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Insulin resistance is independently associated with cardiovascular autonomic neuropathy in type 2 diabetes

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ABSTRACT

Aims/Introduction: Diabetic cardiovascular autonomic neuropathy (DCAN) seriously threatens the prognosis and quality of life of patients with type 2 diabetes mellitus, associated with increased mortality. The present study aimed to investigate the relevant risk factors of DCAN.

Materials and Methods: The present study enrolled a total of 109 patients with type 2 diabetes mellitus. DCAN was defined as a score of at least 2 points in Ewing tests. The updated homeostasis model assessment of insulin resistance (HOMA2-IR) based on fasting C-peptide was calculated to reflect insulin resistance. Logistic regression analysis, interaction and stratified analyses were used to investigate the relationship between HOMA2-IR or other indicators and DCAN. Receiver operating characteristic analysis was carried out to estimate the discriminative value of the variables independently associated with DCAN and to determine the optimal cut-off point of these models to screen DCAN.

Results: The HOMA2-IR levels were significantly higher in patients with DCAN, and tended to be worsened with the progression of the DCAN. Logistic regression analysis showed an independent association between HOMA2-IR (odds ratio 39.30, 95% confidence interval 7.17–215.47) and DCAN. HOMA2-IR (area under the curve 0.878, 95% confidence interval 0.810–0.946; cut-off value 1.735) individually predicted DCAN significantly higher than the other independent risk factors individually used, whereas models combining HOMA2-IR and other risk factors did not significantly boost the diagnostic power.

Conclusions: Insulin resistance is independently associated with DCAN. HOMA2-IR presents to be a highly accurate and parsimonious indicator for DCAN screening. Patients with HOMA2-IR >1.735 are at a high risk of DCAN; thus, priority diagnostic tests should be carried out for these patients for timely integrated intervention.

INTRODUCTION

Diabetic cardiovascular autonomic neuropathy (DCAN) is one of the most common and serious chronic complications of diabetes mellitus, which damages autonomic fibers innervating the heart and blood vessels, leading to abnormal heart rate and hemodynamics[1–3]. DCAN has an insidious onset with hidden early clinical symptoms and is easily overlooked. Diverse clinical manifestations gradually become obvious and severe with the progress of DCAN, such as severe orthostatic hypotension, painless myocardial infarction and even sudden cardiac death, causing increased mortality[4–7]. Importantly, longitudinal studies have reported that the progress of DCAN could be effectively delayed by early intensive controlling of cardiovascular risk factors, such as hyperglycemia and hyperlipidemia[8]. A wide variety of research methods were applied in DCAN diagnosis, of which the Ewing tests are recommended as the gold standard for DCAN diagnosis[6] with superior reproducibility, sensitivity and specificity. However, considering the device, time, venue and technicians required for the Ewing tests, it is not practical to have all patients examined. Therefore, it is urgent to find an

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indicator for screening the high-risk population of DCAN, resulting in early identification and, consequently, early intervention.

Insulin acts as a neurotrophic factor, regulating the growth, survival and differentiation of neurons[9,10]. Insulin resistance is defined as a reduction of biological reactivity to insulin stimulation in target tissues. Progression of insulin resistance is the primary pathogenic process underlying type 2 diabetes mellitus and metabolic syndrome. It is speculated that the sympathetic nervous system predominance and hyperinsulinemia associated with insulin resistance can promote each other, leading to the development of early DCAN in diabetes patients[11–13]. Lifestyle intervention has been proved to help to reverse early DCAN, probably by reducing insulin resistance and insulin-mediated sympathetic activation[12,14]. Thus, insulin resistance might play an important role in the development of DCAN. However, different authors reached different conclusions on the association between insulin resistance and DCAN. The relationship between insulin resistance and DCAN in type 2 diabetes mellitus has not been fully elucidated.

Therefore, the present study was carried out to investigate the relationship between insulin resistance or other risk factors and DCAN, and explore whether the updated homeostasis model assessment of insulin resistance (HOMA2-IR) is an effective and cost-effective index for the screening of DCAN by applying receiver operating characteristic (ROC) analysis.

MATERIALS AND METHODS

Study population

The present cross-sectional study was carried out at the Department of Endocrinology of the Nanfang Hospital of Southern Medical University, Guangzhou, China. from 1 January 2019 to 30 June 2019. A total of 109 inpatients with type 2 diabetes mellitus were enrolled in the present study (the flow chart is shown in Figure S1). Participants were diagnosed with type 2 diabetes mellitus according to the World Health Organization 1999 diagnosis criteria[15] and 2019 classification[16] of diabetes mellitus. Patients were excluded based on the exclusion criteria as follows: (i) age <18 years; (ii) pregnant or lactating women; (iii) proliferative diabetic retinopathy; (iv) recent diabetic acute complications, including diabetic ketoacidosis, hyperosmotic coma, lactic acidosis and so on; (v) arrhythmia, moderate-to-severe heart failure (NYHA class III and IV) and acute coronary syndrome; (vi) with a history of major stroke; (vii) with diseases and behaviors known to affect the autonomic system, such as thyroid dysfunction, electrolyte disorders, vitamin B_{12} deficiency, Parkinson's disease, multiple system atrophy, alcohol abuse and so on; (viii) with a physical disability or mental illness; (ix) under acute stress conditions, such as surgery and severe infection; and (x0) taking drugs that affect autonomic function or heart rate for the past 2 weeks, such as β-blockers, glucocorticoids and so on. The present study was approved by the Medical Ethics Committee of Nanfang Hospital (NFEC-2018-115), and was registered at the Chinese Clinical Trials Registry (ChiCTR1900020491). All participants provided written informed consent before voluntary participation.

Evaluation of general characteristics

All participants received a detailed assessment of their medical history, including age, sex, history of type 2 diabetes mellitus, history of diabetic complications and comorbidities, current medication use, drug use history, smoking, drinking, and other events in the exclusion criteria. The participants also underwent standardized physical examination, including heart rate, systolic blood pressure (SBP) and diastolic blood pressure (DBP), bodyweight, height. Body mass index was calculated as bodyweight divided by the square of the height.

