

Plasma Proteomics Study Between the Frequent Exacerbation and Infrequent Exacerbation Phenotypes of Chronic Obstructive Pulmonary Disease

Chengyu Yang^{1,2,*}, Li Yang^{3,4,*}, Lei Yang⁵, Shuiming Li⁵, Ling Ye^{1,6}, Jinfeng Ye⁵, Chengshui Chen^{3,4,7}, Yiming Zeng⁸, Mengchan Zhu¹, Xiaoping Lin⁸, Qing Peng⁹, Yun Wang^{5,10}, Meiling Jin^{1,6}

¹Department of Pulmonary and Critical Care Medicine, Zhongshan Hospital, Fudan University, Shanghai, 200032, People's Republic of China;

²Department of Pulmonary and Critical Care Medicine, Huadong Hospital, Fudan University, Shanghai, 200040, People's Republic of China;

³Department of Pulmonary and Critical Care Medicine, the First Affiliated Hospital of Wenzhou Medical University, Wenzhou, 325015, People's Republic of China; ⁴Key Laboratory of Interventional Pulmonology of Zhejiang Province, the First Affiliated Hospital of Wenzhou Medical University, Wenzhou, 325015, People's Republic of China; ⁵Longhua Innovation Institute for Biotechnology, Shenzhen University, Shenzhen, Guangdong, 518055, People's Republic of China; ⁶Department of Allergy, Zhongshan Hospital, Fudan University, Shanghai, 200032, People's Republic of China;

⁷Department of Pulmonary and Critical Care Medicine, the Quzhou Affiliated Hospital of Wenzhou Medical University, Quzhou People's Hospital, Zhejiang, 324000, People's Republic of China; ⁸Department of Pulmonary and Critical Care Medicine, the Second Affiliated Hospital of Fujian Medical University, Respiratory Medicine Center of Fujian Province, Quanzhou, Fujian, 362000, People's Republic of China; ⁹Department of Pulmonary and Critical Care Medicine, Minhang Hospital, Fudan University, Shanghai, 201199, People's Republic of China; ¹⁰Guangdong Provincial Key Laboratory of Infectious Diseases and Molecular Immunopathology, Shantou University Medical College, Shantou, Guangdong, 515041, People's Republic of China

*These authors contributed equally to this work

Correspondence: Meiling Jin, Department of Pulmonary and Critical Care Medicine, Zhongshan Hospital, Fudan University, Shanghai, 200032, People's Republic of China, Email jin.meiling@zs-hospital.sh.cn; Yun Wang, Longhua Innovation Institute for Biotechnology, Shenzhen University, Shenzhen, Guangdong, 518055, People's Republic of China, Email yunw@szu.edu.cn

Background: Frequent exacerbation (FE) and infrequent exacerbation (IE) are two phenotypes of chronic obstructive pulmonary disease (COPD), of which FE is associated with a higher incidence of exacerbation and a serious threat to human health. Because the pathogenesis mechanisms of FE are unclear, this study aims to identify FE-related proteins in the plasma via proteomics for use as predictive, diagnostic, and therapeutic biomarkers of COPD.

Methods: A cross-sectional study was conducted in which plasma protein profiles were analyzed in COPD patients at stable stage, and differentially expressed proteins (DEPs) were screened out between the FE and IE patients. FE-related DEPs were identified using data-independent acquisition-based proteomics and bioinformatics analyses. In addition, FE-related candidates were verified by enzyme-linked immunosorbent assay.

Results: In this study, 47 DEPs were screened out between the FE and IE groups, including 20 upregulated and 27 downregulated proteins. Key biological functions (eg, neutrophil degranulation, extracellular exosome, protein homodimerization activity) and signaling pathways (eg, arginine and proline metabolism) were enriched in association with the FE phenotype. Receiver operating characteristic (ROC) analysis of the 11 combined DEPs revealed an area under the curve of 0.985 ($p < 0.05$) for discriminating FE from IE. Moreover, correlation and ROC curve analyses indicated that creatine kinase, M-type (CKM) and fat storage-inducing transmembrane protein 1 (FITM1) might be clinically significant in patients with the FE phenotype. In addition, plasma expression levels of CKM and FITM1 were validated to be significantly decreased in the FE group compared with the IE group (CKM: $p < 0.01$; FITM1: $p < 0.05$).

Conclusion: In this study, novel insights into COPD pathogenesis were provided by investigating and comparing plasma protein profiles between the FE and IE patients. CKM, FITM1, and a combinative biomarker panel may serve as useful tools for assisting in the precision diagnosis and effective treatment of the FE phenotype of COPD.

Keywords: chronic obstructive pulmonary disease, frequent exacerbation, data-independent acquisition, proteomics, bioinformatics analysis

Background

Chronic obstructive pulmonary disease (COPD) is a common and progressive lung disease leading to high morbidity and mortality globally.¹ Acute exacerbation leads to rapid deterioration of pulmonary function and a significant decline in the quality of life of COPD patients, resulting in severe economic and social burdens.²⁻⁴ Based on the frequency of acute exacerbations per year, the COPD population can be divided into two clinical phenotypes: frequent exacerbation (FE) and infrequent exacerbation (IE). Hurst et al reported that FE is a unique phenotype of COPD that appears to be relatively stable.⁵ This status implies the presence of many complex underlying factors regulating the pathophysiological processes, such as genetics, biology, pathology, and behavior, which can determine susceptibility or resistance to recurrent episodes, regardless of the severity of this disease.⁶ Frequent exacerbators have a higher risk of hospitalization and a worse prognosis compared with infrequent exacerbators. Despite the importance of the FE phenotype, its pathogenesis has not been sufficiently studied. The identification of the FE phenotype is currently based on clinical assessment and records of previous treatments, which is complicated by the time-consuming process of establishing a unified standard. Therefore, the discovery of special, functional, and feasible biomarkers is needed to guide clinical practice and improve early interventions for patients.

The proteomics can provide additional bioinformatics information for understanding the pathophysiological processes beyond the genome and transcriptome. Proteomics is one of the rapidly developing high-throughput technologies that has gained popularity in recent years. It is used to explore the protein expression profiles of cells, tissues, and organisms during disease development. Therefore, in-depth investigation of the pathogenesis of COPD with the multi-level discovery of related protein biomarkers has become an important subject of research in modern precision medicine.

In the past, most liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) methods relied on data-dependent acquisition (DDA). Traditional DDA selects peptide ions for fragmentation based on their abundance on MS1 scans, which can be hampered by interference from chemical noise,^{7,8} resulting in the loss of some low-abundance precursors.⁹ Data-independent acquisition (DIA) has emerged to overcome this limitation. In contrast with DDA, DIA systematically parallelizes the fragmentation of all possibly generated precursor ions within a wide *m/z* range and quantifies peptides at the MS2 level, thereby providing better sensitivity, precise quantification, and improved reproducibility.¹⁰ The convoluted or multiplexed fragment ion spectra generated by DIA require more sophisticated processing algorithms than DDA. Therefore, by using DIA, bioinformatics, and statistics in this study, we aimed to investigate FE-related protein profiles and reveal their pathophysiological functions during the disease development to enhance the precision treatment and management of COPD.

Materials and Methods

Subject Enrollment

This research project and procedures were approved by the Ethics Committee of Zhongshan Hospital Affiliated with Fudan University and complied with the ethical guidelines and standards set by the Declaration of Helsinki (No. B2017-022R). Written informed consent was signed by every subject.

This cross-sectional study was conducted at Zhongshan Hospital Affiliated with Fudan University (Shanghai, China). Patients with COPD at stable stage (no history of acute exacerbation within 4 weeks prior to enrollment) were recruited from the outpatient department with no additional intervention from March 2017 to September 2018. All patients maintained the original prescription of therapeutic drugs when sampling.

The inclusion criteria of COPD patients were age of 40 to 80 years old, history of smoking (≥ 10 pack-years), and history of biofuel use (ie, frequent use of charcoal, wood, animal manure, or crops for home heating and cooking) or occupational exposure to dust and chemicals (eg, mining, quarrying, casting, grain dust, paint, chemical industry, and other occupational dust/gas smoke longer than one year).

