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ORIGINAL ARTICLE

Circulating netrin-1 levels are reduced and related to corneal nerve fiber loss in patients with diabetic neuropathy

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ABSTRACT

Aims/Introduction: Deficiency of neurotropic factors is implicated in diabetic neuropathy (DN). Netrin-1 is a neurotropic factor, but its association with DN has not been explored. We have assessed the association between serum netrin-1 levels and early diabetic neuropathy assessed by quantifying corneal nerve fiber loss using corneal confocal microscopy.

Materials and Methods: A total of 72 participants with type 2 diabetes, without and with corneal nerve fiber loss (DN– $n = 42$, DN+ $n = 30$), and 45 healthy controls were studied. Serum netrin-1 levels were measured by enzyme-linked immunosorbent assay, and corneal nerve morphology was assessed using corneal confocal microscopy.

Results: Corneal nerve fiber density, branch density, fiber length and serum netrin-1 levels were significantly lower in the DN– and DN+ groups compared with controls ($P < 0.001$). Netrin-1 levels correlated with corneal nerve fiber length in the DN+ group ($r = 0.51$; $P < 0.01$). A receiver operating characteristic curve analysis showed that a netrin-1 cut-off value of 599.6 (pg/mL) had an area under the curve of 0.85, with a sensitivity of 76% and specificity of 74% ($P < 0.001$; 95% confidence interval 0.76–0.94) for differentiating patients with and without corneal nerve loss.

Conclusions: Serum netrin-1 levels show a progressive decline with increasing severity of small nerve fiber damage in patients with diabetes. Netrin-1 could act as a biomarker for small nerve fiber damage in DN.

INTRODUCTION

Diabetic neuropathy (DN) is associated with painful neuropathy and foot ulceration[1]. The underlying pathogenesis is complex, and there is currently no US Food and Drug Administration-approved disease-modifying treatment for DN[2]. There is also an unmet need for the early diagnosis of DN[3]. Although nerve conduction studies are considered to be the gold standard for evaluating DN, they do not evaluate small fibers, which are the earliest to be damaged in DN[4]. Intra-epidermal nerve fiber density assessment enables quantification of small nerve fiber damage, but skin biopsy is an invasive procedure[5]. Corneal confocal microscopy (CCM) is a rapid, non-invasive ophthalmic imaging method that allows quantification of small nerve fibers[6] that has good diagnostic utility for identifying DN[7] and painful DN[8], but also predicts the development and progression of DN[9,10].

Netrins play an important role in regulating cell migration, cell adhesion and cell survival, and netrin-1 has been linked to diabetic retinopathy[11] and nephropathy[12]. However, a recent meta-analysis showed no change in circulating netrin-1 levels in patients with type 2 diabetes, and increased levels in those with microalbuminuria and macroalbuminuria[13]. Netrin-1 was first discovered as the main cue for axonal guidance[14], and recent studies show that it plays an important role in Schwann cell proliferation, migration and peripheral nerve regeneration[15]. A study of skin and sural nerve biopsies from patients with peripheral neuropathies showed reduced gene expression of the

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netrin family, which correlated with intra-epidermal nerve fiber density, and was lowest in painful compared with painless neuropathy[16]. However, in a recent study from the same group, netrin-1 gene expression was found to be higher in keratinocytes of patients with small fiber neuropathy, and was shown to reduce murine sensory neurite outgrowth *in vitro*[17].

The role of netrin-1 in DN has not been studied to date. We have undertaken a detailed analysis of circulating netrin-1 levels in relation to the severity of small nerve fiber loss in patients with diabetes.

