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Protein Network Analysis Identifies Changes in the Level of Proteins Involved in Platelet Degranulation, Proteolysis and Cholesterol Metabolism Pathways in AECOPD Patients

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ABSTRACT

Chronic obstructive pulmonary disease (COPD) is characterised by a progressive pulmonary and systemic inflammation. Acute exacerbations of COPD (AECOPD) are associated with acute inflammation and infection, increase in the rates of morbidity and mortality. Previous proteomic studies have focussed on identifying proteins involved in COPD pathogenesis in samples collected from the lung (e.g. lung tissue biopsies, bronchoalveolar lavage and sputum) but not from blood of patients who experienced AECOPD. In this study, plasma was analysed by two independent quantitative proteomics techniques; isobaric tag for relative and absolute quantitation (iTRAQ) and multiple reaction monitoring (MRM) to identify differential expression of circulating proteins in patients with stable COPD (sCOPD) and AECOPD. Firstly, iTRAQ performed on pooled plasma samples from AECOPD, sCOPD, and healthy non-smoking controls (HC) revealed 15 differentially expressed proteins between the 3 groups. MRM subsequently performed on a separate cohort of AECOPD, sCOPD, and HC patients confirmed 9 proteins to be differentially expressed by AECOPD compared to HC (Afamin, alpha-1-antichymotrypsin, Apolipoprotein E, Beta-2-glycoprotein 1, Complement component C9, Fibronectin, Immunoglobulin lambda like polypeptide 5, Inter-alpha-trypsin inhibitor heavy chain H3, Leucine rich alpha-2-glycoprotein 1). Network analysis demonstrates that most of these proteins are involved in proteolysis regulation, platelet degranulation and cholesterol metabolism. In conclusion, several potential plasma biomarkers for AECOPD were identified in this study. Further validation studies of these proteins may elucidate their roles in the development of AECOPD.

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Introduction

Chronic obstructive pulmonary disease (COPD) is the most common global respiratory disease and is characterised by progressive airflow limitation and chronic inflammation. The common risk factors for COPD are cigarette smoking, air pollution and genetic predispositions [1]. Patients may experience acute exacerbations of COPD (AECOPD) which is defined as an acute worsening of respiratory symptoms requiring a change in therapy [1]. Aetiological factors of AECOPD include bacterial and viral infections, as well as environmental stresses [2–5], while 30% of cases are idiopathic [6]. Frequent AECOPD is associated with a poorer prognosis and is increasingly recognised as a multi-systemic disease [1]. However, little is known about the pathways and molecules that drive AECOPD and its systemic complications. Identifying pathogenic molecules will be useful in further delineating AECOPD and developing markers and therapeutic targets for this condition.

To this end, high throughput screening of proteins will provide an unbiased assessment of molecules in AECOPD. Development of proteomic-based screening techniques such as two-dimensional gel electrophoresis (2DGE) and mass spectrophotometry (MS) have identified markers of COPD [7]. Bronchoalveolar lavage fluid (BALF) showed increased neutrophil defensins 1 and 2, S100A8 (calgranulin A) and S100A9 (calgranulin B) while lung tissue demonstrated increased matrix metalloproteinase (MMP)-13, and thioredoxin-like 2 and surfactant protein A (SPA) in COPD patients compared to smoking and non-smoking HC [8–10]. Studies examining induced-sputum found lipocalin and apolipoprotein A1 reduced in COPD [11]. BALF and lung tissue from AECOPD are difficult to obtain due to the severity of presentations, while induced sputum is influenced by confounding secretions such as mucus. Therefore, we sampled plasma of patients with AECOPD, stable COPD and healthy non-smoking controls (HC) and examined differences in protein expression by iTRAQ (MRM).

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Table 1. Validation of differentially expressed proteins by MRM.

Proteins	UniProt ID *(peptides per protein)*	AECOPD *vs.* HC	*p*-value	AECOPD *vs.* sCOPD	*p*-value	sCOPD *vs.* HC	*p*-value
Proteins downregulated compared to controls							
Afamin	P43652 *(2)*	**0.71**	**0.004**	0.84	0.17	0.84	0.17
Beta-2-glycoprotein 1	P02749 *(2)*	**0.74**	**0.020**	0.84	0.16	0.88	0.20
Fibronectin	P02751 *(2)*	**0.36**	**<0.001**	0.69	0.14	**0.52**	**0.005**
Proteins upregulated compared to controls							
Alpha-1-antichymotrypsin	P01011 *(3)*	**1.46**	**0.003**	1.25	0.084	1.17	0.31
Apolipoprotein E	P02649 *(3)*	**1.66**	**0.016**	1.32	0.18	1.26	0.078
Complement component C9	P02748 *(2)*	**1.62**	**<0.001**	1.10	0.43	**1.47**	**0.005**
Immunoglobulin lambda-like polypeptide 5	B9A064 *(1)*	**2.49**	**0.001**	1.01	0.99	**2.47**	**0.013**
Inter-alpha-trypsin inhibitor heavy chain H3	Q06033 *(2)*	**1.59**	**0.007**	1.14	0.47	1.39	0.063
Leucine-rich alpha-2-glycoprotein	P02750 *(2)*	**1.67**	**0.009**	1.15	0.45	**1.45**	**0.035**

