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Proteomic study of acute respiratory distress syndrome: current knowledge and implications for drug development

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Abstract

The acute respiratory distress syndrome (ARDS) is a common cause of acute respiratory failure, and is associated with substantial mortality and morbidity. Dozens of clinical trials targeting ARDS have failed, with no drug specifically targeting lung injury in widespread clinical use. Thus, the need for drug development in ARDS is great. Targeted proteomic studies in ARDS have identified many key pathways in the disease, including inflammation, epithelial injury, endothelial injury or activation, and disordered coagulation and repair. Recent studies reveal the potential for proteomic changes to identify novel subphenotypes of ARDS patients who may be most likely to respond to therapy and could thus be targeted for enrollment in clinical trials. Nontargeted studies of proteomics in ARDS are just beginning and have the potential to identify novel drug targets and key pathways in the disease. Proteomics will play an important role in phenotyping of patients and developing novel therapies for ARDS in the future.

Keywords

ARDS; therapy; phenotype; proteomics; inflammatory

Introduction

The acute respiratory distress syndrome (ARDS) is a common cause of respiratory failure in adults and is defined by the acute onset of bilateral lung infiltrates and a low arterial to fraction of inspired oxygen ratio ($\text{PaO}_2/\text{FIO}_2 < 300$) absent isolated hydrostatic edema typical of congestive heart failure [1,2]. While the current definition is pragmatic, it continues to rely on nonspecific clinical criteria to group patients with a diverse array of pathophysiologies and very different prognoses.

ARDS occurs in the setting of a variety of risk factors, which are commonly characterized as ‘direct’ lung injury (e.g. pneumonia, aspiration, or chest trauma) or ‘indirect’ lung injury

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(e.g. sepsis, pancreatitis, or burns) [3]. The estimated incidence of ARDS is as high as 190,600 cases in the US per year, with >70,000 associated deaths [4].

Despite therapeutic advances in ARDS management, including widespread adaptation of low-tidal volume ventilation and restrictive fluid strategies, mortality from ARDS remains high, with estimates ranging from 20% to 40% [5,6]. Dozens of randomized clinical trials of drugs directly targeting ARDS have failed. The only drug therapy in widespread use for ARDS is neuromuscular blockade, which was shown to improve mortality in patients with moderate-to-severe ARDS in a multicenter RCT, but likely functions more as an adjuvant to lung protective ventilation than as a pharmacologic treatment of lung injury [7].

The reasons for failure in drug development in ARDS are likely multifactorial. As mentioned above, ARDS is a syndrome that is a final common pathway for various lung injury mechanisms (damage to lung endothelium, epithelium, cytokine injury, mechanical stretch by injurious ventilator settings, etc.) [5]. Most large-scale drug trials in ARDS enroll patients with many etiologies; if disease mechanisms vary with subclass, trials in all comers will be substantially underpowered. Patients with ARDS are by definition critically ill, and the primary cause of death in ARDS, especially in an era of lung protective ventilation, is often not respiratory failure, but multiorgan dysfunction [8]. Thus, mortality is often driven not by lung disease but other factors (family decisions to limit care, terminal illness prior to development of ARDS, etc.), which also leads to underpowered studies. However, established surrogate endpoints of ARDS severity are lacking. The empirically derived Berlin Definition found that measures of radiographic severity, respiratory system compliance, dead space, and level of positive end-expiratory pressure (PEEP) did not improve mortality prediction beyond classification by the $\text{PaO}_2/\text{FIO}_2$ ratio [2]. However, baseline $\text{PaO}_2/\text{FiO}_2$ ratio may not correlate with the degree of lung injury and substantial heterogeneity can occur in serial measurements of $\text{PaO}_2:\text{FIO}_2$ ratio depending on ventilator settings and degree of lung recruitability [9].

Timing of enrollment is also important in ARDS. ARDS is recognized to occur in several phases, including an early exudative/inflammatory phase (typically defined in the first 7 days) which may resolve, but in a subset of patients evolves into a later fibroproliferative phase [3]. Given profound changes in biomarker profiles even within the first few days of disease, more recent trials target ever earlier enrollments to limit this heterogeneity [7,10].

Given the focus of this review is on proteomics, we acknowledge that we cannot cover advances in genetic risk factors for ARDS nor focus on other genomic biomarkers, including gene expression and metabolomics. Instead, this review will discuss the role of proteomics in identifying biologic pathways important in the pathophysiology of ARDS. Targeted proteomics has already identified multiple potential ARDS biomarkers. We then review recent work incorporating multiple biomarkers into multivariable models that both improve ARDS prognostication and suggest the presence of novel ARDS subphenotypes, not always identifiable by clinical phenotyping. We emphasize the challenges inherent in differentiating protein signals of lung injury from underlying predisposing conditions of sepsis and trauma. Finally, we postulate a critical role for proteomics in informing drug development by

enhancing phenotyping of clinical subgroups of ARDS subjects with the greatest potential to benefit from targeted pharmacologic therapy.

Individual proteomic derangements identify relevant biologic pathways in ARDS

Limitations of single-marker proteomic studies in ARDS to date

Several inherent challenges have limited the utility of protein biomarkers in ARDS relative to other diseases, and currently available biomarkers continue to lack sufficient validity to be incorporated into clinical practice for either the diagnosis or prognosis of ARDS. First, as noted above, ARDS is a syndrome that occurs in response to disparate pathophysiologic disturbances, limiting the value of individual biomarkers specific to one biologic pathway. Models combining multiple biomarkers from different biologic pathways may be needed to establish reliable biologic criteria for ARDS.

In addition to heterogeneity of underlying pathophysiologies, studies attempting to validate protein biomarkers have suffered from heterogeneity in the timing of sample collection relative to time of onset of ARDS. The underlying pathologies contributing to the development of ARDS are dynamic processes with rapidly changing protein signals between early versus late sepsis or early lung injury versus repair adding significant noise obscuring background signals of ARDS. Biobanks of reliably timed samples collected earlier in the development of ARDS may enhance efforts to derive a biologic signal of ARDS.

