Diabetes & Metabolism 45 (2019) 47–52

Available online at ScienceDirect www.sciencedirect.com

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Original article

Low serum bilirubin levels contribute to the presence and progression of distal symmetrical polyneuropathy in Chinese patients with type 2 diabetes

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ARTICLE INFO

Article history:
Received 29 October 2017
Received in revised form 10 February 2018
Accepted 18 February 2018
Available online 22 February 2018

Keywords:
Bilirubin
Distal symmetrical polyneuropathy
Indicator
Type 2 diabetes
Unconjugated bilirubin

ABSTRACT

Aim. – To investigate the association between serum bilirubin and distal symmetrical polyneuropathy (DSPN) in Chinese patients with type 2 diabetes (T2D).

Methods. – A total of 1800 inpatients with T2D (including 68 with 1-year follow-ups) were consecutively enrolled between June 2014 and March 2017. DSPN was diagnosed according to criteria recommended by the Toronto Diabetic Neuropathy Expert Group in 2010. Clinical data were retrospectively collected.

Results. – Patients with vs. without DSPN had low levels of serum total bilirubin (9.9 ± 3.1 μmol/L vs. 10.7 ± 2.8 μmol/L; $P < 0.01$) and unconjugated bilirubin (6.7 ± 2.2 μmol/L vs. 7.3 ± 2.1 μmol/L; $P < 0.01$), respectively. Patients in the lowest tertiles of bilirubin had the highest rates of DSPN and slowest nerve conduction velocities (NCVs). After multivariate adjustment, low levels of unconjugated bilirubin were a risk factor (OR: 0.696, 95% CI: 0.494–0.981; $P = 0.038$, highest vs. lowest) for the presence of DSPN. Furthermore, in the 68 patients who had 1-year follow-ups, those with the lowest tertiles of serum bilirubin showed maximum deterioration in NCVs. These changes remained significant even after multivariate adjustment.

Conclusion. – Low levels of serum bilirubin, especially unconjugated bilirubin, contributed to the presence and progression of DSPN in Chinese patients with T2D. Thus, serum unconjugated bilirubin may be used as an additional indicator of risk and progression of DSPN.

Introduction

Distal symmetrical polyneuropathy (DSPN) is one of the most common chronic complications of diabetes [1], presenting in at least 10–15% of newly diagnosed diabetes patients [2,3] and possibly reaching 50% in those with diabetes for > 10 years [4]. DSPN is the main contributor to several severe diabetes complications, such as foot ulcers and even amputation [5].

Oxidative stress can induce tissue injury in peripheral nerves in patients with diabetes and, in fact, participates in the pathogenesis of DSPN [6]. Bilirubin is the end product of haem catabolism and is an endogenous antioxidant, and the beneficial effects of elevated serum bilirubin on the cardiovascular system at physiological concentrations have recently been reported [7]. In the general population, low levels of total bilirubin have been correlated with subclinical atherosclerotic changes [8], and associated with the incidence of coronary artery disease, stroke and peripheral artery disease [9].

In patients with type 2 diabetes (T2D), low serum levels of total bilirubin have been reported in association with microvascular complications such as microalbuminuria [10] and retinopathy [11], as well as macrovascular complications such as coronary heart disease, peripheral vascular disease [12] and higher risk of amputation [13]. Similarly, an inverse relationship between total

Abbreviations: DSPN, distal symmetric polyneuropathy; AST, aspartate aminotransferase; ALT, alanine aminotransferase; BMI, body mass index; HbA1c, glycosylated hemoglobin; TC, total cholesterol; LDL, low-density lipoprotein; SD, standard deviation.

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https://doi.org/10.1016/j.diabet.2018.02.007
1262-3636/

bilirubin and the presence of DSPN was observed in a Korean population [14]. However, that study explored only the effect of total bilirubin on DSPN. Indeed, no prospective study or analysis has yet been conducted to evaluate the effect of serum bilirubin, including total bilirubin, unconjugated bilirubin and conjugated bilirubin, on progression of DSPN.

Thus, the aim of the present study was to investigate the roles of the different components of serum bilirubin in the presence and progression of DSPN (diagnosed according to nerve conduction studies and neurological assessment) in Chinese patients with T2D.

