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A journey to unravel the pathophysiology of stable and exacerbated Chronic obstructive pulmonary disease through erythrocyte proteomics: A combined mass spectrometry/bioinformatics approach

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Abstract

Chronic Obstructive Pulmonary Disease (COPD) is a progressive lung disorder with high mortality. The present study, explores the novel and highly enriched protein networks differentially expressed in stable and exacerbated COPD variants to elucidate the disease pathophysiology. A label free relative quantification of erythrocyte cytosol proteome based on LC-MS/MS was performed on hemodepleted erythrocyte lysate samples of stable and exacerbated COPD with respect to healthy controls.

Five highly enriched protein clusters in stable and seven in exacerbated COPD were observed, on the application of MCODE algorithm and hierarchical clustering. Functional annotation and over-representation analysis (ORA) of the differentially expressed proteins brought to light the dysregulation of molecular events such as ERAD pathway, MAPK signalling, ciliogenesis, hypoxia, apoptosis and neutrophil migration resulting in the chronic inflammatory response characteristic to COPD. This study is a first-time report of the differential expression of unique proteins such as kyphoscoliosis peptidase, sperm associated antigen-1, calpastatin and LINE-1 in exacerbated COPD, which would lead to chronic bronchitis, bronchiectasis and bacterial or viral infections, contributing to increased severity in exacerbated COPD. The identified proteins could serve as potential candidates for biomarkers in unravelling the pathophysiology of COPD.

1. Introduction

Chronic Obstructive Pulmonary Disease (COPD) is a progressive and non-reversible lung disease that is projected to become the third leading cause of global mortality by the year 2030.Currently at 3 million deaths per year, mortality associated with COPD is expected to rise to 5.4 million annually by the year 2060, owing to population aging, smoking, urbanisation and its related risks (1).

COPD is characterized by irreversible airway obstruction and related respiratory symptoms like difficulty in breathing, cough, and excessive sputum production and could be either stable or exacerbated depending on the substantial variation in its progression and severity. Stable COPD are interspersed with episodes of exacerbation, defined by The Global Initiative for Chronic Obstructive Lung Disease (GOLD) as “an event in the natural course of the disease that is characterized by a change in the patient’s baseline dyspnoea, cough, and sputum that is beyond normal day-to-day variations, is acute in onset and warrants a change in regular medication” (2).

Cell-based or cell free proteomics is a highly reliable technique that can be used to explore and characterize the pathways that are involved in the pathophysiology of various diseases. Many of the previous studies pertaining to erythrocyte characteristics, have reported impaired hemorheology in a COPD pathophysiology. These hemorheological alterations in response to COPD could be a reflection of the differential expression at the protein level, which besides affecting the erythrocyte morphology and ultrastructure, also brings about functional alterations (3). Moreover, erythrocytes are highly differentiated cells fundamental to nearly all basic physiological mechanisms in the human body which makes them

increasingly susceptible to oxidative stress and cytokine upregulation, both of which are hallmarks of chronic inflammation (4, 5).Hence erythrocyte proteoform analysis approach in COPD could prove highly beneficial.

There have been previous reports of the downregulation of erythrocyte membrane proteins such as chorein and methemoglobin reductase in COPD samples when in comparison to healthy controls. Nevertheless, these studies failed to explain or answer essential queries connecting COPD pathophysiology to altered erythrocyte proteome conclusively (6).This could be attributed to many factors such as the use of a labelled proteomic approach or projecting a single protein as a biomarker. These studies also leave many queries unadressed such as the i) masking of less abundant erythrocyte proteins by abundant proteins such as hemoglobin ii) whether cytosolic erythrocyte proteomics have been explored well enough? iii) can erythrocyte protein profiling explain the altered downstream signalling mechanisms that could distinguish stable COPD from COPD exacerbations?

The present study, by addressing these queries explores the highly enriched protein networks differentially expressed in stable and exacerbated COPD. Unmasking the differential expression of previously unexplored novel proteins along with highly enriched protein clusters could prove useful in better understanding the pathophysiological mechanism of COPD and its effect on the hemorheological properties of erythrocytes. This study has combined high throughput protein profiling techniques with bioinformatic approaches to make reliable conclusions connecting erythrocyte proteome with the pathophysiology of COPD. This study thus has immense future prospects in that it could facilitate the development of protein panels that can lead to the effective prognosis, diagnosis and therapeutic management of COPD.

2. Materials And Methods

2.1 Subjects and samples

This study is a comparative cross-sectional erythrocyte proteomic profiling study carried out in a tertiary care hospital in compliance with the Institutional Ethics committee and the Helsinki Declaration of 1975, revised in 2000. All the study group participants were in the age group of 40 to 80, whose blood samples were collected after obtaining written informed consent.

The groups for the proposed study were classified as,

Group A : Exacerbated COPD (AECOPD)

Group B : Stable COPD

Group C : Healthy control.

The stable and exacerbated COPD patients were diagnosed with the help of a pulmonologist, in accordance with the GOLD guidelines. Control samples were collected from healthy volunteers. COPD

patients with other auto-immune diseases and/or chronic conditions were excluded from the study.

