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Pathogenetic and predictive value of biomarkers in patients with ALI and lower severity of illness: results from two clinical trials

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Agrawal A, Zhuo H, Brady S, Levitt J, Steingrub J, Siegel MD, Soto G, Peterson MW, Chesnutt MS, Matthay MA, Liu KD. Pathogenetic and predictive value of biomarkers in patients with ALI and lower severity of illness: results from two clinical trials. Am J Physiol Lung Cell Mol Physiol 303: L634–L639, 2012. First published August 3, 2012; doi:10.1152/ajplung.00195.2012.—Plasma and bronchoalveolar lavage (BAL) biomarkers related to the pathogenesis of acute lung injury (ALI) have previously been associated with poorer clinical outcomes and increased disease severity among patients with ALI. Whether these biomarkers have predictive value in a less severely ill population that excludes septic patients with high APACHE II scores is currently unknown. We tested the association of plasma and BAL biomarkers with physiological markers of ALI severity or clinically relevant outcomes in a secondary analysis of a clinical trial of activated protein C (APC) for the treatment of ALI. Plasma plasminogen activator inhibitor-1 (PAI-1) and mini-BAL protein were both significantly associated with increased oxygenation index (P = 0.02 and 0.01, respectively), whereas there was a trend toward an association between IL-6 and oxygenation index (P = 0.057). High plasma IL-6, thrombomodulin, and mini-BAL protein were all significantly associated with fewer ventilator-free days (VFDs) (P = 0.01, 0.01, and 0.05, respectively); no markers were associated with mortality, but we hypothesized that this was due to the small size of our cohort and the low death rate. To confirm these associations in a larger sample, we identified a restricted cohort of patients from the ARDS Network ALVEOLI study with similar baseline characteristics. We retested the associations of the significant biomarkers with markers of severity and clinical outcomes and studied IL-8 as an additional biomarker given its important predictive value in prior studies. In this restricted cohort, IL-6 was significantly associated with oxygenation index (P = 0.02). Both IL-6 and IL-8 were associated with decreased VFDs and increased 28-day mortality. Future studies should be focused on examining larger numbers of patients with less severe ALI to further test the relative predictive value of plasma and mini-BAL biomarkers for clinically relevant outcomes, including VFDs and mortality, and for their prospective utility in risk stratification for future clinical trials.

IN THE UNITED STATES, acute lung injury (ALI) and the acute respiratory distress syndrome (ARDS) affect ~200,000 patients per year, resulting in a burden of 3.6 million hospital days per year and a mortality rate of 30–40% (20, 26). We recently completed a phase II clinical trial of activated protein C (APC) in nonseptic patients with ALI and patients with sepsis-associated ALI with an APACHE II score of <25 (10). Strikingly, the 28-day mortality rate was only 11% in this study, in contrast to recent clinical trials in the United States, which have reported a mortality rate of 22–30% in the era of low-tidal volume ventilation (12, 18, 19, 28, 29). This highlights the heterogeneity of ALI and suggests that there is a significant population with ALI with relatively low mortality that can be defined based on simple clinical criteria, as in our clinical trial of APC.

Low mortality rates are problematic for clinical trials because sample size accordingly increases; for example, to demonstrate a reduction in mortality from 11 to 9% with 80% power and a two-sided P value of <0.05, a clinical trial would need to enroll more than 3,600 individuals/arm. Therefore, understanding whether or not there is a subset of patients within this less severely ill group who are more likely to have poor outcomes is critical for the design of adequately powered randomized clinical trials.

Furthermore, understanding how the pathogenesis of ALI is similar (and potentially different) in this less severely ill cohort of patients with ALI/ARDS is critical to the development and targeting of novel therapies. It has been shown that ALI pathogenesis involves procoagulant and inflammatory mechanisms as well as damage to the epithelial and endothelial compartments (26). Biomarkers that reflect damage to these cellular compartments, such as markers of inflammation [interleukin-6 (IL-6), IL-8] (13, 15, 25), coagulation [plasminogen activator inhibitor-1 (PAI-1), protein C, thrombomodulin] (13, 27), endothelial cell injury [von Willebrand factor (vWF)]
(22, 23), and epithelial cell injury [surfactant protein D (SP-D) and receptor for advanced glycosylation end products (RAGE)] (4, 6, 8), have all been linked to increased disease severity and poorer clinical outcomes in patients with ALI. Similarly, the concentration of protein in the distal air spaces in patients with ALI obtained through bronchoalveolar lavage fluid (BAL) is an excellent indicator of the degree of combined lung epithelial-endothelial permeability and has also been linked to mortality among patients with ALI (7, 11, 16). However, despite multiple studies showing associations between biomarker levels and outcomes in all patients with ALI, whether these biomarkers continue to have predictive value in less severely ill patients with ALI is unknown.

