Antigen Profiling of Field Metalworking Fluids

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Background

- **Metalworking fluids (MWFs):** complex mixtures of oils, emulsifiers, anti-weld agents, corrosion inhibitors, extreme pressure additives, buffers (alkaline reserve), biocides, and other additives such as tramp oil, hydrolytic fluids and particulate matter from grinding and machining operations.

- **Types:** They can be straight oils (such as petroleum oils) to water-based fluids, which include soluble oils and semisynthetic/synthetic fluids.

- **Metalworking fluids (MWFs)** are used to reduce heat and friction and to remove metal particles in industrial machining and grinding operations.

- According to **NIOSH** about **1.2 million occupational workers** in machine finishing, machine tooling, and other metalworking and metal-forming operations are potentially exposed to the aerosol of MWFs.

- In-use Metal working fluids support microbial growth and repeated exposure to the occupational contaminated MWFs can lead to a variety of health effects such as **hypersensitivity pneumonitis (HP), chronic bronchitis, impaired lung function, and asthma.**
- **Interestingly, In use metal working fluids are heavily contaminated with *Mycobacterium immunogenum* species and has been implicated in Machinists HP.**

Hypothesis and Aims

Hypothesis: Machinists' HP is a cell-mediated immune disease associated with repeated exposure to the mycobacterial species *Mycobacterium immunogenum* frequently isolated from MWFs. However, the specific T-cell antigens/epitopes responsible for causing the MWF exposure-associated HP in the exposed machinists are not yet defined. **Prevailing field conditions in MWF can lead to the induction and release of specific antigens from mycobacteria that are critical for HP etiology.**

AIM 1: Identification of Mycobacteria-specific proteins from the field in-use Metal working fluid (MWF).
1a: Isolation of total proteins from the field samples.
1b: Identification of Mycobacteria-specific immune-reactive proteins.

AIM 2: Identification of MWF-specific T-cell antigens of *M. immunogenum*.
2a: Identify immunoreactive Proteins by LC-MS/MS.
2b: In-silico identification of T-cell antigen epitopes and functional validation.

Experimental Design

Isolation of total proteins from the field samples

MI Contaminated MWF (Test sample)

Non- MI Contaminated MWF (Control sample)

Centrifugation

Biomass

Cell lysis

Cell lysate Fraction

Supernatant

TCA precipitation

Secretory Fraction

Protein separation 2D-Gel/ 1D Gel

Immunoblotting

Identification of peptides by LC-MS/MS

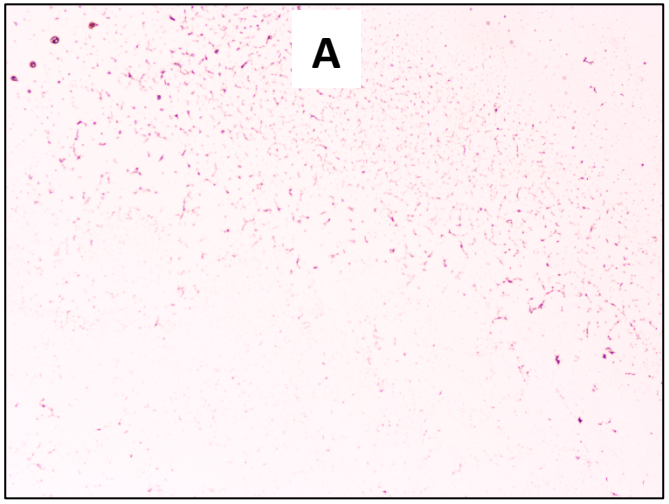
***In-silico* identification of T-cell antigen epitopes and functional validation**

***In-silico* analysis of peptides by IEDB and Propred and Propred I server**

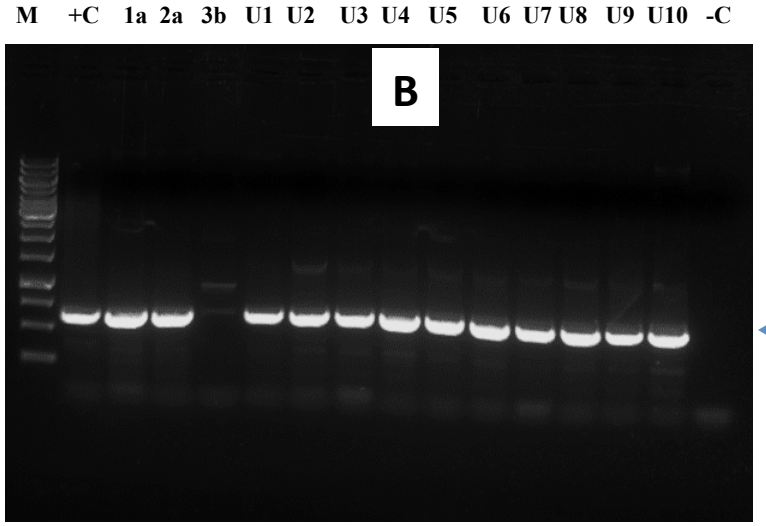
Functional Validation of T cell antigens by T cell response assay