According to the 2017 Global Initiative for Chronic Obstructive Pulmonary Disease (GOLD) guidelines,¹¹ the key diagnostic criterion of COPD is a post-bronchodilator forced expiratory volume at 1 second (FEV₁)/forced vital capacity (FVC) < 0.70 . The COPD patients were assigned to either the FE or IE group based on their exacerbation history. Acute exacerbation was defined as the acute deterioration of respiratory symptoms leading to the requirement for additional

treatment (emergency or hospitalization with antibiotics and/or systemic glucocorticoid therapy).¹¹ The FE subjects were defined as ≥ 2 acute exacerbations of COPD per year or ≥ 1 acute exacerbation leading to hospitalization.¹¹

The exclusion criteria were (a) history of acute pulmonary disease (eg, pneumonia, acute lung injury) within 3 months before enrollment, (b) history of other chronic lung diseases (eg, bronchiectasis, pulmonary interstitial fibrosis), (c) history of malignant tumor, and (d) pregnancy or lactation. The research records included the patients' demographic data, smoking history, history of biofuel use/occupational exposure, laboratory tests, and lung function.

Plasma Specimen Collection

After enrollment, 5 mL of peripheral venous blood per patient was collected into a collection tube containing ethylenediaminetetraacetic acid anticoagulant. Low-temperature centrifugation (4°C, 1000 rpm, 10 min) was immediately performed on the blood sample, and the supernatant was divided into enzyme-free Eppendorf tubes and stored at -80°C.

To detect lower concentrations of plasma proteins, high-abundance and structural proteins were removed using the Human 14 Multiple Affinity Removal Kit (Agilent, Santa Clara, CA, USA) following the manufacturer's instructions.

Protein Digestion and Peptide Fractionation via High-pH Reverse-Phase High-Performance Liquid Chromatography

The protein concentration in the pretreated sample was measured using a Bradford assay kit (Bio-Rad, Hercules, California, USA), and 100 μg of protein from each sample was subjected to trypsin digestion based on an aided filter sample preparation protocol. Briefly, the proteins were denatured by the addition of 8 M urea and vortexed for 5 min, and the proteins were reduced with dithiothreitol at 37°C for 2 hr followed by alkylation with iodoacetamide for 30 min and incubation in the dark. The mixture was then transferred to a 10-kDa filter (Sartorius, Gottingen, Niedersachsen, Germany) and centrifuged at 14,000 $\times g$ for 10 min until the buffer was spun down completely into the collection tube. The proteins were kept on the ultrafiltration membrane and redissolved using 50 mM ammonium bicarbonate. After centrifugation, the redissolved proteins were digested with trypsin (protein: enzyme = 40:1) overnight, and tryptic peptides were subsequently centrifuged and transferred into a fresh tube for peptide collection.

All samples (10 μg /sample) were redissolved and fractionated using the Ultimate 3000 high-performance liquid chromatography system (Thermo Fisher Scientific, Waltham, Massachusetts, USA) coupled with an Agilent 300 Extend-C18 high pH column (2.1 \times 250 mm, 3.5 μm). One sample was loaded onto a column, and the elute was collected at a flow rate of 0.3 mL/min by following gradient mobile phase B (95% acetonitrile (ACN), pH 10.0) at (1) 5% for 3 min, (2) 5–35% for 50 min, (3) 35–95% for 7 min, and (4) 100% for the last 4 min. An additional 5% mobile phase B was flowed for 6 min for equilibration. The elution peak was monitored at a wavelength of 214 nm, and the sample fractions were collected every 1 min. Finally, all components per sample were combined into a total of 25 fractions and vacuum-dried.

DDA and DIA Analyses Based on Nano-Liquid Chromatography-Mass Spectrometry

DDA and DIA analyses were performed using a Q-Exactive mass spectrometer (Thermo) coupled with an Easy-nanoLC 1200 system. The peptide digests were reconstituted in nano-LC mobile phase A (0.1% formic acid), loaded onto a NanoViper C18 trap column (3 mm, 100 \AA), and separated on an analytical column (75 mm \times 25 cm C18-2 mm 100 \AA) via a 2-hr linear gradient from 5 to 35% mobile phase B (95% ACN with 0.1% formic acid) at a flow rate of 300 nL/min, followed by a linear increase to 95% mobile phase B for 2 min and 95% for 6 min. The Q-Exactive mass spectrometer was operated with a spray voltage of 1.9 kV and a capillary temperature of 275°C in a 350–1500 m/z scan range and 70,000 resolution, and the maximum ion injection time was 100 ms. Twenty of the most abundant precursor ions from each DDA cycle were selected for higher-energy collision dissociation fragment analysis with a maximum ion injection time of 50 ms, collision energy of 28 eV, and dynamic exclusion of 25 s. For each sample in the DIA analysis, MS1 and MS2 were set with a maximum ion injection time of 50 ms. MS1 was set as mentioned above (350–1500 m/z scan range and 70,000 resolution) and was separated into 34 acquisition windows with a mass range of 14–152 Da. The liquid conditions were consistent with the DDA model for separation. Raw DIA data were analyzed using Spectronaut Pulsar X.

Spectral Library Construction and Protein Quantification

The spectral library of plasma samples was constructed from raw MS files obtained from the Human UniProt protein database (20,336 protein sequences) and analyzed using Proteome Discoverer (V2.1.0.81). The search was conducted with a precursor tolerance of 10 ppm and a fragment ion tolerance of 0.05 Da. Identified peptides were filtered with a false discovery rate of <1% and contained at least one unique peptide segment. Protein abundance was calculated using the peak areas of the samples. Raw data from the spectral library and DIA were loaded with the Spectronaut Pulsar X software via protein relative quantification analysis. Comparative analyses of the datasets were performed after quantile normalization.

Bioinformatics Analysis

The identified proteins were screened out as differentially expressed proteins (DEPs) between the FE and IE groups. A fold change of relative expression >1.2 or <0.83 with a *p*-value <0.05 served as a criterion for processing the quantitative DIA data. Principal component analysis (PCA) and partial least squares discrimination analysis (PLS-DA) were performed using R package ggplot2 and mixOmics (version 4.2.0). The Metascape online website (<http://metascape.org>) was used for Gene Ontology (GO) enrichment and Kyoto Encyclopedia of Genes and Genomes (KEGG) signal pathway analyses.¹² The protein–protein interaction (PPI) networks of DEPs were constructed using the online database of the Search Tool for the Retrieval of Interacting Genes (STRING; <http://string-db.org>) (version 11.5) and visualized by Cytoscape (version 3.7.1).

Validation of DEPs by Enzyme-Linked Immunosorbent Assay

To validate the accuracy of proteomics results, the expression levels of the key DEPs were detected in plasma samples from an additional 40 FE and 40 IE patients by enzyme-linked immunosorbent assay (ELISA). The candidate proteins were selected based on several features: (a) their biological processes and signaling pathways related to the pathogenesis of COPD, according to previous research; (b) their expression levels related to lung function, according to the correlation analysis; and (c) their areas under the curve (AUC) >0.7, according to receiver operating characteristic (ROC) analysis. The ELISA kits were purchased from Jianglai Bio (Shanghai, China). Each sample was tested with two standard replicates at the same time. The ELISA assays were performed according to the manufacturer's instructions.

Statistical Analysis

Statistical analysis was performed using SPSS 24.0 (IBM Corporation, Armonk, NY, USA). Continuous variables were presented as means (standard deviation) or medians (interquartile range). Independent sample *t*-tests and Mann–Whitney *U*-test were used to determine differences between two groups for continuous variables. Categorical variables were presented as percentages and compared via chi-squared test. Spearman correlation analysis was used to assess the correlation between DEP expression and lung function. The ROC curve was applied to evaluate the diagnostic value of DEPs. A *p*-value <0.05 was considered statistically significant.

Results

Clinical Characteristics of FE and IE Subjects

The summarized workflow of the study is presented in [Figure 1](#). A total of 76 patients with COPD were enrolled, including 34 and 42 patients in the FE and IE groups, respectively. The clinical information of the patients is summarized in [Table 1](#). There were no differences in sex, age, body mass index, smoking history, or hemoglobin levels between the two groups. FEV₁ and FEV₁% predicted were significantly lower in the FE group than in the IE group (*p* <0.05). In addition, there was a significant difference in the GOLD classification between the two groups (*p* <0.05).

