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Decreased plasma neuregulin 4 levels are associated with peripheral neuropathy in Chinese patients with newly diagnosed type 2 diabetes: A cross-sectional study

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ABSTRACT

Neuregulin-4 (Nrg4) is a novel adipokine associated with obesity, hyperglycemia, insulin resistance, dislipidemia, inflammation, and oxidative stress in mice and humans. However, no report has demonstrated the relationship of circulating Nrg4 with diabetic peripheral neuropathy (DPN). The objective of our study was to investigate the relationship between circulating Nrg4 and DPN in a cross-sectional study. Circulating Nrg4 levels were determined with an enzyme-linked immunosorbent assays kit in 132 newly diagnosed type 2 diabetes mellitus (nT2DM) patients and 41 normal controls (NC group). The associations of circulating Nrg4 with other parameters were also analyzed. Circulating Nrg4 levels were significantly lower in nT2DM patients with no DPN than in NC subjects, and were further markedly decreased in nT2DM patients with DPN ($P < 0.01$ or $P < 0.05$). Circulating Nrg4 levels were progressively decreased with an increasing number of abnormal DPN screening (P for trend < 0.01). Circulating Nrg4 levels correlated negatively with 8-iso-prostaglandin $F_{2\alpha}$ (8-iso-$PGF_{2\alpha}$), high-sensitivity C-reactive protein (hs-CRP) and vibration perception threshold (VPT) (all $P < 0.01$), and 8-iso-$PGF_{2\alpha}$, hs-CRP, glycated hemoglobin A1c and VPT were independently related factors to circulating Nrg4 in nT2DM patients ($P < 0.01$ or $P < 0.05$). Moreover, circulating Nrg4 was significantly associated with the development of DPN even after controlling for anthropometric, biochemical and clinical parameters. Additionally, the analysis of receiver operating characteristic curves revealed that the best cutoff value for circulating Nrg4 to predict DPN was 1.58 ng/mL (sensitivity 90.91%, specificity 54.55%, and area under the curve 0.716). These findings together suggested that circulating Nrg4 levels were reduced in DPN patients and Nrg4 may be a novel adipokine associated with inflammation, oxidative stress, and long-term glycemic control in nT2DM patients.

1. Introduction

Diabetic peripheral neuropathy (DPN) is consequence of diabetes-induced large and small, myelinated and unmyelinated, and nerve fiber injury, and is one of the most common and perplexing chronic complications of diabetes, which occurs in up to 50% of patient with diabetes [1]. DPN is a leading cause of foot ulcers and amputations as well as falls among the elderly, which results in escalating health care costs, substantial socioeconomic consequences including loss of work time, and poor quality of life [2,3]. Thus, DPN is now becoming an increasingly important global alarming public health concern. The pathogenetic mechanisms underlying DPN remain poorly understood, although several mechanisms, including metabolic, microangiopathy, inflammation, oxidative stress, mitochondrial dysfunction, autoimmune, and insufficiency of neurotrophic support, have been proposed to be involved in the development of DPN [1,4,5]. Currently, therapy is limited to intensive glycemic control and symptomatic treatments. Therefore, it is critical to identify novel risk factors associated with DPN to facilitate the early detection of individuals at risk, and further prevent or reverse DPN.

Neuregulin-4 (Nrg4) has recently been recognized as a novel and predominantly brown adipose tissue (BAT)-secreted adipokine. It is also reported that Nrg4 is a member of the epidermal growth factor (EGF) family of extracellular ligands, which binds to and activate the receptor tyrosine kinases ErbB3 and ErbB4, and acts via paracrine and/or autocrine and/or endocrine mechanisms after the proteolytic cleavage of the EGF-like domain [6,7]. Nrg4 expression level was significantly enhanced by cold exposure in white fat tissue, highly expressed during

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brown adipocyte differentiation, and further increased by adrenergic receptor activation in brown adipocytes [8,9]. Increasing evidence has shown that Nrg4 exerts a key role in regulating systemic energy balance and glucose and lipid metabolism, and reducing chronic inflammation, and decreased Nrg4 levels have been reported to contribute to obesity, insulin resistance (IR), hyperglycemia, dislipidemia, oxidative stress and inflammation, macrovascular in animals and humans [6,7,9,10–15], all of which have been proposed to be implicated in the pathogenesis of DPN [1,16]. More importantly, neuregulins along with ErbB-3 and ErbB-4 receptors are highly expressed in the developing and mature nervous system, and several studies demonstrate that axon-derived neuregulin promotes oligodendrocyte survival in the developing rat optic nerve and that neuregulin in the central nervous system diminishes autoimmune demyelination, promotes oligodendrocyte progenitor expansion, and enhances remyelination [17]. It is also reported that neuregulin affects neuronal survival and neurite outgrowth of developing rat retina [17]. These data strongly support the idea that neuregulins have cyto- and neuroprotective properties in multiple cell types including neurons and glia. Nrg4 is a member of the neuregulin family (Nrg1-Nrg4), and may play a relevant role in neuronal and oligodendrocyte survival, neurite outgrowth, and myelin protection. In line with the this hypothesis, there is preliminary evidence that Nrg4-contained medium from differentiated brown adipocytes promotes neurite outgrowth in vitro studies [18]. Similar findings were obtained in PC12-HER4 cells (a cell line derived from a rat adrenal medullary pheochromocytoma and stably expressing ErbB-4) [19]. Furthermore, a neurotrophic role for Nrg4 is supported by studies of the related Nrg1 that found it affected the development of neuronal progenitor stem cells [20]. Additionally, Paramo et al. found that Nrg4 was expressed in developing brain and was a major physiologically relevant regulator of the growth and elaboration of pyramidal neuron dendrites in the developing neocortex [21]. On the basis of the aforementioned findings, we hypothesized that plasma Nrg4 levels would be negatively associated with the development of DPN, however, no study has explored whether decreased plasma Nrg4 level may serve as a risk marker for DPN and to what extent it is associated with DPN.

Therefore, in the current study, we aimed to explore the relationship between plasma Nrg4 levels and the risk of DPN by conducting a cross-sectional population study of 132 Chinese patients with newly diagnosed T2DM (nT2DM). Additionally, the association between plasma Nrg4 levels and various risk factors for DPN was evaluated in nT2DM patients.

