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Quantitative proteomic analysis by iTRAQ for identification of candidate biomarkers in plasma from acute respiratory distress syndrome patients

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ABSTRACT

Acute respiratory distress syndrome (ARDS) is a major cause of morbidity and mortality in critical patients. Proteomic analysis of plasma from individuals with ARDS could elucidate new biomarkers for diagnosis and pathophysiology and identify potential ARDS treatment targets. In this study, we recruited 26 patients (15 controls, 11 ARDS). The ARDS group was subdivided into two groups depending on the type of injury: (1) direct lung injury (AD) and (2) indirect lung injury (AI). Using iTRAQ (isobaric tags for relative and absolute quantitation) analysis, we identified 2429 peptides representing 132 plasma proteins. Among these, 16 were differentially expressed in ARDS patients, including 11 overlapping proteins between the AI and AD group and 5 AI-specific proteins. Protein annotation revealed that lipid transport and complement activation were significantly enriched in the biological process category, and lipid transporter, transporter, and serine-type peptidase activities were significantly enriched in the molecular function category. IPA (Ingenuity Pathway Analysis) signaling pathways revealed that the overlapping proteins were involved in a variety of signaling pathways, including those underlying acute phase response; liver X receptor/retinoid X receptor (LXR/RXR) and farnesoid X (FXR)/RXR activation; clathrin-mediated endocytosis; atherosclerosis; interleukin (IL)-12; complement system; and cytokine, nitric oxide, and reactive oxygen species production in macrophages. We present the first proteomic analysis of ARDS plasma using the iTRAQ approach. Our data provide new biomarker candidates and shed light on potential pathological mechanisms underlying ARDS.

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1. Introduction

Acute respiratory distress syndrome (ARDS) is a life-threatening condition characterized by breathing failure. In critically ill patients ARDS can cause death; the mortality rate is 30–60% in developed countries [1]. Current ARDS diagnostic criteria recommended by the 1994 American-European Consensus Conference (AECC) include the onset of clinically significant hypoxemia, diffuse bilateral pulmonary infiltrates, no evidence of left atrial hypertension, and a partial pressure of arterial oxygen/fraction inspired oxygen (PaO2/FiO2) ratio $\leq$ 200 mmHg [2]. Although these criteria are easy to test clinically, they do have disadvantages, especially in regard to variability in chest radiograph interpretation. In addition, clear evidence suggests that low-tidal volume ventilation and conservative fluid management could significantly increase the number of ventilator-free days and reduce ARDS mortality [3,4]. Therefore, early diagnosis with objective criteria, such as biomarkers, could improve treatment and survival rates in patients with ARDS.

Measuring plasma biomarkers is an effective means of evaluating ARDS status and could be used to elucidate underlying pathological mechanisms, which are poorly understood. Over the past two decades, a large number of ARDS-associated biomarkers have been discovered, including epithelium-specific surfactant proteins (SP-A, SP-D), endothelium-specific proteins (von Willebrand factor antigen [vWf-Ag], E-selectin, and vascular endothelial growth factor [VEGF]), cytokines (tumor necrosis factor-alpha [TNF-a], IL-6, and IL-8) and matrix metalloproteinases [5]. Clinical investigations have shown that plasma levels of these proteins are closely related to ARDS severity and mortality. Unfortunately, none of the current plasma biomarkers are completely reliable for diagnosing ARDS, which highlights the need for novel biomarkers.