We used biospecimens from our recently completed clinical trial of APC for ALI to test whether previously validated biomarkers with predictive value in broad populations of ALI patients would still have prognostic value in this ALI population with a low observed mortality rate, possibly providing insight about pathways of injury that might be particularly relevant in this population and furthermore identifying patients who are most likely to benefit from novel therapies. In a companion analysis, we used completed biomarker measurements from the ARDS Network ALVEOLI study to confirm the predictive value of these biomarkers in a similar cohort by restricting our analysis to patients with sepsis or pneumonia and an APACHE II score <25 and patients with other primary causes of ALI.

MATERIALS AND METHODS

Participants. Eligible subjects were critically ill patients at eight university medical centers who met the American/European consensus conference criteria for ALI (2). Major reasons for exclusion included the presence of ALI for more than 72 h; the presence of sepsis with an APACHE II score greater than or equal to 25 (a group in whom APC was approved by the United States Food and Drug Administration at the time of the study); increased risk of bleeding due to trauma, liver dysfunction, or a known coagulation abnormality; inability to obtain consent; and irreversible medical conditions for which the estimated 6-mo mortality exceeded 50% (10). Written informed consent was obtained from the study subject or their surrogate. The institutional review board at each site approved the study, as informed consent was obtained from the subject or their surrogate.

Study design and interventions. Subjects were randomly assigned to receive APC or placebo. Study participants and investigators were blinded throughout the treatment and follow-up period. All subjects were ventilated and weaned from mechanical ventilation using the ARDS Network low-tidal-volume ventilation protocol (1).

Mini-BAL. Mini-BAL was performed at the patient’s bedside in the Intensive Care Unit by trained study personnel at baseline after informed consent was obtained from the subject or their surrogate; informed consent was obtained within 72 h of meeting all three criteria for ALI. A blind Combicath (KOL Bio, Chantilly, VA) catheter was placed into the patient’s endotracheal tube through a standard bronchoscopy adapter, and the inner catheter was advanced into a distal airway. Normal saline (40 ml) was injected and then gently aspirated to retrieve as much of the instilled fluid as possible.

Measured outcomes. The primary outcome of the study was ventilator-free days (VFDs), defined as the number of days to day 28 that the subject achieved unassisted breathing, assuming that a patient survived to day 28 and remained free of assisted breathing. Subjects who did not survive to 28 days were assigned zero VFDs. Secondary outcomes included 28-day mortality, oxygenation index, and PaO2/FIO2 ratio. Oxygenation index was calculated as the product of the mean airway pressure and the fraction of inspired oxygen divided by the partial pressure of arterial oxygen (14). PaO2/FIO2 ratio was calculated by dividing the partial pressure of arterial oxygen with the fraction of inspired oxygen.

Biological sample collection and processing. Blood and mini-BAL specimens were obtained at baseline and on study day 3. In brief, EDTA and citrate anticoagulated plasma as well as mini-BAL lavage specimens were centrifuged immediately after collection at 3,000 rpm for 10 min, divided, and frozen immediately at −80°C. All specimens were transferred to UCSF for long-term storage.

Biomarker measurements. Biomarker measurements were made in duplicate on stored frozen plasma samples from the day of study enrollment and on day 3 of the study. The following plasma biomarkers were measured using commercially available two-antibody sandwich enzyme linked immunosorbent assays (ELISA): IL-6 (R&D Systems, Minneapolis, MN); PAI-1 (American Diagnostica, Stamford, CT) (17); Protein C (Helena Laboratories, Beaumont, TX) (24); SP-D (Yamasa, Japan); vWF (Diagnostica Stago, Parsippany, NJ); RAGE (R&D Systems); Thrombomodulin (American Diagnostica, Stamford, CT). Mini-BAL protein was measured on stored frozen samples from the day of study enrollment and on day 3 of the study using a commercially available protein assay from Bio-Rad (Hercules, CA). Patients with biomarker levels below the limit of detection of the assay were analyzed as if they had a biomarker level at the lower limit of detection.