Evaluation of laboratory parameters

Blood and urine samples were collected in the morning after an overnight fast for at least 8 h. Fasting plasma glucose, postprandial plasma glucose, serum concentration of total cholesterol, triglycerides, low-density lipoprotein (LDL), high-density lipoprotein, uric acid (UA), cystatin C (Cys-C), creatinine and homocysteine (Hcy) were measured through a biochemical automatic analyzer (Beckman Coulter, Brea, CA, USA). Fasting C-peptide (FCP) was measured on an ADVIA Centaur CP Immunoassay System (Bayer, Pittsburgh, PA, USA). Urinary albumin, such as urinary albumin excretion rate (UAER) and urinary protein quantity, was measured using a Siemens Dade Behring BN II Nephelometer (Siemens AG, Munich, Germany). Glycated hemoglobin was obtained through a high-performance liquid chromatography (Tosoh Corp., Tokyo, Japan). Urinary albumin-to-creatinine ratio (UACR) was calculated as UAER divided urinary creatinine concentration. The estimated glomerular filtration rate (eGFR) was calculated using the abbreviated Modification of Diet in Renal Disease formula[17]. HOMA2-IR, a complex, non-linear formula that is difficult to compute manually, applied to reflect insulin resistance, was computed by inputting FCP into the HOMA Calculator software v2.2.3 (The Oxford Centre for Diabetes, Endocrinology and Metabolism, Headington, Oxford, England)[18]. HOMA-IR (HOMA-IR = (fasting plasma glucose × fasting insulin)/22.5[19]) was also used to evaluate the insulin resistance in non-insulin-treated patients. The C-peptide index (CPI = FCP/fasting plasma glucose[20]) was used to evaluate the β-cell function.

Evaluation of cardiovascular autonomic neuropathy

In the present study, cardiovascular autonomic neuropathy was assessed by Ewing tests. The cardiovascular parasympathetic function was evaluated by the heart rate tests to deep breathing (E/I test), to orthostatic change (30:15 test) and to the Valsalva maneuver (Valsalva ratio). The cardiovascular sympathetic function was assessed by measuring the blood pressure response to orthostatic change (orthostatic hypotension test). The specific operation, and the normal, borderline and abnormal values in every test of Ewing tests were as Serhiyenko

et al.[11] previously described. Each of the Ewing tests was scored as 0 for normal, 0.5 for borderline and 1 for abnormal[21] (Table S1). DCAN was defined as a score of ≥2. The severity of DCAN was stratified as non-CAN (all tests normal or only one borderline test), possible or early CAN (one abnormal cardiovagal test result), confirmed diagnosis of CAN (at least two abnormal cardiovagal tests results) and severe or advanced CAN (orthostatic hypotension on the basis of the diagnosis of DCAN[6]). Ewing tests were carried out in the early morning after fasting for at least 8 h using an electromyography machine (Dantec Keypoint 9033A, Copenhagen, Denmark) and a non-invasive blood pressure monitoring system (TASK FORCE MONITOR; Finometer Pro, Enschede, the Netherlands).

Evaluation of other diabetic complications and comorbidities

In the present study, nerve conduction (NC) testing, symptoms and signs of neuropathy were carried out to evaluate diabetic peripheral neuropathy. According to standard procedures previously described by Kimura *et al.*,[22] NC testing was carried out bilaterally on the median, ulnar, posterior tibial and peroneal nerves, by measuring the amplitude to the motor or sensory responses and conduction velocity. Data points less than two standard deviations from the normal limit were defined as outliers. NC was considered abnormal if there was at least one abnormal nerve conduction parameter in at least two nerves[23]. NC testing was carried out using Viking Quest (Nicolet VIASYS Healthcare, Madison, WI, USA). Confirmed diabetic peripheral neuropathy was defined as the presence of abnormal NC with an abnormality of at least one neuropathic symptom or sign[6].

Diabetic retinopathy (DR) was diagnosed after the dilation of the pupils by an experienced ophthalmologist before the Ewing tests. According to the International Clinical Diabetic Retinopathy Severity Scale[24], DR severity was classified as no DR, mild, moderate and severe non-proliferative diabetic retinopathy.

Diabetic kidney disease (DKD) was diagnosed in accordance with the National Kidney Foundation-Kidney Disease Outcomes Quality Initiative clinical practice guidelines[25].

Hypertension was defined as SBP >140 mmHg and (or) DBP >90 mmHg, or self-reported use of antihypertensive medications. Dyslipidemia was defined as the presence of at least one abnormality in serum lipid concentrations as follows: triglycerides ≥2.3 mmol/L, total cholesterol ≥6.2 mmol/L, LDL ≥4.1 mmol/L, high-density lipoprotein <1.0mmol/L, or self-reported dyslipidemia history or on lipid-lowering therapy.

Statistical analysis

An estimated 104 events would be required to provide 90% power to detect a difference of 0.15 between the area under the ROC curve (AUC) under the null hypothesis of 0.70 and an AUC under the alternative hypothesis of 0.85 using a two-sided *z*-test at a significance of 0.05. Data are presented as the mean ± standard deviation (normal distribution) or median (minimum, maximum; non-normal distribution) for continuous variables, and as frequency (percentages) for categorical variables. Lacking uniform classification criteria, cut-points for tertiles of FCP, HOMA2-IR and Cys-C levels were calculated at the 33rd and 67th percentiles, respectively. Differences among the groups were evaluated by the Student's *t*-test, the analysis of variance (ANOVA test; normal distribution) and by the Kruskal–Wallis rank sum test (non-normal distribution) for continuous variables, and the χ^2-test for categorical variables. Univariate logistic regression analyses were applied to evaluate the correlation between the clinical variables and DCAN or the scores of each test in Ewing tests. Multivariate logistic regression analyses were applied to test the independent associations between HOMA2-IR or other covariates and DCAN. Non-adjusted and different multivariate-adjusted models were carried out. Stratified and interaction analyses were also carried out. ROC analysis was carried out to estimate the discriminative value of HOMA2-IR or other variables independently associated with DCAN by calculating AUC and to determine the optimal cut-off point of these models to screen DCAN. To compare the diagnosis value among the HOMA2-IR independent model and different models of other single or multiple variables independently associated with DCAN, and to compare the diagnosis value of HOMA2-IR and other indexes reflecting islet function or insulin resistance, a non-parametric approach was used to analyze the AUCs of these models. A *P*-value <0.05 showed statistical significance. All of the statistical analyses were carried out using the statistical software packages R (http://www.R-project.org, The R Foundation for Statistical Computing, Vienna, Austria) and EmpowerStats version 3.4.3 (http://www.empowerstats.com; X&Y Solutions, Inc., Boston, MA, USA).