ARDS proteomics studies to date have overwhelmingly focused on plasma, which is relatively easily obtained and readily available in many large cohorts, but may poorly reflect conditions in the lung. Conversely, several nontargeted proteomic analyses have assessed bronchoalveolar lavage fluid (BAL) fluid which may be more relevant to lung pathology, but are relatively harder to obtain, and hard to obtain sequentially. We note that direct sampling of edema fluid is possible early in the course of ARDS [11]. Alternatively, as proteomic evaluations of exhaled breath improve [12], this may be an appealing option to enhance timing/longitudinal assessment for future studies.

Studies of biomarkers from unselected populations of ARDS suffer from poor specificity of markers of ARDS relative to underlying risk factors – notably sepsis and trauma – for ARDS. Future efforts will likely need to study more homogeneous subgroups of ARDS to identify biomarkers or therapeutic targets that may not be applicable to all patients with ARDS.

Finally, identifying proteomic changes specific to lung injury is limited by lack of specific clinical criteria for ARDS and also confounding of major biologic disturbances inherent to critical illness. Validity of biomarkers for the diagnosis of ARDS in current large multicenter populations is likely limited by misclassification of cases primarily with severe sepsis or trauma with relative minor or no diffuse lung injury. In the era of lung protective ventilation, risk for death in ARDS patients only partly relates their degree of ARDS severity. Therefore, in regard to prognostication, biomarkers that correctly identify more severe cases of ARDS

may misclassify patients who lack other comorbidities and recover from their ARDS relative to patients with relatively minor ARDS who succumb from other significant comorbidities.

Individual proteomic changes in ARDS identify relevant pathways

Despite the difficulties in identifying robust biomarkers in ARDS, targeted proteomics studies have identified important pathways in the disease. The protein biomarkers highlighted below are associated with either development of ARDS or mortality in patients with ARDS. Figure 1 summarizes the current knowledge of key biologic pathways and their associated proteomic biomarkers in the pathobiology of ARDS.

A summary of key biomarkers and their proposed biologic pathways is summarized in Table 1. Other reviews have provided a more comprehensive summary of the current literature [13–16]. Instead of recreating these efforts, we will highlight well-validated protein biomarkers representing critical pathways in the pathophysiology of ARDS. In particular, we will discuss markers of inflammation, epithelial injury, endothelial injury or activation, and disordered coagulation and repair with specific emphasis on their differential performance in subtypes of ARDS (e.g. with sepsis or trauma as their ARDS risk factor).

Endothelial injury and activation

Loss of integrity of the endothelium with efflux of protein rich edema fluid is an important step in the development of ARDS. Endothelial injury in response to inflammatory cytokines leads to increased capillary permeability while activation of endothelial binding proteins induces transmigration of inflammatory cells into alveoli controlling local infection but also propagating ongoing inflammation and alveolar epithelial injury [3].

von Willebrand factor antigen (VWf-Ag)

VWf is a large multimeric glycoprotein synthesized and stored in Weibel–Palade bodies in endothelial cells and megakaryocytes. VWf plays an important role in hemostasis by bridging platelet binding to vascular endothelium and serving as a carrier protein for factor VIII [77]. VWf is secreted by endothelial cells throughout the body in response to mediators of inflammation and clotting cascade including fibrin, histamine, thrombin, and trypsin [32,78,79]. However, this response is not specific to lung endothelium potentially limiting the role of VWf for identifying lung specific injury.

Studies of circulating levels of plasma VWf-Ag have produced mixed results for predicting both development of ARDS in at-risk patients and mortality in patients with ARDS [17–19]. Moss et al., studying both septic (pulmonary and nonpulmonary) and nonseptic (trauma, aspiration, massive transfusion, and pancreatitis) patients, found plasma VWf-Ag levels were elevated in both groups relative to normal controls but did not distinguish between patients who did and did not develop ARDS. Importantly, while the study separately evaluated both septic and nonseptic patients, both groups contained a mix of patients with potential for systemic endothelial activation (nonpulmonary sepsis in the septic group and trauma, pancreatitis, and massive transfusion in the nonseptic patients) versus lung-specific endothelial activation (pneumonia in the septic patients and aspiration in the nonseptic

patients) which may have contributed to the lack of specificity of Vwf-Ag for identifying patients who progressed to ARDS.

In a large multicenter analysis of 559 patients enrolled in the NHLBI ARDS Network multicenter trial of lower tidal volumes (ARMA) by Ware et al., higher baseline plasma VWF-Ag levels were associated with increased mortality despite controlling for severity of illness, sepsis, and ventilator strategy [20]. VWF-Ag levels were similar in septic and nonseptic patients but were lower in patients with trauma as a primary risk factor. Day 3 levels of VWF-Ag were also lower in survivors but were not different between the high and low tidal volume groups, suggesting that the beneficial effects of lower tidal volume ventilation may not be mediated primarily through effects on the lung endothelium. In analysis of 931 patients in the ARDS Network Fluid and Catheter Treatment Trial, Calfee et al. found a similar association of higher baseline VWF-Ag levels and mortality in both septic and nonseptic patients independent of severity of illness and fluid management strategy [21].

Angiopoietin-2 (Ang-2)

Ang-2 is a ligand for the Tie2 receptor, which blocks phosphorylation and promotes vessel destabilization [23]. In experimental models of ARDS, Ang-2 promotes alveolar epithelial cell death and extracellular gap formation in endothelial cells [23,24]. In humans, Ang-2 is elevated in sepsis and higher Ang-2 levels are associated with the development of ARDS in both septic and nonseptic patients, and with increased mortality in surgical patients with ARDS [23,25,26].

