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ORIGINAL RESEARCH

Proteome Profiling of Red Blood Cells from Patients with COPD Links Proteasome Activation with Abnormal Cell Morphology and Function

Xi-Long Wang[1,*], Kai Zhong[1,*], Rui Li[1,*], Lin-Hui Huang[1,2], Guan-Jin Chen[1], Jin-Wei Chai[1], Xin Chen[1]

[1]Department of Pulmonary and Critical Care Medicine, Zhujiang Hospital, Southern Medical University, Guangzhou, People's Republic of China; [2]Department of Pulmonary and Critical Care Medicine, Hainan General Hospital (Hainan Affiliated Hospital of Hainan Medical University), Haikou, Hainan, People's Republic of China

*These authors contributed equally to this work

Correspondence: Jin-Wei Chai; Xin Chen, Department of Pulmonary and Critical Care Medicine, Zhujiang Hospital, Southern Medical University, Guangzhou, 510282, People's Republic of China, Tel +86 20 62782296, Fax +86 20 61643010, Email vivian20@i.smu.edu.cn; hxkd006@smu.edu.cn

Introduction: Chronic obstructive pulmonary disease (COPD) is characterized by progressive airflow obstruction and persistent respiratory symptoms. Molecular and cellular changes identified in red blood cells (RBCs) of COPD patients may contribute to the pathophysiology of COPD, impacting oxygen transport and systemic inflammation.

Methods: We performed a comparative proteomic analysis on RBCs from 15 male COPD patients and 15 age- and sex-matched control subjects. For the proteomic analysis, individual samples were randomly pooled into 3 biological replicates per group (n = 3). Total RBC proteins were analyzed using tandem mass tag (TMT) labeling followed by LC-MS/MS. Differentially abundant proteins (DAPs) were identified and subjected to Gene Ontology (GO), KEGG pathway, and protein-protein interaction (PPI) network analyses.

Results: We identified 160 DAPs (70 up-regulated, 90 down-regulated) in the RBCs of COPD patients. GO analysis revealed enrichment in processes related to protein stability regulation and immune response. KEGG pathway analysis showed that up-regulated proteins were most significantly enriched in the proteasome pathway, while down-regulated proteins were enriched in complement and coagulation cascades. Notably, a PPI network analysis highlighted a core complex of 10 up-regulated proteins that are all components of the proteasome regulatory particle.

Conclusion: This study provides the in-depth RBC protein profile in COPD, identifying proteasome activation as a key molecular signature. These findings reveal novel biomarkers linked to RBC dysfunction that may contribute to the systemic pathology of COPD and offer potential new therapeutic targets.

Keywords: COPD, red blood cell, proteasome, proteomics

Introduction

Chronic obstructive pulmonary disease (COPD), a common and complex disease characterized by persistent airflow limitation and persistent respiratory symptoms, is a leading cause of mortality worldwide.[1] While cigarette smoking is the leading cause of COPD, other environmental exposures and genetic factors also contribute to its development.[1] It is known that the severity and prognosis of COPD are resulted from lung involvement and multiple comorbidities including cardiovascular disease, musculoskeletal impairment, diabetes mellitus, etc.[2–4] Previous evidences suggest that multiple factors including hypoxic condition, oxidative stress, inflammation, infection and aging are associated with the increased occurrence of COPD comorbidities which then promote COPD development.[5–9] Of these factors, systemic hypoxia might be both a cause and a consequence of COPD development. Therefore, further investigations on the cellular and molecular causes of hypoxia in COPD should help to understand the pathogenesis of this disease and its comorbidities.

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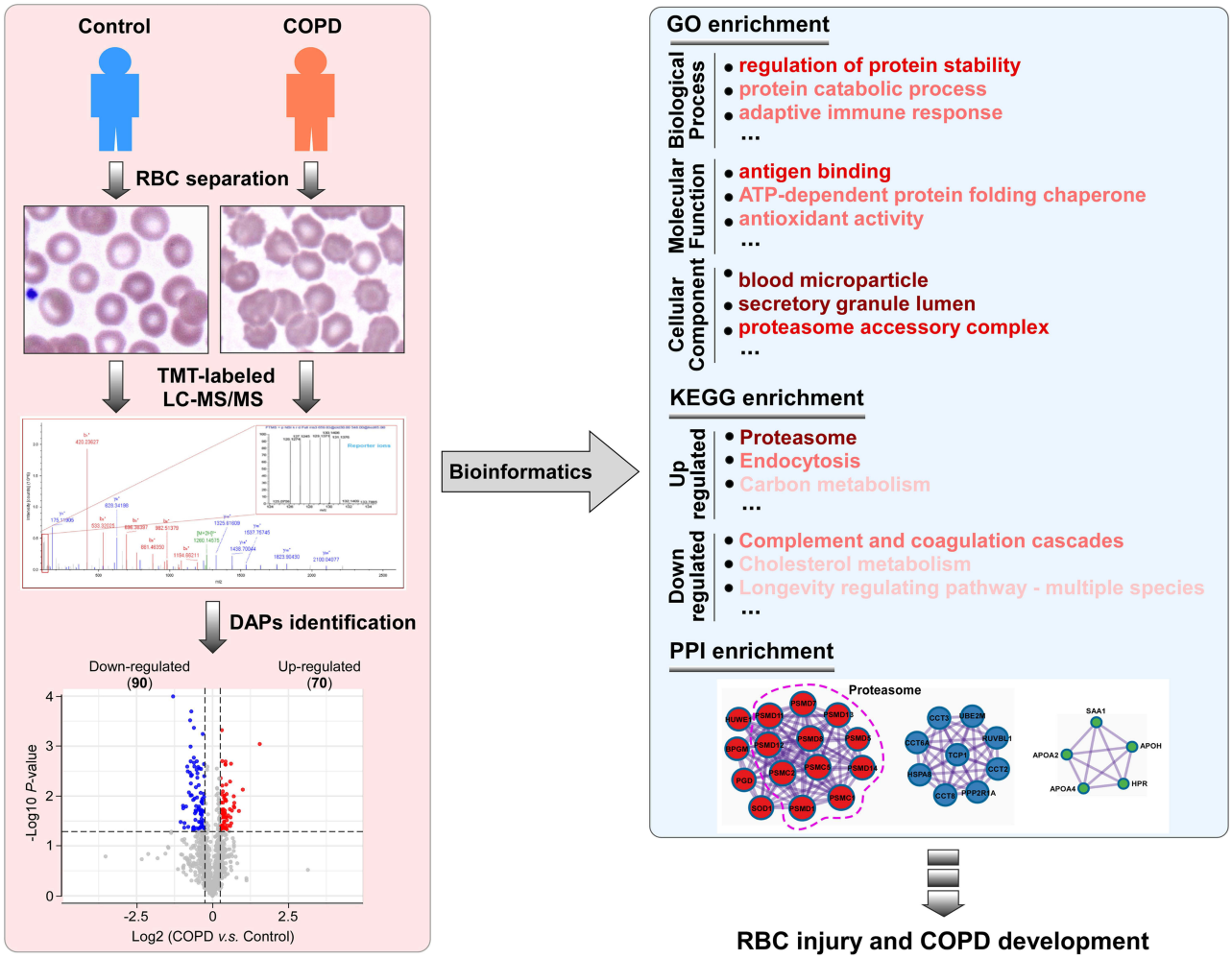
Graphical Abstract



