Compartment-specific protein interactions in beryllium lung disease

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To the Editor:

Chronic beryllium disease (CBD) is a granulomatous lung disorder caused by beryllium (Be) exposure. Of those exposed, up to 20% will develop Be sensitisation (BeS), the precursor to CBD, and 50–100% of BeS workers will develop CBD, at a rate of 6–8% per year [1].

In this pilot study, we investigated proteome-wide changes in bronchoalveolar lavage (BAL) cells in four controls at one time point, three BeS subjects sampled at two timepoints ⩾2 years apart, and three subjects with BeS at the first timepoint and CBD at the second timepoint at least 2 years later. Mixed BAL cell proteins were processed using in-solution digestion. Trypsin-digested peptides were labelled with 16-plex tandem mass tags (TMT) reagent similar to prior studies [2]. The TMT-labelled peptides were fractionated off-line into 32 peptide fractions on a C18 column (pH=10.0) and concatenated into 16 pairs. Aliquots of each concatenated fraction were analysed by liquid chromatography/mass spectrometry on Orbitrap Eclipse system with high-field asymmetrical waveform ion mobility spectrometry. The spectral data (available as MassIVE MSV000090914) were analysed using Sequest in Proteome Discoverer 2.5 for the sequence database search against the human UniProt merged with the contaminant protein database (74,234 sequences). Peptides meeting a false discovery rate (FDR) [3] <0.01 were selected for protein identification and quantification. Unique and razor peptides were included, using pairwise ratio-based mode, similar to the MaxLFQ [4] method. Normalised data (all results described below) and other supplementary materials are available at https://github.com/stop-pre16/Li-ERJ-Supplemental-Tables.

We identified 4580 proteins with >two peptides (supplementary table 1 “Proteins identified”). Comparing the quantile-normalised and log-transformed levels using linear mixed models with a random intercept to account for the repeated measures, we identified 1970 differentially abundant proteins (DAPs; $p<0.05$) in BeS *versus* controls, 1584 in CBD *versus* controls and 745 in BeS *versus* CBD (figure 1a, supplementary table “Pairwise comparison”). While several of the proteins were unique in discriminating the three comparison groups, 1644 proteins differentiated more than two groups (figure 1a, supplementary table “Venn overlapping”). Several of these proteins demonstrated significant monotonic increases or decreases from control to BeS to CBD (figure 1b). The DAPs in BeS *versus* CBD are significantly overrepresented (FDR <0.05) in diverse canonical pathways such as mitochondrial dysfunction, autophagy, oestrogen receptor signalling, sirtuin signalling, glucocorticoid receptor signalling, clathrin-mediated endocytosis pathway and others (supplementary table “Biological processes DAP”). To determine the relationship of these pathways, we also conducted an “overlapping analysis” suggesting common regulation (figure 1c). Sirtuin signalling is a highly connected node, indicating it may be critical to sub-network function involved in oestrogen receptors [5], glucocorticoid receptor signalling [6], mitochondrial dysfunction [7] and autophagy [8]. Some preclinical and clinical studies associate sirtuins with autoimmune [9–12] and granulomatous lung diseases, including tuberculosis [13]. Furthermore, upregulation of sirtuin signalling (SIRT-1, SIRT-2) may reduce autoimmunity and inflammatory responses, probably by suppressing Th1 and Th17 differentiation [13]. With these findings we speculate that sirtuins could function as biomarkers and/or potential therapeutic targets in CBD. We compared the pathways mapping to the DAPs in the CBD *versus* controls with pathways mapping proteins, comparing controls and sarcoidosis cases [2]. We identified shared pathways in the two comparisons, such as interleukin-8 (IL-8), RHOA signalling, integrin signalling, *etc*. as well as some differences, such as HMGB1 signalling (data not shown).

Shareable abstract (@ERSpublications)
The study provides insights into proteins that may be relevant in BeS and CBD. It provides a framework to investigate the global changes in lung compartment-specific inflammatory cells to better understand the potential interplay of proteins in CBD. https://bit.ly/3PLNTXC

