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Urinary Netrin-1 as an Early Marker for Diabetic Nephropathy

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ABSTRACT:

Background: Netrin-1, a laminin-related secreted protein, is a proximal tubular injury urinary marker. It is released in the urine of both humans and mice and highly stimulated after acute and chronic renal diseases.

Objective: The current study aimed to assess the value of urinary netrin-1 level as an early marker for diabetic nephropathy.

Patients and Methods: A total of 60 diabetic patients and 20 healthy controls were enrolled in this case-control study. Diabetic patients were subdivided into normoalbuminuria, microalbuminuria, and macroalbuminuria. Urinary netrin-1 levels were analyzed by enzyme-linked immunosorbent assay (ELISA).

Results: Urinary netrin-1 excretion was significantly higher in the diabetic group (1418.3±733.6 pg/mg creatinine) compared to the control group (477.4±283.6 pg/mg creatinine) with the highest value in the macroalbuminuria group (1919.4±573.4 pg/mg creatinine) and the lowest value in normoalbuminuria group (833.7±595.3 pg/mg creatinine). ROC curve analysis showed that urinary netrin 1/creatinine at a cutoff point of >630.75 pg/mg with AUC of 0.899 had 83.3% sensitivity and 85% specificity for prediction of diabetic nephropathy (P<0.001).

Conclusion: Our study is suggesting that urinary netrin-1 may be a useful biomarker for early detection of diabetic nephropathy.

Keywords: Diabetic nephropathy, Netrin-1, Albuminuria.

INTRODUCTION

Egypt is considered as the 9th leading country in the world for the incidence of diabetes. The prevalence of diabetes in 2019 was around 8.9 million which accounts for 17.2% among adults between 20 and 79 years of age, with an annual death of 76,262 related to diabetes that accounts for 41.6% of deaths under 60 years [(1)].

Diabetic nephropathy (DN) is a serious microvascular complication of diabetes which may be complicated with renal failure, cardiovascular disease, and premature mortality [(2)]. Most diabetic nephropathy patients will die from cardiovascular disease before they reach end-stage renal disease (ESRD) and start on dialysis [(3)].

Traditionally, microalbuminuria is considered the hallmark of early diagnosis of DN. However, a significant renal injury arises among normoalbuminuric diabetic patients that are accompanied by more advanced glomerular lesions [(4)].

Although diabetic nephropathy is classically believed to be a glomerular disease, tubulointerstitial injury is suggested to precede obvious glomerulopathy [(5)].

Netrin-1 is a laminin-related protein of 50–75 KD weight that has been stated as a neuronal guidance cue [(6)]. It is principally expressed in the central nervous system (CNS), but also in non-neural tissues such as kidneys, liver, pancreas, lungs, and vascular endothelium [(7)].

Also, netrin-1 has an anti-angiogenic effect that improves blood flow to hypoxic tissue and has a promising cardioprotective value in the prevention of ischemia-reperfusion injury through the subsequent production of nitric oxide in animal models [(8)].

The current study aimed to assess the value of urinary netrin-1 level as an early marker for diabetic nephropathy.

SUBJECTS AND METHODS

Sixty patients with type 2 diabetes, at the diabetic outpatient clinic of Zagazig University Hospitals between February 2018 and February 2019, as well as 20 healthy controls, were enrolled for this case-control study. All laboratory investigations were done in the Clinical Pathology Department in Zagazig University Hospitals. A total of 80 subjects had a mean age of 49.7 ± 11.5 years with 40 males and 40 females. Diabetes mellitus was diagnosed according to The American Diabetes Association criteria [(9)].

Ethical Considerations:

Informed consent was obtained from all participants and **the study protocol was approved by the Internal Medicine Department and The Institutional Review Board (IRB) of the Faculty of Medicine at Zagazig University.** The work has been carried out following The Code of Ethics of the World Medical Association (Declaration of Helsinki) for studies involving humans.

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Inclusion criteria: Age ≥ 18 years, estimated GFR (eGFR) ≥ 60 mL/min/1.73 m² by MDRD 6 variable equation, stable renal functions without doubling of serum creatinine in the last 5 months, and diabetic patients on oral hypoglycemic medications or insulin therapy.