Material and methods

Subjects

Our study consisted of two parts: a cross-sectional study; and a retrospective follow-up study. For the cross-sectional part, a total of 1800 patients with T2D were enrolled from among inpatients at Drum Tower Hospital, Nanjing, China, between June 2014 and March 2017. T2D was diagnosed according to American Diabetes Association (ADA) criteria, using both fasting and 2-h plasma glucose from an oral glucose tolerance test (OGTT) [15]. Inclusion criteria were: confirmed T2D; and patients aged 18–75 years. Exclusion criteria were:

- patients with chronic liver disease, renal disease, arrhythmias, malignant disease, severe respiratory disease, heart failure and infection;
- patients with serum bilirubin levels above the upper limit of normal (ULN; total bilirubin $>$ 20.5 μmol/L, conjugated bilirubin $>$ 6.8 μmol/L);
- patients with abnormal liver function tests [defined as aspartate aminotransferase (AST) or alanine aminotransferase (ALT) $\geq$ 2 times the ULN];
- patients with stroke, hypothyroidism, alcohol addiction ($\geq$ 21 standard drinks containing 10–12 g of pure alcohol/week in the past 12 months) [16] and any causes of neuropathy other than diabetes.

For the retrospective follow-up study, 68 patients who underwent two tests of nerve conduction velocity (NCV) respectively before and after a 1-year interval were selected from the 1800 enrolled subjects. These 68 patients were assessed for diabetic complications annually at our hospital, and NCV tests were routinely and annually performed in the T2D inpatients at our hospital.

The study protocol was approved by the ethics committee of Drum Tower Hospital (No. 2017-006-01), and all patients gave their informed consent to participate.

Neurological symptoms and examination

All enrolled patients had a complete history of neurological symptoms and examination. Symptoms were documented using a neuropathy symptom scoring system [17] that included feelings of burning, numbness, tingling, fatigue, cramping and aching. An experienced neurologist performed the clinical examinations. Touch sensation was assessed by a 10-g monofilament test (on five sites per foot), ankle reflex was tested by a tendon hammer, vibration sensation by a standard 128-Hz tuning fork, temperature sensation by a TIP THERM test and pain sensation with a pin.

Nerve conduction examination

A skilled technician carried out all of these tests at a room temperature of 24 °C, with the skin temperature of tested patients stabilized to around 31 °C. The following NCVs were measured on both sides:

- median nerve, and both motor and sensory branches;
- ulnar nerve, and both motor and sensory branches;
- peroneal nerve;
- tibial nerve;
- superficial peroneal nerve;
- sural nerve.

If three or more of the tested NCVs were abnormal, then nerve conduction was considered abnormal and diagnosed on the basis of reference values specific to the Chinese population [18]. The presence of abnormal nerve conduction and symptoms or signs of neuropathy was defined as DSPN [19].

Clinical assessment

Patients' clinical data were collected by reviewing their electronic medical records. Data included demographic information, diabetes duration, body mass index (BMI) scores, glycosylated haemoglobin (HbA1c), fasting blood glucose, fasting C-peptide, postprandial C-peptide, triglycerides, total cholesterol (TC), high-density lipoprotein (HDL), low-density lipoprotein (LDL), AST, ALT, total bilirubin, conjugated bilirubin, unconjugated bilirubin, history of hypertension and/or coronary heart disease, and the presence of diabetic complications, including diabetic retinopathy, diabetic nephropathy and cardiovascular autonomic neuropathy. Serum levels of total and conjugated bilirubin were measured by the oxidation method [Zhuhai Senlo (Senlong) Biotech Co., Ltd., Zhuhai, China] and analyzed using an automated biochemistry analyzer (Beckman Coulter, Inc., Indianapolis, IN, USA). Diabetic complications were diagnosed by senior doctors according to the standard definitions in the relevant guidelines. Diabetic nephropathy (DN) was defined according to ADA criteria [15] based on the presence of albuminuria (urinary albumin-to-creatinine ratio $\geq$ 30 mg/g) and the absence of signs or symptoms of other primary causes of kidney damage. Cardiovascular autonomic neuropathy (CAN) was defined as having two or more abnormalities in four reflex tests: heart rate variation during deep breathing; Valsalva manoeuvre; lying-to-standing test; and blood pressure variability in response to standing. Each test was compared against normative values in a previous report [20].

