IS PENTRAxin-3 AN EARLY BIOMARKER OF PRIMARY ACUTE RESPIRATORY DISTRESS SYNDROME IN THE INTENSIVE CARE UNIT? A PROsPECTIVE STUDY

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ABSTRACT

Background: To examine the role of pentraxin-3 (PTX-3) and other biomarkers increasing in the early stages of primary acute lung injury/acute respiratory distress syndrome (ALI/ARDS), we measured the plasma levels of the indicators early in the disease process and at the end of treatment.

Methods: We recorded: age, weight, gender, APACHE-II (Acute Physiology and Chronic Health Evaluation) and SOFA (sequential organ failure assessment) scores for 36 patients hospitalised and intubated with a diagnosis of primary ALI/ARDS in our intensive care unit between August 2009 and December 2010. We measured the plasma levels of tumour necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β), C-reactive protein (CRP), and PTX-3 within the first 24 hours and on days 4 and 8. We collected bronchoalveolar lavage fluid within the first 24 hours of hospitalisation in the intensive care unit and attempted to identify the responsible pathogen. We also recorded the patients' total leukocyte, neutrophil and platelet values and PaO2/FiO2 rates.

Results: PTX-3, CRP, TNF-α, and IL-1β levels, which were noticeably high during hospitalisation, dropped significantly in patients clinically recovering. PTX-3 values of the surviving patients that were elevated at the time of hospitalisation dropped significantly on days 4 and 8 compared with day 1 (p = 0.001). (76.02±10.77, 24.18±11.01, 1.18±0.61 days 1, 4 and 8 respectively). In deceased patients these values remained high on day 4 and 8. All patients PTX-3 levels on day 1 was higher in severe lung injury (ARDS and ALI) in comparison with mild-to-moderate lung injury, as defined by LIS (Lung injury score) (p < 0.001, LIS 0.1-2.5 and LIS >2.5, respectively). Specific pathogens such as Klebsiella pneumoniae and Pseudomonas aeruginosa, with which PTX-3 is known to have affinity, were identified in bronchoalveolar lavage fluids.

Conclusions: Our findings indicate that PTX-3 is closely associated with mortality and severity of lung. PTX3 may be useful in early evaluation and prediction of the primary ALI/ARDS.

Key words: acute lung injury/acute respiratory distress syndrome, tumour necrosis factor alpha, interleukin-1 beta, C-reactive protein, pentraxin-3.

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Introduction

Acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) are disorders characterised by pulmonary alveolar proteinosis and oedema and acute inflammation, which develop together with increased permeability of the alveolo-capillary membrane, leading to respiratory insufficiency and hypoxemia(1). The incidence of acute respiratory insufficiency has been established at 70-140/100,000(2,3).

In the severe inflammation seen with ALI/ARDS, pentraxin has recently been recognized as playing an important role separate from C-reactive protein (CRP), an active infection criterion that is produced by the liver, and inflammatory markers such as tumour necrosis factor-alpha (TNF-α), interleukin (IL)-1 and interleukin-6. Pentraxin-3 (PTX-3) is an acute phase protein that is produced by several tissues and which plays an important role in the regulation of inflammation, removal of apoptotic cells, and resistance of pathogens.
It may provide information about the severity of the disease in intensive care unit patients diagnosed with ALI/ARDS\(^{(4,5)}\). PTX-3 has important effects on epithelial cells\(^{(4,5)}\) and the PTX-3 gene has been identified on human chromosome 3q24-28\(^{(5)}\). PTX3 values, low in healthy subjects (<2 ng/ml), increase rapidly during multiple inflammatory conditions\(^{(6-8)}\). Increased levels of PTX3 have been recently reported in ARDS,\(^{(4,5)}\) and the PTX3 levels are well correlated with the severity of lung injury and a useful predictor of survival. Our hypothesis that high levels of plasma PTX3 in the first days may represent an early marker and have specific role of severity and mortality in ARDS from pneumonia. Our objective was to investigate the effects of increasing PTX-3 levels in the early stages of primary ALI/ARDS, to compare PTX-3 levels with other inflammatory indicators (TNF-\(\alpha\), IL-1\(\beta\), CRP), and to examine the changes in these levels at the end of treatment.