DEPs Screened Between the FE and IE Groups

A total of 1036 proteins were identified from both the FE and IE groups. The quantile normalization of the datasets is presented in [Supplementary Figure 1](#). Forty-seven DEPs were screened out (with a threshold of fold change >1.2 or <0.83, and *p* <0.05) as candidate biomarkers by comparing FE patients to IE patients, including 20 upregulated and 27 downregulated DEPs ([Figure 2](#)). The information of the DEPs is presented in [Table 2](#).

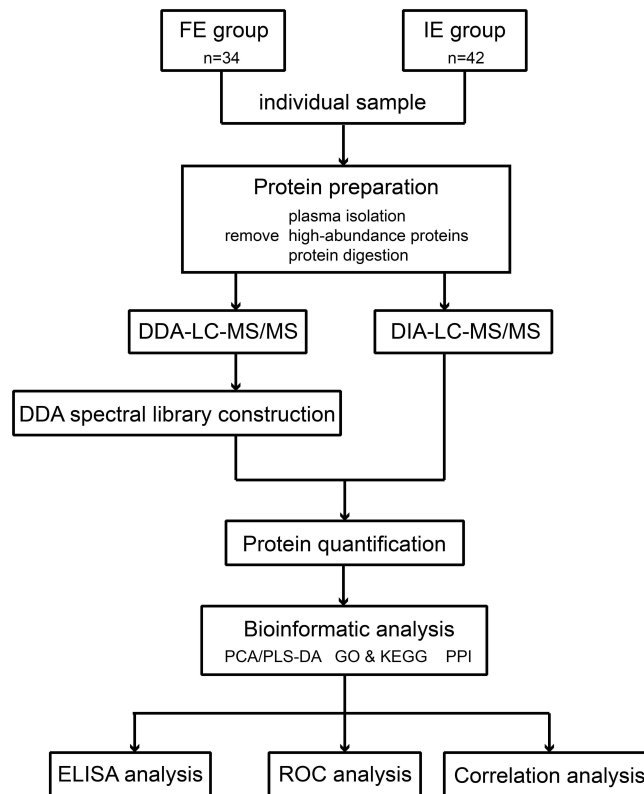


Figure 1 Workflow of the study. The study involved two groups (FE and IE) of COPD patients. An individual specimen was processed following a typical proteomics sample preparation protocol. The plasma protein expression profile of each sample was evaluated using LC-MS/MS based on DIA. Following bioinformatics analysis, correlation and ROC analyses were performed to investigate the clinical importance of DEPs. Candidate proteins were verified using ELISA.

Abbreviations: COPD, chronic obstructive pulmonary disease; DEP, differentially expressed protein; DDA, data-dependent acquisition; DIA, data-independent acquisition; FE, frequent exacerbation; IE, infrequent exacerbation; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; PPI, protein–protein interaction; PCA, principal component analysis; PLS-DA, partial least squares discrimination analysis; LC-MS/MS, liquid chromatography coupled with tandem mass spectrometry; ELISA, enzyme-linked immunosorbent assay; ROC, receiver operating characteristic.

Principal Component Analysis (PCA) and Partial Least Squares Discrimination Analysis (PLS-DA) of the DEPs

To identify the protein expression profiles, PCA was performed based on the DEPs via a comparison assay between the FE and IE groups. The results were visualized using a two-dimensional coordinate map (Figure 3A). The result indicated

Table 1 Clinical Characteristics of Enrolled COPD Patients

	Total	IE Group	FE Group	p-value
Patient, n	76	42	34	-
Male, n (%)	72 (94.74)	40 (95.24)	32 (94.12)	0.828
Female, n (%)	4 (5.26)	2 (4.76)	2 (5.88)	
BMI (kg/m ²)	22.76 (19.84–24.06)	22.86 (20.02–25.54)	22.08 (19.28–23.71)	0.292
Age, years	69.03±6.64	69.05±6.4	69±7.02	0.975
Smoking history, pack-years (n) ^a	41 (19.37–50.25) (n=70)	40.5 (18.38–48.5) (n=38)	44 (32–55.38) (n=32)	0.183
Hb, g/L	146.06±14.19	144.92±11.44	147.08±16.4	0.586
FEV ₁ , L	1.16 (0.83–1.64)	1.4 (0.98–1.86)	0.95 (0.69–1.32) ^b	0.001
FEV ₁ % predicted	47 (32.38–60.1)	53.5 (39.53–67.5)	37.13 (27.75–49.75) ^b	0.001
GOLD stage I, n	6	6	0	0.032
GOLD stage II, n	24	16	8	
GOLD stage III, n	32	16	16	
GOLD stage IV, n	12	4	8	

Note: ^an, number of smokers. ^b2 cases missing data of FEV₁ and FEV₁% were not included in analysis.

Abbreviations: COPD, chronic obstructive pulmonary disease; BMI, body mass index; FE, frequent exacerbation; FEV₁, forced expiratory volume in 1 s; GOLD, Global Initiative for Chronic Obstructive Lung Disease; Hb, hemoglobin; IE, infrequent exacerbation.

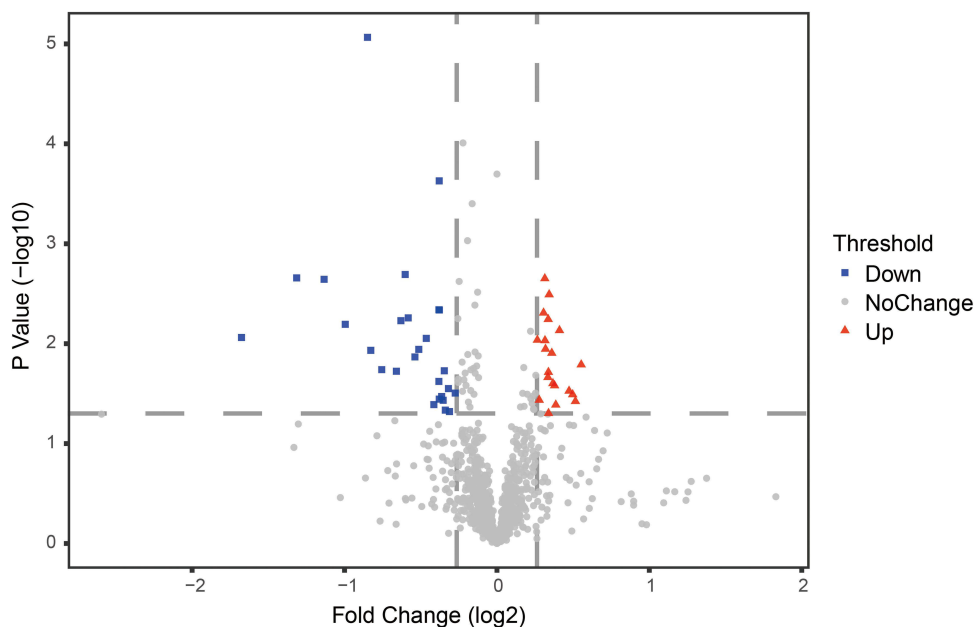


Figure 2 Volcano plot of the protein expression analysis. DEPs with fold change >1.2 or <0.83, and $p < 0.05$ were selected. Red triangles represent upregulation and blue squares represent downregulation.

Abbreviation: DEP, differentially expressed protein.

that there were only a few FE-related features in the plasma protein samples between the two groups, suggesting that strict and sensitive detection of the protein candidates was required. PLS-DA was performed to identify the differences by comparing the protein expression profiles of the FE and IE groups, and the results indicated that two groups could be

Table 2 Differentially Expressed Proteins Between FE and IE Groups of COPD patients

Accession	Description	Gene Name	FC (FE/IE)	p-value
P06702	Protein S100-A9	S100A9	1.466961704	0.01621789
P18065	Insulin-like growth factor-binding protein 2	IGFBP2	1.42872785	0.037737317
O15511	Actin-related protein 2/3 complex subunit 5	ARPC5	1.411043273	0.032264326
Q8WVN6	Secreted and transmembrane protein 1	SECTM1	1.388086318	0.029759724
P11684	Secretoglobin family 1A member 1	SCGB1A1	1.328953745	0.007363425
P07998	Ribonuclease A family member 1, Pancreatic	RNASE1	1.306707667	0.040941696
P02750	Leucine-rich alpha-2-glycoprotein	LRG1	1.299793423	0.026275944
P07339	Cathepsin D	CTSD	1.28985048	0.024966043
Q99784	Olfactomedin 1	OLFM1	1.283235264	0.012416208
P0COL5	Complement C4-B	C4B	1.267995051	0.00322225
P31944	Caspase-14	CASPI4	1.264295723	0.049699006
Q8IUK8	Cerebellin-2	CBLN2	1.263707826	0.019296181
Q9Y5C1	Angiotensin-related protein 3	ANGPTL3	1.262405481	0.005698631
P02649	Apolipoprotein E	APOE	1.260973709	0.02169423
Q02985	Complement factor H-related protein 3	CFHR3	1.247026669	0.011310579
P12109	Collagen alpha-1(VI) chain	COL6A1	1.244188934	0.009289705
Q06033	Inter-alpha-trypsin inhibitor heavy chain H3	ITIH3	1.243101127	0.002228673
P61769	Beta-2-microglobulin	B2M	1.236108358	0.004915119
Q92743	Serine protease HTRA1	HTRA1	1.212044089	0.036663982
P39060	Collagen alpha-1(XVIII) chain	COL18A1	1.201699974	0.009195374
Q93091	Ribonuclease A family member K6	RNASE6	0.827209516	0.031231799
P36980	Complement factor H-related protein 2	CFHR2	0.805801157	0.047835387
P00488	Coagulation factor XIII A chain	F13A1	0.801968846	0.028173442

(Continued)

Table 2 (Continued).

