PLASMA MATRIX METALLOPROTEINASE-3 IS A MARKER OF ACUTE RESPIRATORY DISTRESS SYNDROME AND MORTALITY

By

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(Under the direction of Somanath P.R Shenoy)

ABSTRACT

Diagnosis and prognosis of acute respiratory distress syndrome (ARDS) depend on clinical criteria that are confounded by ARDS heterogeneity. Clinical diagnostics miss 40% of ARDS diagnoses and only 34% of ARDS are recognized at the time of ARDS diagnostic criteria fulfillment. Biomarker-based approaches may improve diagnostic/prognostic strategies and tailor patient-specific therapies, but no validated biomarkers exist. Matrix metalloproteinase-3 (MMP3) is a proteolytic enzyme involved in the pathophysiology of acute respiratory distress syndrome (ARDS) that may serve as a biomarker in ARDS. This study characterized MMP3 in plasma from patients enrolled in the Albuterol for the Treatment of Acute Lung Injury (ALTA) trial to determine the prognostic value of MMP-3 in ARDS. MMP-3 was measured in plasma samples by enzyme-linked immunosorbent assay. MMP-3 discriminated between healthy controls and ARDS. MMP-3 levels were elevated on day three among non-survivors, and increased levels from enrollment to day three predicted mortality.

INDEX WORDS: Acute respiratory distress syndrome; acute lung injury; biomarker, matrix metalloproteinase-3; mortality; prognosis

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DEDICATION

This thesis is dedicated to my wife, Savannah, and my parents

Their love and selfless support have carried me along in all my endeavors

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CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1. Acute respiratory distress syndrome definitions and overview

Acute respiratory distress syndrome (ARDS) is a deadly yet frequently unrecognized lung disease.[1,2] ARDS describes a heterogeneous acute clinical syndrome of acute hypoxemic respiratory failure from various causes of lung injury that lead to inflammation, vascular permeability, pulmonary edema, and subsequent fibrosis. These pathologies result in decreased effective lung volumes (reduced alveolar aeration).[3] ARDS occurs in 10% of all intensive care unit admissions and occurs in nearly one in every four intubated patients, with a mortality rate averaging 40%.[4] Not surprisingly, the global incidence of ARDS increased with COVID-19, as up to 75% of COVID-19 patients admitted to the ICU develop ARDS.[5]

ARDS has been defined and redefined since the first formal definition in 1988[6], with two societies releasing updated definitions and guidelines in 2023 that illustrate the broad and heterogeneous nature of ARDS. Guidelines have moved the ARDS discourse with advocacy for phenotyping to explain the ARDS heterogeneity.

1. The new Global Definition of ARDS from the American Thoracic Society provides updates and modifications to the previous 2012 Berlin Criteria. The Global definition includes the same four categories as the Berlin Criteria.[3]
    a. **Risk factor and origin of edema**: Caused by acute risk factor for lung injury (can be pulmonary or extrapulmonary injury), and cardiogenic pulmonary edema is not

suspected to be a major contributor to pulmonary edema and atelectasis (collapsing alveoli) is not suspected to be a major contributor to gas exchange dysfunction.

b. **Timing**: Acute onset or worsening within one week of the presumed injury/risk factor

c. **Chest imaging**: Bilateral opacities on chest radiography and computed tomography or bilateral B lines and/or consolidations on ultrasound not explained by effusions, atelectasis, or nodules/masses.

d. **Oxygenation**: Now divided into categories based on the situation

   i. Non-intubated ARDS; PaO2:FiO2 $\leq$ 300 mm Hg or SpO2:FiO2 $\leq$ 315 (if SpO2 $\leq$ 97%) on HFNO with flow of $\geqslant$30 L/min or NIV/CPAP with at least 5 cm H2O end-expiratory pressure

   ii. Intubated ARDS: Mild (PaO2:FiO2 200-300), Moderate (PaO2:FiO2 100-199), Severe (PaO2:FiO2 $\leq$ 100)

   iii. Modified definition of resource-limited settings: SpO2:FiO2 $\leq$ 315 (if SpO2 $\leq$ 97%).

2. The European Society of Intensive Care Medicine (ESCIM) released new ARDS guidelines in 2023 focusing on the heterogeneity of the syndrome and advocating for phenotyping approaches. A disconnect persists between the conceptual model of ARDS, where an injury leads to pulmonary inflammation and the physiological response to the injury propagates more inflammation and any discrete measure of inflammation. Despite the clear linkage of ARDS to pulmonary inflammation, there remains no direct measure of this inflammation that can prognosticate the disease or predict response to treatment. To

address the ambiguity of ARDS disease processes, the ESCIM guidelines describe ARDS phenotypes and other terminology as a method to understand ARDS heterogeneity.

a. Phenotype: a clinically observable trait or set of traits resulting from an interaction of genotype and environment. ARDS is considered a phenotype as its diagnostic criteria establish traits unique to a particular syndrome.
b. Subgroup: a subset of patients within a phenotype and may use any variable as a cut-off, such as severe ARDS patients having a PaO2:FiO2 of 100 or less.
c. Sub-phenotype: a distinct subgroup that can be reliably discriminated against from other subgroups based on a set or pattern of observable or measurable properties. In ARDS two sub-phenotypes have been described, hyperinflammatory and hypoinflammatory.
d. Endotype: a sub-phenotype with a distinct functional or pathobiological mechanism predictive of a response to certain treatments.

These updated guidelines recognize the vast clinical and biological heterogeneity of patients presenting with clinical ARDS and provide a framework for expanding the scope of biologic markers and uncovering treatment-responsive subgroups and subphenotypes. Previously, post-hoc analyses of large, randomized controlled trials have shown that phenotyping approaches using phenotypes described as hyper- and hypo-inflammatory have shown variations in treatment response.[7,8] Recently, latent-class analysis revealed dexamethasone's treatment effect occurs only in hyperinflammatory subtypes of COVID-19 ARDS.[9] Simvastatin was associated with improved survival in the hyperinflammatory phenotype, but not in the hypo-inflammatory phenotype or the combined groups.[10] Famous et al observed a benefit from conservative fluid management in ARDS in the non-inflammatory phenotype while worse outcomes occurred with conservative fluid

management in the hyperinflammatory phenotype.[11] Together, these studies represent a dramatic change to modern critical care literature, whereby researchers can use existing trial data and biobanks to uncover unique biologic profiles linked to clinical traits to measure ARDS heterogeneity and form treatment-responsive subphenotypes. However, the ideal biologic markers in ARDS remain unverified, and discovery is ongoing.