Proteomics is a powerful tool for characterizing protein expression during different physiological and pathological states [6]. It is widely used to identify disease biomarkers and for investigating mechanisms underlying pathogenesis in cancer biology and immunology, as well as ARDS or acute lung injury (ALI). Bowler et al. compared the protein profile of distal lung fluid from ALI patients to normal control (NC) subjects using electrophoresis-based

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proteomics and found that plasma is the main source of pulmonary edema fluid proteins, indicating that pulmonary capillaries in ALI patients exhibit increased permeability. Abnormal changes in protein expression and post-translational modifications have also been identified using proteomics [7]. Chang et al. used two-dimensional gel electrophoresis to identify 37 ARDS-related proteins in bronchoalveolar lavage fluid (BALF) that indicated inflammation, infection, and injury [8]. By employing shotgun proteomics, Schnapp et al. identified 870 BALF proteins from three ARDS patients that consisted of several interesting proteins. Further investigation revealed that insulin-like growth factor binding protein-3 and insulin growth factor (IGF) expression levels were correlated with ARDS progression [9].

One challenge in analyzing plasma samples is the dynamic range of protein concentrations [10]. For instance, in human plasma, approximately 20 highly abundant proteins, including albumin, immunoglobulin Gs (IgGs), transferrin, apolipoprotein (Apo) A1-2, and haptoglobin, make up more than 99% of total plasma proteins. Because researchers consider low-abundance proteins to be more likely biomarkers, removal of high-abundance proteins is an important step in proteomic profiling [11,12]. However, it should be noted that some high-abundance proteins, such as albumin, are cargo proteins and their depletion can cause loss of binding of low abundance proteins. Moreover, recent data showed that some of these proteins are themselves indicators of disease. Lai et al. found that plasma samples from mice with oral squamous cell carcinoma had increased levels of haptoglobin and Apo A1 precursor [13]. Others reported that haptoglobin expression is abnormal in the plasma of patients with ovarian cancer and hypertrophic cardiomyopathy [14,15]. As such, removal of high-abundance proteins should be performed with caution.

Narrow-range isoelectric focusing (NR-IEF) is an effective approach for pre-fractioning peptides. Previous reports showed that most human proteins have tryptic peptides at a pH range of 3.5–4.5 [16]. NR-IEF in this pH range will significantly reduce the complexity of peptides without a significant loss of proteome coverage. In this study, we removed two high-abundance proteins and performed NR-IEF and identified plasma proteins comparable to those reported by others. Using iTRAQ (isobaric tags for relative and absolute quantitation), we identified a set of differentially expressed proteins between ARDS samples and NCs. The results will aid in the discovery of new biomarkers and provide information that could reveal potential pathological mechanisms of ARDS.

2. Materials and methods

2.1. Patients and sample collection

This study was approved by the Ethics Committee of Fuxing Hospital, Capital Medical University. Informed consent was obtained from patients or their closest relatives in accordance with the revised Helsinki declaration of 1983. We collected samples from 26 patients, including 11 ARDS patients and 15 NCs. All ARDS patients were defined using 1994 AECC criteria, including PaO2/FIO2 $\leq$ 200 mmHg, bilateral infiltration on the frontal chest radiograph and pulmonary capillary wedge pressure $\leq$ 18 mmHg or no clinical evidence of left atrial hypertension and congestive heart failure. ARDS groups were divided according to the type of lung injury into an AD group (n = 6) and an AI group (n = 5). NCs were 50 years of age or older and did not have lung disease or fever. Clinical parameters are listed in Table 1. The blood was obtained by an experienced nurse using heparin vacuum blood collection tube within 24 h after the onset of ARDS. All of the blood was immediately processed by centrifuging at 3000 rpm for 10 min. The supernatant was aliquoted, and then stored at −80 °C.

Table 1
The clinical parameters of subjects.

Clinical parameters	NC[a]	ARDS		*P* value
		AD[b]	AI[c]	
Age (mean ± SD)	58.2 ± 9.4	82.8 ± 6.2	82 ± 8.8	0.0002[d]
Gender	7 (47%)	5 (83%)	4 (80%)	0.1092[d]
PO_2/FiO_2 (mean ± SD)		151.2 ± 24.8	135.6 ± 19.5	0.2712[e]
APACHEII (mean ± SD)		26.2 ± 7.5	14.6 ± 7.4	0.0311[e]
Total	15	6	5	

[a] Normal control.
[b] ARDS from direct lung injury.
[c] ARDS from indirect lung injury.
[d] *P* value derives from the comparison between ARDS group and normal control group.
[e] *P* value derives from the comparison between AD group and AI group.