Accession	Description	Gene Name	FC (FE/IE)	p-value
P61160	Actin-related protein 2	ACTR2	0.790670435	0.046269439
Q9Y5Y7	Lymphatic vessel endothelial hyaluronin receptor 1	LYVE1	0.786975411	0.018680133
P24387	Corticotropin-releasing factor-binding protein	CRHBP	0.78301915	0.036883957
P54289	Voltage-dependent calcium channel subunit alpha-2/delta-1	CACNA2D1	0.777693317	0.033797462
P21549	Alanine-glyoxylate and Serine-pyruvate aminotransferase	AGXT	0.769660688	0.00460265
Q9NQW7	X-Prolyl aminopeptidase 1	XPNPEP1	0.769262834	0.035896261
P27169	Paraoxonase 1	PON1	0.76905574	0.000234736
AOA0A0MRZ8; P04433	Immunoglobulin kappa variable 3D-11; Immunoglobulin kappa variable 3-11	IGKV3D-11; IGKV3-11	0.768073707	0.004581196
Q15019	Septin-2	SEPTIN2	0.767863045	0.023877145
O75874	Isocitrate dehydrogenase (NADP(+)) cytoplasmic	IDH1	0.750556515	0.040731374
P05534	Major histocompatibility complex, Class I, A	HLA-A	0.725063142	0.008853159
Q13790	Apolipoprotein F	APOF	0.700742081	0.011417254
P09172	Dopamine beta-hydroxylase	DBH	0.68862249	0.013575044
P06732	Creatine kinase M-type	CKM	0.667977227	0.005519924
Q96KN2	Carnosine dipeptidase 1	CNDP1	0.65876237	0.00203092
P0DOY2; P0DOY3	Immunoglobulin lambda constant 2; Immunoglobulin lambda constant 3	IGLC2; IGLC3	0.646063445	0.005887027
Q86VP6	Cullin associated And neddylation dissociated 1	CAND1	0.63285152	0.018880978
P01591	Immunoglobulin J chain	JCHAIN	0.592173243	0.018199333
Q96RD9	Fc receptor-like protein 5	FCRL5	0.563336109	0.011657218
Q8IXL6	Extracellular serine/threonine protein kinase FAM20C	FAM20C	0.555111256	8.59692E-06
Q15386	Ubiquitin-protein ligase E3C	UBE3C	0.501582287	0.006398
A5D6W6	Fat storage-inducing transmembrane protein 1	FITM1	0.455565972	0.00226977
P49641	Mannosidase alpha class 2A member 2	MAN2A2	0.402170931	0.002196441
Q9UKY7	Protein CDV3 homolog	CDV3	0.312907312	0.008671611

Abbreviations: FE, frequent exacerbation; IE, infrequent exacerbation; COPD, chronic obstructive pulmonary disease; FC, fold change.

distinguished (Figure 3B). Compared with the unsupervised PCA model, the supervised PLS-DA model better distinguished the FE group from the IE group, which indicated that remarkable FE-related DEPs existed in the plasma protein profiles of above two groups.

GO and KEGG Enrichment Analyses of FE-Related DEPs

To further understand the biological roles of the 47 FE-related DEPs that were screened out as candidate biomarkers by comparing the FE group with the IE group, GO and KEGG enrichment analyses were performed using Metascape (Figures 4A and B). By GO analysis, the biological processes of the DEPs were mainly enriched in the following terms: neutrophil degranulation, movement of cell or subcellular component, immune response, and lipid storage. In addition, changes in cellular components were associated with the extracellular exosome, extracellular space, and extracellular region. Moreover, changes in molecular function were relevant to protein homodimerization activity, ribonuclease activity, and endonuclease activity. GO enrichment analysis was also performed in the upregulated and downregulated DEPs, respectively (Supplementary Figure 2).

Concurrently, KEGG analysis revealed that the DEPs were involved in complement and coagulation cascades, bacterial invasion of epithelial cells, and arginine and proline metabolism.

Establishment of a Possible PPI Network of FE-Related DEPs

A total of 47 FE-related biomarkers were uploaded to the STRING database. The minimum required interaction score was set to medium confidence (0.3), and disconnected nodes were removed from the network. The PPI network graph is visualized in Figure 5 by Cytoscape to present the possible interrelationships of the DEPs and signaling pathways they involved.

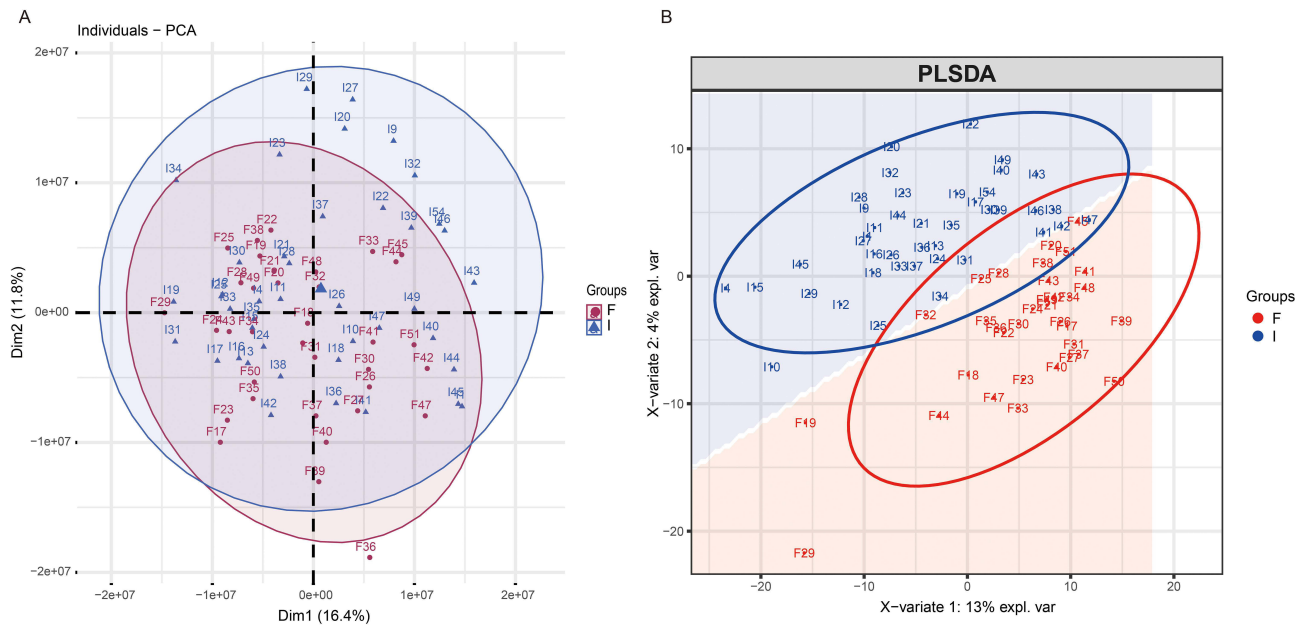


Figure 3 PCA and PLS-DA of the DEPs. **(A)** PCA: red dots represent the FE group and blue triangles represent the IE group. **(B)** PLS-DA: red dots represent the FE group and blue dots represent the IE group.

