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Diagnosis and management of diabetic nephropathy using novel biomarkers, CXCL16 and TNF-α receptors 1and 2

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Abstract

CXCL16 is expressed at a low level in epithelial cells in the normal kidney and play a crucial role in regulating inflammation and tissue injury. The function of TNFα is mediated by two structurally distinct receptors, TNFR1 and TNFR2. The reliability of current tests in predicting the onset, progression and response to various regimens for diabetic nephropathy is still under debate; and it has engendered a search for more sensitive and specific biomarkers. This study was aimed to evaluate the role of some relevant biomarkers in the pathogenesis of DN and that potentially may be used to predict the onset and/or monitor the progression of nephropathy. A total of Seventy Egyptian subjects, including 28 patients with diabetic nephropathy (DN group), 20 type 2 diabetes mellitus patients (T2DM group), and 22 healthy controls of similar age and gender (control group), were enrolled in this study. Serum levels of CXCL16 and TNFR1 & TNFR2 were measured using ELISA technique. Diabetic and renal biomarkers were measured in all subjects. The results showed drastic elevation in the levels of CXCL16, TNFR1, TNFR2, kidney function tests (except eGFR was highly significant decreased) and lipid profile parameters in diabetic nephropathy patients when compared to the control group. CXCL16, TNFR1 and TNFR2 showed high accuracy in the nephropathy group. TNFRs 1 & 2 and CXCl16 may be early, highly sensitive and specific markers and they could be used as a useful promising biomarker for early detection of diabetic nephropathy.

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Introduction

Diabetic nephropathy (DN) is an important microvascular complication of uncontrolled diabetes. The features of DN include albuminuria, extracellular matrix alterations, and progressive renal insufficiency. The major features of DN include glomerular hyperfiltration, urinary albumin excretion, mesangial expansion, basement membrane thickening, and extracellular matrix (ECM) alterations. This phenomenon eventually leads to renal failure (Boonloh *et al.,* 2018). C-X-C motif chemokine Ligand 16 (CXCL16) is a cytokine belonging to the C-X-C motif chemokine family and is unique in that it combines scavenger receptor functions with properties of an inflammatory chemokine (Norlander *et al.,* 2013).

CXCL16 is expressed at a low level in epithelial cells in the normal kidney and play a crucial role in regulating inflammation and tissue injury. CXCL16 contributes to chronic renal injury and fibrosis by recruiting fibrocytes, macrophages and T cells into the kidney. CXCL16 is a chemokine that plays an important role in regulating inflammation, tissue injury, and fibrosis *(*Liang *et al.,* 2016).

Several lines of evidence indicate that CXCL16 plays an important role in the pathogenesis of lupus nephritis, diabetic nephropathy and chronic kidney diseases *(*Wang *et al.,* 2015).

TNF is a pleiotropic cytokine that plays an essential role in mediating inflammatory processes. Generated by many cells, including fat, endothelial, and white blood cells. Subsequently, TNF and its receptors are shed from the cell surface by a disintegrin and metalloproteinase 17. In plasma, TNF appears as free or bound to circulating TNF receptor 1 (TNFR1) and TNF receptor 2 (TNFR2) (collectively referred to as markers of the TNF pathway) *(*Niewczas *et al.,* 2012)**.** TNFR1 and TNFR2 belong to the TNF receptor superfamily, a group of type I single transmembrane glycoproteins *(*Speeckaert *et al.,* 2012)**.** Serum tumor necrosis factor receptor 1 (TNFR1), and receptor 2 (TNFR2) concentrations are strong independent predictors of renal function decline leading to end-stage renal disease (ESRD) in Caucasians and American Indians with diabetes. Elevated serum concentrations of TNFR1 or TNFR2 in patients with type 2 diabetes are associated with early glomerular structural lesions *(*Pavkov *et al.,* 2016). The present study was constructed to evaluate the role of CXCL16, TNFR1 and TNFR2 as predictor markers for DN.

Materials and methods

Materials

This study included 70 patients together with 22 healthy control subjects; their mean age is 40±5 years. The patients were referred to the outpatient clinics of Diabetic Clinic at Ain Shams University Hospitals (Cairo-Egypt). The healthy controls subjects were volunteers from the laboratory staff of Biochemistry Department, Faculty of Science, Ain Shams University. A full medical history was taken with special attention to any associated medical problems.

