Diagnostic efficacy of serum and urinary netrin-1 in the early detection of diabetic nephropathy

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ABSTRACT

This study aimed to assess the diagnostic value of serum and urinary netrin-1 in patients with type 2 diabetes mellitus (T2DM) at different stages of diabetic nephropathy (DN) and to compare its efficacy of estimation in serum with that in the urine. This study was carried out on 135 patients with T2DM and 45 healthy subjects. The patients with diabetes were divided according to urinary albumin creatinine ratio (UACR) into: T2DM with normoalbuminuria, incipient DN with microalbuminuria, and overt DN with macroalbuminuria groups. Serum and urinary levels of netrin-1 were measured by ELISA. The mean levels of serum and urinary netrin-1 were significantly higher in the microalbuminuric and macroalbuminuric patients with DN than those in the normoalbuminuric patients with T2DM, with the highest values detected in macroalbuminuric patients with DN. Urinary netrin-1 level was significantly higher in the normoalbuminuric T2DM group than control group, whereas no significant difference existed regarding serum netrin-1 level. In T2DM groups, the urinary and serum netrin-1 correlated with each other and were independently related to fasting blood glucose, UACR, and estimated glomerular filtration rate. Receiver operating characteristic curve analysis showed that the area under the curve of urinary netrin-1 was 0.916 which is significantly higher than that of serum netrin-1 (0.812) for the detection of incipient DN and reached 0.938 on coestimation of both urinary and serum netrin-1. In conclusion, netrin-1 is a potential diagnostic marker for early detection of DN with its estimation in urine has higher accuracy than that of serum.

Significance of this study

What is already known about this subject?

- Diabetic nephropathy (DN) is a progressive disorder in which inflammation plays a major contributing effect.
- Netrin-1 promotes angiogenesis and has anti-inflammatory effect via inhibition of leucocyte emigration.

What are the new findings?

- First study to estimate statistically the diagnostic power of serum and urinary netrin-1 in DN stages.
- First study to compare and combine the diagnostic value of serum versus urinary netrin-1 in early DN detection.
- For early DN detection:
  - The area under the curve (AUC) of urinary netrin-1 was 0.916 which is higher than that of serum netrin-1 (0.812).
  - An AUC of 0.938 has been achieved on coestimation of both urinary and serum netrin-1.

How might these results change the focus of research or clinical practice?

- Urinary netrin-1 can be used as a potential marker for the detection of early stage of DN in which treatment can inhibit its progression. Coestimation of both serum and urinary netrin-1 enhances their diagnostic power.

INTRODUCTION

Diabetic nephropathy (DN) is a progressive kidney disease characterized by modulation in kidney architecture and function with progressive damage of renal glomeruli and tubules. It is the most common complication of diabetes and results from progressive advanced glycation and inflammation.[1] It comprises one of the major causes of end-stage renal disease (ESRD).[2]

DN is a progressive disease that can pass to an irreversible stage of kidney damage and the patient developed ESRD. However, the treatment of DN at an early stage can help in prohibiting the progress of diabetic kidney disease.[3] Therefore, it is important to recognize patients at the venture of manifesting DN. The renal biopsy is used to detect the histopathological changes of DN and considered as a standard method for its diagnosis. However, this method cannot be used for screening or diagnosis of early DN due to its invasive nature.[4]

Microalbuminuria is considered the first detectable sign of renal involvement in diabetes and used for screening of early DN.[5] However, the risk of progression to DN is considered a continuum, starting even in the range of normal urinary albumin excretion.[6 7] Therefore, searching for new biomarkers is required

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to detect patients at risk of developing DN and upgrade the performance of preventive strategies.

Inflammation plays an important role in the pathogenesis of diabetes and evidence suggested that before its onset, diabetes shows features of inflammation.[8 9] The increased oxidative stress, chemokines, and proinflammatory cytokines, including tumor necrosis factor-alpha and interleukin-6, affect insulin action, and promote insulin resistance, obesity, and type 2 diabetes mellitus (T2DM).[8]

Netrins, a class of laminin-like proteins of 50–75 kD weight,[10] were initially recognized as axonal guidance cues during embryonic development.[11 12] Netrin-1 is first expressed in the central nervous system, but also in non-neural tissues such as vascular endothelial cells, pancreas, lung, liver, spleen, intestine, and kidneys.[13] It is found to be released after acute and chronic kidney insult and excreted in the urine of both humans and mice.[14] It exerts its action by binding to 2 classic receptor families, such as deleted in colorectal cancer (DCC) subfamily (eg, DCC and Neogenin) and uncoordinated 5 (UNC5) subfamily (eg, UNC5A–UNC5D) expressed by the target cells.[12] Netrin-1 plays a role in the migration of vascular endothelial cells and accelerating angiogenesis,[15 16] tumor progression and growth, and regulation of inflammation.[13 17 18] Anti-inflammatory actions of netrin-1 were also reported as suppression of emigration of leucocytes and guard against vascular inflammation, inflammatory peritonitis, and pancreatitis through UNC5B receptor.[13 19 20]

The inflammation is a major contributing factor in the development of DN.[21] Previous studies have investigated different inflammatory and oxidative stress mediator levels in serum and urine as biomarkers for the detection of early DN and reported variable efficiencies.[22] Moreover, netrin-1 had been studied as a biomarker for DN in the urine as well as in the serum in some previous studies with controversial results. Also, it is not clear whether its diagnostic efficacy is better whenever estimated in the serum or urine. Therefore, we performed this study to assess the diagnostic value of serum and urinary netrin-1 in patients with T2DM at different stages of DN and to compare its efficacy of estimation in serum with that in the urine.