Abbreviations: PCA, principal component analysis; PLS-DA, partial least squares discrimination analysis; DEP, differentially expressed protein; IE, infrequent exacerbation; FE, frequent exacerbation.

Correlation Analysis of FE-Related DEPs

Correlation analyses of FEV₁% and FE-related DEPs were performed to investigate the association between the expression of DEP and pulmonary function (Figure 6), individually. The results indicated that pulmonary function was correlated with the expression levels of seven proteins: actin-related protein 2/3 complex subunit 5 (ARPC5), complement C4-B (C4B), creatine kinase, M-type (CKM), Fc receptor-like 5 (FCRL5), fat storage-inducing transmembrane protein 1 (FITM1), immunoglobulin lambda constant 2 (IGLC2)/IGLC3, and ubiquitin protein ligase E3C (UBE3C). Among these proteins, expression levels of ARPC5 and C4B were negatively correlated with FEV₁%, while expression levels of the other proteins were positively correlated with FEV₁% ($p < 0.05$).

ROC Analysis of FE-Related DEPs

Among the 47 FE-related DEPs, 11 proteins had AUCs > 0.7 according to ROC analysis, including inter-alpha-trypsin inhibitor heavy chain 3 (ITIH3), lymphatic vessel endothelial hyaluronan receptor 1 (LYVE1), paraoxonase 1 (PON1), alanine-glyoxylate and serine-pyruvate aminotransferase (AGXT), immunoglobulin kappa variable 3D-11 (IGKV3D-11)/IGKV3-11, septin-2 (SEPTIN2), apolipoprotein F (APOF), dopamine beta-hydroxylase (DBH), CKM, extracellular serine/threonine protein kinase FAM20C (FAM20C), and FITM1 (Figure 7). The sensitivity, specificity, optimal cutoff, 95% confidence interval, and p-values are presented in Table 3. ROC analysis of the 11 combined DEPs resulted in an AUC of 0.985 (95% CI: 0.966–1.000, $p < 0.05$) for discrimination of FE from IE (Figure 8). The max Youden index was 0.752, with a sensitivity of 90.9% and a specificity of 100%, which was superior to the results of any individual DEP, suggesting that the optimal approach was the measurement of a combined biomarker panel.

Validation of Candidate DEPs as FE-Related Biomarkers

To confirm the results obtained from the proteomics, bioinformatics, correlation, and ROC curve analyses, two key candidate biomarkers (CKM and FITM1) were selected and individually validated in plasma samples from an additional 40 FE and 40 IE patients in a validation cohort using ELISA assays. Expression levels of both CKM and FITM1 were significantly decreased in the FE group compared with the IE group, which was consistent with the proteomics results (Figure 9).

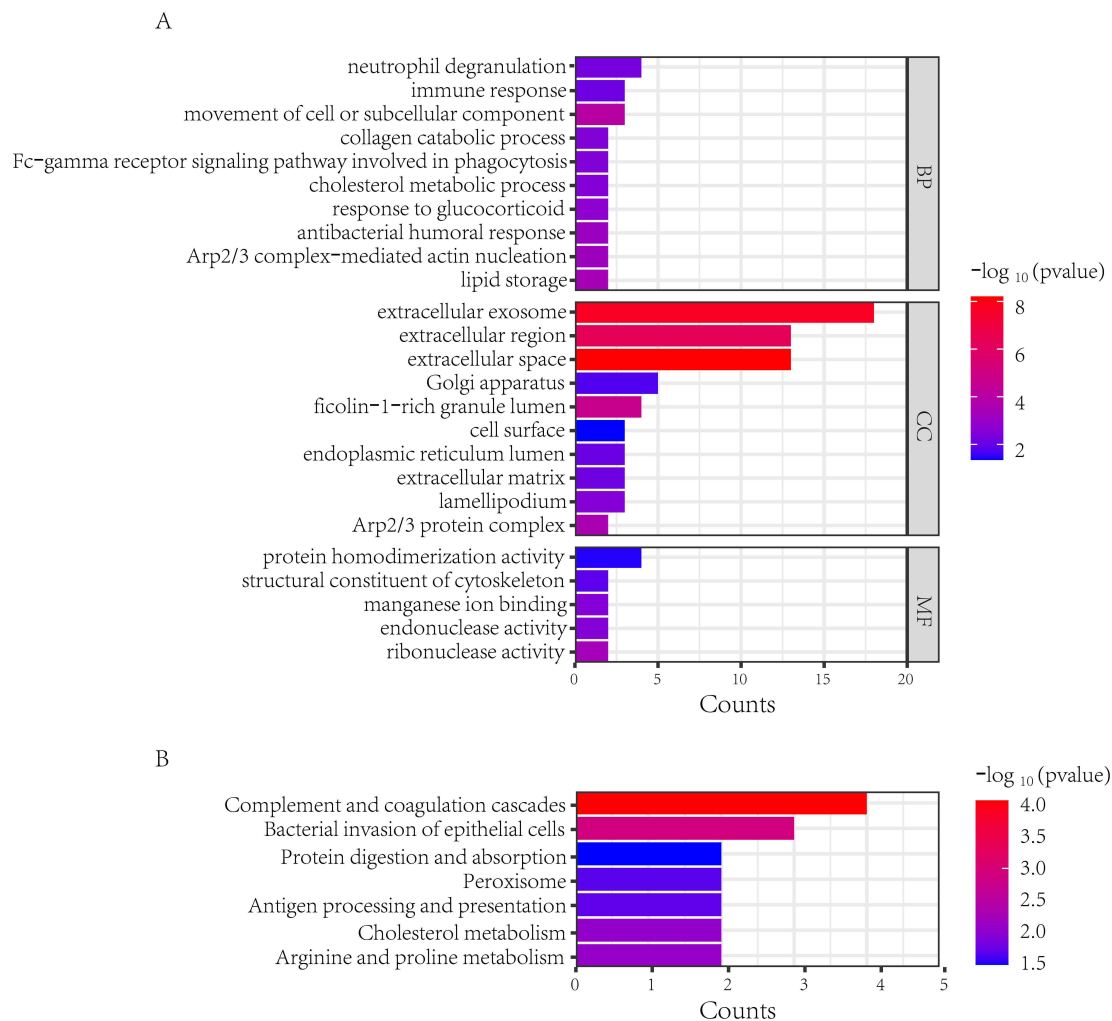


Figure 4 Functional enrichment analyses of FE-related DEPs. **(A)** GO categories. **(B)** KEGG pathways.

Abbreviations: DEP, differentially expressed protein; IE, infrequent exacerbation; FE, frequent exacerbation; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes.

Discussion

In recent years, a growing number of studies have focused on the occurrence and development of COPD. However, few reports have investigated the phenotypes of COPD.^{13–15} The FE phenotype significantly impacts patient's quality of life and disease prognosis. Therefore, it would be of great importance to identify patients with FE phenotype and deliver appropriate monitoring and intervention at the earliest occasion. Singh et al investigated gene expression profiles with microarray and polymerase-chain reaction analysis of sputum and blood samples from subjects in the ECLIPSE COPD cohort and screened out B3GNT, LAF4, and ARHGEF103 as biomarkers predicting FE.¹⁶ Sun et al detected changes in the lung proteome of COPD patients via tandem mass tag-labeled quantitative proteomics combined with LC-MS/MS, which revealed that major histocompatibility complex class II, DQ alpha 1 (HLA-DQA1), polymeric immunoglobulin receptor (PIGR), and biglycan (BGN) were significantly increased in FE patients compared with IE patients.¹⁷ Although the lung is the main target organ of COPD, the complex condition and severe hypoxia can cause a variety of complications in the body. Therefore, blood biomarkers may provide a better systematic assessment of the nature of COPD. The advantage of blood biomarkers is that obtaining blood samples is associated with less risk than sampling from the lung, and these biomarkers can be sampled repeatedly and dynamically to monitor disease progression. Therefore, in this study, peripheral blood samples were collected and individually tested to determine the protein expression profiles of FE and IE patients via DIA-MS.

