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The Role of Calprotectin and Alpha-Defensin in the Diagnosis of Pneumonia in Ventilated Patients

Ventilasyon Uygulanan Hastalarda Pnömoni Tanısında Kalprotektin ve Alfa-Defensininin Rolü

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ABSTRACT

Introduction: *Hospital-acquired pneumonia and ventilator-associated pneumonia are the major causes of death in hospitalized patients, particularly in the intensive care unit, and early diagnosis may contribute to the survival of the patients. Our aim in this study was to contribute to the rapid treatment of ventilator-associated pneumonia by providing an early diagnosis of pneumonia with alfa-defensin, and calprotectin as inflammation biomarkers.*

Materials and Methods: *The study was designed as a single-center, prospective observational study involving mechanically ventilated patients who were admitted to the Internal Medicine Intensive Care Unit at Çukurova University Hospital between May 2018 and July 2019 and were above 18 years of age. Patients' demographics and clinical parameters were noted. Serum alpha-defensin levels were measured with the Human Alpha-defensin ELISA kit (Bioassay Technology Laboratory, Jiaxing, China). Serum calprotectin levels were measured with the Human Calprotectin ELISA kit (Bioassay Technology Laboratory, Jiaxing, China). Deep tracheal aspirates (DTA) and blood specimens were collected on the day of ventilation, as well as on the first, third, and seventh days, prospectively. The patients were monitored for the development of ventilator-associated pneumonia (VAP). Infections other than ventilator-associated pneumonia were also noted.*

Results: *During the study period, 822 patients were admitted to the intensive care unit, accumulating 5101 patient days and 1966 ventilator days. Of the included 88 patients who were intubated and mechanically ventilated, 59.1% were male and the mean age was 59.9 ± 18.4. Mean alpha defensin levels were higher in patients with pneumonia than those without (1679.21 ± 3398.17 vs 552.32 ± 243.67 respectively, p= 0.012). As for the ROC curve analysis, the area under the curve for alpha-defensin in pneumonia patients was 0.583 (p= 0.239). Mean calprotectin levels were higher in patients with pneumonia than those without (230.40 ± 150.6819 ng/mL vs 163.80 ± 73.5819 ng/mL, p= 0.001). As for the ROC curve analysis, the area under the curve for calprotectin in pneumonia patients was 0.621 (p= 0.086).*

Conclusion: *Serum and bronchoalveolar fluid levels of alpha defensin and calprotectin exhibited higher values in patients with pneumonia compared to those without pneumonia. However, due to the absence of statistical significance, larger-scale studies are necessary*

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to ascertain the clinical utility and benefits. In conclusion, it is recommended to plan a study with a larger number of patients, in which serum and bronchoalveolar fluid alpha defensin levels are measured simultaneously and molecular methods are used for more accurate diagnosis.

Key Words: *Alpha defensin; Biomarker; Calprotectin; Inflammation; Ventilator-associated pneumonia*

ÖZ

Ventilasyon Uygulanan Hastalarda Pnömoni Tanısında Kalprotektin ve Alfa-Defensininin Rolü

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Giriş: *Hastane kaynaklı pnömoni ve ventilatör ilişkili pnömoni, özellikle yoğun bakım ünitesinde yatan hastalarda başlıca ölüm nedenlerindendir ve erken tanı, hastaların hayatta kalmasına katkıda bulunabilir. Bu çalışmadaki amacımız alfa-defensin ve inflamasyon biyobelirteci olan kalprotektin ile pnömoninin erken tanısını sağlayarak ventilatör ilişkili pnömoninin hızlı tedavisine katkıda bulunmaktır.*

