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ORIGINAL ARTICLE

Association of serum CTRP9 levels with cardiac autonomic neuropathy in patients with type 2 diabetes mellitus

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ABSTRACT

Aims: Cardiac autonomic neuropathy (CAN) is a serious complication of diabetes and is associated with adipokines. The C1q tumor necrosis factor-related protein 9 (CTRP9) is a newly discovered adipokine. This study aimed to evaluate the association of serum CTRP9 levels with the prevalence and severity of CAN in patients with type 2 diabetes mellitus.

Materials and Methods: We enrolled 262 patients (aged ≥18 years) with type 2 diabetes mellitus into this study. Standard cardiovascular autonomic reflex tests (CARTs) were used to assess CAN and patients were divided into three groups accordingly: a non-CAN group, an early CAN group, and a definite CAN group. Serum CTRP9 levels were measured by enzyme-linked immunosorbent assay, and the tertiles were calculated.

Results: Serum CTRP9 levels decreased significantly in the early CAN and definite CAN groups ($P < 0.05$). The percentage of definite CAN was the highest at the minimum tertile of serum CTRP9 level (T1; $P < 0.05$). Additionally, serum CTRP9 levels were negatively correlated with age, DM duration, hemoglobin A1c (HbA1c), and fasting plasma glucose (FPG) while positively correlated with high-density lipoprotein cholesterol (HDL; $P < 0.05$). The level of CTRP9 was also significantly associated with the four indexes of CARTs ($P < 0.05$). Furthermore, CTRP9 was a protective factor for definite CAN ($P < 0.05$). Compared with the maximum tertile (T3) of the serum CTRP9 levels, a decreased level of serum CTRP9 in T1 significantly increased the prevalence ratio of definite CAN in patients with type 2 diabetes mellitus ($P < 0.05$).

Conclusion: Serum CTRP9 levels were independently associated with definite CAN. CTRP9 represents a reliable biomarker for exploring CAN in patients with type 2 diabetes mellitus.

INTRODUCTION

Cardiac autonomic neuropathy (CAN) is a serious complication of diabetic neuropathy; although it is by far one of the most studied complications, it still has a high prevalence rate[1]. The prevalence of CAN in type 2 diabetes mellitus increases with the duration of diabetes mellitus and may be as high as 60% in both western and Chinese subjects[1,2]. Because it causes dysfunction of the autonomic nerves innervating the heart and blood vessels in young and adult patients[3], CAN is an independent risk factor for cardiovascular mortality, arrhythmia, silent ischemia, any major cardiovascular event, and myocardial dysfunction[4]. Although many risk factors such as old age, long duration of diabetes mellitus, poor glycemic control, hypertension, and smoking have been reported to be closely related with the development of CAN, the mechanism for CAN is still not fully understood[1,5].

Adipokines are bioactive substances secreted by adipose tissue, which are important mediators of various metabolic processes such as glucose uptake, insulin signaling, and fatty acid oxidation[6]. The main adipokines such as adiponectin, omentin,

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leptin, and resistin are highly associated with type 2 diabetes mellitus and its cardiovascular and microvascular complications[6,7]. Studies found that the serum levels of adiponectin and omentin were associated with CAN in patients with type 2 diabetes mellitus[7,8].

The C1q tumor necrosis factor-related protein (CTRP) family is a newly discovered family of adipokines and has 15 members (CTRP1–CTRP15)[9]. Among them, CTRP9 has the highest degree of amino acid identity to adiponectin, which plays an important role in regulating the blood glucose levels in diabetes mellitus[10]. Because CTRP9 has a potent vasorelaxation effect, anti-inflammatory action, and inhibitory effect on the proliferation of vascular smooth muscle cells, it has become a potential biological marker and pharmacological target in patients with type 2 diabetes mellitus and coronary artery disease (CAD)[11,12].

To our knowledge, no study has evaluated the association between serum CTRP9 levels and CAN in patients with type 2 diabetes mellitus. Therefore, this study aimed to investigate the association of serum CTRP9 levels with the prevalence and severity of CAN in this patient population.

MATERIALS AND METHODS

Study subjects

A total of 262 patients (aged ≥18 years) with type 2 diabetes mellitus were recruited randomly from the Department of Endocrinology, Henan Provincial People's Hospital, from August 2018 to October 2020. Type 2 diabetes mellitus was diagnosed according the criteria of the 1999 World Health Organization[13]. The exclusion criteria were: (1) type 1 diabetes mellitus; (2) arrhythmia, coronary heart disease, and heart failure; (3) taking beta-blockers or glucocorticoid for the past 2 weeks; (4) coexisting major psychiatric disorders; (5) acute complications of diabetes mellitus such as diabetic ketoacidosis and diabetic hyperosmolar coma; (6) severe infection, severe kidney dysfunction, or liver dysfunction; (7) abnormal thyroid function; and (8) pregnancy.

