

Changes in TLR-4 expression level and CD14+CD16+ monocyte ratio in the peripheral blood of patients with early diabetic nephropathies

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ABSTRACT: Cytokine-mediated activation of chronic microinflammatory and nonspecific immune responses plays an important role in the progression of early diabetic nephropathy (EDN). The purpose of this study was to investigate Toll-like receptor 4 (TLR-4) levels in monocytes and the CD14+CD16+ monocyte ratios in peripheral blood from diabetic patients with EDN. One hundred and eighty-eight patients with type 2 diabetes mellitus were recruited and divided into 3 groups according to their microalbumin (mALB) content: (1) A-group (mALB < 30 mg/24 h, n=60); (2) B-group (mALB 30–300 mg/24 h, n=64), which was further divided into the losartan potassium treatment group (B-T-group) and the untreated group (B-NT-group); and (3) C-group (mALB > 300 mg/24 h, n=64). Additionally, samples from 50 healthy patients were collected as the control group (N-group). Immune turbidimetric assays were used to measure high-sensitivity C-reactive protein (hsCRP) levels in serum. The levels of interleukin 6 (IL-6) in serum were detected by ELISA. CD14+CD16+ monocyte ratios and TLR-4 levels in monocytes were assessed by flow cytometry. There was no significant difference in the levels of hsCRP, IL-6, and TLR-4 and the CD14+CD16+ monocyte ratios between the A-group and N-group. The hsCRP, IL-6, TLR-4 and CD14+CD16+ monocyte levels in the B-group and C-group were notably higher than those in the A-group or N-group. Furthermore, compared with levels in the B-NT-group, TLR-4 and CD14+CD16+ monocyte levels were significantly decreased in the B-T-group. TLR-4 and CD14+CD16+ monocyte levels were increased in patients with EDN and restored with losartan potassium treatment, and the monocyte levels were positively correlated with the decrease in mALB. The TLR-4 level in monocyte and CD14+CD16+ monocyte ratio can be used as new targets for the early diagnosis of clinical diabetic nephropathy.

KEYWORDS: early diabetic nephropathy, TLR-4, CD14+CD16+, chronic immune microinflammation

INTRODUCTION

Type 2 diabetes (T2DM) is a chronic metabolic disease characterized by hyperglycemia, insulin resistance and a relative lack of insulin [1]. Type 2 diabetes is caused by complex genetic-environment interactions as well as other risk factors such as obesity and a sedentary lifestyle [2, 3]. T2DM and its complications, such as cardiovascular disease, diabetic neuropathy and nephropathy, seriously threaten human life and health [4–7]. With the incidence of increasing T2DM, finding an ideal treatment has become one of the primary tasks in combating this disease. Diabetic nephropathy is one of the most common and serious microvascular complications in diabetic patients [8].

Inflammation is associated with insulin resistance, diabetes and diabetes-related complica-

tions [9, 10]. Monocytes and Toll-like receptor (TLR) family are associated with inflammatory responses [11, 12]. Monocytes in the peripheral blood play an important role in innate immunity and response to infection. Monocytes include 3 subtypes: CD14+CD16–; CD14+CD16+; and CD14–CD16+ [13]. In healthy people, 90–95% of typical monocytes have high expression of CD14 (CD14+) and low expression of CD16 (CD16–). However, inflammation and infection lead to co-expression of CD14 and CD16 (CD14+CD16+) on the surface of monocytes [14]. CD14+CD16+ monocytes secrete high levels of TNF- α and low levels of anti-inflammatory factors such as interleukin-10 (IL-10). In addition, the TLR family is also very important for innate immunity [15]. TLR-4 is associated with atherosclerosis, diet-induced obesity and obesity associated insulin

resistance [16]. Previous studies have shown that monocytes and TLRs in diabetic patients have a proinflammatory effect [10]. However, the correlation between TLR-4 level in monocytes and the CD14+CD16+ monocyte ratio in peripheral blood from diabetic patients with EDN has been rarely reported.

In this study, the levels of TLR-4 and the CD14+CD16+ monocyte ratios in EDN patients were investigated by immunoturbidimetry, ELISA and flow cytometry. The results of this study demonstrate that the TLR-4 level in monocyte and CD14+CD16+ monocyte ratio can be used as new targets for the early diagnosis of clinical diabetic nephropathy.