Isolation of Mycobacterial strain from MWF

Acid fast staining for Mycobacteria



PCR amplification of Hsp65 product



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C

>Hspc1-Hsp667F_A08.ab1

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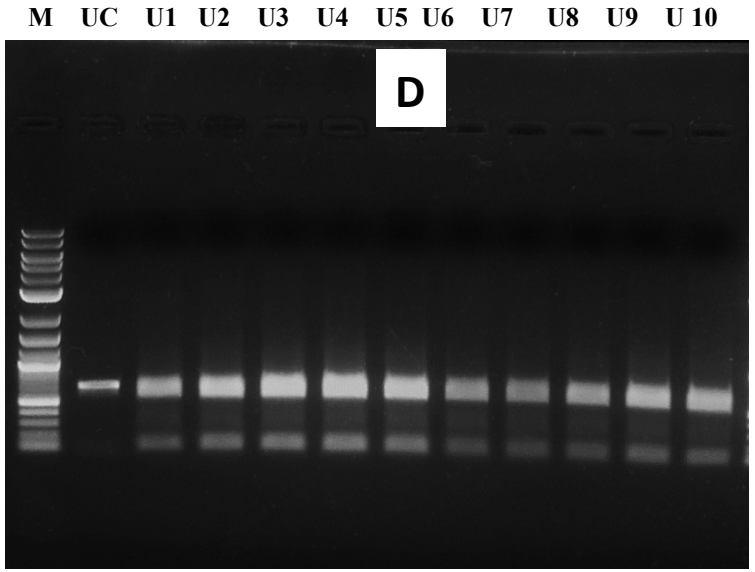
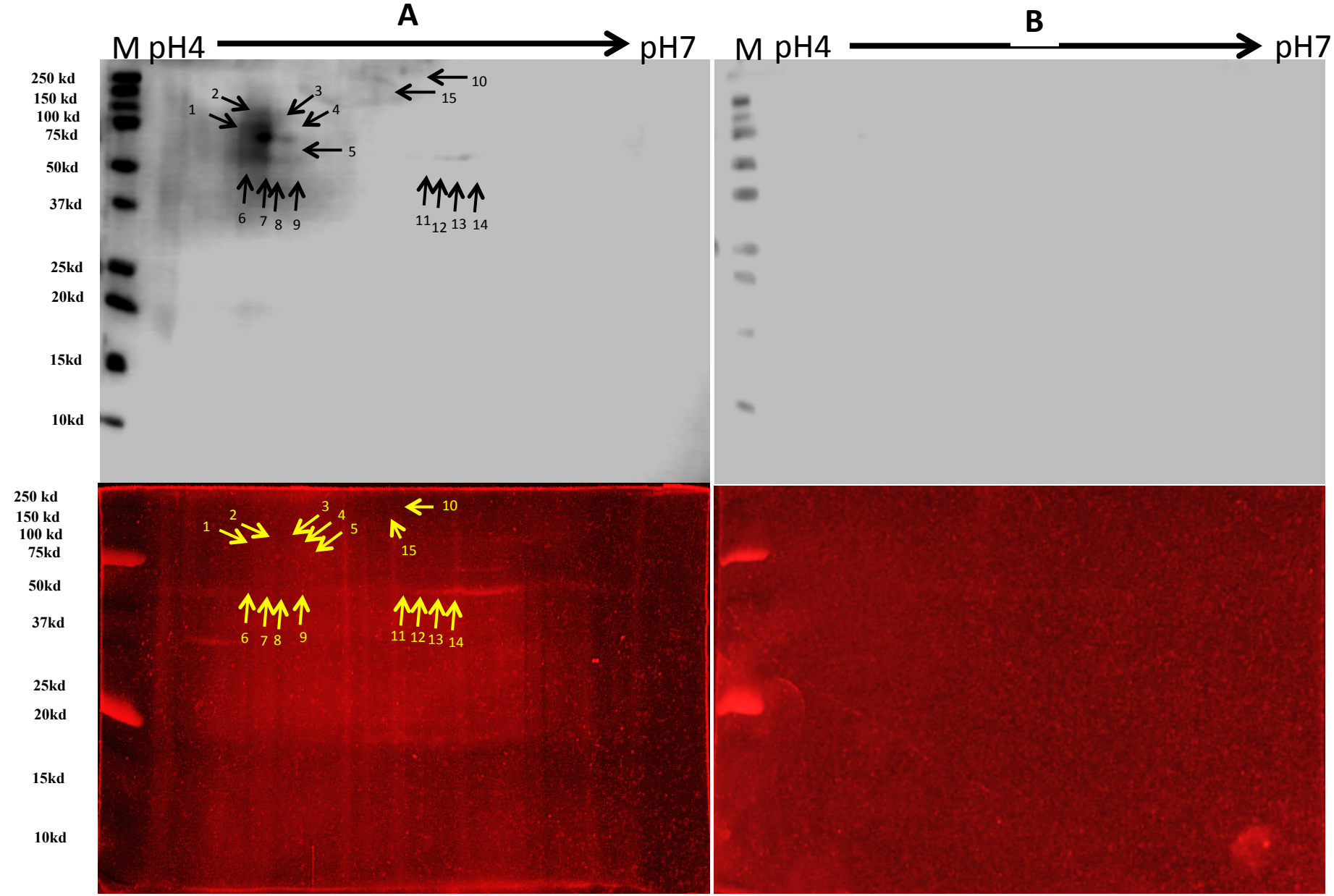
BbvI restriction analysis of 667 bp product