MATERIALS AND METHODS

Participant selection

A total of 117 participants (control, $n = 45$, type 2 diabetes without small nerve fiber loss [DN–], $n = 42$ and with small nerve fiber loss [DN+], $n = 30$) attending the diabetes clinic of IPGME&R and SSKM Hospital, Kolkata, India, participated in the present study. Participants with type 1 diabetes, pregnancy, thyroid disease, gastrointestinal disease, chronic kidney disease, cardiovascular disease, history of a neurological disorder due to a non-diabetic cause, as well as past corneal surgery or corneal dystrophy were excluded. The study protocol was approved by the institutional ethics and research oversight committee. All study participants provided written informed consent in their own vernacular.

Demographic and medical data

All participants underwent physical examination, including measurement of height, weight, body mass index, body fat percentage (bioelectrical impedance method) and blood pressure. Glycated hemoglobin was analyzed by high-performance liquid chromatography and fasting plasma glucose was measure by the glucose oxidase–peroxidase method. Liver function tests and lipid profile were assessed using the Randox kit on a semi autoanalyzer (Randox Laboratories India Pvt. Ltd, Bangaluru, India). The Douleur Neuropathique 4 (DN4) questionnaire was applied, with a DN4 score <4 showing the absence of painful polyneuropathy. Vibration perception threshold (VPT) and warm and cold perception thresholds were evaluated on each foot.

Corneal confocal microscopy and small nerve fiber analysis

All participants underwent examination with the Heidelberg Retina Tomograph 3 Rostock Cornea Module. A trained expert who was blinded to the clinical and biochemical profile of the study participants undertook CCM. The participants' eyes were anesthetized with a drop of proparacaine hydrochloride (0.5% w/v) ophthalmic solution. A drop of hydroxypropyl methylcellulose (hypromellose) 0.3% ophthalmic gel was placed between the lens and the TomoCap to ensure air-free contact. The lens was slowly moved toward the cornea until it was touched, and then, by adjusting the focal plane ring, images were captured from various depths and multiple points. Three non-overlapping, high-quality, high-contrast images from the central corneal sub-basal nerve plexus from each eye were selected for analysis using established methodology[18].

Quantification of corneal nerve parameters was undertaken using fully automated image analysis software (ACCMetrics, V.2.0; University of Manchester, Manchester, UK)[19]. Corneal nerve fiber density (CNFD; the number of main nerve fibers/mm^2), corneal nerve branch density (CNBD; the total number of main nerve branches/mm^2) and corneal nerve fiber length (CNFL; corneal nerve fiber length/mm^2) were analyzed. A representative image and its analyzed version are shown in Figure S1. The threshold value for abnormal CNFL was 2 standard deviations below the mean of the control group. Participants with type 2 diabetes were stratified using this threshold into those with (DN+) and without (DN–) corneal nerve fiber loss.

Measurement of serum netrin-1 level

Serum netrin-1 levels were determined using an enzyme-linked immune sorbent assay method with a commercial kit from Cusabio (Wuhan Huamei Biotech Co., Ltd., Wuhan, China) following the manufacturers protocol. Absorbances were read using a 450-nm filter on a microplate reader by Erba Lisa Scan EM (Transasia Bio-Medicals Limited, Mumbai, India).

Statistical analysis

The 'Kolmogorov–Smirnov test' was carried out to determine the assumption of normality for the data, obtained from the three groups. Data are presented as the mean ± standard deviation. The one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test (parametric) or Kruskal–Wallis (non-parametric) test. Dunn's multiple comparisons test (non-parametric) and unpaired *t*-test (parametric) were used to test for significant differences between groups. The Pearson's or Spearman's correlation test was used to determine the correlation between variables. Receiver operating characteristic (ROC) curve and area under the curve (AUC) analysis was carried out to assess the diagnostic utility of netrin-1 for DN. Statistical analysis was carried out using GraphPad Prism software (V.9.1.0; San Diego, CA, USA). A value of $P < 0.05$ was considered to be statistically significant.