ARDS Network ALVEOLI study. This is a previously described prospective, randomized, multicenter trial of higher end-expiratory pressure/lower FIO2 vs. lower end-expiratory lung volume/higher FIO2 in ALI/ARDS (3). We selected a subcohort of patients who did not have sepsis or pneumonia or who had sepsis or pneumonia and an APACHE II score <25 (n = 259) for the current analyses. Methods for biomarker measurements have been previously described (3, 25). Patients who had IL-8 levels below the lower limits of detection were assigned a value of 15.6 (the lower limit of detection) to allow for log-transformed analyses of the biomarkers.

Statistical methods. Continuous variables were expressed as means ± SD or median with interquartile range and were compared using Student’s t-test, Wilcoxon rank-sum test, or Spearman’s correlation coefficient, where appropriate. Categorical variables were compared using a Chi-square test or Fisher’s exact test where appropriate. Analysis of covariance was used to analyze the impact of APC treatment on the biomarker levels at day 3, controlling for baseline level as in our previous studies (8, 10). All other biomarker comparisons were performed with only the baseline biomarker data. A two-sided P value <0.05 was considered statistically significant. Statistical analysis was performed with STATA/IC 12 (College Station, TX).

RESULTS

Baseline characteristics. Baseline characteristics for the APC cohort are shown in Table 1. Given the modest size of our phase II clinical trial, we sought to validate our findings in another cohort. For this analysis, we used completed plasma biomarker measurements from the ARDS Network ALVEOLI study (3) although there are no BAL samples available from these subjects. To compare similar populations, we created a restricted ALVEOLI cohort that excluded subjects who had APACHE II scores ≥25 and either sepsis or pneumonia. This restricted cohort had 259 subjects (from the original n = 549) who had a mortality rate of 14%, comparable to the relatively low 11% mortality rate of the ALVEOLI cohort.
observed in our APC trial. Other common measures of severity are also comparable between these two populations (Table 1).

No effect of APC treatment on outcome or biomarker levels.

We previously reported that there was no statistically significant difference in VFDs (the primary outcome variable) or mortality in nonseptic patients with ALI or septic patients with ALI with an APACHE II score 25 treated with APC or placebo (10). We also reported that treatment with APC increased levels of circulating protein C but not levels of PAI-1 or IL-6 (10). Similarly, APC treatment had no effect on the additional pathogenic biomarkers presented here: coagulation markers (thrombomodulin and PAI-1) endothelial injury markers (vWF), epithelial injury markers (RAGE, SP-D), inflammatory markers (IL-6), and total protein levels in BAL fluid obtained through a blind mini-BAL procedure (data not shown). In pilot measurements, 50% of subjects had an IL-8 level below the limit of detection, and, given our small initial sample size, we concluded that any analysis of IL-8 in the cohort would lack power for meaningful analysis. Rather, we focused on measurements of IL-8 in the restricted ALVEOLI cohort; although 56% of subjects similarly had measurements below the lower limit of detection, the larger sample size allowed for analysis of the results. Table 2 presents all biomarker levels, by cohort.

Mini-BAL protein, PAI-1, and IL-6 are associated with oxygenation index. We tested the association of biomarkers with physiological indices of ALI severity and focused our analysis on oxygenation index (Table 3) because it has been shown in studies of patients ventilated with a low-tidal-volume strategy to be predictive of mortality, in contrast to the PaO2/FiO2 ratio (21). Plasma PAI-1 (rho = 0.28, P = 0.02; Fig. 1A) and mini-BAL protein (rho = 0.35, P = 0.009; Fig. 1B) both had a significant positive correlation with oxygenation index. IL-6 showed a trend toward correlation as well (rho = 0.23, P = 0.057; Fig. 1C). None of the other biomarkers showed a significant association with oxygenation index.