2. Pathophysiology of ARDS

The pathophysiology of ARDS is broadly characterized by the permeability of the alveolar-capillary membrane, resulting in inflammation and edema; (2) non-aeration of lung tissue decreasing lung compliance; and (3) increased venous admixture and dead space, leading to hypoxemia and hypercapnia.[12] ARDS has been broadly categorized into three pathologically distinct phases after acute lung injury (ALI): the exudative phase (up to 7 days), the proliferative phase (days 7-21), and the fibrotic phase (>21 days). [13] These phases lack discrete time courses and elements of their pathologic changes are often coexistent. For example, a cohort of clinical autopsies of ARDS patients demonstrated that even before day seven, roughly 90% of patients exhibit exudative changes, while 50% show proliferative changes and only 4% show fibrotic changes.[14]

The exudative phase represents the initiation and propagation of acute inflammation. The insult causes epithelial cell injury and subsequent recognition of pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharide, or damage-associated molecular patterns (DAMPs) such as mitochondrial DNA by toll-like receptors on type II alveolar cells and lung macrophages. NFkB signaling polarizes macrophages to a hyperinflammatory state leading to proinflammatory cytokines production and attraction of neutrophils into the alveolar space across the now damaged capillary endothelium and alveolar epithelium. Other mediators like Angiopoetin-2 are produced

from activated endothelial cells and destabilize vascular junctions. Fluid translocates from the vasculature into the lung interstitial space and then the alveolar space. Red blood cells also move into the alveolar space and contribute to injury through the release of hemoglobin and subsequent oxidation.[2,15] Diffuse alveolar damage (DAD) occurs during the exudative phase and is the hallmark of ARDS histopathology. DAD's most characteristic feature is the presence of hyaline membranes, eosinophils clumped with cell debris, surfactants, and various proteins.[16]

The subsequent proliferative phase represents and transitionary period usually beginning about seven days after injury. This phase attempts to restore homeostasis to the disrupted barriers. Type II alveolar cells undergo hyperplasia and differentiation into type I alveolar cells to replace lost epithelial cells.[2] Fibroblasts proliferate and produce a collagenous provisional matrix. The tight junctions of epithelial cells are re-established, and alveolar cells reabsorb the excess fluid while macrophages destroy cell debris. Macrophages take on an anti-inflammatory state and aid in the resolution of inflammation. Normally, the proliferative phase acts as way to achieve homeostasis after injury, but the prolongation of the proliferative phase may lead to the fibrotic phase.[15]

The fibrotic phase is poorly understood and does not occur uniformly across ARDS patients but remains a marker of poor outcomes. The extent of fibrosis may be linked to the degree of damage to the alveolar basement membrane. The phase displays rapid expansion of fibroblasts into myofibroblasts due to heavy local concentration of pro-fibrotic signaling such as PDGF, TGF-β, and IGF-1.[2] The extent of persistent alveolar edema may also contribute to pushing the proliferative phase to fibrosis implying that severe ARDS (more pulmonary edema) may predict fibrotic progression.[17] These three phases are largely generalizations of a dynamic process and patients may show signs of all three phases as the disease progresses, but the exudative phase

remains the most important for understanding ARDS diagnosis and management due to it's early occurrence, and early recognition and management is key in a syndrome with high mortality.

3. Diagnostics challenges of ARDS

Despite 10% of all intensive care patients developing ARDS and a mortality rate nearing 40%, approximately 40% of ARDS cases go unrecognized and two-thirds of patients have a delayed diagnosis.[4] The 2012 Berlin Criteria have been cited as lacking diagnostic specificity and sensitivity that fails to capture the dynamic nature of the clinical variables involved in ARDS diagnosis.[18,19] There are routine barriers for each element of the Berlin Criteria that can obscure diagnosis in clinical practice. For example, ARDS timing criteria may be confounded by acute on chronic disease and recent medical history, ventilator criteria lose reliability with multiple modifications to ventilator settings and rapidly fluctuating settings leading to instability of the PaO2:FiO2 ratio during short periods, radiographic interpretation varies widely, and determining non-cardiogenic pulmonary edema is often dubious in the absence of advanced cardiac monitoring and cardiac ultrasound expertise.[20]

The challenges of clinical diagnosis are exacerbated by the absence of any testing linked to the hallmark of ARDS histopathology, DAD. In an autopsy study over 20 years, DAD was present in only 45% of patients diagnosed with ARDS by clinical criteria, with rates increasing across severity (mild - 12%, moderate - 40%, severe – 58%). This lack of concordance between ARDS and DAD is important as ARDS with DAD has higher mortality than ARDS without DAD (odds ratio 1.81, 95% CI: 1.14-2.80) .[21] The clinical criteria most predictive of DAD have been severe hypoxemia and opacities in all four lung quadrants. DAD was found in two-thirds of patients with Berlin Criteria severe ARDS and opacities in four quadrants.[22] Together this data suggest that more severe ARDS is more specific for DAD pathology and all other ARDS patients

are more likely to meet clinical criteria and lack the pathologic changes originally associated with ARDS.

4. The importance of early ARDS recognition

The high mortality of ARDS makes the missed and delayed diagnoses and subsequent omission of survival-improving interventions a crisis within critical care medicine. The LUNG-SAFE study reported high rates of adjunctive treatment omission due to misdiagnosis, with only 19% of moderate and 43% of severe unrecognized ARDS cases receiving adjunctive treatment (e.g., prone positioning, neuromuscular blockade).[4] A single-center observational study found that clinicians identified 47.5% of ARDS cases and more aggressively managed fluids and volume status among those identified.[23]

The strongest argument in favor of more accurate and earlier diagnosis is that ARDS trials reporting positive interventions all required inclusion within 24 to 48 hours of ARDS recognition. This timing establishes interventions' effectiveness exclusively in an early phase of the disease, likely during the exudative phase pathophysiology.[24-28] For example, when patients with persistent ARDS (>7 days from diagnosis) received methylprednisolone, no benefit was observed and mortality increased among those receiving methylprednisolone after 14 days of ARDS.[29] These factors suggest early ARDS interventions have a better chance of positive outcomes. This phenomenon can be attributed to the suspected attenuation of the hyperinflammatory exudative phase by interventions. Blunting the initial inflammatory peak may prevent the propagation of the inflammatory cascade. The lack of an appropriate biomarker presents a substantial roadblock in the diagnosis and prognosis of ARDS in the clinical setting, delaying prompt intervention.[2,18,19] The way forward in ARDS diagnosis and management will come through establishing a biological definition of ARDS to pair with its clinical criteria. This goal can be pursued using biobanks of

plasma and alveolar samples from large ARDS trials. Biologic variables from patient samples can be used for establishing treatment effects in ARDS and aiding in the prognostic enrichment of future trials.[30]

5. Matrix-metalloproteinase-3 in ARDS pathophysiology

Matrix metalloproteinases (MMPs) are extracellular proteinases that degrade the extracellular matrix and modulate the inflammatory response in a wide array of innate immunologic and pathologic processes. Most MMPs have functional overlap as many can degrade all elements of the extracellular matrix. MMPs are classified by substrate specificity with MMP-1, -8, and -13 as collagenases, MMP-2 and -9 as gelatinases, MMP-3 and -10 as stromelysins, MMP-7 and-26 as matrilysins, and MMP- 14, -15, -16, -17, -24 and -25 as membrane-bound.[31,32] MMPs are produced by neutrophils, macrophages, monocytes, fibroblasts, and epithelial cells and augment cytokine release by unbinding membrane-bound cytokines and carry out diverse functions in both the pathogenesis and repair processes of ARDS. Several MMPs are linked most strongly with pathologic function, collagenases, MMP-1 and -8, and gelatinases, MMP-2 and -9, and stromelysin, MMP-3. MMP-3 and MMP-9 are released in response to local proinflammatory stimulation after an injury occurs. [33]

The main effect of MMPs on ARDS pathophysiology is the degradation of alveolar epithelial-endothelial junctional proteins and basement membrane. MMP-3 demonstrates the ability to both degrade the basement membrane extracellular matrix and disrupt tight junctional proteins claudins and occludins and adherens junctional proteins, E-cadherin. Notably, the cleaving of E-cadherin release and cellular invasion promoting fragment.[34] MMP-3 also contributes to phenotypic disruption of epithelial cells through beta-catenin and E-cadherin degradation, leading them to an invasion-promoting phenotype that persists even when MMP-3

expression decreases.[35] These mechanisms have clear pathologic significance in ARDS as a syndrome characterized by endothelial and epithelial barrier breakdown. Moreover, in ALI models, mice deficient in *Mmp-3* have attenuated neutrophil-mediated lung injury, possibly due to a blunting of their invasion.[36,37]

The cellular mechanisms behind ARDS pathologic changes have been partially examined related to MMP-3: threonine kinase Akt1 regulates endothelial barrier protection and loss of the Akt1 gene produces increased vascular permeability.[38-40] The knockdown of Akt or Akt downregulation through LPS-induced ALI increases the expression and activity of MMP-3 mouse lungs. LPS-induced ALI reverses when treated by an MMP-3 inhibitor.[41] Taken together, these data suggest a distinct role of MMP-3 in the early pathologic processes of ARDS and its role as a diagnostic, prognostic marker, or treatable target remains unknown.