**Materials and methods**

Between August 2009 and December 2010, we studied 36 patients diagnosed with primary ARDS/ALI caused by pneumonia who were admitted to the single centre anaesthesiology and reanimation intensive care unit. The American-European Consensus Conference (AECC) ALI and ARDS criteria are used most commonly to diagnose ALI and ARDS in adults. The AECC criteria for diagnosis of ALI/ARDS are as follows: a) acute onset; b) severe arterial hypoxemia resistant to oxygen therapy alone (PaO\(_2\)/FiO\(_2\) ratio \(\leq 200\) for ARDS and \(\leq 300\) for ALI); c) diffuse pulmonary inflammation (bilateral infiltrates on chest radiographs); and d) no evidence of left atrial hypertension\(^{(2)}\).

The study was approved by Ethics Board of the University of Ataturk, Erzurum (approval on May 15th 2009, registration number 4/135). Signed informed consent was obtained from all patients who took part in the study and/or their next of kin.

We excluded patients transferred from other intensive care units, patients under 18 years of age or over 75 years of age with malignancy, those with chronic liver failure, immunosuppression, previous radiotherapy or chemotherapy, secondary ALI/ARDS and patients with a disease that could affect the level of PTX-3 (e.g., vasculitis, systemic sclerosis, systemic lupus erythematosus, rheumatoid arthritis, Wegener granulomatosis, or Churg-Strauss syndrome).

Patients were ventilated using the synchronized intermittent mandatory ventilation volume control mode according to the Acute Respiratory Distress Syndrome Network (ARDSNet) study low tidal volume (VT) strategy. VT was set at 6 ml/kg predicted body weight and additional ARDSNet strategies were applied\(^{(9)}\). Patients required support for arterial oxygenation with a combination of increased FiO\(_2\) and positive end-expiratory pressure (PEEP). Conservative IV fluid therapy and empirical antibiotics were also used.

**Collection of clinical data and plasma/serum**

The age, gender, weight, aetiology and APACHE-II and SOFA scores of the patients meeting the pre-set criteria were recorded at the time of hospitalisation. Plasma samples were collected for measurement of TNF-\(\alpha\), IL-1\(\beta\), CRP, and PTX-3 within the first 24 hours following intubation and on days 4 and 8 until patients were moved from the intensive care unit. During sample collection, arterial blood gases, lung injury status, leukocyte (white blood cell, WBC), thrombocyte and neutrophil levels of the patients were recorded.

Bronchoalveolar lavage fluid (BAL) was collected using a standard technique within the first 24 hours of hospitalisation in the intensive care unit and we attempted to identify the pathogen responsible for ALI/ARDS, if any. The injured area was established radiographically and reached using bronchoscopy, via a sterile endotracheal tube. A 20 mL sample of 0.9 % saline was then dropped into the respective lobe and aspirated. This was repeated 4 times. Lavage fluids samples were sent to microbiology, evaluated appropriately and the results were recorded.

36 patients included in the study, blood samples were collected and tested. We measured routine biochemical tests, complete blood count, arterial blood gases, CRP, IL-1\(\beta\), TNF-\(\alpha\), and PTX-3 values in all patients. Measurements were performed immediately after sample collection except for PTX-3, IL-1\(\beta\), and TNF-\(\alpha\) for which blood samples were collected into anticoagulant tubes and centrifuged for 3 minutes at 3000 rpm. Plasma was separated and samples were stored at-80°C until analysis. TNF-\(\alpha\) and IL-1\(\beta\) levels were measured using commercially available enzyme-linked immunosorbent assay (ELISA) kits (Invitrogen, Carlsbad, CA). Samples were removed from-80°C, warmed to -20°C and then to 4°C to be suitable for use with the kits. After adding 100 microliter of biotin conjugate
and incubating at room temperature for 2 hours, samples were washed with washing solution 4 times and aspirated. They were then incubated with 100 microliter Streptavidin-HRP for 30 minutes at room temperature. After washing 4 times and aspirating, samples were incubated at room temperature in 100 microliter of stabilised chromogen for 25 minutes. After adding 100 microliter of stop solution and completing the final procedure, the optic density of all samples and standards was established with a 450 nm microplate. Concentrations were established according to standard curves.