SUBJECTS AND METHODS

Study population and design

This was a prospective cross-sectional study in which 135 patients with T2DM were recruited in a consecutive manner from those attending the diabetes outpatient clinic at Tanta University Hospital as illustrated in the flow chart (figure 1). They were diagnosed according to the criteria of the American Diabetes Association[23] as having fasting blood glucose (FBG) >126 mg/dL and HbA1c level >6.5%. They were aged ≥30 years with diabetes duration of more than 10 years. Patients with other autoimmune or inflammatory disorders, superimposed nephropathy due to other renal tubular diseases, and ESRD were excluded. The included patients with T2DM were further classified into 3 groups according to their urinary albumin creatinine ratio (UACR) levels based on the criteria endorsed by the Joint Committee of Diabetic Nephropathy[24] as follows: group (1): T2DM with normoalbuminuria (UACR <30 mg/g, n=45, 21 males and 24 females), group (2): incipient DN with microalbuminuria (UACR=30–300 mg/g, n=45, 27 males and 18 females), group (3): overt DN with macroalbuminuria (UACR >300 mg/g, n=45, 26 males and 19 females). In addition, 45 healthy subjects (24 males and 21 females) were recruited from those attending Tanta University Hospital for routine check-up and served as a control group (group 4). They were non-diabetic subjects with FBG <100 mg/dL, HbA1c level <5.7%, and normal renal

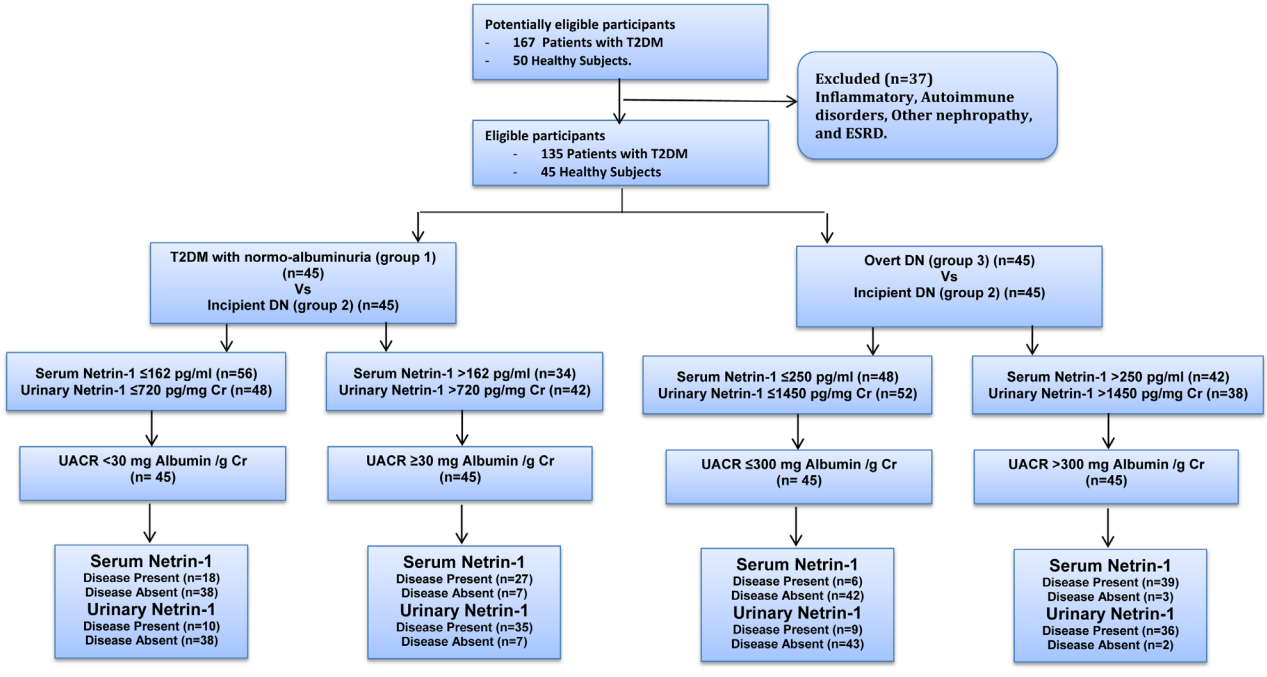

Figure 1 Standards for Reporting of Diagnostic Accuracy (STARD) flow chart of the studied groups. DN, diabetic nephropathy; ESRD, end-stage renal disease; T2DM, type 2 diabetes mellitus; UACR, urinary albumin creatinine ratio.

function and UACR level with no history of autoimmune or inflammatory disorders.