RESULTS

Demographic characteristics

The characteristics of the studied patients in the DCAN and non-DCAN groups are presented in Table 1. Individuals with DCAN were older, had longer durations of diabetes, and had higher values of SBP, serum Cr, Cys-C, UA, Hcy, UAER, urinary protein quantity and UACR, lower eGFR, higher prevalence of DKD, and higher use of insulin and antihypertensive medication than those without DCAN. In addition, FCP, CPI and HOMA2-IR levels were significantly higher in participants with DCAN. HOMA-IR tended to be higher in participants with DCAN, but did not reach significance. The prevalence of possible, confirmed and severe DCAN were 27.5% (n = 30), 17.4% (n = 19) and 16.5% (n = 18), respectively. Age, diabetic durations, SBP, and levels of FCP, HOMA2-IR, CPI, LDL, Cr, Cys-C, Hcy, UAER, urinary protein quantity and UACR, and the prevalence of DKD of the participants tended to be higher with the progression of the DCAN (Table S2). Age, SBP, FCP, HOMA-IR, CPI, triglycerides, Cys-C, UA, Hcy, UAER and UACR tended to be higher in participants with an increased HOMA2-IR level. The prevalence of DCAN tended

Table 1 | Characteristics of patients with type 2 diabetes with or without diabetic cardiovascular neuropathy

	DCAN–	DCAN+	*P*-value
n	72	37	
Male, *n* (%)	36 (50.0%)	25 (67.6%)	0.080
Age (years)	53.2 ± 12.3	62.9 ± 10.2	<0.001***
<50	23 (31.9%)	3 (8.1%)	0.006**
50–59	26 (36.1%)	11 (29.7%)	
60–69	18 (25.0%)	15 (40.5%)	
≥70	5 (6.9%)	8 (21.6%)	
Diabetic duration (years)	8.0 (3.0–11.2)	10.0 (4.0–17.0)	<0.001***
<5	25 (34.7%)	1 (2.7%)	<0.001***
5–10	28 (38.9%)	16 (43.2%)	
11–15	9 (12.5%)	7 (18.9%)	
>15	10 (13.9%)	13 (35.1%)	
Smoking, *n* (%)	23 (31.9%)	11 (29.7%)	0.813
BMI (kg/m^2)	23.20 ± 3.32	24.01 ± 3.64	0.247
SBP (mmHg)	138.6 ± 20.2	148.7 ± 24.5	0.024*
DBP (mmHg)	83.1 ± 10.5	81.9 ± 12.7	0.619
Smoking, *n* (%)	23 (31.9%)	11 (29.7%)	0.813
FPG (mmol/L)	8.48 ± 3.19	8.34 ± 2.97	0.824
PPG (mmol/L)	15.21 ± 5.82	15.29 ± 6.90	0.945
HbA1c (%)	10.0 ± 2.7	9.2 ± 2.9	0.139
FCP (ng/mL)	1.37 (1.02–2.15)	2.64 (1.62–3.26)	<0.001***
HOMA2-IR	1.30 ± 0.50	2.46 ± 1.00	<0.001***
HOMA-IR	1.61 (0.95–3.58)	2.47 (1.00–3.65)	0.872
CPI (ng/mg)	0.95 (0.65–1.57)	1.70 (1.30–2.60)	<0.001***
TC (mmol/L)	5.02 ± 1.37	4.71 ± 1.81	0.320
TG (mmol/L)	1.54 (0.98–2.13)	1.22 (0.83–2.61)	0.800
HDL (mmol/L)	1.05 ± 0.26	1.05 ± 0.29	0.971
LDL (mmol/L)	3.17 ± 1.00	2.95 ± 1.23	0.311
Cr (μmol/L)	63.50 (53.00–79.25)	82.00 (68.00–108.00)	0.011*
Cys-C (mg/L)	0.97 (0.80–1.12)	1.23 (1.05–1.69)	0.010*
UA (μmol/L)	346.01 ± 104.31	412.19 ± 117.41	0.003**
eGFR (mL/min/1.73 m^2)	105.09 ± 36.95	77.16 ± 31.94	<0.001***
Hcy (ng/mL)	10.87 ± 5.05	13.98 ± 4.78	0.009**
UAER (mg/24 h)	16.00 (7.00–118.00)	115.00 (15.00–1272.00)	<0.001***
UTP (g/24 h)	0.11 (0.08–0.78)	0.35 (0.16–1.90)	0.011*
UACR	1.40 (0.70–11.25)	14.20 (1.20–210.00)	0.003**
DPN, *n* (%)	38 (52.8%)	18 (48.6%)	0.683
DKD, *n* (%)	27 (37.5%)	25 (67.6%)	0.003**
DR, *n* (%)	48 (66.7%)	27 (73.0%)	0.501
Hypertension, *n* (%)	31 (43.1%)	22 (59.5%)	0.105
Dyslipidemia, *n* (%)	44 (61.1%)	24 (64.9%)	0.702
Hyperuricemia, *n* (%)	17 (23.6%)	16 (43.2%)	0.035*
Obesity, *n* (%)	18 (25.0%)	13 (35.1%)	0.267
Antidiabetes treatment, *n* (%)			
Non-insulin-treated	45 (62.5%)	14 (37.8%)	0.014*
Insulin-treated	27 (37.5%)	23 (62.2%)	
Anti-hypertensive medication, *n* (%)	13 (18.1%)	14 (37.8%)	0.023*
Anti-dyslipidemia medication, *n* (%)	2 (2.8%)	3 (8.1%)	0.208
Ewing's score	0.50 (0.50–1.50)	3.00 (2.50–3.00)	<0.001***

Continuous data are shown as the mean ± standard deviation or median (Q1–Q3), and categorical data as *n* (%). BMI, body mass index; CPI, C-peptide index; Cr, creatinine; Cys-C, cystatin C; DBP, diastolic blood pressure; DKD, diabetic kidney disease; DPN, diabetic peripheral neuropathy; DR, diabetic retinopathy; eGFR, estimated glomerular filtration rate; FCP, fasting C-peptide; FPG, fasting plasma glucose; HbA1c, glycated hemoglobin; Hcy, homocysteine; HDL, high-density lipoprotein; HOMA-IR, homeostasis model assessment of insulin resistance; HOMA2-IR, updated homeostasis model assessment of insulin resistance; LDL, low-density lipoprotein; OHA, oral hypoglycemic agents; PPG, postprandial plasma glucose; SBP, systolic blood pressure; TC, total cholesterol; TG, triglycerides; UA, urid acid; UACR, urinary albumin-to-creatinine ratio; UAER, urinary albumin excretion rates; UTP, urinary protein quantity. **P*-value <0.05. ***P*-value <0.01. ****P*-value <0.001.

to increase in participants with higher HOMA2-IR levels (Table 2).