2. Patients and methods

2.1. Study participants

132 patients with nT2DM between 30 and 89 years of age, attending the inpatient department of Endocrinology at the Affiliated Hospital of Southwest Medical University between January 2016 and January 2017, were enrolled in the cross-sectional study. The diagnosis of T2DM was based on 75 g oral glucose tolerance tests (OGTT) and the American Diabetes Association criteria. For the current analysis, subjects with nT2DM were subsequently divided into two groups: patients with diabetes but without peripheral neuropathy (no DPN group, n = 66) and patients with diabetes and peripheral neuropathy (DPN group, n = 66). The subjects with nT2DM didn't receive any anti-diabetic therapy, including diet control, exercise, hypoglycemic agents and insulin. Excluded were patients with type 1 diabetes and other endocrine disorders, acute complications of diabetes such as ketoacidosis and hyperosmolar state, absent foot pulses, foot ulcers, limb amputation, hypertension, cardiovascular and cerebrovascular disease, nonalcoholic fatty liver disease, hepatic and renal failure, liver cirrhosis, inflammatory diseases, active infection, cancer, pregnancy or lactation, current treatment with immunosuppressive medications and systemic corticosteroids, prior history of knee or back surgery, any other etiological causes of peripheral neuropathy (e.g., toxin exposure, hereditary, carpal tunnel syndrome, peripheral polyneuritis, infectious polyneuritis, and vasculitis, cervical spondylosis, and lumbar spondylosis), cigarette smoking, alcohol consumption, and patients with history of use of lipid-lowering agents, and use of any drugs known to interfere with oxidant/antioxidant system and peripheral nerve function.

41 sex-, age- and body mass index (BMI)-matched healthy, non-diabetic subjects served as normal control group (NC group). All control subjects were confirmed to have normal glucose tolerance via 75 g OGTT. These subjects were in good general health and had normal kidney, liver and cardiovascular function, and have no family history of T2DM, and other metabolic disorders. None of the control subjects were taking medications known to affect glucose tolerance, lipid metabolism, oxidant/antioxidant system and peripheral nerve function.

The study was performed in accordance with the ethical guidelines of the 1975 Declaration of Helsinki and was reviewed and approved by the human research ethics committee of the Affiliated Hospital of Southwest Medical University, and informed consent was obtained from all nT2DM patients and nondiabetic subjects before any study-related procedure.

2.2. Anthropometric and biochemical measurements

All participants completed a standardized medical history and lifestyle factors (smoking and alcohol consumption) questionnaire and conducted a comprehensive physical examination according to standard procedures. Data regarding age and gender were collected and recorded. Anthropometric measurements, including body weight, height, body mass index (BMI), and blood pressure, were performed with the use of standard methods, as described previously [22]. Blood samples were collected from study participants in the morning either after an overnight fast of at least 8 h or 2 h after a 75 g OGTT. Blood samples were collected by centrifugation at 3500 rpm for 10 min at 4 °C and stored at −80 °C until analytical processing.

Fasting blood glucose (FBG), postprandial 2 h blood glucose (PBG), and glycated hemoglobin A1C (HbA1c) were measured by the glucose-oxidase method and anion exchange high performance liquid chromatography, respectively (arkray ELUENT 80A, Japan). Plasma concentrations of fasting insulin (FIns) were measured using an electrochemiluminescence immunoassay (Roche Elecsys Insulin Test, Roche Diagnostics, Mannheim, Germany). The homeostasis model assessment of insulin resistance (HOMA-IR), a marker of IR, was calculated using the following formula: HOMA-IR = FIns (μU/ml) × FBG (mmol/L)/22.5 [23]. Blood lipid profiles, including total cholesterol (TC), triglyceride (TG), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), total bilirubin (TBIL), blood urea nitrogen (BUN), and creatinine (Cr) were analyzed enzymatically using a 7060 full-automatic biochemical analyzer (Hitachi, Tokyo, Japan) at a certified laboratory. Circulating high- sensitivity C-reactive protein (hs-CRP) levels were measured by the latex-enhanced immunoturbidimetric assay.

2.3. Measurements of plasma 8-iso-$PGF_{2\alpha}$ and Nrg4

Plasma 8-iso-prostaglandin $F_{2\alpha}$ (8-iso-$PGF_{2\alpha}$) levels were determined with an enzyme-linked immunosorbent assay (ELISA) (CUSABIO, Wuhan, China; Catalog No. CSB-E12100h) in all subjects. The intra- and inter-assay coefficient of variations (CVs) were both less than 15%. The assay has a sensitivity of 125 pg/ml, and the linear range of the standard was 125–5000 pg/ml. Plasma Nrg4 concentrations were also measured with an ELISA (Aviscera Biosciences, Santa Clara, CA). The intra- and inter-assay CVs were both less than 10%. The assay has a sensitivity of 0.125–0.25 ng/mL, and the linear range of the standard was 0.25–16.0 ng/mL.

Table 1
Main clinical features and circulating Nrg4 levels in NC, no DPN and DPN subjects.

