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Original Article

PAI-1 and TNF-α profiles of adipose tissue in obese cardiovascular disease patients

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Abstract: Obesity as a leading preventable cause of death worldwide is closely linked to cardiovascular disease (CVD). Plasma plasminogen activator inhibitor (PAI)-1, a potent inhibitor of plasminogen activation and fibrinolysis, is increased in many clinical situations associated with high incidence of CVD. In the obesity-linked elevation of PAI-1, evidence points to TNF-α as an important regulator of PAI-1 expression in adipose tissue. Background: This study aims to evaluate mediastinal PAI-1 and TNF-α mRNA levels in adipose tissues (AT) and compare serum levels in obesity with and without coronary artery disease (CAD). Patients and methods: Obese patients with (n=37) and without CAD (n=20) were included in the study. Results: The serum levels of PAI-1 and TNF-α were significantly higher in obese patients with CAD compared to obese patients without CAD. PAI-1 mRNA expression was significantly increased in mediastinal adipose tissue (MAT) of obese patients with CAD compared to those without CAD, TNF-α mRNA expressions were found to be higher in EAT (epicardial AT), MAT and SAT (subcutaneous AT) of obese patients with CAD. Conclusions: The study demonstrated a close direct relationship between TNF-α and PAI-1. PAI-1 mRNA expression strongly correlated positively with serum TNF-α in MAT, and TNF-α expressions with PAI-1 serum levels.

Keywords: PAI-1, TNF-α, adipose tissue, cardiovascular disease

Introduction

Obesity is a leading preventable cause of death worldwide, and obesity rates are rising in adults and children [1, 2]. Obesity reduces life expectancy and is associated with numerous comorbidities, like hypertension (HTN), type II diabetes mellitus (T2DM), dyslipidemia, obstructive sleep apnea and sleep-disordered breathing, certain cancers, and major cardiovascular diseases (CVD). In adults, overweight is defined as a body mass index (BMI) 25 to 29.9 kg/m^2 and obesity as BMI ≥ 30 kg/m^2. BMI scores ≥ 40 are obese class III and this condition is called "morbid obesity".

Various findings indicate links of obesity to cardiovascular disease, adding weight to classical risk factors. Obesity also increases the prevalence of less conventional risk factors. Adipose tissue is an active endocrine and paracrine organ secreting various cytokines and bioactive mediators, including leptin, adiponectin, interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α), which not only influence body weight, but also insulin resistance, diabetes, lipid levels, blood pressure, coagulation, fibrinolysis, inflammation and atherosclerosis [3, 4].

Impaired fibrinolysis may occur during atherothrombotic CVD in metabolic syndrome or T2DM. Plasma plasminogen activator inhibitor (PAI)-1, a potent inhibitor of plasminogen activation and fibrinolysis, is increased in many clinical situations associated with high incidence of CVD. Impaired fibrinolysis caused by high plasma PAI-1 can lead to excessive fibrin accumulation within the vessels, leading to atherothrombosis. Increased PAI-1 expression is reported in atherosclerotic lesions in humans, especially atherosclerotic plaques in patients with T2DM. High vascular PAI-1 expression induces neointima formation via accumulation of fibrin or fibrinogen caused by inhibited clearance of platelet fibrin thrombin. PAI-1 acting as an acute phase protein, also mediates vascular inflammation, PAI-1 may not only be linked to local but also to systemic development of CVD.

TNF-α is one of many defined physiological regulators and a strong agonist for PAI-1 expression, and has been found to have important function in PAI-1 regulation in many diseases. In the obesity-linked elevation of PAI-1, evidence also points to TNF-α as an important regulator of PAI-1 expression in adipose tissue. Furthermore, TNF-α may contribute to elevated plasma PAI-1 levels in obesity [5].

Increased PAI-1 levels may predispose patients to the formation of atherosclerotic plaques prone to rupture with a high lipid-to-vascular smooth muscle cells ratio as a result of decreased cell migration [6]. PAI-1 polymorphisms are also shown to contribute to PAI-1 plasma levels in obese patients [7].

This study aims to evaluate mediastinal PAI-1 and TNF-α mRNA levels and compare serum levels in obesity with and without coronary artery disease (CAD).