Relationship between HOMA2-IR and DCAN

Correlations between the clinical variables and DCAN are presented in Table S3. The univariate logistic analysis showed that DCAN was positively correlated with age, diabetic duration, SBP, FCP, HOMA2-IR, CPI, Cr, Cys-C, UA, Hcy, UAER, UACR and DKD, and negatively correlated with eGFR ($P < 0.05$). After adjusting for age, sex, body mass index, HbA1c, eGFR, dyslipidemia, hypertension, diabetes duration, DKD and diabetic peripheral neuropathy (model 3), the association between age (odds ratio [OR] 1.07, 95% confidence interval [CI] 1.02–1.13; $P = 0.008$), diabetic duration (OR 1.11, 95% CI 1.13–1,57; $P = 0.007$), FCP (OR 2.96, 95% CI 1.60–5.48; $P < 0.001$), HOMA2-IR (OR 39.30, 95% CI 7.17–215.47; $P < 0.001$), CPI (OR 2.35, 95% CI 1.25–4.40; $P = 0.008$) and DCAN remained statistically significant (Table 3).

The logistic regression analysis of the association between risk factors and scores of each test in the Ewing tests showed that diabetic duration and HOMA2-IR significantly correlated with the heart rate variability to deep breathing, to orthostatic change and to the Valsalva maneuver, and blood pressure response to orthostatic change ($P < 0.05$), whereas age, FCP and CPI correlated with only some of them (Table S4).

Interaction and stratified analyses showed that positive relationships between DCAN and HOMA2-IR levels were noted for all strata with no significant interaction effects observed (Table S5).

Screening value of HOMA2-IR for DCAN

To determine the most effective and cost-effective screening model for DCAN, ROCs were plotted for the independent risk factors identified by the multivariate analysis. We computed and compared the AUC of the HOMA2-IR model with models that additionally included respective individual or multiple risk factors. As outlined in Figure 1a, the area under the ROC curve for HOMA2-IR model (AUC 0.878, 95% CI 0.810–0.946) was superior to the age (AUC 0.732, 95% CI 0.635–0.829, $P = 0.010$), diabetic duration (AUC 0.716, 95% CI 0.620–0.813, $P = 0.009$) and FCP (AUC 0.751, 95% CI 0.655–0.847, $P = 0.003$) models. Compared with other indexes reflecting the islet function and insulin resistance, such as CPI (AUC 0.711, 95% CI 0.608–0.815, $P = 0.001$) and HOMA-IR (AUC 0.517, 95% CI 0.333–0.701, $P < 0.001$), HOMA2-IR showed significantly better diagnostic performance (Figure 1b). None of the models that included the individual or multiple risk factors based on HOMA2-IR had a significantly better diagnosis performance compared with the AUC for HOMA2-IR alone ($P > 0.05$; Figure 1c; formulas are shown in Table S6). The optimal cut-off value of the HOMA2-IR model was 1.735 in screening DCAN, with a specificity of 0.819 and a sensitivity of 0.811 (Figure 2).

DISCUSSION

Because of the atypical and insidious symptoms at the early stages of DCAN, the majority of type 2 diabetes mellitus patients tend to neglect the symptoms and leave it untreated clinically until the DCAN reaches a serious stage, by which time DCAN shows multiple complex and severe clinical manifestations, in turn increasing morbidity and mortality associated with a high risk of cardiac arrhythmias and sudden death[3]. Although DCAN progresses with diabetes progression[26], early intervention and modification of risk factors could delay or reverse the progression of DCAN[27]. Therefore, early and correct identification for patients with a high risk of developing DCAN is crucial to initiate, escalate or intensify treatment.

As HOMA-IR or other indicators based on insulin used frequently in previous studies might be influenced by insulin use and many factors, we calculated HOMA2-IR based on FCP in the present study. HOMA2-IR has been proved to be well correlated with the gold standard hyperinsulinemic-euglycemic clamp method, showing a better performance on reflecting the real insulin resistance than insulin[28] and the indicators based on insulin, and is therefore more convincing. The present study showed that insulin resistance was independently associated with DCAN, and had the potential to reflect the severity of cardiovascular autonomic dysfunction in patients with type 2 diabetes mellitus. In addition, we found that HOMA2-IR individually diagnosed DCAN significantly higher than the other three independent risk factors individually used, whereas models combining HOMA2-IR and other risk factors did not significantly boost the diagnostic power. In particular, HOMA2-IR showed better diagnosis power for DCAN compared with the other two indexes reflecting endogenous insulin secretion and insulin resistance, CPI and HOMA-IR. Therefore, HOMA2-IR appears to be a highly accurate, feasible and cost-effective index for screening DCAN, with reasonably good specificity and sensitivity. These findings show that patients with type 2 diabetes mellitus are supposed to be classified as the high-risk population of DCAN if the value of HOMA2-IR is >1.735. Priority diagnostic tests should be carried out for patients at high risk of DCAN for timely integrated intervention, such as strict control of blood glucose, blood pressure and blood lipids, lifestyle modifications, and exercise adherence, to enhance insulin sensitivity, reduce the risk of DCAN and even slow the progression of DCAN[11].

At present, the insulin resistance-related pathogenesis on the progression of DCAN remains largely unknown. The Toronto Consensus emphasized that autonomic dysfunction is possibly presented in prediabetes already, which is represented as suppression of sympathetic nerves and activation of parasympathetic nerves[29]. Insulin resistance has been proved to cause sympathetic hyperactivity. The attribution of sympathetic overactivity in insulin-resistant conditions is the activation of insulin-driven sympathetic nerves, acting through peripheral mechanisms in acute states; that is, insulin causes endothelium-

Table 2 | Characteristics of patients with type 2 diabetes according to updated homeostasis model assessment of insulin resistance level tertiles

