

## RESEARCH ARTICLE

# The preoperative activity of Th1 and Th17 cytokine axes in prediction of sepsis after radical cystectomy

Cane Tulic<sup>1,a</sup>, Miodrag Lazic<sup>2,a</sup>, Emin Savic<sup>3,a</sup>, Dusan Popadic<sup>3</sup>, Jelena Djukic<sup>1</sup>, Dusan Spasic<sup>2</sup>, Milos Markovic<sup>3</sup>, Zorica Ramic<sup>3</sup>, Marija Mostarica-Stojkovic<sup>3</sup>, Vladimir Trajkovic<sup>3</sup>

<sup>1</sup> Institute of Urology and Nephrology, School of Medicine, University of Belgrade, Belgrade, Serbia

<sup>2</sup> Department of Urology, University Hospital Center "Dr Dragisa Misovic", University of Belgrade, Belgrade, Serbia

<sup>3</sup> Institute of Microbiology and Immunology, School of Medicine, University of Belgrade, Belgrade, Serbia

**Correspondence:** V. Trajkovic, Institute of Microbiology and Immunology, School of Medicine, University of Belgrade, Dr. Subotica 1, 11000 Belgrade, Serbia  
 <vtrajkovic@med.bg.ac.rs>

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**ABSTRACT.** The aim of the study was to correlate the preoperative activity of Th1 and Th17 cytokine axes with the development of sepsis after radical cystectomy. The study involved twenty patients with the infiltrative transitional cell carcinoma of the urinary bladder without previous radiotherapy/chemotherapy, who underwent open radical cystectomy with urinary diversion. Preoperative plasma concentrations of Th1 cytokines interleukin 12 (IL-12) and interferon gamma (IFN- $\gamma$ ), and Th17 cytokines IL-23 and IL-17, were measured using ELISA. Preoperative expression of mRNA for IL-12p35, IFN- $\gamma$ , IL-23p19 and IL-17 was quantified by real-time RT-PCR using mRNA extracted from peripheral blood mononuclear cells. Eight patients developed postoperative sepsis, diagnosed within two weeks post-operation as systemic inflammatory response syndrome in the presence of local or systemic infection. The preoperative basal plasma concentrations of Th1 and Th17 cytokines were slightly above the detection limits, with a tendency toward lower concentrations in patients who developed sepsis, but the difference was not significant ( $p>0.05$ ). The preoperative expression of mRNA encoding IL-12p35 and IL-17 was significantly lower in patients who developed sepsis ( $p=0.003$  and  $p=0.028$ , respectively). The similar trend was observed for IL-23p19 and IFN- $\gamma$ , but the differences did not reach the statistical significance ( $p=0.051$  and  $p=0.172$ , respectively). These data suggest that determination of preoperative Th1 and Th17 cytokine mRNA levels might be useful in predicting sepsis development after radical cystectomy.

**Key words:** radical cystectomy, sepsis, cytokines, Th1, Th17

## INTRODUCTION

Radical cystectomy remains a mainstay of treatment for high-risk superficial bladder cancer [1], but despite relatively low mortality (1-3%), its morbidity remains fairly high ( $\approx 30\%$ ) [2]. One of the most frequent severe complications is sepsis [3, 4], an infection-associated immune activation followed by compensatory immune depression, eventually resulting in multiple organ failure and death [5, 6]. In addition to immune dysfunction related to surgical trauma-induced stress, anaesthesia, hemorrhage/transfusion and ischaemia-reperfusion [7], the underlying illness, comorbidity, age or gender could also play a role in development of postoperative sepsis [5, 8]. In a search for additional markers needed for better stratification within the groups at risk, several predictors of sepsis development/severity have been proposed, including the changes in plasma lactate, procalcitonin, C-reactive protein, protein-C, interleukin 6 (IL-6), IL-18 or D-dimer

levels, the changes in prothrombin time and monocyte expression of NF- $\kappa$ B [6, 9, 10]. However, the usefulness of these markers is restricted to the postoperative period, thus emphasizing the need for the efficient preoperative predictors of sepsis that will enable surgeons to decide upon the best treatment strategy.

