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# Cell-Free mRNA Concentrations of Plasminogen Activator Inhibitor-1 and Tissue-Type Plasminogen Activator Are Increased in the Plasma of Pregnant Women with Preeclampsia

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***Background:*** **Detection of placental mRNA in maternal plasma has been reported in high-risk pregnancies. We attempted to investigate the concentrations of plasminogen activator inhibitor-1 (PAI-1) and tissue-type plasminogen activator (tPA) mRNA in maternal plasma in preeclampsia.**

***Methods:*** **Peripheral blood samples were obtained from healthy pregnant women before and after delivery and also from women with or without preeclampsia. Plasma was isolated from these samples, and RNA was extracted. Plasma PAI-1 and tPA mRNA concentrations were then measured by use of reverse transcription PCR assays. The concentrations were converted into multiples of the median (MoM) of the controls adjusted for gestational age. Data were stratified and analyzed according to the clinical severity of preeclampsia and quantitative distribution of blood pressure and proteinuria.**

***Results:*** **The median (minimum–maximum) PAI-1 mRNA MoM values for women with preeclampsia and controls were 2.48 (0.82–8.53) and 1.00 (0.41–2.33), respectively, whereas the median (minimum–maximum) tPA mRNA MoM values were 3.33 (1.01–10.58) and 1.00 (0.95–1.20), respectively. The concentrations of both PAI-1 and tPA mRNA were significantly increased in cases of preeclampsia, compared with controls ($P < 0.0001$). The MoM values of both mRNA species were directly correlated with the severity of preeclampsia and were greatest among a subgroup of hemolysis, increased liver enzymes, and low platelets pregnancies.**

***Conclusion:*** **Maternal plasma PAI-1 and tPA mRNAs are significantly increased in patients with preeclampsia and are positively correlated with the severity of preeclampsia.**



Preeclampsia remains a leading cause of fetomaternal morbidity and mortality in the developed and developing worlds *(1)*. Incomplete or absent transformation of spiral arteries by replacement with endothelial cells and mural vascular smooth muscle cells has been observed in the placental beds of patients with preeclampsia, as well as in severe cases of intrauterine growth restriction *(2)*.

Normal placental development depends on proper trophoblast invasion, vascular remodeling, and maintenance of intervillous blood flow *(2)*. These processes involve degradation of the extracellular matrix, which is highly dependent on a regulated production of proteolytic enzymes, such as plasmin, a key enzyme of the fibrinolytic system. Particularly important to the regulated production of plasmin are tissue-type plasminogen activator (tPA)[4] and urokinase plasminogen activator

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[4] Nonstandard abbreviations: tPA, tissue-type plasminogen activator; uPA, urokinase plasminogen activator; PAI, plasminogen activator inhibitor; GA, gestational age; HELLP, hemolysis, increased liver enzymes, and low platelets; RT, reverse transcription; MoM, multiples of the median.

(uPA). Inactivation of uPA by various plasminogen activator inhibitors (PAIs) leads to reduced trophoblast invasion *(3)*. Within the placenta, uPA and PAI-2 are expressed in villous trophoblast tissue, whereas tPA and PAI-1 are primarily found at the interface where detachment from maternal tissue occurs *(4)*.

In 2000, Lo et al. *(XX)* described that cell-free mRNA derived from the placenta circulates in the plasma of pregnant women and established an approach independent of gender and genetic polymorphisms *(5)*. They also quantified human chorionic gonadotropin and human placental lactogen gene expression in maternal plasma, both of which are produced only by placental trophoblasts. They showed that the mRNAs are specific for pregnancy plasma and reflect the placental gene expression. Thus, evaluating placental mRNA expression in maternal plasma seems a potentially feasible method by which to monitor placental function. Ng et al. *(6)* also demonstrated that corticotropin-releasing hormone mRNA concentrations in the plasma of pregnant women with preeclampsia are greater than in normal pregnant women. They therefore suggest that plasma corticotropin-releasing hormone mRNA might represent a new molecular marker for preeclampsia *(6)*.

Although it is possible that some circulating cell-free mRNA species might come from other tissue sources, only a few studies have explored aberrant levels in pathological conditions of pregnancy *(6–8)*. In the present study, we attempted to investigate the mRNA expression of PAI-1 and tPA in maternal peripheral blood from women suffering from preeclampsia, compared with age-matched controls.