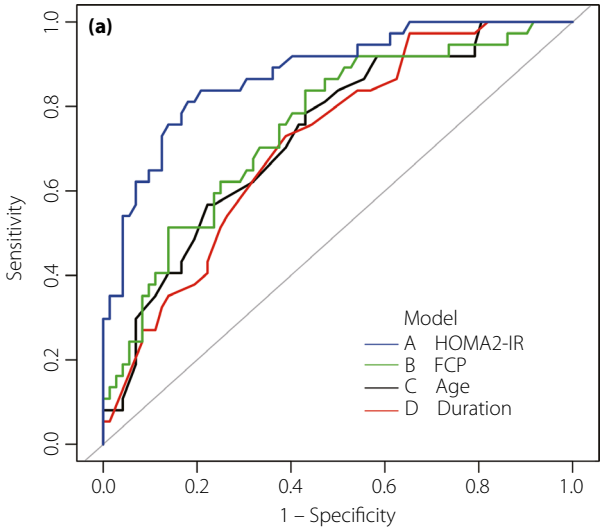
HOMA2-IR	Total	Tertile 1 (<1.20)	Tertile 2 (1.20-1.86)	Tertile 3 (>1.86)	*P*-value for trend
Male, *n* (%)	109	36	37	36	–
Age (years)	56.5 ± 12.5	51.5 ± 10.7	56.6 ± 14.6	61.5 ± 9.8	0.002**
<50	26 (23.9%)	13 (36.1%)	9 (24.3%)	4 (11.1%)	0.006**
50–59	37 (33.9%)	15 (41.7%)	8 (21.6%)	14 (38.9%)	
60–69	33 (30.3%)	7 (19.4%)	15 (40.5%)	11 (30.6%)	
≥70	13 (11.9%)	1 (2.8%)	5 (13.5%)	7 (19.4%)	
Diabetic duration (years)	10.0 (5.0–15.0)	8.0 (3.0–12.0)	10.0 (6.0–12.0)	10.0 (5.8–17.5)	0.459
<5	26 (23.9%)	13 (36.1%)	8 (21.6%)	5 (13.9%)	0.238
5–10	44 (40.4%)	12 (33.3%)	18 (48.6%)	14 (38.9%)	
11–15	16 (14.7%)	12 (33.33%)	4 (10.8%)	6 (16.7%)	
>15	23 (21.1%)	5 (13.9%)	7 (18.9%)	11 (30.6%)	
Smoking, *n* (%)	34 (31.19%)	12 (33.33%)	11 (29.73%)	11 (30.56%)	0.941
BMI (kg/m^2)	23.47 ± 3.44	23.16 ± 3.43	22.96 ± 3.49	24.31 ± 3.33	0.198
SBP (mmHg)	142.0 ± 22.2	133.7 ± 14.7	144.4 ± 25.0	147.9 ± 23.5	0.016
DBP (mmHg)	82.7 ± 11.2	83.1 ± 10.3	82.5 ± 11.9	82.6 ± 11.8	0.973
Smoking, *n* (%)	34 (31.2%)	12 (33.3%)	11 (29.7%)	11 (30.6%)	0.941
FPG (mmol/L)	8.43 ± 3.10	7.99 ± 2.73	9.16 ± 3.68	8.14 ± 2.75	0.215
PPG (mmol/L)	15.24 ± 6.17	15.89 ± 6.03	15.43 ± 6.52	14.38 ± 6.03	0.574
HbA1c (%)	9.71 ± 2.78	9.8 ± 2.8	10.0 ± 3.3	9.3 ± 2.2	0.470
FCP (ng/mL)	1.62 (1.10–2.71)	1.08 (0.91–1.21)	1.69 (1.54–2.16)	2.99 (2.31–3.51)	<0.001***
HOMA2-IR	1.50 (1.07–2.05)	0.91 (0.74–1.04)	1.50 (1.29–1.67)	2.45 (2.05–2.94)	<0.001***
HOMA-IR	1.19 (0.70–1.92)	0.96 (0.90–1.44)	1.65 (0.95–3.08)	3.68 (2.16–4.24)	<0.001***
CPI (ng/mg)	1.19 (0.70–1.92)	0.76 (0.52–1.07)	1.13 (0.76–1.92)	1.94 (1.55–2.79)	<0.001***
TC (mmol/L)	4.91 ± 1.53	5.12 ± 1.67	5.05 ± 1.66	4.57 ± 1.20	0.260
TG (mmol/L)	1.48 (0.91–2.25)	1.12 (0.96–1.74)	1.98 (1.08–3.03)	1.23 (0.83–2.08)	0.026*
HDL (mmol/L)	1.05 ± 0.27	1.14 ± 0.28	1.00 ± 0.26	1.02 ± 0.26	0.053
LDL (mmol/L)	3.10 ± 1.08	3.27 ± 1.19	3.13 ± 1.15	2.89 ± 0.88	0.327
Cr (μmol/L)	70.00 (56.00–89.00)	63.50 (52.50–77.50)	70.00 (56.00–97.00)	75.00 (64.75–92.25)	0.369
Cys-C (mg/L)	1.04 (0.85–1.41)	0.91 (0.79–1.14)	1.04 (0.93–1.45)	1.14 (0.97–1.45)	0.022*
UA (μmol/L)	368.48 ± 112.87	323.75 ± 113.48	397.11 ± 101.07	383.78 ± 112.95	0.012*
eGFR (mL/min/1.73 m^2)	95.61 ± 37.61	100.56 ± 30.73	98.46 ± 45.56	87.73 ± 34.45	0.301
Hcy (ng/mL)	11.90 ± 5.15	9.50 ± 3.68	13.00 ± 6.24	12.76 ± 4.25	0.023
UAER (mg/24 h)	24.00 (7.50–341.50)	12.00 (6.00–102.00)	27.50 (8.75–491.25)	64.00 (9.50–943.50)	0.015*
UTP (g/24 h)	0.18 (0.08–1.20)	0.11 (0.08–0.88)	0.15 (0.08–1.34)	0.26 (0.09–1.83)	0.981
UACR	2.10 (0.80–44.05)	1.30 (0.50–6.53)	2.90 (0.88–52.55)	5.50 (0.95–134.82)	0.044
DCAN, *n* (%)	37	3 (8.3%)	7 (18.9%)	27 (75.0%)	<0.001***
DPN, *n* (%)	56 (51.4%)	19 (52.8%)	15 (40.5%)	22 (61.1%)	0.209
DKD, *n* (%)	52 (47.7%)	14 (38.9%)	17 (45.9%)	21 (58.3%)	0.247
DR, *n* (%)	75 (68.8%)	24 (66.7%)	27 (73.0%)	24 (66.7%)	0.797
Hypertension, *n* (%)	53 (48.6%)	13 (36.1%)	19 (51.4%)	21 (58.3%)	0.155
Dyslipidemia, *n* (%)	68 (62.4%)	18 (50.0%)	24 (64.9%)	26 (72.2%)	0.140
Hyperuricemia, *n* (%)	33 (30.3%)	7 (19.4%)	12 (32.4%)	14 (38.9%)	0.188
Obesity, *n* (%)	31 (28.4%)	6 (16.7%)	11 (29.7%)	14 (38.9%)	0.110
Antidiabetes treatment, *n* (%)					
Non-insulin-treated	59 (54.1%)	17 (47.2%)	24 (64.9%)	18 (50.0%)	0.265
Insulin-treated	50 (45.9%)	19 (52.8%)	13 (35.1%)	18 (50.0%)	
Antihypertensive medication, *n* (%)	27 (24.8%)	9 (25.0%)	8 (21.6%)	10 (27.8%)	0.830
Antidyslipidemia medication, *n* (%)	5 (4.6%)	1 (2.8%)	0 (0.0%)	4 (11.1%)	0.062
Ewing's score	1.50 (0.50–2.50)	1.00 (0.50–1.50)	1.00 (0.50–1.50)	2.50 (1.75–3.00)	<0.001***