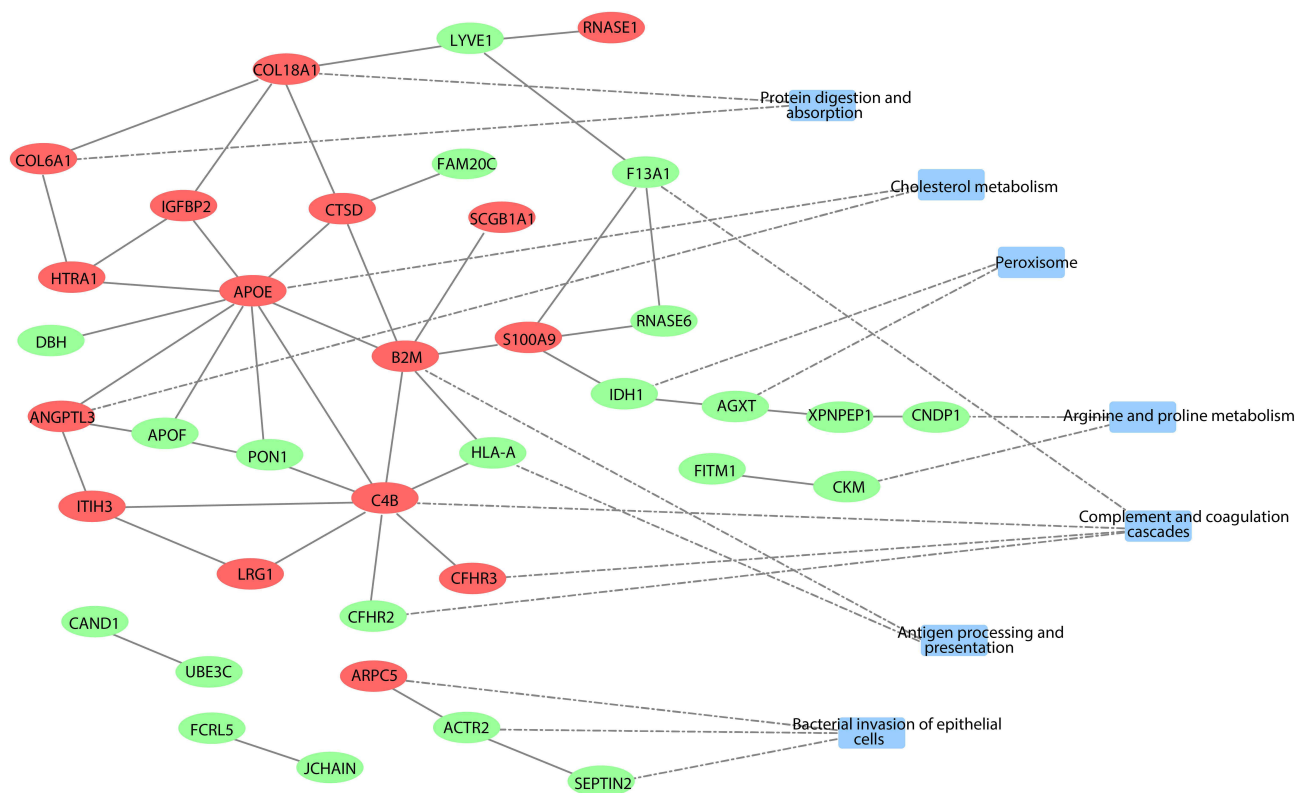


Figure 5 Construction of protein–protein interaction networks for FE-related DEPs. Proteins were indicated with circle nodes in red (upregulation) and green (down-regulation), and KEGG pathways were indicated with the rectangles.

Abbreviation: FE, frequent exacerbation; DEP, differentially expressed protein; KEGG, Kyoto Encyclopedia of Genes and Genomes..

After DIA-MS measurement and comparison analysis, 47 DEPs were screened out between the FE and IE groups, including 20 upregulated and 27 downregulated proteins. The PCA data indicated slight differences in plasma protein profiles between the two groups because of individual risk factors in each sample, further demonstrating that COPD is a heterogeneous disease. Since the two groups of patients only differed in phenotype of COPD, careful and profound analyses were needed to shed more light on these DEPs. A supervised PLS-DA model was then applied to obtain better insights into the data. FE and IE groups were relatively well separated in the PLS-DA score plot. The relationships among the DEPs were analyzed through functional and pathway enrichment analyses, which revealed that neutrophil degranulation, extracellular exosome, protein homodimerization activity, and complement and coagulation cascades were associated with FE. Hoenderdos et al reported that hypoxia augmented neutrophil degranulation and conferred enhanced injurious potential that might be relevant to neutrophilic airway inflammation in COPD.¹⁸ Lodge et al found that hypoxia promoted neutrophil degranulation and neutrophil-induced endothelial damage, which might contribute to increased cardiovascular risk in patients with COPD.¹⁹ Recently, an accumulating amount of research demonstrated the active involvement of complement and coagulation cascades pathway in COPD. Circulating C1q was found to be related to the pulmonary function of COPD and might be a biomarker to predict the risk of COPD deterioration.²⁰ A clinical study showed that coagulation system was activated during COPD exacerbations and coagulation markers were potential predictors of COPD exacerbations.²¹ The results obtained from the present bioinformatics analyses were consistent with data from previous studies.

According to analyses of biological processes and signaling pathways, and results of correlation and ROC curve analyses, the downregulation of plasma CKM and FITM1 may be clinically significant in COPD patients of FE phenotype. The expression levels of CKM and FITM1 detected by ELISA were significantly decreased in FE patients compared with IE patients, which was consistent with the proteomics results. Moreover, PPI network analysis showed that there was a close interaction between the two proteins.

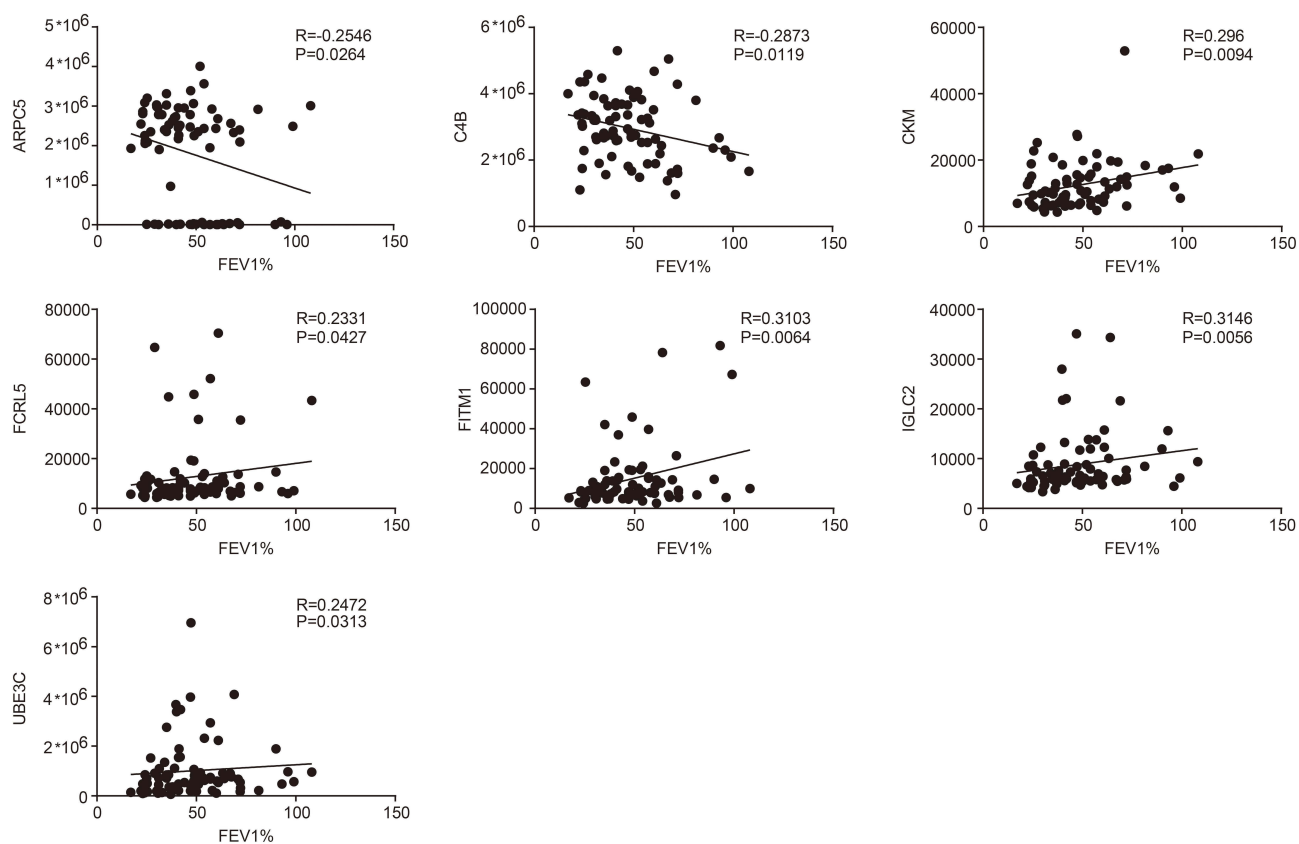


Figure 6 Correlation analyses of FEV₁% and FE-related DEPs. $p < 0.05$ denotes a statistically significant difference.