The complicated interaction of sepsis with Ang-2 levels in ARDS is highlighted by Calfee et al.'s analysis of the ARDS Network Fluid and Catheter Treatment Trial [21]. Higher levels of Ang-2 at baseline were associated with mortality only in nonseptic patients, suggesting a blunting of the signal for lung-specific endothelial injury with systemic increases of Ang-2 in sepsis. However, higher Ang-2 levels at day 3 were associated with mortality in both septic and nonseptic patients and rising Ang-2 levels in septic patients identified a particularly poor prognosis. In addition, a conservative fluid strategy was associated with lower Ang-2 levels in septic (particularly those not in shock and thus managed per protocol) but not nonseptic patients. In contrast, baseline Vwf-Ag levels were lower in survivors independent of sepsis but change in Vwf-Ag to day 3 was not associated with mortality, and Vwf-Ag levels were not affected by fluid management strategy.

The response of Ang-2 levels to conservative fluid management in septic patients – which may simply reflect larger fluid balance shifts in patients who initially received volume loading during early goal-directed therapy – suggests that, in contrast to low tidal volume ventilation, the benefit of conservative fluid management may in part be mediated by decreased endothelial injury. Targeting patients with higher levels of Ang-2 could allow selection of patients with the most potential to benefit from future trials of fluid management in patients with or at-risk for ARDS. Interestingly, as with lower tidal volumes, fluid management did not impact Vwf-Ag levels, and an explanation of this discordant effect on two makers of endothelial injury and activation is unclear.

Intercellular adhesion molecule-1 (ICAM-1)

ICAM-1 is a cell surface glycoprotein expressed on leukocytes, vascular endothelial cells, and type 1 alveolar epithelial cells in response to inflammatory cytokines and is an important mediator for binding and transmigration of leukocytes into organs during inflammation or injury [80]. ICAM-1 levels appear to be elevated in BAL relative to serum in patients at-risk for ARDS or with ARDS but not with hydrostatic edema [27,28]. However, neither plasma nor BAL levels predict development of ARDS in at-risk patients nor distinguish patients with ARDS from at-risk patients [28].

ICAM-1 may have a greater role in prognosis of ARDS. Higher serum levels have been associated with worse outcomes in patients with ARDS [29–31]. In a large multicenter analysis of the ARDS Network ARMA trial, higher baseline plasma levels of ICAM-1 were independently associated with mortality and fewer ventilator- and organ-failure free days. As with Ang-2, rising levels of ICAM-1 on day 3 were associated with increased odds of death. Importantly, none of these studies specifically analyzed the interaction of sepsis on ICAM-1 levels, which may explain some of the lack of signal for identifying patients with ARDS. Also, the association with worse outcomes in ARDS may simply be a marker for more severe or nonresolving sepsis and not specifically worse lung injury.

Epithelial injury

Epithelial injury leading to sloughing of type 1 pneumocytes with loss of barrier function and reduced active resorption of edema fluid via ATPase-dependent sodium pumps is a hallmark of the pathophysiology of ARDS [3]. Protein biomarkers specific to alveolar epithelial injury have a theoretical benefit of less interaction with systemic inflammation and endothelial activation, making them highly sought as potentially more specific markers of lung injury, similar to troponin level for identifying myocardial injury. However, identification of a marker with sufficient sensitivity and specificity in peripheral blood for alveolar injury remains elusive.

Surfactant proteins

Surfactant proteins A through D are amphiphilic lipoproteins secreted by type II pneumocytes with important functions for lung homeostasis as highlighted by infantile respiratory distress syndrome which develops in premature infants born prior to development of sufficient surfactant production. Hydrophobic surfactant proteins B and C reduce alveolar surface tension at the air–fluid interface preventing alveolar collapse at expiratory lung volumes [81]. Hydrophilic surfactant proteins A and D (SP-A and SP-D) also contribute to innate immunity by coating bacteria and viruses, and promoting phagocytosis by macrophages [82].

Studies of surfactant proteins highlight the complexity of differing results depending on which surfactant proteins are measured and which compartments are sampled. Studies measuring SP-A and SP-D suggest levels of these proteins are decreased in the alveolar compartment in patients at-risk for and with ARDS while plasma levels are increased in ARDS [40,41]. In multicenter data from the ARDS Network ARMA trial, higher plasma

levels SP-D but not SP-A were independently associated with death, and fewer ventilator- and organ-failure free days [42]. In contrast to Vwf-Ag, analyzed in a separate analysis of the same trial, lower tidal volume ventilation attenuated the increase in SP-D levels on day 3 of mechanical ventilation. Together these results suggest surfactant proteins levels are reduced in alveoli due to impaired production or secretions by pneumocytes while increased plasma levels reflect loss of epithelial barrier integrity with systemic release.

More recently, plasma SP-D level was one of the best performing biomarkers for identifying cases of ARDS in patients with severe sepsis enrolled in the validating acute lung injury biomarkers for diagnosis (VALID) study and results were more predictive in analysis limited to more severe cases of ARDS [43]. This intriguing result suggests plasma SP-D may be particularly useful for diagnosing ARDS in sepsis since its presence in peripheral blood is likely more specific for alveolar epithelial injury rather than endothelial proteomic changes that may reflect systemic inflammation and injury in sepsis.

Receptor for advanced glycation end products (RAGE)

RAGE is a multiligand-binding transmembrane immunoglobulin that is heavily expressed in the lung and was previously thought to be specific to type 1 epithelial cells making it a compelling biomarker for ARDS [83]. However, RAGE expression has subsequently been found in a wide variety of cells including vascular endothelial cells [84]. Smaller studies have found higher plasma RAGE levels predict worse outcomes following lung transplant [44] and distinguished patients with ARDS from intubated patients with severe sepsis without ARDS [85].

In a multicenter analysis from the ARDS Network ARMA trial, higher baseline RAGE levels were only independently associated with worse outcomes in patients randomized to higher tidal volumes [86]. Also, RAGE levels declined more in patients randomized to lower tidal volumes and the mortality benefit of lower tidal volumes occurred in patients with the highest baseline RAGE levels.