Red blood cells (RBCs) are critical for transporting oxygen from the alveolus to other tissues, and their dysfunction has been associated with the severity and prognosis of COPD.[10–12] In the hypoxic and high-oxidative-stress environment of COPD, RBCs undergo structural changes that impair oxygen transport.[12,13] Several studies have shown that structural and functional alterations in RBC in COPD play an important role in muscle weakness and vascular damage through promoting oxidative stress and inflammation.[14,15] RBC-derived iron significantly contributes to pulmonary iron overload in COPD, dysregulating alveolar macrophage polarization.[16] Furthermore, changes in protein levels and/or modifications in COPD have been shown to promote RBC injuries due to defects in adenosine metabolism, arginine availability and antioxidant defense system.[17–19] Therefore, it is possible that some dysregulated proteins in RBCs of COPD patients may impair oxygen transport and other cellular functions which are associated with COPD development and its comorbidities. By a comparative proteomics focusing on the RBC membrane of COPD with smoking behavior, a previous study identified that a subset of differentially abundant proteins (DAPs) was associated with RBC deformation and methemoglobinemia.[20] Mature RBCs are incapable of de novo protein synthesis, due to the lack of cell nucleus and most of the organelles. Given the high abundance of proteasome in mature RBCs, proteasome-mediated proteolysis may represent the primary mechanism for maintaining protein homeostasis.[21] In end-stage COPD patients (GOLD 4), lung tissue proteasomes exhibit severe functional impairment compared to healthy controls,

concomitant with significant aggregation of ubiquitinated proteins.[22,23] But upregulated expression of proteasome in peripheral blood mononuclear cells (PBMCs), triggering inflammatory cytokine stimulation.[24] These findings collectively indicate systemic dysregulation of the ubiquitin-proteasome system (UPS) in COPD pathogenesis. Although several studies have explored the proteomic profiles of RBCs in various diseases,[25–27] a comprehensive proteomic analysis of RBCs specifically in COPD patients has not been previously reported. Given the emerging role of RBC dysfunction in systemic manifestations of COPD, including oxidative stress and inflammation, a deeper understanding of RBC proteomic alterations in this disease may provide novel insights into disease pathogenesis and potential therapeutic targets.

Using comparative proteomics, this study identified 160 DAPs from the total RBC proteins of enrolled COPD patients with an increased proportion of echinocytes. Bioinformatical analyses indicated that some of the DAPs are mainly involved in regulation of protein stability, proteasomal complex, immune and inflammatory response, which might play important roles in RBC deformations and functions linking to COPD development. Thus, this study provides some RBC-specific biomarkers and therapeutic targets for COPD and its related disorders. Furthermore, while COPD has historically been more prevalent in men, recent evidence suggests that women may have different susceptibility and clinical manifestations.[28] The present study focuses on male patients to establish a baseline proteomic profile in a hormonally consistent group, acknowledging that future research will be crucial to investigate potential sex-specific differences in RBC pathology in COPD.

Materials and Methods

Participants

The general process of this study was summarized in Figure 1. A total of 15 male COPD patients and 15 age- and sex-matched control subjects were recruited in the Zhujiang Hospital between June 2021 and May 2022. As shown in Table 1, these COPD patients with normal weight and forced expiratory volume in the first second (FEV_1)/ forced vital capacity (FVC) < 0.7 after bronchodilator inhalation. The COPD-free control group (control subjects) was smoking-history-matched individuals. Exclusion criteria were additional respiratory diseases, malignant diseases, diabetes mellitus, thyroid disease, severe psychiatric illnesses, mental disorders, unstable cardiovascular disease, liver diseases, kidney diseases, inflammatory diseases, hematological diseases, and drug abuse. Baseline demographics were summarized in Table 1. The study was approved by the ethics committee of the Zhujiang Hospital (no. 2021-KY-060-01) and was conducted in accordance with the Declaration of Helsinki. Informed consent was given before the inclusion of subjects in the study.

Morphological and Biochemical Detections of RBCs

Blood film samples were stained with Modified Giemsa Staining Solution (Beyotime, China) and were observed for RBC morphology under light microscope. The images scanned with a Pannoramic Digital Slide Scanner (3DHistech, Hungary) using CaseViewer 2.3 (3DHistech). The abnormal morphs were named according to the international nomenclature of RBC morphological features.[29] Blood routine examinations were performed using the whole blood by a routine blood test instrument (RJ-0C107223, Mindray, China). Blood routine examinations parameters (including red blood cell count, hemoglobin, red cell distribution width (RDW), hematocrit, etc) were determined in the laboratory department of the Zhujiang Hospital of Southern Medical University.

Sample Collection and Preparation

Fresh venous blood samples from the COPD patients and non-COPD control subjects were collected into tubes containing EDTA, and centrifuged at 1500×g for 5 min. The plasma and buffy coat were removed and the RBCs were washed with phosphate-buffered saline (PBS) four times as previous descriptions.[30] Then the samples were lysed with Red Blood Cell Lysis Buffer (Biosharp, China) according to Prabakaran et al.[31] The erythrocytic lysates were centrifuged at 1500×g for 5 min and the supernatants were collected. Hemoglobin in the samples were removed by Hemovoid depletion columns (Biotech support group, Monmouth Junction, NJ, USA) and then were immediately stored at −80°C.