Variables	NC	no DPN	DPN	*P*
	(n = 41)	(n = 66)	(n = 66)	
Male/Female	23/18	40/26	39/27	0.899
Age (years)	59.15 ± 16.45	63.14 ± 8.64	64.56 ± 10.28	0.196
BMI (kg/m^2)	24.23 ± 3.95	24.87 ± 4.11	25.84 ± 3.17	0.082
SBP (mmHg)	121.00 ± 11.01	123.86 ± 10.70	123.30 ± 9.26	0.355
DBP (mmHg)	70.98 ± 7.57	73.64 ± 8.86	72.30 ± 8.77	0.288
FBG (mmol/L)	4.80 ± 0.40	10.59 ± 3.25**	11.47 ± 3.56**	0.000
PBG (mmol/L)	6.08 ± 0.84	18.32 ± 6.28**	18.96 ± 5.27**	0.000
HbA1c (%)	5.44 ± 0.38	9.87 ± 2.22**	9.92 ± 2.24**	0.000
FIns (μU/ml)	7.42 ± 3.74	11.57 ± 6.38**	9.95 ± 6.00	0.001
HOMA-IR	1.59 ± 0.80	5.34 ± 3.45**	4.94 ± 3.15**	0.000
TC (mmol/L)	4.47 ± 1.13	4.33 ± 1.28	4.50 ± 1.16	0.687
TG (mmol/L)	1.74 ± 1.11	2.15 ± 1.82	2.13 ± 2.06	0.467
HDL-C (mmol/L)	1.21 ± 0.47	1.12 ± 0.27	1.10 ± 0.33	0.289
LDL-C (mmol/L)	2.74 ± 0.85	2.59 ± 1.00	2.79 ± 0.91	0.466
BUN (mmol/L)	6.13 ± 2.38	6.29 ± 1.66	6.79 ± 2.48	0.246
Cr (μmol/L)	71.57 ± 17.18	70.27 ± 19.36	72.71 ± 20.69	0.770
TBIL (μmol/L)	13.10 ± 4.60	13.34 ± 6.23	13.45 ± 7.07	0.747
8-iso-PGF$_{2\alpha}$ (μmol/L)	142.30 ± 43.11	170.38 ± 70.00*	205.73 ± 70.45$^{**\#}$	0.000
hs-CRP (mg/L)	2.35 ± 1.66	4.21 ± 3.21**	6.25 ± 4.77$^{**\#}$	0.000
VPT (v)	–	14.65 ± 7.49	23.55 ± 11.91$^{\#\#}$	–
VPT > 25 V, n(%)	–	7 (10.61)	37 (56.06) $^{\#\#}$	–
Abnormal pricking sensation, n (%)	–	24 (36.36)	66 (100.00) $^{\#\#}$	–
Abnormal nylon monofilament, n (%)	–	0 (0)	33 (50.00) $^{\#\#}$	–
reflex abnormalities, n (%)	–	0 (0)	11 (16.67) $^{\#\#}$	–
Two abnormal DPN screening, n (%)	–	0 (0)	53(80.30) $^{\#\#}$	–
Three or more abnormal DPN screening, n (%)	–	0 (0)	13(19.70) $^{\#\#}$	–

Data are mean ± SD. SD standard deviation, BMI body mass index, SBP systolic blood pressure, DBP diastolic blood pressure, FBG fasting blood glucose, PBG postprandial 2 h blood glucose, HbA1c glycated hemoglobin A1c, FIns fasting plasma insulin, HOMA-IR homeostasis model assessment of insulin resistance, TC total cholesterol, TG triglyceride, HDL-C high-density lipoprotein cholesterol, LDL-C low-density lipoprotein cholesterol, BUN blood urea nitrogen, Cr creatinine, TBIL total bilirubin, 8-iso-PGF$_{2\alpha}$ 8-iso- prostaglandin F$_{2\alpha}$, hs-CRP high-sensitivity C-reactive protein, Nrg4 Neuregulin-4, VPT vibration perception threshold, DPN diabetic peripheral neuropathy. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. NC group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. no DPN group.

2.4. Definition of DPN

All nT2DM patients were asked whether they had symptoms including distal, symmetric, “glove and stocking” distributed loss of sensation or decreased sensation, numbness, pain, paresthesia, tingling, and burning in patients’ feet, legs and upper-limb, and underwent physical examination such as ankle and knee reflexes (as being either presence or weakening or loss). Then, quantitative sensory testing (QST) was performed to screen for peripheral neuropathy in all nT2DM subjects. Vibrating perception threshold (VPT) values were determined by a neurothesiometer (Bio-Thesiometer; Bio-Medical Instrument Co., Newbury, OH, USA) according to previously published methods. A VPT value of > 25 V on either limb was considered abnormal [24]. Moreover, 10 g of light touch pressure on the plantar surface of of the foot was determined using a 5.07-gauge Semmes-Weinstein monofilament (SWM) according to standard testing procedures. If the subject can't perceive 10 g of pressure on any one of four test sites on the plantar surface of both feet (great toe, first metatarsal head, third metatarsal head, and fifth metatarsal head), indicative of their having abnormal light touch pressure sensation. Also, 40 g needle test was performed to examine the pricking sensation on the local skin in the lower limbs and legs, and rated as normal or abnormal. The same experienced physician performed all the above measurements. Patients were diagnosed as having DPN, as described previously [24–28]. The nT2DM subjects were subsequently divided into two groups (66 nT2DM subjects with no DPN, and 66 nT2DM subjects with DPN).

2.5. Statistical analysis

All analyses were performed with the Statistical Package for Social Sciences version 20.0 (SPSS, Chicago, IL). All data were first analyzed for normality of distribution using the Kolmogorov-Smirnov test of normality. Data are expressed as mean ± standard deviation (SD) for continuous variables or percentages for categorical variables.

Two-group comparisons were performed with χ^2 test for categorical variables or Student’s *t* test for normally distributed continuous variables or Mann–Whitney *U* test for nonparametric distributed continuous variables. Comparisons among more than two groups were performed with one-way analysis of variance (ANOVA) for normally distributed continuous variables, and Kruskal-Wallis test for nonparametric distributed covariates. The association between plasma Nrg4 and other variables was investigated by univariate correlation analysis. Multiple stepwise linear regression was subsequently performed to examine the independent variables associated with plasma Nrg4 concentrations in nT2DM patients. Multivariable logistic regression analysis was also performed to ascertain the association of plasma Nrg4 levels with risk of DPN. Odds ratios (OR) and 95% confidence intervals (CI) were estimated. Receiver operating characteristic (ROC) curve analysis was performed to determine the optimal cut-off point of plasma Nrg4 levels for the diagnosis of DPN. In all statistical tests, a *P*-value of < 0.05 was considered to be statistically significant (two sided).