These results suggest that the benefit of lower tidal volumes was mediated in part through decreased epithelial injury. RAGE was also one of the best performing biomarkers for diagnosing ARDS in the VALID cohort [43]. Thus, RAGE may have a role in identifying subgroups of patients most likely to benefit from future trials comparing strategies for lung protective ventilation, or as a surrogate endpoint for reduced epithelial injury in future phase II trials. However, in a separate analysis of the ARMA trial, RAGE was not associated with worse outcomes when the analysis was limited to patients with an APACHE II < 25 [87]. Along with the lack of association of baseline RAGE levels and outcomes in patients managed with lower tidal volumes in ARMA, this result questions the reliability of RAGE in less severely injured patients, especially in an era of lung protective ventilation.

Krebs von Lundgren-6 (KL-6)

KL-6 is a MUC1 mucin protein with increased expression on the surface of type II pneumocytes in response to injury or during repair [88]. In a recent meta-analysis of plasma biomarkers, KL-6 had the highest odds ratio (OR 6.1, 95%CI 3.3–12) for diagnosing ARDS and higher levels were also associated with increased mortality in patients with ARDS [15].

However, KL-6 has only been studied in relatively few patients and needs further validation in large, multicenter patient populations.

Inflammatory mediators of lung injury

Inflammation, either in response to primary lung injury or secondary to systemic inflammation, is an important mediator of ARDS. In addition, injurious mechanical ventilation can induce inflammation and contribute to multiorgan failure in ARDS regardless of the inciting etiology of the underlying lung injury.

Interlukin-6 and interlukin-8 (IL-6 and IL-8)

In the ARMA trial, higher baseline levels of proinflammatory cytokines, IL-6 and IL-8, were independently associated with fewer organ-failure and ventilator-free days but only IL-8 was associated with greater mortality [47]. Importantly, IL-6 and IL-8 were higher in sepsis and pneumonia relative to other causes of ARDS. However, lower tidal volumes were associated with a more rapid decline in both cytokines suggesting that change in IL-6 and IL-8 could serve as surrogate endpoints independent of the cause of lung injury. Importantly, IL-6 and IL-8 levels have also been found to be independently associated with subsequent studies of patients managed with lower tidal volume ventilation [30,48].

Tumor necrosis factor-alpha (TNF- α) and soluble tumor necrosis factor receptors I and II (s-TNFr-I and II)

As with IL-6 and IL-8, baseline levels of s-TNFr-I and II, but not TNF- α , were independently associated with worse outcomes in the ARMA trial [51]. However, only s-TNFr-I levels were significantly lower in patients treated with lower tidal volumes. Experimental human epithelial (A549) cells secrete s-TNFr-I, but not s-TNFr-II in response to inflammatory cytokines TNF- α , interlukin-1 β (IL-1 β), and interferon- γ , suggesting that s-TNFr-I may also serve as a surrogate endpoint for alveolar epithelial injury. In a single-center analysis, Meduri found baseline levels of IL-1 β were also higher in nonsurvivors and, along with IL-6, persistent elevation of was a strong predictor of mortality [49]. Importantly, elevated cytokine levels were also associated with sepsis in this study, but were more highly associated with outcomes of ARDS than with underlying etiology.

Relevant to drug development, Meduri subsequently found that methylprednisolone improved mortality in a small trial of 24 patients with late (7–21 days), nonresolving ARDS [89]. However, in a subsequent multicenter clinical trial by the ARDS Network in a similar patient population, methylprednisolone reduced duration of mechanical ventilation but there was no improvement in mortality. In addition, there was a higher rate of re-intubation in patients treated with methylprednisolone and a trend toward worse mortality in patients enrolled between 14 and 21 days [90]. Importantly, cytokine profiles were not assessed as inclusion criteria for either trial. Also, a phase II trial of anti-TNF- α therapy with a fragment antigen-binding (Fab) dimer for patients with severe sepsis found rapid reduction in TNF- α levels and reduced ventilator- and ICU-free days [91]. However, a subsequent phase IIb trial of a related polyclonal Fab fragment found no clinical benefits despite efficient reduction in

TNF- α concentrations [92]. Other targeted anti-inflammatory therapies have not been studied in ARDS.

Disordered coagulation

Activation of the coagulation cascade and impaired fibrinolytic activity leading to *in-situ* microvascular thrombosis are thought to contribute to organ failure in severe sepsis [93] and are specifically implicated in the pathogenesis of pneumonia and ARDS [94–97]. Activation of protein C by thrombo-modulin is an important step in inhibiting clot formation while plasminogen activator inhibitors released by endothelial cells and platelets are important inhibitors of fibrinolysis. Small studies have demonstrated lower plasma levels of activated protein C, and higher levels of plasminogen activator inhibitor-1 (PAI-1) and thombomodulin are associated with worse outcomes in ARDS [30,60,61].

In a large multicenter analysis from the ARMA trial, lower enrollment levels of protein C and higher levels of PAI-1 were independently associated with greater mortality and fewer organ-failure and ventilator-free days [98]. However, PAI-1 was not associated with outcomes in two separate multicenter analyses in patients with ARDS [48,62]. In a clinical trial of patients with ARDS but without severe sepsis, treatment with activated protein C reduced pulmonary dead space fraction but did not impact other clinically relevant of outcomes [99].

Apoptosis, fibrosis, and impaired healing

Activation of mesenchymal cells and collagen deposition is an important step in restoring the alveolar gas exchange apparatus following epithelial cell death in ARDS [3]. However, tight regulation of a complicated recovery process is needed and disordered repair and ongoing injury can lead to fibrosis and progressive loss of lung function [67]. The extent of fibrosis, either as a measure of the severity of injury or as a measure of disordered repair, correlates with outcome in ARDS [67].