Exclusion criteria:

Age < 18 years, patients known to have acute vasculitis, malignancy or severe liver dysfunction, evidence of current inflammation or infection, recent or current use of steroids, chronic kidney disease (CKD) other than diabetic nephropathy, pregnancy, history of acute myocardial infarction, recent stroke or occlusive peripheral vascular disease within last 6 months and history of renal transplantation.

Based upon urinary albumin/creatinine ratio, diabetic patients were categorized into 3 groups: normoalbuminuria group (albumin/creatinine ratio < 30 mg/g), microalbuminuria group (albumin/creatinine ratio 30-299 mg/g) and macroalbuminuria group (albumin/creatinine ratio ≥ 300 mg/g). 20 patients were involved in each group.

All subjects in the study underwent: full history taking, thorough clinical examination, and routine laboratory investigations including fasting blood sugar (FBS), HbA1C, complete blood count, renal function tests, liver function tests, and lipid profile. Assay of urinary microalbumin was done using Immunometric Enzyme Immunoassay kits (ORGENTEC Diagnostika GmbH, Mainz, Germany). Spot morning urine samples were collected and stored at -20 ℃ till assay. Urinary microalbumin level was divided by urinary creatinine level to calculate urinary albumin/creatinine ratio in mg/gm. Assay of the urinary netrin-1 level was done using Human Netrin-1 ELISA Kits (Elabscience, Catalog No: E-EL-H2328, USA) according to the manufacturer's protocol. Urine samples were centrifuged for 20 minutes at 1000 rpm. Supernatants were collected and kept at -20 ℃ to preserve till assay. Urinary netrin-1 is expressed in pg per mg of creatinine.

Statistical analysis

The collected raw data was analyzed using Statistical Package for Social Science **(SPSS 25)** for Windows (SPSS Inc., Chicago, IL, USA). Mean, standard deviation (± SD) was used for parametric numerical data, while median and interquartile range (IQR) was used for non-parametric numerical data. Frequency and percentage were used for non-numerical data. Kolmogorov-Smirnov test was done to assure the normal distribution of data. An independent t-test was used to calculate the difference between quantitative variables in two groups for parametric variables. A one-way ANOVA test was used to calculate the difference between quantitative variables in more than two groups for parametric variables. The Least Significant Difference (LSD) posthoc test was used to compare the two groups. A Chi-Square test was used to examine the relationship between two qualitative variables. Fisher's exact test was used to examine the relationship between two qualitative variables when the expected count is less than 5 in more than 20% of cells. Receiver operating characteristic (ROC) curve analysis was used to identify optimal cut-off values of urinary netrin-1 with maximum sensitivity and specificity as a predictor for diabetic nephropathy. P-value≤0.05 is considered significant and P-value<0.001 is considered highly significant, while P-value>0.05 is considered non-significant.

RESULTS

Regarding baseline characteristics, we found that the percentage of hypertensive patients, fasting blood sugar level (FBS), HbA1C, and urinary albumin/creatinine ratio was significantly higher (P<0.001), while hemoglobin (Hb), hematocrit, serum albumin, and urinary creatinine were significantly lower in diabetic group compared to controls (table 1). Moreover, there were statistically significant differences among diabetic subgroups regarding their sex, duration of DM, and type of treatment (table 2). The macroalbuminuria group had the longest duration of DM and the highest proportion of patients on insulin therapy.

Regarding the urinary netrin-1 level, it was significantly higher in the diabetic group compared to controls (P<0.001) (table 1).

In the posthoc test (table 3), urinary netrin-1 levels were significantly higher in normoalbuminuria (P<0.05), microalbuminuria, and macroalbuminuria groups (P<0.001) compared to controls. Moreover, urinary netrin-1 levels were significantly higher in microalbuminuria and macroalbuminuria groups compared to the normoalbuminuria group. However, there was no significant difference between macroalbuminuria versus microalbuminuria groups.

In the ROC curve, our study revealed that urinary netrin 1/creatinine more than 630.75 pg/mg had 83.3% sensitivity and 85% specificity for diabetic nephropathy which was statistically highly significant (P<0.001) (table 4, figure 1).