Baseline IL-6 is associated with oxygenation index in the restricted ALVEOLI cohort. Of the three markers that showed an association with oxygenation index in our APC study, mini-BAL protein was unavailable in ALVEOLI because mini-BAL samples were not collected. However, plasma IL-6 was significantly associated with oxygenation index (rho = 0.17, P = 0.01). Plasma PAI-1 and IL-8 were not significantly correlated with oxygenation index (rho = 0.11, P = 0.10 and rho = 0.10, P = 0.14, respectively) although there was a trend toward significance. However, because trauma patients can have different biomarker profiles (5, 9), we performed a sensitivity analysis excluding trauma patients; in this analysis, PAI-1 and IL-8 were not significantly correlated with oxygenation index (rho = 0.04, P = 0.63 and rho = 0.07, P = 0.38, respectively), whereas IL-6 retained its association (rho = 0.16, P = 0.03).

Thrombomodulin, IL-6, and mini-BAL protein are associated with decreased VFDs. None of the plasma or mini-BAL biomarkers were significantly associated with 28-day mortality in our patients in the APC clinical trial. However, given the very low mortality rate and small size of our study, we had extremely limited power to detect differences with mortality as an outcome. We therefore tested a priori the association of biomarker levels with VFDs. Increased plasma levels of thrombomodulin (rho = −0.32, P = 0.01, Table 3), IL-6 (rho =...
Baseline IL-6 and IL-8 are associated with decreased VFDs and increased mortality in the ALVEOLI subcohort. Increased IL-6 (P = 0.04) and IL-8 (P < 0.001) were significantly correlated with increased 28-day mortality in this ALVEOLI subset. Patients with detectable IL-8 levels had a mortality rate of 19% compared with 9% among those with undetectable IL-8 (P = 0.02). Furthermore, baseline increased IL-6 (rho = −0.22, P < 0.001) and IL-8 (rho = −0.23, P < 0.001) were both associated with decreased VFDs. Plasma thrombomodulin and mini-BAL protein were not available for analysis. The association between IL-6 or IL-8 and VFDs or mortality was unchanged in the sensitivity analysis where trauma patients were excluded (rho = −0.22, P = 0.001 and P = 0.05 for the association of IL-6 with VFDs and mortality, respectively; rho = −0.24, P = 0.002 and P = 0.001 for the associations of IL-8 with VFDs and mortality).

Fig. 1. Plasma and mini-bronchoalveolar lavage (BAL) biomarkers are associated with severity of acute lung injury (ALI). Baseline plasma plasminogen activator inhibitor-1 (PAI-1) (A, rho = 0.28, P = 0.02), mini-BAL protein (B, rho = 0.35, P = 0.009), and baseline plasma interleukin (IL)-6 (C, rho = 0.23, P = 0.057) are associated with increased oxygenation index. ○, Patients who died by day 28; ●, those who survived to day 28.

DISCUSSION

Although biomarkers that predict disease severity and patient outcomes have been validated in broad populations of patients with ALI, this is one of the first studies to examine the prognostic value in a more homogeneous population that has a low mortality rate, in the range of 11–14%, by excluding patients with sepsis and APACHE II scores ≥25. In this context, we found that plasma PAI-1 and mini-BAL protein were both significantly associated with increased oxygenation index, whereas IL-6 approached significance. In the restricted ALVEOLI cohort, IL-6 was significantly associated with oxygenation index.

We also found that increased baseline plasma IL-6, thrombomodulin, and mini-BAL protein were significantly associated with fewer VFDs and poorer outcomes. The restricted ALVEOLI cohort confirmed the association of increased IL-6 with decreased VFDs and also showed that IL-8 has a similar relationship; thrombomodulin and mini-BAL protein were not measured in this population. In addition, in the larger ALVEOLI analysis, both IL-6 and IL-8 were significantly associated with increased 28-day mortality.

Of note, IL-8, which has been shown to be a strong predictor of mortality in the overall ALI population, was undetectable in a significant fraction of our clinical trial population as well as in the subset of ALVEOLI patients with lower APACHE II scores. Although we could effectively analyze IL-8 in the ALVEOLI subcohort because of the larger sample size, it could not be analyzed in the smaller APC cohort. Notably, patients with detectable IL-8 had significantly higher mortality than those with undetectable IL-8, indicating that presence of a detectable IL-8 level alone may be a simple and effective way to identify higher-risk patients in this generally low-risk population.

