A Novel Method For Discovery of Peripheral Blood Biomarkers in Idiopathic Pulmonary Fibrosis Using Extensive Depletion and TMTcalibrator™ Tissue-Enhanced Plasma Proteomics

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Introduction

Peripheral biomarkers related to the pathogenesis of idiopathic pulmonary fibrosis (IPF) are urgently needed to improve diagnosis, selection and assessment of treatment, particularly in the context of new drug development. Recent improvements in the depletion of high and medium abundant proteins and development of tissue-enhanced fluid proteomics have increased the breadth and depth of plasma proteome coverage. We have now combined these methods to obtain unparalleled coverage of the IPF plasma proteome.

Method

Longitudinal archival plasma samples (n=25) from six individuals with IPF and five control individuals were obtained from the UCSF Biobank and processed as shown in Figure 1.

- Super-depletion of the top ~70 high and medium abundant proteins using Seppro® IgY14 and Supermix® columns (Merck).
- Plex #1-5 each comprised 6 plasma samples with 4 tissue trigger/calibrant samples. Plex 6 comprised 10 plasma samples only.
- Each TMT® 10plex was analysed using 30 x 2 hour gradient on an Orbitrap® Fusion Tribrid® mass spectrometer with in-line uHPLC.
- MS files were processed with a sequential SEQUEST search strategy in Proteome Discoverer v1.4 (Thermo Scientific) with a standard search followed by a bespoke method in the residual unmatched spectra with variable modification for less-common post-translational modifications such as hydroxylation of proline and lysine.
- Data integration, normalisation, quantitation and statistical analyses were performed within Proteome Sciences' in-house bioinformatics workflows.
- Reported plasma abundance of select IPF-related proteins was obtained through the human plasma proteome database and/or PubMed searches.

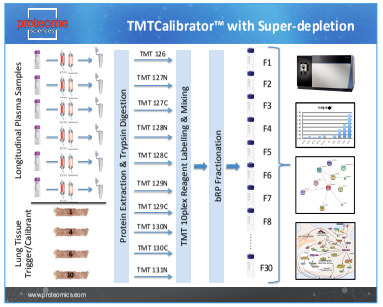

Figure 1 – TMTcalibrator™ workflow with plasma Super-depletion and incorporation of four-point trigger/calibrant. TMT126 – 131N = Tandem Mass Tag reporter ion mass-to-charge.

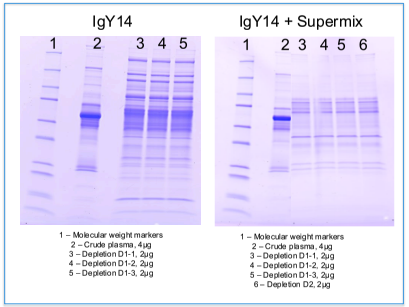

Figure 2 – SDS-PAGE gels showing consistent plasma depletion – Left panel = IgY14 depletion; Right panel = IgY14 + Supermix depletion

	TMTcalibrator™					TMT®MS2
10plex Number	TMT01	TMT02	TMT03	TMT04	TMT05	TMT06
PSMs [≥1 plasma channel]	104,217	164,242	124,941	178,938	172,052	116,725
PSMs [All 10 channels]	51,091	100,387	77,479	125,373	118,110	98,220
Peptides	57,890	76,698	66,954	84,543	81,053	40,938
ProteinGroups	8,903	10,640	9,783	11,027	10,909	4,617
Unique Gene Names	7,536	8,946	8,230	9,238	9,146	3,968

	TMTcalibrator™					TMT®MS2
10Plex Number	TMT01	TMT02	TMT03	TMT04	TMT05	TMT06
Hydroxyproline (P)	621	1,165	979	0	24	0
Hydroxylysine (K)	17	28	62	0	0	0
3-Nitrotyrosine (Y)	71	150	42	0	1	0
Allysine (K)	22	38	19	0	0	0
Phosphorylation (STY)	175	423	317	415	394	339