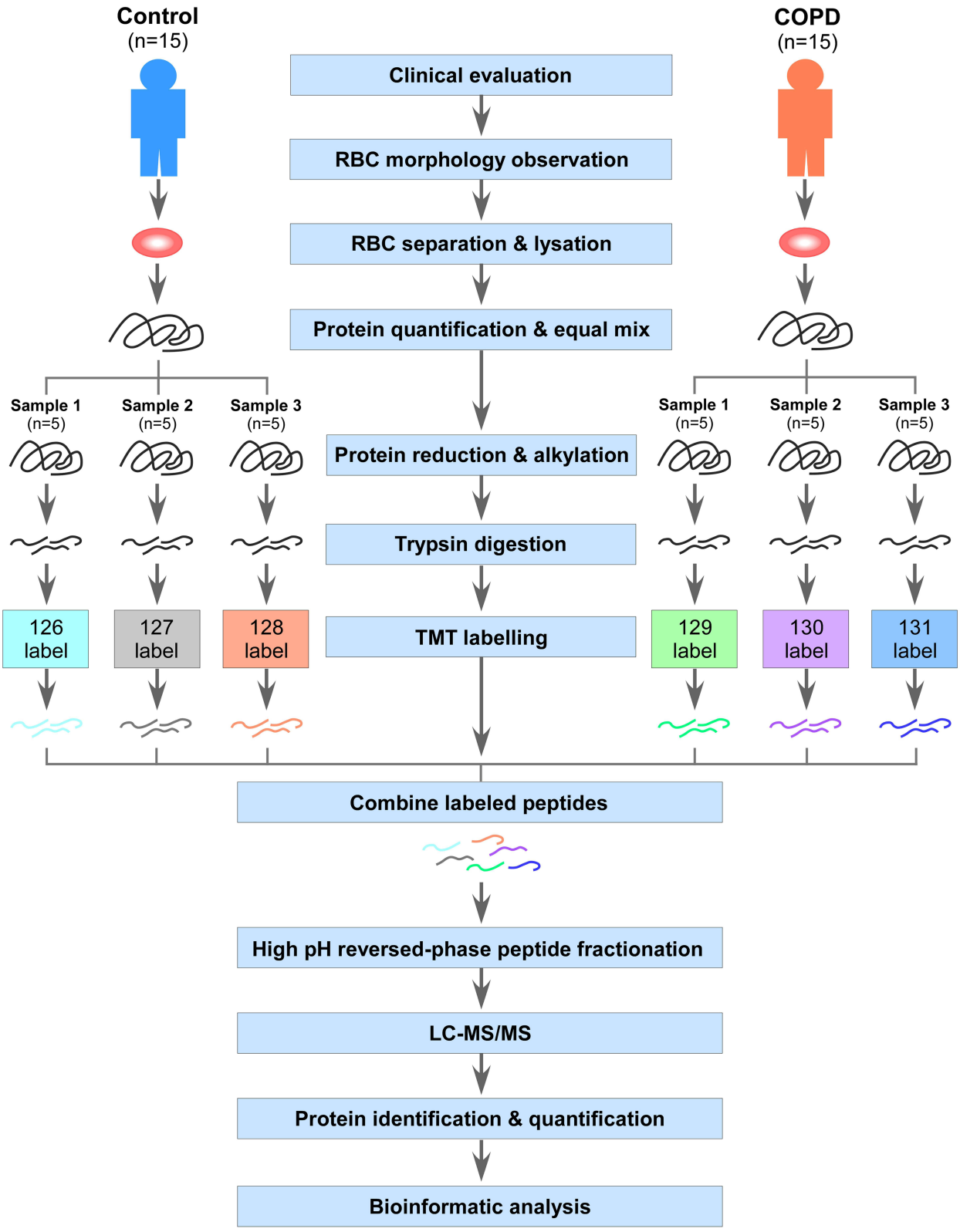


Figure 1 An experimental workflow for protein profiling of RBCs from pooled samples (n = 3) of 15 COPD patients and 15 control subjects.
Abbreviations: COPD, chronic obstructive pulmonary disease; RBC, red blood cell.

Table 1 Demographic and Baseline Characteristics of All Subjects

Characteristics	Control	COPD	*P* value
Number	15	15	
Age	68.1±9.4	69.6±8.6	0.595
Height	1.68±0.05	1.63±0.04	**0.022**
Weight	62.1±7.7	57.6±8.5	0.136
BMI	22.1±1.9	21.7±3.4	0.727
Smoking history	13 (86.7%)	13 (86.7%)	
Current smokers	9 (60%)	6 (40%)	
Pack year	37.5±14.7	46.7±19.7	
Pulmonary function test			
FEV_1% pred (%)	95.9±12.1	37.62±17.9	**<0.001**
FVC% pred (%)	94.9±11.9	61.92±16.0	**<0.001**
FEV_1/FVC (%)	78.4±4.6	46.4±13.1	**<0.001**

Notes: Data are expressed as mean ± standard deviation and number (%). Results were considered significant at *P*-value < 0.05 (displayed in bold).
Abbreviations: BMI, body mass index; FEV_1, forced expiratory volume in 1 second; FVC, forced vital capacity; FEV_1% pred, predicted percentage value of forced expiratory volume in one second; FVC% pred, predicted percentage value of forced vital capacity.

Labeling and Fractionation

To reduce the individual differences within the group, the 15 COPD proteins and 15 control proteins were randomly pooled into 3 samples (5 subjects per sample), respectively. Therefore, the subsequent proteomic analysis was conducted with a sample size of n = 3 for the COPD group and n = 3 for the control group. The random pooling strategy was employed to identify a core proteomic signature by averaging out biological variance within each group. The pooled samples were labeled by TMT reagent according to the manufacturer's instructions. The samples of the control groups were labeled as TMT-126, TMT-127 and TMT-128, while the COPD groups were labeled as TMT-129, TMT-130 and TMT-131.

Fractionation was performed using Reversed-Phase Peptide Fractionation Kit (Thermo Scientific) according to the manufacturer's instructions. Briefly, the labeled peptides were firstly dried, reconstituted, acidified and transferred into fractionation spin column. And then, the fractionated peptides were bound to the hydrophobic resin under aqueous conditions and desalted with water. Finally, the bound peptides were eluted by volatile high-pH elution solution and desalted on Empore™ SPE Cartridges C18 (Sigma).

LC-MS/MS Analysis

LC-MS/MS analysis was performed on a Q Exactive mass spectrometer (Thermo Scientific) with Easy nLC (Proxeon Biosystems, now Thermo Fisher Scientific). The peptide mixtures were loaded onto an Acclaim PepMap100 nanoViper C18 column connected to the C18 reversed-phase analytical column (Thermo Scientific Easy Column, 10 cm long, 75 μm inner diameter, 3 μm resin) and separated using a linear gradient of buffer B (84% acetonitrile and 0.1% formic acid). The mass spectrometer data were acquired using the higher-energy collision dissociation (HCD) method. The survey scan (300–1800 m/z) was produced from the survey scan (300–1800 m/z) to choose the most abundant precursor ions. The MS1 scans were acquired at a resolution of 70,000 at 200 m/z with Automatic gain control (AGC) target at 3e6, maximum inject time at 10 ms, and dynamic exclusion duration at 40.0 s. MS2 scans were acquired by a "Top20" data-dependent method using the following parameters: resolution 17,500 at 200 m/z, isolation width 2 m/z, normalized collision energy 30 eV, and underfill ratio 0.1%.

Protein Identification and Quantitation

MASCOT engine (Matrix Science, London, UK; version 2.2) embedded into Proteome Discoverer 1.4 software was used for identification and quantitation. Following parameters and instructions were applied: the database searching was

performed from Swissprot (Swissprot_human_20368_20200217). The specified digesting enzyme was trypsin and the max missed cleavages were 2. Carbamidomethylation (C) and TMT6 plex (N-terminal and lysine, K) were defined as fixed modifications. The methionine oxidation was set as variable modification. Peptides mass tolerance was set within a window of 20 ppm and fragment mass tolerance was set at 0.1 Da. The peptide FDR was adjusted to ≤ 0.01. Proteins were considered differentiated at the ratio of COPD/ Control≥ 1.2 or ≤ 0.8 and *P*-value < 0.05.

Bioinformatics and Statistical Analysis

Hierarchical clustering analysis was performed using Cluster 3.0 (http://bonsai.hgc.jp/~mdehoon/software/cluster/software.htm) and Java Treeview software (http://jtreeview.sourceforge.net) by selecting Euclidean distance algorithm for similarity measure and average linkage clustering algorithm. The hierarchical clustering analysis was presented visualized by a global heat map. The gene ontology (GO) analysis was performed using the software program Blast2GO to identify the Biological Process, Molecular Function and Cellular Component of genes and gene products. To assess pathways, the KAAS (KEGG Automatic Annotation Server) was used to perform KEGG annotation. The protein-protein interaction (PPI) information was retrieved from Metascape (http://metasape.org) to predict protein-protein interactions and visualized and analyzed by Cytoscape software (http://www.cytoscape.org/, version 3.2.1).[32]

Numeric data were presented as mean ± standard deviation (SD) and categorical data were presented as numbers (percentages). Student's *t*-test was used to calculate the statistical significance of age and BMI. Fisher's exact test was used to analyze sex, past medical history and family history. All statistical analyses were performed with SPSS 20.0. Statistical significance was considered when the *P*-value was < 0.05.

Result

Characterization of the Enrolled COPD Patients and Control Subjects

To minimize the analyzed biases as much as possible, 15 male COPD patients and 15 age- and sex-matched control subjects were enrolled in this study. As shown in Table 1. No significant difference in body parameters and smoking behavior was observed between the two groups. The FEV_1% pred, FVC% pred and FEV_1/FVC of the patients were significantly lower ($P < 0.001$) than those of the control subjects, suggesting that the candidates meet the main clinical characteristics of COPD.