General clinical and biochemical measurements

General clinical information including age, sex, duration of diabetes mellitus, smoking history (current smoking or not), drinking history (current drinking or not), hypertension, dyslipidemia, coronary artery disease, stroke, and medical history for both diabetes mellitus and hypertension were recorded. The patients' blood pressure (BP) was measured three times, and the mean value was recorded. Moreover, waist circumference (WC), height, and weight were assessed, and the body mass index (BMI) was calculated by dividing the weight (kg) by the square of the height (m). After all subjects had fasted for at least 8 h, venous blood samples were collected.

Serum CTRP9 levels were assayed by enzyme-linked immunosorbent assay kits (USCN Life Science, Wuhan, China) according to the manufacturer's instructions. The levels of fasting plasma glucose (FPG) were evaluated by the glucose oxidase method using a biochemical analyzer (ADVIA 2400; Siemens, Berlin, Germany). The levels of hemoglobin A1c (HbA1c) were measured by high-performance liquid chromatography with a VARIANT II Hemoglobin A1c analyzer (Bio-Rad Laboratories, Hercules, CA, USA). The levels of serum fasting C-peptide were determined using a chemiluminescence immunoassay analyzer (Bayer ADVIA Centaur; Bayer, Leverkusen, Germany). The levels of urinary albumin/creatinine ratio (ACR) were measured by turbidimetric inhibition immunoassay and Behre-Benedict's test using a biochemical analyzer (DCA Vantage; Siemens). The levels of total cholesterol (TC), triglyceride (TG), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), and creatinine (Cr) were measured by standard enzymatic methods using a biochemical analyzer (ADVIA 2400; Siemens). According to the Chronic Kidney Disease Epidemiology Collaboration equation, the estimated glomerular filtration rate (eGFR) was calculated based on the creatinine level[14].

The diagnosis of diabetic kidney disease (DKD) was mainly based on any of the following standards: (1) the patient has a history of confirmed diagnosis; (2) ACR > 30 mg/g and(or) eGFR < 60 mL/min/1.73 m^2[15].

Diabetic retinopathy (DR) was assessed by a specialist examination, including fundus photography, fluorescein fundus angiography, or optical coherence tomography (OCT). The subjects were considered to have diabetic retinopathy if they were in the non-proliferative or the proliferative retinopathy stage or had macular edema[16].

Diabetic peripheral neuropathy (DPN) was diagnosed by the following criteria: (1) had typical symptoms and signs of diabetic peripheral neuropathy; (2) abnormal Toronto Clinical Scoring System (TCSS) scores; (3) and/or abnormal nerve conduction test (NCT). For a detailed description, see our previously published literature[17].

Measurement of cardiac autonomic function

Cardiac autonomic neuropathy was diagnosed using standard cardiac autonomic reflex tests (CARTs)[18]. CARTs were performed following a standard protocol and scored a total of four points. First, the heart rate (HR) response to deep breathing was measured by six deep breathing cycles in 1 min, the maximum and minimum R-R intervals were recorded, and the heart rate was then calculated. Then, the difference between the maximum and minimum heart rate was determined. A difference value ≥15 indicated normal, 11–14 signified borderline, and ≤10 indicated abnormal results. Second, the Valsalva R-R ratio was determined by the maximum and minimum R-R interval after the Valsalva maneuver. The following criteria were used: differences in value ≥1.21, 1.11–1.20, ≤1.10 indicated normal, borderline, and abnormal results. Moreover, the heart rate while standing (30/15) was evaluated by R-R interval in the 30th heart beat and 15th heart beat after patients shifted from lying to standing and then converted to heart rate. Differences in value of ≥1.04, 1.01–1.03, and ≤1.0 signified normal, borderline, and abnormal responses to standing, respectively. Finally, the

postural blood pressure change was estimated by the difference in the change in systolic blood pressure between lying down and standing up after 2 min. A difference of ≤10 mmHg indicates normal, 11–29 mmHg indicates borderline, and ≥30 indicates abnormal results. According to the scoring system, for the four tests, a score of 0 represents normal, a score of 1 represents borderline, and a score of 2 represents abnormal function. The total score was the summation of the score of each test and was then considered as the reference for the severity of cardiac autonomic neuropathy. A total score of 0–1, 2–3, and 4–8 represented as non-CAN, early CAN, and definite CAN, respectively.

Statistical analysis

Statistical analyses of data were performed using SPSS version 21.0 (SPSS Inc., Chicago, IL, USA) and SAS version 9.4 (SAS Institute Inc., Cary, NC, USA). Continuous variables with a normal distribution are expressed as the mean ± standard deviation. Variables with a non-normal distribution are presented as the median (25th–75th percentile). Categorical variables are presented as frequencies and proportions. The serum CTRP9 level was categorized based on tertiles, which were calculated at the 33rd and 67th percentiles as follows: tertile 1 (T1), CTRP9 ≤ 2.98 ng/mL; tertile 2 (T2), CTRP9 2.98–3.65 ng/mL; and tertile 3 (T3), CTRP9 > 3.65 ng/mL. One-way analysis of variance was used to evaluate the differences of continuous variables with a normal distribution between groups. The Kruskal–Wallis *H* test was used to compare continuous variables with a non-normal distribution between groups. The Chi-square test was used to assess the rates between groups. Pearson correlation analysis was used to assess the relationship between CTRP9 and other parameters. The above statistical analyses were conducted using SPSS. Moreover, multinomial logistic regression analysis was performed to evaluate the independence of association of CTRP9 with different stages of CAN. Binary logistic regression analysis was used to assess: (1) the independence of association between CTRP9 and the presence of any CAN; (2) the association between tertiles of serum CTRP9 levels and definite CAN. These logistic analyses were performed by SAS, and the prevalence ratio (PR) was calculated. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Clinical characteristics of participants according the severity of CAN