Statistical analysis

Data are presented as means $\pm$ standard deviation (SD) for continuous variables and as percentages for dichotomous variables. All analyses were performed with IBM SPSS version 22.0 software (IBM Corp., Armonk, NY, USA). For continuous variables, Student's *t*-test was used for normal distributions and the Wilcoxon rank-sum test for abnormal distributions (fasting C-peptide, postprandial C-peptide). The Chi^2 or Fisher's exact test was used for dichotomous variables. Linear regression analysis was used for continuous variables, with logistic regression for dichotomous variables as a trend test for tertiles of serum bilirubin. These tests were performed by numbering each tertile (1, 2, 3) of serum bilirubin and treating the variable as continuous in regression models.

Multivariable logistic regression was used to investigate whether serum bilirubin was associated with DSPN after adjusting for age, gender, diabetes duration, BMI, HbA1c, TC, AST, ALT, diabetic retinopathy (DR), DN and CAN. Furthermore, age, duration of diabetes and gender were used as variables for propensity score matching to perform a 1:1 matched-pair analysis, which was followed by conditional logistic regression analysis to investigate independent factors of serum bilirubin in relation to the presence

Table 1
Characteristics of type 2 diabetes patients with (+) and without (−) distal symmetrical polyneuropathy (DSPN) in the present study.

	DSPN (−)	DSPN (+)	*P*
Patients (*n*)	1228	572	–
Age (years)	55.0 ± 12.2	59.6 ± 12.1	0.000
Gender (male, %)	60.9	62.9	0.411
Diabetic duration (years)	7.4 ± 6.5	10.8 ± 8.3	0.000
Body mass index (kg/m^2)	25.3 ± 3.4	24.3 ± 3.1	0.000
Glycosylated haemoglobin (%)	8.2 ± 1.9	9.7 ± 2.3	0.000
Fasting blood glucose (mmol/L)	7.4 ± 2.4	8.4 ± 2.8	0.000
Fasting C-peptide (pmol/L)	687 ± 332	582 ± 433	0.000
Postprandial C-peptide (pmol/L)	1852 ± 1124	1148 ± 853	0.000
Aspartate aminotransferase (U/L)	26.1 ± 15.5	20.9 ± 11.8	0.000
Alanine aminotransferase (U/L)	20.6 ± 9.6	19.2 ± 8.4	0.005
Triglycerides (mmol/L)	1.7 ± 1.2	1.6 ± 1.1	0.113
Total cholesterol (mmol/L)	4.2 ± 1.0	4.2 ± 1.1	0.421
High-density lipoprotein cholesterol (mmol/L)	1.2 ± 0.5	1.2 ± 0.5	0.995
Low-density lipoprotein cholesterol (mmol/L)	2.28 ± 0.91	2.26 ± 0.93	0.645
Hypertension (%)	35.7	39.2	0.152
Coronary heart disease (%)	8.3	9.1	0.586
Diabetic retinopathy (%)	20.4	45.6	0.000
Diabetic nephropathy (%)	20.8	37.9	0.000
Cardiovascular autonomic neuropathy (%)	27.9	50.0	0.000
Total bilirubin (μmol/L)	10.7 ± 2.8	9.9 ± 3.1	0.000
Conjugated bilirubin (μmol/L)	3.3 ± 1.1	3.2 ± 1.2	0.081
Unconjugated bilirubin (μmol/L)	7.3 ± 2.1	6.7 ± 2.2	0.000

Data are presented as means ± SD or %.

of DSPN after adjusting for BMI, HbA1c, TC, AST, ALT, DR, DN and CAN. Linear regression analysis was used to test the trend for serum bilirubin across the number (from 0 to 4) of diabetic complications.

Results

DSPN patients and serum levels of bilirubin

Among the 1800 patients with T2D, 572 were diagnosed as having DSPN. The detailed characteristics of these patients are shown in Table 1. Patients with DSPN had older age, longer durations of diabetes, and larger proportions of DR, DN and CAN. Also, the NCVs in both upper and lower limbs were slower in patients with than without DSPN (Table S1; see supplementary data associated with this article online). In addition, lower levels of serum bilirubin, including total bilirubin (9.9 ± 3.1 μmol/L vs. 10.7 ± 2.8 μmol/L; $P < 0.001$) and unconjugated bilirubin (6.7 ± 2.2 μmol/L vs. 7.3 ± 2.1 μmol/L; $P < 0.001$), were observed in patients with DSPN compared with those without DSPN (Table 1).