## Materials and Methods

### PARTICIPANTS

The study population included women with preeclampsia and normal pregnant women who visited the Department of Obstetrics and Gynecology, University of Indonesia at Cipto Mangunkusumo National Hospital. Participants were recruited between December 2005 and February 2006. All women were informed and agreed to participate in the study, which was approved by the Research Ethics Committee.

Peripheral blood samples were obtained from (*a*) 43 pregnant women with preeclampsia, and (*b*) 41 control pregnancies. There was a median gestational age (GA) of 39 (35–41) weeks for both groups of patients. Preeclampsia was defined as gestational hypertension (systolic pressure >140 mmHg or diastolic blood pressure >90 mmHg on ≥2 occasions after 20 weeks gestation) with proteinuria (>0.3 g/day). Severe preeclampsia was defined by the presence of ≥1 of the following: (*a*) severe gestational hypertension (systolic pressure >160 mmHg or diastolic blood pressure >110 mmHg on 2 occasions after gestational week 20), or (*b*) severe proteinuria (≥5 g protein in a 24-h urine specimen or ≥3 g in 2 random urine samples collected ≥4 h apart). Fetal growth restriction was defined as an estimated fetal weight 2.0 SD below the mean expected weight for GA, as determined by ultrasonographic evaluation. Hemolysis, increased liver enzymes, and low platelets (HELLP) syndrome was defined by the Mississippi classification *(9)*. The control group included pregnant women with no preexisting medical diseases or antenatal complications.

To assess clearance of mRNA expressions, we also obtained samples from 9 women who delivered by elective cesarean section, before delivery and 60 and 120 min after delivery. None of the 9 women had any complication before or after cesarean section.

### PROCESSING OF BLOOD SAMPLES

Peripheral blood samples were taken before the onset of labor. Plasma harvesting was performed immediately after blood collection. In brief, 7-mL blood samples were collected in EDTA-containing tubes and centrifuged at 1600*g* for 10 min at 4°C. Plasma was carefully transferred into plain propylene tubes and stored until they were transported to Japan at −20°C. Molecular analysis was performed in the Department of Obstetrics and Gynecology at Showa University School of Medicine, Tokyo, Japan.

### RNA EXTRACTION

Total RNA was extracted from 1.6 mL of harvested plasma. The plasma was mixed with 2 mL of Trizol LS reagent (Invitrogen) and 0.4 mL of chloroform. This mixture was centrifuged at 12,000*g* for 15 min at 4°C, and the aqueous layer was then transferred to new tubes. After 1 volume of 700 mL/L ethanol was added to 1 volume of the aqueous layer, the mixture was applied to a QIAamp MinElute Virus column (Qiagen) and processed according to the manufacturer's recommendations. Total RNA was eluted with 20 μL of RNase-free water and directly reverse transcribed.

### REAL-TIME QUANTITATIVE REVERSE TRANSCRIPTION PCR

Reverse transcription (RT) was performed using an Omniscript RT reagent set (Qiagen) following the manufacturer's recommendations. Total RNA from the samples (12 μL) was reverse transcribed in a reaction volume of 20 μL, using 2 μL 10× RT buffer, 2 μL 25 × dNTPs, 1 μL 10× RT random primer, 1 μL oligo(dT) primer, 1 μL RNase inhibitor, and 1 μL Omniscript RT. The thermal profile of RT performed in a GeneAmp PCR System 9600 thermal cycler was as follows: 60 min at 37 °C, followed by 5 min at 93°C. After this, cDNA products were amplified by real-time quantitative PCR according to the manufacturer's instructions (QuantiTect Probe PCR reagent set; Qiagen) using a 2-μL aliquot of cDNA and the reagent set's components in a reaction volume of 20 μL. TaqMan PCR analyses for PAI-1 and tPA were performed using predeveloped and commercially available primers and probe sets (cat. no. Hs00167155_m1 for PAI-1 and cat. no.