In total, 262 patients with type 2 diabetes mellitus were recruited, consisting of 83 women and 179 men. The average patient age was 55 (48–61) years, and the mean duration of diabetes mellitus was 10 (4–15) years. According to the CARTs score, there were 91 patients in the non-CAN group, 86 patients (32.82%) in the early CAN group, and 85 patients (32.44%) in the definite CAN group. The anthropometric characteristics, clinical parameters, and serum CTRP9 levels are presented in Table 1.

Compared with patients without cardiac autonomic neuropathy, patients with early CAN and definite CAN were older, had a longer duration of diabetes mellitus, with higher levels of fasting plasma glucose and HbA1c ($P < 0.05$). Compared with the non-CAN group, the levels of systolic blood pressure, diastolic blood pressure increased in early CAN while decreased in the definite CAN group ($P < 0.05$). The prevalence of diabetic retinopathy, diabetic peripheral neuropathy, CAD, and stroke enhanced the aggravation of CAN ($P < 0.05$). Although the level of eGFR also differed among the three groups ($P < 0.05$), no significant differences were found in ACR and the prevalence of diabetic kidney disease among the three groups. The percentage of oral hypoglycaemic drugs use decreased in the definite CAN group while the proportion of using both oral hypoglycemic drugs and insulin was the highest in the definite CAN group ($P < 0.05$). Notably, the serum CTRP9 levels showed significant differences among the three groups, which decreased in the early CAN and definite CAN groups compared with the non-CAN group ($P < 0.05$).

Comparisons of clinical variables according to the tertiles of serum CTRP9 levels

The participants were divided into three groups according to the serum CTRP9 levels. The CARTs scores and the anthropometric and clinical parameters according to the tertile of serum CTRP9 levels are shown in Table 2. Compared with the T3 group, the CARTs scores increased significantly in the T1 and T2 groups [5.0 (2.7–6.0) vs 3.0 (1.0–4.0) vs 1.0 (0.0–2.0), $P < 0.05$]. Patients with higher tertiles of serum CTRP9 levels were younger, had a lower duration of diabetes mellitus, diastolic blood pressure, fasting plasma glucose, HbA1c, triglyceride and percentage of diabetic peripheral neuropathy, CAN ($P < 0.05$).

Prevalence of CAN in different tertiles of CTRP9

The prevalence of CAN in different tertiles of serum CTRP9 levels were evaluated and are shown in Figure 1. At T1, the percentage of non-CAN was lowest, while that of the definite CAN was highest compared with the other groups. In contrast, at T3, the prevalence of non-CAN was highest and the definite CAN was lowest (both $P < 0.05$). However, the percentage of early CAN showed no obvious differences among the tertile groups.

Correlation of CTRP9 with clinical parameters and complications of DM

The correlation between serum CTRP9 levels and clinical variables was analyzed by Pearson correlation. The serum CTRP9 level was negatively correlated with age ($r = –0.225$, $P < 0.001$), duration of diabetes mellitus ($r = –0.165$, $P = 0.007$), HbA1c ($r = –0.220$, $P < 0.001$) and fasting plasma glucose ($r = –0.410$, $P < 0.001$). While serum CTRP9 levels

Table 1 | Characteristics of participants according to the presence and severity of cardiac autonomic neuropathy