In addition to "classic" proinflammatory cytokines such as tumour necrosis factor (TNF), IL-1 and IL-6, the cytokines produced by distinct subpopulations of T helper (Th) lymphocytes, designated Th1 and Th17, have been implicated both in antimicrobial defence and tissue damage during inflammation [11]. Heterodimeric cytokines IL-12(p35/p40) and IL-23 (p19/p40) secreted by monocytes/macrophages and dendritic cells, play principal roles in differentiation of IFN- $\gamma$ -producing Th1 and IL-17-producing Th17 cells, respectively [11, 12]. Both IFN- $\gamma$  and IL-17 stimulate production of proinflammatory cytokines (TNF, IL-1, IL-6) and nitric oxide (NO), a free radical involved in microbial killing, but also in pathology accompanying autoimmunity and sepsis [13]. T cell-deficient mice are more susceptible to acute septic peritonitis [14], and the studies on mice lacking genes

for Th1 and Th17 cytokines or their receptors suggest the importance of both Th1 and Th17 cytokine axes in conferring resistance to experimental polymicrobial sepsis [15-17]. Accordingly, the low capacity for IL-12, IFN- $\gamma$  and IL-23 production in septic patients was found to correlate with higher severity/letality of postoperative sepsis [18-20]. Moreover, the impaired preoperative production of IL-12 correlated with higher incidence and/or severity of sepsis after major abdominal surgery [20-22], suggesting that Th1 suppression predisposing to sepsis cannot solely be attributed to surgical trauma, but may exist before operation. While these findings indicate that preoperative measurement of Th1 cytokines might be useful in predicting sepsis development and/or outcome, no study thus far has examined whether determination of Th17 cytokines might have similar potential.

We here investigated if the preoperative assessment of the activity of Th1 and Th17 cytokine axes at both protein and mRNA levels could be used to predict sepsis development in cystectomized patients.

## PATIENTS AND METHODS

### *Patients and samples*

The study included 20 consecutive patients (February-July 2008) with the second or third grade infiltrative transitional cell carcinoma of the urinary bladder, who underwent open radical cystectomy with urinary diversion at the Institute of Urology and Nephrology, Clinical Center of Serbia and Department of Urology, University Hospital Center "Dr Dragisa Misovic". The preoperative exclusion criteria were previous radiotherapy/chemotherapy, treatment with anti-inflammatory drugs and the existence of systemic inflammatory response syndrome (SIRS). Antibiotic prophylaxis and treatment of preoperative urinary infections were performed as previously described [23]. Plasma was obtained from blood samples collected before anaesthesia and peripheral blood mononuclear cells (PBMC) were isolated by centrifugation on a Ficoll-Paque density gradient (Amersham Biosciences, Piscataway, NJ). Postoperative sepsis was diagnosed within two weeks post-operation as SIRS in the presence of infection (local or systemic), according to established criteria [24]. All the patients gave informed consent. The research has been approved by institutional ethics committee and performed in accordance with the ethical standards of the 1964 Declaration of Helsinki (2008, 6th revision).

### *Cytokine and CRP determination*

Plasma cytokine levels of IL-12(p35/p40), IFN- $\gamma$ , IL-23(p19/p40) and IL-17 were measured by enzyme-linked immunosorbent assay (ELISA) according to manufacturer's instructions (eBioscience, San Diego, CA). Lower limit of detection (4 pg/ml) was reported by the manufacturer (IL-17, IL-12, IFN- $\gamma$ ) or determined in our laboratory (IL-23). Serum CRP was measured by an immunonephelometric method.

### *Real-time RT-PCR*

Total RNA extracted from PBMC using TRIZOL (Invitrogen, Carlsbad, CA) was reverse transcribed with M-MuLV

reverse transcriptase and random hexamers (Fermentas, Vilnius, Lithuania). Primers were designed using Primer Express software and purchased from Metabion International AG (Martinsried, Germany). The sequences of forward primer, reverse primer and Taqman probe (5'FAM-TAMRA3' except 5'VIC-TAMRA3' for GAPDH) were:

IL-12p35 (CTCCCAAAACCTGCTGAGGG; CAATCTTTCAGAAGTGCAAGGG; CAACATGCTCCAGAAGGCCAGACAAACTC),

IFN- $\gamma$  (TGTAGCGGATAATGGAACCTTTTC; AATTGGCTCTGCATTATTTCTG; CACTCTCTTTCATTCTCA AAATGCCTA),

IL-23p19 (TGTGGAGATGGCTGTGACCC; TCATAAAAAATCAGACCCCTGGTGG; CTCAGGGACAACA-GTCAGTTCTGCTTGA),

IL-1 (CATCCATCCCCAGTTGATTGG; GATTTC-GTGGGATTGTGATTC; AAGACCTCATTGGTGTCA-CTGCTACTGCTG)

GAPDH (CATCCATGACAACCTTGGTATCG; CCA-TCACGCCACAGTTCC; AAGGACTCATGACCACAGTCCATGCC).