A total of 10 healthy controls and 10 patients each in stable and exacerbated COPD, were analysed for hemorheological parameters and markers of inflammation such as ESR and CRP. The proteomics analysis was carried out in biological replicates of 3 healthy controls and a total of 7 COPD-3 stable COPD and 4 exacerbated COPD samples. The sample size in each group were reached based on the homogeneity in hemorheological parameters among the 30 analysed samples and based on Cairn's formula (7).

Blood samples were collected in fasting conditions in EDTA tubes and kept frozen until analysis to minimize proteolysis. The samples were centrifuged at 2800 rpm for 10 minutes and the plasma separated. The erythrocyte portion was then washed in PBS and stored resuspended in PBS containing EDTA-free protease inhibitor cocktail tablets, 4ºC, until further processing.

2.2 Erythrocyte lysis and fractionation

Erythrocyte lysates were prepared in accordance with the erythrocyte lysis protocol by Ana Sofia Carvalho et al (8). Ice-cold lysis buffer (5mM Na_2HPO_4, 8mM EDTA, pH-8) was added to the PBS washed erythrocyte samples and centrifuged at 25,000g for 30 minutes at 4ºC. 0.5 mM PMSF was added to sample before centrifugation to prevent protease activity. The cytosolic proteins upon RBC lysis moves to the supernatant, which is collected and stored at

-80°C for further processing.

2.3 Haemoglobin depletion of the erythrocyte cytosolic fraction

Haemoglobin by virtue of its abundance can mask the expression of other low abundance cytosolic proteins in the supernatant. HemoVoid™, a silica-based protein enrichment matrix from Biotech Support Group USA, was used to remove hemoglobin from erythrocyte lysate samples to unmask low abundance, and/or low molecular weight proteins according to the manufacturer's protocol.

2.4 Protein concentration and normalization

Eluate obtained after haemoglobin depletion was further filtered using Amicon Ultra 0.5 centrifugal 5-kDa molecular weight cut-off filters (Millipore) to remove salts and other molecules larger than 5 kDa. Bichinchoninic acid assay (BCA) was performed for protein quantitation and the samples were normalized to a final concentration of 1mg/ml.

2.5 LC MS/MS

The proteomic profiling was performed by liquid Chromatography tandem Mass Spectrometry (LC–MS/MS) at the Mass spectrometry & Proteomics Core facility of Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram, Kerala. Relative protein quantification was performed using ESI-nanoLC-MS/MS (nanoACQUITY UPLC® chromatographic system, Waters, Manchester, UK). Trypsin digestion was carried

out using standard protocols, ie, the samples were reduced using 100 mM 1, 4-Dithiothreitol (Sigma Aldrich), alkylated using 200 mM iodoacetamide (Sigma Aldrich) and digested over night with MS grade trypsin (Sigma Aldrich) in the ratio 1:25 (1 µg of trypsin to 25 µg of protein). All the samples were injected in duplicate (2 injections/ sample). The injection volume was 2.0 µl.

Aliquots were chromatographed on a column with specifications (Trap column: Symmetry® 180µm x 20mm C18 5µm, waters; Analytical column: 75 µm * 200 mm HSS T3 C18 1.8 µm, waters). Peptide separation was carried out at 300 nL/min for 90 min using a Five-step acetonitrile gradient 5–40% over the first 55min and 40–95% for the following 35min. The backpressure varied between 450 and 650 bar. The temperature of the column oven was 35 ºC.

2.6 Mass spectrometry conditions

MS runs were performed in MSE mode in combination with ion mobility (HDMSE). MS system: Synapt G2 High Definition MS™ System (HDMS^E System) Waters, Calibration : Sodium iodide, Acquisition mode: ESI positive, Online mass correction: Using Leucine encephalin in positive ion mode (m/z = 556.2766),Nano ESI capillary voltage: 3.4 KV, Sample cone: 40 V ,Extraction cone: 4 V,IMS gas (N_2) flow: 90 (mL/min),IMS T-Wave™ pulse height: 40 V,IMS T-Wave™ velocity: 800 m/s, IMS voltage: 8 V and 20 V, Mode of operation: Resolution mode ,Data acquisition format: continuum ,Collision energy: 20 eV to 45 eV

2.7 Data analysis conditions

Analysis software: Progenesis QI for Proteomics V4.2 (Non Linear Dynamics, Waters) ,Organism for protein identification: “Homo sapiens” – Reviewed entries only, Database source: UniProt DB (https://www.uniprot.org/),False positive rate: 1, Missed cleavage: 1,Number of fragments per peptide: 1,Number of fragments per protein: 3,Number of peptides per protein: 1, Fixed modification: Cysteine carbamidomethylation, Variable modification: Oxidation of methionine, p -value-0.05.

2.8 Bioinformatic analysis

Two groups were defined for the relative quantitation of stable and exacerbated COPD with respect to control.