Morphological and Biochemical Assessments of the RBCs in COPD

Morphological analyses showed that ~30% RBCs from the COPD patients presented as echinocytes (Figure 2A), significantly higher than that of the control group (Figure 2B). In addition, the red cell distribution width (RDW) and hematocrit in the COPD patients was relatively high and low compared to that of the control subjects, respectively (Figure 2C and D), suggesting the increased heterogeneity and disruption of RBCs in COPD. Whereas no significant difference in RBC number, mean cell volume and hemoglobin concentration was observed between the two groups (Figure 2E–G).

Evaluation of the RBC Proteomes of the COPD and Control Subjects

We firstly evaluated the basic indexes of the identified peptides from the total proteins of RBCs. The LC-MS/MS data showed that the mass errors of most peptides were distributed within 10 ppm (Figure 3A), and the IonScores of 75.7% of peptides were more than 20 (Figure 3B). In addition, the COPD/Control ratios of the number of peptides were nearly equal (Figure 3C), suggesting high quality of the MS1 and MS2 spectra. By searching proteins in the database, we found that a total of 5846 peptides identified from the RBCs were matched to 1138 proteins at a cutoff FDR ≤ 0.01 (Table S1). Of them, 160 DAPs were identified from the COPD RBCs with 70 up-regulated and 90 down-regulated proteins (Figure 3D, Table S1). Additionally, hierarchical cluster analysis of all the DAPs indicated a credible biological repeatability in this study (Figure 3E). To determine the similarities and differences between the identified RBC proteome and other datasets, we firstly compared our data against two previously identified RBC proteomes. We found that 716 and 778 of all the 1138 identified proteins were overlapped with the datasets from Sae-Lee et al[21] and Bryk et al,[33] respectively; and more than half (654 proteins) of them were in common with the two datasets (Figure 4A, Table S2).

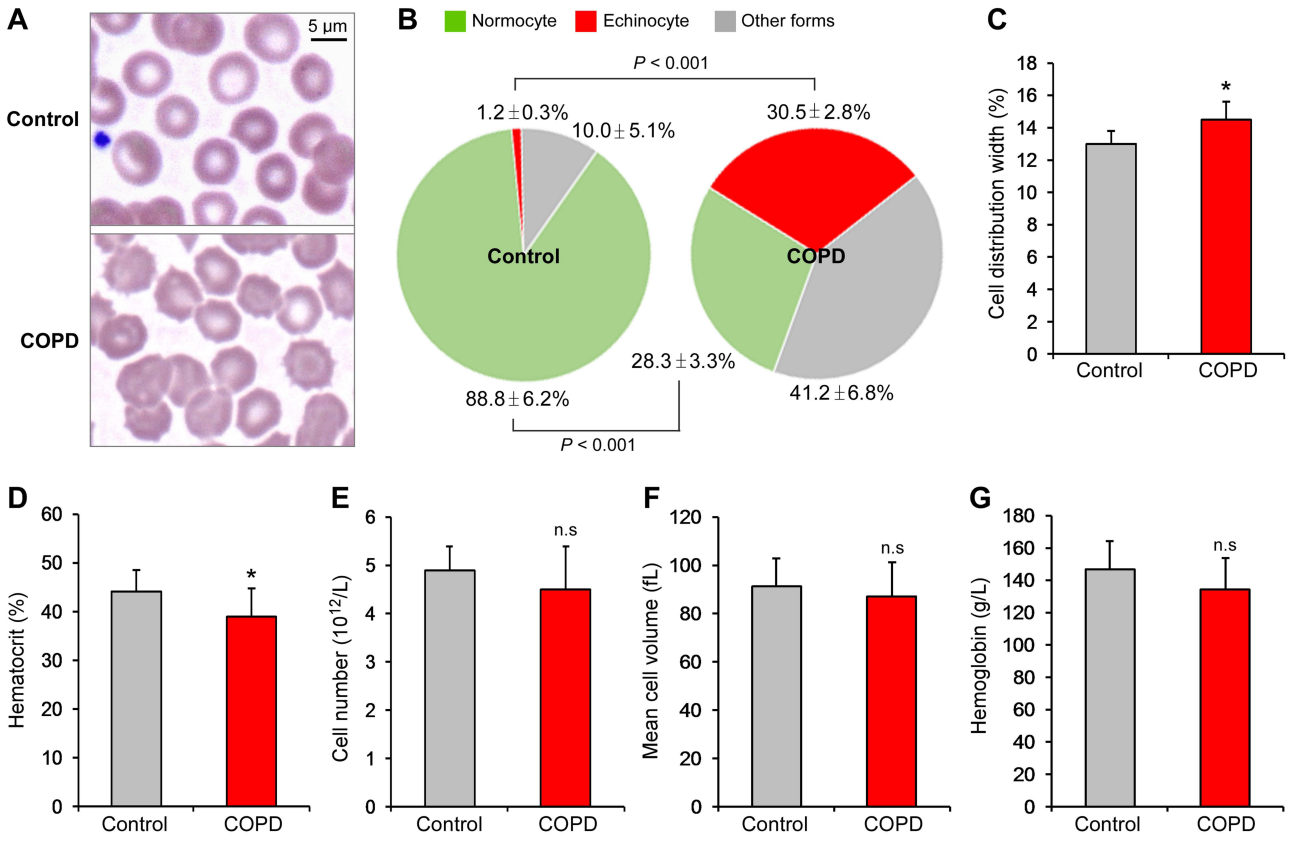


Figure 2 Increased dysmorphology of the erythrocytes from COPD patients. (**A**) Representative images of the erythrocytes using a light microscope. (**B**) Percentage of normocytes, echinocytes and other forms of erythrocytes. (**C–G**) Cell distribution width and hematocrit and were significantly lower in COPD patients (n = 15), compared to COPD-free control (n = 15). The red cell number, mean cell volume, and hemoglobin were lower in COPD patients (but not statistically different). * $P < 0.05$. Scale bars = 5 μm.
Abbreviations: ns, not significant; COPD, chronic obstructive pulmonary disease.

Furthermore, 86 and 93 of 160 DAPs in our study were respectively overlapped with the previous dataset by Sae-Lee et al[21] and by Bryk et al[33] (Figure 4B, Table S2). Comparisons of our dataset against RBC cytoplasmic proteins identified by Roux-Dalvai et al[30] and membrane proteins identified by Alexandre et al[20] found that 72 and 9 DAPs were shown as cytoplasmic protein and membrane protein, respectively (Figure 4C, Table S2).

Functional Characterization of the DAPs in COPD

We firstly characterized all the 160 DAPs by GO enrichment analysis. As shown in Figure 5 and supplementary Tables S3–S5, regulation of protein stability (16 proteins), protein catabolic process (21 proteins), and adaptive immune response (19 proteins) represented the top 3 enriched terms of biological process (Figure 5A); and the most enriched terms of molecular function were antigen binding, ATP-dependent protein folding chaperone, and antioxidant activity (Figure 5B). For cellular component, The DAPs were mainly enriched in blood microparticle, secretory granule lumen, and proteasome accessory complex (Figure 5C). Together, GO functional enrichment analyses suggest that the COPD-associated DAPs are primarily involved in the regulation of protein stability and immune and oxidative responses.