Table 1 – Output metrics for TMTcalibrator™ and TMT®MS2 analysis of IPF plasma – Standard Search (upper panel); Sequential PTM search (lower panel)

Gene Symbol	TMTcal+SD (# peptides)	SD (# peptides)	Plasma Concn
POSTN	195	162	200 ng/ml
Collagen I/IV	1240	371	~100 ng/ml
CHI3L1	10	11	40 ng/ml
CCL18	3	0	31 ng/ml
SP-A	70	31	30 ng/ml
TGFB1	8	20	5 ng/ml
KRT18	24	0	4 ng/ml
MMP7	2	1	4 ng/ml
KRT8	42	1	3 ng/ml
TGFB2	2	0	0.2 ng/ml
LOXL2	1	0	0.1 ng/ml
TGM2	30	1	NR
ITGAV	55	6	NR
ITGB6	22	0	NR
MUC1	19	2	NR
TGFBR1	1	2	NR

Table 2 – Peptide coverage of select IPF-related proteins. TMTcal+SD = Super-depleted plasma with four-channel trigger/calibrant; SD = Super-depletion only; Concentrations from *plasmaproteomedatabase.org*; NR = not reported

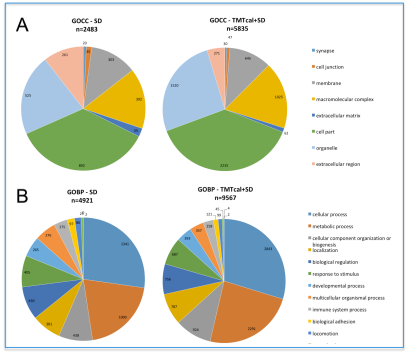

Figure 3 – Biological coverage of TMTcalibrator™ vs Super-depletion alone. A – GO Cellular Compartment; B – GO Biological Process. TMTcal+SD = Super-depleted plasma with four-channel trigger/calibrant; SD = Super-depleted plasma only

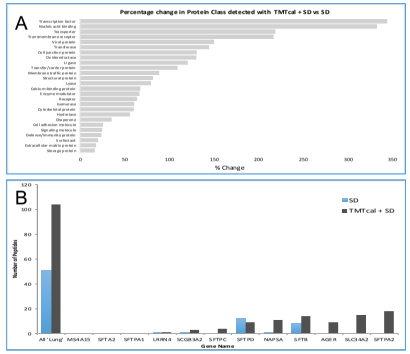

Figure 4 – Increased coverage of the proteome using TMTcalibrator™ with Super-depletion. A – Protein class; B – Lung proteins (based on 186 genes defined as overexpressed in lung at *proteinatlas.org*). TMTcal+SD = Super-depleted plasma with four-channel trigger/calibrant; SD = Super-depleted plasma

Results & Conclusion

- Super-depletion removed ~99% of total plasma protein (Figure 2).
- Detection limit in mid-pg/ml range in plasma channels when using the tissue trigger.
- ~4,000 proteins quantified with Super-depletion only, more than doubled to ~9,000 when tissue trigger added (Table 1).
- Key post-translationally modified proteins only detected when using tissue trigger (Table 1.)
- Improved coverage of select IPF-related proteins (Table 2).
- Distributions for GO cellular components (Figure 3A) or biological processes (Figure 3B) were similar with or without tissue trigger.
- Proportionally greater impact on detection of cellular vs secreted protein classes (Figure 4A) and for lung-associated proteins with TMTcalibrator™ than in Super-depleted plasma alone (Figure 4B).
- TMTcalibrator™ with Super-depletion provides outstanding proteome coverage for plasma biomarker discovery offering deeper insights into IPF disease processes and response to treatment.
- Larger studies required for replication/validation of novel candidate biomarkers.

IP & MB are paid employees and hold stock and/or stock options in Proteome Sciences plc. IP is an inventor on patents covering the TMTcalibrator™ technology

ST & MD are paid employees and hold stock and/or stock options in Pliant Therapeutics Inc. who funded the study