Characteristic	Type 2 diabetes mellitus non-CAN (n = 91)	Type 2 diabetes mellitus early CAN (n = 86)	Type 2 diabetes mellitus definite CAN (n = 85)	F value	P value
Age (years)	53 (46–57)	55 (49–62)	57(49–63)	11.35	0.003
Female/male (n)	34/57	19/67	30/55	5.523	0.063
Duration of DM (years)	7 (2–12)	10 (4–15)	10 (6–18)	8.529	0.014
WC (cm)	86.75 ± 8.53	88.67 ± 9.41	85.60 ± 9.16	3.125	0.086
BMI (kg/m^2)	24.88 ± 3.39	24.69 ± 2.37	25.26 ± 3.43	0.753	0.472
Smoking [n (%)]	34 (37.4)	43 (50.0)	37 (43.5)	2.873	0.238
Drinking [n (%)]	38 (41.8)	48 (55.8)	45 (52.9)	3.929	0.140
Hypertension [n (%)]	38 (41.8)	44 (51.2)	42 (49.4)	1.788	0.409
Systolic BP (mmHg)	131 (120–142)	132 (123–145)	125 (120–134)	7.370	0.025
Diastolic BP (mmHg)	80 (72–89)	82 (76–90)	79 (72–85)	6.570	0.037
FPG (mmol/L)	7.0 (6.7–7.2)	7.8 (7.3–8.6)	9.0 (8.2–9.8)	86.21	<0.001
HbA1c (%)	7.2(6.7–7.9)	7.2 (6.7–7.9)	7.6 (7.1–8.2)	7.263	0.026
Fasting C-peptide (ng/mL)	1.34 ± 0.17	1.48 ± 0.35	1.26 ± 0.18	0.289	0.751
Dyslipidemia [n (%)]	38 (41.8)	34 (39.5)	35 (41.2)	0.435	0.804
TC (mmol/L)	4.43 ± 1.06	4.33 ± 1.02	4.49 ± 1.05	0.506	0.603
TG (mmol/L)	1.67 (1.17–2.46)	1.76 (1.22–2.64)	1.58 (1.11–2.28)	2.292	0.318
HDL-C (mmol/L)	1.05 (0.91–1.33)	1.01 (0.90–1.17)	1.11 (0.93–1.22)	2.200	0.333
LDL-C (mmol/L)	2.37 ± 0.86	2.32 ± 0.73	2.45 ± 0.76	0.534	0.587
ACR (mg/g)	10.7 (6.2–35.7)	15.3 (7.9–34.4)	13.2 (6.8–59.7)	3.965	0.138
eGFR (mL/min/1.73 m^2)	103.48 ± 29.76	101.54 ± 31.22	98.61 ± 21.74	3.985	0.037
CTRP9 (ng/mL)	3.69 ± 0.86	3.37 ± 0.65	2.85 ± 0.72	27.52	<0.001
DKD [n (%)]	20 (22.0)	24 (7.9)	30 (35.3)	3.852	0.146
DR [n (%)]	12 (13.2)	27 (31.4)	34 (40.0)	16.52	<0.001
DPN [n (%)]	10 (11.0)	30 (34.9)	43 (50.6)	32.44	<0.001
CAD [n (%)]	7 (7.7)	15 (17.4)	20 (23.5)	8.378	0.015
Stroke [n (%)]	5 (5.5)	14 (16.3)	18 (21.2)	9.403	0.009
Use of antidiabetic agents [n (%)]	85 (93.4)	83 (96.5)	85 (100.0)	5.761	0.056
Oral hypoglycemic drugs	39 (45.9)	39 (47.0)	24 (28.2)	7.780	0.020
Insulin	20 (23.5)	13 (15.7)	16 (18.8)	1.688	0.430
Both	26 (30.6)	31 (37.3)	45 (52.9)	9.277	0.010
Use of antihypertensive drug [n (%)]	35 (92.1)	40 (90.0)	38 (90.5)	0.706	0.703
ACEI/ARB	21 (60.0)	22 (55.0)	18 (47.4)	1.196	0.550
CCB	14 (40.0)	20 (50.0)	19 (50.0)	0.970	0.616
Beta-blocker-	8 (22.9)	7 (17.5)	12 (31.6)	2.154	0.341
Other	5 (14.3)	6 (15.0)	4 (10.5)	0.384	0.825

ACEI, angiotensin-converting enzyme inhibition; ACR, urinary albumin creatinine ratio; ARB, angiotensin receptor blockers; BMI, body mass index; BP, blood pressure; CAD, coronary artery disease; CAN, cardiac autonomic neuropathy; CCB, calcium-channel blocker; CTRP9, C1q tumor necrosis factor-related protein 9; DKD, diabetic kidney disease; DM, diabetes mellitus; DPN, diabetic peripheral neuropathy; DR, diabetic retinopathy; eGFR, estimated glomerular filtration rate; FPG, fasting plasma glucose; HbA1c, hemoglobin A1c; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; TC, total cholesterol; TG, triglyceride; WC, waist circumference.