Materials and methods

Study populations

37 obese patients with CAD and 20 obese patients without CAD admitted to the Istanbul Bilim University Florence Nightingale Hospital Department of Cardiovascular Surgery for mitral valve replacement and coronary artery bypass surgery were included in our study.

Samples of epicardial, subcutaneous and pericardial adipose tissue were collected during the operation by biopsy and was immediately frozen in liquid nitrogen and stored at -80°C until processing. Blood samples were also taken prior the operation. All specimens were taken after obtaining informed consent and the study was conducted prospectively. The study was approved by the Local Ethical Committee. The protocol followed was consistent with the World Medical Association Declaration of Helsinki (Ethical Principles for Medical Research Involving Human Subjects).

Anthropometric and biochemical measurements

Fasting glucose was measured using enzymatic reference method with glucose oxidase, total cholesterol (TC) was measured using the enzymatic, colorimetric method with cholesterol esterase, HDL-C and LDL-C were directly determined with homogeneous enzymatic colorimetric assay, TGs determination was made by the enzymatic colorimetric method (GPO/PAP) with cholesterol phosphate oxidase and 4-aminophenazone method on the opeRA analyzer. All analyses were performed on Cobas 6000/ Roche. Abdominal and epicardial fat volumes were evaluated by ultrasonography.

RNA extraction and cDNA synthesis

RNA was extracted by the homogenization of adipose tissues under cold conditions, in Trizol Reagent (Roche Diagnostic, Germany) and total RNA from epicardial, pericardial and subcutaneous adipose tissues were extracted with all prep kit (Qiagen, Germany). 1 μg of total RNA was reverse transcribed in 20 μl total volume using Oligo (dT) 18 primers and Revert Aid First Strand cDNA Synthesis Kit (Fermentas, Canada). Following the cDNA synthesis, the quality of cDNA was assessed by PCR amplification of a housekeeping gene (β-globin gene). Then, the cDNA was stored at -80°C until progressing.

Gene expression analysis by quantitative real-time PCR (QRT-PCR)

The comparative analysis of PAI-1 and TNF-α mRNA expression was carried out by means of QRT-PCR using a LightCycler 480 (Roche-Germany). The technique was performed according to the instructions of the manufacturer, in 20 μL, using 2 μL of the cDNA obtained from retrotranscription, 0.25 μmol/L of the corresponding primers, 3 mmol/L $MgCl_2$, 2 μL SYBR Green (Roche Diagnostics SL, Barcelona, Spain). The primers were designed using the software Primer3 v.0.4.0 (http://frodo.wi.mit.edu/primer3/) and synthesized by TIB-MOLBIOL (Berlin-Germany). The following primers were used for the amplification of the PAI-1, TNF-α and cyclophilin (housekeeping gene) genes: PAI-1, forward 5'-TTTGGTGAAGGGTCTGCTGTG-3' reverse 5'-TGCTGCCGTCTGATTTGTGGAA-3'; TNF-α, forward 5'-CCCAGGGACCTCTCTCTAAATCA-3' reverse 5'-AGCTGCCCCTCAGCTTGAG-3'; Cyclophilin forward 5'-TATCTGCACTGCCAAGACTGA-3', reverse 5'-CTTCTTGCTGGTCTTGCCATT-3'.

Measurement of serum PAI-1 and TNF-α by ELISA

Serum samples were kept at -80°C before determination of serum PAI-1 and TNF-α level in

Table 1. Demographic and clinical characteristics of obese patients with and without CAD

	Obese patients with CAD (n=37)	Obese patients without CAD (n=20)	*p* value
Age (years)	60.6±8.9	54.85±7.3	0.012
Waist (cm)	106.5±9.6	88.4±13.2	0.001
BMI	31.4±3.9	27.7±4.2	0.002
Fasting glucose (mg/dl)	157.4±51.5	106.5±12.3	0.001
Fasting insulin (μU/ml)	9.9±4.6	8.1±6.2	0.230
HOMA-IR	3.7±1.9	2.1±1.5	0.001
TC (mmol/L)	178.1±38.1	206.7±48.5	0.019
TGs (mmol/L)	160.2±72.4	145.7±68.8	0.477
SBP (mm/Hg)	131.9±16.4	124.4±11.7	0.076
DBP (mm/Hg)	78.5±8.9	76.5±7.8	0.431
Abdominal fat (cm^3)	246.6±71.9	202.6±75.9	0.041
Epicardial fat (cm^3)	8±4.5	3.9±2.1	0.001