Hs00263492_m1 for tPA; Applied Biosystems). As an initial step, we verified that each PCR assay was specific to mRNA and not to genomic DNA. Amplification data were collected and analyzed with an ABI Prism 7900 Sequence Detector (Applied Biosystems). Each sample was analyzed in duplicate, and multiple negative water blanks were included in every analysis. The thermal profile used was as follows: 15 min of denaturation at 95°C, followed by 15 s of annealing at 94°C and 1 min of extension at 60°C. Quantification of gene expression was performed, with investigators blinded to study group.

### CALIBRATION CURVES

The amount of mRNA per sample was expressed in terms of copies/mL. To quantify the mRNA concentrations of PAI-1 and tPA, we prepared plasmid DNA for a standard dilution curve. Briefly, PCR amplification of PAI-1 and tPA primers was performed using RT products of placental mRNA and a Takara Taq Polymerase reagent set (TaKaRa), according to the manufacturer's instructions and recommended thermal profile. Each PCR product was cloned into a pCR 2.1-TOPO vector (Invitrogen), which transformed the recombinant vector into competent *Escherichia coli*. After culture, DNA was extracted a using QIAprep Spin Miniprep reagent set (Qiagen), and the DNA concentration was calculated. The DNA was then serially diluted to make calibration curves of $10^5$, $10^4$, $10^3$, $10^2$, and 10 copies/mL for each mRNA product.

### STATISTICAL ANALYSIS

Maternal plasma PAI-1 and tPA mRNA concentrations were expressed as multiples of the median (MoM) for unaffected pregnancies. A log linear regression of these median values (weighted according to the number of samples) against GA was performed. The normal median marker values for each GA were estimated from the regression analysis, and concentrations of PAI-1 and tPA mRNA were expressed as MoMs. Inspection of probability plots revealed that the MoMs for PAI-1 and tPA mRNA could be approximated by a log gaussian distribution. MoM values were stratified according to severity of preeclampsia and manifestation of features of the HELLP syndrome. Finally, regression analysis was performed by plotting MoM values vs systolic and diastolic pressure, as well as proteinuria, in both groups of patients.

## Results

A total of 84 pregnant women, including those with preeclampsia (n = 43) and controls (n = 41), were eligible for this study. Table 1 shows characteristics of both groups. No significant differences between preeclamptic and control pregnancies were observed with regard to age, body mass index, or GA.

PAI-1 and tPA MoMs (Table 1) were 2- to 3-fold higher in women with preeclampsia than in controls ($P < 0.001$), and both increased with increasing severity of disease (Fig. 1), with highest results in the patients with HELLP syndrome.

Only within the preeclampsia group did the severity of proteinuria and hypertension strongly correlate with PAI-1 and tPA mRNA MoM concentrations (Figs. 2 and 3). Proteinuria was most strongly associated with increased mRNA concentrations of PAI-1 and tPA, followed by systolic and diastolic blood pressure. Table 2 demonstrates how well the values fit the calibration curves. Among controls, no significant correlations between mRNA species and hypertension or proteinuria were observed.

We analyzed clearance of PAI-1 and tPA mRNA in maternal plasma (GA, 38–40 weeks) to assess the origin of the gene expressions. PAI-1 and tPA mRNA were detected in all 9 samples before delivery and were significantly decreased ($P < 0.01$) at 120 min after delivery (Fig. 4).

## Discussion

In preeclampsia and other pathological conditions of pregnancy, maternal uPA, tPA, and PAI-1 plasma concentrations have been examined by immunoassay for a

**Table 1. Demographics and distribution of clinical variables.**

| Variables[a] | Control (n = 41) | Preeclampsia (n = 43) | *P*[b] |
|---|---|---|---|
| Age, years | 28 (16–45) | 26 (17–37) | NS[c] |
| Gestational age at delivery, weeks | 39 (35–41) | 39 (35–41) | NS |
| Body mass index, kg/m$^2$ | 22 (20–28) | 22 (16–32) | NS |
| Diastolic blood pressure, mmHg | 75 (70–85) | 100 (80–140) | |
| Systolic blood pressure, mmHg | 110 (100–130) | 150 (130–200) | |
| Birth weight, g | 3100 (2500–4200) | 2882 (542) | <0.001 |
| Small for GA, <p 2.3 | 0 | 6 | |
| Corticosteroid given | 0 | 8 | |
| PAI-1 MoM | 1.00 (0.41–2.33) | 2.48 (0.82–8.53) | <0.001 |
| TPA MoM | 1.00 (0.95–1.20) | 3.33 (1.01–10-58) | <0.001 |

[a] Data are presented as medians (minimum–maximum).
[b] Mann–Whitney *U*-test for comparison.
[c] NS, not significant.

