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*Article*

# The Relationship Between Salivary Oxidized Thymosin β4 and Thymosin β10 and Oxidative Stress-Related Diseases in Preterm Infants

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**Abstract:** The study investigates the role of oxidative stress in preterm infants (<30 weeks) and the potential protective function of the oxidized forms of thymosin β4 (Tβ4) and β10 (Tβ10). A total of 149 saliva samples from 18 infants were collected and analyzed. The intact salivary proteome was analysed by nano-HPLC-ESI-MS. The different salivary proteins and their proteoforms investigated were characterized by means of an integrated proteomic platform. Relative quantification of salivary peptides was based on the extracted ion current (XIC) peak area. A significant correlation was found between Post-Menstrual Age and total Tβ4 (p=0.001), oxidized Tβ4 percentage (p=0.025), and total Tβ10 (p=0.043). Higher $FiO_2$ values were associated with lower levels of oxidized Tβ10 (p = 0.005) and with lower percentages of oxidized Tβ10 (p<0.001). Thymosin levels did not differ between infants with or without retinopathy of prematurity, but higher levels of oxidized Tβ10 were found in those who did not develop bronchopulmonary dysplasia (p=0.024). The reduced oxidized Tβ10 in moderate-severe BPD may indicate a protective role in inflammation and tissue repair. This study lays the groundwork for future research on using saliva as a non-invasive matrix to monitor oxidative stress and the therapeutic potential of oxidized thymosins in preterm neonates.

**Keywords:** proteomics; preterm newborns; saliva proteome; thymosin; oxidative stress; bronchopulmonary displasya

## 1. Introduction

Since intrauterine life, there is a delicate balance between the production of reactive oxygen species (ROS) and the body's antioxidant defense mechanisms. When this situation becomes unbalanced and free radical production exceeds the capacity of antioxidant systems, a condition referred to as "oxidative stress" occurs. [1] When produced in limited amounts, ROS have positive

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action, stimulating cell regeneration and immune function. At high concentrations, however, they result in an imbalance that can damage DNA, lipids, and proteins. Protein irreversible oxidative damage comes from covalent modifications, including carbonylation of arginine, lysine, proline and threonine, and oxidation of thiols, which can result in misfolding, fragmentation. [2, 3]

Neonates, especially those born preterm (before 37 weeks of gestational age) have antioxidant systems not fully developed and therefore they are particularly susceptible to oxidative stress. Maternal conditions such as diabetes, obesity and pre-eclampsia, which are more prevalent in preterm births, can worsen the situation by exposing the fetus to increased oxidative stress. [4-5] Furthermore, intrauterine growth restriction and macrosomia appear to be associated with mitochondrial dysfunction and subsequent increased production of ROS. [6] In preterm infants, the increased risk of resuscitation in the delivery room respect to term infants, associated with episodes of apnea, infections, and longer duration of mechanical ventilation and oxygen therapy in the Neonatal Intensive Care Unit (NICU), contribute to an higher incidence of oxidative stress-related diseases [7-8] such as bronchopulmonary dysplasia (BPD), retinopathy of prematurity (ROP), intraventricular hemorrhage (IVH), periventricular leukomalacia (PVL), and necrotizing enterocolitis (NEC). [7, 9]. The mechanisms by which oxidative stress contributes to the development of "oxidative stress related pathology" are only partially known. In PVL, ROS increasing during reperfusion after hypoxic events are implicated in apoptosis of oligodendrocytes precursors. [10, 11] In NEC, free radicals produced by inflammation, ischemia and reperfusion contribute to the disruption of the immature gut barrier. [12, 13]

The pathogenesis of ROP has been extensively studied. The development of the retinal vasculature is completed at 40 weeks gestational age, so preterm infants have incomplete development of the retinal vasculature and a peripheral zone lacking vasculature. During prenatal life, the fetus develops in a relatively hypoxic environment; at birth, the preterm infant is exposed to a condition of relative hyperoxia that results in inhibition of vascular endothelial growth factor (VEGF) and hypoxia-induced transcription factor (HIF) expression. This results in altered retinal vasculature with arrested peripheral vessel development. Subsequently, the resulting retinal ischemia triggers the release of growth factors that might be responsible for the development of aberrant vasculature. [14, 15]