RESULTS

Clinical demographic parameters

The general characteristics of study participants are shown in Table.1 The mean age was significantly higher in the DN+ (53.20 ± 8.02 years, $P = 0.01$) and DN– (49.36 ± 10.34 years, $P < 0.001$) groups compared with controls (43.07 ± 10.53 years). There was no difference in diabetes duration (years) between the DN– and DN+ groups. Fasting plasma glucose and glycated hemoglobin were significantly higher in the DN– and DN+ groups compared with controls. There was no correlation between age, glycated hemoglobin, fasting plasma glucose, duration of diabetes and serum netrin-1 levels (Table S1).

Table 1 | Clinical and metabolic parameters among the study participants

Parameters	Control (n = 45)	DN– (n = 42)	DN+ (n = 30)	P-value
Age (years)	43.07 ± 10.53	49.36 ± 10.34	53.20 ± 8.01	0.0001
Height (cm)	159.4 ± 9.27	161.4 ± 7.59	156.8 ± 9.00	NS
Weight (kg)	65.58 ± 12.86	68.17 ± 10.15	61.59 ± 9.34	NS
BMI	25.86 ± 4.932	26.18 ± 3.720	24.61 ± 2.55	NS
Body fat%	29.74 ± 9.81	28.99 ± 8.56	29.88 ± 8.87	NS
SBP (mmHg)	122.7 ± 11.94	131.9 ± 17.00	126.9 ± 18.21	NS
DBP (mmHg)	80.00 ± 7.754	85.14 ± 10.19	79.32 ± 9.89	0.0396
FPG (mg/dL)	79.37 ± 11.31	134.3 ± 48.59	151.6 ± 67.81	<0.0001
HbA1c (%)	5.21 ± 0.28	7.57 ± 1.18	7.94 ± 1.79	<0.0001
HbA1c mmol/mol	33 ± 3.1	59 ± 12.9	63 ± 19.6	
Duration of diabetes (years)	–	6.750 ± 5.528	9.519 ± 6.87	NS
SGOT (U/L)	28.35 ± 13.31	35.90 ± 26.17	27.38 ± 9.77	NS
SGPT (U/L)	25.72 ± 21.85	32.95 ± 22.05	21.61 ± 11.05	NS
Alkaline phosphatase (U/L)	76.05 ± 20.77	83.98 ± 32.21	92.33 ± 39.01	NS
TG (mg/dL)	127.7 ± 59.78	141.6 ± 59.94	154.7 ± 94.53	NS
Cholesterol (mg/dL)	185.4 ± 41.09	184.8 ± 47.69	170.6 ± 77.07	NS
LDL (mg/dL)	130.9 ± 45.82	112.6 ± 43.20	104.2 ± 55.25	NS
HDL (mg/dL)	42.02 ± 10.20	42.12 ± 13.96	40.64 ± 14.19	NS
DN4 score	0.04 ± 0.21	0.18 ± 0.45	0.30 ± 0.75	NS
VPT (V)	7.923 ± 2.75	15.44 ± 10.94	19.28 ± 10.52	<0.0001
CNFD (n/mm^2)	29.34 ± 4.78	22.87 ± 5.341	16.14 ± 4.41	<0.0001
CNBD (n/mm^2)	31.81 ± 10.07	23.01 ± 12.11	11.76 ± 5.46	<0.0001
CNFL (mm/mm^2)	16.73 ± 2.57	13.82 ± 2.05	9.588 ± 1.61	<0.0001
Netrin-1 (pg/mL)	1,034 ± 352.0	810.2 ± 308.6	522.3 ± 182.0	<0.0001

Clinical and demographic data of the participants in the study. Data are presented as the mean ± standard deviation. BMI, body mass index; CNBD, corneal nerve branch density; CNFD, corneal nerve fiber density; CNFL, corneal nerve fiber length; DN4, Douleur Neuropathique en 4 Questionnaire; FPG, fasting plasma glucose; HbA1c, glycated hemoglobin; HDL, high-density lipoprotein; LDL, low-density lipoprotein; NS, not significant; SGOT, serum glutamic-oxaloacetic transaminase; SGPT, serum glutamate pyruvate transaminase; TG, triglyceride; VPT, vibration perception threshold.