Subjects were classified into the following groups: Group I, Includes 22 healthy subjects, Group II: Includes 20 patients with type 2 diabetes mellitus (DM) with ages range from (42-55) years and Group III: Includes 28 DN patients with age range from (38-60) years. Written informed consent was obtained from all patients after full explanation of the procedure used. The diagnosis of type 2 DM was performed according to the World Health Organization (WHO) criteria. DN was diagnosed by measuring the estimated glomerular filtration rate (e-GFR< 60 ml/min/1.73 m^2) and the urinary albumin excretion rate (UAER) at baseline. The exclusion criteria were: infectious diseases, liver diseases, cancer, smoking and alcoholism, and pregnancy. Before starting, informed consent was obtained from all participants. Ethical approval for this study was taken from Ain Shams University.

Methods

Urine Specimens

Twenty four hours urine sample was collected for each subject in clean container for quantitative determination of protein and creatinine.

Blood Specimens

Ten ml of venous blood samples were collected from patients and healthy controls in the morning after an overnight fasting. 2 ml of blood was collected on EDTA coated tube for HbA1c according to Trivelli (1971). The rest of blood sample was kept in clean glass tube without additives to clot at 37°C for 20 minutes, and then centrifuged at 3000 rpm for 10 minutes. The serum were then separated into aliquots and stored at -20°C to be thawed only once on demand, other serum samples were kept at 4-8°C and used for the estimation of serum lipid parameters within 5 days. The sera of all studied groups were subjected to the following investigations, plasma glucose (Trinder, 1969). kidney function tests included: urea according to Kaplan, (1984) to obtain BUN, creatinine (Butler, 1975), eGFR using Modification of Diet in Renal Disease (MDRD) study equation: (mL/min/1.73m2)= 186 × (Scr)−1.154 × (Age)−0.203 × (0.742 if female) (Woodhouse *et al.,* 2006), urinary protein (Watanabe *et al.,* 1986), urinary creatinine according to Jafe kinetic method (Hare, 1950), and protein/creatinine ratio (Sharma *et al.,* 2013). Total cholesterol (TC) (Trinder, 1969), triacylglycerol (TAG) (Young and Pestaner, 1975), high-density lipoprotein cholesterol (HDL-c), low-density lipoprotein cholesterol (LDL-c) & very low-density lipoprotein cholesterol (VLDL-c) (Friedewald, 1972). Inflammatory markers: chemokine CXCL16 by ELISA assay described by Matloubian *et al.,* (2000), TNFR1 by ELISA assay described by Chan *et al.,* (2000), and TNFR2 according to Santee *et al.,* (1996) by ELISA technique.

Statistical analysis

Statistical analysis was performed using the Statistical Package for the Social Science (SPSS) for Windows (version 23.0, Chicago, IL, USA). Data are presented as means ± SD. The data were analyzed by one-way analysis of variance (ANOVA). A P value ≤ 0.05 was considered statistically significant. Pearson's correlation coefficient analysis was used. Receiver operating characteristic (ROC) curve was performed to define the sensitivity and specificity.

Results

Diabetic biomarkers in Table 1 demonstrate highly significant elevation of fasting plasma glucose, postprandial plasma glucose, and HbA1c ($p<0.001$) in DM group by 105.8%, 156.3% and 56.4%, respectively when compared to control group. In DN group a highly significant elevation of GF, GPP, and HbA1c ($p<0.001$) by 134.4%, 120.8% and 47.9%, respectively were observed when compared to control group.

Table 1. Levels of fasting and postprandial plasma glucose, and insulin glycosylated hemoglobin, in all studied groups.

Groups	Parameters		
	GF (mg/dl)	GPP (mg/dl)	HbA1c%
NC mean ±SE	90.77 ±3.8	108.95±4.5	5.85±0.1
DM mean ±SE %change from C	186.85±10.1 105.8	279.3±14.8 156.3	9.15±0.36 56.4
DN mean ±SE %change from C	212.75±17.8 134.4	240.57±12.7 120.8	8.65±0.38 47.9

Values are given as means ±SE. NC: Normal control. DM: Diabetics without nephropathy. DN: Diabetics with clinical nephropathy. GF: Glucose fasting. GPP: Glucose postprandial. HbA1c: glycosylated hemoglobin.