MATERIALS AND METHODS

Patients

One hundred and eighty-eight patients with T2DM (from January to December 2015, Department of Endocrinology, Second Hospital of Tianjin Medical University) were recruited for this study. Inclusion criteria are as follows: pathologically confirmed T2DM with EDN; there was no use of glucocorticoids, anti-infection and anti-inflammatory drugs during the first three months of treatment. This study was subject to approval by the ethics committee of the Second Hospital of Tianjin Medical University and conducted in accordance with the provisions of the Declaration of Helsinki, Good Clinical Practice guidelines and local laws and regulations. All participants provided written informed consent and confirmed their willingness to perform glucose self-monitoring. Exclusion criteria are as follows: patients with disease of the brain, stomach, lung or other important organs; patients with hematological diseases; and patients with allergies to this treatment drug. The T2DM patients were divided into 3 groups according to their microalbumin (mALB) content: (1) A-group (mALB < 30 mg/24 h, n=60); (2) B-group (mALB 30–300 mg/24 h, n=64), which was further divided into the losartan potassium treatment group (B-T-group, n=32, oral losartan potassium 50 mg once a day) and the untreated group (B-NT-group, n=32); and (3) C-group (mALB > 300 mg/24 h, n=64). Losartan potassium (H20080371) was produced by the Yangtze River Pharmaceutical Group (Jiangsu, China). Patients in the losartan potassium treatment group took the losartan potassium according to the drug instructions. Additionally, 50 healthy participants were recruited (N-group), and all healthy

participants met the inclusion and exclusion criteria of this study. All volunteers were observed and monitored for one year. The characteristics of the study participants are shown in Table 1.

Detection of general biochemical indexes in patients

Fasting blood glucose (FBG), total cholesterol (TC), mALB and serum creatinine (Scr) levels in patients were measured using a BS2000M automatic biochemical analyzer (Mindray, Shenzhen, China), and Hemoglobin A1c (HbA1c) level was detected using a D-10 glycosylated hemoglobin analyzer (Bio-Rad, California, USA) according to the manufacturer's instructions.

Immunoturbidimetric assay

The level of hsCRP in serum was assessed by immunoturbidimetric assay. A C-reactive protein assay kit-CRP (immunoturbidimetry) (Leadman, Beijing, China) and an IMMAGE 800 specific protein analyzer (Beckman Coulter, Inc., California, USA) were used in this study.

Enzyme-linked immunosorbent assay (ELISA)

Levels of IL-6 in serum were measured by ELISA. Human IL-6 ELISA kit was purchased from Abcam (Cambridge, USA) and used according to the manufacturer's protocol. Serum was collected by centrifugation at $2000 \times g$ for 10 min and diluted with Sample Diluent NS. The serum was seeded into 96-well plate (50 μ l/well), then incubated with antibody (50 μ l/well) for 1 h at room temperature on a plate shaker set to 400 rpm. Then, the plates were washed 3 times with PBST (containing 0.05% Tween-20). TMB Substrate (100 μ l) was added to each well and incubated for 10 min in the dark on a plate shaker set to 400 rpm. Then, 100 μ l of stop solution was added to each well, and the absorbance value of each well was measured at 450 nm with the Thermo Fisher Multiskan FC (Thermo Fischer Scientific, Waltham, MA, USA). All samples were measured in duplicate.

Isolation of peripheral blood mononuclear cells (PBMCs) and monocytes

Peripheral blood was collected from patients and diluted at 1:1 using Hanks buffer. The diluted peripheral blood was slowly added into a centrifuge tube containing lymphocyte separation fluid (Solarbio, Beijing, China). The volume ratio of the diluted peripheral blood to lymphocyte separation solution was 2:1. The cells were centrifuged horizontally at

Table 1 The characteristics of the study participants.

Characteristic	A-group	B-T-group	B-NT-group	C-group	N-group
Number	60	32	32	64	50
Sex (M/F)	28/32	13/19	17/15	35/29	24/26
Age (year)*	53 (48–58)	59 (47–65)	56 (45–71)	61 (51–70)	49 (44–62)
Disease duration (month)*	84 (60–96)	90 (70–108)	72 (59–84)	88 (54–98)	

* Data are expressed as median (inter quartile range); M, male; F, female.