Materyal ve Metod: *Çalışma tek merkezli, prospektif gözlemsel bir çalışma olarak planlanmıştır ve Mayıs 2018 ile Temmuz 2019 tarihleri arasında Çukurova Üniversitesi Tıp Fakültesi Hastanesi Dahiliye Yoğun Bakım Ünitesinde yatan, 18 yaşından büyük ve mekanik ventilatöre bağlı hastalar çalışmaya alınmıştır. Hastaların demografik özellikleri ve klinik parametreleri kaydedilmiştir. Serum alfa-defensin düzeyleri Human Alpha-defensin ELISA kiti ile ölçülmüştür. (Bioassay Teknoloji Laboratuvarı, Jiaxing, Çin). Serum kalprotektin düzeyleri Human Calprotectin ELISA kiti ile ölçülmüştür (Bioassay Teknoloji Laboratuvarı, Jiaxing, Çin). Derin trakeal aspiratlar (DTA) ve kan örnekleri, ventilasyon gününde ve prospektif olarak birinci, üçüncü ve yedinci günlerde alınmıştır. Hastalar ventilatör ilişkili pnömoni (VAP) gelişimi açısından izlenmiştir. Ventilatör ilişkili pnömoni dışındaki infeksiyonlar da kaydedilmiştir.*

Bulgular: *Çalışma döneminde 822 hasta, 5101 hasta günü ve 1966 ventilatör günü ile yoğun bakım ünitesine yatırıldı. Entübe edilen ve mekanik olarak ventile edilen 88 hastanın %59.1'i erkekti ve ortalama yaş 59.9 ± 18.4 idi. Ortalama alfa defensin düzeyleri, pnömonisi olan hastalarda olmayanlara göre daha yüksekti (sırasıyla 1679.21 ± 3398.17 ve 552.32 ± 243.67, p= 0.012). ROC eğrisi analizine göre, pnömoni hastalarında alfa-defensin için eğri altında kalan alan 0.583 olarak hesaplandı (p= 0.239). Ortalama kalprotektin düzeyleri, pnömoni hastalarında pnömonisi olmayanlara göre daha yüksekti (230.40 ± 150.6819 ng/mL'ye karşı 163.80 ± 73.5819 ng/mL, p= 0.001). ROC eğrisi analizine göre, pnömoni hastalarında kalprotektin için eğri altında kalan alan 0.621 olarak bulundu (p= 0.086).*

Sonuç: *Alfa defensin ve kalprotektinin serum ve bronkoalveolar sıvıdaki düzeyleri, pnömoni olanlarda olmayanlara göre daha yüksek saptanmıştır fakat istatistiksel anlamlılık saptanmadığından klinik kullanımda yararını tespit etmek için daha geniş çalışmalara ihtiyaç vardır. Daha doğru tanı koyma amacıyla serum ve bronkoalveolar sıvıda, alfa defensin ve kalprotektin düzeylerinin eş zamanlı olarak ölçüldüğü ve moleküler yöntemlerin kullanıldığı daha fazla sayıda hasta içeren bir çalışmanın planlanması önerilmektedir.*

Anahtar Kelimeler: *Alfa defensin; Biyobelirteç; Kalprotektin; İnflamasyon; Ventilatör ilişkili pnömoni*

INTRODUCTION

The second most prevalent nosocomial infection and the leading cause of mortality in critically ill patients is nosocomial pneumonia (NP). Nosocomial pneumonia (NP) occurs in patients who have been hospitalized for more than 48 hours. When it arises in mechanically ventilated intensive care unit (ICU) patients after at least 48 hours of ventilation, it is referred to as ventilator-associated pneumonia (VAP). In contrast to VAP, there are no new radiographic infiltrates in ventilator-associated tracheobronchitis (VAT), despite respiratory infection symptoms[1].

The frequency of NP ranges from 5 to 20 instances per 1000 hospital admissions, with the elderly, immunocompromised, and surgical

patients having the highest rates. According to epidemiological data from the United States, the prevalence of VAP ranges from 2 to 16 incidences per 1000 ventilator days. Cook et al. assessed the likelihood of VAP to be 3% per day for the first five days of mechanical ventilation, 2% per day from Day five to Day ten, and 1% per day beyond that. In a study involving countries in the Middle East, including Türkiye, mortality was 21.5%, and VAP significantly increased death by 1.5 times. While hospital-reported data from the National Healthcare Safety Network suggests a decline in VAP rates, recently published findings from a randomly selected national sample revealed that around 10% of mechanically ventilated patients received a VAP diagnosis, and this rate has remained unchanged over the past decade[1,2].