We then performed KEGG pathway enrichment analyses and found that the down-regulated proteins were mainly enriched in complement and coagulation cascades, cholesterol metabolism and longevity regulating pathway (Figure 6A, Table S6), and the up-regulated proteins were outstandingly enriched in proteasome (Figure 6B, Table S7). An illustration of the proteasome pathway (hsa03050) showed that all the 10 proteasome-associated up-regulated proteins (including PSMC1, PSMC2, PSMC5, PSMD1, PSMD7, PSMD8, PSMD11, PSMD12, PSMD13, PSMD14) presented in the regulatory particles of the proteasome complex (Figure 6C), suggesting an enhancement of proteasome activity under COPD condition (Figure 6B, Table S7).

https://doi.org/10.2147/COPD.S531220

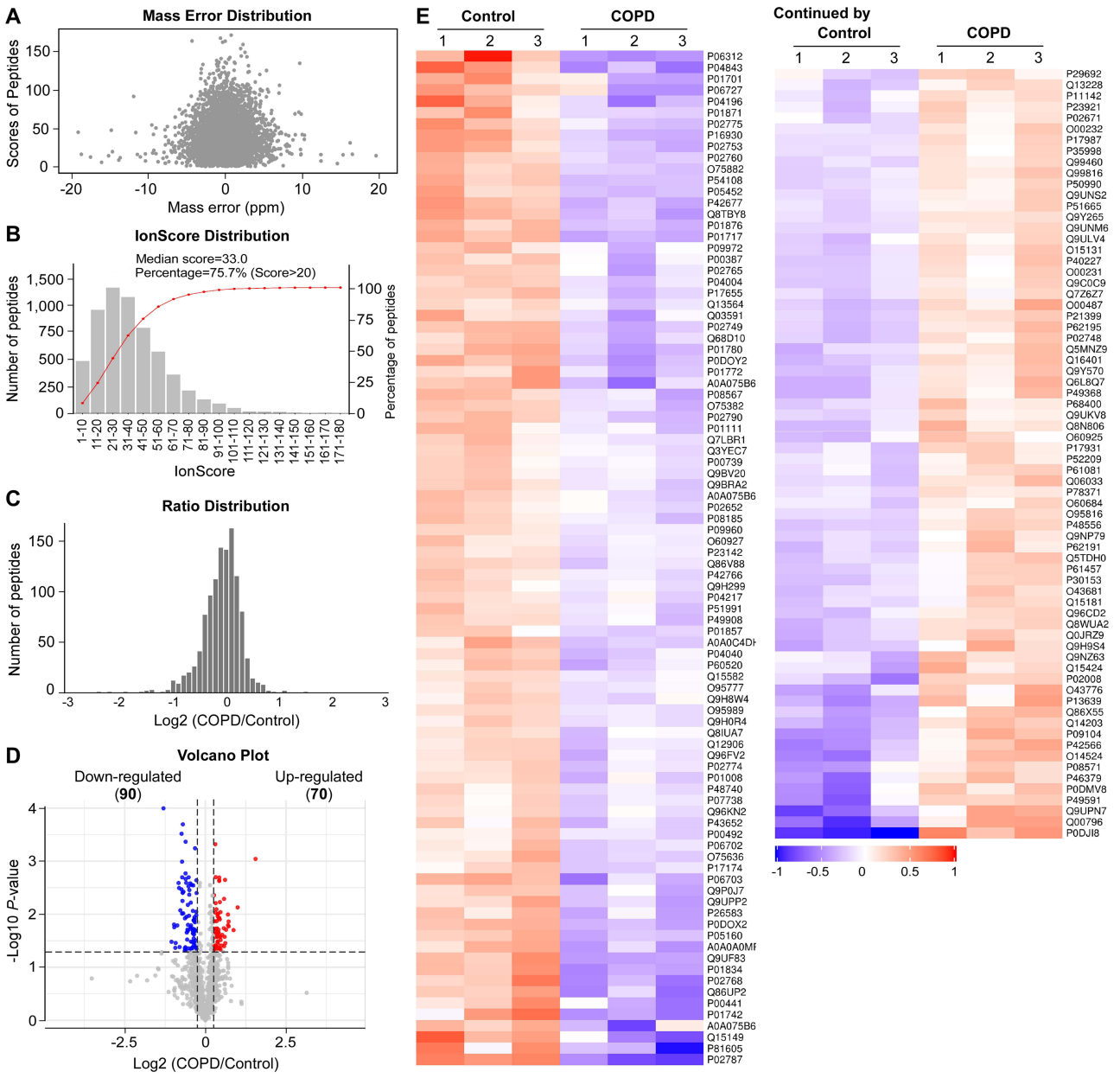


Figure 3 Evaluation of the identified peptides from RBCs. (**A**) Mass error distribution of all identified peptides. (**B**) IonScore distribution of all identified peptides. (**C**) Distribution of the ratio between chronic obstructive pulmonary disease (COPD) patients/ COPD-free control subjects. (**D**) Volcano plot highlights the significant proteins increasing (red nodes) or decreasing (blue nodes) in RBCs from the COPD patients as compared with the controls. (**E**) A heatmap showing the hierarchical clustering analysis of the differentially abundant peptides between COPD patients and controls by *t* test.
Abbreviation: RBCs, red blood cells.

By the enrichment of PPI networks with all the DAPs, we identified 3 enriched PPI networks (Figure 7). Notably, all the proteasomal components presented in the most enriched PPI network (Figure 7).

Discussion

Although several studies have identified human RBC proteomes highlighting some important proteins involved in RBC structure, functions, and disorders,[20,21,31] there lacks an in-depth analysis of RBC proteins upon COPD condition. Therefore, this study for the first time identified an altered protein profile of the RBCs isolated from COPD patients, and these COPD-associated DAPs were mainly associated with the regulation of immune and oxidative response, protein stability and proteasomal activity. Especially, up to 10 up-regulated proteins were identified to be the critical components