Table 2 represent the renal biomarkers where eGFR showed highly significant decrease ($p<0.001$), While BUN and creatinine showed highly significant increase ($p<0.001$) in DN group by -83.2%, 236.3 and 277.9, respectively compared to control group. eGFR, BUN, creatinine, Urinary proteins, Urinary Creatinine and P/C ratio showed non-significant changes ($p>0.05$) in DM group by -13.8%, -7.4%, 15.2%, 28.5%, -6.4% and 90.4%, respectively when compared to control group. Urinary Creatinine showed highly significant decrease ($p<0.001$) in DN group by -48.8%. Urinary proteins and P/C ratio showed highly significant increase ($p<0.001$) in DN group by 992.6% and 2613%, respectively when compared to control group. Data in table (3) revealed. That TAG, TC, HDLc, LDLc and VLDLc showed non-significant changes ($p>0.05$) in diabetic group by 17.4%, 6.5%, -7.8%, 10.1% and 21.5% respectively

compared to NC group. Dyslipidemia (including high serum TAG, TC, LDL-c, and VLDL-c), were observed in DN group (p <0.001) (67.4%, 37.2%, 46.8% and 67.8% respectively) Only HDLc showed a non-significant change (p >0.05) by 2.6% compared to NC group. Table 4 showed non-significant change (p >0.05) in CXCL16 level in DM subjects by 5% while, it showed a highly significant increase (p< 0.001) in DN group by 19.8%, when compared to control group. TNFR1 and TNFR2 showed non-significant changes (p>0.05) DM group by -16.1% and 10.8% respectively, but in DN group they showed highly significant increase (p< 0.001) by 135% and 148.9%, respectively when compared to control group.

Table 3. Levels of different parameters of lipid profile in all studied groups.

Groups	Parameters				
	TAG mg/dl	TC mg/dl	HDL-c mg/dl	LDL-c mg/dl	VLDL-c mg/dl
NC mean ±SE	115.9±9.6	181.8±6.41	50.27±2.33	108.5±5.57	23.09 ±1.88
DM mean ±SE %change from C	136.1±13.49 17.4	193.7±8.58 6.5	46.35±2.54 -7.8	119.4 ±7.2 10.1	28.05 ±2.6 21.5
DN mean ±SE %change from C	194.07±8.58 67.4	249.5±7.23 37.2	51.57 ±3.132.6	159.17±6.73 46.8	38.75±1.72 67.8

Values are given as means ±SE. NC: Normal control. DM: Diabetics without nephropathy. DN: Diabetics with clinical nephropathy.

Table 4. Levels of CXCL16, TNFR1 and TNFR2 in all studied groups.

Groups	Parameters		
	CXCL16 pg/ml	TNFR1 pg/ml	TNFR2 pg/ml
NCmean ±SE	1419.4±33.3	19034.8 ±1512	10206.1±691
DM mean ±SE %change from C	1490.6±43.765	15970.8 ±2221-16.1	11307.7 ±93310.8
DN mean ±SE %change from C	1700±21.919.8	44723.1 ±5337 135	25406.9 ±2302148.9

Values are given as means ±SE. NC: Normal control. DM: Diabetics without nephropathy. DN: Diabetics with clinical nephropathy.

Results of the Receiver Operating Characteristic (ROC) curve displaying that CXCL16 provided the highest diagnostic information followed by TNFR2 & TNFR1 as biomarkers for DN with an AUC of 0.917, 0.828 & 0.778, respectively (P < 0.001) and cut off values of 1485.8 pg/ml, 18703.2 pg/ml and 23728.2 pg/ml (Fig. 1) (Table 5).

Table 5. Area under the curve and cut off values of CXCL16, TNFR1 and TNFR2 in DN group.

Test Result Variable(s)	Area Under the Curve	Cut off value
CXCL16 (pg/ml)	0.917	1485.8
TNFR1 (pg/ml)	0.778	23728.2
TNFR2 (pg/ml)	0.828	18703.2

ROC curves of the combined variables showed that CXCL16, TNFR1 and TNFR2 when used in comination together as diagnostic markers in case of diabetes mellitus Fig. (2) showed moderate accuracy with an AUC of 0.714 (P < 0.05) with cut-off value 0.4699 Table (6). While combined ROC curve in DN group Fig. (3) highest diagnostic performance with an AUC of 0.974 (P< 0.001) with cut-off value 0.8237 as provided in Table (7).