They are: Group C Vs A – Protein profiling of erythrocyte samples in exacerbated COPD with respect to control

Group C Vs B - Protein profiling of erythrocyte samples in stable COPD with respect to control

Only unique peptides with high confidence score were used for the identification of proteins. A Venn diagram was constructed using Venny (bioinfogp.cnb.csic.es/tools/venny/index.html) to identify the proteins unique to and common to the COPD variants using the healthy control group as a reference.

The differentially expressed proteins (DEPs) in stable and exacerbated COPD were mapped and functionally annotated for their biological processes (BP) using the Databases for Annotation,

Visualization and Integrated Discovery (DAVID), a free online bioinformatic resource (https://david.ncifcrf.gov/), and the major functions visually represented.

g:GOST,an over-representation analysis (ORA) tool available in g:Profiler (biit.cs.uf.ee/gprofiler/) was used to perform functional enrichment analysis of the DEPs against REACTOME database. The major biological pathways enriched were visualized in Metascape, a free gene annotation tool that provides automated meta-analysis of expression data.

Protein-protein interaction networking of the DEPS in stable and exacerbated COPD variants with respect to control were constructed using STRING, a biological database of known and predicted Protein-protein interactions (https://string-db.org/) The interaction network was then imported and visualized in Cytoscape 3.9.1, in Yfiles circular layout. The top 20 hub genes in the PPI network were analyzed using Cytohubba plugin in Cytoscape.

Enriched protein-protein interaction clusters were determined based on an MCODE algorithm using Metascape. This algorithm uses vertex weighting, complex prediction and post processing to identify densely connected regions.

Heatmap of differentially expressed erythrocyte proteins in COPD variants with respect to control was constructed using heatmapper (heatmapper.ca), a freely available web server that allows interactive visualization of data in the form of heatmaps.

The DEPS unique to the two groups contributes to the variation in severity and physiological manifestations observed in them and helps distinguish stable COPD pathophysiology from the exacerbated one. Based on the pathway enrichment analysis and MCODE clustering, a pathway exploring the connection between PPI clusters and how they are differentially expressed in stable and exacerbated COPD were illustrated using Biorender, an online scientific illustration platform.

3. Results

The current study is a relative profiling of erythrocyte cytosolic proteome in stable and exacerbated COPD with respect to healthy controls. A schematic representation of the complete workflow is given in Figure.1.

A total of 10 erythrocyte cytosolic samples from 3 stable COPD, 4 exacerbated COPD and 3 healthy controls were analyzed by high-definition mass spectrometry. The baseline characteristics of the COPD patients and healthy controls are as given in supplementary table S1.

A total of 122 unique proteins were identified to be differentially expressed in stable COPD with respect to healthy controls, whereas 136 unique proteins were differentially expressed in exacerbated COPD. After application of FDR 1% and screening based on max fold change set at 1.5 for upregulation and 0.6 for downregulation, 92 DEPs in stable COPD and 95 DEPs in exacerbated COPD were observed with respect to healthy controls as listed in supplementary tables S2 and S3 respectively. 75 differentially expressed proteins were common to both the stable and exacerbated COPD groups, whereas 17 DEPs were unique

to the stable COPD group and 20 DEPs were unique to the exacerbated group, as shown in Fig. 2 (Supplementary table S2).

The GO biological processes that were mostly dysregulated in the differentially expressed proteins were proteosome mediated ubiquitin dependant and independent catabolic process, negative regulation of apoptotic process and response to xenobiotics and oxidative stress, as shown in Fig. 2 (Supplementary table S4).

The most enriched pathways in the g:Profiler Over representation analysis (ORA) performed in the differentially expressed proteins across stable and exacerbated groups (Supplementary table S5), was schematically represented as shown in Fig. 2. The overrepresented pathways were cellular response, response to chemical stress, neutrophil degranulation, cellular detoxification, proteolysis regulation and platelet degranulation. The protein-protein interaction network of the two groups with respect to healthy controls were constructed and visualized in Cytoscape (Supplementary Fig. S1). Of these, the most enriched protein interaction clusters in stable and exacerbated COPD were identified through the application of an MCODE algorithm (Supplementary Fig. S2) respectively and the PPI network model related to the most enriched functional pathways were constructed using Metascape. This PPI network model shines light on how the enriched protein clusters with differential expression leads to stable and exacerbated COPD pathophysiology through the dysregulation of functional pathways as shown in Fig. 3.

To define the expression pattern of the differentially expressed proteins in the stable and exacerbated COPD with respect to control, a heatmap was constructed with hierarchical clustering as shown in Fig. 4.

A pathway modelling of stable and exacerbated COPD, based on the ORA of highly enriched protein clusters were hypothesized and illustrated.

In both the COPD variant conditions, the major PPI clusters enriched are involved in the dysregulation of ERAD pathway, neutrophil degranulation, positive response to stress, platelet degranulation among others. The differential expression of unique proteins such as kyphoscoliosis peptidase, Calpastatin, Sperm associated antigen-1and LINE-1 could be a contributing factor in the increased severity observed in exacerbated variant of COPD.