BPD is the most common morbidity in surviving preterm infants, especially born before 28 weeks of gestation. Among the known risk factors (prenatal and postnatal) that increase the incidence of this disease, the need for oxygen therapy and oxidative stress have a key role. [16] On immature lungs, mechanical ventilation and oxygen administration result in activation of inflammatory cytokines, which in turn implement ROS production through activation of neutrophil degranulation. This condition, associated with the immaturity of antioxidant systems, results in the predominance of oxidative stress. [17, 18] Several studies have shown that premature infants with BPD, already in the early stages of the disease, express a different oxidation pattern than infants who will not develop BPD. [19, 20, 21] Most of these studies have focused on plasma and bronchoalveolar lavage fluid (BALF) analyses [22-27]. Notably, infants who developed BPD showed significantly higher protein oxidation in tracheal aspirates, regardless of gestational age. [22-23] Oxidative inactivation of alpha-1-antitrypsin, measured in BALF samples from preterm infants, was found to correlate with the development of BPD. [20] Increased plasmatic levels of heptanal, 2-nonenal, and 4-hydroxynonenal were found to be good predictors of BPD [24]. Additionally, lipid peroxidation, measured in cord blood, is significantly associated with duration of oxygen treatment and respiratory support. [25] Other promising markers are currently under investigation [27, 28]

Thymosins are a family of small proteins widely distributed across tissues and involved in numerous physiological and pathological processes, including cytoskeletal reorganization, cell migration, immune response modulation, and tissue repair. Among them, thymosin $\beta_4$ ($T\beta_4$) and thymosin $\beta_{10}$ ($T\beta_{10}$) are the most extensively studied proteoforms in the context of cellular stress and inflammation [29, 30] both having a methionine prone to oxidation as the sixth residue in the amino acid sequence. In particular, $T\beta_4$ is known for its role in promoting angiogenesis, protecting against

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oxidative damage, and supporting tissue regeneration. [31] Recent evidence suggests that the selective oxidation of thymosins, leading to sulfoxidized derivatives at methionine residues, does not merely represent molecular damage, but rather a functionally relevant post-translational modification (PTM). Oxidized forms of Tβ4 appear to exert cytoprotective and anti-inflammatory actions, contributing to the regulation of the oxidative stress response. Experimental studies have demonstrated, for instance, that oxidized Tβ4 can modulate inflammatory responses in epithelial and cardiac cells, promoting mechanisms of damage resolution. [32-34] Tβ10 is less studied, but it appears to be involved in cytoskeletal regulation, cellular development and differentiation; moreover, it seems to play an important role in the oncological pathology. [29-30, 35-37]

In preterm neonates, whose early exposure to pro-oxidant conditions may contribute to the development of chronic inflammatory diseases such as BPD, the analysis of oxidized thymosins may offer insight into individual variability in oxidative stress responses. In this context, saliva emerges as an ideal biological fluid for proteomic studies in neonates: it is non-invasive, easily collectable at multiple time points, and allows for the investigation of PTMs such as oxidation. [38, 39]

Therefore, characterization of oxidized derivatives of Tβ4 and Tβ10 and determination of their levels in saliva of preterm infants may offer new insights for the identification of possible early oxidative stress biomarkers and for a better understanding of the pathophysiological mechanisms underlying BPD.

Aim of this study was to investigate the relative amounts of oxidized Tβ4 and Tβ10 in preterm infants oral cavity at birth and later stages of postnatal life by nano-HPLC-ESI-MS analysis of oral fluid, in order to assess their variation in relation to the development of preterm-related diseases such as BPD and ROP, which recognize oxidative stress as one of the pathogenic elements.

## 2. Materials and Methods

### *2.1. Setting*

The enrollment of newborns and the collection of saliva samples were performed at the Division of Neonatology of the Fondazione Policlinico Universitario A. Gemelli IRCCS of Rome. The treatment and proteomic analysis of the collected saliva samples were performed at the Dipartimento di Scienze biotecnologiche di base, cliniche intensivologiche e perioperatorie of the Università Cattolica del Sacro Cuore of Rome.