Abbreviations: FEV₁, forced expiratory volume in l s; FE, frequent exacerbation; DEP, differentially expressed protein.

CKM is involved in arginine and proline metabolism pathway which may play a key role in regulating the formation and metabolism of proteins.²² Anoxic injury in patients with COPD was reported to be accompanied by the production of methylated arginine derivatives, which might be useful for recognizing acute exacerbations of COPD.²³ Activation and expression of methylated arginine derivatives might also play an important role in the extrapulmonary complications of COPD.^{24,25} CKM is believed to play an indispensable role in maintaining energy homeostasis by providing a stable supply of creatine phosphate, which is essential for maintaining the Ca²⁺-ATPase channel in the sarcoplasmic reticulum.²⁶ More than 90% of the CKM in circulation is derived from human skeletal muscle; therefore, as a biomarker of skeletal muscle protein metabolism, it can be used to measure the rate of tissue protein synthesis.²⁷ FITM1 belongs to an evolutionarily conserved family of proteins and is involved in fat storage.²⁸ The binding of FIT protein to triglycerides is important during FIT-mediated lipid droplet (LD) formation.²⁹ A previous study showed that LDs could limit the level of reactive oxygen species and inhibit the oxidation of polyunsaturated fatty acids in glial cells.³⁰ FIT proteins have varying tissue distribution, and FIT1 is mainly expressed in muscle. Yan et al proposed that FIT1 was a direct target of MyoD and participated in muscle development.³¹ Mormeneo et al reported that the overexpression of peroxisome proliferator-activated receptor-gamma coactivator 1 alpha (PGC-1 α) could induce the expression of FITM1 in cultured human skeletal muscle cells, accompanied by the accumulation of LDs.³² There is an imbalance in the rate of muscle protein synthesis and degradation in patients with COPD. The degradation rate of skeletal muscle protein is increased in cachectic patients with COPD, which causes muscle wasting.³³ Muscle wasting was reported to be associated with systemic inflammation and oxidative stress in COPD patients.^{34,35} Hallin et al analyzed 41 patients who were hospitalized due to exacerbation of COPD, and found that patients who lost weight during the 1-year follow-up were more likely to experience acute exacerbations than those who had no weight change or gained weight.³⁶ In addition, it has been reported that there was an association between the occurrence of sarcopenia and deteriorating lung function (FEV₁) in COPD patients.³⁷ Therefore, CKM and FITM1 may be involved in the exacerbation and

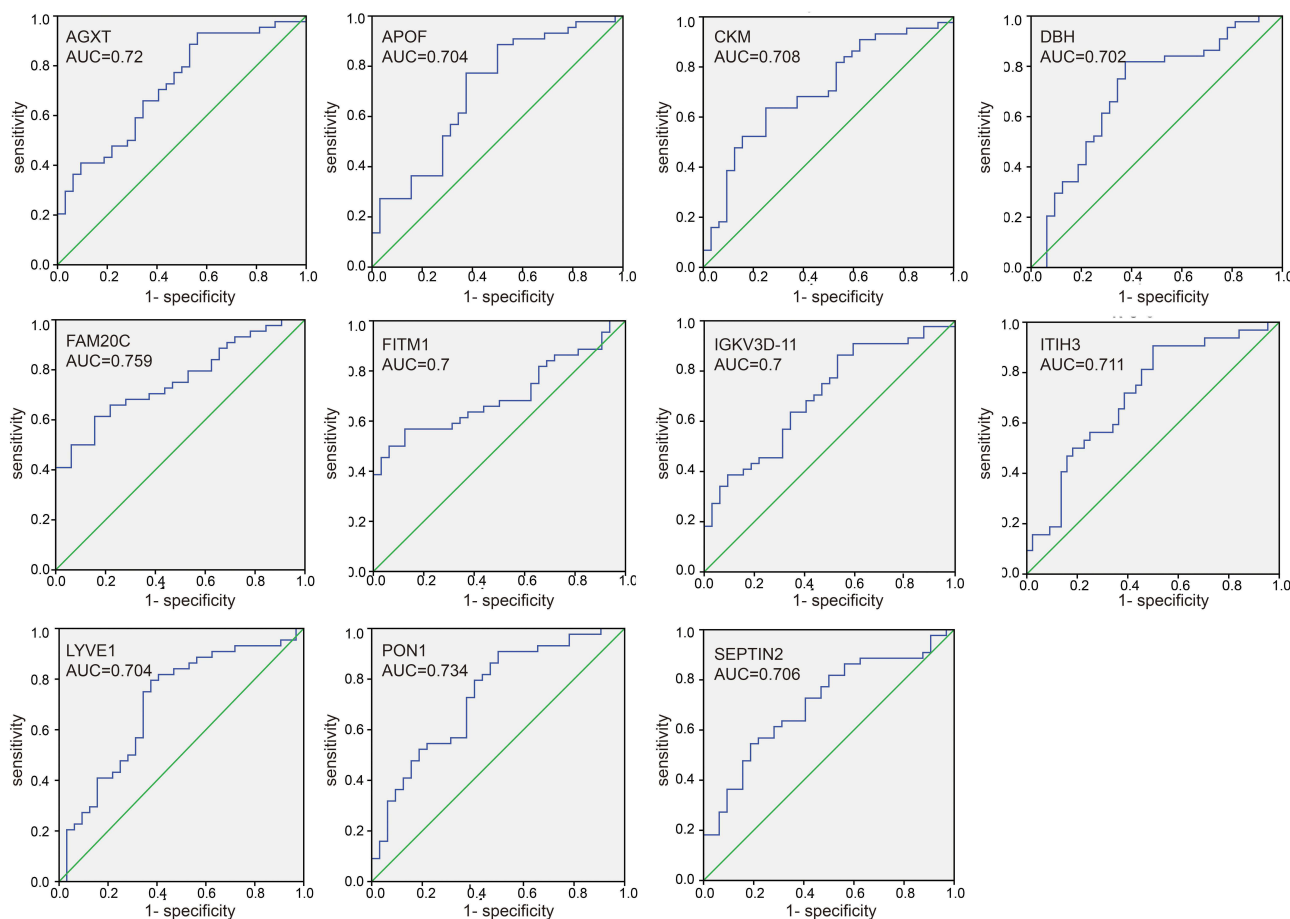


Figure 7 ROC curves for DEPs predicting the FE phenotype. $p < 0.05$ denotes a statistically significant difference.
Abbreviations: AUC, area under the curve; ROC, receiver operating characteristic; DEP, differentially expressed protein; FE, frequent exacerbation.

development of COPD. To date, few studies have investigated the relationship between CKM/FITM1 and COPD. Further studies are needed to explore the mechanisms of the two biomarkers in COPD.

Previous studies have reported that frequent exacerbators tend to have a lower FEV₁% predicted and a rapid decline in FEV₁.^{38–40} Moreover, a lower FEV₁ value was identified as an independent risk factor for future exacerbations.⁴¹ In

Table 3 Areas Under the Curve, Sensitivities, Specificities, and Optimum Cutoff Scores of the DEPs for Predicting Frequently Exacerbated COPD

Protein	AUC	95% CI	Optimal Cutoff	Sensitivity	Specificity	p-value
ITIH3	0.711	0.594–0.828	0.406	0.906	0.500	0.002
LYVE1	0.704	0.582–0.826	0.420	0.795	0.625	0.003
PON1	0.734	0.620–0.849	0.409	0.909	0.500	0.001
AGXT	0.720	0.605–0.853	0.369	0.932	0.437	0.001
IGKV3D-11	0.700	0.582–0.817	0.333	0.864	0.469	0.003
SEPTIN2	0.706	0.589–0.823	0.357	0.545	0.812	0.002
APOF	0.704	0.583–0.824	0.398	0.773	0.625	0.003
DBH	0.702	0.579–0.825	0.443	0.818	0.625	0.003
CKM	0.708	0.590–0.826	0.386	0.636	0.750	0.002
FAM20C	0.759	0.654–0.865	0.458	0.614	0.844	<0.001
FITM1	0.700	0.583–0.817	0.443	0.568	0.875	0.003

Abbreviations: COPD, chronic obstructive pulmonary disease; DEP, differentially expressed protein; AUC, area under the curve; CI, confidence interval.