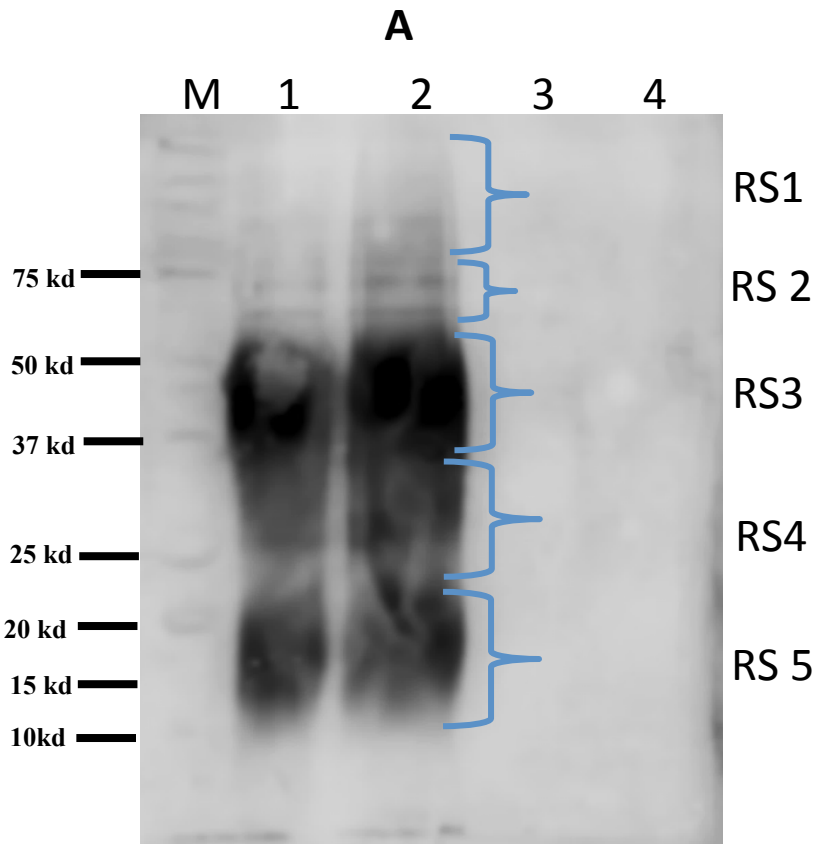
Identification of MI Ags by 2D immuno-proteomics