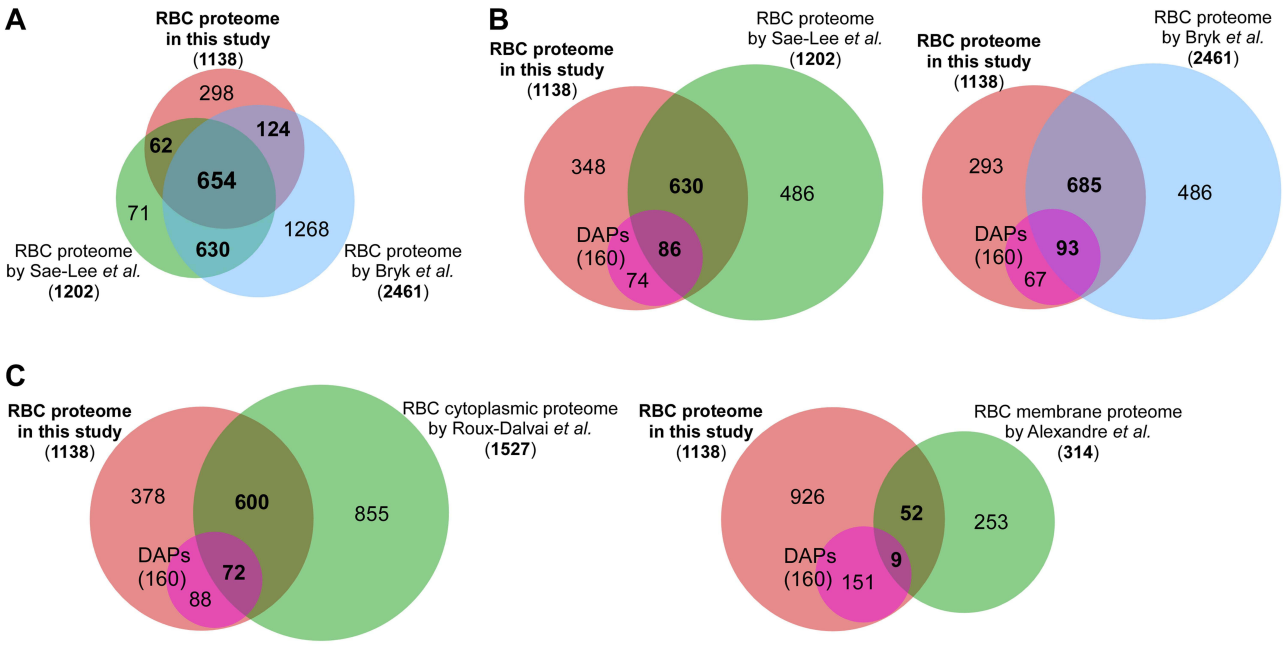


Figure 4 Comparison of RBC differentially abundant proteins (DAPs) in COPD against other datasets using a Venn analysis. (**A**) Comparisons of RBC proteins identified in the present study against previously reported RBC proteins. (**B**) Comparisons of the DAPs in this study against the previously identified RBC proteins from Sae-Lee et al[21] (left) and Bryk et al[33] (right). (**C**) Comparisons of the DAPs in this study against cytoplasmic proteome DAPs from Roux-Dalvai et al[30] (left). Comparisons of the DAPs in this study against RBC membrane proteome DAPs in COPD from Alexandre et al[20] (right). The RBC proteome in this study is in bold.
Abbreviations: RBC, red blood cell; DAPs, differentially abundant proteins.

of the regulatory particle of proteasome complex, suggesting an enhancement of proteasome activity in COPD. Given that abnormal cellular morphology and functions of the same samples from those COPD patients were observed, our findings reveal some COPD-associated molecular biomarkers regarding augmented immune and oxidative responses and proteasome-induced protein degradation, which might contribute to echinocytic formation, decreased oxygen transport and systemic immune inflammation. Therefore, this study should provide some pathophysiological insights into the development of COPD and its related disorders.

In this study, we found that the percentage of echinocytes is elevated in COPD patients by morphology on blood smear. The formation of echinocytes, as observed in our COPD cohort, is a well-documented morphological response of red blood cells to injurious stimuli.[13] A primary trigger for this transformation is increased systemic oxidative stress, a known hallmark of COPD.[12,13] This oxidative environment directly damages cellular components, including proteins and lipids.[34] Using scanning electron microscopy, a previous study also showed increased surface protrusion of the RBCs from COPD patients with a history of smoking, which function as a biosensor for monitoring oxidative imbalance.[12] Our control group consisted of individuals matched a history of smoking. This design was chosen deliberately to isolate the specific effects of the COPD disease state, distinct from the well-documented damage caused by smoking alone. Smoking is a major source of oxidative stress, known to decrease RBC deformability, increase lipid peroxidation, and damage membrane proteins.[35,36] When interacting with environmental factors, such as oxidative stress, RBCs are unable to initiate genetic responses. Instead, RBCs undergo chemical, morphological, and mechanical changes due to cell modifications.[37] In the echinocytes, it has been reported that oxyhemoglobin (oxy-Hb) and deoxyhemoglobin (deoxy-Hb) undergo redistribution, with deoxy-Hb predominantly occupying the central region,[13] resulting in reduced oxygen-releasing capacity. The conversion to methoxy-hemoglobin (met-Hb) and the leakage of oxy-Hb are more obvious in the echinocytes,[13,38] resulting in reduced oxygen-carrying capacity. The redistribution of oxy-Hb and deoxy-Hb in the echinocyte is linked to cytoskeletal rearrangements that form spiny structures, a defining feature of these cells. Increased oxidative stress, which plays a central role in the pathogenesis and progression of COPD, is associated with cytoskeleton rearrangement and reduction in β-sheet content.[38,39] In the COPD erythrocyte, several DAPs may be responsible for morphological abnormalities and conversion of Hb forms in RBCs.[38] Among the proteins in Table S1,

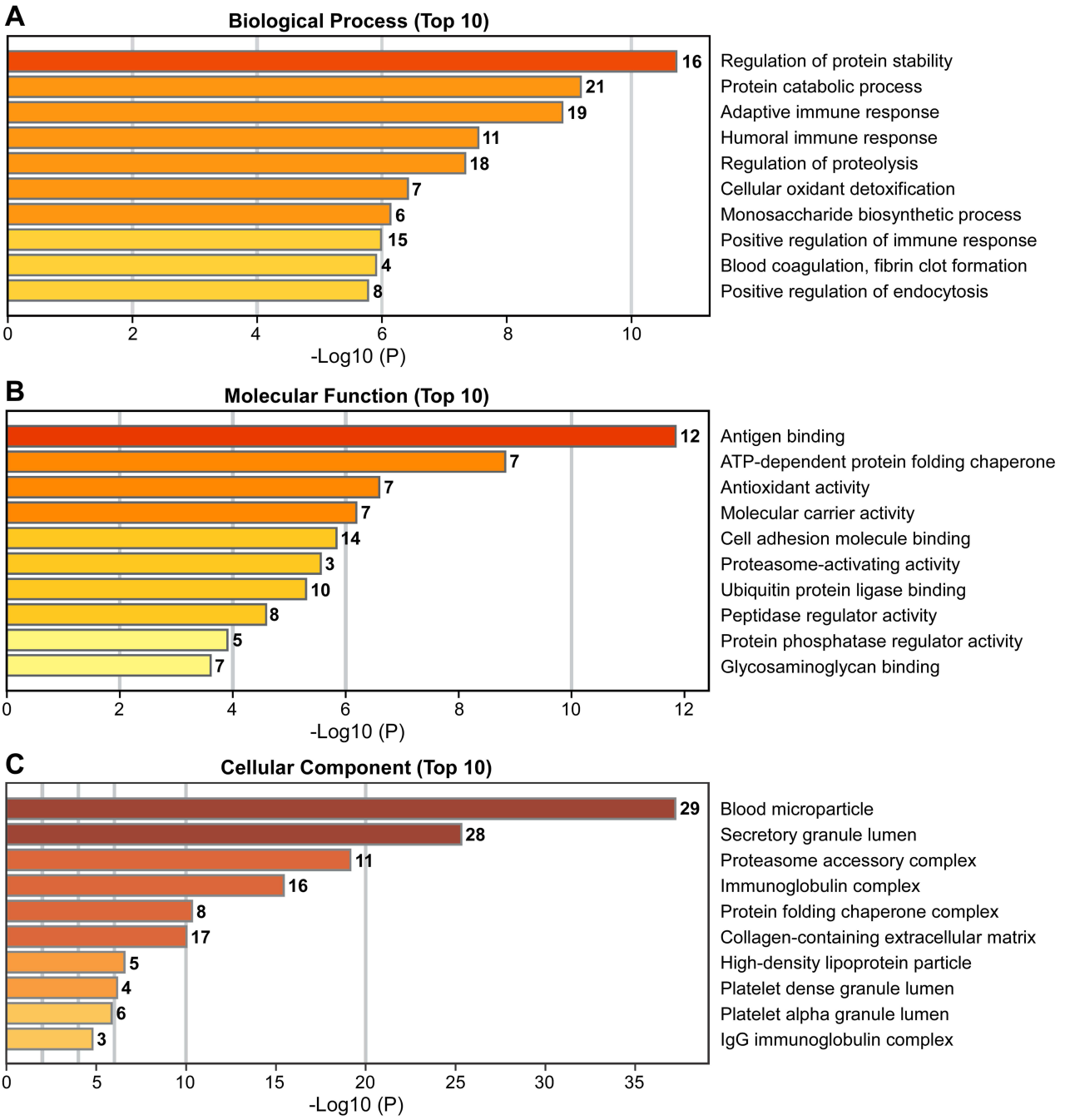


Figure 5 Gene ontology (GO) analysis of the altered RBC proteins in chronic obstructive pulmonary disease (COPD), categorized by GO annotations. GO enrichment showing the -log10 (*P*-value) (below the columns) and the numbers (right of the columns) of the altered proteins of the enriched GO terms including (**A**) biological process, (**B**) molecular function and (**C**) cellular component. Red boxes indicate the *P*-value of the pathway. The redder the color, the larger the -log10 (*P*-value). *P*-value represented the enriched degree.