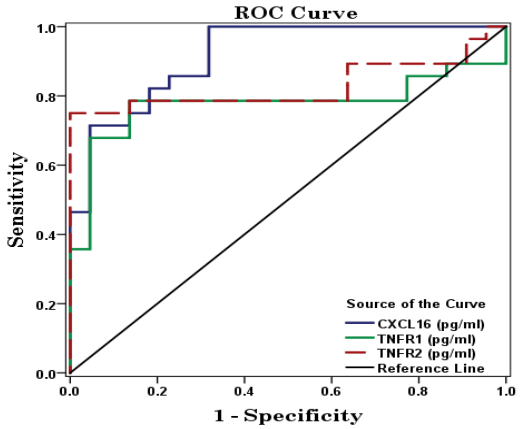

Fig. 1. Receiver operating characteristic (ROC) curves displaying the accuracy of CXCL16, TNFR1and TNFR2 in DN group.

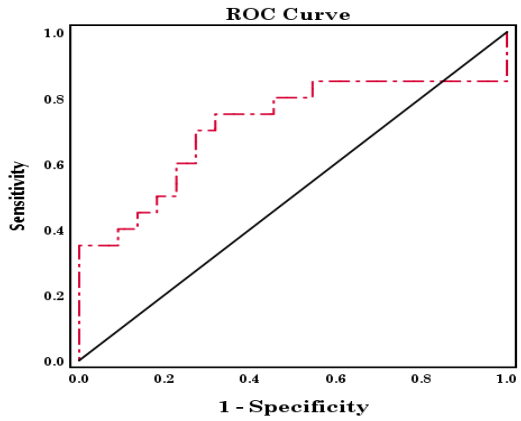

Fig. 2. Combined ROC curve displaying the accuracy of CXCL16, TNFR1and TNFR2 in DM group.

Table 6. Area under the curve and cut off values of combined variables in DM group. Test Result Variable (s).

Area	Std. Error[a]	Asymptotic Sig.[b]	Asymptotic 95% Confidence Interval		Cut off value
			Lower Bound	Upper Bound	
0.714	0.085	0.018	0.547	0.881	0.4699

a. Under the nonparametric assumption

b. Null hypothesis: true area = 0.5.

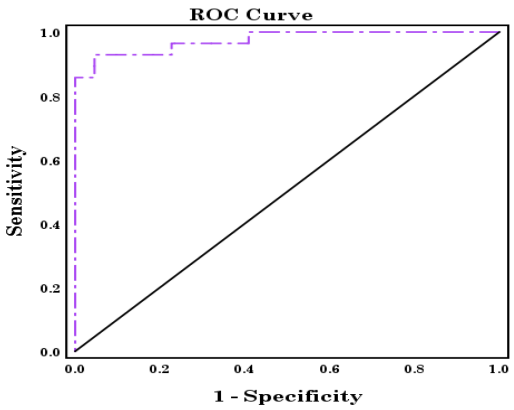

Fig. 3. Combined ROC curve displaying the accuracy of CXCL16, TNFR1and TNFR2 in DN group.

Table 7. Area under the curve and cut off values of combined variables in DN group. Test Result Variable (s).

Area	Std. Error[a]	Asymptotic Sig.[b]	Asymptotic 95% Confidence Interval		Cut off value
			Lower bound	Upper bound	
0.974	0.019	0.000	0.937	1.000	0.8237

a. Under the nonparametric assumption

b. Null hypothesis: true area = 0.5

Discussion

Diabetic nephropathy represents the major cause of end-stage renal disease (ESRD) in Western societies. One of the hallmarks of diabetic nephropathy is the development of proteinuria, which is usually followed by a progressive decline in renal function. The development of diabetic nephropathy is also a major risk factor for cardiovascular disease. From an epidemiological, pathophysiological and clinical perspective, hypertension and poor glycaemic control are usually associated with this condition (Fineberg *et al.,* 2013). CXCL16 exists in both transmembrane-bound and soluble forms. Transmembrane-bound CXCL16 acts as both a cell surface adhesion molecule and a novel scavenger receptor. Soluble CXCL16 can recruit activated immune cells that express CXCR6, the receptor of CXCL16, and mediate immune response-related inflammation (Wang *et al.,* 2015).