The Unità di Proteomica e Metabolomica of IRCCS-Fondazione Santa Lucia of Roma, the Dipartimento di Scienze della Vita e dell'Ambiente and Sezione di Anatomia Patologica of the Dipartimento di Scienze Mediche e Sanità Pubblica of Cagliary University and the Istituto di Scienze e Tecnologie Chimiche "Giulio Natta", Consiglio Nazionale delle Ricerche contributed to the analysis of saliva samples and to data processing.

### *2.2. Study Population and Inclusion Criteria*

The study was carried out in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki. All rules were observed, and written consent forms were signed by the donor or by the parents of each child. For ethical reasons, saliva was collected only when sample collection caused no stress.

Preterm infants with gestational age between 175–216 days (25–30 weeks), admitted to the Neonatal Intensive Care Unit (NICU) from January 2024 to January 2025, were enrolled for this study. Saliva samples were collected at least seven days from the birth date and thereafter every 7-10 days up to 40 weeks (286 days) of Post-Menstrual Age (PMA) or up to discharge if it occurred earlier. Infants with major congenital malformations or prenatal infections were excluded from the study.

As this was a pilot study, in the "pre-discovery" phase of characterization of the peptides, the calculation of the sample size was precluded.

However, it was possible to estimate the number of patients who could be enrolled in an appropriate timeframe: considering that in 2023, 50 newborns with gestational age (GA) between 25

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and 30 weeks were hospitalized in the NICU and among these 13 (26%) died, it was estimated the possibility of studying at least 18 infants over a period of approximately 12 months.

The number of infants we intended to study was in line with the literature evidence for a pilot study. [40]

### *2.3. Sample Collection and Treatment*

The whole saliva was collected with a soft plastic aspirator as it flowed into the anterior floor of the mouth. After collection, each salivary sample was immediately diluted 1:1 (v/v) with 0.2% aqueous 2,2,2-trifluoroacetic acid (TFA) on ice bath. The solution was then centrifuged at 8000g for 5 min (4°C). Finally, the acidic supernatant was separated from the pellet and either immediately analyzed with the HPLC-ESI-MS apparatus or stored at -80 °C until the analysis.

### *2.4. RP–HPLC-ESI-MS Analysis*

The protein content of every sample was determined using the Bradford Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA), and the same quantity of total protein amount was used for mass spectrometry analysis.

An UltiMate 3000 RSLC nano – HPLC System (Thermo Fisher Scientific, Waltham, MA, USA) coupled with a high-resolution Orbitrap Fusion Lumos Tribrid Mass Spectrometer (Thermo Fisher Scientific) equipped with an ESI source was used. Peptides were separated on a PepMap RSLC C18 column (2 µM, 100Å, 50 µm x 15 cm, Thermo Fisher Scientific) using gradient elution. Eluent A consisted of an aqueous solution of 0.1% FA (formic acid), while eluent B was ACN with 0.1% FA. The gradient program was as follows (total runtime: 155 min): 3% B and 97% A (min 0-110), 20% B and 80% A (min 110-120), 40% B and 60% A (min 120-125), 90% B and 10% A (min 125-145), 3% B and 97% A (min 145-155), with a flow rate of 0.3 µL/min. Each injection volume was 5 µL (containing a total of 1 µg of proteins), with an NSI ion source type, positive polarity (voltage 1800 V). MS parameters included data-dependent scan mode (DDS) for acquiring high-resolution MS/MS spectra with an Orbitrap detector, a resolution of 120,000 in the 375 – 1500 m/z range, and HCD fragmentation. Samples were analyzed in analytical triplicate.

### *2.5. Intact Protein/Peptide Characterization and Relative Quantification*

The different salivary proteins and their proteoforms investigated were characterized by means of an integrated proteomic platform.