Rate of DSPN and NCVs according to serum bilirubin tertiles

Further analyses after dividing patients into groups according to tertiles of serum bilirubin levels (total, unconjugated and conjugated bilirubin) and the patients' characteristics are presented in Table S2 (see supplementary data associated with this article online). Those in the highest tertile of bilirubin showed younger age, shorter diabetes durations, a larger proportion of males, higher BMI scores, lower levels of HbA1c, TC and LDL, and higher levels of ALT and AST. Rates of microvascular complications were highest in the lowest tertile of bilirubin, while patients in the lowest tertile of unconjugated bilirubin had the highest prevalence of DSPN (lowest: 40.7%; intermediate: 27.5%; highest: 27.2%; $P < 0.001$). NCVs in both upper and lower limbs increased across all of these tertiles (Table 2). Similar changes and phenotypes were also observed in the tertiles of total and conjugated bilirubin levels (Table 2).

Serum bilirubin and risk of DSPN

After adjusting for age, gender, duration of diabetes, BMI, HbA1c, TC, AST, ALT, DR, DN and CAN, the highest vs. lowest tertiles of unconjugated bilirubin [odds ratio (OR): 0.696, 95% confidence interval (CI): 0.494–0.981; $P = 0.038$] showed a lower OR for the presence of DSPN, thereby indicating that a lower level of unconjugated bilirubin was a risk factor for DSPN (Table 3).

As there were large differences in age and durations of diabetes between the DSPN (+) and DSPN (−) groups, it was considered that multivariate logistic analysis might not be enough. Therefore, a 1:1 matched-pair analysis was also performed, using propensity score matching. After matching by age, duration of diabetes and gender, 543 patient pairs were found. Their general characteristics are presented in Table S3 (see supplementary data associated with this article online). After adjusting for BMI, HbA1c, TC, AST, ALT, DR, DN and CAN in a conditional logistic regression model, the highest vs. lowest tertiles of unconjugated bilirubin (OR: 0.693, 95% CI: 0.486–0.989; $P = 0.043$) still showed a lower OR for the presence of DSPN.

Serum bilirubin and NCVs during the 1-year follow-ups

To study the association between serum bilirubin levels and deterioration of NCV, the 68 patients who underwent two NCV tests with a 1-year interval were analyzed. Their general

Table 2
Presence of distal symmetrical polyneuropathy (DSPN) and parameters of nerve conduction velocity (NCV) by tertiles (T1–T3) of serum bilirubin levels.