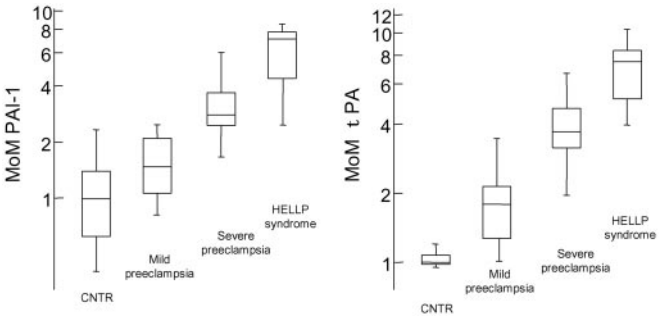


Fig. 1. *Box* and *whiskers plot* of PAI-1 and tPA MoM distribution among controls and women with preeclampsia, stratified according to severity of preeclampsia.

CNTR, control cases.

number of years, but the reports present conflicting results for preeclampsia. In 1984, Wiman et al. *(11)* first described increased plasma protein concentrations of PAI-1 in preeclampsia, after which, similar observations by others followed *(12–16)*. Estelles et al. reported significantly increased plasma PAI-1 in a range of pathological conditions of pregnancy, including complete hydatidiform moles *(17)*, preeclampsia *(14)*, and intrauterine growth retardation *(13)*, compared with normal pregnancies. In contrast, other authors have reported no significant increase in plasma PAI-1 in preeclampsia during late gestation or in cases of intrauterine growth retardation *(18, 19)*. Similarly, some authors report significantly increased tPA concentrations within the plasma of women with preeclampsia *(14, 16, 18)*, whereas others refute these findings *(16, 20)*.

We theorize that the PAI-1 and PA system may play a critical role in the pathogenesis of preeclampsia in early pregnancy and that mRNA expression of PAI-1 and tPA might reflect physiological alterations that occur later in pregnant women with preeclampsia. Thus, in the present study, we compared the mRNA expression of PAI-1 and tPA in maternal plasma among pregnant women with and without preeclampsia. In doing so, we demonstrated significantly increased concentrations of PAI-1 and tPA mRNA in maternal plasma in pregnancies complicated by preeclampsia. PAI-1 and tPA mRNA concentrations (expressed in MoM) were 2.48 and 3.33 times greater in pregnancies complicated by preeclampsia, respectively, than among age-matched control pregnancies.

The present finding of significantly increased PAI-1 and tPA mRNA concentrations in maternal plasma in preeclampsia suggests that increased transcription of mRNA might be related to preeclampsia. There remains a possibility that PAI-1 and tPA mRNA are produced by maternal tissue, rather than the placenta. However, in this study, both mRNA transcripts rapidly decreased after delivery. This finding indicated that majority of mRNA transcript originated from fetus and/or placenta.

Ng et al. *(6)* suggested several theoretical possibilities to explain such quantitative changes in plasma RNA. Apoptotic changes within placental villous trophoblasts among women with preeclampsia *(21)* may be associated with the release of placental mRNA into maternal plasma, since it is postulated that cell death releases nucleic acid into maternal plasma *(22)*. Extensive placental infarction in patients with preeclampsia is associated with marked immunoreactivity for components of the PA system *(23–25)*. A markedly increased PAI-1 concentration in umbilical cord plasma has also been shown in pregnancies complicated by preeclampsia *(19, 26)*. This might also lead to enhanced release of PAI-1 and tPA mRNA into maternal plasma. Lo et al. have reported that the half-life of circulating fetal DNA (placental DNA) is 16.3 min *(27)*. However, a 4-fold increase in the clearance half-life of fetal DNA has been observed in patients with preeclampsia, compared with controls (114 vs 28 min) *(28)*. A similar mechanism might result in higher concentrations of PAI-1 and tPA mRNA in maternal plasma.