Clinical evaluation

All patients with T2DM included in this study underwent careful history taking including the duration of diabetes, presence of hypertension, drug history, and family history of T2DM as well as a full clinical examination.

Sampling

Venous blood samples were collected from all subjects in this study by standard venipuncture under complete aseptic precautions after overnight fasting then delivered in VACUETTE blood collection tubes containing K2EDTA for HbA1c determination and tube containing clot activator/Sep to be used for serum separation. One part of the serum samples was immediately used for estimation of serum FBG and creatinine levels and the other part was stored at −20°C for serum netrin-1 estimation. Early morning spot urine specimens were collected from all subjects, then immediately centrifuged, and the supernatant was used for estimation of urinary creatinine, albumin, and netrin-1 levels.

Laboratory analyses

The HbA1c level was estimated by immunonephelometry on TWIN A1c, Spectrum Diagnostics, Obour City, Cairo, Egypt. The FBG, serum, and urinary creatinine levels were measured on a fully automated chemistry analyzer (Konelab Prime 60I, Thermo Fisher Scientific, Vantaa, Finland). Urinary albumin concentration was estimated by immunoturbidimetry on BTS 350 semiautomated analyzer (BioSystems, Spain), and then UACR (mg/g) was calculated by dividing urinary albumin concentration (mg) by urinary creatinine concentration (g). Estimated glomerular filtration rate (eGFR) was calculated according to the modification of diet in renal disease equation as $186.0 \times (\text{plasma creatinine} \times 1.154) \times (\text{age}^{0.203}) \times 0.742$ (if female) $\times$ 1.210 (if black).[25]

Netrin-1 immunoassay

Serum and urinary netrin-1 levels were measured using a commercially available, quantitative sandwich enzyme immunoassay technique (Human Netrin-1 ELISA kit, NTN1 LifeSpan BioSciences, North America, California, USA, Catalog No: LS-F23473) in accordance with the manufacturer's instructions. A serial dilution was made from a standard stock of 2000 pg/mL. The colorimetric detection in this assay was performed at 450 nm on Tecan Spectra II Microplate Reader (Switzerland). A logit-log standard curve was displayed from which the sample concentration was calculated. The sensitivity is 18.75 pg/mL. Intra-assay coefficient of variation (CV) and interassay CV percentages are both less than 10%. The urinary netrin-1 results were divided by the urinary creatinine results and expressed as pg/mg creatinine. The interpretation of results of this assay was performed by independent investigators with no previous knowledge to the other features of the study subjects.

Statistical analyses

Data were analyzed using IBM SPSS software package V.20.0 (IBM). The Kolmogorov-Smirnov, Shapiro, and D'agostino tests were used to verify the normality of the distribution of variables. Comparisons between groups for categorical variables were assessed using the χ^2 test. Analysis of variance and Kruskal-Wallis tests were used for comparing different groups for normally and non-normally distributed numerical variables. Pearson coefficient was used to correlate between netrin-1 and other DN predictors. The significant variables were entered in multivariable regression analysis. The serum and urinary netrin-1 were combined using binary logistic regression. The receiver operating characteristic (ROC) curve was used to determine the diagnostic performance of the netrin-1 in serum and urine. The area under the curve (AUC) of more than 50% gives acceptable performance and the area about 100% is the best performance for the test. The AUCs of serum and urinary netrin-1 were compared according to DeLong *et al*,[26] using MedCalc statistical software V.15.8 (MedCalc Software, Ostend, Belgium). The optimal cut-off values were determined via Youden's index. The significance of the obtained results was judged at the p value ≤ 0.05.

RESULTS

Basic characteristics of the studied groups

In this study, 180 subjects were included; 135 patients with T2DM and 45 healthy subjects served as a control group. The demographic and laboratory data of the studied groups were shown in table 1. There were no significant differences regarding age and gender among all groups of the study. However, there were statistically significant differences in FBG, HbA1c, serum creatinine, UACR, and eGFR levels between the 4 studied groups except for the creatinine and UACR levels between the normoalbuminuric T2DM and control groups and HbA1c level between the normoalbuminuric T2DM and microalbuminuric DN groups.

Serum and urinary netrin-1 level among studied groups

As shown in table 1, the mean levels of serum netrin-1 were significantly higher in the microalbuminuric and macroalbuminuric patients with DN compared with those of the control group, with the highest values observed in macroalbuminuric patients with DN, and the difference between the groups was statistically significant, while no statistically significant difference was observed between the normoalbuminuric T2DM and control groups. Moreover, there was an increased urinary netrin-1 level in patients with T2DM with normoalbuminuria compared with its level in healthy control with subsequent increase in incipient DN group and more significant elevation in overt DN group.

Netrin-1 and covariables of patients with T2DM

In the T2DM patient groups, serum and urinary netrin-1 showed a significant positive correlation with FBG, HbA1c, serum creatinine, UACR, and a negative correlation with eGFR. Multivariate analysis showed that serum netrin-1 independently related to FBG, serum creatinine levels, UACR, and eGFR (table 2), whereas urinary netrin-1 was independently related to FBG, HbA1c, UACR, and eGFR (table 3).

