Using an iTRAQ Approach to Investigate the Effect of Cigarette Smoke Exposure in a 90-day Inhalation Study Followed by 42-days Recovery Period on Sprague-Dawley Rats Lung Tissues.

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Introduction

In order to update and advance our knowledge regarding the impact of cigarette smoke on the lungs of Sprague-Dawley rats, a system toxicology approach that combines state-of-the-art proteomics with transcriptomics and toxicological endpoints, was developed. A 90-day inhalation study followed by a recovery period of 42 days was conducted as described in the Organization for Economic Co-operation and Development (OECD) Testing Guideline 413. A quantitative proteomics approach using isobaric tags for absolute and relative quantification (iTRAQ®) was performed on the lung tissues of Sprague-Dawley rats to detect changes in protein expression levels between control rats (exposed to air), and rats exposed to mainstream smoke (MS) of the Reference Cigarette (3R4F) at 2 exposure concentrations of 8, and 23 mg/L nicotine. For the recovery period, the same treatment and control groups were used. Six biological replicates were analyzed to assess reproducibility within each group.

The objectives of the study were:

(i) To identify regulated proteins in rat lungs in response to a cigarette-smoke.
(ii) To determine which biological processes are impacted in response to cigarette smoke.
(iii) To analyze and understand the effect of the recovery period on the lung proteome.
(iv) To determine the feasibility of using the iTRAQ approach for product assessment of candidate Modified Risk Tobacco Products (MRTPs) in *in vivo* rat model systems.

MATERIALS & METHODS

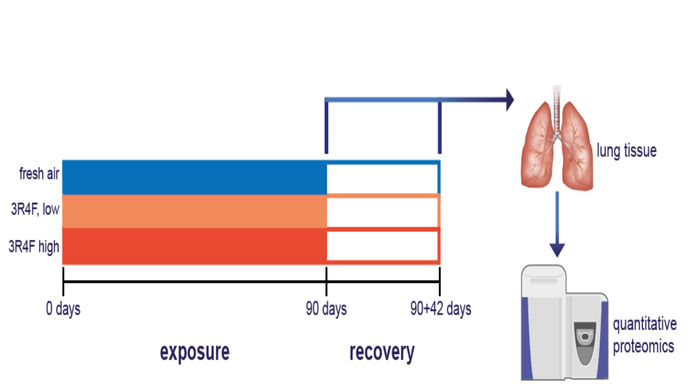

Figure 1: Study design with the different groups and exposure durations.

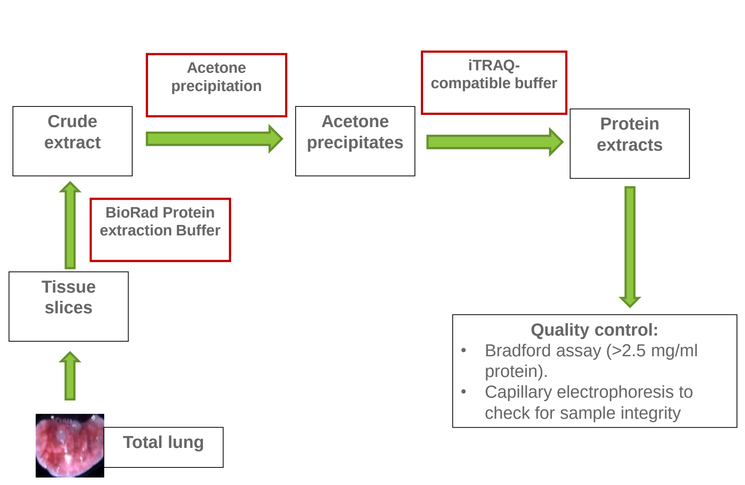

Figure 2: Workflow for the extraction of proteins from rat lungs.

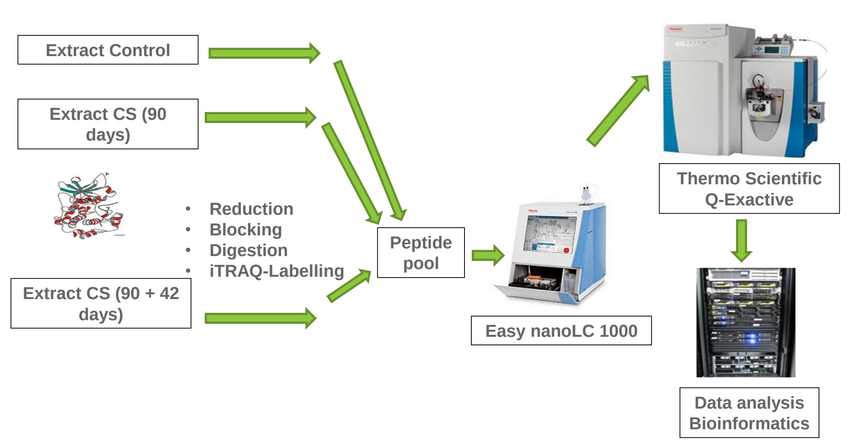

Figure 3: iTRAQ workflow used for the identification of differentially expressed proteins.