VIC (a proprietary product from Applied Biosystems) and FAM (6-carboxyfluorescein) are fluorescent dyes at the 5' end of the Taqman probe, while TAMRA (tetramethylrhodamine) is a fluorescence quencher at the 3' end of the probe. The PCR was performed in a Realplex<sup>2</sup> Mastercycler (Eppendorf, Hamburg, Germany) using RealMasterMix-Probe (Eppendorf). The amplification conditions were 95 °C for 1 min, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. The target gene expression was standardized against that of GAPDH detected in the same well. The results were presented as  $\Delta Ct$  values obtained by subtracting the Ct (threshold cycle) values of GAPDH from the Ct values of target genes.

### *Statistics*

Statistical analysis was performed using SPSS software 11.5 (SPSS Inc., Chicago, IL). Normality of the data was assessed by Kolmogorov-Smirnov test. The differences were analyzed by *t*-test or Fisher's exact significance test where appropriate, while correlations were assessed by Pearson's correlation analysis. Binary logistic regression was used to assess predictive ability of different variables. The  $p<0.05$  was considered significant.

## RESULTS

### *Patients' characteristics and postoperative outcome*

Fifteen out of 20 patients developed SIRS after cystectomy, while sepsis was diagnosed in 8 patients with SIRS. Urinary infections were observed in 4 patients (2 patients with *Escherichia coli*, 1 patient with *Klebsiella* spp. and 1 patient with *Pseudomonas* spp.), 3 patients had wound infections (2 patients with *Enterococcus* spp. and 1 patient with *Pseudomonas* spp.), while positive blood culture (*Klebsiella* spp.) was confirmed in 1 patient. The age, gender distribution, co-morbidity assessed by ASA score, preoperative leukocyte counts and serum CRP levels were not significantly different in septic vs non-septic patients (table 1).

**Table 1**  
Patients' demographic and clinical data.

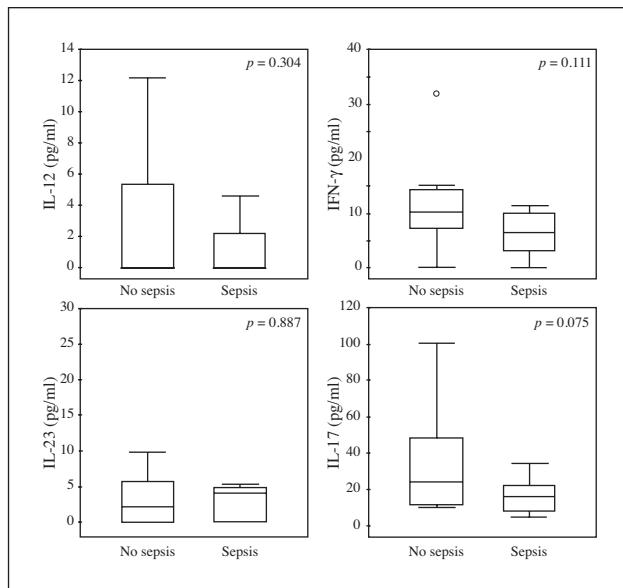
Preoperative characteristic	Septic patients (n=8)	Non-septic patients (n=12)	p
Age (years)	57.5 ± 6.9	62.4 ± 5.4	0.090
Sex (male/female)	7/1	8/4	0.242
ASA	2.13 ± 0.84	1.83 ± 0.58	0.366
CRP (mg/L)	30.3 ± 37.3	22.9 ± 34.2	0.659
Leukocytes ( $\times 10^9/L$ )	16.1 ± 11.0	8.3 ± 1.9	0.083