For sample size calculation, we used the following formula:

$$N = \frac{(q_1^{-1} + q_2^{-1})(t_{\alpha/2} + t_{\beta})^2 S^2}{\delta^2}$$

where N is the total sample size (the sum of the sizes of both comparison groups). In our study, it is assumed that test level $\alpha = 0.05$, $\beta = 0.1$, and the population mean difference between NC group and nT2DM patients without DPN group is 1.5 ($\delta = 1.5$). Also, we assume that the sample size ratio between NC group and nT2DM patients without DPN group is 1:1.5. Accordingly, q_1 (nT2DM patients without DPN group) = 1.5/(1 + 1.5) = 0.6, and q_2 (NC group) = 1/(1 + 1.5) = 0.4. Previous work has proposed that the SD of Nrg4 for NC

group is 2 (S = 2) [29]. Then, all parameters are included in the above formula, and we get the following result: $\mathbf{N} = \frac{[(0.6)^{-1} + (0.4)^{-1}](1.960 + 1.282)^2 \times (2)^2}{1.5^2} = 78$. From the result, we known that the minimum sample size in NC group is 31, and the minimum sample size in nT2DM patients without DPN group is 47. In other words, this study have test efficacy of 0.90 at a two-sided α of 0.05 as long as the sample size in NC group is ≥31 and the sample size in nT2DM patients without or with DPN group is ≥47.

3. Results

3.1. Anthropometric, biochemical, and clinical parameters of all the subjects

Table 1 summarizes the anthropometric, biochemical and clinical parameters of study subjects. The three groups were similar in anthropometric parameters, blood pressure, lipid profiles, renal function, and TBIL (all $P > 0.05$). When compared with the NC subjects, nT2DM patients with or without DPN had significantly higher FBG, PBG, HbA1C, HOMA-IR, 8-iso-$PGF_{2\alpha}$, hs-CRP, and VPT ($P < 0.01$ or $P < 0.05$). Plasma levels of 8-iso-$PGF_{2\alpha}$, hs-CRP, and VPT value were significantly higher in nT2DM participants with DPN as compared with those without ($P < 0.01$ or $P < 0.05$). As expected, nT2DM participants in the DPN group showed greater prevalence of VPT > 25 V, abnormal pricking sensation, abnormal nylon monofilament, reflex abnormalities, two abnormal DPN screening, and three or more abnormal DPN screening than those in the no DPN group (all $P < 0.01$).

3.2. Plasma Nrg4 level and its association with with anthropometric, biochemical and clinical parameters in study subjects

When compared with the NC subjects, nT2DM patients with or without DPN had decreased plasma levels of Nrg4 (2.23 ± 1.31 vs. 4.17 ± 1.97 ng/ml, $P < 0.01$; 3.28 ± 1.50 vs. 4.17 ± 1.97 ng/ml, $P < 0.05$; Fig. 1A). Moreover, nT2DM patients with VPT > 25 V exhibited significantly lower levels of plasma Nrg4 compared with those with VPT ≤25 V (1.61 ± 0.77 vs. 3.32 ± 1.45 ng/ml, $P < 0.01$; Fig. 1B). Likewise, plasma Nrg4 also declined significantly in nT2DM patients with abnormal pricking sensation compared with those with normal pricking sensation (2.33 ± 1.25 vs. 3.65 ± 1.60 ng/ml, $P < 0.01$; Fig. 1C). As shown in Fig. 1D and Fig. 1E, there is a trend of decrease in circulating Nrg4 levels in nT2DM patients with abnormal nylon monofilament and reflex. However, this difference did not reach statistical significance (all $P > 0.05$). To further assess the association of Nrg4 with DPN, we stratified the mean levels of circulating DPN by the number of abnormal DPN screening. The result indicated that circulating Nrg4 levels were progressively decreased with increasing number of abnormal DPN screening (P for trend < 0.01; Fig. 1F). Next, we analyzed the relationship of plasma Nrg4 levels with various other parameters by using simple correlations. In NC participants or nT2DM patients, plasma Nrg4 correlated significantly and negatively with 8-iso-$PGF_{2\alpha}$ and hs-CRP (all $P < 0.01$; Table 2). A strong negative correlation of circulating Nrg4 levels with VPT was also observed in nT2DM patients ($P < 0.01$; Table 2). Furthermore, we performed multiple stepwise regressions models to identify the main determinants of circulating Nrg4 levels in NC participants and nT2DM patients. The results showed that 8-iso-$PGF_{2\alpha}$, HbA1C and DBP were independent related factors to circulating Nrg4 levels in NC participants (Table 2). The multiple regression equation was: $Y_{Nrg4} = 14.053 - 0.040X_{8\text{-iso-}PGF2\alpha} - 1.465\ X_{HbA1C} + 0.053\ X_{DBP}$ ($R = 0.846$, $R^2 = 0.716$). In addition, 8-iso-$PGF_{2\alpha}$, hs-CRP, HbA1C and VPT were independently associated with circulating Nrg4 levels in nT2DM patients (Table 2). The multiple regression equation was: $Y_{Nrg4} = 6.878 - 0.011X_{8\text{-iso-}PGF2\alpha} - 0.072X_{hs\text{-}CRP} - 0.095X_{HbA1C} - 0.042X_{VPT}$ ($R = 0.778$, $R^2 = 0.605$).

3.3. Multivariable-adjusted ORs for the association of plasma Nrg4 concentration with increased presence of DPN

To assess whether plasma Nrg4 concentrations can decrease the risk of development of DPN, multivariate logistic regression analysis was mapped. As shown in Table 3, the risk of presence of DPN decreased by 42.5% per 1 SD increase in plasma Nrg4 levels before various other parameters adjustment. Importantly, plasma Nrg4 concentrations were significantly associated with the development of DPN even after controlling for anthropometric parameters, renal function, blood pressure, lipid profile, glucose metabolic parameters, markers of inflammation and oxidative stress [OR = 0.616 (95% CI: 0.394–0.965, $P < 0.05$)], indicating that there was a 38.4% decrease in the odds of having DPN for each 1 ng/ml increase in Nrg4 levels.

3.4. The predictive value of circulating Nrg4 in detecting DPN

To explore the predictive value of plasma Nrg4 for DPN, we analyzed the receiver operating characteristic (ROC) curves of plasma Nrg4. The results revealed that the best cutoff value for circulating Nrg4 to predict DPN was 1.58 mg/L (sensitivity 90.91%, specificity 54.55%, and AUC 0.716; Fig. 2).