6. MMP-3 in ARDS diagnostics and prognostics

Some literature has reported changes in MMP-3 in the context of clinical ARDS and suggests biofluid concentrations of MMP-3 could serve as a biomarker for early ARDS.[36,41-46] In a pilot study, MMP-3 enzymatic activity was increased in ten plasma samples of ARDS compared to healthy controls.[46] In BAL fluid from patients with ARDS MMP-2, -9, and -8 showed no concentration difference between survivors and non-survivors. MMP-3 was undetected from 78% of BAL samples, but when detected, patients showed a 50% increase in mortality (83% vs 32%) and higher acuity of illness.[45] In patients with COVID-19, an early elevation in MMP-3 correlates with the World Health Organization (WHO) severity stage and to a greater extent than MMP-9.[47]

Research design and specific aims

Acute respiratory distress syndrome (ARDS) is a lethal disease that, prior to the COVID-19 era, affected 200,000 Americans annually, and has since increased. No laboratory-guided diagnostic biomarkers or targeted pharmacologic therapies exist in clinical practice. Only supportive care measures (e.g., low tidal volume ventilation, prone positioning, and fluid management) have improved mortality. ARDS's vast clinical heterogeneity contributes to treatment failures, which has prompted the establishment of ARDS phenotypes to identify treatment-responsive phenotypes. Presently, there is a gap in knowledge in defining the optimal clinical and biological markers best suited for phenotyping, and a paramount issue remains the lack of a biomarker related to ARDS pathophysiology and severity of disease.

Our **central hypothesis** is that MMP-3 concentrations increase in ARDS and patients with higher MMP-3 levels experience an elevated rate of mortality. Additionally, the magnitude of difference in MMP-3 levels throughout ARDS progression signals worse patient outcomes.

Specific Aims

Aim 1: Test the hypothesis that elevated plasma MMP3 concentration is associated with higher patient acuity and worse outcomes in early ARDS. Human plasma samples from the Albuterol for the Treatment of Acute Lung Injury (ALTA) trial (Conducted from 2007 to 2008 and published in 2011) supplied by the National Heart, Lung, and Blood Institute's (NHLBI) Biological Specimen and Data Repository Information Coordinating Center (BioLINCC) will have their MMP3 concentration retrospectively measured by ELISA. Samples from individual patients on day 0 and day 3 of trial enrollment will have their MMP3 concentration related to

patient morbidity (i.e., ventilator-free days from day 1 to day 28, and days not spent in the ICU from day 1-28), and mortality at 30, 60 and 90 days.

Aim 2: Test the hypothesis that MMP-3 levels in ARDS patients are elevated compared to healthy controls. ALTA trial samples of ARDS patients and healthy control samples had MMP-3 concentrations measured by ELISA.

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CHAPTER 2

Plasma matrix metalloproteinase-3 predicts mortality in acute respiratory distress syndrome: A biomarker analysis of a randomized controlled trial

Timothy W Jones, Sultan Almuntashiri, Aaron Chase, Abdullah Alhumaid, Payaningal R Somanath, Andrea Sikora, Duo Zhang. *Respiratory Research* 2023;24(1):166; doi:10.1186/s12931-023-02476-5.

Abstract

Background: Matrix metalloproteinase-3 (MMP-3) is a proteolytic enzyme involved in acute respiratory distress syndrome (ARDS) pathophysiology that may serve as a lung-specific biomarker in ARDS.

Methods: This study was a secondary biomarker analysis of a subset of Albuterol for the Treatment of Acute Lung Injury (ALTA) trial patients to determine the prognostic value of MMP-3. Plasma sample MMP-3 was measured by enzyme-linked immunosorbent assay. The primary outcome was the area under the receiver operating characteristic (AUROC) of MMP-3 at day 3 for the prediction of 90-day mortality.

Results: A total of 100 unique patient samples were evaluated and the AUROC analysis of day three MMP-3 showed an AUROC of 0.77 for the prediction of 90-day mortality (95% confidence interval: 0.67-0.87), corresponding to a sensitivity of 92% and specificity of 63% and an optimal cutoff value of 18.4 ng/mL. Patients in the high MMP-3 group (≥ 18.4 ng/mL) showed higher mortality compared to the non-elevated MMP-3 group (< 18.4 ng/mL) (47% vs. 4%, $p<0.001$). A positive difference in day zero and day three MMP-3 concentration was predictive of mortality with an AUROC of 0.74 correlating to 73% sensitivity, 81% specificity, and an optimal cutoff value of +9.5 ng/mL.

Conclusions: Day three MMP-3 concentration and difference in day zero and three MMP-3 concentrations demonstrated acceptable AUROCs for predicting 90-day mortality with a cut-point of 18.4 ng/mL and +9.5 ng/mL, respectively. These results suggest a prognostic role of MMP-3 in ARDS.

Keywords: acute respiratory distress syndrome, acute lung injury, biomarker, matrix metalloproteinase-3, mortality prediction

Introduction

Acute respiratory distress syndrome (ARDS) is a lethal disease without laboratory-guided diagnostic or prognostic biomarkers.[1,2] The LUNG SAFE study determined clinicians failed to recognize ARDS 40% of the time, and only 34% recognized the disease at the first time fulfillment of ARDS diagnostic criteria.[3] This failure to recognize ARDS is problematic because early treatment has been associated with better response to ARDS therapies.[4,5] Significant clinical heterogeneity exists among ARDS presentations, a factor likely contributing to this high rate of underrecognition.[6] Given that delayed diagnosis of ARDS is common and may result in therapy initiation beyond the window for efficacy, rapid, objective tools for identifying the broad range of ARDS presentations are needed. Additionally, beyond diagnosis, failure to appropriately prognosticate the severity of illness may inhibit clinical-decision making regarding the use of invasive therapies most likely to benefit certain phenotypes (e.g., neuromuscular blockade, prone positioning)

Identification of ARDS sub-phenotypes using biomarkers has been proposed, but these efforts have primarily relied on non-specific biomarkers, such as inflammatory cytokines (e.g., IL-6, IL-1B, TNFa), which may represent general critical illness common to ARDS.[7] Recently, a lung-specific biomarker, club cell secretory protein (CC16), demonstrated reasonable AUROC for prediction of ARDS, as well as 60-day mortality in patients, from the FACTT trial.[8] This finding along with corroborating evidence, suggests phenotyping through combining lung-specific biomarkers, non-specific biomarkers, and physiological parameters may contribute

substantially to bedside diagnostic and prognostic tools.[9,10] The recent decades of ARDS research have sought to establish "biologically treatable traits" to simplify selecting patients likely to benefit from therapy, and single biomarkers, if capable of representing a combination of specific physiologic and biologic traits and readily available, will have clinical application.[11]

Matrix metalloproteinases (MMPs) are extracellular proteases capable of degrading every part of the extracellular matrix and the proteins of the alveolar epithelial-endothelial unit under pro-inflammatory conditions, a process central to ARDS pathophysiology.[12,13] Previous studies suggest serum and bronchoalveolar lavage fluid levels of MMP-3 may serve as a biomarker to inform targeted therapies in early ARDS.[14-19] Mice deficient in *Mmp-3* have less severe lung injury in acute lung injury (ALI) models [16,20], and recently, early elevations in MMP-3 have been identified with COVID-19 observing the most prominent MMP-3 elevations in severe disease.[21,22]

Given the evidence supporting MMPs as contributors to ARDS pathophysiology, this study sought to explore the relationship of MMP-3 changes early in ARDS with patient outcomes in the context of a robust randomized controlled trial of ARDS patients, Albuterol to Treat Acute Lung Injury (ALTA). The study hypothesized that elevated MMP-3 from both static and dynamic measures would be associated with increased mortality.