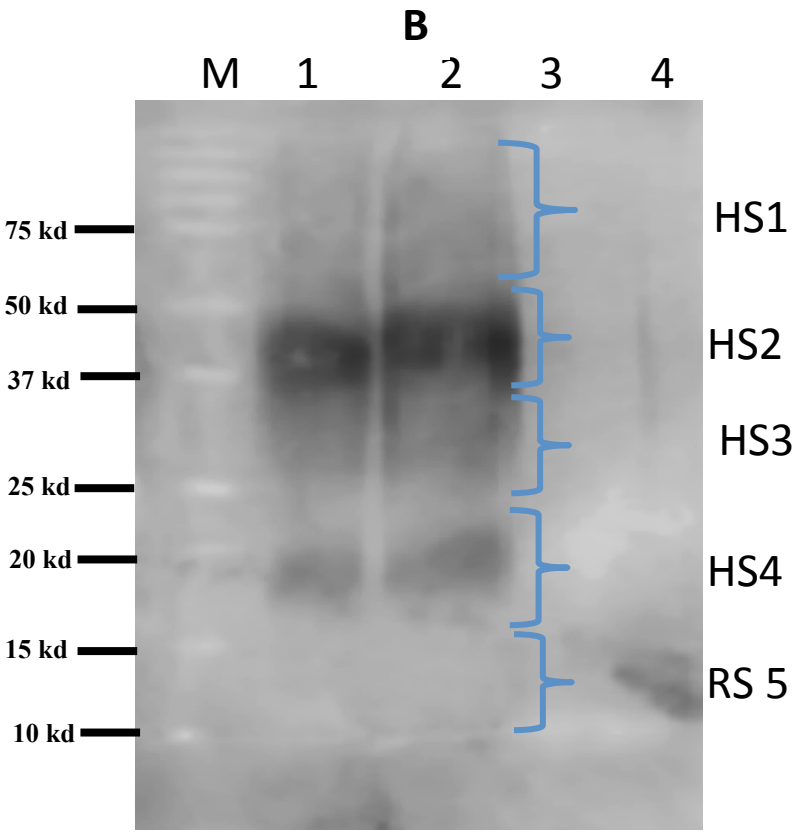
Mycobacteria contaminated MWF

Non-mycobacteria contaminated MWF

1D immunoblot with anti MI Rabbit/HP patients sera

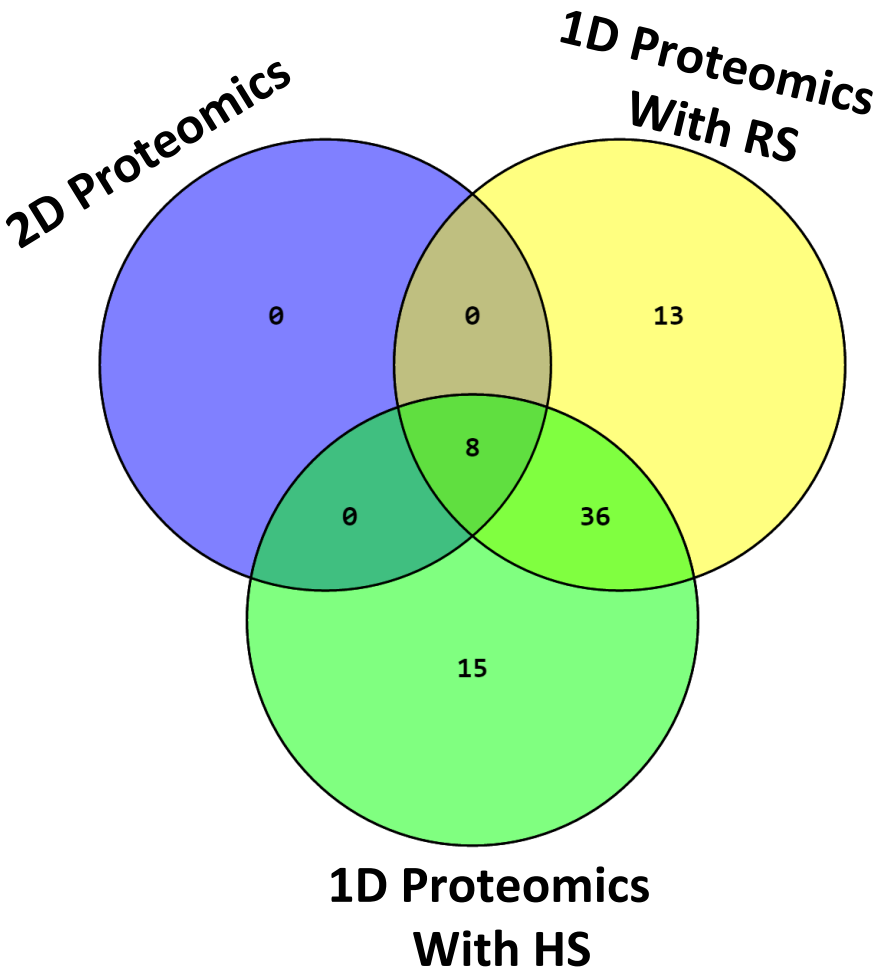


Anti MI Rabbit sera



HP patients sera

Identification of MI antigens by LC-MS/MS

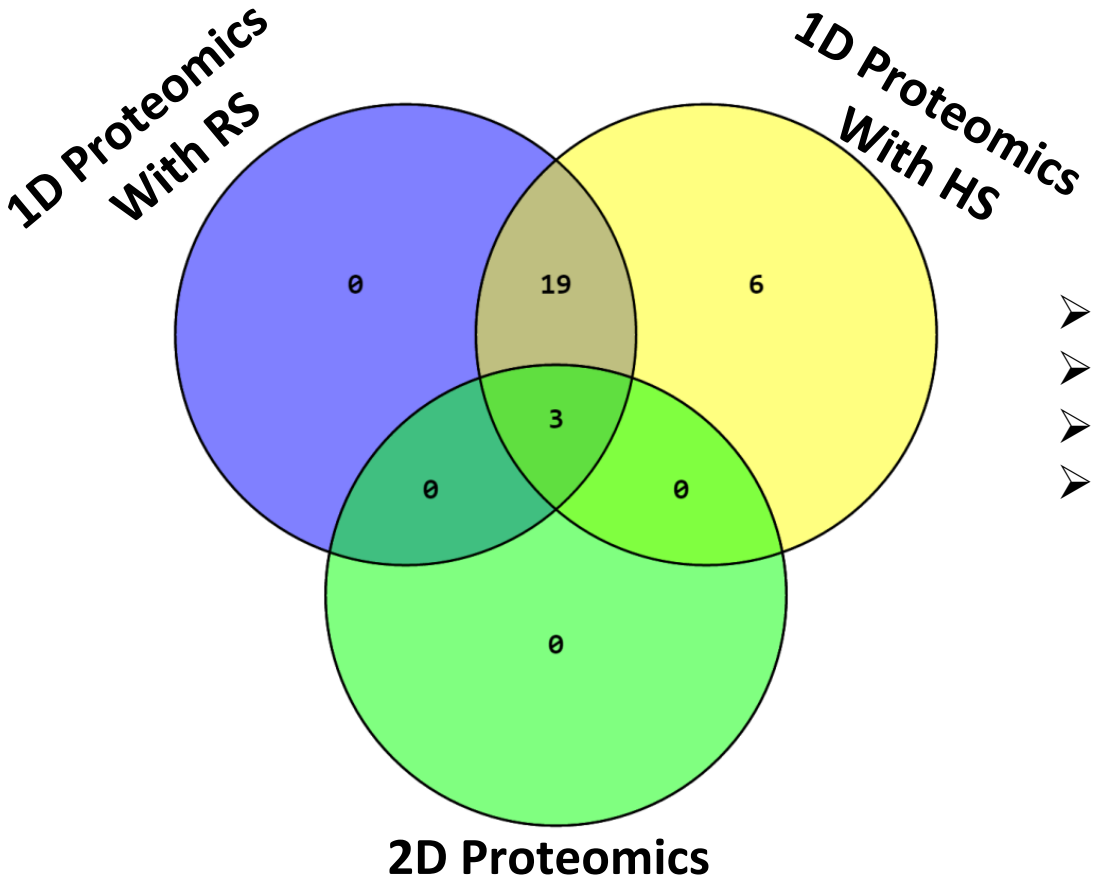


- A total of 72 Antigens were detected
- 13 Ags were uniquely detected with RS
- 15 Ags were uniquely detected with HS
- 8 Ags were detected by 2D separation
- Which were found in HS and RS as well
