Suppression of Phoenixin-14 Predicts Mortality in Sepsis: Evidence from Serum Levels and Gene Expression

Assistant Professor, MD Gülsüm ALTUNTAŞ 30.04.2025

Department of Anesthesiology and Reanimation, Firat University Medicine Faculty Hospital, Elazig 23300, Turkey

Study Protocol

Study Design and Patients

In this prospective observational cohort study, patients aged 18 years and older admitted to our tertiary intensive care unit with diagnoses of sepsis and/or septic shock were evaluated. Ethical approval was obtained from the Fırat University Non-Interventional Research Ethics Committee on March 27, 2024, with the number 23376. The entire study was funded by the Fırat University Scientific Research Projects Coordination Unit (FUBAP), under project number TF.24.11. Our study adhered to the Helsinki Declaration and the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) guidelines.

The treatment protocol for patients was followed according to the Sepsis-3 guidelines (1), and antibiotic therapy was administered in consultation with an infectious disease specialist, aiming at the source of sepsis. Septic shock was also defined according to the Sepsis-3 guidelines (1), based on the requirement for vasopressor support to maintain adequate mean arterial pressure despite adequate fluid resuscitation.

Patients under the age of 18, patients who were discharged/transferred or died within 72 hours after being diagnosed with sepsis, patients/relatives who refused to participate in the study, pregnant women, patients using chronic steroids, patients whose blood samples were collected

under inappropriate conditions, and patients with a history of malignancy or immunosuppression were also excluded from the study.

The study was started with participants who agreed after signing an informed consent form.

Collection of Blood Samples and Data

At the time of ICU admission, parameters such as age, gender, body mass index (BMI), comorbidities, infection focus, complete blood count, CRP, procalcitonin, Acute Physiology and Chronic Health Evaluation (APACHE) II score, Sequential Organ Failure Assessment (SOFA) score, vasopressor requirement, mechanical ventilation need, and renal replacement therapy requirement were recorded. On the first day of hospitalization, 3 mL of blood was routinely collected from patients for biochemical analysis. The levels of IL-6, TNF-α, IL-1β, IL-10, and PNX14 were measured using real-time PCR, while serum levels were determined using ELISA. Blood samples were collected once at ICU admission to assess baseline PNX-14 levels for early prognostic evaluation.

RNA Isolation

Total RNA was isolated from peripheral blood samples using the NucleoSpin RNA Blood kit (Macherey-Nagel, Germany) according to the manufacturer's instructions. RNA concentration and purity were assessed using a Qubit 2.0 Fluorometer (Thermo Fisher Scientific).

RNA Quality Control – Agarose Gel Electrophoresis

RNA integrity was assessed by agarose gel electrophoresis. Briefly, RNA samples were separated on a 1% agarose gel and visualized using a gel documentation system to confirm RNA integrity, according to standard laboratory procedures.

RNA Concentration Measurement – Qubit Fluorometric Method

RNA concentration and quality were assessed using a Qubit 2.0 Fluorometer (Thermo Fisher Scientific, USA) and the Qubit RNA HS Assay Kit, following the manufacturer's recommended protocol.

cDNA Synthesis

Complementary DNA (cDNA) was synthesized from total RNA using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, USA) as per the manufacturer's protocol.

Quantitative Real-Time PCR (qRT-PCR)

Quantitative real-time PCR analysis was performed using SYBR Green Master Mix (AMPLIFYME SYBR Universal Mix) on an Applied Biosystems StepOnePlus system. GAPDH was used as the reference gene due to its reported expression stability in peripheral blood samples under inflammatory conditions. Relative gene expression was calculated using the $2^{-}\Delta\Delta Ct$ method, with GAPDH as the internal control. In the absence of a healthy control group, the survivor group was used as the calibrator for relative expression analysis. Primer sequences are listed in Table 1.

Table 1. Primers used in the study

	Forward Primer	**Reverse Primer**
GAPDH	5'-GAA GGT GAA GGT CGG AGT C-3'	5'-GAA GAT GGT GAT GGG ATT TC-3'
PNX-14	5'-CGG CTT CAT CTC CCT GAT CG -3'	5'ACA GCC CTC TCA TTT CCT GC-3'

GAPDH (glyceraldehyde-3-phosphate dehydrogenase; PNX-14: Phoenixin14

ELISA Protocol

Serum levels of Phoenixin-14 and cytokines were measured using commercially available ELISA kits (BT Lab, Shanghai, China) according to the manufacturer's instructions.

End points

Primary endpoint: the relationship between PNX14 levels and in-hospital mortality in patients with sepsis or septic shock. Secondary endpoints: to evaluate the tendency of PNX-14 levels to be suppressed at the molecular level in patients with sepsis and their potential as a translational biomarker. Additionally, to assess the relationship between PNX14 and SOFA scores, APACHE II scores, septic shock, requirements for mechanical ventilation or renal replacement therapy, and ICU length of stay in patients with sepsis. The correlation between PNX14 levels and those of CRP, procalcitonin, IL-1β, IL-6, IL-10, and TNF-α was also examined as part of the endpoints.

Statistical Analysis

Before the study, calculations were conducted using G*Power 3.1 with 80% power, $\alpha = 0.05$, and an expected medium effect size ($d = 0.5$), indicating that at least 64 patients were needed. This requirement was met by including 77 patients in the study.

The data were analyzed using IBM SPSS Statistics for macOS, version 29.0 (IBM Corp., Armonk, NY, USA). Descriptive statistics for continuous variables are presented as mean ± standard deviation for normally distributed data and median (Q1–Q3) for non-normal data; categorical variables are shown as frequency (percentage). Group comparisons between developing and non-developing patients were conducted with the independent samples t-test for normally distributed continuous data and the Mann–Whitney U test for non-normal data. Normality of continuous variables was checked with the Kolmogorov-Smirnov test. For categorical variables, either the Pearson chi-square test or Fisher's exact test was used, as appropriate.

The relative quantification (RQ, 2^-ΔΔCt) values from qPCR experiments were compared between two groups (Dead and Survived). The distribution of both groups was assessed using the Shapiro–Wilk test prior to analysis, and the assumption of normality was not rejected (D group p=0.12; S group p=0.17). Homogeneity of variances was evaluated with the Levene test, which indicated unequal variances (p=0.018). Therefore, the differences between groups were analyzed using the Welch independent samples t-test. Additionally, the nonparametric Mann–Whitney U test was performed to verify robustness.

The ability of each biomarker to predict mortality was evaluated using ROC (receiver operating characteristic) curve analysis. The area under the curve (AUC), 95% confidence interval, optimal cut-off value, sensitivity, specificity, Youden index, and p-values were calculated. Since PNX-14 levels were inversely related to mortality in the ROC analysis, the analysis was performed with the reversed direction. Comparison of ROC curves for PNX-14 versus procalcitonin and CRP was conducted using the DeLong test.

To identify variables linked to mortality, univariate logistic regression analysis was conducted. Variables that proved significant were included in the multivariate logistic regression model to determine independent risk factors. The Backward-Wald method was employed for variable selection. Multicollinearity among independent variables was evaluated using the variance inflation factor (VIF) and tolerance values; VIF less than 5 and tolerance values between 0.40 and 0.80 were considered acceptable. The relationships between PNX, inflammatory biomarkers, and clinical severity scores were analyzed using Pearson or Spearman correlation tests, depending on the distribution of the variables, with correlation coefficients (r) and p-values reported. In all analyses, a p-value less than 0.05 was regarded as statistically significant.