Fas and Fas ligand

Apoptosis of epithelial cells occurs in both direct and indirect etiologies of ARDS [100]. The Fas/Fas ligand system is the best-studied mechanism regulating epithelial cell death. Soluble Fas and Fas ligand are elevated in pulmonary edema fluid relative to plasma levels in patients with early ARDS and relative to edema fluid from controls with hydrostatic edema [72,73]. Higher baseline Fas and Fas ligand levels in tissue and edema also correlate with increased morbidity and mortality [72,73]. Also, messenger RNA for Fas and Fas ligand were upregulated in BAL fluid during the acute phase of sepsis-related ARDS but not sepsis without ARDS or the late phase of sepsis-induced ARDS [74].

These results suggest epithelial apoptosis occurs early in ARDS and, at least in the alveolar compartment, Fas and Fas ligand may be specific markers for ARDS in patients with sepsis. However, the Fas/Fas ligand system is not unique to lung epithelial cells likely limiting the sensitivity and specificity of Fas and Fas ligand as plasma biomarkers. However, edema fluid from patients with ARDS can induce apoptosis in cultures of epithelial cells [72]. This

process was blocked by inhibiting the Fas/Fas ligand pathway, suggesting a potential novel therapeutic target for the prevention or treatment of ARDS.

Procollagen peptides

Procollagen peptide III (PCP III), a marker of collagen synthesis, is elevated in edema fluid sampled during intubation in patients with ARDS compared to those with hydrostatic edema and higher levels of PCP III are highly associated with mortality, suggesting the fibroproliferative response occurs early and may play an active role in the progression of ARDS [57,68]. Meduri also found plasma levels of PCP I and PCP III were increased at baseline in patients with ARDS and rising levels predicted worse outcomes [69]. Treatment with methylprednisolone led to rapid and sustained reduction in plasma and BAL levels of both proteins. However, as above, the benefits of methylprednisolone for treatment of late, nonresolving ARDS were not confirmed in a multicenter clinical trial by the ARDS Network [89,90].

Keratinocyte growth factor (KGF)

KGF is a member of the fibroblast growth factor family and is a potent mitogen for type II pneumocytes and may be an important mediator of epithelial repair following lung injury [101,102]. In addition to enhancing alveolar epithelial cell migration and repair in wound healing studies of isolated alveolar epithelial cells, KGF protected against lung injury in experimental models of ARDS from hyperoxia, acid aspiration, mechanical ventilation, bacterial pneumonia, radiation injury, and bleomycin-induced lung injury [103]. In *ex vivo* human lung studies, KGF enhanced resolution of alveolar edema by restoring normal alveolar edema fluid clearance and enhanced monocyte phagocytosis of *E. coli*, via a KGF receptor found on monocytes [104]. A phase II study of KGF for the prevention of ARDS in patients with severe sepsis has recently been completed but published results are pending (ISRCTN95690673).

Proteomics studies incorporating multiple biomarkers

In addition to the targeted single biomarker studies described above, which point to multiple distinct mechanisms involved in ARDS pathogenesis, several groups have tested multiple biomarkers within the same cohort to either identify novel aspects of ARDS pathogenesis or improve identification and prediction of ARDS.

Novel ARDS subphenotypes

Calfee et al. [105] used latent class analysis (LCA) to identify subtypes of ARDS using data from two large clinical trials incorporating patients with diverse etiology of ARDS [47,106]. More than 30 baseline clinical and biomarker variables were considered as class-defining variables. In both large clinical trials, the populations were best defined using two classes, or sub-phenotypes. The smaller class (~30% of both ARDS populations) was consistent with a more inflammatory type – higher levels of IL-6 and IL-8, more frequent baseline vasopressor requirement, and comprised of a higher proportion of patients with sepsis as the underlying ARDS risk factor. Importantly, this class of patients could not be inferred by clinical categories, but was associated with both higher mortality and a differential response

to therapy with high PEEP. A parsimonious model incorporating two biomarkers (IL-6 and soluble TNFr-I) and the clinical variable of vasopressor use at baseline correctly classified the phenotype with >90% accuracy in both populations. This suggests that this phenotype could potentially be assessed as an enriched target population for enrollment in future clinical trials.

Direct versus indirect lung injury

Calfee et al. [107] examined how biomarkers differ by lung injury mechanism (e.g. pneumonia or aspiration for direct injury vs. pancreatitis or nonpulmonary sepsis for indirect injury). Five biomarkers were tested simultaneously in two cohorts (a single-center cohort [108] and a multicenter clinical trial [109]), including markers of epithelial injury (SP-D and RAGE), endothelial injury (Ang-2), and inflammation (IL-6 and IL-8). As expected, SP-D was significantly higher and Ang-2 significantly lower in patients with direct versus indirect lung injury. However, the prognostic value of the biomarkers for association with mortality was similar regardless of lung injury etiology, with IL-6, IL-8, and Ang-2 associated with increased odds of death in both populations. RAGE showed a trend toward significance only in patients with direct lung injury (OR 2.3 vs. 1.0 in the single-center cohort and 1.5 vs. 1.2 in the clinical trial, though neither interaction term was significant).

Multimarker protein panels in identifying ARDS

Fremont et al. examined seven biomarkers representing multiple biologic pathways (including RAGE, PCP III, Ang-2, BNP, IL-10, TNF α , and IL-8) in 192 patients admitted to the trauma ICU, of whom 107 developed ARDS [110]. The 7-biomarker panel differentiated ARDS with an AUC of 0.82. In a population of sepsis patients, Ware et al. examined 11 biomarkers in 100 patients with sepsis and ARDS versus 100 with sepsis and no ARDS. They identified a panel of five biomarkers [SP-D, RAGE, IL-6, IL-8, and club cell secretory protein (CC-16)] representing multiple types of injury out-performed any single biomarker, and performed particularly well at identifying severe ARDS [43].

Multimarker protein panels in improving ARDS mortality prediction

Calfee et al. tested multiple biomarkers for risk prediction for mortality in comparison to the widely used APACHE 2 score [111]. They found that a parsimonious 3-biomarker panel (IL-8, TNF- α , and SP-D) substantially improved risk prediction in two independent ARDS cohorts.