RESULTS

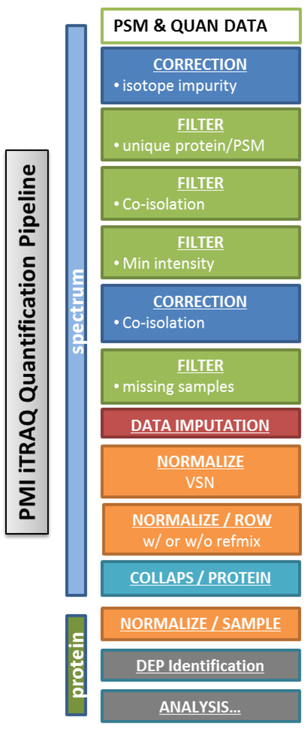

Figure 4: Quantification pipeline for isobaric tag labeled samples. The pipeline is implemented in R. It allows full control over processing steps and settings. The workflow used allowed for sensitive and robust detection of differentially regulated proteins based on unique peptides only. DEP, differentially expressed protein; VSN, Variance stabilizing normalization (Arntzen *et.al.*, 2011).

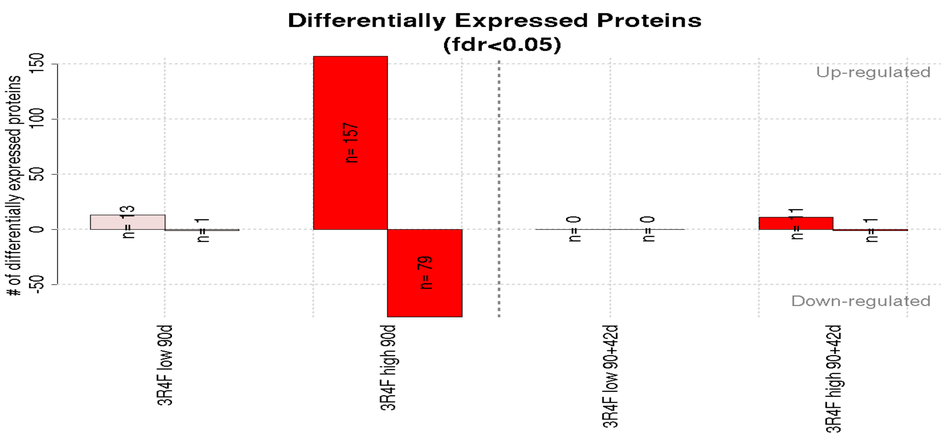

Figure 5: Number of differentially expressed proteins in comparison to fresh-air control for each exposure treatment and exposure duration..

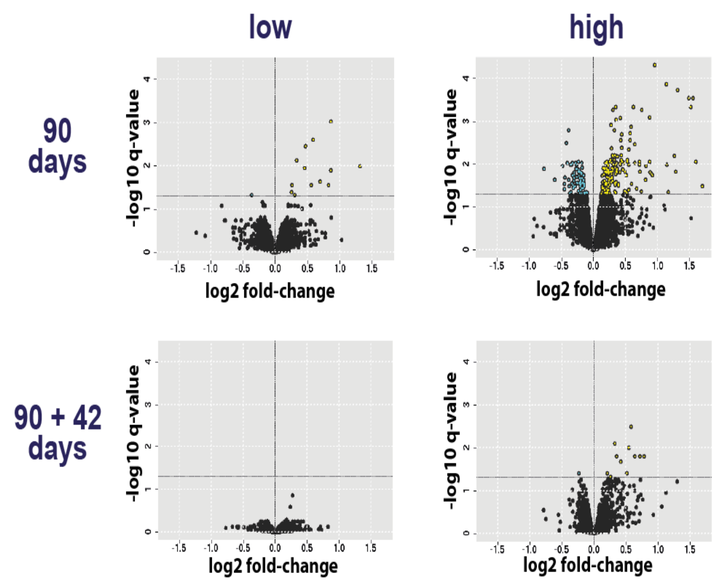

Figure 6 Tobacco smoke exposure showed a strong overall impact on the lung proteome. The volcano plots show the log2 fold-change versus -log10 of the FDR-adjusted p-values. Significantly up-(yellow) or down-regulated (blue) proteins are shown (adjusted p-value < 0.05).