BMI indicates body mass index; TCs, total cholesterol; TGs, triglycerides; SBP, Systolic blood pressure; DBP, Diastolic blood pressure. *P* values represent comparisons using *T* test and one-way ANOVA. The data are expressed as mean values ± standard deviation.

blood. The levels of each serum sample was also evaluated using enzyme-linked immunosorbent assay (ELISA) kits (PAI-1 e-Bioscience, TNF-α e-Bioscience) according to the manufacturer's recommendations.

Each serum sample, (PAI-1 50 μl and TNF-α 50 μl) was directly transferred to the microtest strip wells of the ELISA plate and then assayed according to the manufacturer's instructions. The absorbance was measured (PAI-1 610 nm and 650 nm, TNF-α 610 nm and 650 nm) in a microtest plate spectrophotometer. PAI-1 and TNF-α levels were quantified with a calibration curve using human PAI-1 and TNF-α standard.

Statistical analysis

Statistical analysis was performed with SPSS software (Statistical package for the Social Sciences, version 21.0, SPSS Inc, Chicago, IL, USA). For comparisons of the differences between mean values of both groups unpaired Student t Test was used. The PAI-1 and TNF-α gene expression data were obtained as ct values. The expression of each gene was compared between depots using the ΔΔCt method (ΔΔCT=ct of the target gene minus ct of the housekeeping gene). Baseline differences between obese patients w/o CAD were examined by Mann-Whitney U test. To reveal any differences between groups, data underwent log transformation to satisfy ANOVA criteria and were then subjected to one-way ANOVA with Tukey's post hoc analysis. In all cases differences were considered significant at P<0.05.

Results

The demographic and clinical characteristics of the study groups are summarized in **Table 1**. We found significant differences between obese patients with and without CAD. Waist (*P<0.001*), age (*P=0.012*), BMI (*P=0.002*), HOMA-IR (*P=0.001*), fasting glucose (*P<0.001*), TCs (*P=0.019*), epicardial fat (*P=0.001*) and abdominal fat (*P=0.041*) were significantly different between both study groups. No significant difference was observed in fasting serum insulin, TGs, SBP and DBP (P>0.05).

The serum levels of PAI-1 and TNF-α were significantly higher in obese patients with CAD compared to obese patients without CAD (*P=0.008* and *P=0.013*, respectively) (**Figures 1** and **2**).

Moreover TNF-α serum levels were found positively correlated with the thickness of abdominal fat (r =0.404, P=0.018).

Figure 3 represents mRNA expression levels of PAI-1 and TNF-α. PAI-1 mRNA expression level was found to be significantly increased in mediastinal adipose tissue (MAT) of obese patients with CAD compared to those without CAD (P=0.007), while epicardial adipose tissue (EAT) and subcutaneous adipose tissue (SAT) PAI-1 mRNA expression did not differ significantly among the groups. TNF-α mRNA expressions were found to be higher in EAT (P<0.0001), MAT (P<0.0001) and SAT (P<0.0001) of obese patients with CAD compared to obese patients without CAD.

TNF-α expressions were positively correlated with PAI-1 serum levels in MAT (r=0.402; P=0.007) (**Figure 4A**). Furthermore PAI-1 mRNA expression strongly correlated positively with TNF-α serum levels in mediastinal adipose tissue (MAT) (r=0.306; P=0.026) (**Figure 4B**). There was no significant correlation between