We used a commercially available ELISA kit (Cusabio Biotech Co., Ltd. Wuhan, China) to measure PTX-3 levels. Samples were removed from -80°C, warmed to-20°C and then to 4°C to be suitable for use with the kits. The manufacturer’s procedure was followed for all samples. First, an ELISA plate composed of small wells was covered with PTX-3-specific antibodies. After adding 100 microliter of standard or sample, the plate was covered with adhesive strips and incubated at room temperature for 2 hours. After adding 100 microliter of biotin-antibody solution and incubating at room temperature for 1 hour, samples were washed 3 times with washing solution. After incubating in 100 microliter of horseradish peroxidase solution at room temperature for 1 hour, samples were washed 5 times with washing solution and aspirated. Next, 90 ml of 3,3′,5,5′ tetramethylbenzidine substrate was added, and the samples were incubated at room temperature for 10-30 minutes. Only the samples containing PTX-3 exhibited a change in colour when they were mixed with biotin-labelled antibody and enzyme-labelled avidin. Finally, after 50 ml of sulphuric acid solution was added and the enzyme-substrate reaction was complete, the change in colour was established with the microplate reader optical density at 450 nm. Concentrations were calculated according to the standard curves (microplate reader, microplate washer, and microplate shaker: BioTek, VT, USA).

**Statistical Analysis**

We used SPSS (Statistical Package for Social Sciences, Chicago, IL, USA) for Windows, version 18.0 software for statistical analysis. Data were presented as number, percentage, mean and standard deviation. WBC, neutrophil, and thrombocyte counts, CRP, IL-1β, and PTX-3 comparisons of the patients between days 1 and 4, days 4 and 8, and days 1 and 8 were conducted using the repeated measurement of ANOVA test with Bonferroni correction. The demographic data of all patients were examined using the Mann–Whitney-U test and t-test. P < 0.05 was considered statistically significant.

**Results**

Sixteen of the patients were female (45.5%) and 20 were male (55.5%). Baseline demographic and clinical characteristics are summarized in Table 1.

<table>
<thead>
<tr>
<th>Patients (n=36)</th>
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<tbody>
<tr>
<td>Median Age (years)</td>
<td>54.0±15.76</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>71.4±8.82</td>
</tr>
<tr>
<td>Gender (M/F)</td>
<td>20/16</td>
</tr>
<tr>
<td>Mean APACHE-II score</td>
<td>22.6±7.38</td>
</tr>
<tr>
<td>PaO2/FiO2 scores on admission</td>
<td>111.9±31.1</td>
</tr>
<tr>
<td>Mortality rate no. of patients (%)</td>
<td>20/36 (55.5)</td>
</tr>
<tr>
<td>no. of patients with ARDS/ALI</td>
<td>22/14</td>
</tr>
<tr>
<td>Microbiology</td>
<td></td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>15</td>
</tr>
<tr>
<td>Klebsiella pneumoniae</td>
<td>10</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>11</td>
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</tbody>
</table>

**Table 1**: Distribution of the 36 patients by demographic characteristics, mean APACHE II and PaO2/FiO2 scores on the day of admission, number of patients with ARDS/ALI and mortality rate.

* M, male; F, female; APACHE, Acute Physiology and Chronic Health Evaluation

**Figure 1**: Plasma PTX-3 comparisons. Comparison of plasma PTX-3 levels on days 1, 4 and 8 in the surviving and nonsurviving patients with ALI/ARDS. * D 1–8 p Value of PTX-3 = 0.001
PTX-3 values of the surviving patients that were elevated at the time of hospitalisation dropped significantly on days 4 and 8 compared with day 1 (p = 0.001) (Figure 1). PTX-3 levels in patients who were clinically improved reached normal levels on day 8 (<2 ng/ml). The values that were high at the time of hospitalisation remained high on day 4 and 8 in the patients who died. (Table 2-3).

In the surviving patients, TNF-α values that were high at the time of admission to the intensive care unit decreased significantly on day 4 compared with day 1 (p = 0.001), and decreased further on day 8 compared with day 1 (p = 0.001) (Figure 2-Table 2). In the patients who died, values remained high or increased slightly but there was no statistical difference (Table 3).

CRP levels dropped significantly in the surviving patients, although they did not reach normal limits until the patients were discharged from the intensive care unit. Day 8 CRP values of surviving patients significantly decreased compared to day 1 (p=0.001). In the patients who died, CRP levels remained elevated or increased and there was statistical difference on days 4 and 8 compared with day 1 (p = 0.036 , p = 0.026 respectively).

However it was observed that IL-1β values of the surviving patients decreased on day 4 and day 8 in comparison to day 1, these decreases were statistically insignificant. In the patients who died, values remained high.

PTX-3, TNF and CRP but not IL-1β were significantly correlated with SOFA scores. Ptx-3 and CRP were related to APACHE scores but not IL and TNF. PTX-3 was significantly related to CRP levels but the other two mediators were not. Ptx-3 and CRP were related to WBC count but IL and TNF were not (Table 4).

