**Title:** The proteomic response is linked to regional lung volumes in ventilator-induced lung injury

Seiha Yen1 #, Yong Song2 #, Melissa Preissner3 #, Ellen Bennett1, Richard Wilson4, Macarena Pavez2, Stephen Dubsky3, Peter A. Dargaville 2, Andreas Fouras5, Graeme R. Zosky1,2 \*

*1School of Medicine, College of Health and Medicine, University of Tasmania, Hobart, Tasmania, Australia.*

*2Menzies Institute for Medical Research, College of Health and Medicine, University of Tasmania, Hobart, Tasmania, Australia.*

*3Department of Mechanical and Aerospace Engineering, Monash University, Melbourne, Victoria, Australia.*

*4Central Science Laboratory, University of Tasmania, Hobart, Tasmania, Australia.*

*54Dx Limited, Melbourne, Victoria, Australia.*

#These authors contributed equally to this work.

\*Address correspondence to:

*Graeme R. Zosky (PhD); Address:* *Menzies Institute for Medical Research, College of Health and Medicine, University of Tasmania, Hobart, Tasmania, Australia; Ph: +61 3 6226 6921;* *Email: Graeme.Zosky@utas.edu.au*

**Tissue extraction and peptide sample preparation**

Lung tissue was dissected and proteins extracted in TPER reagent (Thermo Scientific, Rockford, USA). Protein concentrations were determined using the E-Z Quant assay based on absorbance values obtain for BSA over the concentration range x to y µg/ml. Sample volumes corresponding to 50 µg aliquots were cleaned up by precipitation in nine volumes of ethanol. Protein samples were then reduced using 10 mM dithiothreitol overnight at 4 °C and alkylated using 50 mM iodoacetamide in the dark for 2 hours. Proteins were then digested with 2 µg mass spectrometry -grade trypsin/Lys-C mix (Promega, Madison, USA) on 500 µg Sera Beads (GE Healthcare products 65152105050350 and 45152105050350, [Chicago](https://www.google.com.au/search?biw=1522&bih=738&q=Chicago&stick=H4sIAAAAAAAAAOPgE-LUz9U3MM6OT0pT4gAxDeNTTLS0spOt9POL0hPzMqsSSzLz81A4VhmpiSmFpYlFJalFxYtY2Z0zMpMT0_MBZbpejU0AAAA&sa=X&ved=2ahUKEwjxu8qfxsflAhUQ5o8KHdypB_wQmxMoATAXegQIDBAH), USA) per sample, as recommended for the Single-Pot, Solid-Phase Sample Preparation (SP3) method (1). Briefly, samples were digested overnight using a Thermomixer (Eppendorf) set to 37 °C and 1,000 rpm. Peptides were collected after centrifugation at 15,000 rpm for 20 minutes at 4 °C, then transferred to HPLC autosampler vials.

**Peptide analysis by nano-liquid chromatography and Orbitrap tandem mass spectrometry**

Peptide samples equivalent to ~0.5 µg were separated using an Ultimate 3000 nano RSLC system (Thermo Scientific). Peptides were first concentrated on a 20 mm × 75 µm PepMap 100 trapping column (3 µm C18) for 5 minutes then separated using a 250 mm × 75 µm PepMap 100 RSLC column (2 µm C18) at a flow rate of 300 nL/min and held at 45 °C. A 90 minute gradient from 98% mobile phase A (0.1% formic acid in water) to 50% mobile phase B (0.08% formic acid in 80% acetonitrile and 20% water) comprised the following steps: 2-10% B over 12 minutes, 10-25% B over 48 minutes, 25-45% B over 10 minutes, holding at 95% B for 5 minutes then re-equilibration in 2% B for 15 minutes. The nanoHPLC system was coupled to a Q-Exactive HF mass spectrometer equipped with nanospray Flex ion source (Thermo Scientific) and controlled using Xcalibur 4.1 software. Spray voltage was set to 2.0 kV, S-lens RF level to 50 and heated capillary set at 250 °C. MS scans were acquired from 370-1500 m/z at 60,000 resolution, with an AGC target of 3 × 10e6 and a maximum fill time of 100 minutes. Fragment ion scans were acquired at 15,000 resolution (scan range 200-2000 m/z), with an AGC target of 2 × 10e5 and a maximum fill time of 28 minutes. An isolation width of 1.4 m/z was used, and normalized collision energy for HCD set to 30eV. MS/MS spectra were acquired in data-dependent mode using a Top15 method with 30-second dynamic exclusion of fragmented peptides.

**Database searching and criteria for protein identification**

Data files were imported into MaxQuant version 1.6.5.0 (http://maxquant.org/) and MS/MS spectra were searched using the Andromeda search engine against the complete *Mus musculus* UniProt reference proteome (downloaded on 06/04/2017). Default settings for protein identification by Orbitrap MS/MS were used, with the match-between-runs function enabled, including a maximum of two missed cleavages, mass error tolerances of 20 ppm then 4.5 ppm for initial and main peptide searches, respectively, 0.5 Da tolerance for fragment ions, carbamidomethyl modification of cysteine and variable methionine oxidation. A false discovery rate of 0.01 was used for both peptide-spectrum matches and protein identification.

**Determination of relative protein abundance and statistical analysis**

We utilized MaxLFQ, the MaxQuant algorithm for peptide intensity determination and normalization (2), using pair-wise comparison of unique and razor peptide intensities and a minimum ratio count of 2. The proteinGroups output files generated by MaxQuant analysis were processed as follows: The normalised label-free quantification (LFQ) intensity values, MS/MS counts and the numbers of razor and unique peptides for each of the identified proteins were imported into Perseus software version 1.5.031 (<http://perseus-framework.org/>). Protein groups identified either as potential contaminants (prefixed with CON\_), identified by modified site only, by reverse database matching or on the basis of a single matching peptide were removed. LFQ intensity values were then log2–transformed and then a filter applied to include only proteins detected in a minimum of 70% of the samples. Missing values were replaced with random intensity values for low-abundance proteins based on a normal distribution of protein abundances using default MaxQuant parameters.

**References**

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