4. Discussion

At present, the biomarkers for COPD includes proteins such as C-reactive protein (CRP), fibrinogen and RAGE, all of which are indicators of generalized inflammation. A review of previous COPD literature, showed studies pertaining to erythrocyte deformability, red blood cell aggregation and hematocrit (Hct) alterations which were corroborated by our findings in 10 patient samples each in COPD variant groups, with respect to control (9). These alterations prompted us to carry out erythrocyte protein profiling in COPD in a severity dependant manner, as the exact pathophysiological mechanism of COPD remains inconclusive to date. Recent studies in the past have attempted to uncover novel proteins in COPD, but

due to a focus on targeted approach to a single protein biomarker, exploration of pathophysiology and its distinction in the two variants are incomplete (10).

With the removal of hemoglobin from the erythrocyte lysate, the low abundance proteins in the cytosol are unmasked, potentiating the discovery of novel proteins and their role in COPD pathophysiology. Hence this study is a minimally invasive non-targeted profiling of hemodepleted erythrocyte cytosolic proteome.

In both stable and exacerbated COPD variants, S100A8 and S100A9 were upregulated, which are Ca^{2+} binding proteins released actively during inflammation, acting as a scaffold for further physiological events. Functional analysis of the enriched DEPs in stable COPD revealed their contribution to pathways such as the ERAD pathway, wherein differential expression of the proteosomal complex led to accumulation of unfolded proteins which could trigger the activation of inflammatory signalling pathways such as NF-KB and JNK-AP1, contributing to chronic inflammatory response in COPD (11). Associated neutrophil degranulation could activate MAPK signalling; another inflammatory pathway (12).

Differential expression of proteins such as 14-3-3, peroxiredoxin and Rab GDP dissociation inhibitor alpha observed in the study and their cross talk with each other could lead to the dysregulation of ciliogenesis, leading to ciliary dysfunction which explains mucus hyperplasia in COPD. We could map proteins such as Serpina 1, ORM1, transferrin, albumin and hemopexin to platelet degranulation and activation, which could explain the positive modulation of hypoxia signalling pathway in COPD (13). The hypoxic conditions and associated dyspnoea in COPD patients could be attributed to the upregulation of all the proteins involved in platelet activation and aggregation, as observed in our study.

An enriched cluster with proteins such as tubulin folding cofactor and myotrophin was mapped to the activation of semaphorin plexin signalling leading to neutrophil migration and increased vascular permeability, which may contribute to the chronic inflammatory condition observed in stable COPD condition (14).

The differential expression of proteins such as proteosome complex, valosin containing protein and α synuclein in exacerbated COPD variant could lead to a defective CFTR pathway leading to chronic bronchitis in exacerbations (15). This study is a first-time report of the differential expression of proteins such as kyphoscoliosis peptidase, sperm associated antigen-1, Calpastatin and LINE-1 type transposase domain containing protein 2 in exacerbated COPD.

Kyphoscoliosis peptidase, a protein associated with muscle organ development was found to be downregulated in the exacerbated variant of COPD in our study. Abnormal muscle development as a result of this differential expression could manifest as Myofibrillar myopathy, a comorbidity often associated with severe COPD exacerbation(16). Sperm associated antigen-1 was another unique protein found to be upregulated in exacerbation. Functional mapping of this protein shows its vital role in axonemal dynein complex assembly. Its differential expression could lead to severe abnormalities to the motile cilia leading to bronchiectasis and bacterial or viral infections leading to exacerbations (17, 18).

Calpastatin, a calpain inhibitor, was found to be overexpressed in exacerbated COPD samples. This could lead to dysregulated endothelial nitric oxide synthase expression, associated with increased severity in COPD (19). LINE-1 type transposase domain containing protein 2 overexpression in exacerbated COPD was found to initiate oxidative stress induced DNA strand breaks promoting apoptosis, cell senescence and pro-inflammatory response (20).

A few other DEPs common to both the stable and exacerbated variants were biphosphoglycerate mutase, bleomycin hydrolase and hypoxanthine phosphoribosyl transferase (HPRT1). Biphosphoglycerate mutase was found to be downregulated in the COPD variants with respect to control, contributing to dysregulated glucose metabolism, respiratory gaseous exchange, O_2 transport and erythrocyte development in COPD (21). Underexpressed HPRT1 disrupts purine salvage which could lead to high uric acid levels, contributing to rapid decline in lung function observed in COPD variants with respect to control (22–24).

Adapting a systematic and mechanistic strategy that involved the determination of the biological processes and the enriched pathways of the hub proteins helped in hypothesizing the pathophysiology of stable and exacerbated COPD. The identified proteins common and unique to the stable and exacerbated COPD, with respect to control are involved in a wide range of functions from carbohydrate and nucleotide metabolism to inflammation and apoptosis to ciliary function to hypoxia, all playing inevitable roles in the pathogenesis and progression of COPD. Further validation studies in to these unexplored proteins, reported for the first time in exacerbated COPD could help in better understanding the disease pathophysiology, leading to better therapeutic management of COPD variants, through the development of specialised protein panels.