4. Discussion

To the best of our knowledge, this was the first study to examine the relationship between plasma Nrg4 concentrations and the risk of DPN. We found negative associations between plasma Nrg4 levels and risk of DPN and its pathogenic factors such as hyperglycemia (HbA1c), oxidative stress (8-iso-$PGF_{2\alpha}$) and inflammation (hs-CRP) in type 2 diabetes. Moreover, the association between decreased plasma Nrg4 concentrations and DPN in type 2 diabetes is independent of anthropometric parameters, renal function, blood pressure, lipid profile, glucose metabolic parameters, markers of inflammation and oxidative stress.

As mentioned earlier, Nrg4 is a secreted water-soluble protein that has been found in the circulation. Nrg4 is expressed in multiple organs, with the highest expression levels in brown adipose tissue [30]. In animal models, mice lacking Nrg4 developed reduced insulin sensitivity, impaired glucose tolerance, and hepatic steatosis following high-fat feeding, whereas overexpression of Nrg4 was found to attenuate hepatic lipogenic signaling, maintain glucose and lipid homeostasis, prevent weight gain, and improve IR [10]. In humans, Wang et al. and Zhang et al. demonstrated that levels of Nrg4 mRNA in both subcutaneous and visceral adipose tissues, and circulating Nrg4 levels were significantly reduced in patients with T2DM as compared with healthy individuals with normal glucose tolerance [10,29]. Consistent with that, we found that plasma Nrg4 levels were significantly decreased in patients with nT2DM compared with healthy controls, implying that decreased levels of plasma Nrg4 may play a crucial role in the pathogenesis of nT2DM. Importantly, we also showed that circulating Nrg4 levels were further markedly decreased in nT2DM patients with DPN compared with those with no DPN, further implying that decreased Nrg4 concentrations may be implicated in the development of DPN. DPN is often characterized by damage to both large myelinated Aα and Aβ nerve fibers, as well as small, thinly myelinated C fibers [31]. VPT represents dysfunction in large myelinated nerve fibers [2], and T2DM patients with VPT > 25 V on either limb were considered to have DPN [24,32]. Evidence has suggested that VPT test appears to be an appropriate and reliable measure for early screening DPN [24], and is routinely used as one of the QST to reflect the clinical severity of neuropathy in patients with diabetes [32]. Pricking sensation is a small unmyelinated nerve fiber-related symptom [2] and is captured by 40 g needle test. As expected, we found that nT2DM patients with VPT > 25 V and abnormal pricking sensation had significantly lower levels of circulating Nrg4 compared with those with VPT ≤25 V and normal