Table 2 | Characteristics of participants according to CTRP9 tertiles

Characteristic	T1 (≤2.98) *n* = 86	T2 (2.98–3.65) *n* = 89	T3 (>3.65) *n* = 87	*F* value	*P* value
CTRP9 (ng/mL)	2.40 ± 0.43	3.33 ± 0.22	4.18 ± 0.50	231.95	<0.001
CART score	5.0 (2.7–6.0)	3.0 (1.0–4.0)	1.0(0.0–2.0)	26.64	<0.001
Age (years)	58.5 (52.0–64.0)	54.0 (48.0–59.5)	52.0 (48.0–57.0)	14.89	0.001
Female/male (*n*)	31/55	25/64	29/58	1.310	0.519
Duration of DM (years)	12 (7–18)	7 (2–12)	10 (4–15)	16.57	<0.001
WC (cm)	84.35 ± 8.76	87.64 ± 9.62	87.89 ± 9.35	22.33	<0.001
BMI (kg/m^2)	25.25 ± 3.30	24.61 ± 2.99	25.01 ± 3.02	0.950	0.388
Smoking [*n* (%)]	41 (47.7)	36 (40.4)	37 (42.5)	0.980	0.613
Drinking [*n* (%)]	45 (52.3)	49 (55.1)	37 (42.5)	3.034	0.219
Hypertension [*n* (%)]	44 (51.2)	44 (49.4)	36 (41.4)	1.901	0.386
Systolic BP (mmHg)	130 (120–140)	128 (120–139)	132 (122–145)	3.509	0.173
Diastolic BP (mmHg)	79 (72–82)	80 (75–86)	82 (74–91)	9.939	0.007
FPG (mmol/L)	8.8 (7.4–9.8)	7.6 (7.0–8.7)	7.3 (6.7–8.1)	33.49	<0.001
HbA1c (%)	7.7 (6.9–8.3)	7.3 (6.9–7.9)	7.1 (6.5–7.9)	13.97	<0.001
Fasting C-peptide (ng/mL)	1.28 ± 0.14	1.45 ± 0.30	1.25 ± 0.18	0.23	0.796
Dyslipidemia [*n* (%)]	42 (48.8)	38 (42.7)	37 (42.5)	0.906	0.636
TC (mmol/L)	4.54 ± 1.07	4.47 ± 1.10	4.26 ± 0.94	1.697	0.185
TG (mmol/L)	1.93 (1.34–2.84)	1.62 (1.23–1.62)	1.45 (1.10–2.19)	6.976	0.031
HDL-C (mmol/L)	1.05 (0.92–1.23)	1.01 (0.91–1.20)	1.08 (0.93–1.38)	1.968	0.374
LDL-C (mmol/L)	2.47 ± 0.82	2.39 ± 0.82	2.27 ± 0.76	1.542	0.216
ACR (mg/g)	14.6 (6.8–35.2)	10.9 (6.2–48.1)	12.2 (6.9–34.2)	0.512	0.774
eGFR (mL/min/1.73 m^2)	104.50 ± 28.39	96.60 ± 31.75	95.62 ± 28.67	2.332.	0.099
DKD [*n* (%)]	31 (36.1)	25 (28.1)	18 (20.7)	5.034	0.081
DR [*n* (%)]	29 (22.1)	24 (27.0)	20 (23.0)	2.532	0.282
DPN [*n* (%)]	34 (39.5)	30 (33.7)	19 (21.8)	6.513	0.039
CAD [*n* (%)]	20 (23.3)	14 (15.7)	8 (9.2)	6.361	0.042
Stroke [*n* (%)]	14 (16.3)	15 (16.9)	8 (9.2)	2.619	0.270
Use of antidiabetic agents [*n* (%)]	85 (98.8)	85 (95.5)	83 (95.4)	1.994	0.369
Oral hypoglycemic drugs	34 (40.0)	38 (44.7)	35 (42.2)	0.386	0.824
Insulin	12 (14.1)	18 (21.2)	17 (20.5)	1.696	0.428
Both	39 (45.9)	29 (34.1)	31 (37.4)	2.634	0.268
Use of antihypertensive drug [*n* (%)]	40 (90.9)	38 (86.4)	35 (97.2)	2.892	0.236
ACEI/ARB	24 (60.0)	24 (63.2)	13 (37.1)	5.866	0.053
CCB	18 (45.0)	20 (52.6)	15 (42.9)	0.789	0.674
Beta-blocker	10 (25.0)	8 (21.1)	9 (25.7)	0.259	0.878
Other	6 (15.0)	7 (18.4)	2 (5.7)	2.715	0.257

ACEI, angiotensin-converting enzyme inhibition; ACR, urinary albumin creatinine ratio; ARB, angiotensin receptor blockers; BMI, body mass index; BP, blood pressure; CAD, coronary artery disease; CART, cardiac autonomic reflex test; CCB, calcium-channel blocker; CTRP9, C1q tumor necrosis factor-related protein 9; DKD, diabetic kidney disease; DM, diabetes mellitus; DPN, diabetic peripheral neuropathy; DR, diabetic retinopathy; eGFR, estimated glomerular filtration rate; FPG, fasting plasma glucose; HbA1c, hemoglobin A1c; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; TC, total cholesterol; TG, triglyceride; WC, waist circumference.

were positively correlated with high density lipoprotein ($r = 0.122$, $P = 0.048$). In addition, the level of CTRP9 was associated with diabetic kidney disease ($r = –0.12$, $P = 0.052$), diabetic peripheral neuropathy ($r = –0.172$, $P = 0.005$) and CAD ($r = –0.209$, $P = 0.001$).

Correlation of CTRP9 with the four indexes of CARTs

Pearson correlation analysis was used to compare the correlation between CTRP9 and the four points of CARTs, as shown in Figure 2. The serum CTRP9 level was positively correlated with the heart rate response to deep breath ($r = 0.447$, $P < 0.001$), Valsalva ratio ($r = 0.423$, $P < 0.001$) and the heart rate response to standing (30/15) ($r = 0.395$, $P < 0.001$). The serum CTRP9 level was negatively correlated with a postural blood pressure change ($r = –0.360$, $P < 0.001$).