- A total of 44 Ags were common to all.

- **This is the first report of MI antigens detected directly from the MWF**

Identification of immuno-reactive antigens from In use MWF immunoproteomics. Antigens reacting to anti MI antibodies of rabbit (RS)or Human serum (HS). Protein were separated either by 2D or run on SDS PAGE.

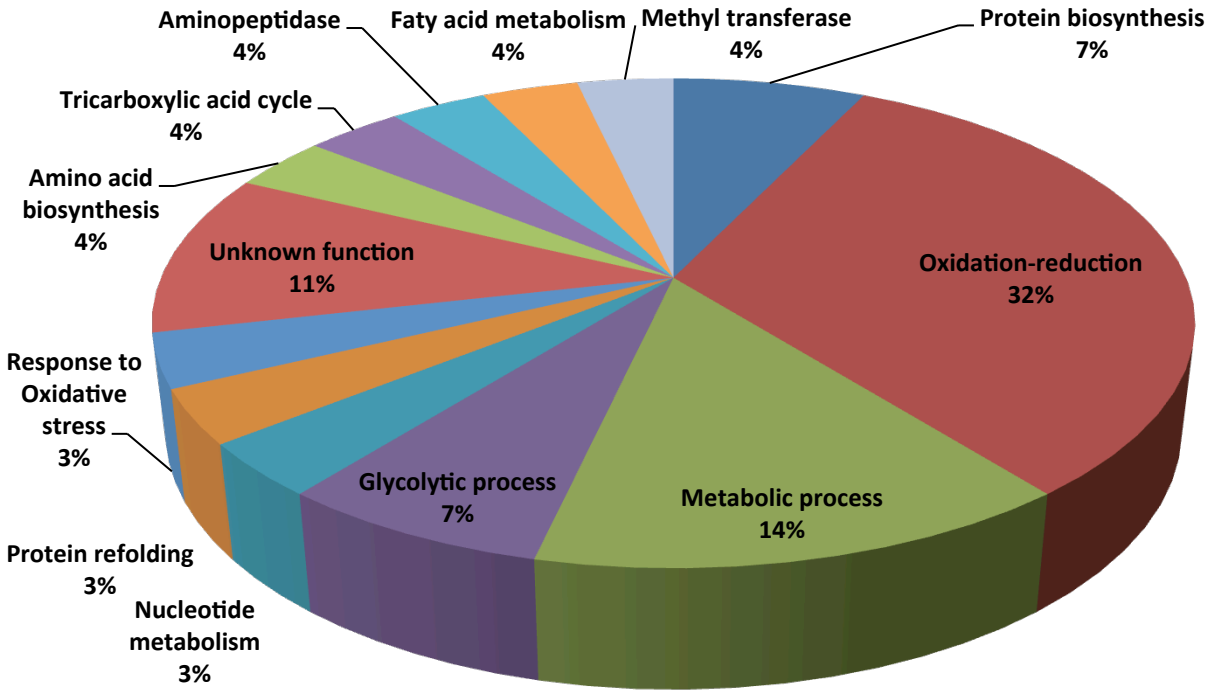
Identification of Abundant antigens



- 22 Antigens detected with RS
- 28 Antigens detected with HS
- 3 Antigens detected with 2D
- **6 Antigens were unique to HS**