5. Conclusion

In summary, this study adopted a mass spectrometric and bioinformatics combined strategy to unravel the pathophysiology of stable and exacerbated COPD. The hemoglobin depleted erythrocyte cytosolic proteins which are unique to exacerbated COPD, such as kyphoscoliosis peptidase, sperm associated antigen-1, LINE 1 and calpastatin could help in differentiating stable and exacerbated COPD clinically. The data obtained from high throughput mass spectrometry was combined with bioinformatic techniques to attempt a pathophysiological modelling of stable and exacerbated COPD. The unmasked proteins and enriched protein clusters would serve as a promising scaffold for future research as further studies into these proteins could provide valuable insights into their potential as a biomarker, thereby aiding in the therapeutic management of stable and exacerbated COPD.

Abbreviations

AECOPD-Acute exacerbated chronic obstructive pulmonary disease, ACD- Anemia of chronic disease, BCA- Bichinchoninic acid, BP-biological process, CFTR-Cystic fibrosis transmembrane conductance regulator, CRP-C reactive protein, DAVID-Databases for Annotation, Visualization and Integrated

Discovery, DEP-Differentially expressed protein, ESR- Erythrocyte sedimentation rate, ERAD-Endoplasmic reticulum associated protein degradation, FDR-False discovery rate, GOLD-The Global Initiative for Chronic Obstructive Lung Disease, HCT-Hematocrit, HPRT1-hypoxanthine phosphoribosyl transferase, JNK-AP1-c Jun N-terminal kinase/activator protein -1, LC-MS/MS – liquid chromatography tandem mass spectrometry, MAPK-Mitogen activated kinase, MCODE-molecular complex detection, NF-κB-Nuclear factor kappa B, ORA-Over representation analysis, PPI-Protein protein interaction, PMSF-Phenylmethylsulfonyl fluoride, STRING-Search tool for the retrieval of interacting genes/proteins.

Declarations

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DECLARATION OF INTEREST

The authors of the article entitled “Unravelling the pathophysiology of stable and exacerbated COPD by erythrocyte proteomics: A mass spectrometric/bioinformatics approach” declare no conflict of interest in this work.