In this study, we did not take into account the possible effects of magnesium sulfate, antihypertensive medica-

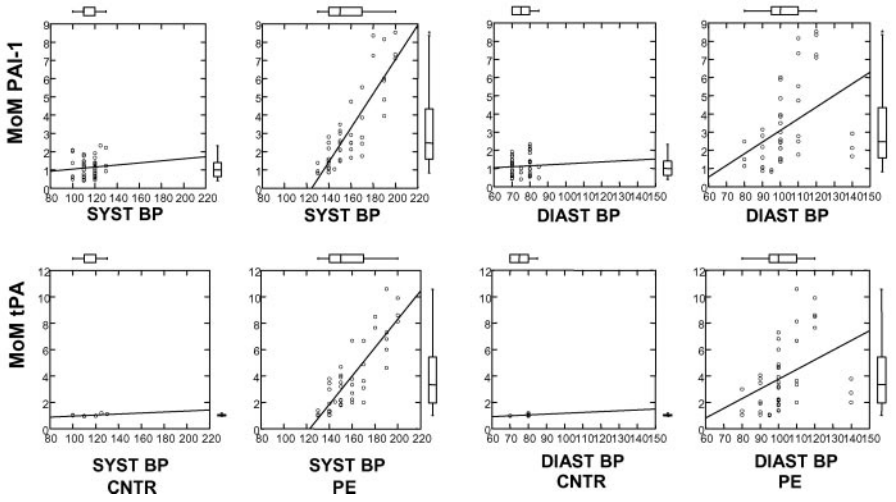


Fig. 2. Correlation between PAI-1 and tPA MoM values and systolic (SYST) and diastolic (DIAST) blood pressure (mmHg) in women with preeclampsia

*BP*, blood pressure; *CNTR*, control cases; *PE*, preeclampsia cases.

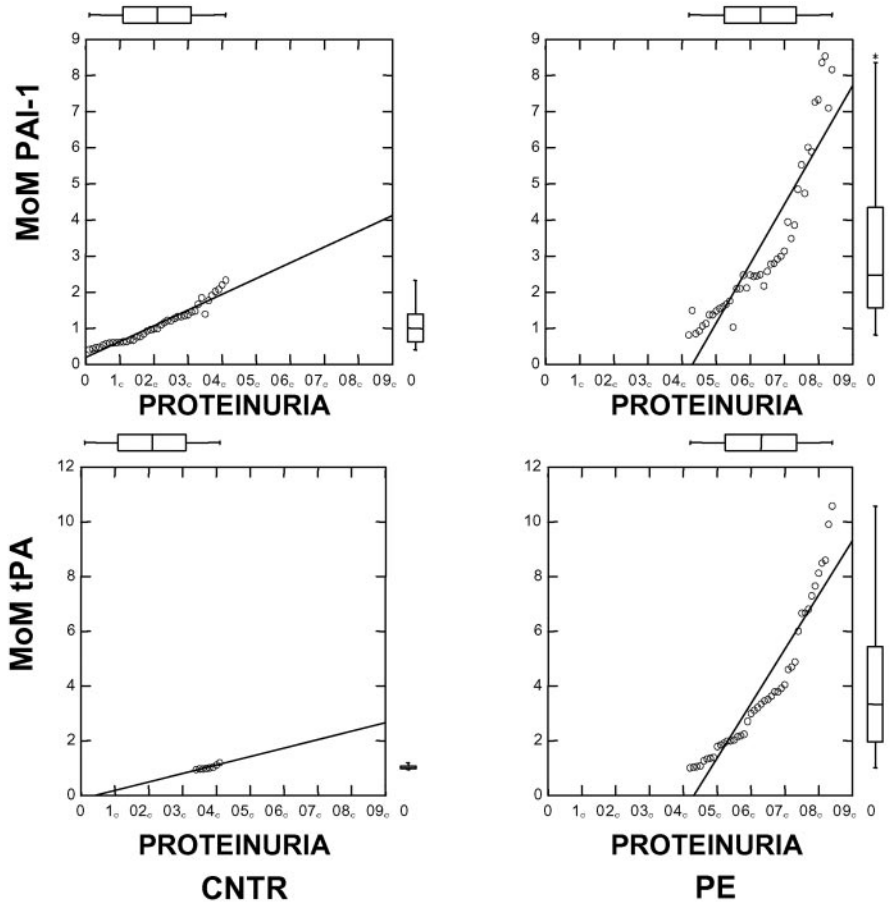


Fig. 3. Correlation between PAI-1 and tPA MoM values and severity of proteinuria (g/mL) in women with preeclampsia

CNTR, control cases; PE, preeclampsia cases.

tions, or methyldopa on concentrations of various components of the PA system *(29, 30)*. Specifically, corticosteroid administration might increase PAI-1 and tPA concentrations. Since only 20% of our patients with preeclampsia received corticosteroid injections, corticosteroid administration likely had minimal effect on our overall results.