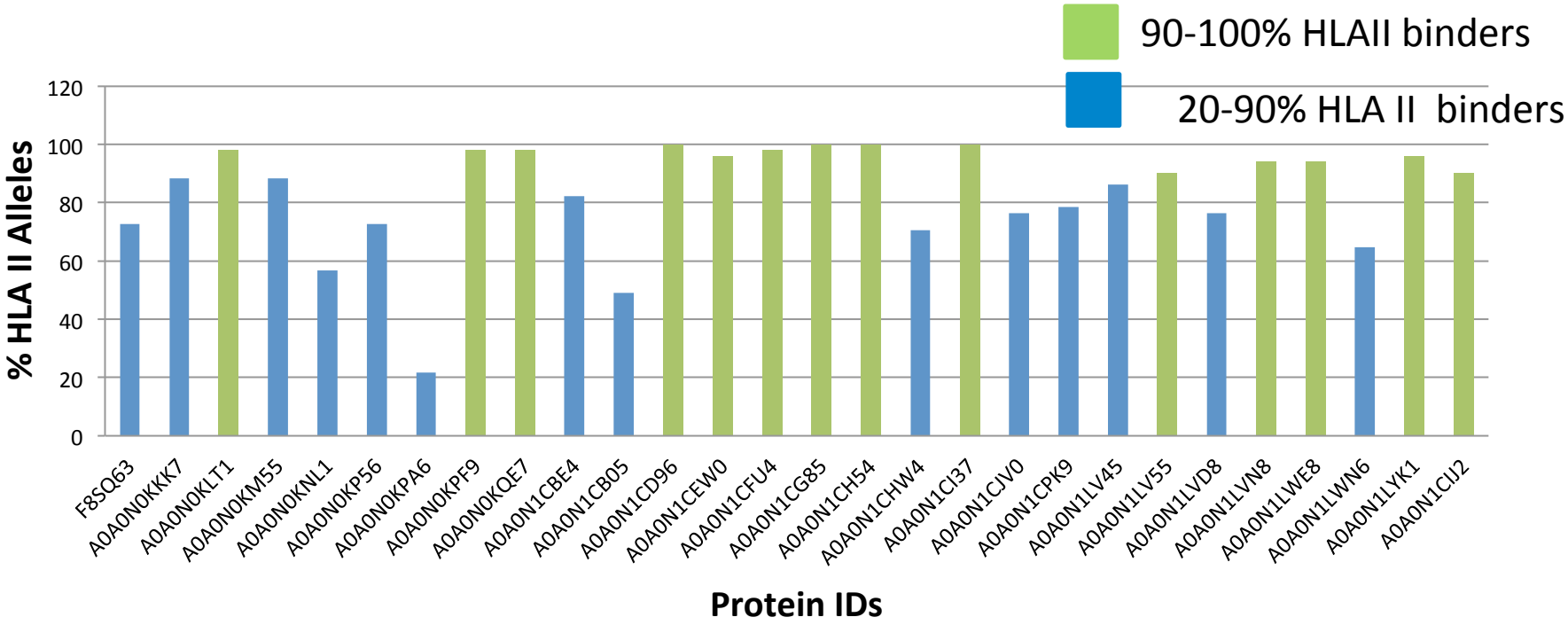
Identification of **Abundant antigens** based on peptides reacting to anti MI antibodies of rabbit (RS), Human serum (HS) and with
RS separated with 2D