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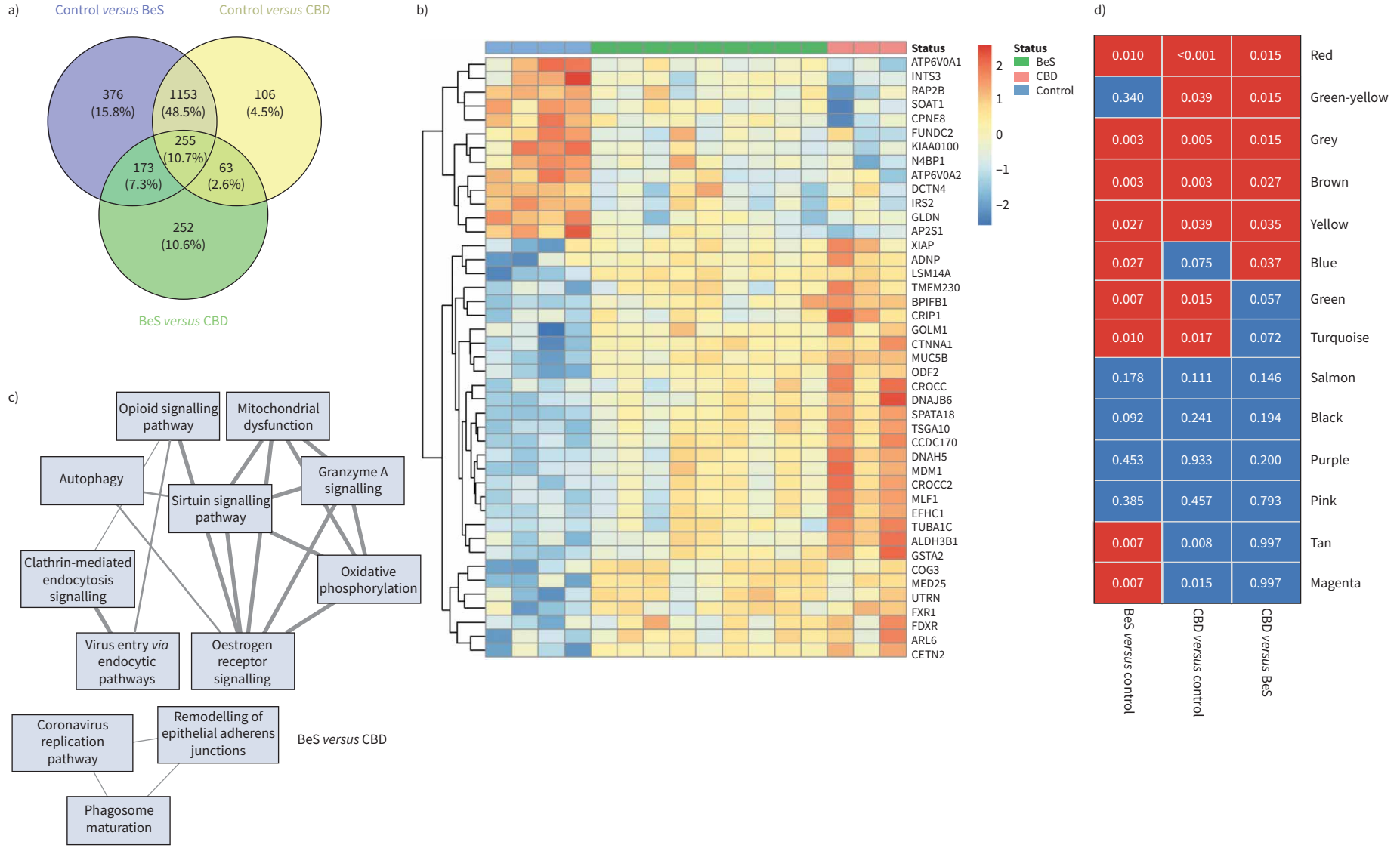

FIGURE 1 Protein pathways and modules linked to beryllium sensitisation (BeS) and chronic beryllium disease (CBD). Quantile normalised and log transformed protein abundances were analysed for identifying differentially abundant proteins (DAPs) in the three comparison groups as well as protein modules that were different in the three groups using Weighted Gene Co-expression Network Analysis in R. a) Overview of the proteins with differential abundance (DA) ($p<0.05$). b) Heatmap of proteins that demonstrated a significant monotonic increase or decrease in their abundance going from controls to BeS to CBD. c) "Overlapping analysis" in Ingenuity Pathway Analysis (IPA) for canonical pathways mapping to DAPs in BeS and CBD comparison. The edges (*i.e.*, the lines drawn between two related pathways/nodes) represent the relationship of these pathways and are shown only between any pair of pathways that have at least four shared proteins. Moreover, the thickness of the edges is proportional to the total number of shared proteins in the connected pathways with thicker lines indicative of a larger number. Highly connected pathways such sirtuin and oestrogen receptor signalling may be critical in BeS and CBD pathogenesis. d) The results of Weighted Gene Co-expression Network Analysis (WGCNA) where 14 distinct modules were identified. Several modules identified by WGCNA demonstrate DA in BeS or CBD compared to controls and BeS compared with CBD. False discovery rate (FDR) adjusted p-values for each pairwise comparison of module eigen-protein loadings as obtained using t-tests on regression coefficients from linear mixed models. Those with an FDR <0.05 are highlighted in red.