On behalf of the authors

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Figures

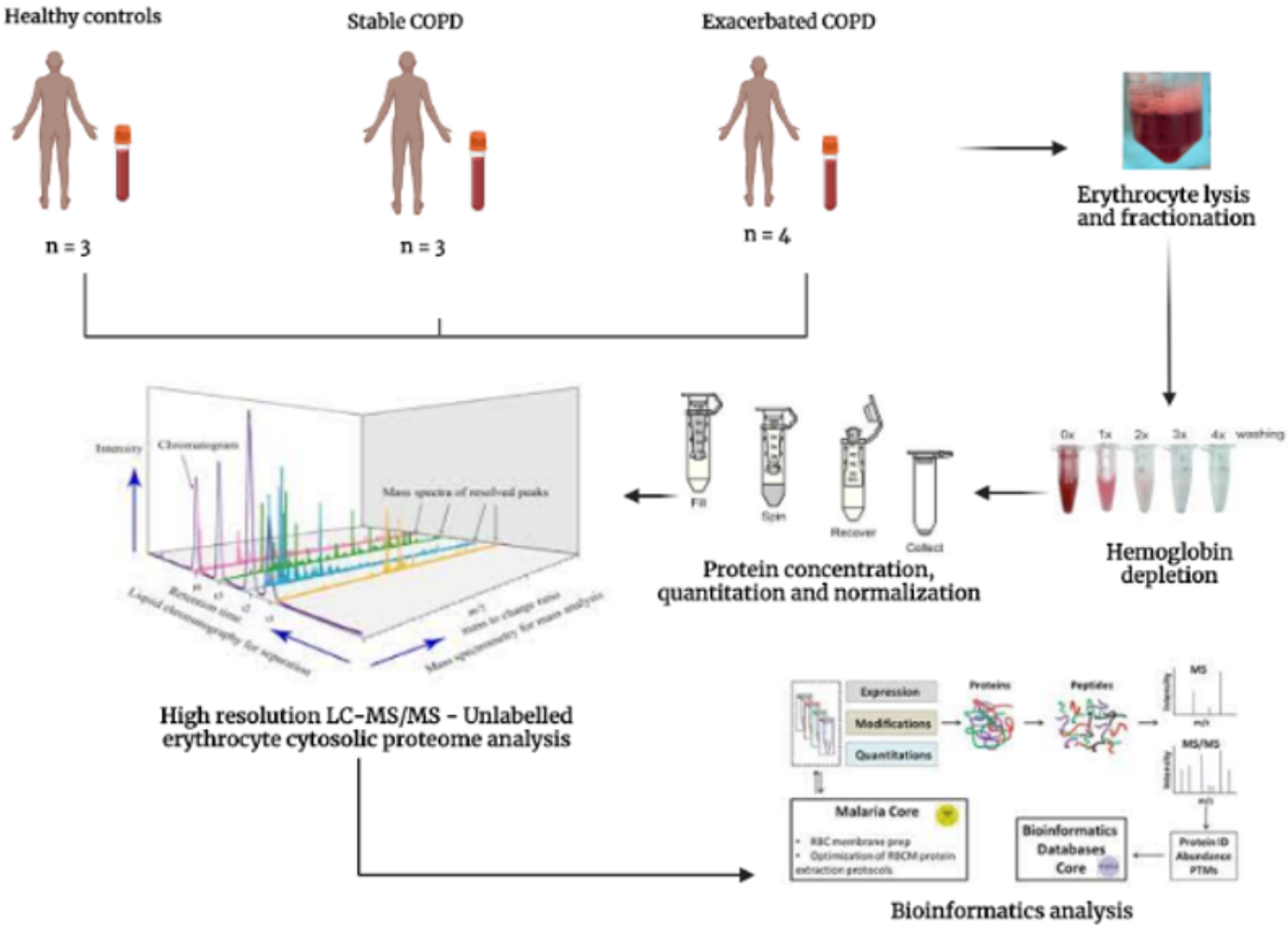
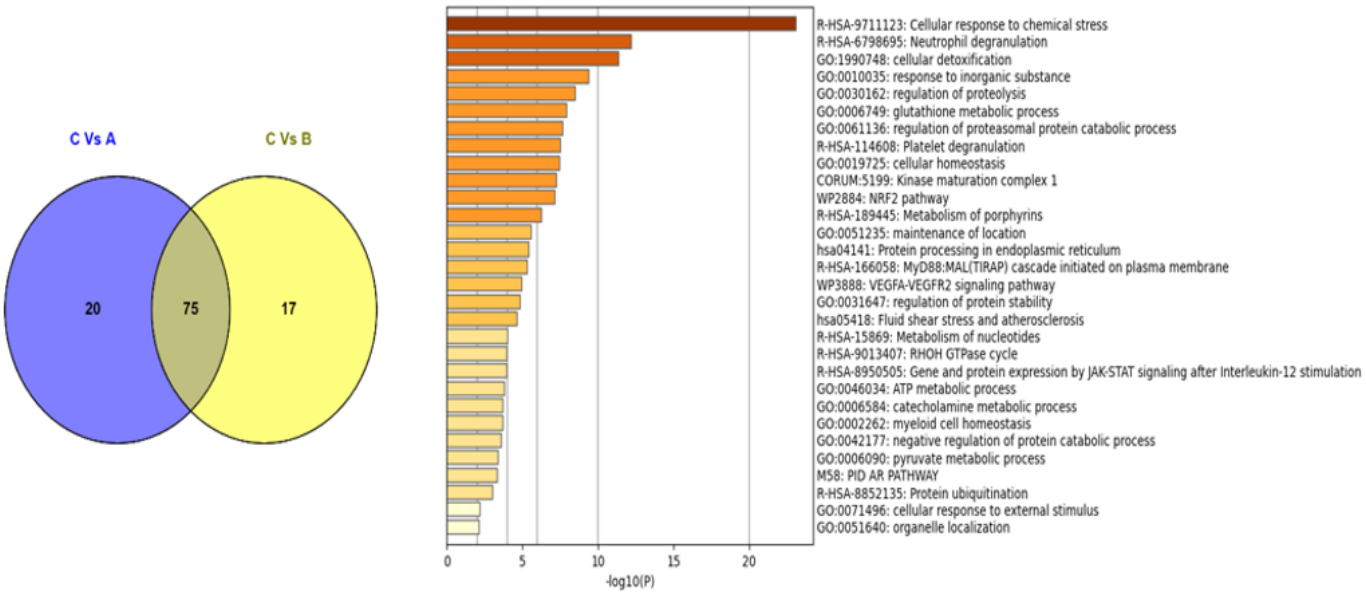


Figure 1

A schematic workflow of the erythrocyte cytosolic proteome analysis

Overview of the study design: the protein profiling of the human erythrocyte cytosolic fraction was performed in the two COPD variant groups with respect to control via LC-MS/MS, after hemodepletion. Then the differentially expressed proteins were functionally annotated and their interaction with each other identified through various bioinformatic techniques.