Associations between CTRP9 and different stages of CAN

Four models were constructed to assess the association between CTRP9 and different stages of CAN, as shown in Table 3. In the binary logistic regression analysis, although no adjustments

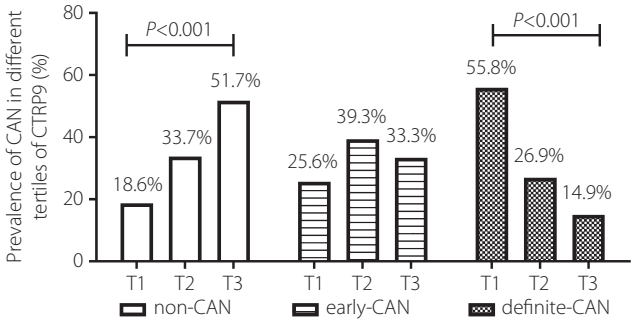

Figure 1 | Prevalence of cardiac autonomic neuropathy by severity in different tertiles of serum CTRP9 levels. CAN, cardiac autonomic neuropathy; CTRP9, C1q TNF-related protein 9.

were made for any other risk factors in model 1, the serum CTRP9 level was significantly negatively associated with the prevalence ratio (PR) of any CAN (including early CAN and definite CAN) in patients with type 2 diabetes mellitus. After adjusting for sex, age, waist circumference, systolic blood pressure, diastolic blood pressure, smoking, and drinking (model 2), the serum CTRP9 level still showed a strong influence on the incidence of any CAN. To explore the role of traditional risk factors associated with CAN, model 3 was adjusted for the duration of type 2 diabetes mellitus, fasting plasma glucose, and HbA1c, and model 4 was further adjusted for the risk factors in model 3, dyslipidemia, and estimated glomerular filtration rate. However, the serum CTRP9 level had no significant effect on the incidence of any CAN in models 3 and 4. Multinomial logistic regression analysis was then performed to evaluate the independence of association of serum CTRP9 level with different stages of CAN. In model 1, the serum CTRP9 level was significantly associated with a decreased prevalence rate of early CAN [PR 0.810, 95% confidence interval (CI) 0.706–0.960, $P = 0.010$] and definite CAN (PR 0.683, 95% CI 0.617–0.758, $P = 0.001$). In model 2, the serum CTRP9 level was associated with the prevalence ratio of definite CAN but not with early CAN. The serum CTRP9 level had no significant effect on the prevalence rate of early CAN in models 3 and 4. For definite CAN, model 3 and model 4 showed significant relationship between serum CTRP9 level and the incidence of definite CAN.

Furthermore, we analyzed the association between the decreased serum CTRP9 level and the prevalence rate of definite CAN. As shown in Table 4, compared with the serum CTRP9 level in T3, the serum CTRP9 level in T1 increased the prevalence rate of definite CAN (PR 3.735, 95% CI 2.279–6.758, $P < 0.001$) in model 1, but not that of T2. After adjustment for age, sex, waist circumference, blood pressure, smoking status, and drinking status, the CTRP9 level in T1 was still significantly associated with an increasing prevalence rate of definite CAN (model 2). Although the prevalence ratio in model 3 (model 2 + duration of diabetes mellitus, fasting plasma glucose and HbA1c level) showed no significant differences among the tertile groups, the prevalence ratio in model 4 (model 3 + TC,

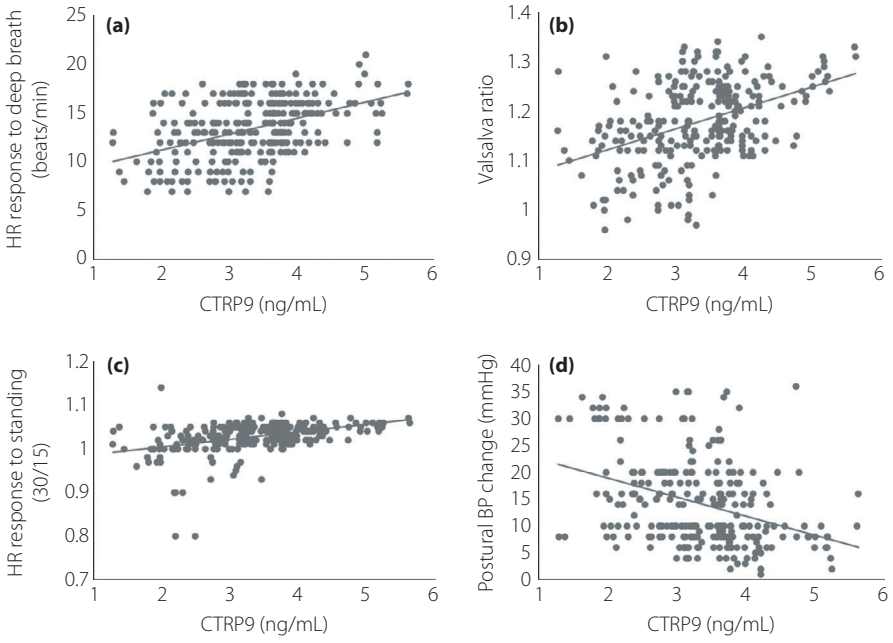

Figure 2 | Correlations of CTRP9 with the four indexes of CARTs. (a) Correlation of CTRP9 with heart rate response to deep breath, (b) correlation of CTRP9 with Valsalva ratio, (c) correlation of CTRP9 with heart rate response to standing, (d) correlation of CTRP9 with a postural blood pressure change. CARTs, cardiac autonomic reflex tests; CTRP9, C1q TNF related protein 9.