Neuropathic symptoms and quantitative sensory testing

There were no significant differences in DN4 score among the study groups. VPT was significantly higher in the DN– (15.44 ± 10.94) and DN+ (19.28 ± 10.52) groups compared with controls (7.92 ± 2.75; $P < 0.001$) and was significantly higher in the DN+ compared with the DN– group ($P < 0.01$). Participants with VPT >15 V had a significantly lower serum netrin-1 level compared with those with VPT <15 V (490.6 ± 160.5 vs 642.9 ± 102.4; $P < 0.05$). Thermal discrimination threshold testing was undertaken in a subset of study participants (control n = 16, DN– n = 22 and DN+ n = 19). Warm perception threshold differed significantly between controls and DN– (35.23° ± 0.58 vs 36.71° ± 0.71; $P < 0.0001$), and controls and DN+ (35.23° ± 0.58 vs 36.31° ± 0.74; $P < 0.0001$), but with no significant difference between the DN– and DN+ groups ($P = 0.07$). Cold perception threshold also differed significantly in the DN– (24.82° ± 1.31 vs 26.39° ± 1.05) and DN+ (24.96° ± 2.34 vs 26.39° ± 1.05) groups compared with controls (both $P < 0.05$), with no significant differences between the DN– and DN+ group ($P = 0.79$). Serum netrin-1 levels did not correlate with either warm ($r = 0.23$; $P = 0.35$) or cold perception ($r = –0.09$; $P = 0.73$) thresholds.

CCM parameters

A representative CCM image from each group is shown in Figure 1a–c. CNFD (22.87 ± 5.34 vs 29.34 ± 4.78, $P < 0.001$), CNBD (23.01 ± 12.11 vs 31.81 ± 10.07, $P = 0.001$) and CNFL (13.82 ± 2.05 vs 16.73 ± 2.57, $P < 0.001$) were significantly lower in the DN– group compared with controls. CNFD (16.14 ± 4.41 vs 29.34 ± 4.78, $P < 0.001$), CNBD (11.76 ± 5.46 vs 31.81 ± 10.07, $P = 0.001$) and CNFL (9.59 ± 1.61 vs 16.73 ± 2.57, $P < 0.001$) were significantly lower in the DN+ group compared with controls. CNFD (16.14 ± 4.41 vs 22.87 ± 5.34, $P < 0.001$) CNBD (11.76 ± 5.46 vs 23.01 ± 12.11, $P < 0.001$) and CNFL (9.59 ± 1.61 vs 13.82 ± 2.05, $P < 0.001$) were significantly lower in the DN+ group compared with the DN– group (Figure 1d–f).

Serum netrin-1 level

The intra-assay coefficient of variation for netrin-1 was <5%, and the inter-assay coefficient of variation was <10%. Serum netrin-1

