (years)	8.1 ± 8.6	42	14.5 ± 12.3	108	<0.001
T2D/T1D/other diabetes (*n*)	30/4/8	42	96/8/4	108	0.007†
HbA1c (%)	8.7 ± 1.8	42	8.9 ± 2.0	108	0.516
Hypertension	23 (54.8)	42	68 (63.0)	108	0.356
Dyslipidemia	33 (78.6)	42	85 (78.7)	108	0.986
Smoking (current and ever-smoker)	17 (40.5)	42	60 (55.6)	108	0.097
Habitual alcohol drinking	15 (35.7)	42	40 (37.0)	108	0.880
Estimated GFR (mL/min/m^2)	86.7 ± 27.2	42	70.2 ± 30.7	108	0.002
UACR (mg/gCre)	22.8 ± 35.2	40	355.9 ± 1109.0	103	0.003
Systolic blood pressure (mmHg)	122.0 ± 16.0	42	125.5 ± 17.9	108	0.256
Diastolic blood pressure (mmHg)	73.3 ± 10.9	42	73.2 ± 14.1	108	0.954
LDL cholesterol (mg/dL)	104.6 ± 30.5	42	103.6 ± 40.7	106	0.878
HDL cholesterol (mg/dL)	43.6 ± 12.4	42	44.3 ± 13.5	107	0.777
Triglyceride (mg/dL)	148.9 ± 87.9	42	81.6 ± 59.2	108	0.276
Total bilirubin (mg/dL)	0.89 ± 0.32	42	0.71 ± 0.32	108	0.003
Lower ABI	1.10 ± 0.10	41	1.10 ± 0.15	104	0.944
Mean baPWV (m/s)	15.4 ± 3.7	40	16.8 ± 4.1	103	0.051
LVEF (%)	69.0 ± 7.2	39	68.1 ± 9.0	104	0.517
E/e'	11.4 ± 4.4	38	12.7 ± 5.0	100	0.146
CVRR (resting)	3.4 ± 2.5	40	1.9 ± 1.2	101	<0.001
CVRR (deep breathing)	6.2 ± 3.4	40	3.6 ± 2.5	100	<0.001

Data are presented as mean ± SD or *n* (%). SNCI, sural nerve conduction impairments; T2D, type 2 diabetes; T1D, type 1 diabetes; GFR, glomerular filtration rate; UACR, urinary albumin/creatinine ratio; ABI, ankle-brachial pressure index; baPWV, brachial-ankle pulse wave velocity; LVEF, left ventricular ejection fraction; E/e', the ratio of early transmitral flow velocity to early diastolic velocity of the mitral annulus; CVRR, coefficient of variation of RR intervals; AP, amplitude potential; CV, conduction velocity. †Fisher's exact test.

Table 3 | Agreement ratios of sural nerve conduction impairments versus diabetic peripheral neuropathy prediagnosis, diabetic retinopathy and diabetic nephropathy

Modified SNCI versus	PPA (%)	NPA (%)
DPN prediagnosis by simplified diagnostic criteria	54.6	90.5
1) Subjective symptoms	47.2	90.5‡
2) Diminished ATR	67.6†	76.2
3) Diminished vibratory sensation	42.6	81.0
Diabetic retinopathy	57.4	76.2
Diabetic nephropathy	50.0	83.3

Total n = 150. Positive percent agreement (PPA; sensitivity) is the proportion of comparative/reference method positive results in which the test method result is positive. Negative percent agreement (NPA; specificity) is the proportion of comparative/reference method negative results in which the test method result is negative. AP, amplitude potential; ATR, Achilles tendon reflexes; CV, conduction velocity; DN, diabetic nephropathy; DR, diabetic retinopathy; SNCI, sural nerve conduction impairments. †The best PPA. ‡The best NPA.