Table 3 | Associations between CTRP9 and different stages of CAN

Model	Independent variable	Early CAN		Definite CAN		Any CAN	
		PR (95% CI)	*P* value	PR (95% CI)	*P* value	PR (95% CI)	*P* value
Model 1	CTRP9	0.810 (0.706–0.960)	0.010	0.683 (0.617–0.758)	0.001	0.820 (0.774–0.878)	<0.001
Model 2	CTRP9	0.875 (0.745–1.038)	0.106	0.686 (0.677–0.727)	0.001	0.871 (0.865–0.888)	0.042
Model 3	CTRP9	0.977 (0.951–1.053)	0.844	0.743 (0.294–0.832)	0.018	0.914 (0.900–0.925)	0.426
Model 4	CTRP9	0.966 (0.935–0.996)	0.781	0.730 (0.714–0.949)	0.047	0.908 (0.894–0.920)	0.442

BP, blood pressure; CAN, cardiovascular autonomic neuropathy; CI, confidence interval; CTRP9, C1q tumor necrosis factor-related protein 9; DM, diabetes mellitus; eGFR, estimated glomerular filtration rate; FPG, fasting plasma glucose; HbA1c, hemoglobin A1c; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; PR, prevalence ratio; TC, total cholesterol; TG, triglyceride; WC, waist circumference. Model 1 was not adjusted; Model 2 was adjusted for sex, age, WC, systolic BP, diastolic BP, smoking and drinking; Model 3 was adjusted for the variables adjusted for in model 2 + duration of DM, FPG and HbA1c; Model 4 was adjusted for the variables adjusted for in model 3 + TC, TG, LDL-C, HDL-C and eGFR.

TG, LDL-C, HDL-C and eGFR) revealed that the decreasing serum CTRP9 level in T1 was still significantly associated with an increasing prevalence rate of definite CAN.

DISCUSSION

In this study, we found that serum CTRP9 levels reduced significantly in cardiac autonomic neuropathy patients with type 2 diabetes mellitus. In addition, the prevalence of definite CAN increased with the decrease in serum CTRP9 levels. The CTRP9 level also correlated with the four test results of CAN. Moreover, after adjusting for other risk factors, decreased serum CTRP9 levels were associated with an increased prevalence ratio of definite CAN.

Cardiac autonomic neuropathy is one of the most serious diabetic complications, as the AACORD trial supported the conclusive evidence that CAN was an independent predictor of mortality in patients with type 2 diabetes mellitus[19]. However, CAN has always been unrecognized by patients and clinicians because its early symptoms were insidious and some cases are even asymptomatic[20,21]. Common symptoms are always detected at the late stage of CAN, including weakness, palpitations, and syncope when the patients stand up suddenly[22].

Table 4 | Associations between tertiles of CTRP9 and definite CAN

Model	Independent variable	β	SE	Wald	*P*	PR (95% CI)
Model 1	CTRP9					
	T1	1.318	0.273	23.27	<0.001	3.735 (2.279–6.728)
	T2	0.590	0.310	3.64	0.057	1.805 (1.003–3.434)
	T3	1 (reference)				
Model 2	CRRP9					
	T1	1.198	0.271	19.55	<0.001	3.313 (2.360–6.173)
	T2	0.500	0.299	2.79	0.095	1.648 (0.859–3.232)
	T3	1 (reference)				
Model 3	CTRP9					
	T1	0.460	0.260	3.12	0.077	1.583 (1.189–1.875)
	T2	0.188	0.255	0.54	0.461	1.217 (1.84–1.938)
	T3	1 (reference)				
Model 4	CTRP9					
	T1	0.604	0.301	4.03	0.044	1.830 (1.634–2.079)
	T2	0.200	0.279	0.51	0.474	1.221 (1.037–1.361)
	T3	1 (reference)				

BP, blood pressure; CAN, cardiovascular autonomic neuropathy; CI, confidence interval; CTRP9, C1q tumor necrosis factor-related protein 9; DM, diabetes mellitus; eGFR, estimated glomerular filtration rate; FPG, fasting plasma glucose; HbA1c, hemoglobin A1c; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; PR, prevalence ratio; TC, total cholesterol; TG, triglyceride; WC, waist circumference. Model 1 was not adjusted; Model 2 was adjusted for sex, age, WC, systolic BP, diastolic BP, diastolic BP, smoking and drinking; Model 3 was adjusted for the variables adjusted for in model 2 + duration of DM, FPG and HbA1c; Model 4 was adjusted for the variables adjusted for in model 3 + TC, TG, LDL-C, HDL-C and eGFR.

Therefore, timely diagnosis and treatment are essential in lowering the mortality of CAN in patients with type 2 diabetes mellitus. Sympathetic and parasympathetic dysfunctions caused a decline in heart rate variability in the early stage of CAN, and orthostatic hypotension emerged in the advanced stage[22,23].