Salivary peptide and protein quantitative analysis of the saliva specimens was based on the measurement of the eXtracted Ion Current (XIC) peak area (signal/noise ratio >5). The XIC search revealed the peak associated with the protein of interest by extracting along the total ion current (TIC) chromatographic profile the intensity of the ion current of specific multiply charged ions ($m/z$) generated by the ESI source. The ions used to quantify the proteins and peptides were chosen roughly in a number proportional to the protein mass and carefully selected to exclude values in common with other co-eluting peptides. The area of the XIC peak was proportional to protein/peptide concentration; therefore, under constant analytical conditions, it was used for quantitative analysis and comparative studies. [41-43] The estimated percentage error of the XIC procedure was <10%.

The percentage of oxidized $T\beta_4$ and $T\beta_{10}$ was calculated as the percentage of the XIC area of the oxidized forms relative to the XIC area of both total Tβ4 and total Tβ10, given by the sum of the oxidized and non-oxidized forms of the protein.

### *2.6. Statistical Analysis*

Statistical analysis was conducted using Statistical Package for Social Science (SPSS®, IBM®) version 25. Categorical data were expressed as number and percentage and numerical data were reported as mean and standard deviation (SD) or median and interquartile range (IQR), depending on their distribution. The Shapiro–Wilk test was employed to assess the normality of distribution for

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continuous variables. Categorical data were compared using Chi-square test or Fisher exact test, as appropriate. Continuous variables were compared using the Student's t-test for normally distributed data or the Mann–Whitney U test for non-normally distributed data.
Correlations between variables were assessed using Pearson's correlation coefficient for parametric data or Spearman's rank correlation coefficient for non-parametric data.
A p-value < 0.05 was considered statistically significant.

## 3. Results

We enrolled 18 preterm infants with a gestational age (GA) of less than 30 weeks. General characteristics of the enrolled neonates are shown in Table 1. Among them, 5 infants (27.8%) developed moderate-to-severe BPD, and 4 (22.2%) developed ROP, stage 3 or 4.

**Table 1.** Data are reported as median [interquartile range] and number (percentage). .

| | **Genaral characteristics** |
|---|---|
| Gestational age, weeks | 28.0 [25.7-29.6] |
| Neonatal weight, grams | 940 [790-1180] |
| Male sex | 11/18 (61.1%) |
| Vaginal delivery | 6/18 (33.3%) |
| ROP 3-4 stage | 4/18 (22.2%) |
| BPD moderate-severe | 5/18 (27.8%) |

A total of 149 saliva samples were analyzed. $T\beta_4$ was detected in 148 samples (99.3%) and $T\beta_{10}$ in 143 samples (96%). Their oxidized forms were present, in varying proportions, in 141 (94.6%) and 131 (87.9%) samples, respectively.

Correlation analysis did not show any significant association between gestational age and the levels of both total $T\beta_4$ and total $T\beta_{10}$ and the percentage of their oxidized derivatives. In contrast, a significant correlation was observed between PMA and total $T\beta_4$ (Spearman's Rho -0.269, $p = 0.001$) as well as the percentage of oxidized $T\beta_4$ (-0.184, $p = 0.025$), and between PMA and total $T\beta_{10}$ (-0.166, $p = 0.043$).

Moreover, correlation analysis did not reveal any significant association between $FiO_2$ (at the time of sample collection) and total levels of either protein, nor between $FiO_2$ and the percentage of oxidized $T\beta_4$. Conversely, higher $FiO_2$ values were associated with lower levels of oxidized $T\beta_{10}$ (-0.230, $p = 0.005$) and with lower percentages of oxidized $T\beta_{10}$ (-0.322, $p < 0.001$).

We compared the levels of both total thymosins and oxidized derivatives, expressed both as absolute values and percentages, between infants who developed oxidative stress-related conditions (moderate-to-severe BPD or ROP stage 3-4) and those who did not.

Levels of both total and oxidized $T\beta_4$ and $T\beta_{10}$, as well as the percentage of oxidized forms, were comparable between infants who developed ROP stage 3-4 and the control group (Table 2).