	Total bilirubin (μmol/L)				Unconjugated bilirubin (μmol/L)				Conjugated bilirubin (μmol/L)			
	T1 < 8.9	T2 8.9–11.8	T3 > 11.8	*P* for trend	T1 < 6.0	T2 6.0–8.0	T3 > 8.0	*P* for trend	T1 < 2.8	T2 2.8–3.7	T3 > 3.7	*P* for trend
Patients (*n*)	596	612	592	–	596	612	592	–	596	612	592	–
Total bilirubin (μmol/L)	7.2 ± 1.2	10.3 ± 0.8	13.9 ± 1.4	0.000	7.4 ± 1.5	10.2 ± 1.2	13.7 ± 1.6	0.000	7.9 ± 1.9	10.6 ± 1.9	13.1 ± 2.1	0.000
Conjugated bilirubin (μmol/L)	2.3 ± 0.6	3.3 ± 0.7	4.3 ± 0.9	0.000	2.6 ± 1.0	3.2 ± 1.0	4.1 ± 1.0	0.000	2.1 ± 0.4	3.2 ± 0.2	4.6 ± 0.8	0.000
Unconjugated bilirubin (μmol/L)	4.9 ± 1.0	6.9 ± 0.9	9.5 ± 1.3	0.000	4.7 ± 1.0	6.9 ± 0.6	9.6 ± 1.1	0.000	4.7 ± 1.0	6.9 ± 0.6	9.6 ± 1.1	0.000
DSPN (%)	41.9	26.1	27.4	0.000	40.7	27.5	27.2	0.000	37.9	30.1	26.7	0.000
Motor NCV (m/s)												
Median	52.8 ± 5.4	54.4 ± 4.5	54.3 ± 3.8	0.000	53.1 ± 5.6	54.1 ± 4.5	54.4 ± 3.7	0.000	53.2 ± 5.1	54.0 ± 4.9	54.3 ± 3.9	0.000
Ulnar	54.9 ± 5.8	55.9 ± 5.5	55.7 ± 4.4	0.015	54.9 ± 5.9	55.8 ± 5.5	55.7 ± 4.3	0.004	55.3 ± 5.3	55.4 ± 5.8	55.8 ± 4.6	0.097
Peroneal	41.7 ± 4.5	42.8 ± 4.2	42.2 ± 4.3	0.058	42.0 ± 4.5	42.4 ± 4.4	42.3 ± 4.2	0.204	42.0 ± 4.5	42.6 ± 4.3	42.2 ± 4.2	0.428
Tibial	41.6 ± 4.5	42.5 ± 4.3	42.6 ± 3.7	0.000	41.8 ± 4.6	42.2 ± 4.2	42.8 ± 3.8	0.000	41.6 ± 4.6	42.7 ± 4.0	42.6 ± 4.5	0.000
Sensory NCV (m/s)												
Median	49.3 ± 7.1	51.4 ± 6.8	50.6 ± 6.5	0.004	50.0 ± 7.1	50.8 ± 7.1	50.6 ± 6.4	0.222	49.7 ± 7.0	50.7 ± 6.8	51.1 ± 6.7	0.000
Ulnar	52.8 ± 4.7	53.6 ± 5.2	53.3 ± 4.6	0.140	53.0 ± 5.1	53.6 ± 4.9	53.2 ± 4.5	0.428	53.1 ± 4.7	53.2 ± 5.1	53.4 ± 4.7	0.369
Sup peroneal	44.5 ± 3.9	44.8 ± 4.9	44.5 ± 4.3	0.960	44.8 ± 4.2	44.5 ± 4.8	44.5 ± 4.3	0.407	44.9 ± 4.3	44.2 ± 4.5	44.6 ± 4.4	0.395
Sural	44.1 ± 4.7	45.0 ± 4.9	44.9 ± 4.0	0.003	44.2 ± 5.2	44.8 ± 4.3	45.0 ± 4.1	0.004	44.2 ± 4.7	44.9 ± 4.7	45.0 ± 4.2	0.004

Data are means ± SD or %.
Sup: superficial.

Table 3
Odds ratios (ORs) for models of distal symmetrical polyneuropathy by tertiles (T1–T3) of serum bilirubin levels.

	Total bilirubin (μmol/L)			Unconjugated bilirubin (μmol/L)			Conjugated bilirubin (μmol/L)		
	OR	95% CI	*P*	OR	95% CI	*P*	OR	95% CI	*P*
Model 1									
T1	1			1			1		
T2	0.500	0.390–0.641	0.000	0.566	0.442–0.724	0.000	0.675	0.528–0.864	0.002
T3	0.515	0.401–0.662	0.000	0.553	0.431–0.709	0.000	0.554	0.429–0.716	0.000
Model 2									
T1	1			1			1		
T2	0.651	0.469–0.904	0.010	0.605	0.435–0.840	0.003	0.885	0.637–1.229	0.466
T3	0.805	0.582–1.113	0.189	0.711	0.515–0.982	0.038	0.850	0.587–1.232	0.392
Model 3									
T1	1			1			1		
T2	0.685	0.484–0.968	0.032	0.648	0.457–0.919	0.015	0.989	0.699–1.401	0.951
T3	0.790	0.539–1.075	0.121	0.696	0.494–0.981	0.038	0.835	0.566–1.234	0.366

Model 1: adjusted for age and gender; model 2: adjusted for variables as in model 1 and for diabetic duration, body mass index, HbA1c, total cholesterol, and alanine and aspartate aminotransferases; model 3: adjusted for variables as in model 2 and diabetic nephropathy, diabetic retinopathy and cardiovascular autonomic neuropathy.
CI: confidence interval.