Materials and Methods

This study was a secondary analysis of the multicenter randomized controlled trial, ALTA). ALTA included 282 mechanically ventilated patients and compared the beta-2-agonist albuterol to placebo for the treatment of acute lung injury (ALI)/ARDS.[23] This study was approved by the Augusta University Institutional Review Board (1128838-14).

Plasma MMP-3 concentrations were measured in 100 plasma samples from ALTA and 20 healthy control plasma samples using enzyme-linked immunosorbent assay (ELISA). The primary outcome was the area-under-the-receiver operating characteristic (AUROC) of day 3 MMP-3 concentrations to predict 90-day mortality in patients with ARDS. Day 0 and 3 were chosen because they approximated the baseline expression close to ARDS diagnosis and then reassessed several days into disease progression to allow discrimination between rapidly improving ARDS phenotypes described as rapidly improving by the 24-hour mark.[3,24] Secondary outcomes included the predictive value of the dynamic change (defined as the positive or negative absolute change) between day 0 (MMP-3 concentration at trial enrollment) and day 3 MMP-3 concentrations (MMP-3 concentration on the third day of trial enrollment) for 90-day mortality measured by AUROC and the association of MMP-3 concentration on APACHE III. Both day 3 and dynamic MMP-3 concentrations were evaluated for other patient outcomes, including ventilator-free days (VFDs) and ICU-free days. The diagnostic value of day 0 MMP-3 was also assessed via AUROC analysis using healthy patient control and ALTA ARDS plasma samples.

Plasma samples

Plasma samples and coded data sheets from patients enrolled in ALTA were obtained from the National Heart, Lung, and Blood Institute's (NHLBI) Biological Specimen and Data Repository Information Coordinating Center (BioLINCC). As negative controls, an additional 20 healthy patient plasma samples were obtained from Innovative Research Inc, Novi, MI. Samples were stored at -80 °C. Plasma MMP-3 concentration was assessed in duplicates on days 0 and 3 by ELISA.

Plasma total MMP-3 Protein Measurement using ELISA:

All plasma samples were stored at -80°C until use. Plasma MMP-3 concentrations were measured with Human Total MMP-3 DuoSet ELISA Kit from R&D Systems, Inc, Catalog #: DY513 (Minneapolis, MN). Briefly, 100 µL of the sample (or control standard) and Reagent Diluent were added to each well. The plate was covered with an adhesive strip and incubated for 2 hours at room temperature. Wells were aspirated and washed with Wash Buffer, repeating the wash process two times for a total of three washes. A 100 µL of the detection antibody in reagent diluent was added to each well. The plate was covered with a new adhesive strip and incubated for 2 hours at room temperature. The aspiration and wash process was repeated three times. Then, 100 µL of the working dilution of Streptavidin-HRP was added to each well, and the plate was covered and incubated for 20 minutes at room temperature, followed by repeat aspiration and wash cycles. Following aspiration and wash, a 100 µL of substrate solution was added to each well and incubated for 20 minutes at room temperature. Lastly, add 50 µL of stop-solution (2N sulfuric acid) to each well. Optical density was determined at 450 nanometers. MMP-3 concentration was calculated based on a linear standard curve.

Statistical Analysis

Statistical analyses and figure development were performed with IBM SPSS Statistics Version 28.0. Statistical significance was assessed by a two-sided alpha of 0.05. Continuous variables were analyzed with Student's t-test or Mann-Whitney U Test for parametric and non-parametric data, respectively. Categorical variables were assessed with Fischer's Exact Test. A Shapiro-Wilk Test was performed to assess for normally distributed data with a significance of $p < 0.05$, indicating non-normal distribution. AUROC was calculated on ALTA samples dichotomized by the presence of 90-day mortality to assess the predictive capability of MMP-3 concentration for mortality. The optimal cutoff value for MMP-3

concentration was determined by calculating Youden's index (YI). Logistic regression was performed in a backward stepwise fashion. The following variables were included in the original model: Apache III score, vasopressor use within the 24 hours before randomization, PaO_2/FiO_2 at randomization, sex, body mass index, and day 3 MMP-3. At each step, the variable with the highest p-value was removed until all remaining variables had a p-value of 0.1 or less. Multicollinearity was excluded with variance inflation factors for each variable and goodness-of-fit was assessed with the Hosmer-Lemeshow test. Kaplan-Meier plots were used to estimate the survival rate in each group.

Results

Patient characteristics

The plasma concentration of MMP-3 was determined at day 0 and day 3 in 100 samples from ALTA (50 in the albuterol treatment group and 50 in the placebo group). Baseline characteristics did not differ between albuterol and treatment groups of the ALTA trial **(Table 1)**. Most samples were derived from patients with pneumonia or sepsis as the ARDS etiology. ARDS severity was moderate in each group and comparable between placebo and albuterol groups (PaO_2/FiO_2 140 vs. 144, $p = 0.77$.) The demographics and outcomes data based on the ALTA trial treatment group (albuterol vs. placebo) are included in the electronic supplement (see Table E1).

MMP-3 as a prognostic marker

For the primary outcome, an AUROC curve analysis of day 3 MMP-3 concentration had an AUROC of 0.77 (95% confidence interval (CI): 0.67-0.87) for the prediction of 90-day mortality with an optimal cutoff value of 18.4 ng/mL (YI: 0.58) yielding a sensitivity of 92% and specificity of 63% (**Figure 1**). Day 3 MMP-3 concentrations were significantly elevated in non-survivors at 90 days compared to survivors (26.4 ng/mL vs. 13.4 ng/mL, $p < 0.001$). Patients with

elevated MMP-3 had fewer VFDs (11 days vs. 18 days, p=0.003) and fewer ICU-free days (11.5 vs. 22, p=0.01). **Table 2** summarizes these results.

Among patients with day 3 MMP-3 ≥ 18.4 ng/mL, 48% died at 90 days, while among those with MMP-3 values below 18.4 ng/mL, 4% died at 90 days ($p < 0.001$). The probability of survival at 90 days was 96% vs. 52% ($p<0.001$) for patients with <18.4 ng/mL. vs. ≥ 18.4 ng/mL day 3 MMP-3 concentrations and 90% vs. 42% for a change in MMP-3 from day 0 to 3 < +9.5 ng/mL and ≥ +9.5 ng/mL, respectively. **Figure 2** displays Kaplan-Meier survival plots. In multivariate linear regression controlling for APACHE III score, MMP-3 concentration on day 3 was associated with 90-day mortality (OR: 1.024 [95% CI, 1.004 – 1.045]), indicating each increase in 1 ng/mL predicted a 2.4% mortality increase (**Table 3**).

The change in baseline MMP-3 was also explored as a predictor of mortality. The change from MMP-3 from day 0 to 3 was elevated among those with mortality at 90 days (+14.5 ng/mL vs. +3.7 ng/mL, $p<0.001$). Day 0 to 3 MMP-3 change was predictive of mortality with an AUROC of 0.74 and an optimal cutoff value of +9.5 ng/mL (YI: 0.54), providing 73% sensitivity and 81% specificity (**Figure 1**). Univariate and multivariate regression did not detect a significant association between Day 0 to 3 MMP-3 change and 90-day mortality.