Table 1 Comparison between the 4 studied groups according to different parameters

	Group 1 (n=45)	Group 2 (n=45)	Group 3 (n=45)	Group 4 (n=45)	P value
Sex					
Male	21 (46.7%)	27 (60%)	26 (57.7%)	24 (53.3%)	0.597
Female	24 (53.3%)	18 (40%)	19 (42.3%)	21 (46.7%)	
Age (y)	52.0±9.9	52.6±9.7	51.7±9.2	50.3±8.1	0.068
FBG (mg/dL)	154.9±21.9†‡§	187.1±47.1†‡	318.6±87.6†	83.8±8.3	<0.001*
HbA1c (%)	7.45±0.80†‡	7.37±1.05†‡	8.80±1.20†	4.73±0.29	<0.001*
Creatinine (mg/dL)	0.88±0.20‡§	1.19±0.22†‡	1.85±0.42†	0.79±0.07	<0.001*
UACR (mg/g)	15.0±6.2‡§	118.8±60.9†‡	505.6±206.9†	13.2±6.3	<0.001*
eGFR (mL/min)	87.1±20.1†‡§	62.4±9.6†‡	37.3±6.5†	102.3±12.4	<0.001*
Serum netrin-1 (pg/mL)	123.2±30.9‡§	190.9±76.5†‡	599.6±312.6†	106.8±29.1	<0.001*
Urinary netrin-1 (pg/mg creatinine)	517.4±174.4†‡§	1081.7±331.3†‡	1689.4±279.5†	282.4±45.9	<0.001*

Data are expressed as mean±SD.
Group 1: T2DM with normoalbuminuria.
Group 2: incipient DN with microalbuminuria.
Group 3: overt DN with macroalbuminuria.
Group 4: healthy control.
*Statistically significant at p≤0.05.
†Significant with group 4.
‡Significant with group 3.
§Significant with group 2.
DN, diabetic nephropathy; eGFR, estimated glomerular filtration rate; FBG, fasting blood glucose; T2DM, type 2 diabetes mellitus; UACR, urinary albumin creatinine ratio.;

Netrin-1 diagnostic efficacy

For detection of incipient DN, ROC curve analysis was performed and showed that serum netrin-1 had an AUC of 0.812 with a sensitivity of 60.0%, a specificity of 84.4% at a cut-off value >162 pg/mL, whereas urinary netrin-1 had an AUC of 0.916 with a sensitivity of 77.8%, a specificity of 84.4% at a cut-off value >720 pg/mg creatinine. The AUC of urinary netrin-1 was significantly higher than that of serum netrin-1 with an AUC difference of 0.104 (95% CI 0.012 to 0.197, p=0.027) (table 4, figure 2A). On the other hand, serum netrin-1 had an AUC of 0.939 with 86.7% sensitivity and 93.3% specificity at a cut-off value >250 pg/mL, whereas urinary netrin-1 had an AUC of 0.931 with 80.0% sensitivity and 95.6% specificity at a cut-off value >1450 pg/mg creatinine for detection of overt DN with no significant difference was observed between AUCs of both serum and urinary netrin-1 (AUC difference=0.008 (95% CI −0.037 to 0.053, p=0.722) (table 4, figure 2B).

Combined efficacy of netrin-1

The Pearson correlation analysis revealed that a significant positive correlation existed between serum and urinary netrin-1 in patients with T2DM (r=0.794, p<0.001) (figure 3). Thus, combined analysis of serum and urinary netrin-1 was performed by means of binary logistic regression and revealed that AUC of combined serum and urinary netrin-1 was 0.938 and higher than that of serum netrin-1 (AUC difference=0.127, 95% CI 0.051 to 0.202, p=0.001) and urinary netrin-1 (AUC difference=0.022, 95% CI −0.009 to 0.054, p=0.168) for the detection of incipient DN (table 4, figure 2A).

DISCUSSION

DN is manifested by progressive damage of renal glomeruli and tubules.[1] The chronic subclinical inflammation may have a serious effect on the expansion and advancement of

Table 2 Correlation and multivariate analysis of the parameters associated to serum netrin-1 in patients with diabetes (n=135)

	Correlation		Multivariate			
Serum netrin-1	r	P value	B	Standardized beta coefficient	t	P value
Age	−0.007	0.468	–	–	–	–
FBG	0.911	<0.001*	1.346	0.440	4.513	<0.001*
HbA1c	0.736	<0.001*	−14.99	−0.065	−1.122	0.264
Creatinine	0.865	<0.001*	177.1	0.317	2.861	0.005*
UACR	0.904	<0.001*	0.467	0.408	4.856	<0.001*
eGFR	−0.672	<0.001*	2.125	0.184	2.701	0.008*

All variables with p<0.05 were included in the multivariate.
Beta: unstandardized coefficients.
*Statistically significant at p≤0.05.
eGFR, estimated glomerular filtration rate; FBG, fasting blood glucose; UACR, urinary albumin creatinine ratio.