Functional clustering of the Abundant antigens



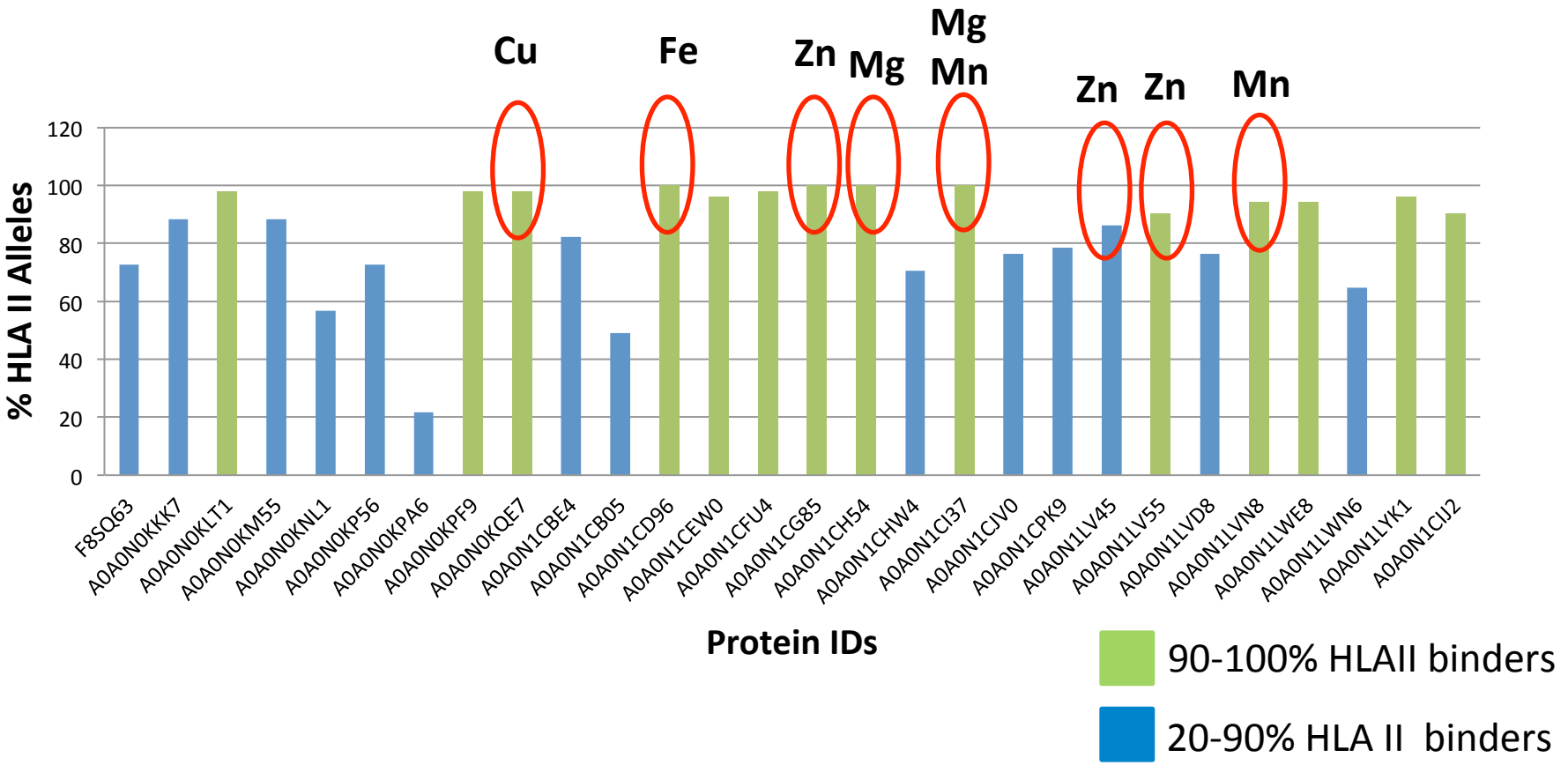
Functional information was retrieved from the UniProt Knowledgebase (UniProtKB) (http://www.uniprot.org/)

T cell antigen analysis by immunoinformatics



- 50% antigens were found to be T cell antigens in the MWF
- Antigenic epitopes were found to bound more 90% of the HLA alleles in Query(51)
- HLAII alleles binding analysis was done by ProPred online server.