### The preoperative blood levels of Th1 and Th17 cytokines

To assess potential usefulness of Th1 and Th17 cytokines in predicting postoperative sepsis, we measured their plasma levels before cystectomy. The detectability of cytokines was 100% (IL-17), 85% (IFN- $\gamma$ ), 55% (IL-23), and 35% (IL-12). The detection frequencies of cytokines did not significantly differ in patients who developed postoperative sepsis in comparison to those with no septic complications (data not shown). The cytokine concentrations (with the exception of IL-17) were only slightly above the detection limits, with a tendency toward lower concentrations in patients who developed sepsis, but the difference did not reach statistical significance ( $p>0.05$ ) (figure 1). Also, there were no significant differences in cytokine blood levels between patients with and without SIRS (data not shown). Therefore, the preoperative measurement of plasma concentrations of Th1 and Th17 cytokines could not be efficiently used to predict development of sepsis after radical cystectomy.

### The preoperative PBMC levels of Th1 and Th17 cytokine mRNA

We next investigated if preoperative PBMC mRNA levels of Th17/Th1 cytokines would perform better than their



**Figure 1**

The preoperative blood levels of Th1 and Th17 cytokines in septic vs non-septic patients. The box-plots contain median values (horizontal line), interquartile range (the box length), largest and smallest values which are not outliers (whiskers), and outliers (values between 1.5 and 3 box lengths) represented as open circles.

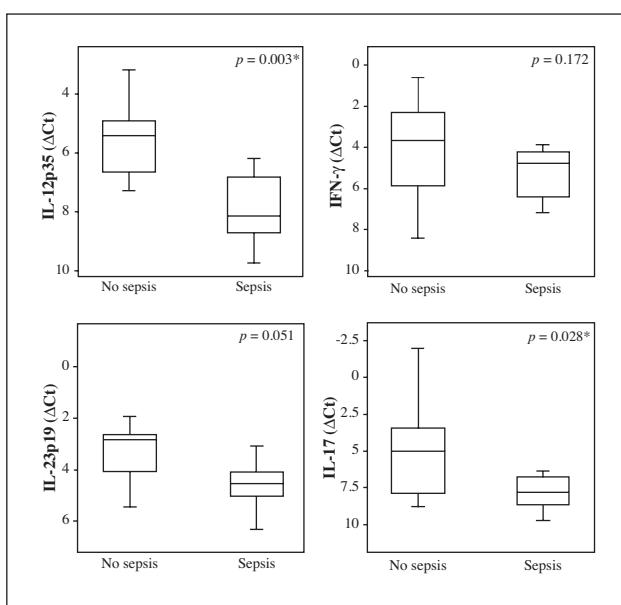
protein concentrations in differentiating septic from non-septic patients. Indeed, the mRNA levels of IL-12p35 chain and IL-17 were significantly higher in PBMC of patients who did not develop postoperative sepsis, while the difference in concentration of IL-23p19 mRNA showed borderline significance (figure 2). The preoperative concentrations of IFN- $\gamma$  mRNA in PBMC were also higher in patients without septic complications, but the difference did not reach statistical significance (figure 2). No significant differences were found in Th17/Th1 cytokine mRNA levels between patients with and without SIRS (data not shown). Thus, the preoperative PBMC mRNA concentrations of Th1 and Th17 cytokines are more efficient than their plasma levels in predicting postoperative sepsis development.

### The correlations between preoperative protein and/or mRNA levels of Th1 and Th17 cytokines

In agreement with the ability of IL-12 and IL-23 to induce IFN- $\gamma$  and IL-17, respectively [11, 12], the plasma levels of IL-12 positively correlated with those of IFN- $\gamma$ , while the blood concentrations of IL-23 were in correlation with those of IL-17 (table 2). Accordingly, the PBMC levels of IL-23p19 mRNA were positively correlated with the concentrations of IL-17 mRNA, while the correlation between IL-12p35 and IFN- $\gamma$  mRNA concentrations did not reach statistical significance (table 2). A positive correlation was also observed between IFN- $\gamma$  and IL-23, both at the protein and mRNA levels (table 2), which is consistent with the previously described capacity of IL-23 to induce IFN- $\gamma$  production in T cells [25]. Interestingly, no correlation at all was observed between the protein and mRNA concentrations of IL-17 or IFN- $\gamma$  (table 2).