Table 3 Correlation and multivariate analysis of the parameters associated to urinary netrin-1 in patients with diabetes (n=135)

	Correlation		Multivariate			
Urinary netrin-1	r	P value	B	Standardized beta coefficient	t	P value
Age	−0.011	0.448	–	–	–	–
FBG	0.809	<0.001*	1.766	0.295	2.329	0.021*
HbA1c	0.610	<0.001*	−71.78	−0.158	−2.112	0.037*
Creatinine	0.846	<0.001*	178.9	0.164	1.136	0.258
UACR	0.843	<0.001*	0.739	0.330	3.021	0.003*
eGFR	−0.794	<0.001*	−6.420	−0.284	−3.208	0.002*

All variables with p<0.05 were included in the multivariate.
Beta: unstandardized coefficients.
*Statistically significant at p≤0.05.
eGFR, estimated glomerular filtration rate; FBG, fasting blood glucose; UACR, urinary albumin creatinine ratio.

DN, which raises the glomerular permeability of intravascular protein in the glomerulus by activating protein kinase cascade and transcription factors.[27] Evidence suggested that acute and chronic kidney diseases are connected with inflammation in which inflammatory mediators play a considerable role in tissue damage.[28] Netrin-1 is one of the anti-inflammatory mediators that found to be highly released after acute and/or chronic kidney injury and excreted in the urine of both human and experimental animals.[29] Our study aimed to investigate the efficacy of netrin-1 for the detection of different stages of DN and to compare its diagnostic value in serum with that in the urine.

In this study, serum netrin-1 was found to be significantly increased with the progression of nephropathy and was higher in microalbuminuric and macroalbuminuric patients with DN compared with normoalbuminuric T2DM and control groups. These findings were in agreement with Ay *et al*,[30] who reported that serum netrin-1 was significantly higher in microalbuminuric patients than normoalbuminuric groups and with Liu *et al*,[31] who found that plasma netrin-1 level was significantly increased in macroalbuminuria group relative to microalbuminuria and normoalbuminuria groups. Moreover, no significant difference in serum netrin-1 level between normoalbuminuric T2DM and control groups was observed in our study which was in accordance with Ay *et al*'s[30] study findings. However, Yim *et al*[32] reported that patients with diabetes had a higher level of serum netrin-1 in comparison with the control group. On contrary, other studies reported that serum netrin-1 level was decreased in newly diagnosed patients with T2DM[33 34] as well as prediabetic counterpart.[34] The contradiction of the results among studies is attributed to the difference in the characteristics of the studied groups and the difference in commercial ELISA kits used in various studies for measuring netrin-1 levels.

Netrin-1 has been detected to be increased in urine as a result of acute kidney injury in many human and animal studies.[14 35] Moreover, urinary netrin-1 has gained attention as a potential biomarker independently predicting the development of diabetes mellitus and DN.[36 37] In this study, the urinary netrin-1 level was significantly higher in incipient DN and overt DN groups compared with the normoalbuminuric T2DM group and healthy control. Interestingly, a significant increase in urinary netrin-1 level was observed in normoalbuminuric T2DM groups relative to the healthy controls which indicated its usefulness for the detection of the early normoalbuminuric stage of DN. In the same context, Jayakumar *et al*[36] detected a significant increase in urinary netrin-1 level in patients with DN with no microalbuminuria.

We found that serum and urinary netrin-1 were independently related to FBG, eGFR, and UACR with a positive correlation with fasting blood sugar, HbA1c, serum creatinine, and UACR, and a negative correlation with eGFR. In the same context, a study of Ay *et al*[30] reported a positive association between HbA1c, UACR, and serum netrin-1, and a negative association was present between eGFR and serum netrin-1, while no association was present with age,

Table 4 Agreement (sensitivity, specificity) with netrin-1 to predict incipient DN

			95% CI				
	AUC	P value	LL	UL	Cut-off	Sensitivity	Specificity
Incipient DN (microalbuminuria) versus T2DM (normoalbuminuria)							
Serum netrin-1 (pg/mL)	0.812	<0.001*	0.715	0.886	>162	60	84.4
Urinary netrin-1 (pg/mg creatinine)	0.916	<0.001*	0.839	0.964	>720	77.8	84.4
Combined netrin-1	0.938	<0.001*	0.867	0.978	–	–	–
Overt DN (macroalbuminuria) versus incipient DN (microalbuminuria)							
Serum netrin-1 (pg/mL)	0.939	<0.001*	0.868	0.978	>250	86.7	93.3
Urinary netrin-1 (pg/mg creatinine)	0.931	<0.001*	0.857	0.973	>1450	80	95.6
Combined netrin-1	0.958	<0.001*	0.893	0.989	–	–	–

Cut-off was chosen according to Youden's index.
*Significant at p<0.05.
AUC, area under the curve; ;DN, diabetic nephropathy; LL, lower limit; T2DM, type 2 diabetes mellitus; UL, upper limit.