Coronin-1C (CORO1C), which is related to organization of the cytoskeleton;[40] NADH-cytochrome b5 reductase 3 (CYB5R3), which is an iron reductase with the activity of recycling met-Hb to oxy-Hb.[41] The damage and dysfunction of RBCs are manifested to be abnormal RBC indices.[11] Our data indicated a significant increase and decrease in proportion of RDW and hematocrit, respectively, suggesting morphological abnormalities of RBCs in COPD patients. These abnormal hematological parameters are associated with a worse prognosis and reduced quality of life in COPD.[11,42] Moreover, the white blood cells and monocytes increased slightly in the COPD group, but not significantly (Table S8).

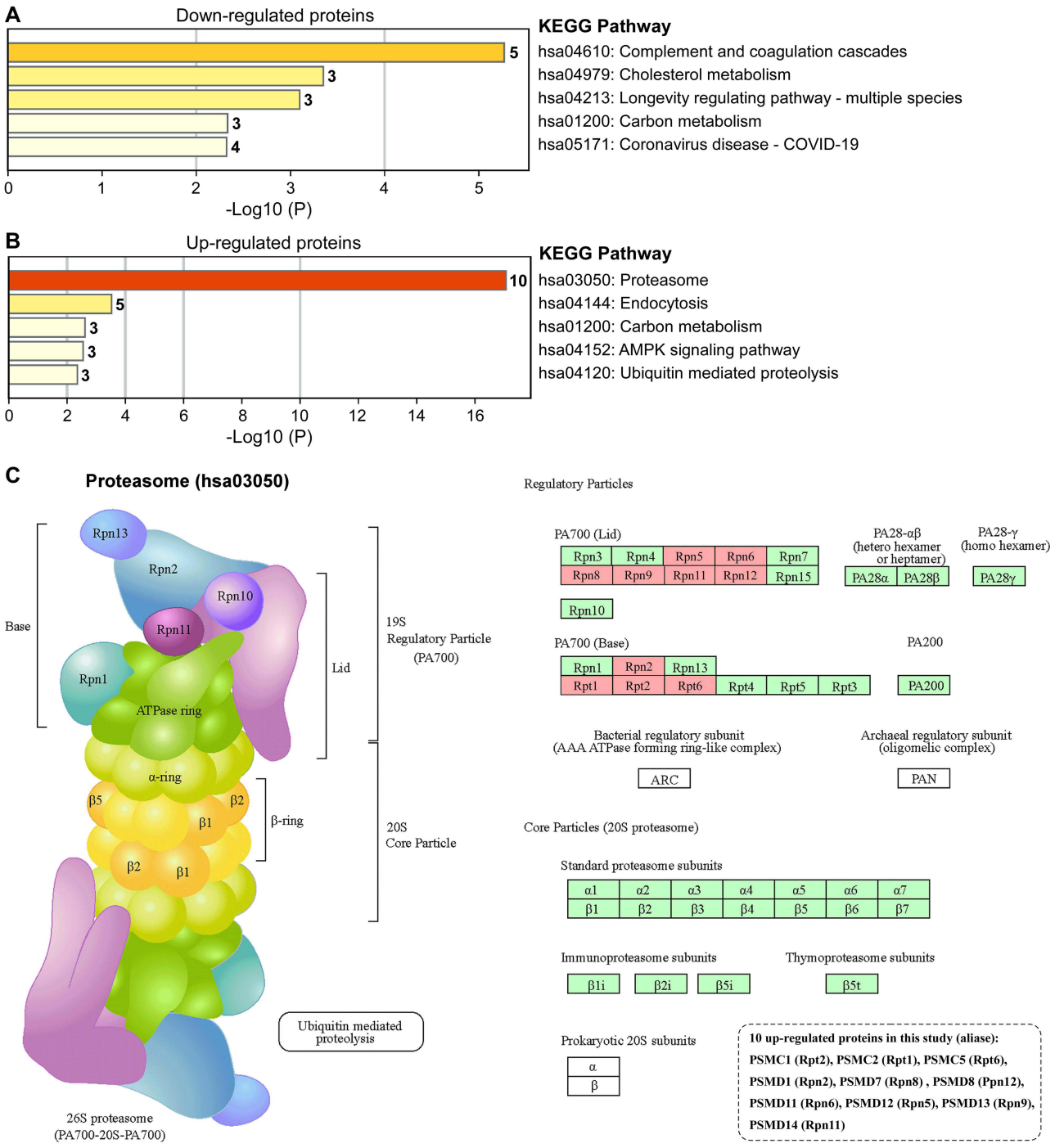


Figure 6 Enrichment of Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway with differentially abundant proteins (DAPs). KEGG enrichment showing the -log10 (*P*-value) (below the columns) and the numbers (right of the columns) of the altered proteins within enriched KEGG pathways for (**A**) down-regulated and (**B**) up-regulated proteins. Red boxes indicate the P-value of the pathway. The redder the color, the larger the -log10 (*P*-value). (**C**) Diagram of proteasome including the COPD-associated DAPs. Red boxes indicate the DAPs in the proteasome pathways. *P*-value represented the enriched degree.

Therefore, identification of the changes of RBC proteins upon COPD should be important to reveal the relationship between molecular change and dysregulation of RBC morphologies and functions.

While previous studies have partially profiled RBC membrane proteins in COPD, this study represents the first comprehensive proteomic analysis encompassing total RBC proteins, identifying novel, distinctively enriched biological pathways. Comparison with previously reported RBC proteomes revealed that ~70% of the identified proteins had been

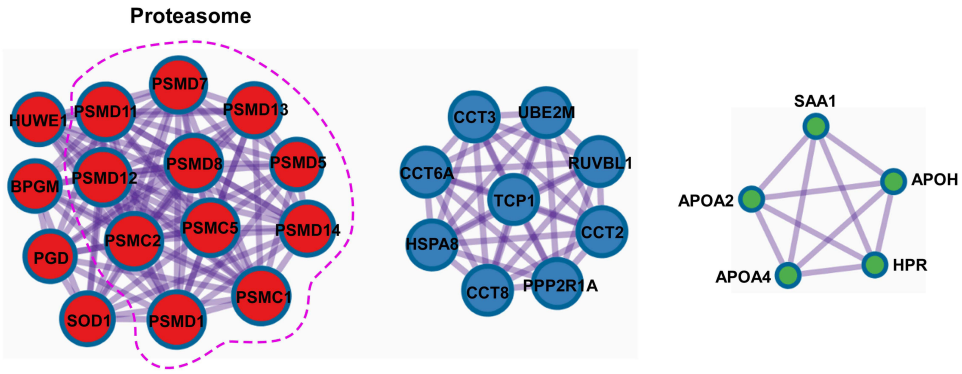


Figure 7 Molecular complex detection (MCODE) identification in the protein-protein interaction (PPI) network. Protein names in bold fonts indicated the components of the MCODE sub-clusters.