2.2. Immunodepletion of high-abundance proteins

Equal volumes of plasma from each subject were pooled and treated with a ProteoExtract Albumin/IgG Removal Kit (Merck, Darmstadt, Germany) to remove albumin and IgG, which accounts for approximately 80% of the total plasma protein concentration, according to the manufacturer's instructions. Plasma proteins concentrations were determined using a bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL, USA). Proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) followed by Coomassie Blue staining to verify removal quality.

2.3. Protein digestion and iTRAQ labeling

Immunodepleted protein samples was reduced and alkylated using 5 mM DL-dithiothreitol (DTT) at 56 °C for 60 min and 10 mM iodoacetamide (IAM) at room temperature for 45 min. The protein samples were dialyzed (cut-off of 3.5 kDa) to remove excess DTT and IAM, and then they were dried. Protein pellets were dissolved in 30 ìl 50% tetraethylammonium bromide (TEAB, Sigma, St. Louis, MO, USA) containing 0.1% SDS. A total of 100 ìg dissolved proteins were digested with sequencing-grade trypsin (Promega, Madison, WI, USA) at a ratio of 1:50 (w/w) overnight. Finally, each group of digested peptides was labeled with the iTRAQ reagents according to the protocol of the 8-plex iTRAQ labeling kit (Applied Biosystems, Foster City, CA, USA).

2.4. IEF of peptides

To prepare the sample for IEF analysis, the digested proteins were pooled and desalted using Strata X-C microcolumns (Phenomenex, Torrance, CA, USA). Samples were evaporated until dry and suspended in IEF buffer containing 8 M urea and 0.5% 3.5–5 pH ampholine. A 24-cm linear immobilized pH gradient (IPG) strip (pH 4–6) was rehydrated overnight with the peptide solution. IEF was performed on an Ettan IPGphor II system (GE Healthcare, Waukesha, WI, USA) with the following conditions: 4 h step at 50 V, 1 h step at 500 V, 1 h step at 1000 V, linear gradient from 100 to 8000 V in 1 h, and 8000 V up to 100 kVh.

After focusing, the strips were cut into 24 pieces starting at the acidic end. A total of 200 ìl water followed by 50% and 100% ACN were successively incubated with the pieces of strip for 1 h. All extracts were pooled and then cleaned on Strata X microcolumns (Phenomenex).

2.5. Proteomics

Based on matrix-assisted laser desorption/ionization time-of-flight/time-of-flight mass spectrometry (MALDITOF/TOF-MS) analysis, the fractions with the lower peptide number were combined with the surrounding fraction to reduce the total number. Each peptide fraction was injected into a C18 analytical reverse-phase column and analyzed using a hybrid quadrupole/time-of-flight MS (MicroTOF-Q II, Bruker, Billerica, MA, USA) in an information-dependent acquisition mode (IDA). Product ion spectra were accumulated for 3 s in the mass range *m/z* 50–2000. The data were collected and analyzed using Data Analysis Software (Bruker).

2.6. Data analysis

The resulting MS/MS spectra were searched against the International Protein Index (IPI) human sequence database (version 3.83) using MASCOT software, version 2.2 (Matrix Science, London, UK). The false discovery rate (FDR) of peptide matches was estimated using automatic decoy searching. Search results were exported into Scaffold (version 3.0) for protein quantification. Protein abundance from the NC group was used as a reference for determining fold change; changes of 2-fold or higher were considered significant. Because all protein iTRAQ ratios were transformed to base 2 logarithm values, a fold change of two is reported as −1 or 1 for down- and up-regulation, respectively.