In the BeS to CBD comparison, we found other upregulated pathways involved in phagolysosome formation and phagocytosis, including phagosome maturation, clathrin-mediated endocytosis and virus entry *via* endocytic pathways. Particle dissolution within antigen-presenting cell phagolysosomes is an important source of dissolved beryllium for input to the cell-mediated immune reaction [14]. Our previous study and others also identified increased phagolysosome activation [15] and clathrin-mediated endocytosis pathway in another granulomatous lung disease, sarcoidosis [2]. Interruption of any of the steps required for phagolysosome formation and acidification was shown to suppress granulomatous cell aggregate formation and inflammatory pathways in sarcoidosis [15].

While we observed several DAPs between BeS and CBD, none of the p-values survived multiple comparison adjustment probably due to the small number of CBD cases. Consequently, we also performed an unsupervised Weighted Gene Co-expression Network Analysis (WGCNA), designed to identify co-expressed proteins with shared regulation; we detected 14 co-expressed protein modules. Using the module eigen-protein loading as a univariate summary for each sample, we found nine modules with significant differences (FDR <0.05) between controls and BeS, nine modules between controls and CBD, and six modules between BeS and CBD (figure 1d, supplemental table "WGCNA protein modules"). The number of proteins in each of six differentially abundant (DA) modules was 39 (Red), 24 (Green-yellow), 428 (Grey), 93 (Brown), 68 (Yellow) and 214 (Blue). The Blue module, which had significant DA in the control *versus* BeS and control *versus* CBD comparisons, included proteins that map to canonical pathways such as IL-4 signalling, apoptosis signalling, granzyme B signalling, antigen presentation pathway, MSP-RON signalling in macrophages, glucocorticoid receptor signalling and glioma signalling (FDR <0.05). Although the Turquoise and Magenta modules demonstrated a trend in BeS *versus* CBD, there was significant DA in control *versus* BeS and *versus* CBD. The canonical pathways in the Turquoise and Magenta modules are outlined in supplementary table "Modules IPA". Enrichment analysis for proteins in other modules did not reach the FDR threshold. For other modules, Ingenuity Pathway Analysis (IPA) failed to detect significantly enriched canonical pathways after FDR correction. However, for two of them (Red and Green-yellow), using just the first timepoint we found significant differential abundance between those that remained BeS and those that progressed to CBD where upregulation was observed in the progressors for both modules. Moreover, both of these modules also saw significant increases from the first to the second samples in the progressors while no significant differences were detected in those that remained BeS (supplementary table "WGCNA others").

Despite limited sample sizes, this study provides insight into proteins that may be relevant in BeS and CBD. It provides a framework to investigate the global changes in lung compartment-specific inflammatory cells to better understand the potential interplay of proteins in BeS and CBD. The promising pilot study indicates: 1) key differences in canonical pathways in CBD, BeS and controls; and 2) protein modules that differed between BeS and CBD that participate in different biological processes. Our findings suggest that: 1) distinct pathways and likely mechanisms are detectable in individuals with BeS *versus* CBD; and 2) modules, likely with shared regulation and function, may play a role in BeS to CBD progression.

These data strongly support our premise that there are biological processes that differ between BeS and CBD and may be linked to progression to CBD. The pathways and networks established here lay the foundation for developing diagnostic and therapeutic tools for CBD. Despite our constrained sample size, we observe interesting associations with BeS and CBD and anticipate that this framework can be leveraged to gain deeper insights into pathogenesis of Be-induced lung disease. This important translational information may also have implications for other granulomatous diseases, such as sarcoidosis, hypersensitivity pneumonitis or Crohn's disease.