characteristics are shown in Table S4 (see supplementary data associated with this article online). Patients in the lowest tertile of unconjugated bilirubin (< 6.0 μmol/L) had significantly decreased sensory NCVs (superficial peroneal nerve: −2.1 ± 1.1, 0.1 ± 1.1, 0.5 ± 0.8 m/s; $P < 0.001$ for trend; sural nerve: −4.1 ± 3.3, −0.2 ± 5.0, 0.4 ± 5.1 m/s; $P = 0.024$ for trend) as well as motor NCVs (ulnar nerve: −1.8 ± 2.9, −1.9 ± 2.2, 1.6 ± 3.5 m/s; $P < 0.001$ for trend) compared with those in the highest tertile (unconjugated bilirubin ≥ 6.0 μmol/L but ≤ 8.0 μmol/L; unconjugated bilirubin > 8.0 μmol/L) after a 1-year follow-up. Similar changes were also observed in tertiles of total and conjugated bilirubin levels (Table 4).

After adjusting for age, gender, duration of diabetes, BMI, HbA1c, TC, AST and ALT, the changes in both motor (ulnar nerve: β = 0.352, $P = 0.003$ for trend) and sensory (sural nerve: β = 0.406, $P = 0.004$ for trend) NCVs remained significant for tertiles of unconjugated bilirubin. NCV changes for tertiles of total bilirubin (peroneal nerve, motor branch: β = 0.327, $P = 0.014$ for trend; superficial peroneal nerve, sensory branch: β = 0.240, $P = 0.016$ for trend) and conjugated bilirubin (peroneal nerve, motor branch: β = 0.590, $P = 0.002$ for trend; ulnar nerve, sensory branch: β = 0.614, $P = 0.002$ for trend; superficial peroneal nerve, sensory branch: β = 0.589, $P < 0.001$ for trend) also remained significant after multivariate adjustment.

Discussion

The present study demonstrates that lower levels of serum-unconjugated bilirubin are a risk factor for the presence of DSPN in a Chinese population with T2D. Furthermore, low basal levels of bilirubin were accompanied by decreased NCVs at a 1-year follow-up, indicating that lower basal levels of bilirubin are also associated with progression of DSPN.

Bilirubin is the end product of haem catabolism, and has antioxidant and anti-inflammatory effects [13]. Over the recent decades, numerous studies have focused on the association between serum bilirubin and diabetic microvascular complications, and several cross-sectional studies indicated that higher total bilirubin levels had protective effects on nephropathy [10], retinopathy [11] and CAN [21,22] in patients with T2D or impaired glucose metabolism. In addition, one prospective study indicated that low serum levels of total bilirubin were a predictor of progression from microalbuminuria to macroalbuminuria at a 1-year follow-up [23]. A recent meta-analysis involving 132,240 subjects from 27 studies found a negative association between total bilirubin concentration and risk of diabetic microvascular complications [24]. Similar to the results reported by Mashitani et al. [23], the present study found proportionally more males than females in the highest category of serum bilirubin levels. Consistent with previous reports, our study has also revealed that lower levels of total and unconjugated bilirubin are associated with higher rates of DR, DN and CAN (Table S2; see supplementary data associated with this article online). Furthermore, concentrations of total and unconjugated bilirubin decreased when the total number of microvascular complications increased (Fig. S1; see supplementary data associated with this article online), thereby indicating that total and unconjugated bilirubin may be additional indicators of microvascular complications in T2D.

Table 4
Changes in nerve conduction velocity (NCV) parameters by tertiles (T1–T3) of serum bilirubin levels at a 1-year follow-up.