Nontargeted proteomics

While the studies above all focused on a single or several individual proteins with an *a priori* hypothesis for involvement in lung injury using highly quantitative techniques like ELISA to measure a specific protein, nontargeted proteomics instead examines many analytes in an unbiased fashion. The benefit of this approach is the potential to identify novel ARDS mechanisms and potential therapeutic targets.

A detailed discussion of the methodology of nontargeted proteomics is beyond the scope of this article; fortunately, multiple outstanding reviews have been published previously

[112,113]. Briefly, mass spectrometry is the most commonly used method of proteomic profiling, which was made possible on a large scale because of advances in electrospray ionization of peptides. The two most commonly used methods of reliable protein identification are also those most widely used in ARDS proteomics studies today. The first is difference in gel electrophoresis (DIGE), in which gel spots are identified and then excised for further characterization by mass spectrometry, often termed a 'bottom-down approach'. In contrast, a 'bottom-up approach' involves proteolysis of the sample to peptides followed by liquid chromatography-mass spectroscopy (LC-MS-MS). Each method has its strengths, with DIGE able to detect a more limited range of high-abundance proteins, while LC-MS-MS was previously thought to be slightly less quantitative. This latter deficit is somewhat overcome with stable isotope tagging (e.g. isobaric tags for relative and absolute quantification (iTRAQ), below).

In Table 2, we have summarized the ARDS nontargeted proteomic literature to date. We note several aspects to these studies. First, no two studies use identical methods for sample handling, let alone proteomic profiling, with some using DIGE, others LC MS-MS, yet others iTRAQ labeling. Thus, given that different methods are optimized to identify different proteins, it is unsurprising that their major conclusions vary widely.

The nontargeted proteomic studies in ARDS conducted to date differ in important ways from the targeted plasma biomarker studies highlighted above. First, proteomic studies in ARDS to date have typically examined a small number of samples (Table 2). Given the extensive heterogeneity in ARDS discussed earlier in this review, small sample sizes will likely contribute to heterogeneous conclusions. Also notable is the fact that most nontargeted proteomics studies have focused on comparing the protein levels in the alveolar compartment, usually BAL, undiluted pulmonary edema fluid or exhaled breath. While most studies to date compared ARDS samples to healthy controls, Bhargava et al. examined BAL fluid from different subtypes of ARDS patients – specifically 15 in early phase (days 1–7), including 7 survivors and 8 non-survivors; and 7 in late phase (day 8–35). They identified 792 proteins, and found that 161 were differentially expressed in early-phase ARDS between survivors and nonsurvivors. Pathways including activation of immune response, wound healing, and multiple pathways involving coagulation are more abundant in survivors, while collagen synthesis and carbohydrate catabolism are more abundant in nonsurvivors. While these results are exploratory given small numbers, they suggest the promise of nontargeted proteomic study in identifying novel mechanisms and thus future drug targets in ARDS.

Proteomics and the future of drug discovery in ARDS

As highlighted above, targeted proteomics has already identified multiple mechanistic pathways that are involved in ARDS, including endothelial and epithelial injury, inflammation, disordered coagulation and proteins involved in lung healing (including apoptosis and fibrosis pathways). The importance of these pathways has been confirmed in human disease, as many of the proteins can serve as biomarkers either in identifying subphenotypes of ARDS or associated with disease prognosis.

In contrast, nontargeted proteomics has been applied to much smaller datasets in ARDS to date, often comparing ARDS edema fluid to that of healthy controls as opposed critically ill or at-risk patients without ARDS. Because of differing proteomic profiling techniques and patient populations, the role of these newly identified proteins as potential biomarkers or as novel ARDS therapeutic mechanisms is less certain.

Despite these advances in ARDS proteomics to date, none of the pathways have specifically been targeted in novel drug trials. We nonetheless argue that proteomics has substantial potential to advance drug development in ARDS.

Proteomics for identifying endophenotypes of ARDS for therapeutic trials

While the vast majority of ARDS clinical trials to date have largely included all patients (mild and severe ARDS, direct and indirect lung injury, and septic and nonseptic patients), proteomics will likely play a major role in identifying enriched subphenotypes of ARDS with the highest likelihood of benefiting from specific targeted therapy. A 2-biomarker panel of IL-6, soluble TNFr-I, and need for vasopressors identified a subgroup of 'inflammatory ARDS' patients who benefited from the intervention of higher levels of PEEP but could not be identified based on clinical factors alone [105]. Thus, a trial targeting this enriched population of 300 patients may have shown a significant benefit where the larger trial of 1000 patients did not. We anticipate that collecting a plasma sample will be the default in future therapeutic clinical trials to test whether therapies are effective in a subset of patients. If a subphenotype is identified repeatedly in clinical trials as particularly likely to benefit from therapeutic interventions, these patients may specifically be targeted in novel therapeutic trials. Equally importantly, patients unlikely to benefit can be excluded from such trials and thus are spared the risk of potential side effects. To enable this important advance, point-of-care tests of proteomic biomarkers would need to be developed to enable identification of eligible subjects at the time of trial enrollment.

Nontargeted proteomics for identifying novel ARDS mechanisms

As reviewed above, nontargeted proteomics studies to date have been limited by small sample size and use of healthy controls as a comparison group. As proteomics technologies improve and become less costly, we anticipate that larger-scale nontargeted proteomics studies of both plasma and BAL will identify novel mechanisms of ARDS pathogenesis, which could themselves serve as targets for future drug development. Of particular interest are therapies with the potential to convert patients from a phenotype of collagen synthesis and carbohydrate catabolism seen in nonsurvivors to one of an activated but regulated immune response, wound healing, and coagulation homeostasis seen in survivors.