**Table 2.** Total values and percentage of oxidized protein in the group of infants with ROP stage 3-4 and in the control group. Mann–Whitney U test.

| | **ROP stage 3-4** | **Control group** | **p** |
|---|---|---|---|
| $T\beta_4$ | $8.30*10^7$ [$4.20*10^7$-$2.60*10^8$] | $1.40*10^8$ [$3.825*10^7$-$6.10*10^8$] | 0.137 |
| $T\beta_{10}$ | $2.70*10^7$ [$5.85*10^6$-$6.10*10^7$] | $3.65*10^7$ [$7.20*10^6$-$1.55*10^8$] | 0.265 |
| **%ox $T\beta_4$** | 7.87 [3.00-13.89] | 6.51 [2.91-16.60] | 0.870 |
| **%ox $T\beta_{10}$** | 4.37 [1.15-10.24] | 5.53 [2.12-11.69] | 0.296 |

Regarding BPD, despite comparable total levels of $T\beta_4$ and $T\beta_{10}$, we observed a higher percentage of oxidized $T\beta_{10}$ in infants who did not develop the condition (Table 3).

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**Table 3.** Levels of total proteins and percentage of oxidized forms in the group of infants with moderate or severe BPD and in the control group. Mann–Whitney U test. .

| | BPD Moderate-severe | Control group | p |
|---|---|---|---|
| Tβ4 | 1.10*10⁸ [4.75*10⁷-3.75*10⁸] | 1.45*10⁸ [3.75*10⁷-6.90*10⁸] | 0.316 |
| Tβ10 | 3.10*10⁷ [8.05*10⁶-1.40*10⁸] | 2.85*10⁷ [7.10*10⁶-1.28*10⁸] | 0.832 |
| **%ox Tβ4** | 5.88 [3.08-11.4] | 8.46 [2.81-17.79] | 0.416 |
| **%ox Tβ10** | 4.32 [1.35-9.13] | 7.16 [2.64-15.86] | 0.024 |

## 4. Discussion

To our knowledge, this is the first study performed to analyze the levels of Tβ4 and Tβ10 and of their oxidized derivatives in the oral fluid of preterm neonates, exploring their potential association with clinical conditions related to oxidative stress, particularly BPD and ROP. The findings offer valuable insights into better understanding the role of oxidized thymosins as potential non-invasive biomarkers of oxidative stress in preterm infants.

The analysis confirmed the high presence of both total and oxidized forms of Tβ4 and Tβ10 in neonatal saliva, with detection rates exceeding 90% for both proteins. This supports the idea that saliva is a suitable biological fluid for proteomic studies in neonates, as it consistently allows for the detection of post-translationally modified proteins, such as oxidized forms. [44]

One of the main findings concerns the inverse correlation observed between postnatal age and the salivary levels of both total and oxidized Tβ4, and to a lesser extent also for Tβ10. This finding is consistent with previous studies, such as the one by Nemolato S. et al. [45], which highlighted that Tβ4 levels increase significantly during early neonatal stages and decrease over time. The increase in Tβ4 concentration in the early postnatal period may reflect its essential role in supporting fetal and neonatal development. The subsequent reduction of thymosin levels could indicate a physiological adaptation of the body's antioxidant and inflammatory responses as the infant matures. This decrease may also result from the progressive consumption of thymosin proteins in response to persistent oxidative stress during early life. The lack of a significant correlation with gestational age suggests that the thymosin profile may depend less on the degree of prematurity and more on factors related to the extrauterine environment.

Another relevant aspect was the association between oxidized thymosin levels and oxygen administration ($FiO_2$). In particular, a significant inverse correlation was observed between $FiO_2$ and both the absolute level and percentage of oxidized Tβ10, but not for Tβ4. This may indicate a greater sensitivity of Tβ10 to oxidative stress induced by high oxygen levels, or its preferential degradation under hyperoxic conditions. The selectivity of this association for Tβ10 rather than Tβ4 is an intriguing finding that warrants further investigation.

When comparing neonates who developed moderate or severe BPD with those who did not, no significant differences were observed in the total levels of either thymosin, but a significant decrease in the percentage of oxidized Tβ10 was determined in infants with BPD. Although preliminary, this finding may suggest a protective role for oxidized Tβ10, where its reduced presence in infants with BPD could reflect an inadequate antioxidant response or greater susceptibility to unchecked oxidative damage. In contrast, the lack of significant differences for Tβ4 could indicate a more generic or less specific role for this isoform in the context of BPD.

