Official Journal of the Asian Pacific Society of Respirology
Respirology

ORIGINAL ARTICLE

Analysis by proteomics reveals unique circulatory proteins in idiopathic pulmonary fibrosis

YUBEN P. MOODLEY,[1,2,3,4] TAMERA J. CORTE,[4,5,6] BRIAN G. OLIVER,[7,8] IAN N. GLASPOLE,[4,9,10] ANDREJA LIVK,[11] JASON ITO,[11] KIRSTEN PETERS,[11] RICHARD LIPSCOMBE,[11] TAMMY CASEY[11] AND DINO B.A. TAN[1,2]

[1]*School of Biomedical Sciences, University of Western Australia, Perth, WA, Australia;* [2]*Stem Cell Unit, Institute for Respiratory Health, Perth, WA, Australia;* [3]*Department of Respiratory Medicine, Fiona Stanley Hospital, Perth, WA, Australia;* [4]*National Health and Medical Research Council Centre of Research Excellence in Pulmonary Fibrosis, University of Sydney, Sydney, NSW, Australia;* [5]*Department of Respiratory Medicine, Royal Prince Alfred Hospital, Sydney, NSW, Australia;* [6]*School of Medicine, University of Sydney, Sydney, NSW, Australia;* [7]*Woolcock Institute of Medical Research, University of Sydney, Sydney, NSW, Australia;* [8]*Faculty of Science, University of Technology Sydney, Sydney, NSW, Australia;* [9]*Department of Allergy and Respiratory Medicine, The Alfred Hospital, Melbourne, VIC, Australia;* [10]*Faculty of Medicine, Monash University, Melbourne, VIC, Australia;* [11]*Proteomics International, Perth, WA, Australia*

ABSTRACT

Background and objective: **Idiopathic pulmonary fibrosis (IPF) is a progressive fibrotic disease that has a poor 3-year median survival rate with unclear pathophysiology. Radiological features include bibasal, subpleural fibrosis and honeycombing while its pathology is characterized by fibroblastic foci and honeycombing. Proteomic analysis of circulating molecules in plasma may identify factors that characterize IPF and may assist in the diagnosis, prognostication and determination of pathogenic pathways in this condition.**

Methods: **Two independent quantitative proteomic techniques were used, isobaric tags for relative and absolute quantitation (iTRAQ) and multiple reaction monitoring (MRM), to identify differentially expressed plasma proteins in a group of IPF patients in comparison to healthy controls with normal lung function matched for age and gender.**

Results: **Five proteins were identified to be differentially expressed in IPF compared to healthy controls (upregulation of platelet basic protein and downregulation of actin, cytoplasmic 2, antithrombin-III, extracellular matrix protein-1 and fibronectin).**

Conclusion: **This study further validates the combinational use of non-targeted discovery proteomics (iTRAQ) with targeted quantitation by mass spectrometry (MRM) of soluble biomarkers to identify potentially important molecules and pathways for pulmonary diseases such as IPF.**

SUMMARY AT A GLANCE

This study utilized a combination of non-targeted discovery proteomics with targeted quantitation by mass spectrometry of soluble plasma biomarkers to identify potentially important molecules and pathways in idiopathic pulmonary fibrosis (IPF). Five proteins were identified to be differentially expressed in IPF compared to healthy controls.

Key words: idiopathic pulmonary fibrosis, isobaric tags for relative and absolute quantitation, multiple reaction monitoring, proteomics.

INTRODUCTION

Idiopathic pulmonary fibrosis (IPF) is a progressive fibrotic disease that has a 50% mortality at 3 years from diagnosis.[1] Increasingly, IPF is recognized to be a disease of the elderly (>65 years) with a male predilection.[1,2] Radiological features of IPF include bibasal, subpleural fibrosis with honeycombing while the pathology of IPF is characterized by fibroblastic foci and honeycombing.[2]

With the advent of '–omics' research, there is growing data showing differences in proteins, RNA, lipids and metabolites between healthy and IPF subjects.[3,4] These differences have been documented in lung tissue, bronchoalveolar lavage (BALF) and the circulation.[5–7] Identifying molecules that characterize IPF would be helpful in the diagnosis, prognostication and determination of pathogenic pathways in this condition.