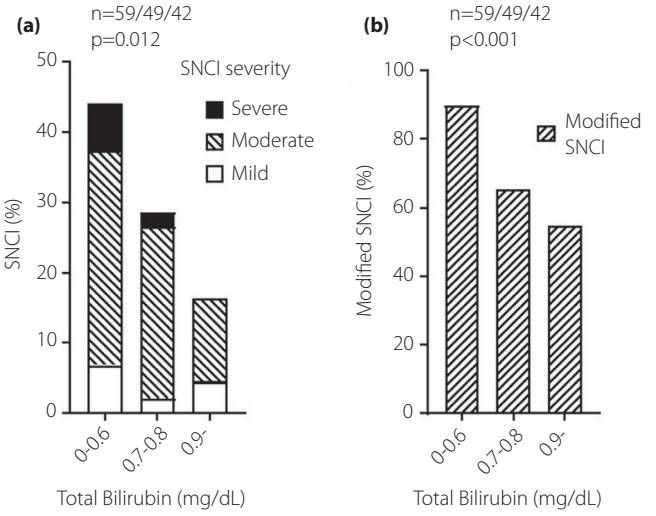

Figure 1 | The relationship between total bilirubin tertiles and sural nerve conduction impairments (SNCI; n = 150). (a) The incidence of SNCI by total bilirubin tertiles, indicated as a stacked bar chart of SNCI severity. (b) The incidence of modified SNCI by total bilirubin tertiles. P-values were calculated by a Pearson's χ^2-test.

current DPN diagnosis. This non-invasive methodology for peripheral nerve conduction was convenient and useful for the evaluation of risks for neuropathy and vascular complications in patients with diabetes. This study also cultivated a further understanding about the protective role of bilirubin against the development of DPN.

As the population of the present study was based on inpatients in the university hospital, the clinical features might be severer than general outpatients, considering not too long diabetes duration. Their high incidences of diabetic complications were statistically preferable to assess the mutual relevance among risk markers. The laboratory data managed in a single center by a limited number of technicians and medical doctors were precise and consistent. The medical records supervised by at least two specialists for internal medicine were also reliable.

The prevalence of modified SNCI using DPN-Check® was 1.7-fold higher than the prediagnosis rate by simplified DPN criteria. It indicates that simplified diagnosis criteria might underestimate DPN frequency, as compared with the estimation by SNCI (42 vs 72%). Indeed, PPA of subjective symptoms and diminished vibratory sensation were considerably low compared with diminished ATR, but their NPA were very high. It implies that there might be a certain amount of asymptomatic neuropathy or pseudo-normal vibratory senses. Thus, DPN-Check® is a valid option in the objective neurological assessment to avoid overlooking severe DPN.

Then, we investigated the risk factors for DPN represented as any SNCI in the current study. As the precedent study reported[17], a longer diabetes duration was the strongest DPN risk in the present study. There was no sex difference. Obesity or the habits of smoking and alcohol, regarded as lifestyle-related factors, were not considered as at DPN risk either. Although type 2 diabetes had a higher prevalence of DPN and more severe SNCIs than type 1 diabetes, their clinical features, such as age, duration and other complications, were too different to be compared directly. HbA1c or lipid profiles were not associated with DPN. Actually, the association of HbA1c and DPN was quite controversial among the previous studies[13,17,30-32]. To say the least, a single measurement of HbA1c might not be a representation for long-term hyperglycemia, so we should not interpret that better glycemic control is not necessary to avoid DPN. Furthermore, the recent studies showed that glucose fluctuation, but not HbA1c, was an independent risk for SNCI[30]. Thereto, the contribution of low-density lipoprotein cholesterol to DPN has been inconsistent[30-32]. CVRRs, generally decreased by autonomic neuropathy in diabetes, were declined in the participants with any SNCI; which substantiated the resemblance of diabetes-induced neural injuries.

We also investigated whether SNCI could be a risk for not only DPN, but also diabetic complications, because diabetic complications are believed to share the causative effect of dysglycemia in most dominant pathogenic theories. Indeed, SNCI was closely related to the incidence of DR/DN, even after adjusted by the other confounding risk factors for diabetic complications, as shown in Table S2. In contrast, modified SNCI did not show any correlation to macrovascular complications. Thus, SNCI can be considered as one of the representatives of dysglycemic memory.

Serum total bilirubin is a potent anti-oxidant and inhibits the oxidative stress enhanced in the diabetic state, which is an established causative theory for diabetic complications[19,20,26]. The increase of bilirubin leads to the risk reduction for diabetic macroangiopathy and microangiopathy[21-23,33,34]. There were a

Table 4 | Comparison of logistic regression models for modified sural nerve conduction impairments