Data is expressed as the geometric mean ratios of peptides that represent the protein of interest and the significant (bold) changes in the protein level of the second study group. Statistical significance was indicated by student's *t*-test, $p < 0.05$.

Methods

Study demographics

The study was approved by the Ethics Committee at the Royal Perth Hospital (RPH), Western Australia (EC 2010/070) and all participants gave informed consent. Blood samples from study participants were collected in lithium heparin tubes. Plasma was separated by centrifugation (1000 *g*, 10 min) and stored at -80 °C. Patients admitted to the RPH Emergency Department with an episode of AECOPD and a separate cohort of stable COPD (sCOPD) patients from a dedicated RPH COPD clinic who were previous smokers (>15 pack-years and ceased smoking >5 years earlier) were recruited into our study. Stable COPD patients did not experience AECOPD for the last 6 months. AECOPD was diagnosed as "an acute worsening of respiratory symptoms requiring additional therapy" by an ED and respiratory physician, whilst the severity of COPD was categorised by a respiratory physician per the GOLD criteria [1]. Study demographics are presented in Supplementary Table 1. Samples were age-matched (Mann–Whitney test; *p*-values = 0.56–0.84) and sex-matched with slight overrepresentation of male subjects in each group (53–60%). All patients had been treated with anticholinergic drugs, long-acting beta agonists and inhaled corticosteroids but none were receiving systemic corticosteroids or had asthma, diabetes, neuromuscular, allergic or rheumatological disease at the time of sampling.

Plasma preparation and iTRAQ labelling

Individual plasma samples were pooled for each group ($n = 20$ in each group). A fourth group was generated by pooling 20 µL plasma from each of the three groups. Technical replicates were generated by splitting each of the groups into two aliquots. Fourteen high-abundant proteins in plasma were immunodepleted using a MARS14 chromatography column (Agilent Technologies, Australia). The iTRAQ technique was carried out as previously described [12]. Peptides were separated by strong cation exchange chromatgraphy (SCX) on an Agilent 1100 HPLC system. Eight fractions containing the peptides were then analysed by electrospray ionisation mass spectrometry using the Shimadzu Prominence nano HPLC system (Shimadzu, Australia) and a 5600 TripleTOF mass spectrometer (Sciex, Australia).

Multiple reaction monitoring (MRM)

Using a separate cohort of patients, MRM was carried out as previously described [12]. Plasma samples (20 µL per sample) from each group ($n = 15$) were immunodepleted, reduced, alkylated and trypsin digested before being desalted on a Strata-X 33 μm polymeric reversed phase column (Phenomenex). Peptides were analysed by electrospray ionisation mass spectrometry (LC/MS) using a Thermo UltiMate 3000 RS nanoflow UHPLC system (Thermo Scientific) coupled to a 5500 Q-TRAP mass spectrometer (Sciex). Unlabelled and ^{18}O-labeled reference standard plasma peptides were loaded onto an Acclaim™ PepMap™ 100 C18 LC Column, 2 µm particle size x 150 mm (Thermo Scientific) and separated with a linear gradient of water/acetonitrile/0.1% formic acid (v/v) over 18 min.

Quantitation of C-reaction protein by ELISA

Plasma levels of C-reactive protein (CRP) were measured as a biomarker of systemic inflammation using an ELISA kit according to manufacturer's instructions (R&D Systems, Minneapolis, MN, USA). A sample with known cytokine concentrations (QC) was assayed on each plate to assess inter-plate variation (coefficient of variance was <10%).

Data analysis

Protein identification and quantification for iTRAQ data were performed using ProteinPilot™ 4.5 Beta Software (AB Sciex, Framingham, USA) and spectra searched against the human SwissProt database. All identified proteins had an Unused Protscore of >1.3 (which corresponds to proteins identified with >95% confidence), and differentially expressed proteins were required to have at least two unique peptides and have significantly different protein ratios in at least one of the technical replicates (*p*-value of <0.05, the software performs a *t*-test between the peak intensities of each peptide in each disease group for a given protein) and a global false discovery rate (FDR) of <0.1% determined at the protein level by the PSPEP algorithm.