250 × g for 20 min at room temperature. A capillary pipette was inserted into the white cell layer to aspirate the cells, and the cells were transferred to another tube. Then, these cells were resuspended in Hanks buffer. The magnetic separation method was used for monocyte isolation as previously described [17].

Flow cytometry assay

Monocytes (1×10^5 cells) were washed with PBS buffer (containing 1% fetal bovine serum) and centrifuged at 250 × g for 15 min. Then, the cells were resuspended in PBS buffer and fixed with 4% formaldehyde for 15 min at room temperature. The cells were permeabilized with 100% and 90% methanol, and then incubated on ice for 30 min. Five microliters of primary antibodies, anti-CD16 (Becton, Dickinson and Company, New Jersey, USA), anti-CD14 (Becton-Dickinson) and anti-TLR-4 (Becton-Dickinson) were added into monocyte suspension sample (100 μl) at 4°C and incubated for 30 min in the dark. Cells were centrifuged at 250 × g for 20 min and washed with PBS buffer. Then, the cells were resuspended in PBS buffer. Then, the samples were analyzed using a Coulter XL flow cytometer (Beckman Coulter).

Statistical analysis

SPSS22.0 (version 22.0; IBM Corp., Armonk NY, USA) was used for statistical analysis. All data are expressed as the mean ± standard deviation (SD). Analysis of variance between groups was performed for variance analysis. Student's *t*-test was applied to compare 2 groups. one-way analysis of variance was applied to compare all pairs of the group. Pearson's correlation coefficients were calculated to analyze the correlation of hsCRP, IL-6 and CD14+CD16+ monocyte levels. In order to estimate the accuracy of the EDN prediction, receiver operating characteristics (ROC) curve was made, and areas under the curve (AUC) was calculated. $p < 0.05$ was considered statistically significant.

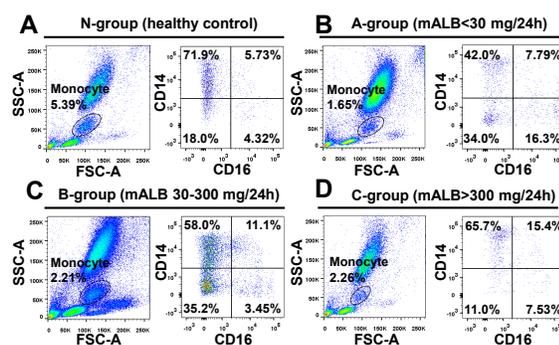


Fig. 1 Flow cytometry analysis of CD14+CD16+ monocyte ratio of: (A) a healthy patient in N-group, (B) a T2DM patient in A-group, (C) a T2DM patient in B-group, and (D) a T2DM patient in C-group.

RESULTS

Blood pressure and general biochemical indexes

The morning blood pressures of the patients in each group were within the range of 120–140/70–90 mmHg. In addition, there were no significant differences in the levels of FBG, TC, HbA1c and Scr among the A-group, B-group and C-group (Table 2, $p > 0.05$).

The levels of hsCRP, IL-6, TLR-4 and CD14+CD16+ monocytes in the A, B, C and N groups

To investigate TLR-4 levels in monocytes and the CD14+CD16+ monocyte ratios in peripheral blood from patients with T2DM, 188 patients with T2DM were recruited and divided into 3 groups according to their microalbumin (mALB) content: (1) A-group (mALB < 30 mg/24 h, n=60); (2) B-group (mALB 30–300 mg/24 h, n=64); and (3) C-group (mALB > 300 mg/24 h, n=64). The normality test showed that the hsCRP, IL-6 and TLR-4 levels in monocytes and CD14+CD16+ monocyte ratios in each group were normally distributed (Fig. 1, Table 3). There were no significant differences in hsCRP, IL-6 and TLR-4 levels in monocytes and

Table 2 General biochemical indexes of patients.