Table (1): Clinical and laboratory parameters among diabetic and control groups

Variables		Controls (n=20)	DM (n=60)	Test	P-value
Age		43.7±11.7	51.7±10.7	1.818	0.061
Sex*	**Male**	14 (70%)	26 (43.3%)	4.28	**0.039**
	Female	6 (30%)	34 (56.7%)		
Smoking#		4(20%)	8(13.3%)	0.52	0.48
Hypertension#		0(0%)	49(81.7%)	42.51	**<0.001**
FBS, mg/dl		92.9 ± 5.5	174.1 ± 43.7	8.25	**<0.001**
HbA1C, %		5.4 ± 0.1	9.1 ± 1.8	9.3	**<0.001**
Hematocrit, %		41.3 ± 4	37.4 ± 3.6	4.07	**<0.001**
WBC, x10^9/L		8 ± 2.6	8.3 ± 1.8	0.63	0.531
Hb, g/dl		14 ± 1.5	12.4 ± 1.6	4.06	**<0.001**
Platelets, x10^9/L		252 ± 7.8	261.4 ± 7.5	0.49	0.622
Total Cholesterol, mg/dl		194 ± 36.5	205.2 ± 44	1.02	0.312
LDL, mg/dl		128.8 ± 33.2	125.5 ± 30.3	0.41	0.684
HDL, mg/dl		28.5 ± 1.7	33.8 ± 3.4	1.58	0.118
Triglycerides, mg/dl		183.7 ± 8.6	229.8 ± 18.2	1.62	0.108
Serum Albumin, g/dl		4.5 ± 0.3	3.8 ± 0.6	4.1	**<0.001**
ALT, IU/L		15.8 ± 3	15.4 ± 6.8	0.21	0.833
AST, IU/L		21.9 ± 3.5	21.7 ± 3.5	0.11	0.913
Total Bilirubin, mg/dl		0.4 ± 0.1	0.5 ± 0.02	1.38	0.171
Creatinine, mg/dl		0.9 ± 0.1	0.9 ± 0.2	0.93	0.353
BUN, mg/dl		14.4 ± 3.3	14.5 ± 4.2	0.05	0.963
eGFR ml/min/1.73 m^2		90.6 ± 13.9	86.9 ± 5.9	0.61	0.543
Urinary creatinine gm/dl		0.16±0.06	0.06±0.05	7.72	**<0.001**
Urinary abumin/creatinine ratio mg/gm		21.6±5.7	539.2±86.9	2.93	**0.004**
Urinary netrin-1/creatinine pg/mg		477.4±283.6	1418.3±33.6	8.26	**<0.001**

- All variables were expressed in Mean±SD except (*) and (#) expressed in their No. (%).
- All variables were compared using independent t-test except (*) and (#) using the Chi-square test and Fisher exact test respectively.

ALT: alanine transaminase, AST: aspartate transaminase, BUN: blood urea nitrogen, eGFR: estimated glomerular filtration rate, FBS: fasting blood sugar, HbA1C: Glycosylated hemoglobin, HDL: high-density lipoprotein, LDL: low-density lipoprotein, WBCs: white blood cells