Theoretical pI calculations of the identified peptides were performed using a BioPerl module, pICalculator. Gene Ontology (GO) functional classifications and GO enrichment analysis that were significantly enriched in differentially expressed proteins were analyzed with Protein ANalysis THrough Evolutionary Relationships (PANTHER) software (http://panther.appliedbiosystems.com/). IPA (Ingenuity System Inc., Redwood, CA, USA) was used to analyze canonical pathways involving the differentially expressed proteins. Significance levels were assessed with Fisher's exact tests.

3. Results

3.1. Subject clinical characteristics

ARDS can be caused by direct or indirect insult to lung cells, and these involve different pathogenic pathways, morphological aspects, and ventilatory strategies. Therefore, the samples from ARDS patients were divided into an AD group (pneumonia or aspiration) and an AI group (sepsis, abdominal infection, or cholecystitis) to reduce the heterogeneity of the ARDS sample data. As shown in Table 1, the ARDS group was significantly younger in age ($P = 0.0002$) but had a similar gender ratio ($P = 0.1092$) compared with the NC. The PO2/FiO2 ratio of the AI group was similar with that of the AD group, while the APACHEII (Acute Physiology and Chronic Health Evaluation II) score of the AI group was significantly lower than that of the AD group ($P = 0.0311$).

3.2. Verification of high-abundance plasma protein removal

To estimate the efficiency of high-abundance protein removal, we performed the analysis using 12% SDS–PAGE followed by Coomassie blue staining. As shown in Fig. 1, most of the albumin and IgG were depleted, and some of the low-abundance proteins were enriched.

3.3. Peptide identification and evaluation of IEF separation

We identified 2429 non-redundant peptides with an FDR of 2.26%, which is within the acceptable limit of 5% [17]. Among these, a total of 403 peptides were unique. To evaluate the performance of peptide fractionation, the pI values of peptides from each fraction were calculated and compared with that of the theoretical pI value (Fig. 2). For the 12 collected fractions, the mean peptide pI values ranged from 3.83 to 6.67. Our results show that the observed pI gradient is close to that of the theoretical pI gradient obtained from the manufacturer. This indicates that the NR-IPG strip fractions plasma peptides appropriately.

3.4. Identification of differentially expressed proteins

A total of 132 non-redundant proteins with at least one unique peptide were successfully identified. Compared with the NC group, there were 11 and 16 differentially expressed proteins for the AD and AI subgroups, respectively (Table 2). Of these, 11 proteins overlapped, 7 of which were up-regulated and 4 were down-regulated. Only Apo B-100, C-II, and A-IV; the high-molecular weight isoform of kininogen-1; and haptoglobin showed obvious changes in the AI group, suggesting that these proteins were associated with different ARDS initiation insults.

3.5. GO of differentially expressed proteins

To gain insight into the biological significance of the 16 differentially expressed proteins in the ARDS patient samples, the proteins were categorized according to GO annotation. All 16 proteins were recognized by PANTHER software, which produced 40 and 22 hits for molecular functions and cellular components, respectively. In the biological process group, they fell into nine categories, including metabolic process (56%), immune system process (50%), transport (31%), cellular process (25%), and response to stimulus (25%). In the molecular function group, they fell into five categories including transporter activity (50%), catalytic activity (31%), binding (25%), receptor activity (19%), and enzyme regulator activity (13%) (Fig. 3). To determine whether the proteins were enriched in certain groups, we employed over-representation