### The value of Th1 and Th17 cytokine protein and mRNA levels in predicting sepsis after cystectomy

Finally, binary logistic regression was employed to evaluate the usefulness of plasma protein and PBMC mRNA



**Figure 2**

The preoperative PBMC levels of Th1 and Th17 cytokine mRNA in septic vs non septic patients (\* denotes a significant difference).

**Table 2**

Correlations between preoperative protein and/or mRNA levels of Th1/Th17 cytokines (\* denotes a significant difference, NA: not assessed).

	IL-12(p35/p40)	IL-23(p19/p40)	IFN- $\gamma$ $\Delta$ Ct	IL-17 $\Delta$ Ct
IFN- $\gamma$	$r=0.693$ $p=0.001^*$	$r=0.450$ $p=0.047^*$	$r=0.102$ $p=0.669$	NA
IL-17	$r=0.278$ $p=0.236$	$r=0.787$ $p < 0.001^*$	NA	$r=-0.066$ $p=0.781$
IL-12p35 $\Delta$ Ct	$r=-0.427$ $p=0.087$	NA	$r=0.411$ $p=0.102$	$r=0.277$ $p=0.238$
IL-23p19 $\Delta$ Ct	NA	$r=0.247$ $p=0.356$	$r=0.540$ $p=0.031^*$	$r=0.702$ $p=0.002^*$

levels of Th1 and Th17 cytokines in predicting sepsis after cystectomy. Collinearity diagnostics returned the VIF values of less than 4, indicating no overt signs of multicollinearity. The model incorporating blood concentrations of IL-12, IFN- $\gamma$ , IL-17 and IL-23 as independent variables did not perform better than the “empty” model with intercept only, as shown by omnibus test for model coefficients ( $p=0.081$ ). On the other hand, the model containing PBMC mRNA levels of IL-12p35, IFN- $\gamma$ , IL-23p19 and IL-17 was significantly better in predicting the postoperative sepsis than the intercept-only model ( $p=0.017$ , Nagelkerke R square=0.735, correct prediction 86.7% vs 53%). The best single independent predictor was the concentration of IL-12p35 mRNA, showing the borderline significance (Wald test  $p=0.05$ , Exp(B)=4.878). It should be noted that the odds ratio values were inverted ( $\text{Exp}(B) > 1$ ) because larger  $\Delta$ Ct values actually mean lower mRNA concentrations. In other words, with each 50% drop in preoperative IL-12p35 mRNA concentration (corresponding to one unit increase in p35  $\Delta$ Ct), the odds for postoperative sepsis development increase almost five-fold. The mRNA levels of IL-17 (Wald test  $p=0.058$ , Exp(B)=1.914), IL-23p19 ( $p=0.080$ , Exp(B)=2.791) and IFN- $\gamma$  ( $p=0.174$ , Exp(B)=1.436) were all below the level of statistical significance as independent predictors. Nevertheless, their joint predictive value was indicated by finding that the model including all four variables (IL-12p35, IFN- $\gamma$ , IL-23p19, IL-17) performed better than the model containing IL-12p35 only (Nagelkerke R square 0.735 vs 0.611, correct prediction 86.7 vs 76.5%).

## DISCUSSION

Both IL-12-induced Th1 and IL-23-induced Th17 responses are important for controlling experimental sepsis [15-17], but their involvement in human sepsis is still not clear. Our results indicate that the lower preoperative activity of Th1 and Th17 cytokine axes is associated with the increased risk for a development of septic complications after radical cystectomy.

The low preoperative capacity of LPS-stimulated monocytes to express IL-12p40 mRNA and produce IL-12 has previously been found to correlate with the higher incidence and/or severity of sepsis after major abdominal surgery [20-22]. Accordingly, we here demonstrated that the preoperative levels of IL-12p35 mRNA in unstimulated PBMC were significantly lower in patients who developed septic complications after radical cystectomy. Furthermore, the present report for the first time indicates