In this preliminary study, we demonstrate significant differences in circulatory protein expression between

Correspondence: Yuben P. Moodley, School of Medicine, University of Western Australia, Level 2, Harry Perkins Institute of Medical Research, Fiona Stanley Hospital Campus, 5 Robin Warren Drive, Murdoch, Perth, WA 6150, Australia. Email: yuben.moodley@uwa.edu.au

Received 2 September 2018; invited to revise 15 November 2018; revised 13 April 2019; accepted 4 June 2019 (Associate Editor: Michael Keane; Senior Editor: Chris Grainge)

healthy and IPF patients when analysed by a proteomic biomarker discovery and validation workflow.

METHODS

Plasma samples were obtained from patients attending interstitial lung disease (ILD) clinic at the Alfred Hospital (Melbourne), Royal Prince Alfred (RPA) Hospital (Sydney) and Royal Perth Hospital (Perth). IPF was diagnosed at ILD multidisciplinary discussion (MDD) at each site. Blood from all IPF patients was collected into Ethylenediaminetetraacetic acid (EDTA) tubes at the time of initial diagnosis and plasma was isolated by centrifugation and stored immediately at −80°C. Blood from healthy volunteers at the Royal Perth Hospital was collected into lithium heparin tubes and plasma was isolated by centrifugation and stored immediately at −80°C. The proteomic analysis was done in two phases: discovery using isobaric tags for relative and absolute quantitation (iTRAQ) on pooled samples followed by validation using targeted mass spectrometry with multiple reaction monitoring (MRM) on individual samples. All subjects were not currently smoking, and none had received anti-fibrotic treatment. Study demographics are presented in Table S1 in Supplementary Information. This study had ethical approval from the Royal Perth Hospital Ethics Committee (HREC 2011-138) for Western Australia (WA) plasma collection and proteomic analysis, and for plasma collection at RPA (HREC/15/RPAH/28) and Alfred (Alfred 417/11).

Plasma preparation and iTRAQ labelling

Individual plasma samples were pooled for each group (n = 10 in each group) and technical replicates generated. Fourteen highly abundant proteins in plasma were immunodepleted using a MARS14 chromatography column (Agilent Technologies, Mulgrave, VIC, Australia). The iTRAQ technique was carried out as previously described.[8] Peptides were separated by strong cation exchange (SCX) chromatography on an Agilent 1100 HPLC system. A total of 95 fractions were then analysed by electrospray ionization mass spectrometry using the Agilent 1260 Infinity HPLC system coupled to an Agilent 1260 Chipcube Nanospray interface on an Agilent 6540 mass spectrometer (Agilent Technologies). Peptides were loaded onto a ProtID-Chip-150 C18 column (Agilent Technologies) and separated with a linear gradient of water/acetonitrile/0.1% formic acid (v/v).

Multiple reaction monitoring

Using a separate cohort of patients, MRM was carried out as previously described.[8] Samples 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, Torrance, CA, USA). Peptides were analysed by electrospray ionization liquid chromatography/mass spectrometry (LC/MS) using a Thermo UltiMate 3000 RS nanoflow UHPLC system (Thermo Scientific, Scoresby, VIC, Australia) coupled to a 5500 Q-TRAP mass spectrometer (Sciex, Mt Waverley, VIC, Australia). Unlabelled and ^{18}O-labelled reference standard plasma peptides were loaded onto an Acclaim PepMap 100 C18 LC Column, 2 μm particle size × 150 mm (Thermo Scientific) and separated with a linear gradient of water/acetonitrile/0.1% formic acid (v/v) over 18 min.

Data analysis

Protein identification and quantification for iTRAQ data were performed using ProteinPilot 4.5 Beta Software (AB Sciex, Framingham, MA, 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, as calculated by the software) 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). Unlabelled/^{18}O-labelled peptide ratios were exported from Skyline for further statistical analysis using SPSS for Windows (version 22; SPSS Inc., Chicago, IL, USA). Peptide ratios for each target protein were combined to provide a 'protein-based' ratio that was then compared between IPF patients and healthy controls using a Student's t-test. Age was compared using the Mann–Whitney U-test, whilst the ratio of male : female (M:F) was compared using the Fisher's exact 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

The IPF patients were well matched for age and gender with the controls (Table S1 in Supplementary Information). A non-targeted approach (iTRAQ) was first performed on pooled samples (n = 10 in each group) and a total of 189 proteins were detected with >95% confidence level in protein detection. Of these, 12 proteins demonstrated significant differences (>10%) between IPF patients and healthy controls. A targeted mass spectrometry approach (MRM) was then performed in a separate cohort of IPF patients versus healthy controls (n = 15 in each group) for validation and only five proteins (actin, cytoplasmic 2; antithrombin-III; extracellular matrix protein-1, fibronectin and platelet basic protein) were confirmed as significantly different in levels of expression (Table 1). The only significant correlations between the level of these proteins versus age or lung function data were between plasma levels of antithrombin-III with forced vital capacity (FVC) (L) (r = 0.68, P = 0.005).