These infections negatively impact significant patient outcomes. While all-cause mortality associated with VAP has been reported to range from 20% to 50%, a recent meta-analysis estimated the attributable mortality as 13%. Even HAP, generally considered to be less severe than VAP, causes serious complications in approximately 50% of patients, including respiratory failure, pleural effusions, septic shock, renal failure, and empyema. These issues are particularly prominent among ICU patients, with mortality rates approaching those of patients with VAP. Furthermore, there appears to be some debate regarding the impact of VAP on the length of stay. Two recent studies estimated that VAP prolongs the length of mechanical ventilation by 7.6 to 11.5 days and prolongs hospitalization by 11.5 to 13.1 days compared to similar patients without VAP. The excess cost associated with VAP was estimated to be high at approximately $40.000 per patient[3].

To avoid problems, it is critical to diagnose these dangerous illnesses as soon as possible. However, due to restricted diagnostic tools and a broad differential diagnosis for patients in the intensive care unit (ICU) who develop rising oxygen requirements, leukocytosis, and secretions, HAP and VAP can be difficult to diagnose quickly[4]. Several biomarkers, including CRP, procalcitonin, prohormones, presepsin, and TREM-1 are used in the diagnosis, severity determination, and prognosis for patients with community-acquired pneumonia (CAP). Despite recent extensive research, none of them appear to be optimal, and the quest for novel biomarkers that will most adequately predict the severity and treatment response in pneumonia continues.

Antimicrobial peptides and proteins are among the earliest innate immune molecular effectors. One of these compounds is alpha defensin, specifically human neutrophil peptide (HNP)-1. This peptide has been detected in varying proportions within bronchoalveolar lavage (BAL) fluid and is likely a consequence of neutrophil degranulation in infected airways[5]. Another one is calprotectin which is mainly expressed in neutrophil granulocytes and stored in the cytosol[6]. There is growing evidence that alpha defensin and calprotectin levels are elevated in patients with various neutrophil-dominated inflammatory diseases. Patients with sepsis or meningitis, as well as noninfectious diseases such as idiopathic pulmonary fibrosis, have elevated defensin and calprotectin levels in their plasma. Alpha defensin and calprotectin are also used in the diagnosis of bacterial infections including prosthetic joint infections, and gastrointestinal infections[7-9].

Early diagnosis and treatment of pneumonia contribute significantly to the reduction of morbidity and mortality[10]. Therefore, this study aimed to contribute to the rapid and effective treatment by providing an early diagnosis of pneumonia with alfa-defensin or calprotectin in ventilated patients.

MATERIALS and METHODS

The study was planned as a single-center, prospective observational study. Ethical approval was obtained from the University Ethical Board for Noninterventional Studies (Approval Number: 75/18; 2 Mar 2018). The study encompassed randomly selected mechanically ventilated patients who were admitted to the Internal Medicine Intensive Care Unit of Çukurova University Hospital between May 2018 and July 2019 and were aged 18 years or older.

Deep tracheal aspirates (DTA) and blood specimens were collected on the day of ventilation and the first, third, and 7th days prospectively. The patients were monitored for the development of VAP. Infections other than ventilator-associated pneumonia were also noted. Patients were clinically diagnosed based on the criteria outlined in the IDSA 2016 guidelines for hospital-acquired pneumonia and VIP guidelines[3]. The same pulmonologist and Infectious Diseases Specialist evaluated all the patients.

Patients' demographics, vital signs, clinical and chest radiography findings, underlying conditions such as immunosuppression, diabetes mellitus, etc., medications, complete blood count values, procalcitonin, and CRP values were noted. Clinical pulmonary infection score (CPIS), acute physiology and chronic health evaluation (APACHE), sequential organ failure assessment (SOFA), and the simplified acute physiology score II (SAPS II) scores were calculated.

Clinical samples were obtained from the patient's peripheral blood and indwelling catheters, tracheal and bronchial aspirates, and urinary catheters. They were cultured in appropriate culture media (obtained from bioMйrieux, France) at Çukurova University Faculty of Medicine Hospital Central Laboratory Microbiology Unit. Gram and Giemsa-stained DTA samples were also evaluated by the same microbiologist in terms of leukocytes and microorganisms for all specimens.