the association between the lower preoperative expression of mRNA encoding the Th17 cytokines (IL-17 and IL-23p19) and higher incidence of the postoperative sepsis. The sepsis-predicting potential of preoperative determination of Th1 and Th17 cytokine mRNA, particularly IL-12p35 and to the lesser extent IL-17 and IL-23p19 mRNA, was demonstrated by a binary logistic regression. These data support the protective role of Th1 and Th17 cytokines in postoperative sepsis, together with findings that impaired postoperative production of Th1 (IL-12 and IFN- $\gamma$ ) and Th17 cytokines (IL-23) is related to increased sepsis severity/mortality after abdominal surgery [18, 19]. While IFN- $\gamma$  and IL-17 directly promote anti-bacterial defence through stimulation of macrophages and neutrophils [25, 26], the role of IL-12 and IL-23 is mainly restricted to induction of IFN- $\gamma$  and IL-17, respectively [11, 12]. Accordingly, we have observed positive correlations between IL-12 and IFN- $\gamma$ , as well as between IL-23 and IL-17, both at the protein and mRNA level. In agreement with the ability of IL-23 to induce IFN- $\gamma$  [27], a positive correlation was also observed between the IL-23 and IFN- $\gamma$  protein/mRNA concentrations.

It has been proposed that cancer itself, as well as neoadjuvant therapy, particularly radiation, can cause immunosuppression predisposing to sepsis [22, 28, 29]. To avoid the possible influence of neoadjuvant therapy on the activity of Th1 and Th17 cytokine axes, we examined only the patients who did not receive anticancer therapy before the operation. However, our study did not include healthy controls, so we could only speculate whether the Th1 and Th17 activity in our patients was altered by underlying malignancy. Since the IL-12-producing ability of LPS-stimulated PBMCs did not differ between cancer patients and patients with non-malignant disease [20, 22], it seems that impaired IL-12 production in sepsis-prone patients undergoing major abdominal surgery was not cancer-related. Of note, the lower preoperative activity of Th1 and Th17 cytokine axes coinciding with postoperative septic complications was apparently not due to generalized immunosuppression, but rather to a specific immune status predisposing to sepsis development. Namely, in contrast to results obtained with IL-12, the PBMCs of patients who developed sepsis after abdominal surgery did not produce lower amounts of proinflammatory cytokine IL-1 and even secreted more TNF than PBMCs of non-septic patients [20]. Similarly, our data indicate that the higher preoperative levels of the proinflammatory cytokines IL-6 and macrophage migration inhibitory factor are associated with the increased risk for sepsis development after cystectomy (Savic et al., unpublished). These results suggest a

preferential suppression of Th1 and Th17 cytokine axes and exclude a general defect in cytokine secretion in patients predisposed to sepsis after major surgery, including radical cystectomy.

Determination of cytokine blood levels might be a less complicated, less time-consuming and less expensive equivalent of the in vitro PBMC stimulation. However, contrary to the results obtained with cytokine mRNA, the protein concentrations of Th1 and Th17 cytokines did not significantly differ between septic and non-septic patients in our study. This discrepancy might be due to a higher sensitivity and accuracy of the real-time RT-PCR measurement of cytokine mRNA in comparison with ELISA for cytokine protein determination [18]. Accordingly, the observed absence of correlation between the protein and mRNA levels of Th1 and Th17 cytokines in our study can be explained by the technical imprecision of the methods used to determine the protein levels [30]. However, one could not completely exclude the possibility that the blood-circulating mononuclear cells were not the only source of Th1 and Th17 cytokines and that some other cells (e.g. endothelial cells, tissue-residing macrophages/dendritic cells, fibroblasts) could have contributed to cytokine concentrations in the blood.

Because of the small number of patients, the results presented here should be considered somewhat preliminary, thus awaiting further confirmation. While the SIRS incidence after radical cystectomy in our study (75%) is comparable to previously reported data [31], one might argue that the Bone's criteria we used may overestimate sepsis incidence in comparison with some more stringent criteria for sepsis/SIRS definition [32]. Nevertheless, our data indicate that the preoperative determination of Th1 and Th17 activity may hopefully allow for the stratification of patients for novel immunomodulatory therapeutic strategies aimed at reducing the risk of postoperative infection and sepsis. It also seems worthy of investigation whether the incidence of septic complications in patients with low preoperative Th1 and Th17 activity could be reduced by less aggressive surgical procedures such as laparoscopic cystectomy.

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The authors declare no conflict of interest.

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