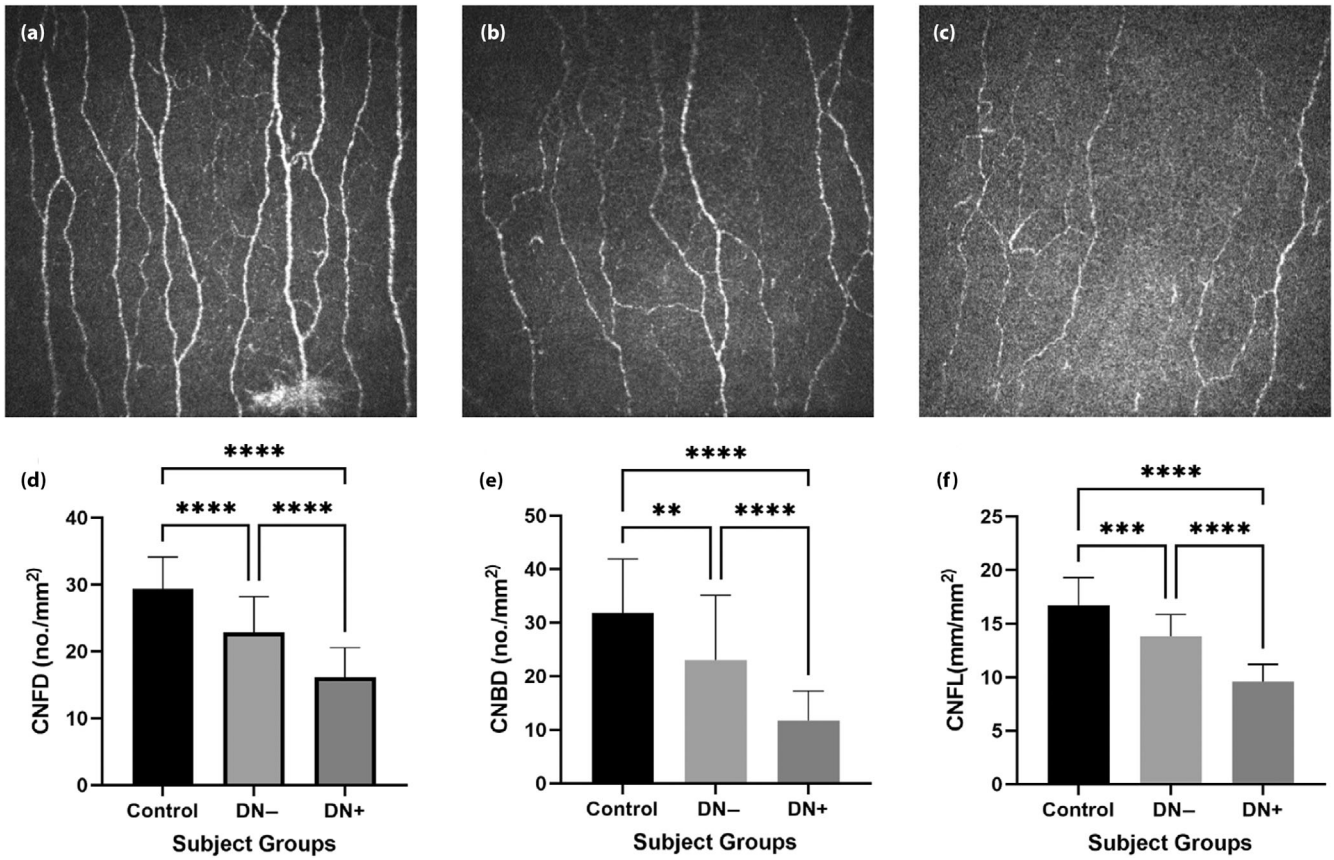


Figure 1 | Corneal confocal image from (a) controls, (b) type 2 diabetes patients without small nerve fiber loss (DN–) and (c) type 2 diabetes patients with small nerve fiber loss (DN+). Bar charts with mean and standard deviation showing a progressive significant decrease in (d) corneal nerve fiber density (CNFD), (e) corneal nerve branch density (CNBD) and (f) corneal nerve fiber length (CNFL) in patients with DN– and DN+ compared with controls. ****$P < 0.0001$, ***$P < 0.001$, **$P < 0.01$.

levels were significantly lower in the DN– (830.4 ± 311.2; $P < 0.05$) and DN+ (476.5 ± 148.8) groups compared with controls (1,034 ± 352.0), and was significantly lower in the DN+ group compared with the DN- group ($P < 0.01$; Figure 2a).