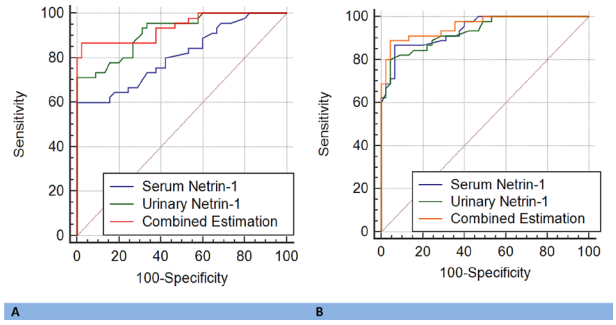

Figure 2 Receiver operating characteristic (ROC) curve analysis of netrin-1 for prediction of diabetic nephropathy (DN) stages: (A) incipient DN stage, (B) overt DN stage.

FBG, and creatinine. Also, the study done by Yim *et al*[32] reported that netrin-1 was in a positive association with FBG, HbA1c, and creatinine, and in a negative association with eGFR. On the other hand, Liu *et al*[33] reported that a negative association was present between FBG, HbA1c, and netrin-1. Recently, Gao *et al* showed that netrin-1 directly stimulates insulin secretion in isolated mouse islets of high-fat diet/streptozotocin-induced diabetic mice by promoting calcium ion influx into the beta cell with subsequent generation of cyclic adenosine 5'-monophosphate with improvement of beta-cell function and islet vascularization.[38] In addition, netrin-1 was detected to attenuate the oxidative stress induced by high glucose and thus protecting against diabetes-induced vascular damage.[39] However, limited data about the half life of netrin-1 in the serum and the mechanism by which hyperglycemia could induce netrin-1 secretion are unclear. Taken together, the correlation between FBG and serum netrin-1 suggests that elevated serum netrin-1 is probably attributable to a compensatory response of hyperglycemia[32] in order to stimulate insulin secretion and attenuate the oxidative stress damage induced by hyperglycemia. However, further functional evidence is needed to confirm these speculations.

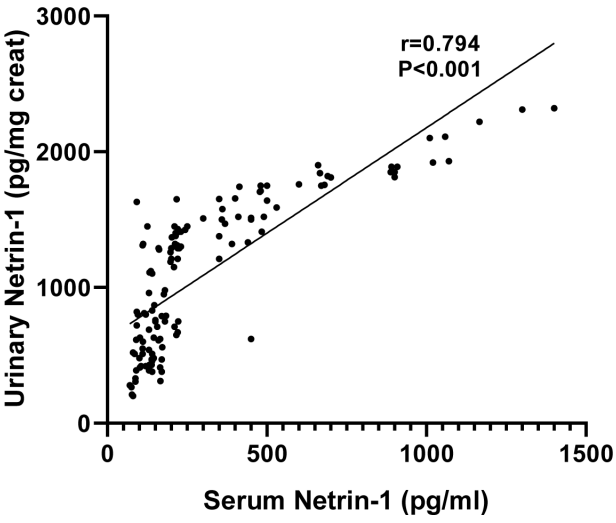

Figure 3 Correlation analysis between serum and urinary netrin-1 in patients with type 2 diabetes mellitus (T2DM).

Netrin-1 has a crucial regulatory effect on hyperglycemia and diabetes as it promotes islet beta-cell remodeling and pancreatic morphogenesis.[40] Moreover, it attenuates the inflammation via regulating cyclo-oxygenase-2 expression and stimulating macrophage differentiation to its anti-inflammatory M2 type.[40 41] Furthermore, the progress of DN is associated with increased netrin-1 levels,[31] and there are different possible explanations for this finding. Netrin-1 stimulates albumin uptake from proximal tubular cells. The excretion of high levels of albumin in DN enhances the production of netrin-1 from proximal tubular epithelial cells via activation of extracellular signal-regulated kinase and Akt kinase pathways to promote its translation.[42 43] Thus, netrin-1 production is a compensatory mechanism for the defective capacity of the proximal tubules in early DN.[44] Another possible cause for increased urinary netrin-1 in DN is the tubular epithelial injury which occurs in the context of DN as netrin-1 is mainly expressed in proximal renal tubular cells. Based on the results of this study and other reports, elevated netrin-1 in urine supported the fact that tubular dysfunction usually precedes the glomerular dysfunction in DN; however, its elevation in serum may indicate the progression of glomerular damage.

Although the diagnostic accuracy of netrin-1 for T2DM had been investigated in many previous studies,[32 34] to the best of our knowledge, no previous studies have statistically evaluated the diagnostic value of serum or urinary netrin-1 in DN. It is worth mentioning that this study reported for the first time that the diagnostic accuracy of urinary netrin-1 was high (AUC=0.916) and significantly exceeded that of serum netrin-1 (AUC=0.812) for the detection of DN at the early incipient stage. Therefore, we suggested that the estimation of netrin-1 in serum is not as helpful as that in urine to show the tubular dysfunction that might occur in patients with DN with no microalbuminuria. However, it may be related to the glomerular dysfunction and can be used as another tool for the detection of microalbuminuria. Therefore, we suggested that the combined estimation of both serum and urinary netrin-1 is beneficial and could increase the diagnostic efficiency of netrin-1 as a marker for early DN.