Proteomic change as intermediate endpoint in clinical trials

While large, multicenter trials in ARDS often target mortality as an endpoint, as mortality of ARDS has improved from 40% to 50% 10 years ago to 20% to 25% in recent clinical trials [121], powering trials for a mortality endpoint is becoming increasingly expensive. In addition, patients with ARDS are by definition critically ill, and death is often attributed to multisystem organ failure rather or other comorbidities rather than refractory ARDS. In this review, we have highlighted protein biomarkers that have been associated with important

endpoints like ARDS mortality, development of ARDS, and even response to fluid management strategy. The discriminatory ability of these biomarkers has not yet been sufficiently established; thus, they have yet to be incorporated into clinical practice or ARDS trial design. However, if proteomic biomarkers (from plasma or BAL) are repeatedly found to be associated with key pulmonary outcomes, these could serve as important intermediate variables and surrogate endpoints in clinical trials.

Trials targeting multiple pathways

Targeted proteomics has shown that ARDS involves multiple pathways leading to a distinct clinical syndrome. Therapeutic trials in ARDS usually target only a single pathway (e.g. statins or corticosteroids for anti-inflammatory properties, or beta-agonists to enhance alveolar fluid clearance). Given the many dysregulated pathways, it seems reasonable to consider that a multipronged therapeutic approach, using multiple agents (e.g. similar to the approach used in cancer or HIV, among other diseases) may be necessary to effect meaningful change in ARDS pathogenesis. A 'personalized medicine' approach to ARDS, in which the most dysregulated pathways for a given individual could be targeted by specific therapies, is likely years away, but may ultimately improve outcomes of ARDS.

Summary

In summary, ARDS is a disease that carries substantial mortality and morbidity, and therapeutic options are lacking. Targeted proteomic approaches have identified multiple proteins that can serve individually and together as biomarkers of both subphenotypes of disease and disease prognosis. We anticipate that in the future, proteomics will play a major role in ARDS by identifying novel targets for drug development and distinguishing enriched subphenotypes of patients with the highest likelihood of benefitting from specific target therapies.

Expert commentary

ARDS is an important disease with substantial attributable morbidity and mortality and very limited therapeutic options. Proteomics has already improved our understanding of ARDS pathophysiology in important ways. Targeted proteomics has confirmed the importance of various pathways of lung injury (notably inflammation, epithelial injury, endothelial injury or activation, and disordered coagulation and repair). Protein changes, alone or in models that combine multiple proteomic changes with clinical features, can serve as potential biomarkers for ARDS pathogenesis. Most excitingly, protein alterations can help to identify novel subphenotypes of disease that may differentiate patients most likely to benefit from novel therapeutic options.

Five-year view

We anticipate that proteomics will advance our understanding of ARDS pathophysiology in several important ways in the next 5 years. First, nontargeted proteomic studies of ARDS are just beginning; as proteomic methods improve and become cheaper, larger studies of carefully phenotyped patients will identify novel pathophysiology in the disease. These

novel pathways could serve as fruitful targets for drug development. Second, we anticipate that proteomic biomarkers will improve ARDS phenotyping and enable identification of subsets of patients most likely to benefit from novel therapeutic options. This should in turn enable better-powered ARDS trials because of a lack of misclassified subject enrollment, and hopefully maximize our likelihood of identifying novel therapeutic options for this devastating disease.

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Key issues

- ARDS is an important disease with substantial attributable morbidity and mortality and very limited therapeutic options.
- Marked heterogeneity in pathogenesis and prognosis of existing populations of ARDS have challenge the identification of protein biomarkers with sufficient validity for clinical use.
- While no proteomic biomarkers are currently in widespread clinical practice, targeted proteomics has confirmed the importance of several pathways of lung injury, including inflammation, epithelial injury, endothelial injury or activation, and disordered coagulation and repair
- Nontargeted proteomic studies in ARDS have used small sample sizes and methods between studies (including phenotype investigated) has varied, limiting the reproducibility between studies to date.
- Single and multi-proteomic biomarker studies are enabling identification of novel subphenotypes of disease, which may enable enrollment of homogenous populations of ARDS patients who are particularly likely to respond to therapy in future clinical trials

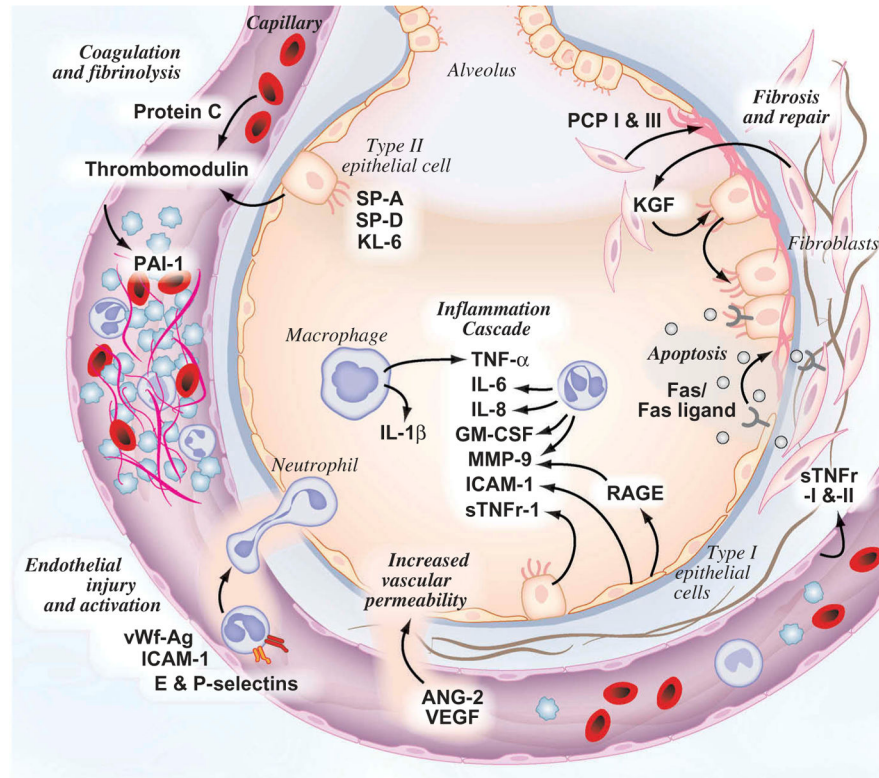


Figure 1.
Key pathways and known proteomic changes in ARDS pathogenesis.