Table (2): Clinical and laboratory parameters among the studied groups

Variables		Category				Test	P-value
		Control	Normoalbuminuria	Microalbuminuria	Macroalbuminuria		
Age		43.7±11.7	51.75±8.15	49.85±14	53.35±9.5	0.523	0.595
Sex *	Male	14 (70%)	5 (25%)	7 (35%)	14 (70%)	8.78	**0.003**
	Female	6 (30%)	15 (75%)	13 (65%)	6 (30%)		
Smoking#		4 (20%)	3 (15%)	2 (10%)	3 (15%)	0.42	0.997
Duration of DM		-	3.8±2.57	7.85±2.89	17.1±5.42	62.97	**<0.001**
Hypertension*		0 (0%)	17 (85%)	15 (75%)	17 (85%)	0.89	0.641
Treatment #	Oral	-	20 (100%)	16 (80%)	2 (10%0	38.49	**<0.001**
	Insulin	-	0 (0%)	4 (20%)	18 (90%)		
FBS, mg/dl		92.9 ± 5.5	166.7 ± 50.6	175.8 ± 4	179.9 ± 4	22.9	**<0.001**
HbA1C, %		5.4 ± 0.1	8.4 ± 1.7	9.2 ± 1.8	9.6 ± 1.7	32.5	**<0.001**
Hematocrit, %		41.3 ± 4	38.2 ± 3.8	38.2 ± 3.9	35.8 ± 2.4	7.9	**<0.001**
WBC, x10^9/L		8 ± 2.6	8 ± 1.9	8.8 ± 1.8	8.3 ± 1.6	0.7	0.577
Hb, g/dl		14 ± 1.5	12.9 ± 1.4	13.1 ± 1.5	11.2 ± 1.1	14.9	**<0.001**
Platelets, x10^9/L		252 ± 7.8	253.1 ± 8.7	254.2 ± 7.4	276.9 ± 7.2	0.5	0.665
Total Cholesterol mg/dl		194 ± 36.5	203.9 ± 41.7	205.2 ± 53.5	206.4 ± 37.6	0.3	0.79
LDL, mg/dl		128.8 ± 33.2	130.6 ± 33.8	122.8 ± 34.3	123.1 ± 22.3	0.3	0.811
HDL, mg/dl		28.5 ± 1.7	36 ± 5.8	32.2 ± 2.2	33.2 ±2.2	1.1	0.343
Triglycerides, mg/dl		183.7 ± 8.6	187.7 ± 8.1	250.7 ± 40.8	251 ± 18.5	2.4	0.072
Serum Albumin, g/dl		4.5 ± 0.3	4.2 ± 0.2	4.2 ± 0.4	3.1 ± 0.5	49.3	**<0.001**
ALT, IU/L		15.8 ± 3	13.5 ±3.2	14.2 ± 3.7	18.6 ± 4.4	2.3	0.083
AST, IU/L		21.9 ± 3.5	20.7 ± 3.7	19.8 ± 3.4	24.6 ± 6.9	1.5	0.22
Total Bilirubin, mg/dl		0.4 ± 0.1	0.4 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	1.4	0.258
Creatinine, mg/dl		0.9 ± 0.1	0.8 ± 0.2	0.8 ± 0.2	1.1 ± 0.1	12.5	**<0.001**
BUN, mg/dl		14.4 ± 3.3	12.2 ± 2.8	12.6 ± 2.3	18.5 ± 4	16.5	**<0.001**
eGFR ml/min/1.73m^2		90.6 ± 13.9	93.7 ± 16.5	102 ± 3	65 ± 7.2	13.8	**<0.001**
Urinary creatinine g/dl		0.16±0.06	0.12±0.06	0.05±0.01	0.03±0.01	35.03	**<0.001**
Urinary albumin/creatinine ratio mg/gm		21.6±5.7	19.7±4.3	92±2	1505.8±66.4	94.75	**<0.001**
Urinary netrin-1/creatinine ratio pg/mg		477.4±83.6	833.7±95.3	1501.9±77	1919.4±73.4	17.306	**<0.001**

- All variables were expressed in Mean±SD except (*) and (#) expressed in their No. (%).
- All variables were compared using the ANOVA test except (*) and (#) using the Chi-square test and Fisher exact test respectively.

ALT: alanine transaminase, AST: aspartate transaminase, BUN: blood urea nitrogen, DM: diabetes mellitus, eGFR: estimated glomerular filtration rate, FBS: fasting blood sugar, HbA1C: Glycosylated hemoglobin, HDL: high-density lipoprotein, LDL: low-density lipoprotein, WBCs: white blood cells

Table (3): Least Significant Difference (LSD) Posthoc test.