Although our study is the first to statistically assess the diagnostic power of netrin-1 in DN and compare the efficacy of netrin-1 estimation in serum in relation to the urine, there are some limitations that need further work on. First, the sample size is relatively small. Second, the results were obtained using a single method (ELISA) with a commercial kit; however, the precision of the kit is acceptable. Therefore, further studies on a large number of cases and using other methodologies or other commercial ELISA kits are required to confirm our results.

In conclusion, netrin-1 is a potential marker for the detection of DN at an early stage. Urinary netrin-1 was detected to be significantly elevated in normoalbuminuric T2DM cases than the healthy subjects with normal UACR. Therefore, we suggested that urinary netrin-1 in normoalbuminuric patients with diabetes may predict future development of albuminuria. Moreover, estimation of netrin-1 in urine presents a high diagnostic efficacy and exceeds that whenever estimated in the serum.

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Competing interests None declared.

Patient consent for publication Not required.

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Data availability statement All data relevant to the study are included in the article or uploaded as supplementary information.

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REFERENCES

1 Zeni L, Norden AGW, Cancarini G, *et al*. A more tubulocentric view of diabetic kidney disease. *J Nephrol* 2017;30:701–17.
2 Mora-Fernández C, Domínguez-Pimentel V, de Fuentes MM, *et al*. Diabetic kidney disease: from physiology to therapeutics. *J Physiol* 2014;592:3997–4012.
3 Hostetter TH. Prevention of end-stage renal disease due to type 2 diabetes. *N Engl J Med* 2001;345:910–2.
4 Biesenbach G, Bodlaj G, Pieringer H, *et al*. Clinical versus histological diagnosis of diabetic nephropathy--is renal biopsy required in type 2 diabetic patients with renal disease? *QJM* 2011;104:771–4.
5 Gross JL, de Azevedo MJ, Silveiro SP, *et al*. Diabetic nephropathy: diagnosis, prevention, and treatment. *Diabetes Care* 2005;28:164–76.
6 Zachwieja J, Soltysiak J, Fichna P, *et al*. Normal-range albuminuria does not exclude nephropathy in diabetic children. *Pediatr Nephrol* 2010;25:1445–51.
7 MacIsaac RJ, Tsalamandris C, Panagiotopoulos S, *et al*. Nonalbuminuric renal insufficiency in type 2 diabetes. *Diabetes Care* 2004;27:195–200.
8 Zeyda M, Stulnig TM, Obesity STM. Obesity, inflammation, and insulin resistance--a mini-review. *Gerontology* 2009;55:379–86.
9 Tsalamandris S, Antonopoulos AS, Oikonomou E, *et al*. The role of inflammation in diabetes: current concepts and future perspectives. *Eur Cardiol* 2019;14:50–9.
10 Basnakian AG. Netrin-1: a potential universal biomarker for acute kidney injury. *Am J Physiol Renal Physiol* 2008;294:F729–30.
11 Kennedy TE, Serafini T, de la Torre JR, *et al*. Netrins are diffusible chemotropic factors for commissural axons in the embryonic spinal cord. *Cell* 1994;78:425–35.
12 Serafini T, Kennedy TE, Galko MJ, *et al*. The netrins define a family of axon outgrowth-promoting proteins homologous to C. elegans UNC-6. *Cell* 1994;78:409–24.
13 Ly NP, Komatsuzaki K, Fraser IP, *et al*. Netrin-1 inhibits leukocyte migration in vitro and in vivo. *Proc Natl Acad Sci U S A* 2005;102:14729–34.
14 Reeves WB, Kwon O, Ramesh G. Netrin-1 and kidney injury. II. netrin-1 is an early biomarker of acute kidney injury. *Am J Physiol Renal Physiol* 2008;294:F731–8.
15 Navankasattusas S, Whitehead KJ, Suli A, *et al*. The netrin receptor UNC5B promotes angiogenesis in specific vascular beds. *Development* 2008;135:659–67.
16 Nguyen A, Cai H. Netrin-1 induces angiogenesis via a DCC-dependent ERK1/2-eNOS feed-forward mechanism. *Proc Natl Acad Sci U S A* 2006;103:6530–5.
17 Arakawa H. Netrin-1 and its receptors in tumorigenesis. *Nat Rev Cancer* 2004;4:978–87.
18 Fitamant J, Guenebeaud C, Coissieux M-M, *et al*. Netrin-1 expression confers a selective advantage for tumor cell survival in metastatic breast cancer. *Proc Natl Acad Sci U S A* 2008;105:4850–5.
19 Chen J, Cai Q-P, Shen P-J, *et al*. Netrin-1 protects against L-arginine-induced acute pancreatitis in mice. *PLoS One* 2012;7:e46201.