![Figure 2](image2.png)

**Figure 2: Plasma TNF-α comparisons.**
Comparison of plasma TNF-α levels on days 1, 4 and 8 between surviving and nonsurviving patients with ALI/ARDS. * D 1-8 p Value of TNF-α = 0,001

![Figure 3](image3.png)

**Figure 3: Pentraxin 3 (PTX3) and Lung injury score (LIS).** PTX3 was higher in severe lung injury (ARDS and ALI) in comparison with mild-to-moderate lung injury, as defined by LIS (LIS 0.1–2.5 and LIS >2.5, respectively). *p < 0.001 in comparison with mild-to-moderate class. The 10th and 90th percentiles are signified by whisker caps.
concluded that acting with neutrophils, CRP has a role in ARDS, and examined CRP levels and its role in ARDS, and change in alveolar macrophage counts. The authors found that the increased plasma CRP value decreased in the clinically improved patients and the increased neutrophil percentage and leukocyte counts also decreased. We concluded that CRP is an inflammatory marker in primary ARDS patients.

Microbiology
Several different microorganisms were found in the bronchoalveolar lavage fluid. Of the patients who were discharged from the intensive care unit (ICU), Pseudomonas aeruginosa was cultured in 3 patients, Klebsiella pneumoniae was cultured in 7 patients, and Staphylococcus aureus was cultured in 9 patients. Of the patients who died, Pseudomonas aeruginosa was cultured in 6 patients, Klebsiella pneumoniae was cultured in 7 patients, and Staphylococcus aureus was cultured in 3 patients and Staphylococcus aureus was cultured in 3 patients (Table 1).

Discussion
In our study, in the surviving patients, following high levels of PTX-3 on the first day, we found that PTX-3 levels dropped in the early periods and values reached normal levels before the patients were discharged from the intensive care unit. In the patients who died, high PTX-3 values remained unchanged. PTX-3 levels were significantly higher on day 4 in the patients who died compared with those who survived. IL-1β and TNF-α values of the surviving patients decreased on day 4 and day 8 in comparison to day 1. PTX-3 levels are associated with parameters that describe both the severity of lung injury. PTX-3 and CRP levels are correlated to APACHE and SOFA scores.

Heuertz et al. (10) examined the effects of CRP in a rabbit model of experimentally-induced alveolitis. When the bronchoalveolar lavage fluid from these rabbits was examined, it was shown that the total leukocyte and neutrophil counts increased with no change in alveolar macrophage counts. The authors examined CRP levels and its role in ARDS, and concluded that acting with neutrophils, CRP has a protective role in lung injury and that increased CRP levels are closely associated with reduced mortality. In our study, we found that the increased plasma CRP value decreased in the clinically improved patients and the increased neutrophil percentage and leukocyte counts also decreased. We concluded that CRP is an inflammatory marker in primary ARDS patients.

Cytokines, which are polypeptides produced and secreted by various cells, regulate the immune system and inflammation, cell growth, healing and the systemic response to injury. The role of inflammatory cytokines has also been widely investigated in the natural history of pulmonary diseases (13). Armstrong et al. (12), in a study conducted in patients diagnosed with ARDS and at risk of ARDS, measured levels of TNF-α and IL-10 in bronchoalveolar lavage fluid, plasma and alveolar macrophage cultures. The authors found that IL-10 concentration in the BAL fluid and plasma of patients diagnosed with ARDS was lower than in those at risk, with no significant difference in the alveolar macrophage cultures in the BAL fluid of the patients diagnosed with ARDS despite a tendency to produce TNF-α in these cells. It is believed that TNF-α and IL-10 secretion may change relative to each other in ARDS and that there is a balance between pro-inflammatory and anti-inflammatory cytokines in the lungs.

Donnelly et al. (13), in a study conducted in early phase ARDS patients, examined the association between pro-inflammatory cytokines (TNF-α, IL-1β, IL-8) and anti-inflammatory cytokines (IL-10 and IL-1 receptor antagonist) and the association between mortality and these values. They found that pro-inflammatory and anti-inflammatory cytokines were higher in patients diagnosed with ARDS compared with the controls. While there was a significant correlation between the mortality rates of the patients and the anti-inflammatory cytokines at low concentration, no significant correlation was found between pro-inflammatory cytokines and mortality.