In this study, four non-invasive CARTs were used to diagnose and evaluate the severity of CAN. CARTs were recommended by the American Diabetes Association and Cardiovascular Autonomic Neuropathy Subcommittee of the Toronto Consensus Panel[24], which has been conducted in many previous studies[18,25]. In our study, based on the CARTs score, the prevalence of both early CAN and definite CAN exceeded 30%, and the total morbidity rate was more than 60%, which was similar to those reported in previous studies[8,24,26]. Because of its high morbidity rate, undetectable symptoms in early stage, and the poor prognosis in the advanced stage, it is necessary to explore clinical parameters and biological markers associated with CAN.

Apart from the traditional and validated risk factors such as age, duration of diabetes mellitus, lifestyle, and poor glucose control, emerging evidence shows that adipokines may critically affect the occurrence of CAN in patients with type 2 diabetes mellitus[8,14]. CTRP9 is a member of the CTRP family discovered in 2009, which is a secreted glycoprotein and has the highest similarity to adiponectin[5]. Increasing evidence shows that CTRP9 plays a cardioprotective role in cardiovascular disease given its multiple mechanisms such as in anti-inflammation[27], regulation of glucose and lipid metabolism[28], and anti-atherosclerosis function[29]. In addition, the relationship between CTRP9 and type 2 diabetes mellitus has also been studied. For patients with newly diagnosed type 2 diabetes mellitus, circulating CTRP9 levels are increased and correlated with insulin resistance[30].

Emerging evidence has recognized that CTRP9 may be a promising biomarker for microvascular and macrovascular complications of type 2 diabetes mellitus. Animal experiments demonstrated its role in attenuating diabetic retinopathy and nephropathy in db/db mice, which is in accordance with the vascular endothelial protective function of CTRP9[31,32]. Studies have also observed changes in serum CTRP9 levels in patients with type 2 diabetes mellitus and coronary artery disease, which is a macrovascular disease. One study has demonstrated that compared with those in healthy individuals, CTRP9 levels significantly decreased in individuals with type 2 diabetes mellitus, CAD, and type 2 diabetes mellitus combined with CAD[11]. Another study revealed that CTRP9 levels in patients with type 2 diabetes mellitus and CAD were increased compared with the control group[12]. These opposing results may be due to the differences in age, BMI, and insulin resistance between the two studies.

As an autonomic neuropathy complication of diabetes mellitus, CAN also enhanced the mortality risk of patients with type 2 diabetes mellitus[24]. However, the relationship between CTRP9 and CAN has not been studied. In this study, the serum levels of CTRP9 showed a significant decrease in patients with CAN, regardless of its stage. We further divided all the subjects into three subgroups by the tertile of serum CTRP9 levels and found that the ratio of non-CAN decreased in the middle and lower tertiles of serum CTRP9 levels compared with the upper tertile, while the ratio of definite CAN showed an increased tendency with a decrease in serum CTRP9 level. Furthermore, the level of CTRP9 was negatively correlated with the four detailed results of CARTs. These results revealed that the serum CTRP9 levels may be associated with the prevalence and severity of CAN in patients with type 2 diabetes mellitus.

One study has reported that the serum CTRP9 level was related to adhesion molecules (intercellular adhesion molecule-1 and vascular adhesion molecule-1) and inflammatory cytokines (interleukin-6 and tumor necrosis factor-α) in type 2 diabetes mellitus[12], and both of them were associated with the pathogenesis of CAN in patients with type 2 diabetes mellitus. Notably, the present study demonstrated that serum CTRP9 levels were negatively associated with fasting blood glucose, HbA1c, duration of diabetes mellitus, and age. The above parameters have previously been proved as risk factors of CAN[5]. Moreover, the levels of CTRP9 showed a significant positive correlation with HDL, a protective factor for cardiovascular disease. The above results showed CTRP9 may be a protective factor for CAN; therefore, logistic regression was conducted to test this. After adjustment for sex, age, waist circumference, smoking, drinking, duration of diabetes mellitus, fasting plasma glucose, HbA1c, blood pressure, LDL, HDL, triglyceride, total cholesterol, and estimated glomerular filtration rate, CTRP9 was found to be a protective factor for definite CAN, but not for early CAN and any CAN. Furthermore, a decreased serum CTRP9 level from T3 to T1 significantly increased the prevalence ratio of definite CAN in patients with type 2 diabetes mellitus. In this study, no independent associations were found between CTRP9 and early CAN after adjustment for the risk factors, but additional studies with larger cohorts are needed to further assess this effect.

In addition to the relatively small sample size, this study also had some other limitations. Firstly, although this cross-sectional study revealed the association between CTRP9 and CAN in patients with type 2 diabetes mellitus, longitudinal studies are needed to observe the temporal relationship fate of these variables. Secondly, we used only one criterion to evaluate CAN in type 2 diabetes mellitus. Thirdly, other CTRP family members and adiponectin were not studied. In addition, only the waist circumference but not the visceral fat was tested in this study.

In conclusion, this study suggests that serum CTRP9 was associated with CAN in patients with type 2 diabetes mellitus. Especially, its level was independently associated with definite CAN. CTRP9 could represent a reliable and promising biomarker for exploring CAN in patients with type 2 diabetes mellitus.

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DISCLOSURE

The authors declare that they have no conflict of interest.

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