Table 1 The relative expression levels of proteins in IPF versus healthy controls by iTRAQ and MRM

Molecules	UniProt ID	iTRAQ†	*P*-value†	MRM‡	*P*-value‡
Actin, cytoplasmic 2	P63261	0.09	0.08	0.17	<0.001
		0.04	0.03		
Antithrombin-III	P01008	0.95	0.009	0.59	0.002
		0.16	0.005		
Extracellular matrix protein	Q16610	0.25	0.80	0.51	<0.001
		0.15	0.019		
Fibronectin	P02751	0.73	0.02	0.42	0.002
		0.51	<0.001		
Platelet basic protein	P02775	4.29	0.29	2.28	<0.001
		22.7	0.005		

†Fold change expression levels and *P*-values in IPF in comparison to healthy controls by iTRAQ performed in technical replicates.
‡Adjusted fold change expression levels and *P*-values by MRM from multiple peptides that represented a protein of interest.
IPF, idiopathic pulmonary fibrosis; iTRAQ, isobaric tags for relative and absolute quantitation; MRM, multiple reaction monitoring.

DISCUSSION

This study identified five proteins that were differentially expressed in IPF disease compared to healthy controls when employing two independent quantitative proteomic techniques. There was upregulation of platelet basic protein, which is derived from pro-platelet basic protein, a chemokine (CXCL7) enriched in platelets, neutrophils and macrophages.[9] No previous studies have identified this protein as being upregulated in IPF; however, it has derivatives such as connective tissue activating peptide III that acts as a fibroblast mitogen and hence may promote fibrotic pathways.[10]

There was significant downregulation of actin, cytoplasmic 2, which strengthens crosslinks between the cytoskeleton and the sarcolemmal membrane in myocytes.[11] The downregulation of this protein may correspond with the increased myofibril formation and increased smooth muscle actin formation by myofibroblasts in IPF,[11] although this protein has not been identified in the circulation of IPF patients.

We also demonstrate downregulation of antithrombin-III, extracellular matrix protein-1 and fibronectin in IPF patients. Antithrombin-III is a molecule that inhibits clotting by binding to thrombin. The coagulation pathway has been implicated in the pathogenesis of IPF and a fall in antithrombin-III may be due to its anticoagulant effects through the inhibition of thrombin-mediated cleavage of fibrinogen to fibrin in fibrosis.[12] Extracellular matrix protein-1 is implicated in angiogenesis and increased proliferation of endothelial cells.[13] There are no current data for extracellular matrix protein-1 in relation to IPF but it is known to inhibit matrix metalloproteinase-9 which is implicated in lung remodelling.[14,15] Fibronectin is a mediator of cell matrix adhesions by binding extracellular matrix proteins (e.g. collagen). It promotes myofibroblast differentiation and has been shown to be elevated in the lungs of IPF patients.[16] Lower levels of circulating antithrombin-III, extracellular matrix protein-1 and fibronectin may reflect their consumption or increased homing of these molecules to the lungs as they play a major in fibrosis.

Our study shows the power of a proteomic workflow for biomarker discovery in relatively small cohorts. First, 12 protein candidates were shown to be differentially expressed in pooled samples using iTRAQ mass spectrometry. Of these, five proteins were validated in individual patients with IPF compared to healthy controls using targeted (MRM) mass spectrometry assays. Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) analysis demonstrated enrichment in biological processes involved in defence, inflammatory and platelet pathways as well as extracellular components that potentially promotes IPF (Fig. S1 in Supplementary Information).[17]

Proteins (e.g. platelet basic protein and actin, cytoplasmic 2) that were identified in this study but not described in previous IPF studies may be due to several factors such as differences in the experimental design and instrumentation used (e.g. this study validated by MRM vs Niu *et al.* validated by ELISA)[4] and differences in collection and type of samples used (plasma vs serum). The challenge is how to interpret and contextualize the new molecules identified by proteomic analysis and to determine the significance of new pathways and inter-relationships.[5,6,17–19] Furthermore, changes in the level of proteins identified in our study may be influenced by co-morbidities prominent in IPF (e.g. increased platelet activation or plasma antithrombin-III in patients with hypertension or cardiovascular diseases). The influence of co-morbidities will require a larger study.