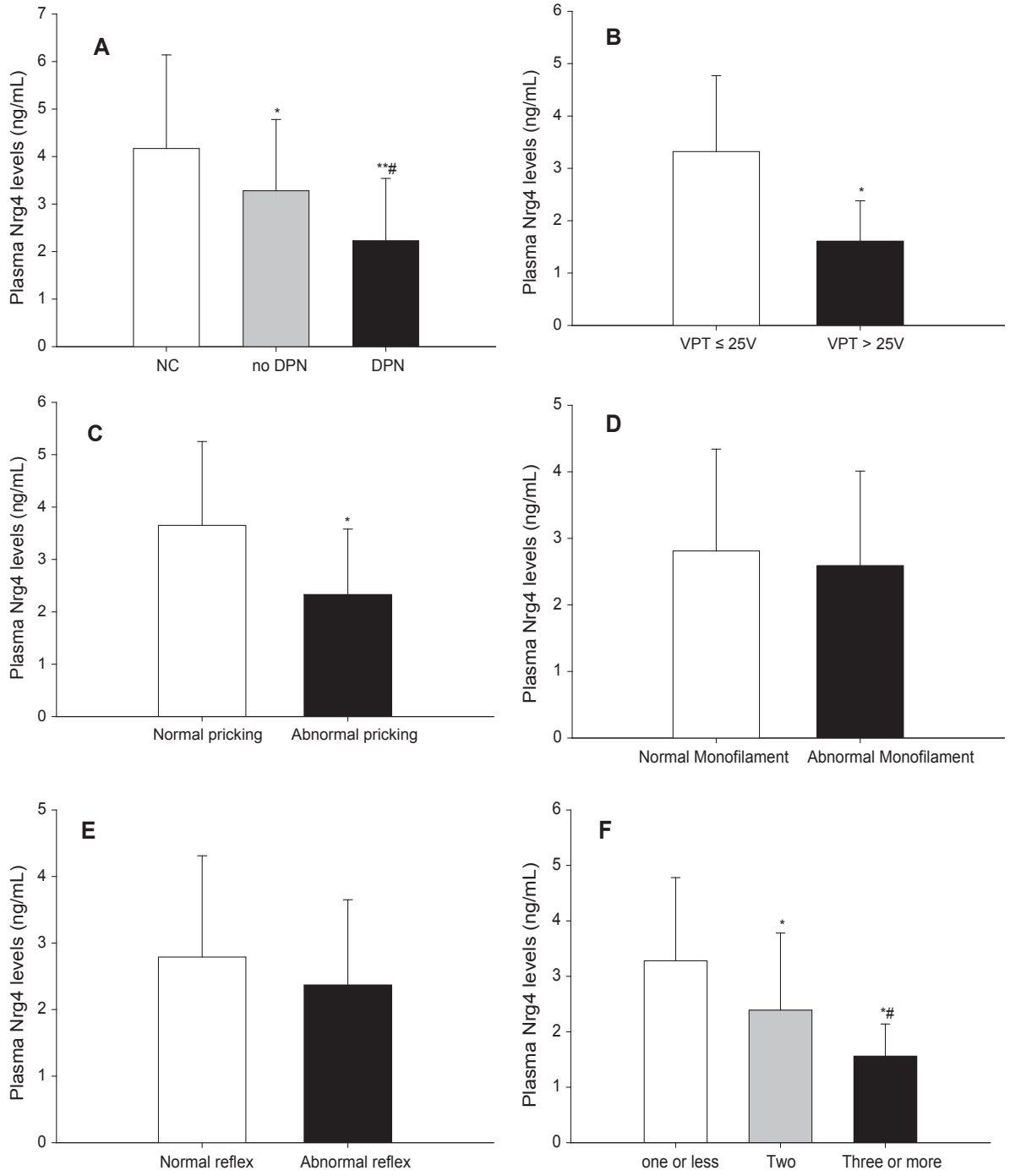

Fig. 1. Circulating Nrg4 levels in study population. (A) Circulating Nrg4 levels in NC, no DPN and DPN subjects. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. NC group; $^{\#}P < 0.01$ vs. no DPN group. (B) Circulating Nrg4 levels in nT2DM patients with VPT > 25 V and VPT ≤25 V. $^{*}P < 0.01$ vs. nT2DM patients with VPT > 25 V. (C) Circulating Nrg4 levels in nT2DM patients with normal and abnormal pricking sensation. $^{*}P < 0.01$ vs. nT2DM patients with normal pricking sensation. (D) Circulating Nrg4 levels in nT2DM patients with normal and abnormal Nylon Monofilament. (E) Circulating Nrg4 levels in nT2DM patientswith normal and abnormal ankle and knee reflexes. (F) Circulating Nrg4 levels in nT2DM patients with one or less, two, and three or more abnormal DPN screening. $^{*}P < 0.01$ vs. nT2DM patients with one or less abnormal DPN screening; $^{\#}P < 0.01$ vs. nT2DM patients with two abnormal DPN screening.

pricking sensation, respectively. Moreover, circulating Nrg4 levels were progressively decreased with an increasing number of abnormal DPN screening. In addition, bivariate correlations showed that circulating Nrg4 levels inversely correlated with VPT, and VPT was independently related factor to circulating Nrg4 in nT2DM patients in multiple linear regression analysis. More importantly, multivariate logistic regression analysis indicated that circulating Nrg4 was significantly associated with the development of DPN even after controlling for anthropometric, biochemical and clinical parameters. Consistently, further analysis of ROC curves revealed that the cutoff point of circulating Nrg4 concentration to predict DPN was 1.58 ng/mL. Collectively, these data demonstrate that, as expected, decreased levels of circulating Nrg4 may be associated with both large and small nerve fibers damage, and may play an important role in the development of DPN. This hypothesis is strongly supported by three previous studies [18–20]. Rosell et al. demonstrated that Nrg4-contained medium from differentiated brown

Table 2
Linear correlation and multiple regression analysis of variables associated with plasma Nrg4 levels in study subjects.

Variable	Simple				Multiple			
	NC		nT2DM		NC		nT2DM	
	r	*P-value*	*r*	*P-value*	*β*	*P-value*	*β*	*P-value*
Sex	−0.258	NS	−0.027	NS				
Age	−0.015	NS	−0.045	NS				
BMI	−0.089	NS	−0.135	NS				
SBP	0.085	NS	0.014	NS				
DBP	−0.022	NS	0.096	NS	0.053	0.032		
FBG	0.023	NS	−0.116	NS				
PBG	0.042	NS	0.013	NS				
HbA1c	−0.155	NS	−0.010	NS	−1.465	0.003	−0.095	0.014
FIns	0.220	NS	0.034	NS				
HOMA-IR	0.217	NS	−0.027	NS				
TC	0.016	NS	0.163	NS				
TG	−0.049	NS	0.025	NS				
HDL-C	−0.140	NS	0.146	NS				
LDL-C	0.190	NS	0.126	NS				
BUN	0.138	NS	−0.150	NS				
Cr	0.062	NS	−0.072	NS				
TBIL	0.034	NS	−0.074	NS				
8-iso-$PGF_{2\alpha}$	−0.778	0.000	−0.674	0.000	−0.040	0.000	−0.011	0.000
hs-CRP	−0.419	0.006	−0.558	0.000			−0.072	0.002
VPT			−0.552	0.000			−0.042	0.000

In multiple linear stepwise regression analysis, values included for analysis in patients of NC group were sex, age, BMI, SBP, DBP, FBG, PBG, HbA1C, FIns, HOMA-IR, TG, TC, HDL-C, LDL-C, BUN, Cr, TBIL, 8-iso-$PGF_{2\alpha,}$ hs-CRP, while values included for analysis in nT2DM patients were sex, age, BMI, SBP, DBP, FBG, PBG, HbA1C, FIns, HOMA-IR, TG, TC, HDL-C, LDL-C, BUN, Cr, TBIL, 8-iso-$PGF_{2\alpha,}$ hs-CRP, and VPT.

Table 3
Association of plasma Nrg4 concentrations with DPN in fully adjusted models.

Model adjust	DPN		
	OR	95% CI	*P-value*
Unadjusted	0.575	0.432–0.765	< 0.001
sex, age, BMI	0.586	0.440–0.782	< 0.001
sex, age, BMI, BUN, Cr	0.593	0.444–0.792	< 0.001
sex, age, BMI, BUN, Cr, SBP, DBP, FBG, PBG, HbA1C, FIns, HOMA-IR, TG, TC, HDL-C, LDL-C	0.574	0.421–0.783	< 0.001
sex, age, BMI, BUN, Cr, SBP, DBP, FBG, PBG, HbA1C, FIns, HOMA-IR, TG, TC, HDL-C, LDL-C, hs-CRP	0.605	0.420–0.873	0.007
sex, age, BMI, BUN, Cr, SBP, DBP, FBG, PBG, HbA1C, FIns, HOMA-IR, TG, TC, HDL-C, LDL-C, hs-CRP, TBIL, 8-iso-$PGF_{2\alpha}$	0.616	0.394–0.965	0.034

Results of multivariate logistic regression analysis are presented as the odds ratio of being in DPN status decrease in plasma Nrg4. OR odds ratio; CI, Confidence interval.