For the biochemical analysis, a 10 ml sample of DTA was centrifuged at 10.000 cycles per minute, then stored at 4° C for 30 minutes. The resulting supernatant was subsequently preserved at -80° C until measurement of alpha-defensin and calprotectin concentrations. Alpha-defensin and calprotectin levels were measured with the Human Alpha-defensin ELISA kit and Human Calprotectin ELISA kit. Both the Human Alpha-defensin and Calprotectin ELISA kits utilize the Enzyme-Linked Immunosorbent Assay (ELISA) method. The Alpha-defensin ELISA kit's plate has been pre-coated with Human A-DF1 antibody, while the Calprotectin ELISA kit's plate has been pre-coated with Human CAL antibody, both from Bioassay Technology Laboratory in Jiaxing, China.

For statistical analysis of the studied samples, the highest value of alpha defensin and calprotectin was used. Continuous variables were analyzed by Student t test and Chi-square Fisher's exact test was used to compare categorical variables. Pearson correlation was used for comparing two continuous variables. A p value of <0.05 was considered statistically significant. The efficacy of alpha-defensin and calprotectin values was also demonstrated by ROC curve analysis. SPSS v.20.0 was used for statistical analysis.

RESULTS

In the study period, 822 patients were admitted to the ICU, accumulating 5101 patient days and 1966 ventilator days. Of the included 88 patients who were intubated and mechanically ventilated, 59.1% were male and the mean age was 59.9 ± 18.4 years.

The most common underlying disease was malignancy at 46.6% and was followed by acute renal failure at 43.2%, coronary heart disease at 21.6%, and diabetes mellitus at 12.5%. The most common malignancies were lung cancer and hematological cancers (34.1% and 31.7% among cancers respectively).

At the time of intensive care unit admission and during the seven-day study period, 12 patients were diagnosed with community-acquired pneumonia, three patients with hospital-acquired tracheobronchitis, 42 patients with hospital-acquired pneumonia, six patients with ventilator-associated tracheobronchitis and 12 patients with ventilator-associated pneumonia. One patient had both CAP and VAP and a total of 65 patients (73.9%) were diagnosed with pneumonia. While two patients were diagnosed with urinary tract infections and seven patients with bloodstream infections, sepsis of undetermined origin was detected in one patient. The crude mortality rate was 83%.

Table 1. Comparison of the continuous variables in patients with or without pneumonia

	Pneumonia (n= 65)		No pneumonia (n= 23)		Total (n= 88)		
	Mean ± SD**	Median (IQR[ŸŸ])	Mean ± SD**	Median (IQR[ŸŸ])	Mean ± SD**	Median (IQR[ŸŸ])	p-value
Age (years)	61.9 ± 17.78	66 (27.5)	62.14 ± 14.83	63 (14.75)	61.96 ± 16.96	65 (24)	0.962
LOS* (days)	22.10 ± 16.69	17 (20)	21.27 ± 14.62	18.5 (21)	25.38 ±15.37	21 (20)	0.845
ICU-LOS[ŸŸ] (days)	15.44 ± 14.41	12 (11)	11.77 ± 9.81	10 (13)	17.29 ± 13.10	12 (11)	0.410
APACHE[ŸŸ]	46.68 ± 48.66	35 (19.9)	35.22 ± 14.6	32 (6)	32.7 ± 10.5	31 (9)	0.222
SOFA[§]	11.31 ± 4.91	11 (6)	10.18 ± 3.67	10 (6)	11.05 ± 3.61	11 (0.6)	0.329
CPIS[‖]	4.25 ± 1.66	4 (2)	2.73 ± 1.61	2.5 (2)	2.76 ± 1.30	3 (1)	<0.001
SAPS II[¶]	65.17 ± 18.35	66 (23.3)	58.84 ± 13.23	57.90 (15.22)	64.69 ± 19.14	65 (25.15)	0.142

Student t-test was used.
*: Hospital length of stay, [ŸŸ]: Length of stay in intensive care unit, [ŸŸ]: Acute physiology and chronic health evaluation score, [§]: Sequential organ failure assessment score, [‖]: Clinical pulmonary infection score, [¶]: The simplified acute physiology score II score, **: Standard deviation, [ŸŸ]: Interquartile range.