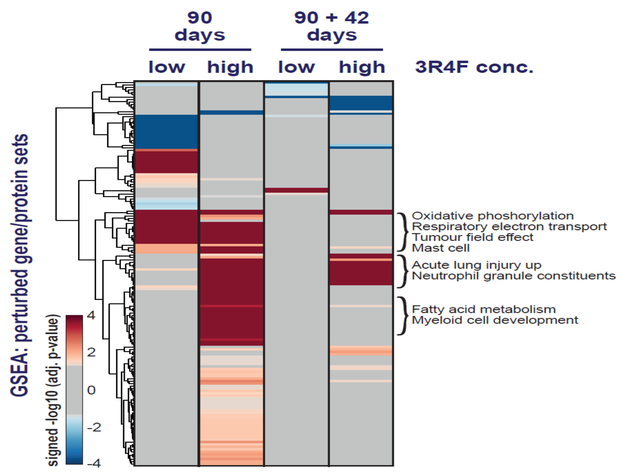

Figure 7 Gene set enrichment analysis (GSEA) shows a concentration-dependent gene set perturbation by cigarette smoke and a partial recovery after 42 days of fresh air exposure. The heatmap shows the significance of association (-log10 adjusted p-value) of up- (red) and down- (blue) regulated proteins with gene sets. Select gene sets enriched for up-regulated proteins by cigarette smoke exposure are highlighted for three different clusters.

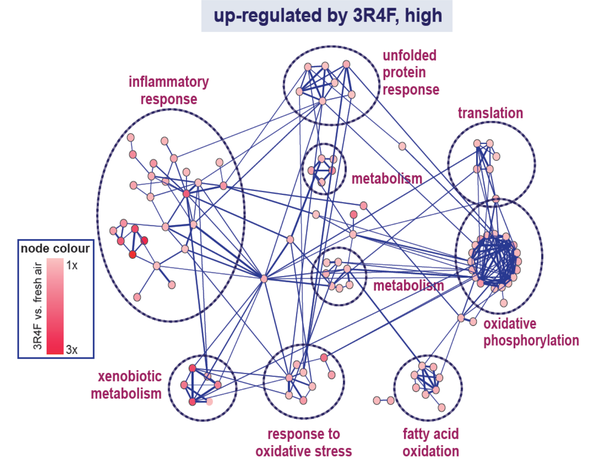

Figure 8 Functional clustering of the significantly up-regulated proteins upon cigarette smoke exposure. Functional interactions linking the up-regulated proteins upon 90-day cigarette smoke exposure (high concentration) were obtained from the STRING database (Franceschini et al., 2013). Clusters in the resulting functional association networks were identified and annotated. The affected functional clusters include xenobiotic metabolism, response to oxidative stress, and inflammatory response.

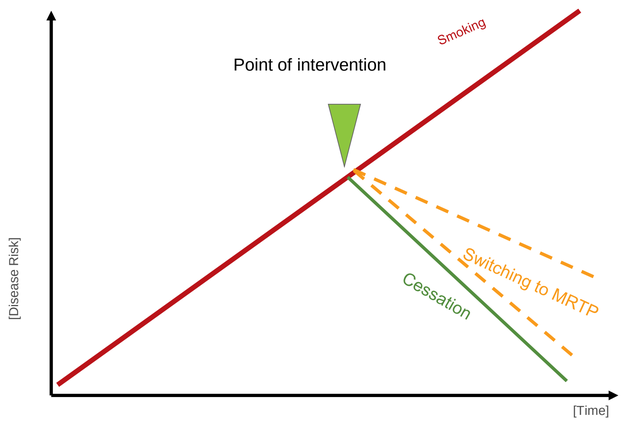

Figure 9 Assessment framework to compare switching to MRTPs with ongoing smoking and using smoking cessation as the benchmark.

Conclusions

- Exposure of rat lung tissues to cigarette smoke produced a biological effect on the proteome that is concentration dependent, i.e. showed a dose response effect as measured by iTRAQ. **(Figure 5 and 6)**
- Exposure of rat lungs to high dose of 3R4F showed up-regulation of biological functions, such as the inflammatory response, oxidative stress, xenobiotic metabolism, fatty acid oxidation, oxidative phosphorylation, unfolded protein response. **(Figures 7 and 8)**
- After the 42 days recovery period, the smoke induced perturbation of the rat proteome was reduced.
- The rat proteome alterations measured by iTRAQ reflected the smoke exposure effects and will provide the foundation for future assessment of candidate Modified Risk Tobacco Products (MRTPs) that are currently developed by PMI. **(Figure 9)**

References

- Arntzen, M. O., *et al*. (2011), IsobariQ: software for isobaric quantitative proteomics using IPTL, iTRAQ, and TMT. J Proteome Res **10**(2): 913-920.
- Franceschini, A., *et al*. (2013), STRING v9.1: protein-protein interaction networks, with increased coverage and integration. Nucleic Acids Res **41** (Database issue): D808-815.

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HUPO Conference, Madrid, Spain
October 5-8, 2014.