Our results revealed that there is a highly significant increase in serum concentration of CXCL16 in diabetic nephropathy subjects when compared to control group. While, in T2DM group non-significant change were observed when compared to healthy subjects. These results are in agreement with Zhao *et al.* (2014) who suggested that CXCL16 is involved in the pathogenesis of renal dysfunction in diabetes patients as supported by two findings. First, serum CXCL16 levels were significantly increased in diabetes patients with renal disease when compared with healthy subjects. Furthermore, no significant changes in serum CXCL16 levels were found between healthy and T2DM subjects. Second, HDL and LDL cholesterol levels were significantly different only in subjects with DN and not in T2DM patients without renal injury in comparison with healthy subjects. These data suggest that CXCL16 may be a biomarker involved in the onset and deterioration of renal injury in diabetic patients, and the increased serum CXCL16 levels may be related to the abnormality of cholesterol metabolism in DN subjects.

Zhao *et al.* (2014) speculate that the elevation of CXCL16 expression in subjects with DN may be related to the abnormalities of cholesterol metabolism. This is supported by the facts that

elevation of CXCL16 following with higher levels of oxLDL were found in streptozotocin-induced diabetic mice, increased glomerular CXCL16 expression was also accompanied by high levels of oxidized low-density lipoprotein in subjects with glomerular kidney diseases in human (Gutwein *et al.,* 2009).

Also the present results were in agreement with Wang *et al.* (2015) who reported that, in the presence or absence of a broad spectrum metalloproteinase inhibitor, treatment of human podocytes with IFN-γ promoted the uptake of ox-LDL, and the application of a CXCL16 blocking antibody strongly reduced the uptake of ox-LDL in human podocytes, suggesting that CXCL16 could also act as a scavenger receptor for ox-LDL in podocytes. Results of the present study were in agreement with Elewa *et al.* (2016) who reported that, there were no significant differences in serum CXCL16 levels between healthy and type 2 diabetes mellitus (DM) subjects.

Izquierdo *et al.* (2012) reported that, CXCL16 has a proinflammatory effect on renal proximal tubular cells and potentiates TWEAK-induced inflammatory responses. Also, previous studies have shown that CXCL16 is upregulated in the kidney following unilateral ureteral obstruction and angiotensin induced renal injury, and genetic disruption of CXCL16 attenuates renal fibrosis and preserves kidney function.

The present data indicated that serum CXCL16 levels were strongly associated with eGFR in DN patients. This finding also is in agreement with Zhao *et al.* (2014) who reported that, serum CXCL16 levels were strongly associated with eGFR in DN patients suggesting that elevated CXCL16 levels are closely related to glomerular injury and declining renal function in DN patients and serum CXCL16 may be an indicator of renal injury in subjects with T2DM.

CXCL16 is an important chemokine that regulates renal injury and fibrosis in renal artery stenosis (RAS). In response to RAS, CXCL16 recruits macrophages, T cells, and myeloid fibroblasts into the kidney, leading to renal injury and fibrosis. These results suggest that inhibition of CXCL16 could constitute a novel therapeutic strategy for renovascular disease (Ma *et al.,* 2016).

Hasegawa *et al.* (1991) were the first to implicate TNFα in the pathogenesis of diabetic nephropathy. TNF achieves its pleiotropic cellular and pathologic effects by binding to its 2 surface receptors: TNFR1 and TNFR2. These receptors also bind lymphotoxin-α (formerly TNF-α), but no other members of the TNF ligand superfamily. TNFR1 (p55, CD120a) and TNFR2 (p75, CD120b) are related structurally, but are functionally distinct receptors that are co-expressed on the surface of most cell types. They are single transmembrane glycoproteins with 28% homology mostly in their extracellular domain (Vielhauer & Mayadas, 2007). Type 2 diabetes is associated with activation of the innate immune system and chronic low-grade inflammation (increased concentrations of TNF, IL-6 and C-reactive protein) (Donath & Shoelson, 2011).