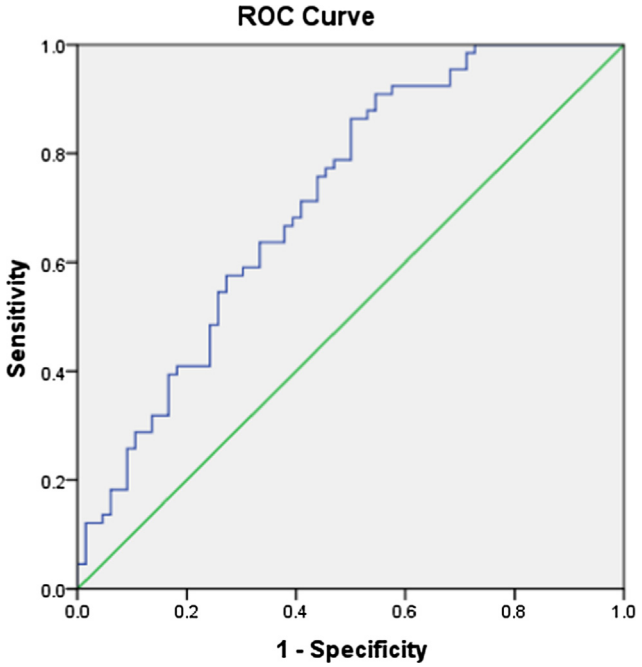

Fig. 2. Receiver operating characteristic (ROC) curve analysis was performed for the prediction of DPN in all nT2DM patients.

adipocytes promotes neurite outgrowth in vitro studies [18]. Another report, performed in conditioned medium from transfected Cos7 cells or a chemically synthesized and refolded peptide, also revealed that Nrg4 can elicit neuronal outgrowth in PC12-HER4 cells [19]. These results, together, indicate that Nrg4 may play a crucial role in the development of the innervation net in adipose tissues. Additionally, a neurotrophic role for Nrg4 is also supported by the study of the related Nrg1 that found it affected the development of neuronal progenitor stem cells [20], prompting us to speculate that Nrg4 deficiency may be involved in the development of DPN because insufficiency of neurotrophic support is closely related to the risk of DPN. More prospective longitudinal studies should be performed to confirm our findings and further dissecte the specific mechanism of action.

Numerous experimental and clinical studies have provided solid evidence of an important role for chronic low-grade inflammation in the pathogenesis of DPN [1,5]. There are numerous reports demonstrating altered Nrg4 expression during intestinal inflammation [33–35]. In an early report, Bernard et al. and Schumacher et al. described decreased expression levels of Nrg4 in patients with inflammatory bowel disease (Crohn's disease and ulcerative colitis) and colitic animals compared with normal controls and wild type controls, respectively, and Nrg4 administration reduced the severity of colitis induced by acute dextran sulfate sodium through reducing macrophage numbers and levels of inflammatory factors such as tumor necrosis factor α (TNFα), interferon (IFN) γ, and interleukin-6 (IL-6) in C57Bl/6 mice [33,34]. Similar results were obtained for changes in Nrg4 levels in a mouse model of small intestinal inflammation induced by total

parenteral nutrition [35]. Additionally, the study by McElroy and coworkers showed that Nrg4 can reduce the incidence and severity of necrotizing enterocolitis induced by dithizone/klebsiella in CD-1 mouse and formula feeding/hypoxia in newborn Sprague-Dawley rats [14]. These findings raise the possibility that Nrg4 may be protective against induced intestinal inflammation, and inflammation may also contribute to the decline in Nrg4 expression. In support of this, Ma and colleagues conducted preventive and treatment studies, and stated that overexpression of Nrg4 can reduce chronic inflammation induced by a high fat diet in C57BL/6 mice through inhibiting macrophage marker monocyte chemotactic protein 1 (Mcp1) gene expression, and enhancing M2 macrophage marker gene Cd163 expression [36]. Moreover, Wang et al., Chen et al. and Nugroho et al. revealed that differentiated 3T3-L1 adipocyte Nrg4 expression was suppressed by pro-inflammatory cytokines such as TNFα, and antiinflammatory agents such as rosiglitazone exert an opposite effect [10,15,37]. Recently, Guo et al. found that mRNA expression of genes involved in hepatic inflammation such as Tnfa, Il1b, Il12b, Nos2, Ccl2, Ccl5, and Adgre1 (F4/80) were significantly elevated in the livers from Nrg4-null mice upon nonalcoholic steatohepatitis diet feeding, whereas the abundance of F4/80+ macrophage was significantly reduced in Nrg4-transgenic mice [38], illustrating that Nrg4 may attenuate liver inflammation. More recently, a clinical study from our laboratory revealed that lower plasma Nrg4 levels may be associated with elevated levels of hs-CRP, an indicator of inflammation, in nT2DM patients, and hs-CRP was the factor most strongly associated with plasma Nrg4 levels [30]. In accordance with previous researches above, in this study, we found that nT2DM subjects with DPN had significantly higher inflammatory marker such as hs-CRP than those with no DPN, and plasma Nrg4 correlated negatively with hs-CRP in nT2DM patients. Furthermore, multiple linear regression analysis revealed that hs-CRP was independently associated with circulating Nrg4 levels in nT2DM patients. Based on these findings in our study and previous ones, we extrapolate that the association between circulating Nrg4 and inflammatory markers may provide new insights into the potential involvement of anti-inflammatory effect of Nrg4 in the pathogenesis of DPN in type 2 diabetes.

Hyperglycemia is an important mechanism in the pathogenesis of DPN, and control of hyperglycaemia is fundamental for long-term prevention and management of DPN [16,39]. It is well established that long-term hyperglycaemia can lead to functional and structural deficits in peripheral nervous system in a progressive manner, and increase the likelihood of DPN [1,40]. HbA1c as an index of long-term glycemic control has been shown to be related to the incidence and prevalence of diabetic peripheral polyneuropathy in both cross-sectional and prospective epidemiological studies that primarily include patients with type 1 diabetes [16,32]. It has been reported that Nrg4-deficient mice following high-fat feeding exhibited significant increases in body weight, FBG levels, and worsening of IR, but Nrg4-overexpressing mice displayed the opposite results [10], suggesting that Nrg4 may maintain glucose homeostasis, and improve IR, and the positive association between decreased circulating Nrg4 and DPN may be attributed to elevated blood glucose associated with IR. Unfortunately, our results did not demonstrate significant associations between plasma Nrg4 and glucose metabolic parameters (FBG, PBG, HbA1C, FIns, and HOMA-IR) in simple correlation analysis, which was coincident with those of studies by Dai et al. [8] and we et al. [30], and was in contrast with the results from Zhang et al. [29] and Kralisch et al. [41]. The conflicting findings may be partly explained by differing study designs, ELISA measurements, selection of patients, duration of diabetes, medication use, and the presence or absence of complications. Of great interest is the finding that HbA1C was negatively and independently related factor to circulating Nrg4 in NC subjects or nT2DM patients in multiple linear regression analysis, demonstrating that long-term glycemic control may affect the levels of plasma Nrg4 in NC subjects or nT2DM patients, and providing further evidence that degree of blood glucose control (as assessed by HbA1c) may, at least in part, mediate the association between plasma Nrg4 and DPN in type 2 diabetes. However, the underlying mechanism is not fully understood, and warrants further study.