MMP-3 as a marker of ARDS

Additionally, MMP-3 concentrations in 20 healthy control patient samples were analyzed as a negative control. AUROC analysis of healthy controls and ALTA subjects, day 3 MMP-3 showed a high predictive value for ARDS with an AUROC of 0.86 (95% CI, 0.76–0.93) and an optimal cutoff value of 9.9 ng/mL (YI, 0.75) with 80% sensitivity and 95% specificity (Figure E1). The 20 healthy samples showed significantly lower MMP-3 concentration than day 0 MMP-3 (6.5 ng/mL vs. 12.1 ng/mL, $p <0.001$).

Discussion

In this first analysis of the biomarker of MMP-3 from a randomized controlled trial of ARDS, MMP-3 performed well as a prognostic biomarker in ARDS, appropriately classifying patients with a higher risk of mortality and morbidity as measured by AUROC. Plasma MMP-3 levels as both static and dynamic measures showed marked elevations in non-survivors versus survivors, and multivariate regression identified a positive association with MMP-3 day concentrations and 90-day mortality when controlling for severity of illness. Moreover, MMP-3 was elevated in ARDS vs. non-ARDS patients.

The prognostic performance of MMP-3 was similar to a previous latent class analysis (LCA) of two randomized controlled ARDS trials (AUROCs ~0.75).[25] This similar performance of a *single* biomarker is compared to a validated panel of clinical and biomarker variables, which may pose a superior strategy for diagnosing and prognosticating ARDS both as a single variable and an addition to current models.[25] Notably, the complex and heterogenous pathophysiology characterized by numerous acute phase reactants makes identification of a single, highly efficacious marker that is sufficiently powerful (i.e., AUROC > 0.9) for diagnosis and prognosis unlikely.[25,26] However, these results support the hypothesis that lung-specific biomarkers may improve predictive power and/or model parsimony. Indeed, such a lung-specific biomarker may serve as an early (if imperfect) marker for disease that can reduce time to diagnosis (and thus time to intervention, particularly those that show maximal benefit in the early stages of ARDS), especially if used in the context of existing models and phenotyping efforts.

Beyond diagnosis, phenotyping using a biomarker, transcriptomic, and clinical data has shown promise to improve prognostication efforts.[26] Specifically, a dichotomous classification system has emerged with hyperinflammatory and hypoinflammatory phenotypes. The

hyperinflammatory ARDS phenotype is characterized by shock, sepsis, and worse outcomes, while the hypoinflammatory phenotype occurs commonly in trauma-associated ARDS with better outcomes owed to features of rapidly improving ARDS.[27,28] Across five separate phenotyping studies, hyperinflammatory phenotypes were suggested to have a 90-day mortality rate of 38%-51%, while hypoinflammatory phenotypes showed a rate of 17%-23%.[29] Compared to the current study, the mortality rate in the high MMP-3 arms was similar to the hyperinflammatory phenotype, whereas the low MMP-3 arm had only 4% mortality despite comprising 50% of the cohort. Patients with more pronounced changes in MMP-3 from baseline to day 3 also had an increased risk of 90-day mortality, potentially implying a function of the intensity of MMP-3 elevations on disease progression; however, this study is unable to assess if MMP-3 is marker or a mediator for lung damage.

Differences in treatment response based on phenotype may explain the litany of negative results characteristic of ARDS treatment studies. Famous et al. showed the benefit of the fluid restriction intervention in ARDS occurs only in the hyperinflammatory phenotype and potentially worse outcomes in the non-inflammatory phenotype.[30] Using the same two phenotypes, Calfee et al. found simvastatin was associated with improved survival in the hyperinflammatory phenotype.[31] Recently, after the ROSE trial challenged routine use of neuromuscular blockade in ARDS, a reanalysis of the ROSE trial data suggested the inflammatory ARDS phenotype may benefit from neuromuscular blockade.[32] The present study did not aim to evaluate or establish phenotypes and phenotypic responses to treatments as no differences were observed with albuterol treatment in the overall cohort and this study used a small sample size of the larger study. An evaluation of albuterol's effects on MMP-3 was beyond the scope of this investigation. Albuterol has repeatedly shown minimal clinical effects on

mechanically ventilated patients, and thus even with larger sample sizes, no benefit is likely to exist.[23,33,34] However, biomarkers like MMP-3 related to ARDS pathophysiology and disease progression may aid in evaluating responses to treatment and support clinical trial enrichment by identifying patients most likely to benefit from a therapy, especially when combined with additionally clinical variables and biomarkers.[26]

Multiple mechanisms linking MMP-3 to lung injury have been identified. Multiple MMPs contribute to ARDS pathogenesis, and MMP-3 has been shown as the primary driver of inflammatory MMP profiles.[35] MMP-3 is also mechanistically associated with ARDS outcomes as the impetus for MMP-3 production in lung endothelial cells is hyperinflammatory states,[20,36] the phenotype associated with worse outcomes. The mechanisms of MMP-3 mediated injury includes induction of epithelial-mesenchymal transition in lung epithelial cells,[37] TGF-β1 activation,[38] and junctional protein degradation, which are components of ARDS progression.[14] Additionally, MMP-3 has been associated with the progression of COVID-19 severity, and inflammatory cytokines are known to increase dramatically with COVID-19.[21,39]

While many biomarkers have been associated with ARDS, few ARDS biomarkers have been suggested as therapeutic targets, including the receptor for advanced glycation end products (RAGE),[40,41] club cell secretory protein (CC16),[8] and MMP-3.[14,35] Distinct from other ARDS biomarkers, MMP-3 has been linked preliminarily to a mainstay intervention in ARDS as neuromuscular blockade with *cis*atracurium reduced lipopolysaccharide induction of MMP-3 in human endothelial cells.[42] Interestingly, dexamethasone has an inhibitory effect on MMP-3 and other MMP activity.[43-45] Investigations into treatment effects of dexamethasone based on MMP-3 levels are an intriguing avenue for study given dexamethasone's mortality reducing effects in ARDS.[5,46]

Finally, most investigations have evaluated variables at a single time point, assuming that early presentation is a reasonable predictor of overall outcome and treatment response. Yet, critical illness is known to be a highly dynamic state. [47,48] However, Bhavani et al. recently published novel sepsis phenotyping that captured the dynamic nature of critical illness.[49] These models studied changes in vital signs over time (termed group-based trajectory changes) and identified a differential treatment response favoring balanced crystalloids compared to normal saline in one of the four subphenotype groups most characterized by persistent hypotension.[49] In the present study, dynamic assessments also yielded insights, as change over time of MMP-3 may provide an assessment of disease progression as increases in MMP-3 from baseline to day 3 were ubiquitous among non-survivors at 90 days. The period from baseline to day 3 may represent the early exudative phase of ARDS during which diffuse alveolar damage occurs, and MMP-3 pathogenesis is most present,[1,14] and importantly, the time crucial to initiate mortality-reducing ARDS interventions (e.g., lung protective ventilation, corticosteroids).[50,51]