Interestingly, biomarkers such as vWF, RAGE, and SP-D, which were associated with both severity and outcome in previously published studies (4, 6, 8, 23), were not associated with either severity or outcome in this novel population. Because patients with ALI and severe systemic disease were excluded from this trial, it is possible that these biomarkers are useful predictors primarily in more severely ill patients. This may be due to either the lack of sensitivity of these markers in less severe illness or differing contributions of various physiological compartments to less severe vs. more severe ALI. Indeed, because inflammatory markers (IL-6 or IL-8) were consistent predictors of oxygenation index, VFDs, and even 28-day mortality in the ALVEOLI restricted cohort,
inflammation may be an important pathophysiological avenue to prioritize in future studies because it is consistently disrupted across the spectrum of ALI severity. Similarly, our results in the APC cohort with thrombomodulin and mini-BAL protein, markers of dysregulated coagulation and epithelial/endothelial permeability, respectively, warrant repetition in larger cohorts of patients if appropriate biospecimens are available. These may represent additional pathophysiological mechanisms that appear to be of greater relevance in this population of less severe ALI; indeed, PAI-1, a second marker of dysregulated coagulation, was associated with oxygenation index, a marker of ALI severity, in our APC cohort. We consistently failed to observe an association of markers of direct endothelial and epithelial injury with either ALI severity or outcomes.

The results presented here are significant to the field given the heterogeneity of etiology, severity, and outcome in ALI. First, a detailed understanding of the pathogenesis of less severe ALI may allow for improved risk stratification of patients. Currently, clinical trials are designed to focus on patients with high mortality to best evaluate the effect of a given novel therapeutic. Using well-tested plasma and BAL biomarkers to identify nonseptic patients with ALI who have high mortality or low VFDs would allow us to enrich clinical trial populations without diluting the effect of a novel therapeutic. Second, study of this patient population may improve the development of new therapeutics. Identifying pathophysiological contributions that are consistent across many subgroups may provide for more promising therapeutic targets than those that are only relevant in specific subgroups, such as the most severely ill patients that are commonly studied.

Overall, our study has several strengths. Our patient population is novel; the strict inclusion criteria for the original clinical trial created a more homogeneous population of patients with ALI, less subject to the variability of severe comorbid conditions, e.g., sepsis. Thus the results may inform our understanding of mechanisms that are directly and consistently disrupted in ALI. Furthermore, the use of the restricted ALVEOLI cohort wherever possible to confirm our findings reduces the chance of type 1 error and allows us to extend our work to IL-8, an additional inflammatory biomarker.

There are also some limitations to this study. First, the study population was selected to exclude the sickest patients, which limits its generalizability. However, there has been concern that some clinical trials in patients with ALI have included too many patients with both mild and moderate lung injury, and our study attempts to address this important question by providing biomarkers that may help with risk stratification in future clinical trials. Second, the number of patients in the APC trial (n = 75) was modest, and the study was not specifically powered to discern true differences in biomarker levels. However, many of the biomarkers that did not show significant association with either severity or outcomes were not close to significance, so additional patients would have been unlikely to have changed the fundamental conclusions. Furthermore, we confirmed our major findings in a larger cohort of patients with the ARDS Network ALVEOLI clinical trial.

In summary, our previously reported trial of APC for ALI allowed us to test well-established biomarkers for their predictive value in a less severely ill population of patients with ALI who did not have sepsis with an APACHE II ≥25. Elevated mini-BAL protein, IL-6, and PAI-1 were associated with the physiological severity of lung injury (as measured by the oxygenation index), and elevated plasma IL-6, thrombomodulin, and mini-BAL protein were associated with a decreased number of VFDs. We subsequently validated the associations of IL-6 with oxygenation index and VFDs in a restricted ALVEOLI cohort; IL-6 was further associated with mortality in this larger cohort. A second marker of inflammation, IL-8, was similarly associated with outcomes in this analysis. The associations of IL-6 and IL-8 with outcomes were robust in a sensitivity analysis where trauma patients were excluded from the ALVEOLI cohort. Future studies should be focused on expanding the number of patients with less severe ALI to further test the relative predictive value of plasma and mini-BAL biomarkers for clinically important outcomes, including VFDs and mortality.

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