Li Li[1,2], Brian Vestal[3,4], Margaret M. Mroz[1], Sucai Liu[1], Kristyn MacPhail[1], Tim J. Griffin[5], Ivana V. Yang[2,6,7], Lisa A. Maier[1,2,8] and Maneesh Bhargava[9]

[1]Division of Environmental and Occupational Health Sciences, Department of Medicine, National Jewish Health, Denver, CO, USA. [2]Division of Pulmonary and Critical Care Sciences, Department of Medicine, School of Medicine, University of Colorado Anschutz Medical Campus, Aurora, CO, USA. [3]Center for Genes, Environment and Health, National Jewish Health, Denver, CO, USA. [4]Department of Biostatistics and Bioinformatics, Colorado School of Public Health, University of Colorado Anschutz Medical Campus, Aurora, CO, USA. [5]Department of Biochemistry, Molecular Biology, and Biophysics, Center for Mass Spectrometry and Proteomics, University of Minnesota, Minneapolis, MN, USA. [6]Division of Biomedical Informatics and Personalized Medicine, Department of Medicine, School of Medicine, University of Colorado Anschutz Medical Campus, Aurora, CO, USA. [7]Department of Epidemiology, Colorado School of Public Health, University of Colorado Anschutz Medical Campus, Aurora, CO, USA. [8]Department of Environmental and Occupational Health, Colorado School of Public Health, University of Colorado Anschutz Medical Campus, Aurora, CO, USA. [9]Pulmonary, Allergy, Critical Care and Sleep, Department of Medicine, University of Minnesota, Minneapolis, MN, USA.

Corresponding author: Li Li (lil@njhealth.org)

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References

1 Newman LS, Mroz MM, Balkissoon R, *et al.* Beryllium sensitization progresses to chronic beryllium disease: a longitudinal study of disease risk. *Am J Respir Crit Care Med* 2005; 171: 54–60.
2 Bhargava M, Viken KJ, Barkes B, *et al.* Novel protein pathways in development and progression of pulmonary sarcoidosis. *Sci Rep* 2020; 10: 13282.
3 Benjamini Y. Discovering the false discovery rate. *J R Stat Soc B* 2010; 72: 405–416.
4 Cox J, Hein MY, Luber CA, *et al.* Accurate proteome-wide label-free quantification by delayed normalization and maximal peptide ratio extraction, termed maxlfq. *Mol Cell Proteomics* 2014; 13: 2513–2526.
5 Elangovan S, Ramachandran S, Venkatesan N, *et al.* SIRT1 is essential for oncogenic signaling by estrogen/estrogen receptor alpha in breast cancer. *Cancer Res* 2011; 71: 6654–6664.
6 Chiu HC, Chiu CY, Yang RS, *et al.* Preventing muscle wasting by osteoporosis drug alendronate in vitro and in myopathy models via sirtuin-3 down-regulation. *J Cachexia Sarcopenia Muscle* 2018; 9: 585–602.

7 Wan X, Garg NJ. Sirtuin control of mitochondrial dysfunction, oxidative stress, and inflammation in Chagas disease models. *Front Cell Infect Microbiol* 2021; 11: 693051.
8 Zhao Y, Yang J, Liao W, *et al.* Cytosolic FoxO1 is essential for the induction of autophagy and tumour suppressor activity. *Nat Cell Biol* 2010; 12: 665–675.
9 Li G, Xia Z, Liu Y, *et al.* SIRT1 inhibits rheumatoid arthritis fibroblast-like synoviocyte aggressiveness and inflammatory response via suppressing NF-κB pathway. *Biosci Rep* 2018; 38: BSR20180541.
10 Hisada R, Yoshida N, Umeda M, *et al.* The deacetylase SIRT2 contributes to autoimmune disease pathogenesis by modulating IL-17A and IL-2 transcription. *Cell Mol Immunol* 2022; 19: 738–750.
11 Leblond A, Pezet S, Cauvet A, *et al.* Implication of the deacetylase sirtuin-1 on synovial angiogenesis and persistence of experimental arthritis. *Ann Rheum Dis* 2020; 79: 891–900.
12 Sharma N, Shandilya A, Kumar N, *et al.* Dysregulation of sirt-1 signaling in multiple sclerosis and neuroimmune disorders: a systematic review of sirtuin activators as potential immunomodulators and their influences on other dysfunctions. *Endocr Metab Immune Disord Drug Targets* 2021; 21: 1845–1868.
13 Bhaskar A, Kumar S, Khan MZ, *et al.* Host sirtuin 2 as an immunotherapeutic target against tuberculosis. *Elife* 2020; 9: e55415.
14 Stefaniak AB, Day GA, Hoover MD, *et al.* Differences in dissolution behavior in a phagolysosomal simulant fluid for single-constituent and multi-constituent materials associated with beryllium sensitization and chronic beryllium disease. *Toxicol In Vitro* 2006; 20: 82–95.
15 Crouser ED, Locke LW, Julian MW, *et al.* Phagosome-regulated mtor signalling during sarcoidosis granuloma biogenesis. *Eur Respir J* 2021; 57: 2002695.