Continuous data are shown as the mean ± standard deviation or median (Q1–Q3), and categorical data as *n* (%). BMI, body mass index; CPI, C-peptide index; Cr, creatinine; Cys-C, cystatin C; DBP, diastolic blood pressure; DKD, diabetic kidney disease; DPN, diabetic peripheral neuropathy; DR, diabetic retinopathy; eGFR, estimated glomerular filtration rate; FCP, fasting C-peptide; FPG, fasting plasma glucose; HbA1c, glycated hemoglobin; Hcy, homocysteine; HDL, high-density lipoprotein; HOMA-IR, homeostasis model assessment of insulin resistance; HOMA2-IR, updated homeostasis model assessment of insulin resistance; LDL, low-density lipoprotein; OHA, oral hypoglycemic agents; PPG, postprandial plasma glucose; SBP, systolic blood pressure; TC, total cholesterol; TG, triglycerides; UA, urid acid; UACR, urinary albumin-to-creatinine ratio; UAER, urinary albumin excretion rates; UTP, urinary protein quantity. **P*-value <0.05. ***P*-value <0.01. ****P*-value <0.001.

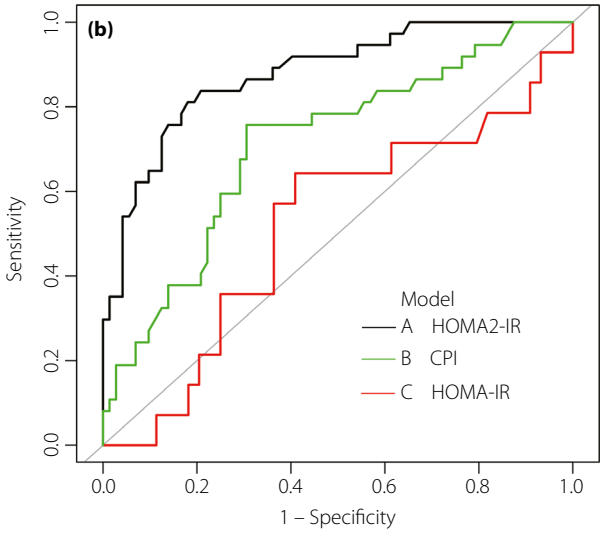
Table 3 | Multivariate logistic regression analysis of risk factors for diabetic cardiovascular neuropathy

	Unadjusted model		Model 1[†]		Model 2[‡]		Model 3[§]	
	OR, 95% CI	*P*-value	OR, 95% CI	*P*-value	OR, 95% CI	*P*-value	OR, 95% CI	*P*-value
Age (per 1 year)	1.08 (1.04, 1.13)	<0.001***	1.09 (1.04, 1.14)	<0.001***	1.09 (1.04, 1.14)	<0.001***	1.07 (1.02, 1.13)	0.008**
<50	1.0 (ref)	–	1.0 (ref)	–	1.0 (ref)	–	1.0 (ref)	–
50–59	3.24 (0.80, 13.08)	0.098	3.58 (0.87, 14.77)	0.078	3.46 (0.82, 14.70)	0.092	3.17 (0.66, 15.07)	0.148
60–69	6.39 (1.60, 25.51)	0.009**	8.62 (2.04, 36.47)	0.003**	8.14 (1.84, 36.06)	0.006**	5.18 (0.94, 28.60)	0.059
≥70	12.27 (2.37, 63.36)	0.003**	15.78 (2.87, 86.95)	0.002**	14.19 (2.52, 79.98)	0.003**	9.37 (1.37, 64.02)	0.023*
Diabetes duration (per 1 year)	1.13 (1.05, 1.20)	<0.001***	1.13 (1.05, 1.21)	<0.001***	1.12 (1.05, 1.20)	0.001**	1.11 (1.13, 1.57)	0.007**
<5	1.0 (ref)	–	1.0 (ref)	–	1.0 (ref)	–	1.0 (ref)	–
5–10	14.29 (1.77, 115.55)	0.013*	16.41 (1.99, 135.04)	0.009**	22.02 (2.39, 203.23)	0.006**	16.64 (1.60, 173.44)	0.019*
11–15	19.44 (2.09, 180.64)	0.009**	26.08 (2.68, 253.42)	0.005**	24.07 (2.37, 244.79)	0.007**	14.45 (1.32, 158.72)	0.029*
>15	32.50 (3.74, 282.24)	0.002**	33.05 (3.75, 291.01)	0.002**	36.83 (3.89, 348.98)	0.002**	32.15 (2.92, 354.02)	0.005**
FCP (1 ng/mL)	2.32 (1.52, 3.53)	<0.001***	2.30 (1.45, 3.64)	<0.001***	2.68 (1.54, 4.68)	<0.001***	2.96 (1.60, 5.48)	<0.001***
<1.27	1.0 (ref)	–	1.0 (ref)	–	1.0 (ref)	–	1.0 (ref)	–
1.27–2.3	7.50 (1.94, 28.99)	0.004**	7.23 (1.71, 30.61)	0.007**	7.50 (1.94, 28.99)	0.004**	9.84 (1.87, 51.77)	0.007**
>2.3	12.29 (3.18, 47.47)	<0.001***	13.06 (3.02, 56.42)	<0.001***	12.29 (3.18, 47.47)	<0.001***	20.19 (3.38, 120.42)	0.001**
HOMA2-IR (per 1 unit)	13.52 (4.91, 37.24)	<0.001***	12.06 (4.07, 35.70)	<0.001***	13.32 (4.27, 41.58)	<0.001***	39.30 (7.17, 215.47)	<0.001***
<1.2	1.0 (ref)	–	1.0 (ref)	–	1.0 (ref)	–	1.0 (ref)	–
1.2–1.86	2.57 (0.61, 10.83)	0.200	1.65 (0.35, 7.71)	0.527	1.81 (0.36, 9.09)	0.470	1.84 (0.29, 11.72)	0.520
>1.86	33.00 (8.12, 134.11)	<0.001***	22.99 (5.28, 100.17)	<0.001***	28.08 (5.79, 136.27)	<0.001***	81.71 (10.51, 635.08)	<0.001***
CPI (1 ng/mg)	2.00 (1.31, 3.05)	0.001**	1.86 (1.17, 2.96)	0.009**	2.04 (1.17, 3.56)	0.012*	2.35 (1.25, 4.40)	0.008**
<0.81	1.0 (ref)	–	1.0 (ref)	–	1.0 (ref)	–	1.0 (ref)	–
0.81–1.66	2.20 (0.71, 6.79)	0.171	2.19 (0.64, 7.44)	0.209	2.61 (0.72, 9.41)	0.143	2.72 (0.64, 11.57)	0.1745
>1.66	5.88 (1.98, 17.48)	0.001**	4.97 (1.52, 16.28)	0.008**	6.25 (1.60, 24.39)	0.008**	8.98 (1.96, 41.16)	0.005**