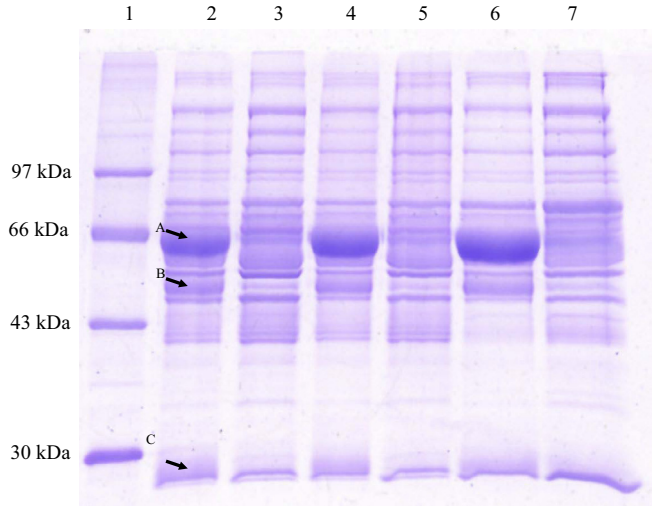

Fig. 1. Albumin and IgG analysis. Lanes 2, 4, and 6 were loaded with pooled plasma samples from AD, AI, and NC groups, respectively. Lanes 3, 5, and 7 were loaded with pooled plasma samples that had albumin and IgG removed from AD, AI, and NC groups, respectively. Lane 1 contains protein marker. The bands corresponding to albumin (A), IgG heavy chain (B), and IgG light chain (C) were significantly reduced in lanes 3, 5, and 7.

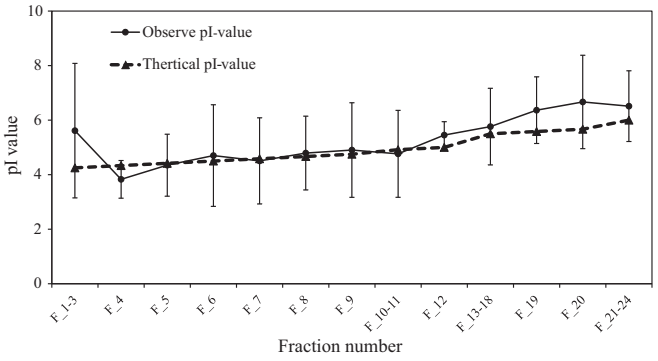

Fig. 2. Average pI values of identified plasma peptide plotted against the IPG fraction numbers. The solid line indicates observed pI values according to the identified plasma peptides in each corresponding fraction. The dashed line indicates the theoretical pI values obtained from the manufacturer. The spots in the solid line are the average peptide pI values. Error bars are standard deviations.

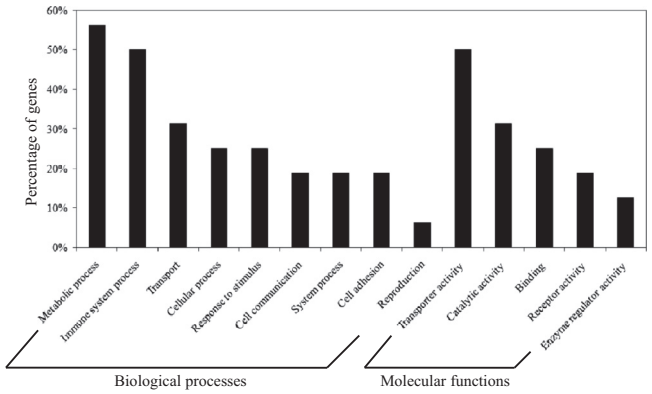

Fig. 3. Enriched GO term distribution of differentially expressed proteins between ARDS samples and NCs.

analysis. We found that lipid transport and complement activation were significantly enriched in the biological process group ($P < 0.01$) and that lipid transporter, transporter, and serine-type peptidase activities were significantly enriched in the molecular function group ($P < 0.01$).