20 Mirakaj V, Gatidou D, Pötzsch C, *et al*. Netrin-1 signaling dampens inflammatory peritonitis. *J Immunol* 2011;186:549–55.
21 Zheng Z, Zheng F. Immune cells and inflammation in diabetic nephropathy. *J Diabetes Res* 2016;2016:1–10.
22 Gluhovschi C, Gluhovschi G, Petrica L, *et al*. Urinary biomarkers in the assessment of early diabetic nephropathy. *J Diabetes Res* 2016;2016:1–13.
23 American Diabetes Association. 2. classification and diagnosis of diabetes. *Diabetes Care* 2017;40:S11–24.
24 Haneda M, Utsunomiya K, Koya D, *et al*. A new classification of diabetic nephropathy 2014: a report from joint Committee on diabetic nephropathy. *J Diabetes Investig* 2015;6:242–6.
25 Levey AS, Coresh J, Balk E, *et al*. National Kidney Foundation practice guidelines for chronic kidney disease: evaluation, classification, and stratification. *Ann Intern Med* 2003;139:137–47.
26 DeLong ER, DeLong DM, Clarke-Pearson DL. Comparing the areas under two or more correlated receiver operating characteristic curves: a nonparametric approach. *Biometrics* 1988;44:837–45.
27 Ioannou K, Stel VS, Dounousi E, *et al*. Inflammation, endothelial dysfunction and increased left ventricular mass in chronic kidney disease (CKD) patients: a longitudinal study. *PLoS One* 2015;10:e0138461.
28 Obermüller N, Geiger H, Weipert C, *et al*. Current developments in early diagnosis of acute kidney injury. *Int Urol Nephrol* 2014;46:1–7.
29 Ranganathan P, Mohamed R, Jayakumar C, *et al*. Guidance cue netrin-1 and the regulation of inflammation in acute and chronic kidney disease. *Mediators Inflamm* 2014;2014:1–13.
30 Ay E, Marakoğlu K, Kizmaz M, *et al*. Evaluation of netrin-1 levels and albuminuria in patients with diabetes. *J Clin Lab Anal* 2016;30:972–7.
31 Liu C, Li Q, Feng X, *et al*. Elevated levels of netrin-1 in the serum of patients with diabetic nephropathy: relationship with renal function and inflammation. *Eur J Inflamm* 2018;16:205873921880928.
32 Yim J, Kim G, Lee B-W, *et al*. Relationship between circulating netrin-1 concentration, impaired fasting glucose, and newly diagnosed type 2 diabetes. *Front Endocrinol* 2018;9:691.
33 Liu C, Ke X, Wang Y, *et al*. The level of netrin-1 is decreased in newly diagnosed type 2 diabetes mellitus patients. *BMC Endocr Disord* 2016;16:33.
34 Nedeva I, Gateva A, Assyov Y, *et al*. Relationship between circulating netrin-1 levels, obesity, prediabetes and newly diagnosed type 2 diabetes. *Arch Physiol Biochem* 2020:1–6.
35 Ramesh G, Krawczeski CD, Woo JG, *et al*. Urinary netrin-1 is an early predictive biomarker of acute kidney injury after cardiac surgery. *Clin J Am Soc Nephrol* 2010;5:395–401.
36 Jayakumar C, Nauta FL, Bakker SJL, *et al*. Netrin-1, a urinary proximal tubular injury marker, is elevated early in the time course of human diabetes. *J Nephrol* 2014;27:151–7.
37 White JJ, Mohamed R, Jayakumar C, *et al*. Tubular injury marker netrin-1 is elevated early in experimental diabetes. *J Nephrol* 2013;26:1055–64.
38 Gao S, Zhang X, Qin Y, *et al*. Dual actions of netrin-1 on islet insulin secretion and immune modulation. *Clin Sci* 2016;130:1901–11.
39 Toque HA, Fernandez-Flores A, Mohamed R, *et al*. Netrin-1 is a novel regulator of vascular endothelial function in diabetes. *PLoS One* 2017;12:e0186734-e.
40 De Breuck S, Lardon J, Rooman I, *et al*. Netrin-1 expression in fetal and regenerating rat pancreas and its effect on the migration of human pancreatic duct and porcine islet precursor cells. *Diabetologia* 2003;46:926–33.
41 Ranganathan PV, Jayakumar C, Ramesh G. Netrin-1-treated macrophages protect the kidney against ischemia-reperfusion injury and suppress inflammation by inducing M2 polarization. *Am J Physiol Renal Physiol* 2013;304:F948–57.
42 Jayakumar C, Mohamed R, Ranganathan PV, *et al*. Intracellular kinases mediate increased translation and secretion of netrin-1 from renal tubular epithelial cells. *PLoS One* 2011;6:e26776.
43 Mohamed R, Jayakumar C, Ranganathan PV, *et al*. Kidney proximal tubular epithelial-specific overexpression of netrin-1 suppresses inflammation and albuminuria through suppression of COX-2-mediated PGE2 production in streptozotocin-induced diabetic mice. *Am J Pathol* 2012;181:1991–2002.
44 White JJ, Mohamed R, Jayakumar C, *et al*. Tubular injury marker netrin-1 is elevated early in experimental diabetes. *J Nephrol* 2013;26:1055–64.