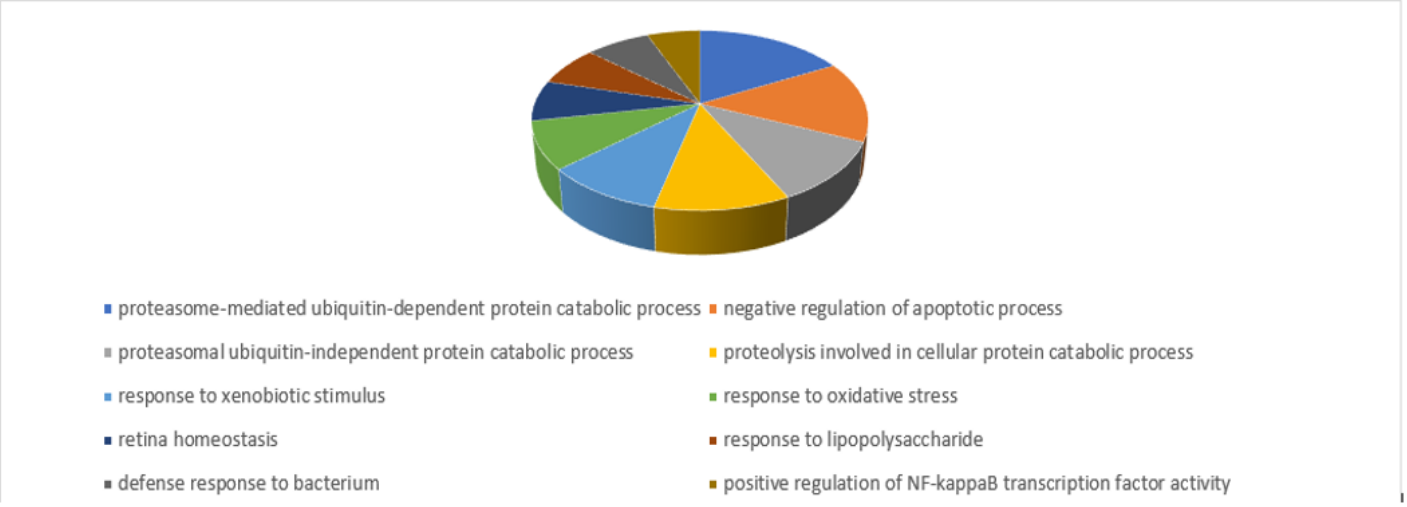


Figure 2

Functional mapping and pathway enrichment analysis of the differentially expressed proteins in stable and exacerbated COPD

a) Identification of differentially expressed proteins common and unique to stable (yellow) and exacerbated (blue) COPD with respect to each other. There are 75 proteins common to both the COPD

variants with respect to control, whereas 20 proteins are unique to exacerbated COPD and 17 unique to stable COPD. b) The major biological processes of the differentially expressed proteins analysed against the reactome database c) The top 30 pathways significantly enriched in the differentially expressed proteins common to both stable and exacerbated COPD with respect to control.

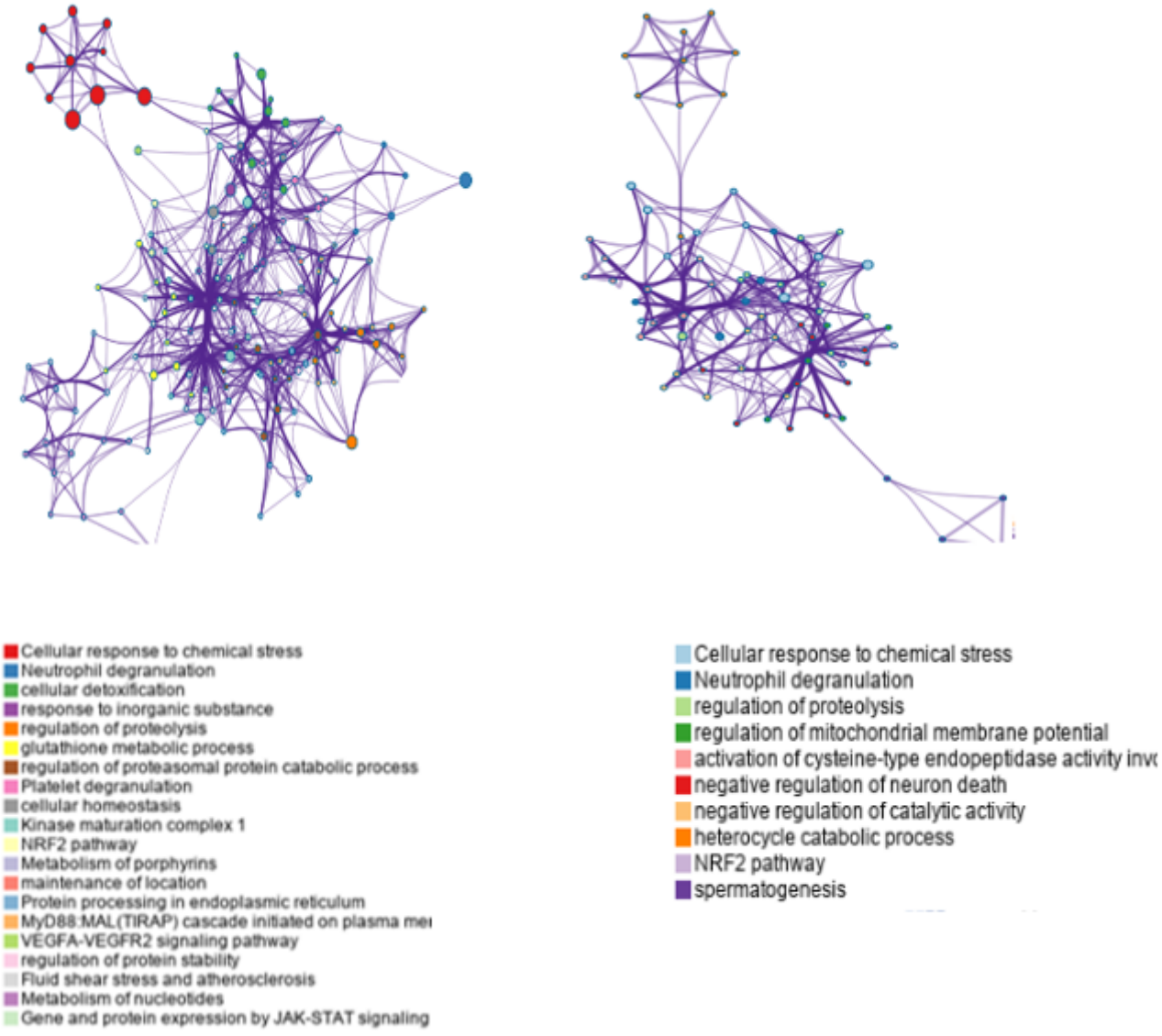


Figure 3

PPI network model related to the most enriched functional pathways in stable and exacerbated COPD