3.6. Canonical signaling pathways associated with differentially expressed genes

To reveal signaling pathways involved in ARDS, we performed IPA analyses. Specifically, we analyzed the proteins that overlapped between the ARDS subgroups to investigate a potential common pathological mechanism underlying ARDS. A total of 10 of the 11 overlapping proteins were successfully recognized by IPA and identified eight canonical signaling pathways that were statistically significant ($P < 0.01$). These included acute phase response signaling, liver X receptor/retinoid X receptor (LXR/RXR) and farnesoid X receptor (FXR)/RXR activation, clathrin-mediated endocytosis signaling, atherosclerosis signaling, IL-12 signaling and production in macrophages, nitric oxide and reactive oxygen species production in macrophages, and complement system signaling (Fig. 4). The results indicate that these pathways may be activated in ARDS.

4. Discussion

Highly abundant plasma proteins are routinely removed prior to proteomic analysis to increase the detection of low-abundance proteins. However, some reports suggest that many of the high-abundance plasma proteins are associated with ARDS. We adopted the strategy of depleting albumin and IgG proteins followed by NR-IEF. In our samples, a total of 132 plasma proteins were detected, which is similar to that reported in previous studies [18,19]. We were able to identify some useful high-abundance proteins in the plasma, such as Apo A-I, complement component C9, and serotransferrin, which would be missed if they were removed by several different types of high-abundance protein affinity columns. This result suggests that our plasma sample preparation allowed for a successful proteomics analysis and also maintained high-abundance proteins within clinically useful ranges.

Apos are predominantly produced in the liver and can transport lipids through the lymphatic and circulatory systems. Plasma levels of Apo are indicators of many diseases, including cancer, cardiovascular disease, and autoimmune disease [20–22]. Abnormal concentrations of some Apos (A-1 and E) are observed in the plasma of ARDS patients and during sepsis and trauma, high-risk factors of ARDS [7,23]. Hence, individual Apo proteins can be used to indicate ARDS but not with complete specifically. In this study, protein enrichment analysis revealed that five lipid transport

Table 2
Information of differentially expressed proteins in ARDS.

Accession numbers	Protein names	Coverage (%)	M.W.[c] (kDa)	Fold change[d]	
				AD[a]	AI[b]
IPI00029739	Isoform 1 of complement factor H	16	31	−1.6	−1.5
IPI00021841	Apolipoprotein A-I	69.7	47	−1.5	−1
IPI00022463	Serotransferrin	43.6	516	−1.3	−1
IPI00021857	Apolipoprotein C-III	27.3	47	−1.1	−1
IPI00022229	Apolipoprotein B-100	23.1	51	−0.7	−1.2
IPI00021856	Apolipoprotein C-II	40.6	77	−0.3	−1.9
IPI00304273	Apolipoprotein A-IV	48	24	−0.3	−1.5
IPI00032328	Isoform HMW of kininogen-1	22.8	14	0.1	−1
IPI00641737	Haptoglobin	38.6	38	0.7	1
IPI00553177	Isoform 1 of alpha-1-antitrypsin	61	11	1	1.2
IPI00022395	Complement component C9	22.4	45	1	1
IPI00022429	Alpha-1-acid glycoprotein 1	40.8	25	1.2	1.5
IPI00022417	Leucine-rich alpha-2-glycoprotein	29.1	63	1.4	1.5
IPI00847635	Alpha-1-antichymotrypsin	47.5	139	1.5	1.5
IPI00022389	Isoform 1 of C-reactive protein	23.2	11	2.3	2.9
IPI00552578	Serum amyloid A protein	40.2	25	2.8	3.3

[a] ARDS from direct lung injury.
[b] ARDS from indirect lung injury.
[c] M.W. molecular weight.
[d] Log 2 based fold change.