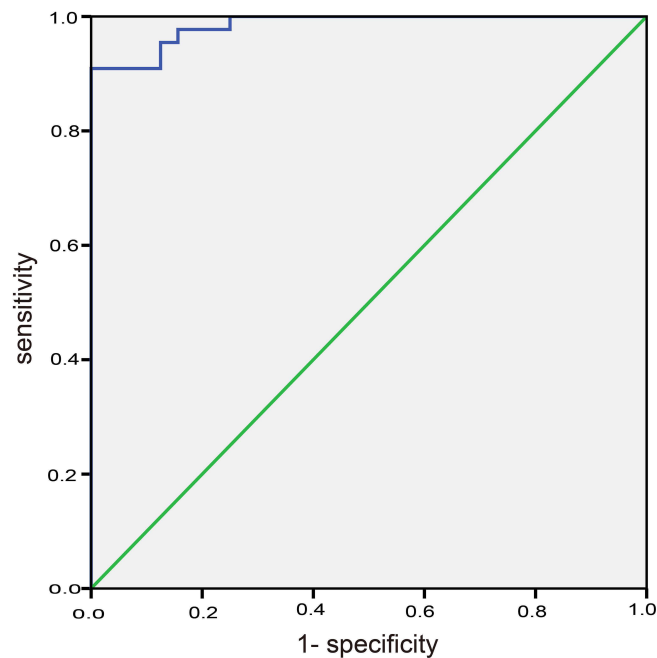


Figure 8 ROC curve for the combination of 11 DEPs predicting the FE phenotype. $p < 0.05$ denotes a statistically significant difference. **Abbreviations:** ROC, receiver operating characteristic; DEP, differentially expressed protein; FE, frequent exacerbation.

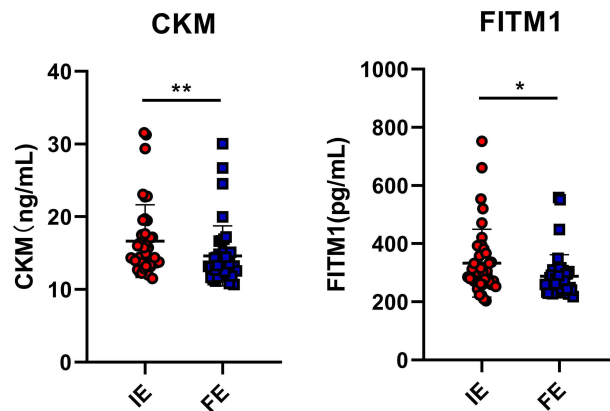


Figure 9 Plasma levels of CKM and FITM1 between the FE and IE subjects. * $p < 0.05$; ** $p < 0.01$.

Abbreviations: IE, infrequent exacerbation; FE, frequent exacerbation; CKM, creatine kinase, M-type; FITM1, fat storage-inducing transmembrane protein 1.

this study, FEV_1 and $FEV_1\%$ predicted were significantly lower in the FE group than in the IE group. In addition to CKM and FITM1, ARPC5, C4B, FCRL5, IGLC2/IGLC3 and UBE3C expression levels were correlated with pulmonary function, suggesting that these proteins might have significant associations with the development of airflow obstruction and be essential in the FE phenotype. FCRL5 belongs to the immunoglobulin receptor superfamily and is expressed on both mature B cells and plasma cells. A recent study identified a novel single-nucleotide polymorphism of FCRL5 that was significantly associated with a risk of developing asthma with comorbid allergic rhinitis.⁴² The UBE3C gene is crucial in antigen presentation and immune invasion. Polymorphisms in this gene could affect NF- κ B activation by modulating I κ B ubiquitination and were involved in the decrease of lung function with airway inflammation.⁴³

ROC analysis of the 11 selected DEPs revealed that a combinative biomarker panel was the optimal approach for discriminating FE from IE in COPD patients. LYVE-1 binds to hyaluronan on the luminal surface of lymphatic vessels. LYVE-1 expression was reported to be significantly higher in the lung tissue of COPD patients compared with non-

COPD smokers and was correlated with FEV₁% predicted.⁴⁴ PON1, a member of the paraoxonase family of enzymes, has antioxidant properties, and is involved in redox homeostasis. Lada et al reported that patients with COPD had lower PON1 activity compared with healthy subjects,⁴⁵ and they found that PON1 polymorphism could contribute to the reduction of PON1 activity in patients with COPD.⁴⁶ SEPTIN2 is a member of a highly conserved GTPase family. It was revealed that SEPTIN2 fundamentally regulated airway epithelial barrier function in response to pathological luminal stimuli by altering cortical actin expression.⁴⁷ The protein encoded by DBH catalyzes the conversion of dopamine to norepinephrine, which is the main neurotransmitter of the sympathetic nervous system.⁴⁸ A DBH polymorphism (rs3025343) was significantly associated with smoking cessation in patients with COPD.⁴⁹

Interactions between COPD and other DEPs (meaningful in the correlation and ROC analysis) have not been widely reported according to our literature retrieval. Further studies will focus on exploring the regulatory roles of those potential biomarkers for the early diagnosis and prevention of the FE phenotype of COPD.

Conclusions

In this study, two novel biomarkers (CKM and FITM1) and a comprehensive perspective on the mechanism of FE phenotype were found by detecting, analyzing, and verifying plasma samples with proteomics in this study. These findings can be applied in the precision diagnosis and treatment of COPD. Furthermore, a panel of 11 plasma biomarkers might be a useful tool for discriminating the FE patients from the IE patients. Further investigations are required to elucidate the pathophysiological function of plasma proteins involved in the FE phenotype of COPD via enlarging the sample size and performing researches in vitro and in vivo.

Abbreviations

COPD, chronic obstructive pulmonary disease; FE, frequent exacerbation; IE, infrequent exacerbation; LC-MS/MS, liquid chromatography-tandem mass spectrometry; DDA, data-dependent acquisition; DIA, data-independent acquisition; AUC, area under the curve; ROC, receiver operating characteristic; ELISA, enzyme-linked immunosorbent assay; GOLD, Global Initiative for Chronic Obstructive Lung Disease; FEV₁, forced expiratory volume in 1 s; FVC, forced vital capacity; DEPs, differentially expressed proteins; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; PPI, protein-protein interaction; PCA, principal component analysis; PLS-DA, partial least squares discrimination analysis; ARPC5, actin-related protein 2/3 complex subunit 5; C4B, complement C4-B; CKM, creatine kinase, M-type; FCRL5, Fc receptor like 5; FITM1, fat storage-inducing transmembrane protein 1; IGLC2, immunoglobulin lambda constant 2; UBE3C, ubiquitin protein ligase E3C; ITIH3, inter-alpha-trypsin inhibitor heavy chain 3; LYVE1, lymphatic vessel endothelial hyaluronan receptor 1; PON1, paraoxonase 1; AGXT, alanine-glyoxylate and serine-pyruvate aminotransferase; IgKV3D-11, immunoglobulin kappa variable 3D-11; SEPTIN2, septin-2; APOF, apolipoprotein F; DBH, dopamine beta-hydroxylase; FAM20C, extracellular serine/threonine protein kinase FAM20C; HLA-DQA1, major histocompatibility complex class II, DQ alpha 1; PIGR, polymeric immunoglobulin receptor; BGN, biglycan; LD, lipid droplet; PGC-1 α , peroxisome proliferator-activated receptor-gamma coactivator 1 alpha.

Data Sharing Statement

The data used to support the findings of this study are available from the corresponding authors upon request.

Ethical Approval

The study has been approved by the Ethics Committee of Zhongshan Hospital affiliated with Fudan University (No. B2017-022R).

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Disclosure

The authors declare that they have no competing interests.

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