Laboratory parameters	Control Vs. Macro	Control Vs. Micro	Control Vs. Normo	Macro Vs. Micro	Macro Vs. Normo	Micro Vs. Normo
FBS, mg/dl	**<0.001**	**<0.001**	**<0.001**	0.733	0.28	0.458
HbA1C, %	**<0.001**	**<0.001**	**<0.001**	0.352	**0.015**	0.126
Hematocrit, %	**<0.001**	**0.009**	**0.009**	**0.034**	**0.035**	0.993
Hb, g/dl	**<0.001**	**0.033**	**0.014**	**<0.001**	**<0.001**	0.733
Serum Albumin, g/dl	**<0.001**	**0.034**	**0.037**	**<0.001**	**<0.001**	0.967
eGFR, ml/min/1.73m^2	**<0.001**	0.066	0.61	**<0.001**	**<0.001**	0.179
Serum Creatinine, mg/dl	**0.013**	**0.006**	**0.016**	**<0.001**	**<0.001**	0.706
BUN, mg/dl	**<0.001**	0.082	**0.031**	**<0.001**	**<0.001**	0.666
Urinary Creatinine, g/dl	**<0.001**	**<0.001**	**0.01**	0.147	**<0.001**	**<0.001**
Albumin/creatinine ratio mg/g	**<0.001**	**<0.001**	0.084	**<0.001**	**<0.001**	**<0.001**
Urinary netrin-1/creatinine pg/mg	**<0.001**	**<0.001**	**0.043**	0.089	**<0.001**	**<0.001**

Macro: macroalbuminuria, micro: microalbuminuria, normo: normoalbuminuria

Table (4): Validity of urinary netrin-1 in diabetic nephropathy.

Variables	Area under Curve	Standard Error	Asymptotic Significant	Asymptotic 95% confidence interval		Cut-off	Sensitivity	Specificity
				Lower Bound	Upper Bound			
Urinary netrin-1/creatinine pg/mg	0.899	0.036	**0.0001**	0.828	0.969	630.75	83.3%	85%

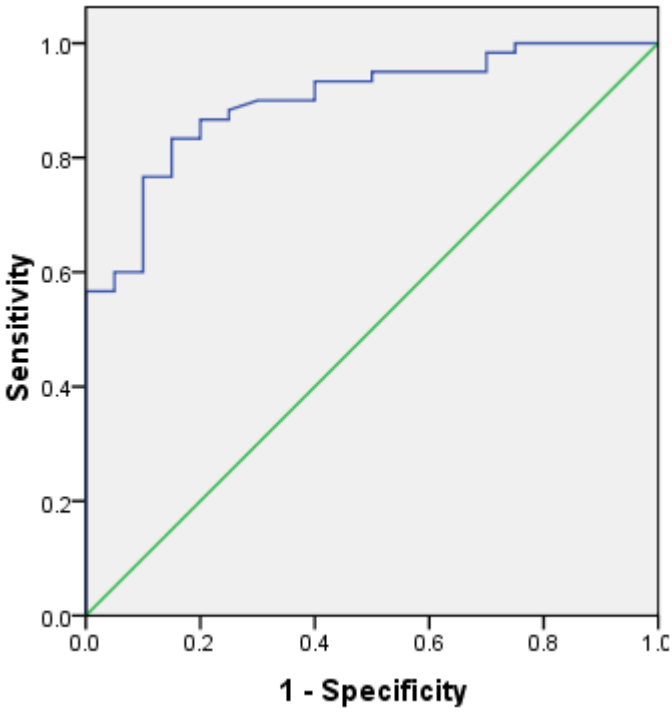

Figure (1): ROC curve of urinary netrin-1/creatinine in diabetic nephropathy.

DISCUSSION

Netrin-1 is a laminin-related protein expressed in the endothelial cells of normal kidneys with very little expression in the tubular epithelial cells. After kidney injury, the expression of netrin-1 protein emerges in the proximal tubular epithelial cells while down-regulates in vascular endothelial cells [(10)].

In our study, it was found that urinary netrin-1excretion (in pg/mg creatinine) was significantly higher in the diabetic group (1418.3±733.6 pg/mg creatinine) compared to the control group (477.4±283.6 pg/mg creatinine) (table 1) with the highest value in macroalbuminuria group (1919.4±573.4 pg/mg creatinine) and the lowest value in normoalbuminuria group (833.7±595.3 pg/mg creatinine) (table 2 and figure 1). Of note, urinary netrin-1 was significantly higher in normoalbuminuria ($P<0.05$), microalbuminuria, and macroalbuminuria groups ($P<0.001$) in comparison with the control group. Moreover, urinary netrin-1 was significantly higher in microalbuminuria and macroalbuminuria cases in comparison with normoalbuminuria cases. Furthermore, in ROC curve analysis, urinary netrin-1/creatinine at a cutoff point of >630.75 pg/mg with AUC of 0.899 had 83.3% sensitivity and 85% specificity for prediction of diabetic nephropathy ($P<0.001$).