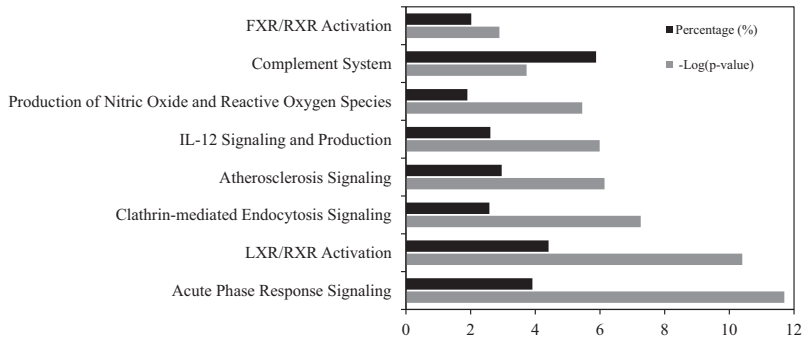

Fig. 4. Enriched canonical IPA pathways of the overlapped differentially expressed proteins within all ARDS samples. Gray bars indicate the negative value of log(*p*-value) for each function. Black bars represent the percentage of proteins in a given pathway that meet cut criteria to the total number of proteins that make up that pathway.

proteins were over-represented in our dataset, which suggests that Apos are correlated with ARDS pathophysiology. There are reports that Apos can regulate the host immune response by a variety of mechanisms, such as lipopolysaccharide (LPS) or endotoxin binding and neutralization, inhibition of adhesion molecule expression, or stimulation of endothelial nitric oxide synthase production [24]. Although the functional role of most Apos during ARDS remains unclear, some reports suggest that Apo A-I is involved binding LPS; these reports are based on analysis of animal models and clinical investigations [25]. Despite the variation in binding affinity, Apos bind LPS in vitro, which indicates that they may function as anti-inflammatory proteins like Apo A-I [24]. Of the five lipoproteins, Apo A-IV, C-II, and B-100 were down-regulated in AI samples. The difference is likely related to with the type of insult that led to ARDS. Direct insults primarily affect the alveolar epithelium causing a local alveolar inflammatory response, while indirect insults affect the vascular endothelium and cause a global inflammatory response [26]. The lower number of Apo proteins in the AI group indicates that a less extensive inflammatory response occurs compared with the AD group.

Typically, activation of the complement system lyses bacteria, recruits immune cells to infected tissue, and increases phagocytic clearance. In fact, complement activation can directly contribute to vascular leakage, which was believed to underlie ARDS pathology [27]. Abnormal concentrations of complement components, including complement C3, C9, and factor B are also observed in plasma and BALF from clinical ARDS samples [7,8]. In our experiment, we found that complement proteins were significantly enriched, indicating that they are correlated with ARDS.

By analyzing the 11 overlapping proteins, we found that "acute phase response" was the most significant canonical pathway. Changes in acute phase proteins (APPs) in the plasma can be triggered by infection, tissue injury, neoplastic growth, and immunological disorders [28]. Therefore, single APPs would not typically be used as disease-specific biomarkers. However, we believe it is necessary to evaluate the usefulness of APP profiles as biomarkers for diagnosing ARDS. AAPs are multifunctional and contribute to both the enhancement and inhibition of inflammation in different disease states. ARDS is most likely to be determined by the orchestrated generation of a specific profile of APPs at unique concentrations and in different molecular forms. This speculation was partially supported by the following observations. APPs, alpha 1-antitrypsin, haptoglobin, serum amyloid A, transferrin, and vWf, are frequently observed in the plasma of ARDS patients, while APPs, transthyretin, C-reactive protein, and haptoglobin are more likely to be detected during sepsis [7,8,29,30].

In conclusion, 16 proteins exhibited abnormal concentrations in the plasma of patients with ARDS. Some of these proteins, including APPs, Apos, and complement components, are related to diseases, such as sepsis, trauma, cardiovascular disease, and bacterial pneumonia. Therefore, we suggest that abnormal concentrations of individual proteins are not specific indicators of ARDS but that profiles of several proteins may be indicators of ARDS. Different profiles should be systemically evaluated for ARDS-specific protein expression in various clinical samples, especially samples from patients at high risk for sepsis and trauma. Such profiles may be useful as part of a diagnostic panel. Finally, the identification of these proteins provides new insight into the potential pathological mechanism underlying ARDS.

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