It will also be important to confirm the proteomic results by an extended population study to determine the robustness of the protein expression in a different IPF cohort with a comparison to cohorts of other non-ILD chronic lung diseases, such as COPD, and by alternative methodologies. The repeated expression of molecules in international multicentre studies will identify the most important molecules and pathways for future studies.

Acknowledgements: We acknowledge Ms Susanne Webster and Ms Karen Symonds for assistance with the collection of blood samples. The proteomic analyses were performed in facilities provided by the Lotterywest State Biomedical Facility-Proteomics node and Bioplatforms Australia at the Harry Perkins Institute for Medical Research. This work has been supported by

the NHMRC Centre of Research Excellence in Pulmonary Fibrosis (CRE-PF).

Disclosure statement: The work was conducted through academic grant funding. There are no financial benefits from the results for Proteomics International as the employment organization of the authors. Furthermore, there is no patent or equivalent relating to the results, from which a financial return can be derived.

Author contributions: Conceptualization: Y.P.M., T.J.C., B.G.O., I.N.G., D.B.A.T., A.L., T.C., R.L. Data curation: T.C., A.L., J.I., K.P., R.L., D.B.A.T. Formal analysis: K.P., T.C., R.L., D.B.A.T., Y.P.M. Funding acquisition: Y.P.M., T.J.C., B.G.O., I.N.G. Investigation: Y.P.M., A.L., R.L., D.B.A.T., T.J.C., B.G.O., I.N.G. Methodology: R.L., A.L., T.C., J.I., K.P. Project administration: Y.P.M., D.B.A.T. and R.L. Resources: Y.P.M., T.J.C., B.G.O., I.N.G. Software: R.L., A.L., J.I., K.P., T.C. Supervision: Y.P.M., D.B.A.T. and R.L. Validation: T.C., R.L., A.L. Visualization: D.B.A.T. Writing—original draft: D.B.A.T., Y.P.M., T.C. Writing—review and editing: D.B.A.T., Y.P.M., T.C., R.L., K.P., T.J.C., B.G.O., I.N.G., A.L., J.I.

Abbreviations: COPD, chronic obstructive pulmonary disease; ELISA, enzyme-linked immunosorbent assay; HPLC, high-performance liquid chromatography; ILD, interstitial lung disease; IPF, idiopathic pulmonary fibrosis; iTRAQ, isobaric tags for relative and absolute quantitation; MRM, multiple reaction monitoring.

REFERENCES

1 Jo HE, Glaspole I, Grainge C, Goh N, Hopkins PM, Moodley Y, Reynolds PN, Chapman S, Walters EH, Zappala C *et al*. Baseline characteristics of idiopathic pulmonary fibrosis: analysis from the Australian Idiopathic Pulmonary Fibrosis Registry. *Eur. Respir. J.* 2017; **49**: pii: 1601592.

2 Raghu G, Rochwerg B, Zhang Y, Garcia CA, Azuma A, Behr J, Brozek JL, Collard HR, Cunningham W, Homma S *et al*.; American Thoracic Society; European Respiratory Society; Japanese Respiratory Society; Latin American Thoracic Society. An official ATS/-ERS/JRS/ALAT clinical practice guideline: treatment of idiopathic pulmonary fibrosis. An update of the 2011 clinical practice guideline. *Am. J. Respir. Crit. Care Med*. 2015; **192:** e3–19.

3 Landi C, Bargagli E, Bianchi L, Gagliardi A, Carleo A, Bennett D, Perari MG, Armini A, Prasse A, Rottoli P *et al*. Towards a functional proteomics approach to the comprehension of idiopathic pulmonary fibrosis, sarcoidosis, systemic sclerosis and pulmonary Langerhans cell histiocytosis. *J. Proteomics* 2013; **83**: 60–75.

4 Niu R, Liu Y, Zhang Y, Wang H, Wang Y, Wang W, Li X. iTRAQ-based proteomics reveals novel biomarkers for idiopathic pulmonary fibrosis. *PLoS One* 2017; **12**: e0170741.

5 Korfei M, von der Beck D, Henneke I, Markart P, Ruppert C, Mahavadi P, Ghanim B, Klepetko W, Fink L, Meiners S *et al*. Comparative proteome analysis of lung tissue from patients with idiopathic pulmonary fibrosis (IPF), non-specific interstitial pneumonia (NSIP) and organ donors. *J. Proteomics* 2013; **85**: 109–28.