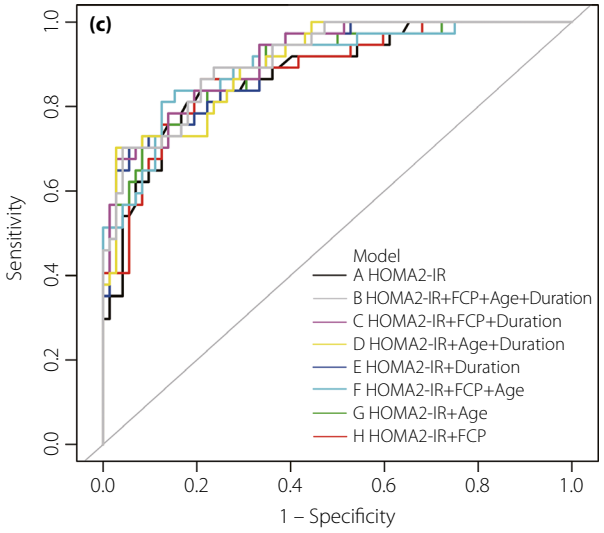
CPI, C-peptide index; FCP, fasting C-peptide. **P*-value <0.05. ***P*-value <0.01. ****P*-value <0.001. [†]Model 1: adjusted by age and sex. [‡]Model 2: adjusted by model 1 plus body mass index, glycated hemoglobin, hypertension and dyslipidemia. [§]Model 3: adjusted by model 2 plus diabetic duration, diabetic peripheral neuropathy, estimated glomerular filtration rate and diabetic kidney disease.



Model	AUC	*P* value
A	0.878 (95%CI 0.810, 0.946)	Reference
B	0.751 (95%CI 0.655, 0.847)	0.003
C	0.732 (95%CI 0.635, 0.829)	0.010
D	0.716 (95%CI 0.620, 0.813)	0.009



Model	AUC	*P* value
A	0.878 (95%CI 0.810, 0.946)	Reference
B	0.711 (95%CI 0.608, 0.815)	0.001
C	0.517 (95%CI 0.333, 0.701)	<0.001



Model	AUC	*P* value
A	0.878 (95%CI 0.810, 0.946)	Reference
B	0.913 (95%CI 0.860, 0.966)	0.050
C	0.912 (95%CI 0.859, 0.966)	0.061
D	0.905 (95%CI 0.849, 0.961)	0143
E	0.904 (95%CI 0.847, 0.961)	0.009
F	0.901 (95%CI 0.840, 0.962)	0.149
G	0.895 (95%CI 0.833, 0.950)	0.264
H	0.884 (95%CI 0.818, 0.950)	0.452

Figure 1 | (a) Comparison of the receiver operating characteristic (ROC) curves for age, diabetic duration, fasting C-peptide (FCP) and updated homeostasis model assessment of insulin resistance (HOMA2-IR) to diagnose diabetic cardiovascular autonomic neuropathy, respectively. Model A (blue) represents the ROC curve of HOMA2-IR; model B (green) represents the ROC curve of FCP; model C (red) represents the ROC curve of age; and model D (red) represents the ROC curve of diabetic duration. (b) Comparison of the ROC curves for C-peptide index (CPI), HOMA-IR and HOMA2-IR to diagnose diabetic cardiovascular autonomic neuropathy. Model A (black) represents the ROC curve of HOMA2-IR; model B (green) represents the ROC curve of CPI; and model C (red) represents the ROC curve of HOMA-IR. (c) Comparison of the ROC curves for models of the individual or multiple risk factors combined with HOMA2-IR to diagnose diabetic cardiovascular autonomic neuropathy. Model A (black) represents the ROC curve of HOMA2-IR; model B (grey) represents the ROC curve of HOMA2-IR combined with age, diabetic duration and FCP; model C (purple) represents the ROC curve of HOMA2-IR combined with FCP and diabetic duration; model D (yellow) represents the ROC curve of HOMA2-IR combined with age and diabetic duration; model E (deep blue) represents the ROC curve of HOMA2-IR combined with diabetic duration; model F (light blue) represents the ROC curve of HOMA2-IR combined with FCP and age; model G (green) represents the ROC curve of HOMA2-IR combined with age; and model H (red) represents the ROC curve of HOMA2-IR combined with FCP.

dependent vasodilation leading to baroreflex-mediated sympathetic activation. The central mechanism mainly manifests in chronic states as hyperinsulinemia operating in the paraventricular nucleus of the hypothalamus and the arcuate nucleus. Notably, in the present study, we also found that CPI used as a marker of endogenous insulin secretion was independently associated with DCAN, strongly confirming that hyperinsulinemia closely associated with insulin resistance[30] manifesting early in type 2 diabetes mellitus plays an important role in promoting the occurrence and progression of DCAN[31]. In addition, insulin receptors have been found on the carotid body in animal models of insulin resistance, confirming insulin-induced hyperactivation[32]. The present study also showed that HOMA2-IR was correlated with the scores of each test in Ewing tests, indicating that insulin resistance might act on both sympathetic and parasympathetic nerves, which also proves that insulin resistance plays an important role in the progression of DCAN.

The precise pathogenesis of DCAN is complex and remains unclear; several molecular mechanisms are involved, including hyperglycemia, oxidative stress, inflammation and endothelial dysfunction[33–35]. These mechanisms interact with each other, of which hyperglycemia is the major pathological factor[12,33,36,37]. It is well established that type 2 diabetes mellitus is characterized by chronic hyperglycemia due to worsening insulin resistance[38]. Furthermore, insulin resistance is closely associated with inflammation, related to the increased expression of several inflammatory factors[39], such as tumor necrosis factor-α, interleukin-6 and so on. Inflammatory cytokines also disrupt insulin signaling, thereby contributing to insulin resistance[40]. Therefore, insulin resistance can be involved in DCAN process by multiple mechanisms.