Table 1

Proteomic pathways associated with ARDS.

Pathway	Biomarker	Source	Role in ARDS	Reference
Endothelium injury and activation	VWF-Ag	Endothelial cells and platelets	Increase platelet binding to vascular endothelium	[17–22]
	Ang-2	Endothelial cells	Regulates capillary permeability	[21,23–26]
	ICAM-1	Endothelial cells, type I alveolar cells, leukocytes	Promotes leukocyte binding and transmigration	[27–31]
	E-selectin & P-selectin	Endothelial cells, platelets & leukocytes	Promotes leukocyte binding and transmigration	[32–34]
	VEGF	Endothelial cells	Increases vascular permeability	[35–39]
Epithelial injury	SP-A & SP-D	Type II alveolar cells	Impaired alveolar recruitment and innate immunity	[40–43]
	RAGE	Type I epithelial cells	Receptor for inflammatory ligands	[44,45]
	KL-6	Type II epithelial cells	Increased expression in injury or repair	[46]
Inflammation	IL-6	Leukocytes	Proinflammatory	[43,47–50]
	IL-8	Leukocytes	Proinflammatory	[43,47–50]
	IL-1 β	Macrophages	Proinflammatory	[49]
	TNF- α	Macrophages	Proinflammatory	[49,51,52]
	sTNFr-I & II	Multiple tissues	Proinflammatory	[51–53]
	Neutrophils	Vascular migration	Promote phagocytosis and cellular injury	[54–56]
	MMP-9	Neutrophils	Mediate neutrophil cellular injury	[57]
	Macrophages	Resident in lung and vascular migration	Promote immunity and repair	[58]
	GM-CSF	Leukocytes and endothelial cells	Promote immunity and repair	[59]
Coagulation	Protein C	Liver	Prevents in-situ thrombosis	[23,30,60]
	PAI-I	Vascular endothelial cells and platelets	Promotes thrombosis	[23,30,48,60–62]
	Thrombomodulin	Type II pneumocytes	Activates protein C	[19,60]
Oxidative stress	Nitric oxide	Endothelial cells and leukocytes	Mediator of oxidative injury	[63]
	Ferritin	Acute phase reactant and product of oxidative stress	Mediator of oxidative injury	[64,65]
	Antioxidants	Ubiquitous	Prevent oxidative injury	[66]
Fibrosis	PCP I & III	Fibroblasts	Marker of collagen synthesis	[67–69]
Disordered repair	TGF- α	Ubiquitous	Endothelial cell migration and angiogenesis	[70,71]
	KGF & HGF	Mesenchymal cells	Epithelial cell mitogens	
Apoptosis	Fas/Fas ligand	Ubiquitous	Promote cell death	[72–74]
	Perforin/granzyme	Ubiquitous	Promote cell death	[74]
Extracellular matrix	Desmosine	Lung interstitium	Marker of disruption of extracellular matrix	[75,76]

GM-CSF, granulocyte/macrophage colony stimulating factor; HGF, hepatocyte growth factor; ICAM-1, intercellular adhesion molecule 1; IL-1 β , interleukin 1 beta; KGF, keratinocyte growth factor; PAI-I, plasminogen activator inhibitor-I; PCP I & III, procollagen peptides I & III; RAGE, receptor for advanced glycation end products; SPA & D, surfactant protein A & D; TGF- α , transforming growth factor alpha; TNF- α , tumor necrosis factor alpha; vWF-Ag, von Willebrand factor antigen.

Table 2

Nontargeted proteomics studies in acute respiratory distress syndrome (ARDS).

First author/year	N	Control population	Fluid type	N proteins	Profiling method	Major conclusions
Bowler [116]	16 ARDS	12 healthy controls	Plasma and pulmonary edema/BAL	158	2-D gel electrophoresis – MALDI TOF	↑ Surfactant protein A isoforms in patients with ARDS & many protein modifications/truncations
De Torre [118]	11 ARDS	33 healthy controls + endotoxin challenge	BAL		SELDI-TOF mass spectrometry	↑ ApoA1, S100 Ca-binding proteins in both ARDS and inflammatory response
Schnapp [115]	3 ARDS	6 healthy controls	BAL	226–659 per patient	Shotgun proteomics, LC-MS-MS	Many proteins differ; focus on IGF pathway changes, validated in independent BAL set
Chang [114]	ARDS by time: D1 (N=7), D3 (N=8), D7 (N=5)	9 healthy controls	BAL	37	2-D gel electrophoresis-MALDI TOF	Proteins in inflammation, infection, and injury differ in ARDS vs. healthy and differ over time
Gessner [120]	24 ARDS	10 healthy 6 pneumonia (no ARDS)	Exhaled breath	3	2-D gel electrophoresis – MALDI TOF	Cytokeratins 2, 9, and 10 present in ARDS. Higher levels correlated with PEEP, PIP, and ARDS
Chen [119]	Direct (6) & indirect (5) ARDS	15 healthy controls	Plasma	132	iTRAQ (isobaric tags for relative absolute quantification) LC-MS-MS	Substantial overlap in ARDS differentially expressed proteins, regardless of direct/indirect; involve 8 canonical pathways
Bhargava [117]	Early ARDS survivors (7) vs. nonsurvivors (8) Late ARDS (7)	N/A	BALF	499	iTRAQ LC MS/MS	Early survivors: ↑ injury response incl. immune response & cation/iron homeostasis; vs. nonsurvivors: collagen synthesis/catabolism

BAL: bronchoalveolar lavage fluid; IGF: insulin-like growth factor; iTRAQ: isobaric tags for relative and absolute quantification; MALDI TOF: matrix-assisted laser desorption/ionization TOF (time of flight); PEEP: positive end-expiratory pressure; PIP: peak inspiratory pressure; SELDI-TOF: surface-enhanced laser desorption/ionization-TOF (time of flight).