	Total bilirubin (μmol/L)				Unconjugated bilirubin (μmol/L)				Conjugated bilirubin (μmol/L)			
	T1 < 8.9	T2 8.9–11.8	T3 > 11.8	*P* for trend	T1 < 6.0	T2 6.0–8.0	T3 > 8.0	*P* for trend	T1 < 2.8	T2 2.8–3.7	T3 > 3.7	*P* for trend
Patients (*n*)	10	24	34	–	12	20	36	–	18	24	26	–
Changes in motor NCV (m/s)												
Median	0.5 ± 3.8	1.2 ± 2.9	0.9 ± 3.4	0.842	0.9 ± 3.7	0.6 ± 2.9	1.1 ± 3.5	0.712	1.7 ± 2.6	0.4 ± 4.3	0.9 ± 2.4	0.490
Ulnar	−2.7 ± 3.4	−0.1 ± 2.8	0.8 ± 3.7	0.009	−1.8 ± 2.9	−1.9 ± 2.2	1.6 ± 3.5	0.000	−0.8 ± 3.9	−0.5 ± 2.2	1.1 ± 3.9	0.054
Peroneal	−1.8 ± 2.5	−0.7 ± 3.8	0.5 ± 2.3	0.039	−2.2 ± 2.4	0.5 ± 2.7	−0.2 ± 3.1	0.350	−1.7 ± 3.7	−0.1 ± 2.9	0.8 ± 2.2	0.015
Tibial	−2.1 ± 1.4	1.7 ± 4.2	0.2 ± 3.4	0.598	−2.8 ± 1.3	2.1 ± 3.2	0.2 ± 3.8	0.444	1.5 ± 4.7	−0.7 ± 3.2	0.8 ± 3.1	0.700
Changes in sensory NCV (m/s)												
Median	−0.9 ± 2.1	0.7 ± 2.2	−0.1 ± 2.2	0.790	−0.1 ± 2.2	−0.8 ± 2.1	0.5 ± 2.2	0.223	0.8 ± 2.0	−0.4 ± 2.2	−0.1 ± 2.4	0.278
Ulnar	−2.6 ± 1.8	−0.1 ± 3.6	0.3 ± 4.2	0.066	−1.4 ± 3.0	−1.2 ± 3.7	0.7 ± 3.9	0.047	−1.6 ± 3.5	−0.3 ± 3.2	0.8 ± 4.2	0.035
Sup peroneal	−3.0 ± 1.2	−0.3 ± 0.5	0.5 ± 0.8	0.000	−2.1 ± 1.1	0.1 ± 1.1	0.5 ± 0.8	0.000	−0.7 ± 1.4	−0.1 ± 0.1	0.4 ± 1.0	0.012
Sural	−4.6 ± 4.5	−0.3 ± 4.6	0.1 ± 5.3	0.101	−4.1 ± 3.3	−0.2 ± 5.0	0.4 ± 5.1	0.024	−0.7 ± 5.3	−0.5 ± 5.0	−0.4 ± 5.1	0.856

Data are means ± SD.
Sup: superficial.

One Korean study examined the association between total bilirubin and DSPN [14]. They enrolled 1207 T2D patients, and DSPN was defined according to the Michigan Neuropathy Screening Instrument rather than NCVs. They found that total bilirubin levels were inversely related to the presence of DSPN after multivariate adjustment, whereas there were no data on the effects of unconjugated bilirubin. In our present study, all components of serum bilirubin (total, unconjugated and conjugated bilirubin) were studied. Unconjugated bilirubin has been recognized as the most active antioxidant in bilirubin. In *db/db* mouse aortas, unconjugated bilirubin treatment improved endothelium-dependent relaxation [25]. Atazanavir, a drug that raises unconjugated bilirubin levels, enhances plasma antioxidant capacity and improves endothelium-dependent relaxation in patients with diabetes [26]. Thus, unconjugated bilirubin could be more influential and be the potential drug for treating oxidative stress in diabetes.

On the other hand, the effect of conjugated bilirubin on peripheral nerves remains to be clarified. In our study, levels of both total and unconjugated bilirubin were significantly decreased in patients with DSPN and showed a positive correlation with NCV. However, unconjugated bilirubin levels showed a negative association with the presence of DSPN after multivariate adjustment. Also, nerve conduction studies rather than questionnaires and physical examinations alone were used to diagnose DSPN, and are the gold standard for making the diagnosis. These objective measures could also be used as indicators in correlation analyses. Notably, NCVs increased in all tertiles of bilirubin levels. In addition, progression of DSPN, defined as deterioration of NCV, was analyzed at the 1-year follow-up. Patients in the lowest tertile of bilirubin (unconjugated, total and conjugated bilirubin) at baseline were found to have significantly decreased NCVs in both motor and sensory nerves on follow-up. Indeed, the reduction of 4.1 m/s in sensory sural nerve NCVs in the lowest tertile of unconjugated bilirubin represented a further deterioration of 56% at the 1-year follow-up. Such changes in NCV remained significant even after multivariate analysis, indicating that lower levels of bilirubin are a risk factor for progression of DSPN. The 1-m/s change in all tertiles of bilirubin represents the relative deterioration of NCV.