The mean and median values of hospital length of stay, length of stay in the intensive care unit (ICU), APACHE, SOFA, CPIS, and SAPS II scores on the day of ICU admission in patients with and without pneumonia are demonstrated in Table 1. There was no difference between patients with and without pneumonia except for CPIS, which was significantly higher in patients with pneumonia. There was also no difference in the presence of pneumonia in terms of gender (73.1% in males vs 75% in females, p= 1.000).

The distribution of biochemical parameters and leukocyte count in patients with or without pneumonia are shown in Table 2. WBC, CRP, and procalcitonin levels were similar in patients with pneumonia and those without. The mean alpha defensin levels were higher in patients with pneumonia than those without (1679.21 ± 3398.17 ng/mL vs 552.32 ± 243.67 ng/mL respectively, p= 0.012). As for the ROC curve analysis, the area under the curve for alpha-defensin in pneumonia patients was 0.583 (p= 0.239). The mean calprotectin levels were higher in patients with pneumonia than those without (230.40 ± 150.6819 ng/mL vs 163.80 ± 73.5819 ng/mL, p= 0.001). As for the ROC curve analysis, the area under the curve for calprotectin in pneumonia patients was 0.621 (p= 0.086). There was no correlation observed between CRP and either calprotectin or alpha defensin (p= 0.579 and 0.325, respectively).

DISCUSSION

Ganz et al. described neutrophil defensins for the first time in 1985 (11). These [human neutrophil peptides (HNP) -1, -2, -3, and -4] are antimicrobial peptides found in neutrophil granules that kill a wide range of bacteria in vitro, including *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Escherichia coli*, as well as many fungi and some enveloped viruses, and play a vital role in innate host defense (12). Human alpha-defensins HNP1-3 are present mostly in neutrophils and are created by their bone marrow precursors as they progress from

promyelocytes to myelocytes and slightly beyond. Immunoreactive HNP1-3 has been detected in natural killer cells, CD T cells, B cells, and monocytes within freshly isolated peripheral blood mononuclear cells (13). They account for 5-7% of the total cellular protein in a human polymorphonuclear leukocyte (PMN) and 30-50% of the protein in primary granules containing myeloperoxidase (MPO)[14]. Given that 10^6 human PMNs carry 4-5 g of HNP1-3, an 80 kg person's bone marrow must generate at least 250 mg of HNP1-3 per day to match the daily neutrophil turnover, and it should be capable of doubling or tripling this quantity during multiple infections[15]. HNP1-3 concentrations are exceedingly low in normal plasma. Even when their levels increase during sepsis, the plasma concentration of HNP1-3 is notably elevated in patients with sepsis or meningitis. However, a considerable portion of these molecules will be bound to proteins (16). Because of their low extracellular level, they are unlikely to kill extracellular bacteria unless the bacteria enter an extracellular environment with a high local alpha-defensin concentration and either provide the ionic conditions required for alpha-defensin-mediated killing activity or provide other factors for their function. The most likely location for this would be at the cell's surface, characterized by an abundance of glycolipid and glycocalyx defensin-docking sites. The mucosal surface of the lungs is a suitable environment for the activity of these peptides[17].

Recent research has shown that human airway epithelial cells can produce antimicrobial peptides and play a multifunctional role in primary immunity. Several investigations have suggested that these peptides involve in pneumonia pathogenesis[18-21]. In a recent study, researchers reported significantly elevated concentrations of alpha-defensins 1-4 in the bronchoalveolar fluid of preterm infants with pneumonia compared to the non-pneumonia group of patients[22]. We investigated the role of HNP-1 in the diagnosis of pneumonia in ventilated patients. In our study, out of the 88 patients, 65 were diagnosed with pneumonia, either acquired in the community or the hospital. The mean alpha-defensin levels were higher in patients with pneumonia than

Table 2. Distribution of biochemical parameters and leukocyte count in patients with or without pneumonia