a)The PPI clusters enriched upon application of MCODE algorithm, classified according to their functionally enriched pathways in stable COPD. b) The PPI clusters enriched upon application of MCODE algorithm, classified according to their functionally enriched pathways in exacerbated COPD.

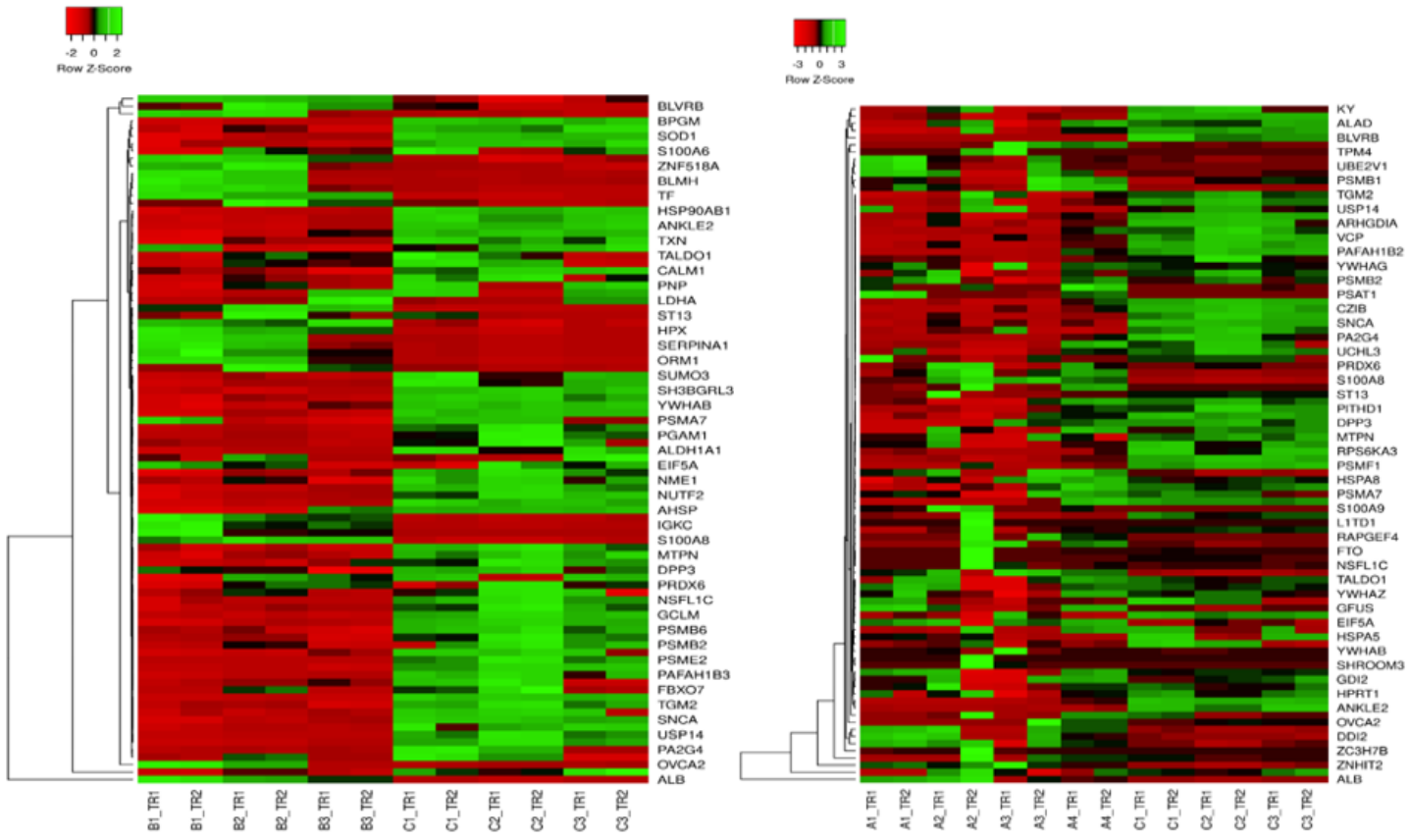


Figure 4

Heatmap of differentially expressed proteins in stable and exacerbated COPD with hierarchical clustering

Hierarchical clustering analysis performed in stable and exacerbated COPD with respect to control respectively, represented as a heatmap. The samples include the normalized abundance of proteins in the samples duplicated.

Supplementary Files

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- SUPPLEMENTARYMATERIAL.docx