The result of the current study showed that TNF receptors 1 & 2 were found to be highly significantly increased in diabetic nephropathy group while no significantly changes were found in diabetic subjects when compared to healthy subjects. These results are in harmony with results of Niewczas *et al.* (2012) who demonstrated that, the risk of ESRD in T2D was strongly associated with elevated concentrations of circulating TNFR1 and TNFR2.

In addition these results are in agreement with the explanation of Fernández-Real *et al.* (2012) who reported that, structural kidney damage in patients with type 2 diabetes is associated with TNF system activity and specifically with plasma sTNFR1 concentrations. Also they hypothesize that elevation of sTNFR1 precedes the development of renal structural damage and that sTNFRs could be more sensitive to predict renal functional and/or structural derangement than albuminuria. In fact, circulating sTNFR1 concentrations was similar in patients with micro albuminuria and norm albuminuria. Fernández-Juárez *et al.* (2017) reported that, high levels of TNFR1 are associated with progression of

renal disease and increased mortality in patients with established diabetic nephropathy. Previous studies have highlighted the role of inflammation, particularly the activity of the TNF system, in the progression of renal disease. In an experimental model, TNFα induced direct glomerular injury, which was markedly attenuated in mice lacking TNFα. It has been reported that exposure of the kidneys to TNFα increases the mRNA expression of TNFα receptors in the renal tubulointerstitium and triggers apoptosis and cell death (Fernández-Juárez *et al.,* 2017).

Moreover a single measurement of the plasma TNFR concentration (TNFR1 > TNFR2) was a predictor of ESRD in patients with type 2 diabetes, even after adjustment for clinical covariates such as urinary albumin excretion. There is a hypothesis that elevated concentrations of TNFRs contribute directly to renal injury and progressive renal function decline (Speeckaert *et al.,* 2012).

In clinical studies, TNFR2 has been independently associated with early loss of glomerular filtration (Izumi *et al.,* 2013). In a cross-sectional study, circulating TNFR2, but not TNFR1, was associated with an early decline in kidney function in non-diabetic Japanese patients (Kurashina *et al.,* 2014). In another study, early loss of glomerular filtration in patients with type 1 diabetes without proteinuria was strongly associated with circulating TNFR2 levels. A systemic rather than a local kidney source of TNFR is contributing to the increased risk for early renal function loss (Gohda *et al.,* 2012).

Gohda *et al.* (2017) demonstrated that, the TNF pathway is involved in the pathogenesis of various types of renal diseases, and that TNF-related biomarkers are also associated with the levels of albuminuria or GFR. Al-Al-Lamki & Mayadas (2014) reported that, TNF and its receptors may be viable biomarkers of DN. In studies of type 1 and type 2 diabetic patients, elevated levels of TNFR1 and TNFR2 emerged as strong, stable predictors of DN progression to chronic kidney disease, stage 3 or ESRD. This correlation was independent of circulating TNF and relevant clinical covariates.

The reported ability to predict ESRD in diabetic patients (high TNFR values preceded ESRD onset by several years), suggests that TNFRs may not simply be biomarkers, but contribute to disease outcome (Gohda *et al.,* 2012; Niewczas *et al.,* 2012). Results of ROC curves describing the diagnostic performance of different biomarkers for DN patients group revealed the following, CXCL16, TNFR1 & TNFR2 showed highest diagnostic information.

Diagnostic accuracy can be improved considerably by combining multiple markers, whose performance in identifying diseased subjects is usually assessed via receiver operating characteristic (ROC) curves. It is now well accepted that single markers may not be sufficiently accurate, while multi-marker combinations can achieve significant specificity and sensitivity values (Mazzara *et al.,* 2017).

Results of combined ROC curves in case of DM showed moderate accuracy while in case of DN group the combined ROC curve showed the highest accuracy and gives excellent diagnostic assay in DN.

Conclusion

In conclusion, TNFR1, TNFR2 and CXCl16 may be early, highly sensitive and specific markers and they could be used as a useful promising biomarker for early detection of diabetic nephropathy. Targeting on reducing their levels may be specific therapeutic interventions to prevent DN progression. Future studies should focus on social and genetic determinants of inflammation and their association with DN in Egyptian diabetic nephropathy patients.

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