Group	n	FBG (mmol/l)	TC (mmol/l)	mALB (mg/24 h)	HbA1c (%)	Scr (μ mol/l)
N	50	4.80 \pm 0.40	4.90 \pm 0.99	5.51 \pm 0.45	4.71 \pm 0.50	67.3 \pm 4.51
A	60	8.94 \pm 2.45	4.97 \pm 1.17	6.71 \pm 0.71	8.66 \pm 1.89	70.4 \pm 8.41
B	64	9.17 \pm 2.31	4.54 \pm 1.52	55.63 \pm 6.93	8.87 \pm 2.29	72.5 \pm 9.34
C	64	10.17 \pm 3.19	5.09 \pm 1.26	552.94 \pm 96.33	9.11 \pm 1.65	75.9 \pm 9.76

Table 3 The levels of hsCRP, IL-6, TLR-4 and CD14+CD16+ monocyte in A, B, C and N groups.

Group	n	hsCRP (mg/l)	IL-6 (pg/ml)	TLR-4 (%)	CD14+CD16+ (%)
N	50	0.791 \pm 0.306	10.787 \pm 3.264	11.024 \pm 3.604	6.10 \pm 2.591
A	60	1.015 \pm 0.408	13.312 \pm 4.246	11.341 \pm 4.095	7.35 \pm 3.736
B	64	2.279 \pm 0.503*	23.456 \pm 4.285*	17.531 \pm 5.385*	11.5 \pm 4.550*
C	64	3.217 \pm 0.967*	32.317 \pm 8.672*	19.961 \pm 5.231*	15.6 \pm 4.807*

* $p < 0.05$ versus the N group.

Table 4 IL-6, TLR-4 and mALB levels and CD14+CD16+ monocyte ratios in B-T-group and B-NT-group.

Index	B-T-group (n=32)	B-NT-group (n=32)
IL-6 (pg/ml)	20.33 \pm 3.66	22.04 \pm 3.86
TLR-4 (%)	14.78 \pm 5.01*	19.81 \pm 2.57
CD14+CD16+ (%)	5.94 \pm 2.04*	9.49 \pm 3.15
mALB (mg/24 h)	20.61 \pm 3.21*	68.37 \pm 5.26

* $p < 0.05$ versus B-NT-group.

CD14+CD16+ monocyte ratio between the A-group and N-group ($p > 0.05$, Fig. 1, Table 3). However, the levels of hsCRP, IL-6 and TLR-4 in monocytes and CD14+CD16+ monocyte ratios in the B-group and C-group were notably higher than those in the N-group ($p < 0.05$, Fig. 1, Table 3). We also performed a one-way analysis of variance, and the results show that the hsCRP, IL-6 and TLR-4 levels in monocytes and CD14+CD16+ monocyte ratios have a significant effect on EDN progress ($p < 0.05$, Table 3).

The levels of hsCRP, IL-6, TLR-4 and CD14+CD16+ monocytes in the B-T-group and B-NT-group

Sixty-four patients in the B-group (mALB 30–300 mg/24 h) were divided into 2 groups with 32 cases in each group: the losartan potassium treatment group (B-T-group, oral losartan potassium 50 mg once per day) and the untreated group (B-NT-group). The levels of IL-6 and TLR-4 in monocytes, mALB and CD14+CD16+ monocyte ra-

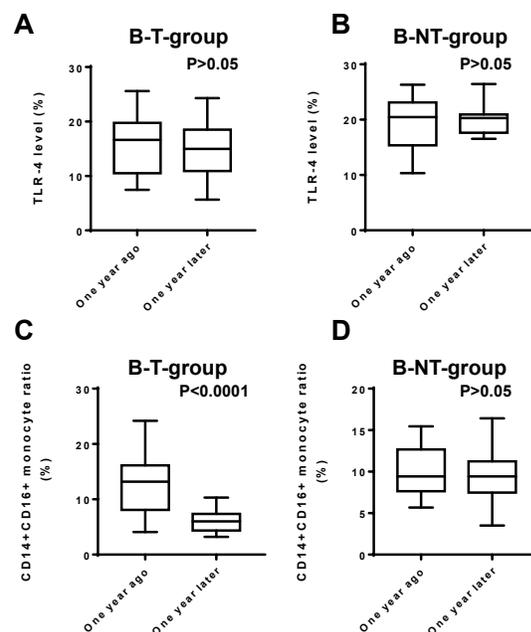


Fig. 2 Time-dependent analysis of TLR-4 and CD14+CD16+ monocytes in: (A,B) the B-T-group and (C,D) the B-NT-group.