Strengths of this study included the use of clinical ARDS samples from a large randomized controlled trial, the evaluation of MMP-3 at multiple time points, and the novelty of using MMP-3 to predict ARDS mortality. The utility of MMP-3, particularly in a biomarker panel, may best be seen in its ability to guide clinically complex decisions: e.g., if patients with high MMP-3 who were treated with *cis*atracurium and/or dexamethasone had better outcomes than similar patients with MMP-3 without *cis*atracurium and/or dexamethasone. This scenario is hypothetical at present but shows the potential of such a biomarker to inform therapy. Despite these strengths, several limitations warrant discussion. First, the sample size and timing of collection may have limited the power to detect a more robust AUROC, especially for dynamic variables. No samples were available from the biorepository on days 1 and 2 (this study used

only day 0 and 3); therefore, change in MMP-3 in the acute exudative phase of ARDS on days 1 through 2 were not captured. Second, the population had a small portion of trauma patients, with most patients having ARDS from infectious causes, which may bias the study towards the hyperinflammatory phenotype and prevent assessment of how MMP-3 responds in non-infectious ARDS (or the hypoinflammatory phenotype). Although MMP-3 showed strong differentiation capacity between ARDS and non-ARDS, the non-ARDS samples came from healthy patient samples, limiting the specificity for ARDS. Future diagnostic studies would be strengthened by evaluating critically ill patients with non-ARDS diagnoses. Finally, the ALTA trial was conducted from 2007 to 2008, and patient samples were frozen for approximately 15 years. Storage time is known to influence protein quality and yield, but the extent is not well described; however, plasma samples stored for 30 years can have ~35% of their protein concentration variation accounted for by storage time.[52] These samples likely have undergone some protein degradation, and concentrations would be expected to be higher than observed in this study.

Conclusion

In conclusion, plasma MMP-3 levels demonstrated a prognostic relationship to ARDS mortality. Additionally, MMP-3 elevations from baseline may represent a phenotype of patients with elevated mortality risk. MMP-3 warrants further evaluation as a lung-specific biomarker for predicting treatment benefits among interventions known to improve mortality in ARDS. Future studies should include MMP-3 as a component in phenotyping and predictive methods.

Table 1. Demographics by MMP-3 level and change in MMP-3 from day 0 to 3

	Day 3 MMP-3 concentration			Day 0 to 3 MMP-3 difference		
	High (≥18.4 ng/mL) (n=50)	Low (<18.4 ng/mL) (n=50)	P-value	High (≥9.4 ng/mL) (n=33)	Low (<9.4 ng/mL) (n=67)	P-value
Characteristic						
Age (years)	55±15	46±15	0.003	57±14	47±15	0.001
Male	32 (64)	22 (44)	0.07	19 (58)	35 (52)	0.67
Body mass index	28 ± 6	28 ± 7	0.73	28±6	28 ±7	0.66
APACHE III, mean (SD)	106 ± 28	79 ± 23	0.001	107±29	85±26	<0.001
Vasoactive use within 24 hours before randomization	29 (58)	23 (46)	0.32	20 (61)	32 (47)	0.29
Time from ALI to randomization (hours), median	26 (13-37)	15 (10-28)	0.025	26.5 (13.2-38.4)	18.4 (10.2-28.7)	0.14
PaO_2/FiO_2	140 ± 63	144 ± 57	0.73	131±60	148±59	0.21
ARDS causes, n (%)						
Pneumonia	18 (36)	20 (40)	0.68	22 (44)	16 (32)	0.22
Sepsis	18 (36)	10 (20)	0.075	12 (24)	16 (32)	0.37
Aspiration	9 (18)	7 (14)	0.59	4 (8)	12 (24)	0.03
Trauma	4 (8)	6 (12)	0.5	8 (16)	2 (4)	0.046
Multiple transfusions	1 (2)	1 (2)	1.0	2 (4)	0	0.56
Other	0	6 (12)	0.047	2 (4)	4 (8)	0.4

All data are presented as n (%), mean ± SD, and median (interquartile range) unless otherwise noted.

ALI = acute lung injury, ARDS = acute respiratory distress syndrome, APACHE III = Acute Physiology and Chronic Health Evaluation III, ICU = intensive care unit, MMP-3 = matrix metalloproteinase-3, Vfd = ventilator free days

Table 2. Outcomes by MMP-3 concentration and change in MMP-3 from day 0 to 3

	Day 3 MMP-3 concentration			Day 0 to 3 MMP-3 difference		
	High (≥18.4 ng/mL) (n=50)	Low (<18.4 ng/mL) (n=50)	P-value	High (≥9.4 ng/mL) (n=33)	Low (<9.4 ng/mL) (n=67)	P-value
Outcome						
Mortality at 30 days	16 (32)	2 (4)	<0.001	12 (36)	6 (9)	0.02
Mortality at 60 days	21 (42)	2 (4)	<0.001	16 (48)	7 (10)	0.001
Mortality at 90 days	24 (48)	2 (4)	<0.001	19 (58)	7 (10)	0.001
ICU free days	11 (0-21)	18 (11-23)	0.003	8 (0-17)	18 (10.5-22)	0.001
VFD	11.5 (0-22)	22 (14-24)	0.01	20 (10-23)	18.5 (0-22)	0.001
MMP-3 concentration						
Day 0	17.2 (11.7–24.3)	8.5 (4.6– 12.1)	0.001	13.6 (9.3–21)	11.3 (5.5– 17)	0.04
Day 3	27.9 (23.4–44.6)	11 (6.4 – 13.4)	0.001	34.4 (24.7–51.7)	12.9 (8.2–21.3)	0.001
Change day 0 to 3	+13.5 (+7.9-+23.3)	+0.7 (-1.6 – +4.2)	0.001	+17.5 (+13.5 – +28)	+2.1 (-1.5 – +6.1)	0.001

All data are presented as n (%), mean ± SD, and median (interquartile range) unless otherwise noted.

ALI = acute lung injury, ARDS = acute respiratory distress syndrome, APACHE III = Acute Physiology and Chronic Health Evaluation III, ICU = intensive care unit, MMP-3 = matrix metalloproteinase-3, Vfd = ventilator free days

Table 3. Association of MMP-3 and APACHE III with mortality by multivariate regression

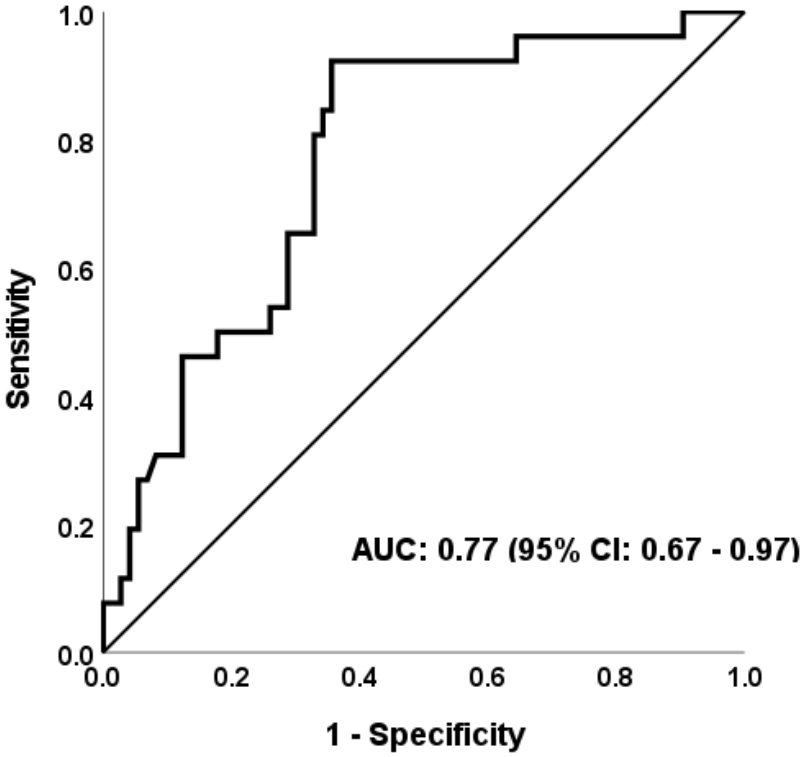
	Univariate		Multivariate	
	OR (95% CI)	p-value	OR (95% CI)	P-value
Day 0 MMP-3 concentration	1.022 (0.983 – 1.052)	0.28	1.001 (0.98 – 1.02)	0.89
Day 3 MMP-3 concentration	1.030 (1.008 – 1.052)	0.007	1.024 (1.004 – 1.045)	0.026
Day 0 to 3 MMP-3 change	1.005 (0.991 – 1.019)	0.46	0.999 (0.981 – 1.017)	0.89
APACHE III	1.032 (1.013 – 1.051)	<0.001	1.028 (1.008 – 1.048)	0.005