It is worth noting that tPA and PAI-1 mRNA concentrations were significantly correlated with the relative severity of preeclampsia. This finding agrees with previous immunoassay results. Belo et al. *(31)* observed a significant correlation between tPA concentrations and severity of proteinuria in women with preeclampsia. Previously, we demonstrated a strong independent association between proteinuria, as well as hypertension, and fetal DNA values within maternal plasma. The estimated fetal DNA concentrations in patients with mild and severe preeclampsia were 2.25 and 5.06 times greater, respectively, than in controls at 34 weeks gestation *(32)*. Similar to concentrations of cell-free DNA, mRNA expression levels of PAI-1 and tPA could be markers to evaluate the pathogenesis of preeclampsia. Interestingly, relative differences in proteinuria and hypertension were correlated with PAI-1 and tPA MoM values in women with preeclampsia, but not in controls, in the present study. These findings indicate that increased gene expression of PAI-1 and tPA might contribute to increased blood pressure and/or proteinuria, 2 manifestations of preeclampsia.

**Table 2. Goodness of fit of the estimated linear equations among the preeclampsia group.**

| Variable | F | *P* |
|---|---|---|
| PAI vs proteinuria | 334.39 | <0.001 |
| tPA vs proteinuria | 312.17 | <0.001 |
| PAI vs systolic BP[a] | 110.77 | <0.001 |
| tPA vs systolic BP | 100.84 | <0.001 |
| PAI vs diastolic BP | 8.17 | 0.007 |
| tPA vs diastolic BP | 7.75 | 0.008 |

[a] BP, blood pressure.

In conclusion, this study demonstrated increased PAI-1 and tPA mRNA concentrations in the plasma of patients with preeclampsia, compared with normal participants. These findings provide further insight into pathologic pregnancy. It might be worthwhile to undertake a prospective systematic investigation of the numerous genes expressed in placental tissue to further understand the mechanism(s) behind various pathological conditions of pregnancy.

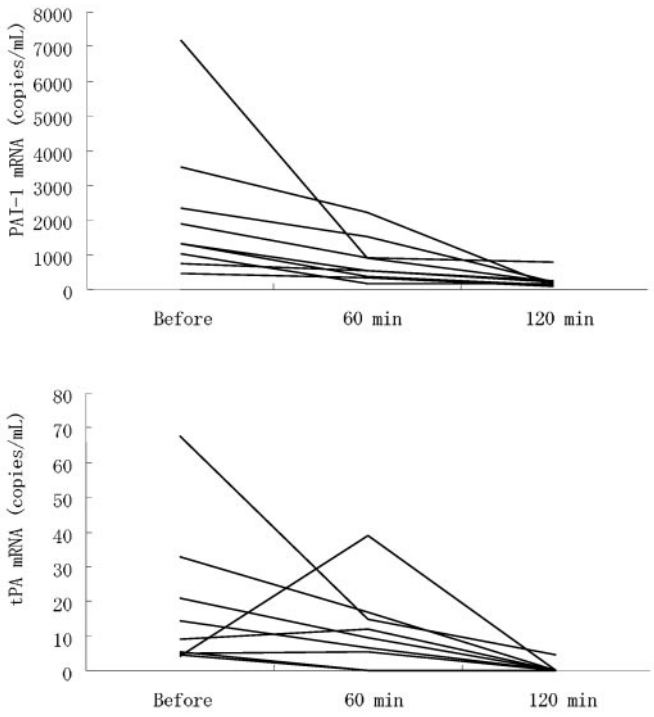


Fig. 4. Clearance of PAI-1 and tPA mRNA concentration before delivery and 60 and 120 min after delivery.

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