Correlation between netrin-1 and CCM parameters

There was no significant correlation between netrin-1 levels and CCM parameters (CNFD $r = 0.13$, CNBD $r = 0.01$ and CNFL $r = 0.11$, respectively) in the whole diabetes group. In the DN+ group, there was a significant positive correlation between netrin-1 levels and CNFL ($r = 0.51$; $P < 0.01$; Figure 2b), but not with CNFD ($r = 0.31$; $P = 0.11$) or CNBD ($r = 0.06$; $P = 0.76$). Multiple regression analysis, which included age, body mass index, diastolic blood pressure and VPT as independent factors, showed that only netrin-1 was a significant predictor for DN ($\beta = 0.004$, $P < 0.038$).

ROC curve for netrin-1

A ROC curve analysis among the patients with type 2 diabetes showed that a netrin-1 cut-off value of 599.6 (pg/mL) had an AUC of 0.85, with a sensitivity of 76% and specificity of 74% ($P < 0.001$; 95% confidence interval 0.76–0.94) for differentiating DN+ from DN– (Figure 3).

DISCUSSION

The present study shows a progressive reduction in serum netrin-1 levels in participants with increasing corneal nerve fiber loss, and the ROC analysis demonstrates good diagnostic utility for netrin-1 in differentiating diabetes patients with and without small nerve fiber damage.

In a seminal paper published almost 20 years ago, it was shown that the reversal of neuropathy and vasculopathy in a diabetic murine model were both attributed to netrins[20]. Netrin-1 plays a key role in a variety of physiological processes, including cell injury, proliferation and migration[21], and is an emerging player in driving inflammation[22] in a variety of tissues, including the liver[23], visceral tissue[24] and brain[25]. In a recent study, gene expression profiles of the netrin family were significantly lower in the skin and sural nerve of 88 patients with polyneuropathy, including those with DN and especially

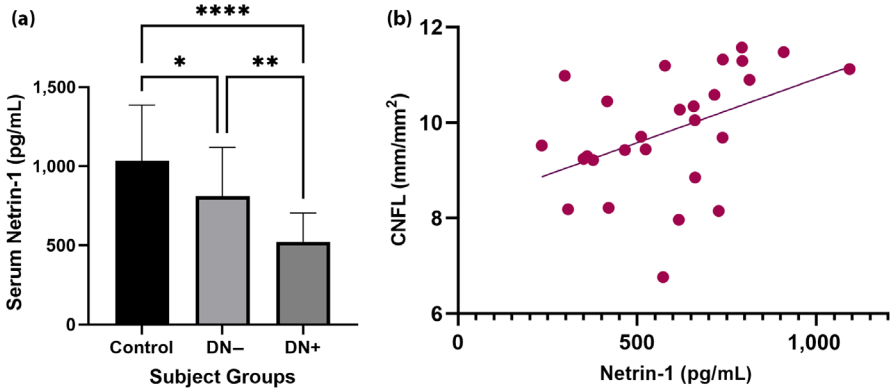
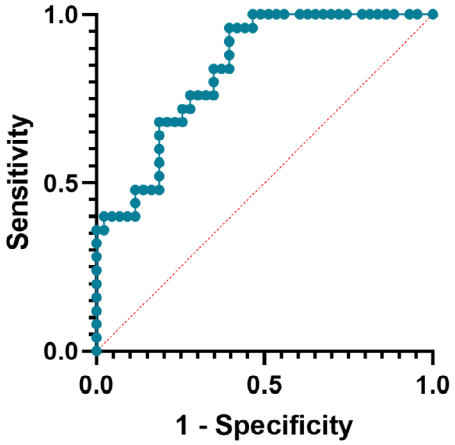


Figure 2 | (a) Bar chart expressed as the mean and standard error of the mean showing a progressive significant decrease in serum netrin-1 levels in type 2 diabetes patients without small nerve fiber loss (DN–) and type 2 diabetes patients with small nerve fiber loss (DN+) compared with controls. ****$P < 0.0001$, **$P < 0.01$, *$P < 0.05$. (b) Correlation between serum netrin-1 levels and corneal nerve fiber length (CNFL) in the DN+ group ($r = 0.51$; $P < 0.01$).