MMP-3 variables were all individually tested in logistic regression with APACHE III score as covariates. Model variables that were iteratively removed stepwise included vasopressor use within the 24 hours before randomization, PaO_2/FiO_2 at randomization, sex, and body mass index,

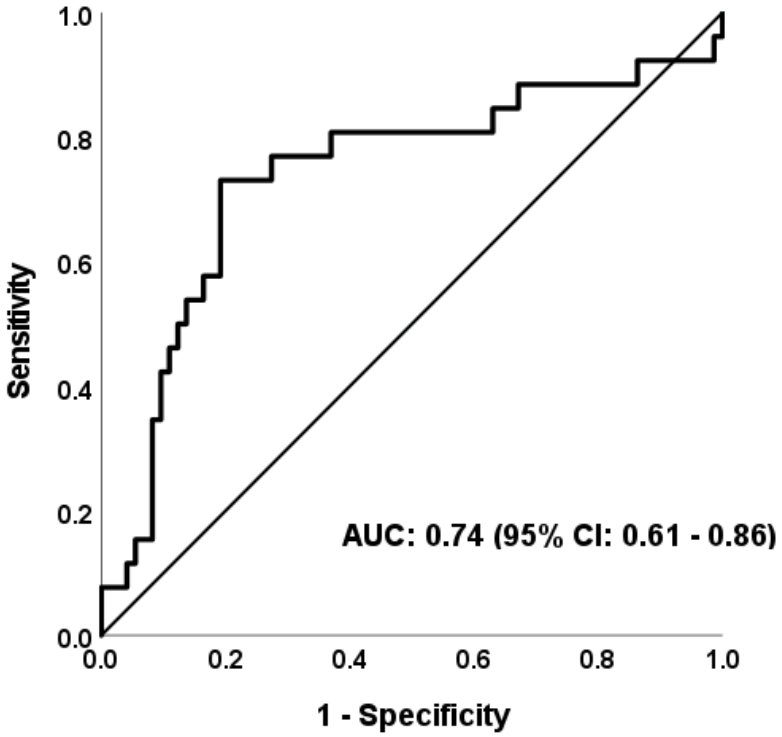
APACHE III = Acute Physiology and Chronic Health Evaluation III, MMP-3 = matrix metalloproteinase-3

Figure 1. Receiver operating characteristic curves for MMP-3 prediction of 90-day mortality in ARDS. Receiver operating characteristics of **A)** MMP-3 concentration on day 3 and **B) c**hange in MMP-3 concentration from baseline to day 3

A.


1.0
0.8
0.6
0.4
0.2
0.0
Sensitivity
AUC: 0.77 (95% CI: 0.67 - 0.97)
0.0
0.2
0.4
0.6
0.8
1.0
1 - Specificity

B.


1.0
0.8
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0.2
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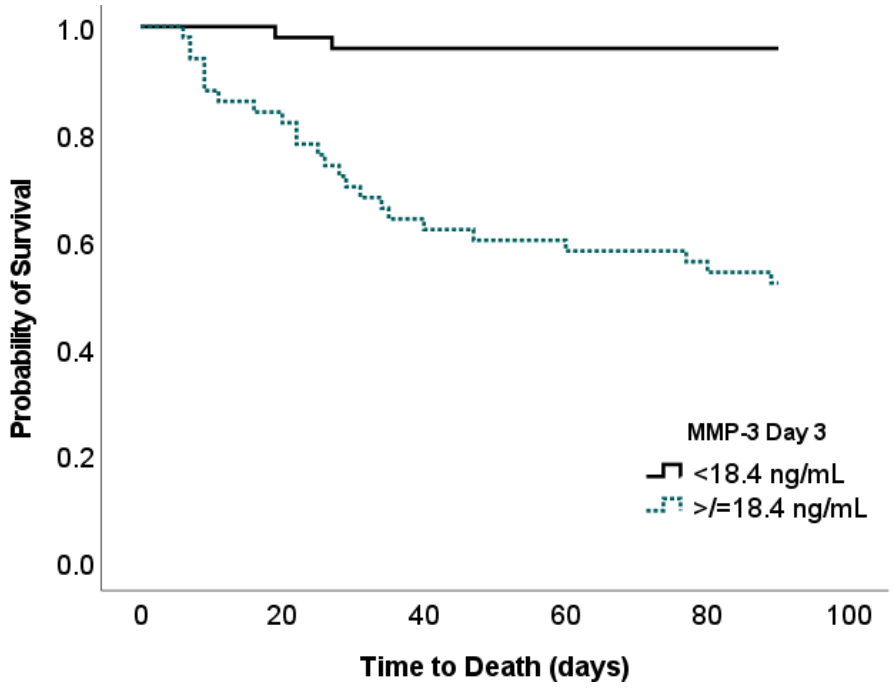
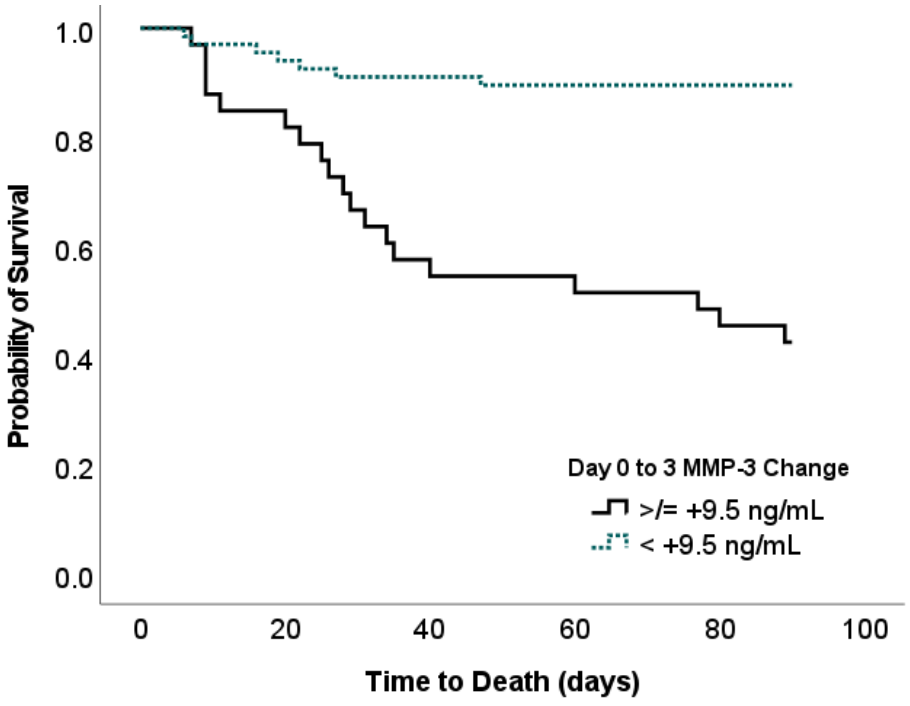
Sensitivity
AUC: 0.74 (95% CI: 0.61 - 0.86)
0.0
0.2
0.4
0.6
0.8
1.0
1 - Specificity

Figure 2. Kaplan-Meier survival curves stratified by MMP-3 concentration and change in MMP-3. **A** Day 3 MMP-3 concentration plotted as a survival curve separated into two groups by using the 18.4 ng/mL cutoff for day 3 MMP-3. **B** Day 0 to 3 MMP-3 concentration change plotted as a survival curve separated into two groups by using the 9.5 ng/mL cutoff for day 0 to 3 MMP-3 change. The probability of survival at 90 days was 95.9% vs 52% ($P<0.001$) for low. vs. high MMP-3 concentration and 90% vs 42% ($P<0.001$) for a change in MMP-3 from day 0 to 3 < +9.5 ng/mL and ≥ +9.5 ng/mL, respectively.