tios were retested after one year. As shown in Table 4, the levels of TLR-4 in monocytes, mALB and CD14+CD16+ monocyte ratios in the B-T-group were significantly higher than those in the B-NT-group ($p < 0.05$). However, the IL-6 level was not significantly different between the B-T-group and the B-NT-group ($p > 0.05$, Table 4). At the same

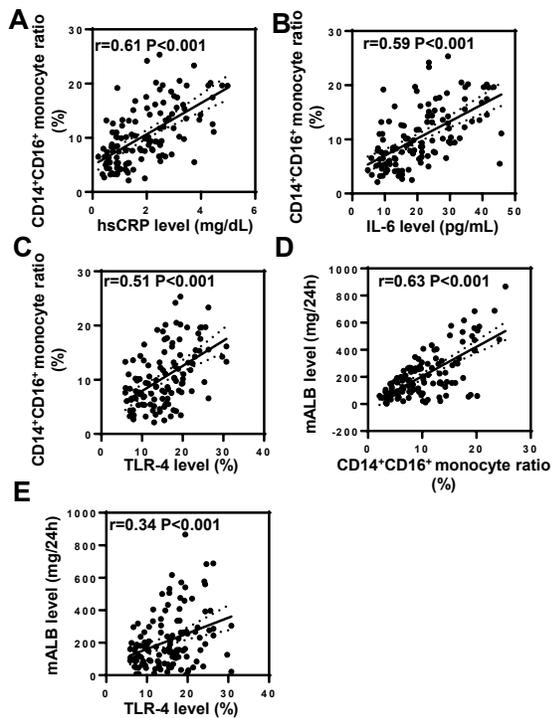


Fig. 3 Pearson's correlation scatter plot of CD14+CD16+ monocyte ratio with (A) hsCRP levels, (B) IL-6 levels, and (C) TLR-4 levels. Pearson's correlation scatter plot of mALB levels with (D) CD14+CD16+ monocyte ratio and (E) TLR-4 levels.

time, we also compared the data of group B one year ago and one year later. However, due to the short follow-up time, we only observed a significant decrease in CD14+CD16+ monocyte ratios in B-T-group (Fig. 2).

Correlation between hsCRP, IL-6 and TLR-4 levels in monocytes and CD14+CD16+ monocyte ratio

Finally, we analyzed the correlation of hsCRP, IL-6 and TLR-4 in monocytes and CD14+CD16+ monocyte levels in 188 T2DM patients. As shown in Fig. 3(A-C), the expression levels of hsCRP, IL-6 and TLR-4 in monocytes were positively correlated with CD14+CD16+ monocyte ratios. In addition, we also compared the correlation between TLR-4 in monocytes and CD14+CD16+ monocyte ratios to mALB levels. The results showed a significant positive correlation between TLR-4 in monocytes and CD14+CD16+ monocyte ratios and mALB levels (Fig. 3DE).

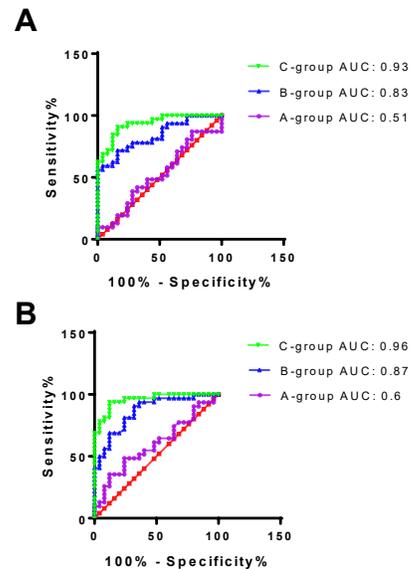


Fig. 4 The receiver operating characteristics (ROC) curves of the levels of TLR-4 in (A) monocytes and (B) CD14+CD16+ monocyte ratios for EDN diagnosis.

The ROC curves of the levels of TLR-4 in monocytes and CD14+CD16+ monocyte ratios for EDN diagnosis

To confirm that the levels of TLR-4 in monocytes and CD14+CD16+ monocyte ratios had high efficacy in predicting EDN, mALB level-dependent ROC analyses were conducted. The A-, B- and C-group AUC values of TLR-4 levels in monocytes for the prediction of EDN were 0.51, 0.83 and 0.93, respectively (Fig. 4a). In addition, the A-, B- and C-group AUC values of CD14+CD16+ monocyte ratios for the prediction of EDN were 0.6, 0.87 and 0.96, respectively (Fig. 4b). The results show that the levels of TLR-4 in monocytes and CD14+CD16+ monocyte ratios can be used as sensitive indicators for EDN diagnosis in patients with mALB level greater than 30 mg/24 h.