For MRM, all transition peaks for each target peptide were analysed using Skyline (University of Washington, Seattle, WA, USA). Un-labelled/^{18}O-labelled peptide ratios were exported from Skyline for further statistical analysis using SPSS for Windows (version 22; SPSS Inc., Chicago, IL). Peptide ratios for each target protein were combined to provide a 'protein-based' ratio that was then compared between AECOPD patients, sCOPD patients and HC using a student's t-test. A two-tailed level of significance of $p < 0.05$ was used throughout. Correlations were assessed using the non-parametric Spearman's rank correlation coefficients.

Results

Differential expression of plasma proteins between AECOPD patients, sCOPD and HC

A non-targeted approach (iTRAQ) was first performed on pooled samples ($n = 20$ in each group) and a total of 220 proteins were detected with >95% confidence. Of these, 15 proteins demonstrated significant differences (greater than 10%) between AECOPD *vs.* HC, AECOPD *vs.* sCOPD or sCOPD *vs.* HC (Supplementary Table 2). A targeted mass spectrometry approach (MRM) was subsequently performed in a separate cohort of AECOPD, sCOPD and HC ($n = 15$ in each group) for validation.

Nine proteins [afamin, α1-antichymotrypsin, apolipoprotein E (Apo-E); β2-glycoprotein 1, complement component C9, fibronectin; immunoglobulin lambda like polypeptide 5 (IGL5), inter-alpha-trypsin inhibitor heavy chain H3 (ITIH3), and leucine rich alpha-2-glycoprotein 1 (LRG1)] were confirmed as significantly different in AECOPD *vs.* HC (Table 1). Of these, 8 proteins correlated with the level of plasma CRP, a reliable marker of systemic inflammation in COPD [13], (afamin, $r = -0.53$, $p < 0.001$; α1-antichymotrypsin, $r = 0.39$, $p = 0.008$, β2-glycoprotein 1; $r = -0.41$, $p = 0.006$; complement component C9, $r = 0.59$, $p < 0.001$; fibronectin, $r = -0.46$, $p = 0.002$; IGL5, $r = 0.31$, $p = 0.04$; ITIH3, $r = 0.48$, $p = 0.001$ and LRG1, $r = 0.49$, $p = 0.001$). This suggests that perturbed levels of these proteins in circulation are related to increased systemic inflammation in COPD.

Three proteins were down-regulated in AECOPD patients (afamin, β2-glycoprotein 1, fibronectin) and 6 proteins were up-regulated (α1-antichymotrypsin, Apo-E, complement component C9, ITIH3, IGL5 and LRG1) relative to HC (Table 1). Level of fibronectin was also lower in sCOPD than HC, whilst complement component C9, IGL5 and LRG1 were also increased in sCOPD compared to HC (Table 1). Gene ontology analysis using the STRING software (https://STRING-db.org/cgi/network.pl) demonstrated enrichment in biological processes involved in proteolysis regulation, platelet degranulation and cholesterol metabolism (Supplementary Figure 1). No significant difference in protein levels was detected in AECOPD *vs.* sCOPD.

Discussion

We examined plasma samples from AECOPD patients (on admission), sCOPD patients and non-smoking HC using proteomic techniques in order to delineate the systemic responses during the intense inflammatory period of AECOPD. Using two independent quantitative proteomics techniques, we identified differential expression proteins between AECOPD *vs.* HC ($n = 9$) and sCOPD *vs.* HC ($n = 4$), but none in AECOPD *vs.* sCOPD.

Afamin and beta-2-glycoprotein were down-regulated in AECOPD compared to HC. Afamin is a human plasma vitamin E-binding glycoprotein primarily expressed in liver and facilitates vitamin E transport across the blood-brain barrier. It has been found strongly associated with the metabolic syndrome [14]. Reduction of afamin is associated with acute medical conditions such as heart failure, pneumonia and sepsis with an inverse correlation with acute phase reactants such as CRP and IL-6. In addition, it has been reported, in line with our study, to be reduced in AECOPD [15]. β2-glycoprotein-1 (or Apolipoprotein H) is involved in the activation of lipoprotein lipase in lipid metabolism, complement regulation, has anti-coagulant properties and is increased in chronic infections. It has not been described in AECOPD but is associated with smoking, males, increasing age, hyperlipidaemia antiphospholipid syndrome and athero-sclerosis [16]. Therefore, this protein may be a link to the increased incidence of cardiovascular diseases in COPD.