A


1.0
0.8
0.6
0.4
0.2
0.0
Probability of Survival
0
20
40
60
80
100
Time to Death (days)
MMP-3 Day 3
<18.4 ng/mL
>/=18.4 ng/mL

B


1.0
0.8
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0.2
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Probability of Survival
0
20
40
60
80
100
Time to Death (days)
Day 0 to 3 MMP-3 Change
>/= +9.5 ng/mL
< +9.5 ng/mL

Figure S1. Receiver operating characteristic curve for Day 3 MMP-3 prediction of ARDS. Data utilized to construct the curve were from 20 healthy non-diseased plasma samples and 100 ARDS samples from the ALTA trial on day 3 of enrollment.

Figure S1.

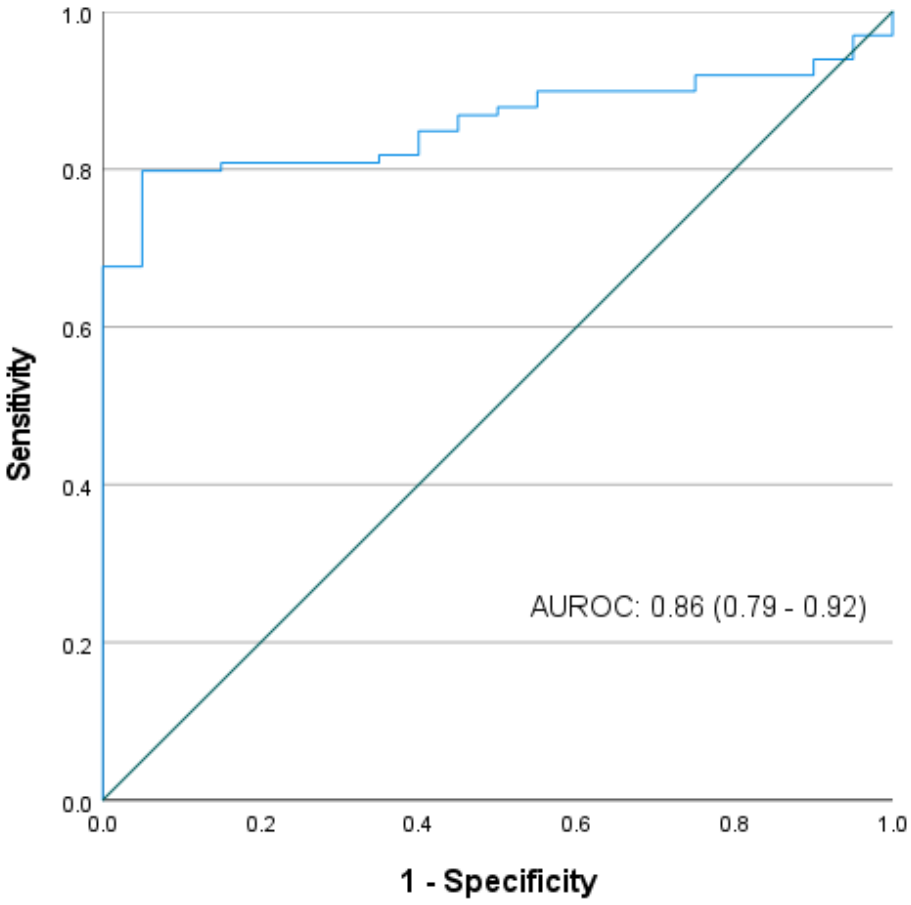
1.0
0.8
0.6
0.4
0.2
0.0
Sensitivity
AUROC: 0.86 (0.79 - 0.92)
0.0
0.2
0.4
0.6
0.8
1.0
1 - Specificity

Table S1. Demographics and outcomes among ALTA trial treatment groups

	Trial treatment group		
	Placebo (n=50)	Albuterol (n=50)	P-value
Characteristic			
Age (years)	52 ± 15	49 ± 16	0.33
Male	26 (52)	28 (56)	0.84
Body mass index	28±6	28 ±7	0.69
APACHE, mean (SD)	90±30	94±27	0.50
Vasoactive use within 24 hours before randomization	24 (48)	28 (56)	0.55
Time from ALI to randomization (hours), median	15 (10-31)	23 (12-35)	0.39
PaO_2/FiO_2	140±60	144±60	0.77
ARDS causes, n (%)			
Pneumonia	22 (44)	16 (32)	0.16
Sepsis	12 (24)	16 (32)	0.37
Aspiration	4 (8)	12 (24)	0.03
Trauma	8 (16)	2 (4)	0.047
Multiple transfusions	2 (4)	0	0.55
Other	2 (4)	4 (8)	0.4
Outcome			
Mortality at 30 days	7 (14)	11 (22)	0.44
Mortality at 60 days	10 (20)	13 (26)	0.64
Mortality at 90 days	11 (22)	15 (30)	0.50
ICU free days	17 (10-22)	15 (0-21)	0.07
VFD	20 (10-23)	18.5 (0-22)	0.07
MMP-3 concentration			
Day 0	11.9 (8.1-18.5)	12.5 (6.4-17.9)	0.93
Day 3	17.2 (11-26.3)	21.2 (10.4-28.8)	0.89
Change day 0 to 3	+4.2 (+0.66 - +14.5)	+6.3 (-0.9 - +13.5)	0.79

All data are presented as n (%), mean ± SD, and median (interquartile range) unless otherwise noted.

ALI = acute lung injury, ARDS = acute respiratory distress syndrome, APACHE III = Acute Physiology and Chronic Health Evaluation III, ICU = intensive care unit, MMP-3 = matrix metalloproteinase-3, Vfd = ventilator free days

Table S2 MMP-3 Concentrations Medians and Means

MMP-3 Concentrations Medians and Means				
MMP-3 concentration	Total MMP-3 ng/mL (n=100) (n=20) healthy	High Day 3 MMP3 (≥18.4 ng/mL) (n=50)	Low Day 3 MMP-3 (<18.4 ng/mL) (n=50)	P-value*
Day 0 ALTA samples	12.08 (7.33 – 18.49) 17.79 ± 36.05	17.2 (11.7– 24.3) 26.2 ± 49.4	8.5 (4.6– 12.1) 8.8 ± 4.7	0.001
Day 3 ALTA samples	19.20 (10.98 – 28.15) 26.10 ± 36.05	27.9 (23.4– 44.6) 41.7 ± 34.6	11 (6.4 – 13.4) 10.2 ± 4.5	0.001
Change day 0 to 3 ALTA samples	6.07 (0.074 – 13.55) 8.52 ± 38.19	+13.5 (+7.9- +23.3) +15.53 ± 52.92	+0.7 (-1.6 – +4.2) 1.37 ± 4.08	0.001
Healthy controls	6.53 (4.93 – 9.50) 7.12 ± 2.46			

*Comparisons are made between high and low MMP-3 day 3 groups
Data represented as medians (interquartile range) and mean ± standard deviation

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