No significant differences were observed between neonates with advanced-stage ROP and the control group, either in total levels or oxidation percentages of the two isoforms. This may suggest that oxidative regulation of thymosins plays a less critical role in the pathogenesis of ROP, or that other pathogenic mechanisms, such as dysregulated angiogenesis, may dominate its development.

The results of our study are in line with previous findings on the biological roles of oxidized Tβ4. In particular, the study by Mark A. Evans et al. [46] showed that oxidized Tβ4, in the form of Tβ4-sulfoxide, exerts protective effects in models of cardiac injury. Tβ4 oxidation appears to attenuate inflammatory cell infiltration and promote cardiac wound healing by reducing tissue damage and

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aiding inflammation resolution. These effects are likely mediated by the ability of oxidized Tβ4 to modulate the inflammatory response, as suggested by JD Young et al. [47], where Tβ4 -sulfoxide was implicated in the resolution of inflammation in various tissues. The inflammation-resolving capacity of oxidized Tβ4 could explain its beneficial effects in improving clinical outcomes in diseases like BPD, where chronic inflammation plays a central role in pathogenesis.

These studies, particularly those highlighting the functional role of oxidized thymosins, suggest that thymosin oxidation may not just be a detrimental modification, but rather a functionally relevant post-translational modification that can modulate cellular and inflammatory responses in a favorable manner.

These results are in line and consistent with what was previously observed by our research group [29], which identified high levels of Tβ4 in saliva of preterm infants and its expression in several fetal organs. Given that preterm birth can interrupt organ development—potentially leading to long-term health risks, as described by the Barker hypothesis—the team explored the role of Tβ4 in promoting fetal growth and organ maturation. Preliminary animal studies [48] showed that administering Tβ4 to pregnant mice enhanced fetal growth and accelerated development of organs like the heart, lungs, and kidneys. These results suggest that Tβ4 could be a promising agent in "physiological regenerative medicine" aimed at improving outcomes in preterm infants, and they warrant further clinical investigation.

Finally, it is important to note that our study has some limitations, including the small sample size, which may have limited statistical power to detect more subtle differences or to conduct multivariate analyses. Additionally, the heterogeneity of sample collection times and individual clinical variability may have influenced the salivary levels of the studied proteins. Lastly, since this is an observational study, no causal link can be established between oxidized thymosin levels and the development of oxidative stress-related diseases.

Despite these limitations, our findings suggest that salivary analysis of oxidized Tβ10 forms represents a promising tool for exploring oxidative status in preterm neonates and could, in the future, help in the early identification of infants at risk for conditions like BPD. Further studies with larger cohorts and extended clinical follow-up will be needed to validate these findings and clarify the functional significance of the observed oxidative modifications.

## 5.Conclusions

In conclusion, the results of our study suggest that oxidized Tβ4 and Tβ10 could serve as potential biomarkers of oxidative stress in preterm neonates, with implications for understanding diseases related to this stress, such as BPD. While no significant correlations were found with the development of ROP, the decrease in oxidized Tβ10 levels in neonates with moderate-severe BPD suggests a potential protective role for the oxidized form in modulating the inflammatory response and resolving tissue damage. Our results align with preclinical studies highlighting the potential of oxidized thymosin in promoting healing and inflammation resolution, indicating that thymosin oxidation may be a physiological strategy to counter chronic inflammation in pathological conditions such as BPD. Although sample size and methodology limitations require further confirmation, this study lays the groundwork for future research on using saliva as a non-invasive matrix to monitor oxidative stress and the therapeutic potential of oxidized thymosins in preterm neonates.

**Author Contributions:** Conceptualization, C.T., G.V., F.I., M.C.; Methodology, F.I, M.C., C.T., M.B., O.A., C.C. and G.G.; Formal Analysis, M.N., DT.D., M.G. and C.S.; Original draft preparation, C.T. and S.F.; Review and editing, M.C., G.V., F.G. and I.M. All authors have read and agreed to the published version of the manuscript.

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**Conflicts of Interest:** The authors declare no conflicts of interest.

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