Explanatory variables	Model 1 (n = 148)		Model 2 (n = 141)		Model 3 (n = 141)	
	OR (95% CI)	P-value	OR (95% CI)	P-value	OR (95% CI)	P-value
Total bilirubin (mg/dL)	0.10 (0.02–0.49)	0.002*	–	–	0.10 (0.01–0.58)	0.010*
Diabetic retinopathy	–	–	2.41 (0.88–6.59)	0.083	2.23 (0.79–6.28)	0.125
Estimated GFR (mL/min/m^2)	–	–	0.97 (0.95–1.00)	0.033*	0.97 (0.94–1.00)	0.021*
UACR (mg/gCre)	–	–	1.01 (1.00–1.02)	0.009*	1.01 (1.00–1.03)	0.013*

All models were additionally adjusted for age, sex, BMI, height, diabetes duration, HbA1c, smoking, alcohol, systolic/diastolic blood pressures, LDL-C, HDL-C, and triglyceride. ORs were based on per a unit increase. OR, odds ratio; 95%CI, 95% confidence interval. Model 1: a basic model including bilirubin. Model 2: a microvascular complication model. Model 3: a microvascular complication model including bilirubin. GFR, glomerular filtration rate; SNCI, sural nerve conduction impairments; UACR, urinary albumin/creatinine ratio. *$P < 0.05$.

few studies that indirectly reported the causative role of bilirubin in the development of diabetic autonomic neuropathy[32,35]. As for DPN, the clinical study carried out by Kim *et al.* was the only evidence so far that showed the inverse correlation of serum total bilirubin levels and DPN in patients with type 2 diabetes[27]. However, their protocol to diagnose DPN was dependent on the inquiries for subjective symptoms and physical examination, which could not eliminate the chances of arbitrary discretion.

We decided to use DPN-Check® because its measurement is easy and reproducible. DPN-Check® assures its objectivity, prevailing as a non-invasive and quantitative large fiber NCS[10-13]. Recently, other quantitative techniques to assess DPN have been developed. Intra-epidermal nerve fiber density is known to correlate well with sural nerve density[36], but invasive skin biopsy is required. Corneal nerve fiber density and length acquired by *in vivo* corneal confocal microscopy are non-invasive markers for DPN[37], whereas they are still not popular, because the equipment is expensive. Both of them target the epidermal small nerve fibers, but not large fibers. Their procedures are complex, essentially requiring trained specialists for nerve pathology.

Therefore, we carried out further study using DPN-Check® to confirm the contribution of bilirubin to DPN in humans. Consistent with the previous report[27], less SNCI was found in the higher bilirubin tertile. Multivariate analysis clarified that the effect of bilirubin was independent of conventional risk factors and microvascular complications. According to the previous findings that showed enhanced oxidative stress in individuals with DPN[32], the inverse relationship of bilirubin and SNCI might be explainable, but we still require further intervention study to prove the causal link.

The present study had certain limitations. First, we included all patients with any type of diabetes in this study to avoid selection bias. In consequence, the heterogeneous population might confuse the effect of each risk factor on SNCI, provably resulting in the loss of glycemic effect on DPN. Preferably, the prevalence of DPN should be validated in future studies based on organized populations with diabetes. Second, although we did not carry out standard NCS, even standard NCS on the sural nerve does not directly evaluate mononeuropathy of the other large fiber nerves or autonomic neuropathy. Mononeuropathy can usually be identified by specific neurogenic signs, such as a shooting/lancinating pain, paresthesia and various dysesthesia, but we found no participants diagnosed as mononeuropathy. Autonomic neuropathy, represented as CVRRs, was well correlated to SNCI in the present study. Therefore, it might not weaken the clinical importance of DPN-Check®. Third, although we recruited an adequate number of participants to carry out multivariate analysis, the present study did not provide longitudinal changes in the serum total bilirubin level over time in patients. Further longitudinal study is required to elucidate the relationship between serum bilirubin and the development of neuropathy.

In conclusion, SNCI guaranteed reasonable agreements with not only subjective DPN diagnosis criteria, but also other diabetic complications. We confirmed older age, longer diabetes duration, surrogate markers for both micro- and macrovascular complications, and total bilirubin as risk factors for DPN. Furthermore, we were the first to show the independent inverse relationship between bilirubin and SNCI. These results show SNCI assessment by DPN-Check® to be a comprehensive marker for diabetic complications in routine medical care for diabetes patients.

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DISCLOSURE

The authors declare no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1 | Sural nerve amplitude potential/conduction velocity versus diabetic peripheral neuropathy prediagnosis (n = 150).

Table S2 | Modified sural nerve conduction impairments in the logistic regression models for diabetic retinopathy and diabetic nephropathy (n = 148).