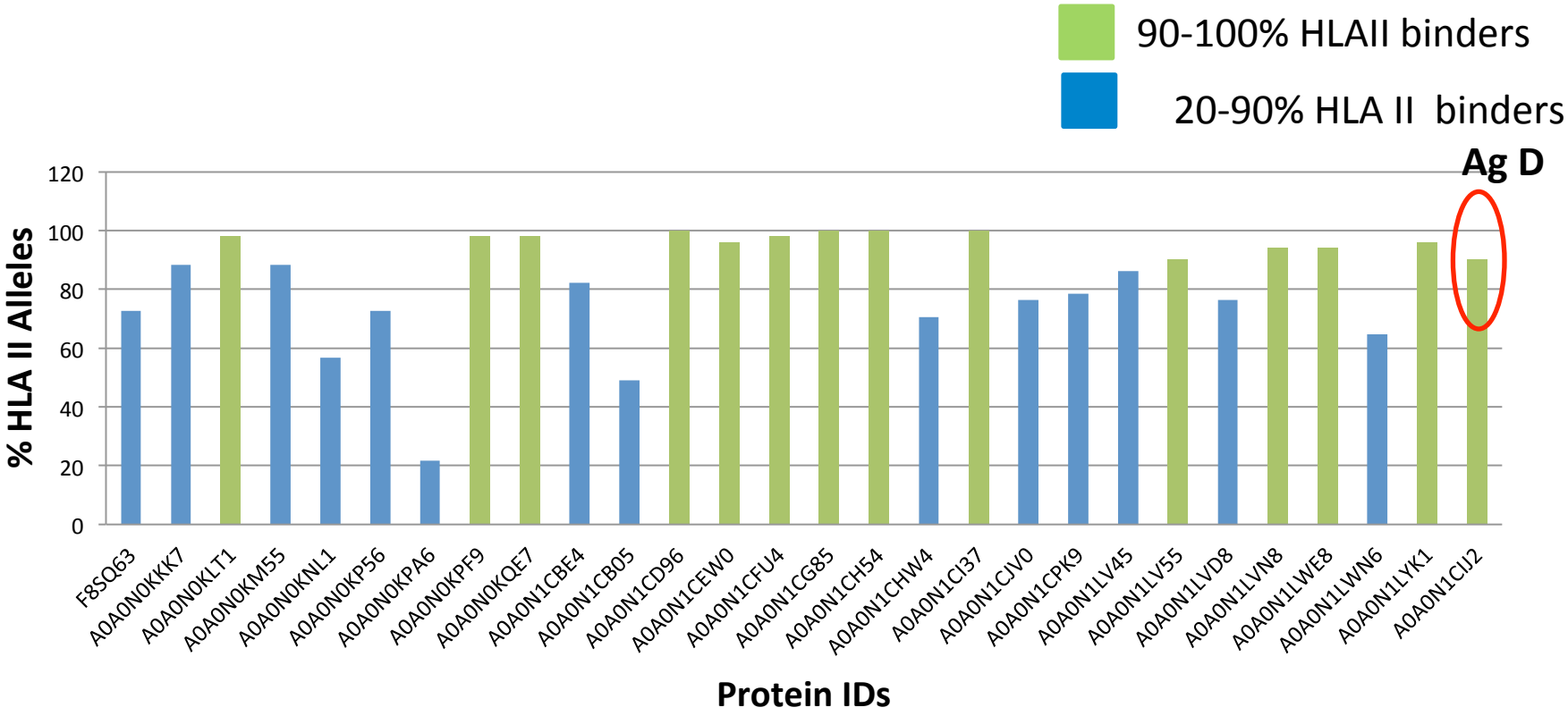
Metal Binding proteins



- **Eight antigens among the T cells antigens were found to be metal binders**
- **There are reports which link different metal exposure and cases of HP and Asthma.**

Functional Validation of the MI antigen

Functional validation of a T cell antigen

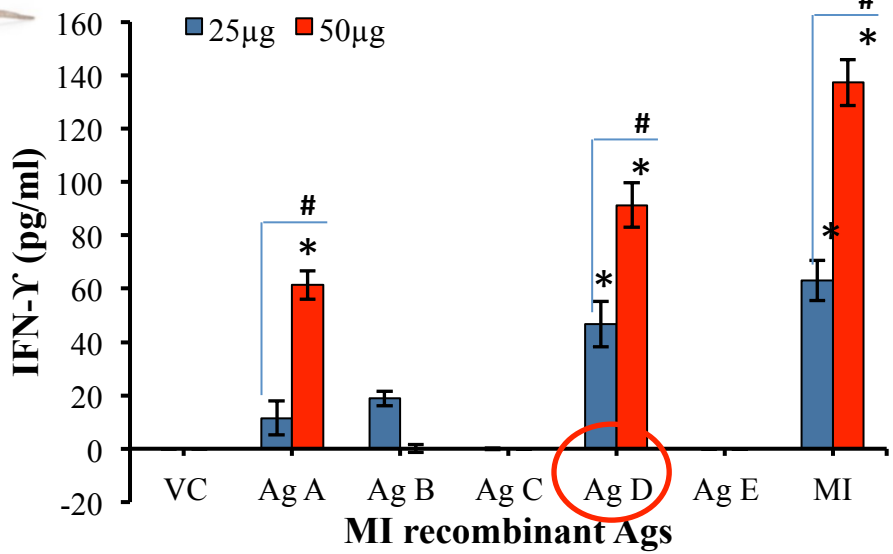


- For functional validation as a T cell Ag, we selected an HLA II binder Named as **Ag D**
- **Gene encoding Ag D was cloned and expressed in *E. coli* expression vectors systems. Protein was NI-NTA purified and made endotoxin free.**

T cell response assay

Mycobacterium immunogenum (MI)

Whole cell lysate exposure for 3 weeks



- Spleens were harvested and Exposed splenocytes were again re- challenged with Ag D and MI lystates

- Ag D also activated the CD4+ T cells isolated from the healthy human donor Using ELISPOT Test for IFN-g response.
- Ag D also induced strong cytokine response in alveolar macrophages and
- In Human dendritic cells..

Chandra et al. Molecular Immunology 2016

Summary and conclusion

- Isolated a novel strain of ***M. immunogenum MJY27*** from In use MWFs.
- **First report of identification of MI antigens directly from the MWFs.**
- Identified a total of **72 MI specific antigens** from the used MWFs.
- Among the abundant antigens **14 antigens were found to be T cell antigens**.
- **Eight antigens** among the T cells antigens were found to be **metal binders**.
- We have functionally validated **Ag D for T cell response in mice and in human DC-T cell assay**
- In conclusion Identified Ags may provide a key to understanding the etiological aspects of the occupational HP disease development and facilitate development of immunodiagnostic and intervention strategies for HP patients in occupational settings.

Future Directions

1) **Screening of field MWF types and conditions** supporting *M. immunogenum* growth and its differential potential for eliciting immunogenicity.

2) **Antigens/epitopes identified in this pilot study** will be used to design expanded studies to investigate different aspects of mycobacterial HP research.

i). **Development of diagnostics:** In short-term, knowledge of these antigenic epitopes will be utilized for the development of immunodiagnostic tools for MOL/HP patients. In long-term, these diagnostic tools could be adapted to **assess the personal occupational risk for developing HP in machinists.**

ii). **Etiology of HP development:** Studies on the etiological role of identified T-cell antigens in HP could be undertaken based on antigen/ T cell interactions and in vivo challenge studies. In this context, the actual T cell subpopulation(s) involved in HP development and progression could be characterized.

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Thanks