These results are suggesting that early tubular lesions may precede glomerular lesions in developing micro- and macroalbuminuria. This is further supported by recent studies showing that the severity of DN depends not only on the severity of glomerular lesions but also on the degree of damage of tubulointerstitium. Brito *et al.* showed that the width of the proximal tubular basement membrane is thickened in diabetics with normoalbuminuria compared with healthy controls [(11)]. In another study in diabetic patients with microalbuminuria and similar eGFR, Fioretto and his colleagues showed that only 29 % had typical histological glomerular features of diabetic nephropathy, while 42 % had severe tubulointerstitial damage disproportionate to the mild glomerular lesion [(12)].

The role of the tubular epithelial cells in the development of albuminuria is supported by the recent observation that a mutation in cubilin, which is a receptor for albumin mediating its endocytosis, caused severe proteinuria in humans [(13)]. Furthermore, a proximal tubular epithelial cell-specific cubilin also developed albuminuria in knockout mice [(14)].

Our results go hand in hand with those reported by Jayakumar and his colleagues **(5)**. It is in agreement also with **Ramesh *et al.*** [(15, 16)] and with **Tu *et al.*** [(17)]. Ramesh and his colleagues found that the urinary netrin-1 level is significantly higher in patients who developed acute kidney injury (AKI) after cardiopulmonary bypass compared to patients who did not experience AKI after surgery [(15)]. Also, urinary netrin-1 was significantly higher in renal transplant recipients postoperatively and in patients who developed AKI induced by radiocontrast, sepsis, ischemia, and drugs as compared to healthy controls [(16)]. Tu and his colleagues found that urinary netrin-1 was significantly higher in septic patients in ICU who developed AKI compared to those who didn't experience AKI and to healthy controls [(17)].

Our results are also consistent with **Ay *et al.*** [(18)] who found that plasma netrin-1 in microalbuminuric diabetic patients was significantly higher than that in normoalbuminuric diabetic patients and control group. However, no significant difference was observed between normoalbuminuric patients and the control group. Moreover, our results are in agreement with Yim and his colleagues. However, they didn't classify diabetic patients according to the degree of albuminuria[(19)].

In contrast to our results, **Liu *et al.*** [(20)] reported that serum netrin-1 level in diabetic patients was significantly lower than that in the control group ($P<0.001$).

There may be three possible explanations for this finding. First, there is a role for netrin-1 in the inflammation that was involved in the pathogenesis of type 2 DM. **Natura *et al.*** [(21)] reported that netrin-1 could play role in the regulation of inflammation that might negatively regulate the secretion of insulin and participate in β-cell dysfunction. Netrin-1 regulates COX-2 expression at the transcriptional level [(22)]. Moreover, overexpression of netrin-1 promotes islet remodeling and differentiation of macrophage to the "alternative" or M2-like phenotype[(23)]. Second, netrin-1 is negatively correlated with hyperglycemia and is linked with islet dysfunction in diabetic patients. It is well known that repeated and prolonged exposure to hyperglycemia leads to β-cell degradation reduces glucose-stimulated insulin secretion and ultimately causes β-cell apoptosis [(24)]. **De Breuck *et al.*** [(23)] had proposed that netrin-1 is expressed and secreted in the pancreas where it plays a key role in regenerating pancreatic morphogenesis. In patients with newly diagnosed type 2 DM, secretion of netrin-1 in the damaged and apoptotic β cells is considerably decreased, in turn encouraging failure of β-cell function. Third, insulin resistance disturbs the circulation of netrin-1. It has proposed that netrin-1 is only selectively upregulated in the visceral white adipose tissue and that a considerable decline in circulating netrin-1 level occurs in obese compared with lean individuals. In obese rats, netrin-

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1 is a macrophage retention signal in adipose tissue during obesity. This may promote chronic inflammation and insulin resistance which subsequently develops in type 2 DM [25].

CONCLUSION

In conclusion, our study is suggesting that urinary netrin-1 may be a useful biomarker for early detection of diabetic nephropathy.

DECLARATION OF INTEREST: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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