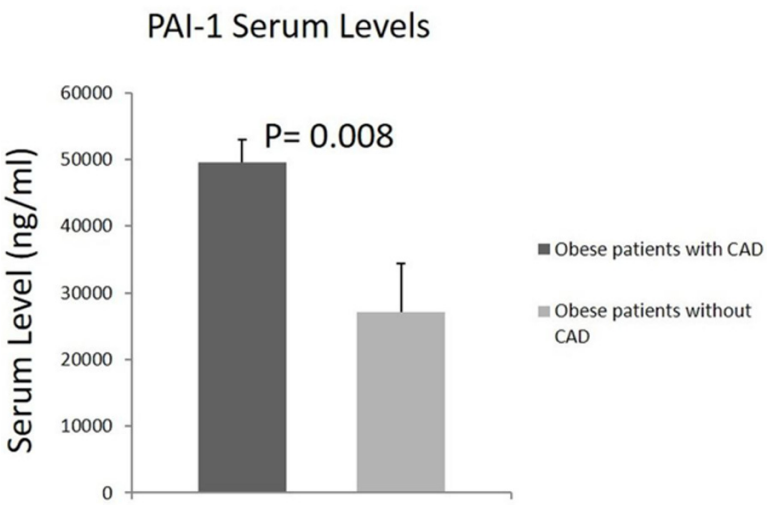

Figure 1. Serum levels of PAI-1 in obese patients with and without CAD. *P* value represents comparisons using Mann-Whitney U test.

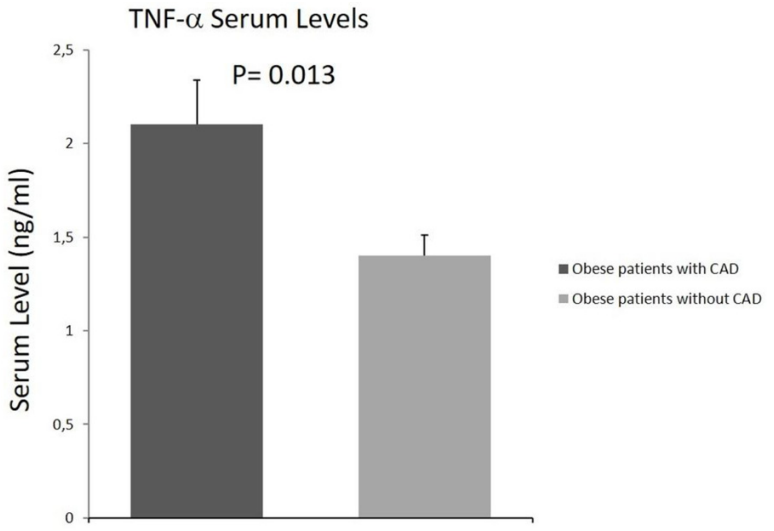

Figure 2. Serum levels of TNF-α in obese patients with and without CAD. *P* value represents comparisons using Mann-Whitney U test.

gene expression levels in epicardial adipose tissue (EAT) and subcutaneous adipose tissue (SAT). In addition, no correlation was found between the gene expressions and descriptive data (metabolic & clinical), including HOMA-IR, BMI, TCs, fasting glucose and waist circumference.

Discussion

Obesity represents a risk factor for the development of diseases such as T2DM, hypertension, metabolic syndrome (MetS), atherosclerosis and coronary artery disease (CAD). Moreover obesity contributes to the pathogenesis of primary atherosclerosis and coronary artery bypass grafts (CABG).

Adipose tissue is located within metabolically explicit depots. Despite its prepotency in total body fat, subcutaneous adipose tissue (SAT) is less metabolic active. Visceral adipose tissue is smaller in size, but more metabolically active [8]. CAD is usually caused by build-up of fatty deposits on the walls of the arteries around the heart. Mediastinal adipose tissue (MAT) is one of the visceral depots located in the thoracic cavity external to the parietal pericardium. It has not been comprehensively studied, but accumulation of fat in this region is a predictor of CAD risk, metabolic syndrome and abdominal aortic calcification [9].

Adipose tissue is an active endocrine organ. Interestingly, it is also associated with a state of chronic low-grade inflammation characterized by elevated plasma concentrations of proinflammatory cytokines and adipokines like PAI-1 and TNF-α [10]. These adipokines have an important role in CAD development. High PAI-1 plasma levels are accepted as an actual component, one of the independent risk factors and biomarkers used for the prediction of CAD [11].

TNF-α is a powerful pro-inflammatory cytokine and its concentration increases with obesity and CAD. TNF-α deteriorates insulin-receptor function, inhibits glucose transporter (GLUT) mRNA synthesis, and leads to development of insulin resistance [12]. Moreover studies indicate TNF-α as an important regulator the obesity-linked elevation of PAI-1 expression in adipose tissue and may contribute to elevated plasma PAI-1 levels in obesity [13].