Fibronectin levels were decreased in both AECOPD and sCOPD patients compared to HC. Fibronectin is a multifunctional extracellular matrix protein that plays an important role in cell proliferation, adhesion, and migration. Decreased circulating fibronectin to CRP ratio increased the risk of all-cause (coronary and cardiovascular) mortality in COPD patients [17].

Proteins that were upregulated in AECOPD (but not in sCOPD) compared to HC were alpha-1-antichymotrypsin, Apo-E and ITIH3. Alpha-1-antichymotrypsin is an acute phase protein which inhibits the activity of proteases and has been shown to be released by activated neutrophils [18]. Although this protein is not well described in COPD, neutrophil inflammation is a hallmark of AECOPD [19], and hence raised levels of alpha-1-antichymotrypsin in our study may relate to increased neutrophil activity during AECOPD. ITIH3 is a serine protease inhibitor involved in hyaluronan metabolism and platelet degranulation. ITIH chain 4 (not ITIH3) has been shown as a novel biomarker for environmental exposure to particulate air pollution (particulate matter ≤10 μm) in COPD patients which correlated with CRP, suggesting circulating ITIH4 to be involved in inflammation [20]. Apo-E is primarily produced by the liver and macrophages, and mediates cholesterol metabolism in an isoform-dependent manner. It has been associated with hyperlipidaemia and hypercholesterolaemia, which lead to atherosclerosis, coronary heart disease and stroke [21].

Levels of complement component C9, IGL5 and LRG were raised in both AECOPD and sCOPD in this study. Complement component C9 forms part of the membrane attack complex downstream of other complement

components such as C3a and C5a [22]. Although C9 has not been described in COPD, complement C5a has been shown to be upregulated in AECOPD which may result in increased C9 levels [23], leading to increased cellular damage observed in AECOPD. The functional role of IGL5 is unknown and has not been described in COPD. Gene ontology analysis suggests that it is involved in B cell receptor signalling, complement activation, innate immune response and phagocytosis. LRG is involved in protein-protein interaction, signal transduction, and cell adhesion and development. This protein is expressed during granulocyte differentiation and shed/secreted by activated cells during inflammation. [24]. In airways disease sputum LRG is higher in asthmatics *vs.* HC [25].

Protein network analysis using STRING demonstrated enrichment in biological processes involved in the regulation of proteolysis, complement activation, platelet degranulation and cholesterol metabolism. As the pathways identified in our study do not relate directly to lung function or pulmonary inflammation, it is anticipated that biomarkers identified in our study are non-specific and informed us the increased risk of co-morbidities (e.g. cardiovascular diseases) especially in COPD patients who are frequent exacerbators. Furthermore, AECOPD increases the risk of subsequent cardiovascular disease events and vice versa [26, 27].

There are currently three previous proteomic studies that has focussed on the identification of AECOPD biomarkers. Leung *et al.* 2016 used MRM-MS to measure peptides discovered from previous iTRAQ analysis performed on the ECLIPSE cohort and compared them in three cohorts of AECOPD patients at acute presentation and during convalescence. They identified five proteins that distinguished AECOPD and convalescence states (Apolipoprotein-A4, Apolipoprotein-C2, Fibronectin, Complement factor C9 and LPS-binding protein) [28]. Of these proteins, we found that fibronectin and complement factor C9 do not distinguish AECOPD from sCOPD. The five proteins that were significantly different in AECOPD *vs.* HC in our study, three of them (afamin, beta-2-glycoprotein-1 and alpha-1-antichymotrypsin) were not differential expressed in their study, but apolipoprotein-E and inter-alpha-trypsin were not measured.

Shi *et al.* 2018 measured 507 pre-determined plasma inflammatory mediators using commercial antibody microarray and showed increased Apo-E in AECOPD than sCOPD and HC and higher complement component C9 levels in both AECOPD and sCOPD which are in line with our study [29]. Chen *et al.* 2012 used the same antibody microarray assay and found 20 proteins (associated with innate immune progression, inflammatory injury and tissue repair) downregulated in AECOPD *vs.* HC [30]. These proteins were not identified in our study and the two other proteomic studies mentioned. ITIH3 (involved in platelet degranulation) elevation in AECOPD is a unique finding in our study and hence warrants further investigation in future studies.

It is important to validate the proteomics results in relation to COPD severity and other chronic lung diseases (e.g. asthma or idiopathic pulmonary fibrosis) before confirming the importance of these molecules as biomarkers or therapeutic targets. The repeated expression of molecules in multicentre studies will identify the most important molecules and pathways for future studies.

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Disclosure statement

The authors declare that they have no competing interests. No financial benefit from the results occurring to Proteomics International as an organisation of the authors employed by Proteomics International. Furthermore, there is no patent or equivalent relating to the results, from which a financial return can be derived.

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