6 Landi C, Bargagli E, Carleo A, Bianchi L, Gagliardi A, Prasse A, Perari MG, Refini RM, Bini L, Rottoli P. A system biology study of BALF from patients affected by idiopathic pulmonary fibrosis (IPF) and healthy controls. *Proteomics Clin. Appl.* 2014; **8**: 932–50.

7 Meltzer EB, Barry WT, Yang IV, Brown KK, Schwarz MI, Patel H, Ashley A, Noble PW, Schwartz DA, Steele MP. Familial and sporadic idiopathic pulmonary fibrosis: making the diagnosis from peripheral blood. *BMC Genomics* 2014; **15**: 902.

8 Bringans SD, Ito J, Stoll T, Winfield K, Phillips M, Peters K, Davis WA, Davis TME, Lipscombe RJ. Comprehensive mass spectrometry based biomarker discovery and validation platform as applied to diabetic kidney disease. *EuPA Open Proteom.* 2017; **14**: 1–10.

9 Holt JC, Harris ME, Holt AM, Lange E, Henschen A, Niewiarowski S. Characterization of human platelet basic protein, a precursor form of low-affinity platelet factor 4 and beta-thromboglobulin. *Biochemistry* 1986; **25**: 1988–96.

10 Paul D, Niewiarowski S, Varma KG, Rucinski B, Rucker S, Lange E. Human platelet basic protein associated with antiheparin and mitogenic activities: purification and partial characterization. *Proc. Natl. Acad. Sci. U. S. A.* 1980; **77**: 5914–8.

11 Adler KB, Craighead JE, Vallyathan NV, Evans JN. Actin-containing cells in human pulmonary fibrosis. *Am. J. Pathol.* 1981; **102**: 427–37.

12 Cavaco RA, Kaul S, Chapman T, Casaretti R, Philips B, Rhodes A, Grounds MR. Idiopathic pulmonary fibrosis associated with pulmonary vein thrombosis: a case report. *Cases J.* 2009; **2**: 9156.

13 Han Z, Ni J, Smits P, Underhill CB, Xie B, Chen Y, Liu N, Tylzanowski P, Parmelee D, Feng P *et al*. Extracellular matrix protein 1 (ECM1) has angiogenic properties and is expressed by breast tumor cells. *FASEB J.* 2001; **15**: 988–94.

14 Lemjabbar H, Gosset P, Lechapt-Zalcman E, Franco-Montoya ML, Wallaert B, Harf A, Lafuma C. Overexpression of alveolar macrophage gelatinase B (MMP-9) in patients with idiopathic pulmonary fibrosis: effects of steroid and immunosuppressive treatment. *Am. J. Respir. Cell Mol. Biol.* 1999; **20**: 903–13.

15 Fujimoto N, Terlizzi J, Aho S, Brittingham R, Fertala A, Oyama N, McGrath JA, Uitto J. Extracellular matrix protein 1 inhibits the activity of matrix metalloproteinase 9 through high-affinity protein/protein interactions. *Exp. Dermatol.* 2006; **15**: 300–7.

16 Upagupta C, Shimbori C, Alsilmi R, Kolb M. Matrix abnormalities in pulmonary fibrosis. *Eur. Respir. Rev.* 2018; **27**: pii: 180033.

17 Ahrman E, Hallgren O, Malmstrom L, Hedstrom U, Malmstrom A, Bjermer L, Zhou XH, Westergren-Thorsson G, Malmstrom J. Quantitative proteomic characterization of the lung extracellular matrix in chronic obstructive pulmonary disease and idiopathic pulmonary fibrosis. *J. Proteomics* 2018; **189**: 23–33.

18 Carleo A, Bargagli E, Landi C, Bennett D, Bianchi L, Gagliardi A, Carnemolla C, Perari MG, Cillis G, Armini A *et al*. Comparative proteomic analysis of bronchoalveolar lavage of familial and sporadic cases of idiopathic pulmonary fibrosis. *J. Breath Res.* 2016; **10**: 026007.

19 Foster MW, Morrison LD, Todd JL, Snyder LD, Thompson JW, Soderblom EJ, Plonk K, Weinhold KJ, Townsend R, Minnich A *et al*. Quantitative proteomics of bronchoalveolar lavage fluid in idiopathic pulmonary fibrosis. *J. Proteome Res.* 2015; **14**: 1238–49.

Supplementary Information

Additional supplementary information can be accessed via the *html* version of this article at the publisher's website.

Figure S1 STRING analysis demonstrated enrichment of extracellular matrix protein pathways.
Table S1 Subject demographics.