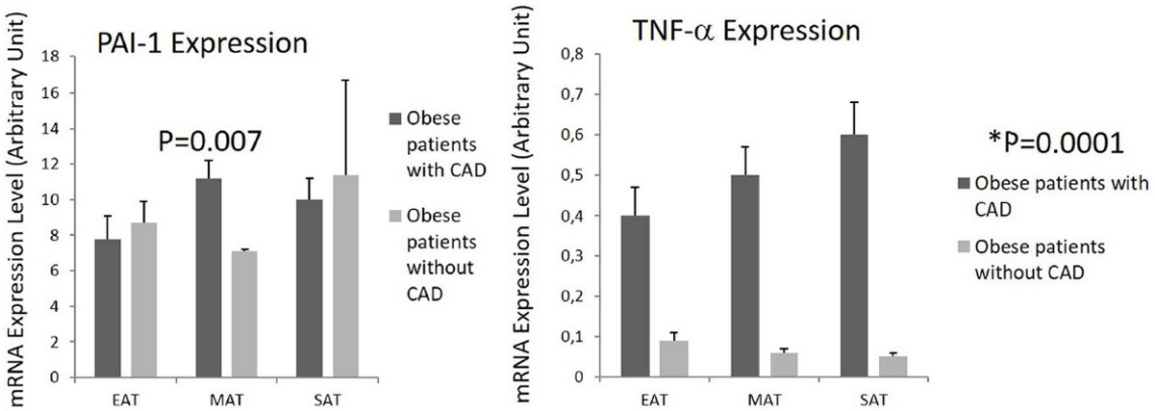

Figure 3. mRNA levels of PAI-1 (A) and TNF-α (B) in EAT, MAT and SAT of both study groups. EAT, epicardial adipose tissue; MAT, mediastinal adipose tissue; SAT, subcutaneous adipose tissue. *P* value represents comparisons using a Mann-Whitney U test.

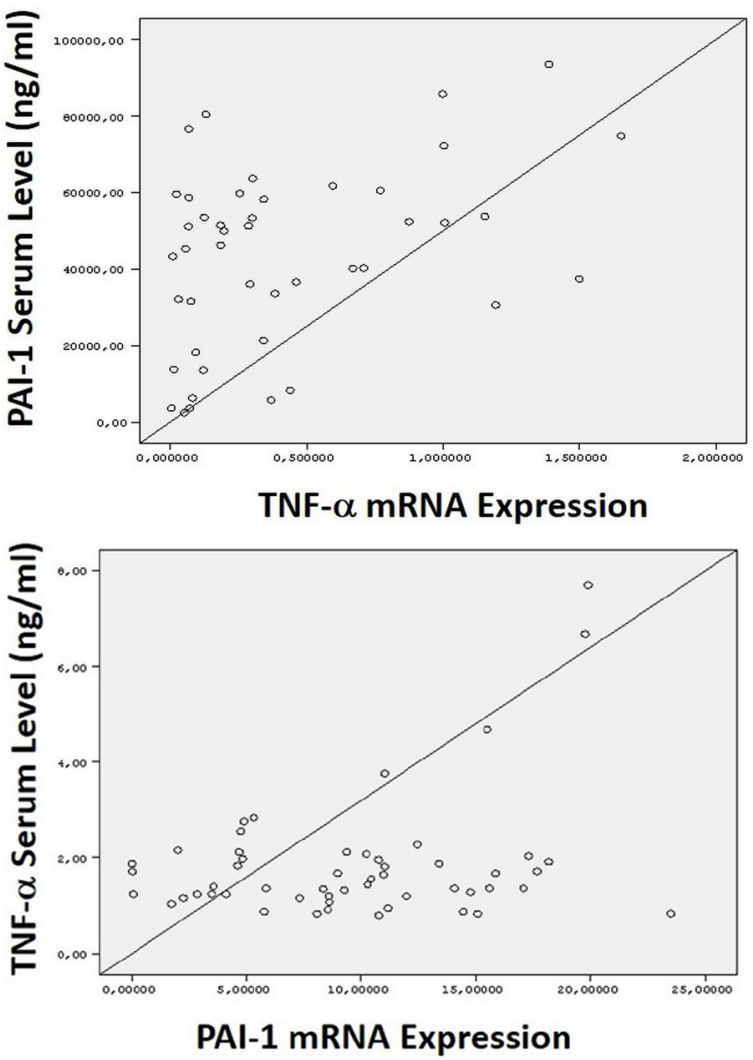

Figure 4. Correlation graph of TNF-α gene expression with PAI-1 serum levels (A) and of PAI-1 gene expression with TNF-α serum levels (B) in MAT.