Serum Netrin-1 Level	Cut-off Value	Sensitivity %	Specificity %	AUC %	*P* value
DN+	<599.6	76	74	85	<0.001

Figure 3 | Receiver operating characteristic curve analysis of serum netrin-1 for neuropathy prediction. Serum netrin-1 cut-off value of 599.6 pg/mL with an area under the curve (AUC) of 85% ($P < 0.01$). DN+, type 2 diabetes patients with small nerve fiber loss.

those with painful neuropathy, and furthermore correlated with intra-epidermal nerve fiber density[16]. However, a skin biopsy study revealed the complex interplay between netrin-1 and nerve repair by showing higher gene expression of netrin-1 in keratinocytes of patients with small fiber neuropathy, emphasizing the complex pathophysiology of pain and small fiber damage[17]. In this study, there was no difference in serum netrin-1 levels between patients with a lower and higher DN4 score, and overall, the patients had a low score, which would not fulfill the diagnosis of painful diabetic neuropathy. Furthermore, in a subset of the present patients, there was no correlation between netrin-1 levels and warm and cold thermal thresholds.

CCM has good reliability and reproducibility in showing corneal nerve loss in individuals with DN[18], and in a systematic review and meta-analysis of >4,000 participants, it showed an early reduction in all corneal nerve parameters, even in patients without DN, emphasizing the ability of CCM to detect early subclinical DN[7]. CNFL has the best reproducibility and strongest association with the severity of DN[26], and is reduced early in children with type 1 diabetes without DN[27]. Accordingly, alongside the assessment of neuropathic symptoms using DN4 and vibration perception threshold, we undertook CCM to help identify early subclinical DN. We showed that diabetes patients with a minimal reduction in CNFL show lower netrin-1 levels, which are further reduced in patients with a greater reduction in CNFL and more severe DN, as evidenced by higher VPT in these patients. Of mechanistic relevance, netrin-1 reduces inflammation and increases corneal nerve fiber regeneration with corneal epithelial wound healing in diabetic mice[28].

We also showed a correlation between serum netrin-1 levels and CNFL, and multiple regression shows an independent

association between netrin-1 levels and DN. Furthermore, ROC analysis showed a moderate to good diagnostic utility, with an AUC of 0.85, and a sensitivity and specificity of 76% and 74%, respectively, for serum netrin-1 differentiating diabetes patients with and without small fiber damage. This is promising, as a previous larger study of 432 patients with diabetes showed that neuron-specific enolase levels had an AUC of 0.73, with a sensitivity and specificity of 66.3% and 72.5%, respectively, for differentiating patients with and without DN[29].

We acknowledge this was a small cross-sectional study, which lacked the assessment of neurophysiology and skin biopsy. However, we used corneal confocal microscopy to identify early and more advanced small fiber damage, which was independently associated with circulating levels of netrin-1. Circulating netrin-1 might be a promising biomarker for identifying early and established DN, but the assay is currently not routinely available, and the sensitivity and specificity for differentiating patients with and without small fiber damage were moderate. Further larger studies are needed to validate the diagnostic utility of netrin-1 in diabetic neuropathy.

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DISCLOSURE

The authors declare no conflict of interest.

Approval of the research protocol: The study was approved by Institutional Ethics Committee.

Informed consent: All study participants provided written informed consent.

Registry and the registration no. of the study/trial: (Ethical approval number: IPGME&R/IEC/2022/069 dated 05th March, 2022).

Animal studies: N/A.

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SUPPORTING INFORMATION

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

Figure S1. (A) Representative CCM image and (B) Its annotated analyzed one using automated ACCMetrics softwere. Red line represents the main fiber, blue line represents nerve branch and green dot represents branch point.

Table S1. Partial correlation between serum netrin-1 and clinical variables.