observed in RBCs from healthy volunteers, indicating the reliability of our proteomic data. The non-overlapping proteins may result from the differences in proteomics platforms and/or thorough removal of hemoglobin prior to proteome analysis in the present study. Additionally, among the 160 DAPs in our dataset, more than half were identified to be RBC cytoplasmic proteins, but only 9 DAPs were RBC membrane proteins, suggesting a substantial proportion of cytoplasmic proteins were dysregulated in response to COPD. Given that mature RBCs lack a nucleus, and transcription- and translation-related organelles for protein synthesis, the observed profile of DAPs may be a composite result of two main factors. Under a chronic hypoxia condition, the initial protein complement established during erythropoiesis can be influenced by the accumulation of hypoxia-inducible factors (HIFs).[43] The COPD-associated DAPs identified in the present study might be resulted from dysregulation of protein degradation. The presence of both up- and down-regulated proteins suggests that both mechanisms are likely at play.

By GO functional enrichment analysis with all the DAPs, this study identified that regulation of protein stability and protein catabolic process represented the most enriched biological processes, suggesting that an imbalance in homeostasis of RBC proteins may serve as a hallmark and underlying pathogenic mechanism for COPD. To some extent, this finding is supported by a previous study that the increases in protein clearance and endoplasmic reticulum-associated degradation were identified in the lung tissue of cigarette smoking-induced COPD mouse model.[44] As a leading risk factor for COPD, smoking has been identified to increase the production of ROS and promote protein oxidation, which result in protein misfolding.[45] To counteract smoking-induced disruptions, chaperones and antioxidant enzymes restore proteostasis and reduce oxidative stress.[46,47] In our study, we found that some DAPs are enriched in ATP-dependent protein folding chaperone and antioxidant activity, and some of these DAPs such as chaperones including chaperonin containing TCP1, heat shock proteins and the proteasome have been identified to facilitate the proper folding and clearance of misfolded proteins in RBCs.[48–50] Furthermore, the reduction of antioxidant enzymes suggests low abilities to scavenge ROS, reduce inflammation and combat oxidative stress.[51] Additionally, GO enrichment data regarding cellular component indicated that some DAPs significantly enriched in blood microparticles, secretory granule lumens, and the proteasome accessory complex, implicating that alterations in endomembrane and proteasome systems may contribute to dysregulation of structure and function of RBCs during COPD development.

By KEGG pathway enrichment analysis using all the up-regulated proteins, we found that the proteasome pathway is the most enriched pathway. The proteasome-associated up-regulated proteins (including PSMC1, PSMC2, PSMC5, PSMD1, PSMD7, PSMD8, PSMD11, PSMD12, PSMD13, PSMD14) are shown to be the components of the regulatory particle of proteasome complex, and all of them present the most enriched PPI network. Due that the regulatory particle regulates some essential processes of protein degradation including substrate recognition, deubiquitylation, unfolding, and translocation into the core particle,[52] the up-regulation of proteasome-associated DAPs in COPD should play a critical role in promoting protein degradation linked to COPD pathogenesis. This opinion is supported by previous evidence. PSMD1 and PSMD14 affect the targeting signal and the process of proteasome gate opening, thereby regulating substrate degradation.[53] A recent study found that proteasome expression was also up-regulated in peripheral

blood mononuclear cells (PBMC) of COPD patients.[24] Our findings suggest that COPD imposes an additional pathological burden that triggers a more pronounced and specific response, such as the coordinated upregulation of the proteasome system, which was not reported in studies focusing solely on smokers without COPD. This highlights that while smoking initiates RBC damage, the progression to COPD involves distinct cellular maladaptations. Given the critical role of the proteasome, especially the 19S subunit, in RBC disorders, our data suggest that 19S proteasome inhibition may represent a novel therapeutic approach for COPD treatment.

This study also has several limitations. To avoid hormonal interference, only male patients were included. Later data from developed countries suggest that women are equally, if not more, susceptible.[54] Nonetheless, COPD prevalence remains higher among men in developing countries.[55] Low abundant proteins, which account for only 2% of the proteins in erythrocyte cytosol, were partially masked by plasma proteins such as albumin (ALB), serum amyloid A-1 protein (SAA), and immunoglobulins. Circulation ALB levels were also significantly decreased in patients with COPD (Table S9), consistent with the proteomics. While our random pooling strategy was designed to identify a robust, common proteomic signature in COPD, we acknowledge that it may obscure protein changes specific to different clinical phenotypes of the disease. Future studies with larger, non-pooled cohorts will be necessary to investigate the proteomic profiles associated with specific COPD subtypes. Patients in this study were not pre-stratified by the severity of airflow limitation and smoking status. An ideal study design to fully dissect the distinct contributions of smoking and disease would involve four cohorts, including COPD patients who smoked, COPD patients who never smoked, healthy smokers, and healthy never-smokers. While studies on never-smoker COPD patients are emerging, comprehensive proteomic comparisons of RBCs across all four groups are currently lacking and represent a vital area for future research. Such work would definitively disentangle the effects of smoking from the intrinsic pathophysiology of COPD.

A key future direction is to functionally validate the findings in the present study. For instance, the pronounced upregulation of the proteasome complex strongly suggests an increased turnover of damaged proteins in response to systemic oxidative stress. Future studies should aim to directly measure proteasome activity in RBCs from COPD patients and correlate it with the levels of oxidized proteins. Mechanistically, this enhanced degradation could target crucial cytoskeletal proteins (eg, spectrin, ankyrin), leading to the loss of membrane integrity and reduced deformability that manifests as echinocytosis. Therefore, a critical next step is to use techniques like ektacytometry to directly measure RBC deformability and link it to proteasome activity and specific protein levels in a larger patient cohort. Such studies will be crucial for translating our molecular findings into a functional understanding of RBC pathology in COPD.

Conclusion

In conclusion, this study is for the first time to determine an in-depth altered RBC proteome from the COPD patients. We identified that the COPD-associated DAPs primarily contribute to the regulation of protein stability and immune and inflammatory responses. Furthermore, the levels of 10 regulatory components of the proteasomal complex significantly increased upon COPD condition, suggesting an enhanced activity of this complex. We propose that these dysregulated biological functions and pathways (especially for proteasome) should be involved in formation of echinocytes, reduction of oxygen transport and dysregulation of the immune system, which then impair other tissues. The upregulated proteasomal proteins represent a novel class of potential blood-based biomarkers. Quantifying these proteins could offer a non-invasive means to monitor the systemic impact and oxidative stress burden in COPD. Furthermore, the proteasome itself may represent a novel therapeutic target for mitigating the systemic effects of COPD. We acknowledge the limitations of this discovery-phase study, including the relatively small sample size and the inclusion of only male subjects. Therefore, several future directions are essential.

Data Sharing Statement

The data that support the findings of this study are available from the corresponding author with reasonable request.

Ethical Approval

The study was approved by the ethics committee of the Zhujiang Hospital (no. 2021-KY-060-01) and was conducted in accordance with the Declaration of Helsinki. Informed consent was obtained before the inclusion of subjects in the study.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

All authors declare no conflicts of interest in this work.

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