In addition to insulin resistance reflected by FCP, HOMA2-IR and CPI, the present study points to two recognized factors that independently associated with DCAN; namely, age and diabetic duration, which is consistent with previous studies[5,12]. However, it was unexpected that HOMA-IR was not significantly correlated to DCAN, but was found to rise with increasing HOMA2-IR, possibly due to the sample size after excluding the insulin-treated patients was too small to detect a statistically significant association with DCAN. Several prospective cohort studies confirmed that hyperglycemia, hyperlipidemia and hypertension have been identified as the major controllable risk factors for progression of DCAN[8,35,41–43]. In the present study, the LDL level increased significantly with the progression of DCAN. SBP was correlated with DCAN. However, the correlation between DCAN and serum glucose was not observed. This might be attributed to the fact that the present study was a cross-sectional study, monitoring the serum glucose at a specific time, and therefore failed to reflect long-term serum glucose fluctuations. Previous studies also found that microvascular complications are the main risk factors for the DCAN process[5,44,45]. The relationship between DKD and the progression of DCAN, and correlation between the indicators reflecting kidney function, such as eGFR, UAER, and UACR, were found in the present study, whereas diabetic peripheral neuropathy and DR were not observed to be significantly associated with DCAN in the present study. These different conclusions might relate with the various study populations, diagnostic criteria and different research methods; for instance, patients with proliferative diabetic retinopathy were not involved in the present study.

The present study had several notable advantages. First, unlike the previous studies of DCAN mostly using *t*-tests or linear regression analysis, the present study further provided more stable and reliable evidence of the correlation between DCAN and insulin resistance by logistic regression analysis, stratified and interaction analyses, and ROC analysis. Second, different from prior studies, we applied HOMA2-IR based on FCP to reflect insulin resistance instead of HOMA-IR or other indicators simply calculated based on insulin in the present study. It is worth noting that HOMA2-IR could be calculated by using FCP, which is not influenced by the route of insulin administration, not being cleared in the liver[46]. Therefore, HOMA2-IR is applicable to both insulin-treated and non-insulin-treated patients, showing better practicability than HOMA-IR. HOMA2-IR has also been shown to have a more effective diagnostic performance for DCAN than CPI and

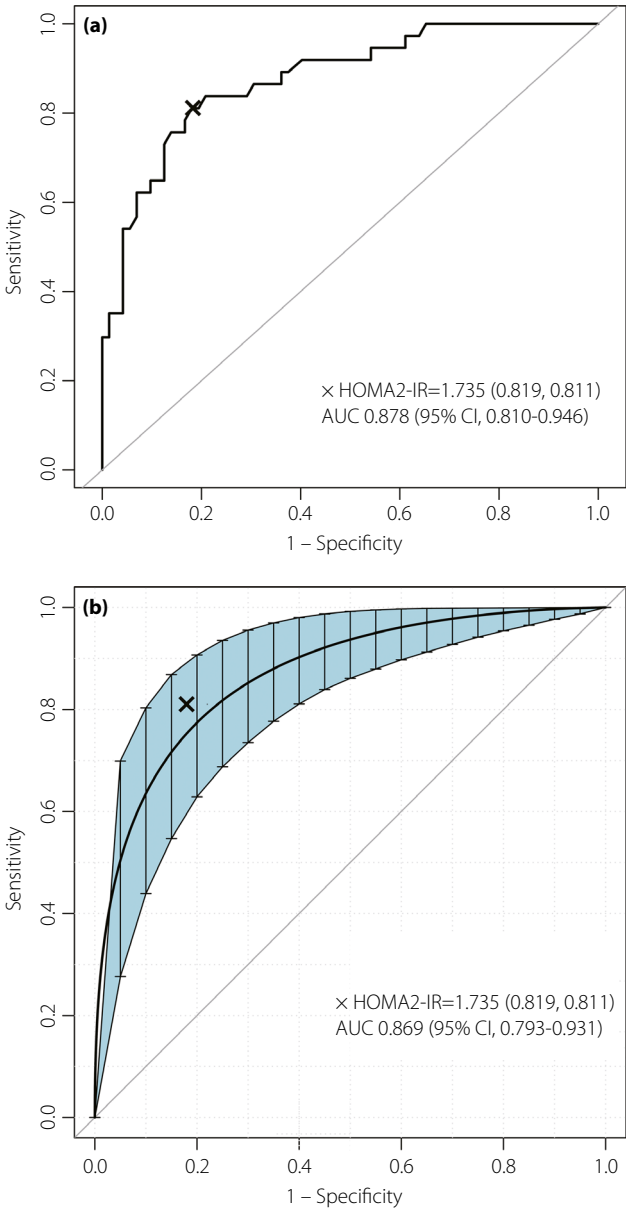


Figure 2 | Receiver operating characteristic curve for the updated homeostasis model assessment of insulin resistance (HOMA2-IR) to diagnose diabetic cardiovascular autonomic neuropathy. (a) The optimal cut-off value for classification is shown by "×" annotating this threshold value followed by specificity and sensitivity. (b) The blue shading denotes the bootstrap estimated 95% confidence interval (CI) with the area under curve (AUC).

HOMA-IR in the present study. Third, the Ewing tests recommended as the gold standard for diagnosis of DCAN were used in the present study, and therefore, the validity of the diagnosis for DCAN was guaranteed. However, there were also some limitations that should be considered when interpreting the present findings. First, this was a cross-sectional study with a relatively small sample size. Although a definite causal association cannot be shown, the present study contributes to further explore the potential DCAN risk factors. Second, the participants enrolled in the present study were all inpatients. Compared with outpatients, inpatients tended to be more likely to be diagnosed with DCAN because of a more comprehensive diagnosis. Therefore, it might limit the extrapolation of the conclusions of this study to outpatients. Another noteworthy point is that Ewing tests comprise five cardiovascular reflex tests when initially proposed. Nevertheless, due to the poor maneuverability, specificity and sensitivity of carrying out the handgrip test, the Toronto Consensus Expert Panel on Diabetic Neuropathy[5] no longer suggested applying the blood pressure response to sustained handgrip as part of the gold standard for clinical CAN testing. Therefore, we applied currently mainstream simplified Ewing tests excluding the handgrip test in the present study. However, based on our results, we remain convinced that HOMA2-IR is an effective and feasible index for screening DCAN. Future prospective large cohort studies including inpatients and outpatients are warranted.

In conclusion, insulin resistance is independently associated with DCAN. HOMA2-IR presents to be a highly accurate and parsimonious index for screening DCAN. Patients whose HOMA2-IR is >1.735 are at a high risk of DCAN; thus, priority diagnostic tests should be carried out for patients at high risk of DCAN for timely integrated intervention.

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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 | Definition of normal, borderline and abnormal in each Ewing test.

Table S2 | Characteristics of patients with type 2 diabetes according to the degree of cardiovascular autonomic neuropathy.

Table S3 | Univariate logistic regression analysis of risk factors for diabetic cardiovascular neuropathy.

Table S4 | Univariate regression analysis between the risk factors and each Ewing test's score.

Table S5 | Association between updated homeostasis model assessment of insulin resistance and diabetic cardiovascular autonomic neuropathy according to baseline characteristics.

Table S6 | Formulas of models combining updated homeostasis model assessment of insulin resistance and other risk factors.

Figure S1 | Flow chart of the study population.