The present study was undertaken to determine PAI-1 and TNF-α mRNA levels in EAT, MAT and SAT, to compare the results with the serum levels of PAI-1 and TNF-α in relation to obesity with and without CAD.

BMI scores (kg/m^2) 20 to 24.9 kg/m^2 are considered normal, scores of 25 to 29.9 are overweight, scores of 30 to 39.9 are obese. BMI scores ≥ 40 are obese class III and it is called morbid obesity. In addition, the size of a person's waist or waist circumference (>102 cm for males, >88 cm for females), increases the risk of obesity. The obese study group with CAD in our study had an average BMI score of 31.4 kg/m^2, but the same score in obese individuals without CAD was 27.7 kg/m^2 (**Table 1**). The waist sizes of obese CAD patients were significantly higher compared to obese individuals without CAD (106.5 cm and 88.4 cm, respectively; P< 0.001). Especially the increase of waist size in obese individuals raises the risk of cardiovascular disease development.

In addition to being a risk marker for CAD, several lines of evidence point to a proatherogen-

ic role for PAI-1. Endothelial PAI-1 seems to be primarily responsible for PAI-1 levels in plasma [14].

We first tested PAI-1 and TNF-α serum levels in obesity with and without CAD. Serum PAI-1 levels were significantly higher in obesity with CAD compared to obesity without CAD. Similarly, a number of studies were already conducted to identify plasma PAI-1 levels as predictive factors for CAD patients [2, 15].

Our results indicate that obese patients with CAD have higher TNF-α serum levels compared to obese patients without CAD. It is fair to assume that our results of TNF-α serum levels are supportive to previous evidence in literature [16, 17]. In addition, our results also indicate the positive relationship between increased TNF-α serum levels and increased abdominal fat in agreement with earlier reports [18]. One of the areas that our study did not focus on is the differential TNF-α serum levels according to age. It is well known that many physiological changes occur in human body with age. Bruunsgaard et al. showed that TNF-α levels associate with age and atherosclerosis, but age was not considered to be a factor in our data analysis [19].

In this study we also determined mRNA levels of PAI-1 and TNF-α of EAT, MAT and SAT in obesity w/o CAD. TNF-α mRNA levels were significantly higher in EAT, MAT and SAT in obesity with CAD compared to obesity without CAD which is in agreement with earlier reports [20-22].

Our previous data demonstrates that the increase of TNF-α expression in adipose tissues (EAT, MAT and SAT) in CAD patients resulted in aggregation of abundant macrophages, suggesting TNF-α to take part in this process [23].

The present study suggests TNF-α as an inflammatory cytokine in adipose tissue (especially MAT) and as one of the main visceral depots in non-CAD and CAD patients. This finding may support that cytokine profile changes in adipose tissue excretion as an important key player in CAD progression.

Our study also reports PAI-1 mRNA levels to be significantly increased in MAT and mRNA levels did not show any difference in EAT and SAT of obese patients with CAD compared to obese patients without CAD. It is well known that PAI-1 plasma levels are increased in obesity and MetS [24]. Consistent with our findings, PAI-1 mRNA levels assessed in visceral adipose tissue are considered as an important source of circulating PAI-1 in obesity and MAT is one of the main visceral depots [25].

We also focused on the role of increased mRNA levels of PAI-1 and TNF-α in MAT in the pathogenesis of coronary atherosclerosis, although previous study has proven that abdominal adiposity might play a more significant role [26]. On the other way, our data suggest that mediastinal fat is an important source of PAI-1 secretion which at least partly influences plasma PAI-1 levels.

Moreover, results of this study demonstrated a close direct relationship between TNF-α and PAI-1. PAI-1 mRNA expression strongly correlated positively with TNF-α serum levels in MAT, and TNF-α expressions were positively correlated with PAI-1 serum levels.

Disclosure of conflict of interest

None.

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