<table>
<thead>
<tr>
<th>Table 4: Correlation among day 1 pentraxin 3, interleukin-1, tumor necrosis factor-α and C-reactive protein and others.</th>
<th>R and p values were computed and tested according to a simple linear regression model</th>
</tr>
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<tbody>
<tr>
<td>PTX-3</td>
<td>CRP</td>
</tr>
<tr>
<td>R = 0.5649*</td>
<td>R = 0.2629</td>
</tr>
<tr>
<td>CRP</td>
<td>R = 0.1567</td>
</tr>
<tr>
<td>R = 0.3159</td>
<td>R = -0.0294</td>
</tr>
<tr>
<td>TNF</td>
<td>R = -0.0680</td>
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</tbody>
</table>

* Statistically significant p value
rates. Meduri et al.\(^\text{(1)}\) found in a study of 27 patients diagnosed with ARDS that TNF-α, IL-1β, IL-6 and IL-8 plasma values remained elevated longer in the patients who died, and that these values rapidly decreased in the patients who survived. Unlike these studies, which did not differentiate primary and secondary ARDS, we measured the levels of the pro-inflammatory cytokines IL-1β and TNF-α during the mortality and treatment process in patients who were diagnosed with primary ARDS/ALI, a severe inflammatory process. Our patients required mechanical ventilation, and we found that increased TNF-α and IL-1β values decreased significantly and rapidly in the surviving patients whereas these values remained high in the patients who died. We found that decreasing TNF-α, IL-1β levels were associated with clinical improvement. We believe that, based on these findings, pro-inflammatory cytokines are closely associated with mortality.

PTX-3 is an acute phase protein that plays an important role in the body’s natural defence against specific pathogens. It has characteristics similar to those of antibodies including microbial activity, complement activation, and facilitating cellular recognition by phagocytes.

Outer membrane protein A (OmpA), which is the main external membrane protein of the gram-negative enterobacters, is recognised by PTX-3\(^\text{(15)}\). Soares et al.\(^\text{(16)}\) examined the physiological duties of PTX-3 in a pulmonary infection model caused by gram-negative Klebsiella pneumoniae in an experimental study. PTX-3 secretion was identified in the lungs following pulmonary infection that developed at the onset of inoculation with K. pneumoniae. Phagocytosis of the bacteria was shown by increased TNF-α, and high neutrophil spread and migration. In our study, we detected microorganisms that could cause inflammation in BAL fluid obtained during bronchoscopy in primary ARDS patients conducted within 24 hours of admission to the intensive care unit. We cultured both Klebsiella pneumoniae and Pseudomonas aeruginosa in different patients. These findings support the fact that PTX-3 has an affinity for gram-negative bacteria and plays an important role in the development of infection with these pathogens.

Mairuhu et al.\(^\text{(17)}\) found that, in 44 patients, plasma PTX-3 levels were much higher compared with CRP and that PTX-3 levels increased within 7 days of the onset of symptoms and returned to normal values by day 8. In our study, we found that, in primary ALI/ARDS, an inflammatory process, PTX-3 levels reached a maximum on the first day and decreased shortly afterward in response to treatment, reaching normal levels on day 8. However, unlike Mairuhu et al., we found that elevated CRP levels on the first day, similar to PTX-3, decreased with clinical improvement, although they reached normal values later than PTX-3 when patients were discharged from the intensive care unit. Based on these results, we believe that there is a significant correlation between ARDS and PTX-3 levels.

Mauri et al.\(^\text{(18)}\) examined CRP and PTX-3 levels in 21 patients diagnosed with ARDS who were monitored in the intensive care unit. The authors found that PTX-3 levels peaked on the first day and decreased subsequently, remaining low until discharge from the intensive care unit and that CRP levels peaked on day 2 and remained high during the entire ICU treatment period. PTX-3 levels in all of the patients who died during the first days remained high. A weak correlation was found between CRP and PTX-3. The authors concluded that PTX-3 levels may be closely associated with the clinical course and the severity of ALI/ARDS. In contrast, we found that patients with primary ARDS had high CRP and PTX-3 levels when entering the intensive care unit, although PTX-3 reached a normal level earlier than CRP. These findings indicate that PTX-3 is an important marker in the mortality and treatment process in patients with direct lung injury.

Our study has some limitations: first, this study included a small sample size from a single centre; second, BAL was used only for microbial cultures we didn’t measure the PTX-3 levels in BAL.

Conclusions

In our study we demonstrate that patients diagnosed with primary ALI/ARDS, increased PTX-3 levels were closely associated with the clinical course and lung severity. Persisting high levels of PTX-3 is associated with mortality. PTX3 showed a stronger correlation with APACHE, SOFA Scores and others biomarkers analyzed (CRP and WBC count).

References

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