	Pneumonia (n= 65)		No pneumonia (n= 23)		Total (n= 88)		
	Mean ± SD[§]	Median (IQR[‖])	Mean ± S[§]	Median (IQR[‖])	Mean ± SD[§]	Median (IQR[‖])	p-value
WBC*-max (cell/mL)	19219.67 ± 16887.84	15600 (13450)	19036.36 ± 9344.18	17350 (15350)	19171.08 ± 15200.32	15900 (13900)	0.989
CRP[ý]-max (mg/dL)	23.48 ± 17.96	18 (19.43)	18.91 ± 13.69	16.1 (25.15)	22.27 ± 16.98	18 (20.6)	0.455
PCT[ý]-max (mg/dL)	20.37 ± 28.79	7.68 (23.78)	20.53 ± 30.94	4.64 (29.12)	20.42 ± 29.19	7.38 (26.36)	0.994
Alpha-defensin (ng/mL)	1679.21 ± 3398.17	491 (556.5)	552.32 ± 243.67	456 (447)	1380.51 ± 2952.13	483 (477)	0.012
Calprotectin (ng/mL)	230.40 ± 150.6819	169 (167)	163.80 ± 73.5819	153 (106)	212.99 ± 137.61	159.50 (137)	0.001

Student t-test was used.
*: White blood count, [ý]: C-reactive protein, [§]: Standard deviation, [‖]: Interquartile range.

in those without (3398.17 ng/mL vs 1679.21 ng/mL), while WBC, CRP, and procalcitonin levels were similar.

C-reactive protein (CRP) has been linked to COVID-19 severity[23,24]. Certain authors have attempted to establish correlations between significant parameters and HNPs, revealing a positive correlation with C-reactive protein (CRP). However, in our study, no correlation was observed between CRP and either calprotectin or alpha defensin.

Calprotectin, a protein found in high concentrations within the cytosol of neutrophil granulocytes, makes up a significant portion (40-50%) of the total protein content. This protein is released when neutrophils are activated or undergo turnover, and it serves as a crucial indicator of inflammation associated with neutrophil activity[25]. It serves as an essential component of the innate immune response, playing a role in antimicrobial defense and regulation of inflammatory processes. Calprotectin is known to exhibit antimicrobial properties and chemoattractant activity, recruiting immune cells to the site of infection[26]. With the activation of neutrophils, markers that indicate their activation are released. These markers are stored within granules or the cytoplasm of the neutrophils and can be quickly released when needed[27]. In a study, plasma calprotectin values were found to be significantly higher in patients with bacterial respiratory tract infections (pneumonia, tonsillitis, or mycoplasma) than in patients with viral respiratory infections, or healthy controls[28,29]. In our study, although a method specifically designed to determine the exact etiology of infection was not utilized, it was found that patients with pneumonia had higher levels of calprotectin compared to those without pneumonia. CRP and calprotectin are both biomarkers reflective of the acute phase response. Multiple studies have provided evidence supporting the observation of increased CRP levels during inflammatory processes. Pneumonia and malignancy are one of these inflammatory processes that promote CRP elevation. In our study, we did not observe significant differences in CRP levels among patients grouped into pneumonia. We hypothesize that the high proportion of patients with malignancy in our study might have influenced these results, as malignancy itself can lead to elevated CRP levels due to leukocyte infiltration in neoplastic tissues[30].

Limitations of our study included the inability to detect alpha-defensin levels in bronchoalveolar lavage fluid (BALF) and the fact that it was conducted at a single site. Furthermore, standard culturing methods were used for diagnosis and molecular methods could have potentially enhanced the accuracy of diagnosis. In our study, elevated levels of alpha defensin and calprotectin were observed in ventilated pneumonia patients. However, based on the ROC curve results, these markers cannot be utilized as standalone diagnostic tools.

CONCLUSION

In conclusion, we recommend the design of a study involving a larger patient cohort, where serum and bronchoalveolar fluid alpha defensin and calprotectin levels are concurrently measured. The incorporation of molecular methods would contribute to a more precise diagnosis.

ETHICS COMMITTEE APPROVAL

This study was approved by the Çukurova University Non-Invasive Clinical Research Ethics Committee (Decision no: 75, Date: 02.03.2018).

CONFLICT of INTEREST

The authors have no conflicts of interest to declare that are relevant to the content of this article.

AUTHORSHIP CONTRIBUTIONS

Concept and Design: AC, ÖÖK, EÖ

Analysis/Interpretation: AC, ÖÖK, EÖ

Data Collection or Processing: ÖÖK, SB, PE, ÖGÖ, EÖ

Writing: AC, ÖÖK

Review and Correction: All of authors

Final Approval: All of authors

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