DISCUSSION

The pathogenesis of EDN is very complex and has not yet been fully elucidated, but many studies have pointed out that the inflammatory pathway is the central link in the progression of EDN, and the extracellular matrix proliferation caused by continuous inflammation promotes the progression of EDN. The research on inflammation in EDN will help develop new therapeutic targets and detection indicators. In this study, TLR-4 levels in mono-

cytes and CD14+CD16+ monocyte ratios, which can reflect the inflammatory state and proinflammatory functions, were used to explore the role of immune microinflammation in the EDN of T2DM. The results showed that there was no significant difference in the levels of hsCRP, IL-6, TLR-4 and CD14+CD16+ monocytes between the N-group and A-group (mALB < 30 mg/24 h), indicating that there was no chronic microinflammatory reaction in the early stage of kidney injury in diabetes mellitus. The levels of hsCRP, IL-6, TLR-4 and CD14+CD16+ monocytes in DN microalbuminuria patients (B-group, mALB 30–300 mg/24 h) or DN patients with a large amount of proteinuria (C-group, mALB > 300 mg/24 h) were significantly higher than those in healthy controls or diabetic patients without renal injury (A-group, mALB < 30 mg/24 h). This trend is consistent with reports by Yang et al [18]. However, compared to the research by Yang et al, our research focuses on EDN and provides a larger sample size. The above results indicated that with the increase in mALB, renal injury was gradually aggravated and the level of chronic microinflammation was increased.

Several studies have shown that the expression of TLR-4 in the kidneys of patients with DN is significantly higher than that in normal people, with the proximal tubule epithelial cells being obvious. Ligands filtered from the glomerulus such as high glucose, high-speed surging family B1 protein and glycosylation products can be more quickly recognized by TLR-4. In patients with T2DM, the TLR-4 receptor may simultaneously recognize high glucose, high free fatty acids and glycosylation products to regulate the immune inflammatory response and promote the development of DN [19]. In addition, Brooks-Worrell et al [20] found that the proportion of CD14+CD16+ monocytes in peripheral blood was elevated in autoantibody-negative diabetic patients with ketosis and could further differentiate into their corresponding subtypes. In this study, we are demonstrating that TLR-4 levels in monocytes and CD14+CD16+ monocyte ratios have a significant effect on EDN progress. However, its specific mechanism still needs further research.

Losartan potassium is a class of angiotensin receptor blocker (ARB) drugs that can reduce urinary protein and inhibit inflammation [21]. Losartan potassium can selectively and competitively bind to angiotensin receptor I subtype (ATI), block ATI-mediated physiological responses and improve the glomerular hypermetabolism [21]. Studies have also confirmed that ARBs inhibit inflammation by

inhibiting the NF-KB pathway [22]. This study also showed a significant decrease in the levels of TLR-4 and CD14+CD16+ monocytes in the B-T-group compared to those in the B-NT-group ($p < 0.05$). These results further confirmed that when the patients were in the microalbuminuria stage of DN, treatment with ARB drugs significantly decreased the levels of TLR-4 and CD14+CD16+ monocytes, decreased chronic microinflammation and the corresponding level of uric microalbumin and played a certain role in alleviating and reversing the early damage in diabetic nephropathy. Moreover, CD14+CD16+ monocytes were positively correlated with the clinical inflammatory markers, hsCRP and IL-6, further indicating their predictive value in DN (including the early stage). However, the IL-6 level was not significantly different after losartan potassium treatment. This result suggests that losartan potassium may not inhibit inflammation through the TLR-4–IL-6 signaling axis. But this still needs further research.

In conclusion, the TLR-4 levels and CD14+CD16+ monocyte ratio were increased in patients with EDN, restored with losartan potassium treatment and were positively correlated with the decrease in mALB level. ROC analyses showed that the TLR-4 level and CD14+CD16+ monocyte ratio can be used as new targets for the early diagnosis of clinical diabetic nephropathy.

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