Nevertheless, the mechanism underlying the association between serum bilirubin, especially unconjugated bilirubin, and DSPN is still unclear, although there are a few plausible explanations. First, bilirubin can decrease oxidative stress in cells. Patients with Gilbert's syndrome, in which serum unconjugated bilirubin levels are persistently mildly elevated, appear to be protected against several diseases, such as diabetes and cardiovascular disease, involving increased oxidative stress [27,28]. In nerve tissue, bilirubin inhibits the protein kinase C and NADPH oxidase pathways; lowers the production of reactive oxygen species (ROS) and uncoupled endothelial nitric oxide synthase (eNOS) resulting in suppression of lipid peroxidation; and alleviates endothelial dysfunction [29]. Second, bilirubin protects nerve tissue against inflammatory injury: 20 μmol of bilirubin inhibited T-cell differentiation and the release of interleukin (IL)-2, tumour necrosis factor (TNF)-α and IL-10, and major histocompatibility complex (MHC) class-II expression in macrophages [30,31]. In one clinical study, serum levels of bilirubin were inversely associated with C-reactive protein, a marker of inflammation [32]. Third, the enzymes involved in the bilirubin metabolism pathway, such as haem oxygenase (HO)-1, may also contribute to the neuroprotective effects of bilirubin. In animal models of diabetes, induction of HO-1 led to improvement in pain perception and peripheral nerve function [33], whereas the overexpression of HO-1 in neurons resulted in improved neuronal viability and reduced neural apoptosis [34]. In addition, increased HO-1 activity boosts expression of neurotrophic factors of the phosphoinositide 3-kinase (PI3K)–protein kinase B (Akt) pathway [35].

However, the present study also has several limitations. First, the enrolled subjects were all inpatients, rendering selection bias inevitable, although the relatively large sample size and use of a standardized method at a single centre raise the reliability of our findings. In addition, the use of both nerve conduction studies and clinical examinations made the diagnosis more precise and convincing. Second, while no prospective study has ever been conducted to investigate the role of serum bilirubin in DSPN progression, a retrospective analysis of patients who underwent two tests of NCV with a 1-year interval was included in our present study, making it possible for the first time to examine the effect of serum bilirubin on the development of DSPN.

Conclusion

Low levels of serum bilirubin, especially unconjugated bilirubin, are significantly associated with the presence and progression of DSPN in Chinese patients with T2D, thereby suggesting that serum unconjugated bilirubin may be used as an additional indicator of DSPN risk and progression. Also, further prospective studies with large sample sizes are now needed to confirm the effect of serum bilirubin on DSPN progression.

Authors' contributions

JWJ, WMW, YB and DLZ designed and conducted the trial. JWJ, WMW, TWG, CHC and WC contributed to data collection. JWJ, TWG, YB and JS analyzed the data. JWJ, WMW and TWG conducted the trial, interpreted the data and wrote the manuscript. YB and DLZ discussed the data and revised the manuscript. All authors edited the manuscript and approved the final version.

Disclosure of interest

The authors declare that they have no competing interest.

Acknowledgments

This work was supported by the National Natural Science Foundation of China Grant Awards (81570737, 81370947, 81570736, 81770819, 81500612, 81400832, 81600637, 81600632, and 81703294), the National Key Research and Development Program of Chin a(2016YFC1304804 and 2017YFC1309605), the Jiangsu Provincial Key Medical Discipline (ZDXKB2016012), the Key Project of Nanjing Clinical Medical Science (BE2015604 and BE2016606), the Jiangsu Provincial Medical Talent (ZDRCA2016062),and the Nanjing Science and Technology Development Project (201605019).

Appendix A. Supplementary data

Supplementary data (Fig. S1, Tables S1–S4) associated with this article can be found, in the online version, at https://doi.org/10.1016/j.diabet.2018.02.007.

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