It is generally recognized that increased oxidative stress is considered the key player in the pathogenesis of DPN [16,42]. The increased oxidative stress in diabetes includes not only oxygen free radical generation due to nonenzymatic glycosylation (glycation), autoxidation of glycation products, and nonenzymatic peroxidation, but also changes in the tissue content and activity of antioxidant defence systems. Evidence suggests that increased oxidative stress may contribute to nerve blood flow and nerve conduction deficits, small sensory nerve fiber dysfunction, and morphological manifestations of DPN [43]. It has also been proved that early neuropathic changes can be reversed not only by antioxidants but also by agents counteracting downstream effectors of oxidative stress, e.g., PARP activation [44]. Bilirubin, an end metabolic product of heme metabolism, has been known to play an important physiologic role as an important endogenous antioxidant through efficiently scavenging of peroxyl radicals and suppression of oxidation [45]. 8-iso-$PGF_{2\alpha}$, a stable end product of nonenzymatic peroxidation of arachidonic acid in membrane phospholipids, is widely regarded as reliable indicator of oxidative stress in vivo [42,46]. A growing body of evidence suggests that both bilirubin and 8-iso-$PGF_{2\alpha}$ are related to the development of DPN. Consistent with a previous study, we found that plasma 8-iso-$PGF_{2\alpha}$ levels significantly elevated in nT2DM patients compared with NC subjects, and plasma levels of 8-iso-$PGF_{2\alpha}$ were further markedly increased in nT2DM participants with DPN, but the difference in TBIL among the three groups was not statistically significant, supporting the notion that increased oxidative stress, as measured by the elevation in 8-iso-$PGF_{2\alpha}$ but not the decrease in TBIL, is involved in the pathogenesis of DPN. Moreover, our study, for the first time, showed that circulating Nrg4 levels correlated negatively with 8-iso-$PGF_{2\alpha}$, and 8-iso-$PGF_{2\alpha}$ was independently related factor to circulating Nrg4 in NC subjects ornT2DM patients. These data, altogether, suggest that decreased levels of circulating Nrg4 seem to could have contributed to an increase in oxidative stress in DPN patients. This hypothesis is strongly supported by one of our early report demonstrating that age- and sex-adjusted plasma Nrg4 concentrations in nT2DM patients were negatively correlated with levels of gamma-glutamyltransferase, a marker of oxidative stress [6]. Also, Slattery et al. recently reported that Nrg4 gene expression was upregulated among patients with high oxidative balance scores (lower oxidative stress) compared to patients with lower scores [11]. Thus, these data suggest that circulating Nrg4 may serve as a novel marker associated with oxidative stress in vivo, and increased oxidative stress may be a pivotal mechanism linking decreased levels of circulating Nrg4 to DPN in nT2DM patients. Future research is required to confirm our findings and to better understand the mechanisms associated with a possible beneficial effect of circulating Nrg4 on DPN.

Our study has some limitations that need to be discussed. Firstly, our findings must be interpreted with caution because the sample size is small, and the cross-sectional study design makes it hard to resolve issues of causality between plasma Nrg4 levels andDPN. Thus, larger prospective studies are needed to confirm our findings. Secondly, our findings may not be generalizable to other ethnic populations because the study was based on Chinese Han nT2DM patients at a single center, and plasma Nrg4 levels may be dependent on several factors associated with ethnicity. Thirdly, the current gold standard for the diagnosis and assessment of the severity of DPN, nerve conduction studies, is less frequently used and difficult to apply in large studies. The QST (VPT, 10 g Semmes-Weinstein monofilament, and 40 g needle test) is not the gold standard for diagnosing or staging DPN, but it has been validated against nerve conduction studies and has been used widely in clinical screening studies. We choosed to use neurological symptoms and the QST (in concert with ankle and knee reflexes) to define neuropathy because it can offer an opportunity to evaluate neurological dysfunction of different types of nerve fibers. Fourthly, all biochemical parameters were measured only once, a common practice in clinical studies to

alleviate the economic burden for patients, which may underestimate the true association because of this variability. Fifthly, we did not use clinical neuropathy rating scores such as Toronto Clinical Scoring System to quantify the severity of diabetic neuropathy because the temperature and joint position sensation are not currently available in this study. Nevertheless, our study has several strengths. First of all, we tentatively defined DPN using a combination of neuropathic symptoms, signs, andQST, which can comprehensively reflect the damage to both large and small nerve fiber- related, and make up for the deficiency of nerve conduction studies that can provide information on only a large-fiber dysfunction, and may not be sensitive enough to detect minor degrees of peripheral nerve impairments early in the course of neuropathy. Most importantly, our study is, to our knowledge, the first to evaluate the association between plasma Nrg4 level and DPN in Chinese nT2DM patients.

In summary, the present study showed a reverse association between plasma Nrg4 levels and DPN in nT2DM patients. Moreover, 8-iso-$PGF_{2\alpha}$, hs-CRP and HbA1C were independently related factors to circulating Nrg4 in nT2DM patients, suggesting that decreased levels of Nrg4 may trigger the development of DPN through its prooxidative, proinflammatory, and elevating blood glucose effects, and plasma Nrg4 may serve as an additional tool in the early detection, prevention, and treatment of DPN. More well-designed prospective longitudinal studies are warranted to confirm our findings and further define the contribution of Nrg4 in the development of DPN.

Conflict of interests

The authors have no relevant conflicts of interest to disclose.

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