Proteomics Analysis of the Lung Proteome in Two Different Strains of Mice Within Systems Toxicology Approach for Product Assessment to Investigate Effects of Switching to Candidate and Prototype Modified Risk Tobacco Products, or To Smoking Cessation.

A. Elamin[1], B. Titz[1] , S. Dijon,[1] C. Nury[1], T. Schneider[1],, F. Martin[1], B. Phillips[2], N.V. Ivanov[1], M.C. Peitsch[1] , J. Hoeng[1].

[1]Philip Morris International, Research & Development, Neuchâtel, Switzerland; [2]Philip Morris International, Research Laboratories, Singapore.

INTRODUCTION

Cigarette smoking is a strong causative element contributing to the development of several diseases, including cardiovascular disease (CVD) and chronic obstructive lung diseases such as COPD/emphysema. Reliable system-wide measurements are key to a meaningful systems-toxicology approach by which impact of toxicants in cells are evaluated (Hoeng et al., 2012). We evaluated the advantages of using proteomics methodologies for the product assessment of candidate modified risk tobacco product (cMRTP), which is already commercialized, and prototype modified risk tobacco product (pMRTP), which is still under further development. The objective was to integrate the proteomics data with other endpoints, mainly transcriptomics and lipidomics datasets, within our systems toxicology approach. Two studies were conducted on 2 different mice strains: 1) an ApoE-deficient mice which are prone to developing premature atherosclerosis and emphysema (using cMRTP), and 2) C57BL/6 mice which are prone to developing emphysema, a form of chronic obstructive pulmonary disease (COPD) (using pMRTP). We evaluated the effects of combustible cigarette smoke (CS) from a reference conventional cigarette (3R4F) and aerosol from two different types cMRTP and pMRTP on mice exposed for up to 7/8 months.

MATERIALS & METHODS

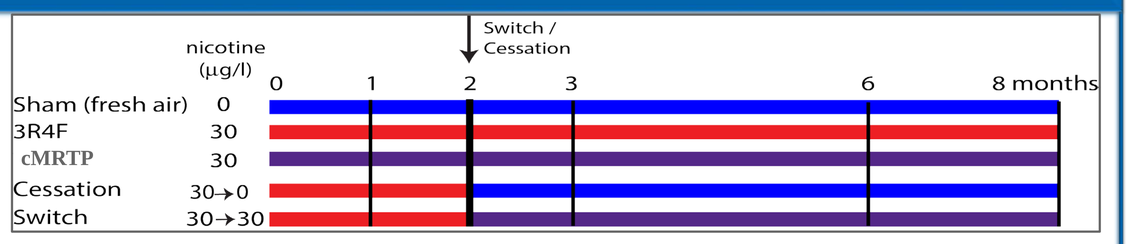

Figure 1. Study design with different exposure groups and durations for the ApoE-/- mice. 3R4F cigarettes were smoked according to the Smoking Intense Puffing Regime. Design for the C57BL/6 is similar except the duration of the study

Female ApoE$^{-/-}$ mice were exposed to 3R4F (600 mg/m^3 TPM), cMRTP (matched to the nicotine in 3R4F – 29.9 µg/l) or filtered air for 3 hours per day, 5 days per week, for up to 8 months. After 2 months of exposure to 3R4F, switching and cessation groups were exposed to aerosol from THS2.2 or filtered air, respectively. Dissections were performed after 1, 2, 3, 6, and 8 months of exposure. At each time point animals were allocated for the following endpoints: bronchoalveolar lavage fluid (BALF), identification of infiltrated inflammatory cells in lungs and multi-analyte (cytokines/chemokines) profiling, histopathological evaluation lung function, plaque surface determination (**data not shown**) and an extensive molecular high-throughput analysis using "Omics" approaches (transcriptomics, proteomics and lipidomics). Only lung proteomics datasets are presented.

Female C57BL/6 mice were exposed to 3R4F (750 mg/m^3), pMRTP ((matched to the nicotine in 3R4F – 34.4 µg/l) or filtered air for 4 hours per day, 5 days per week, for up to 7 months in another study with similar switching after 2 months as described above. Dissections were performed on months 1, 2, 5 and 7 months of exposure for the different endpoints as described above. Only the lung proteomics datasets are presented..

RESULTS

ApoE$^{-/-}$

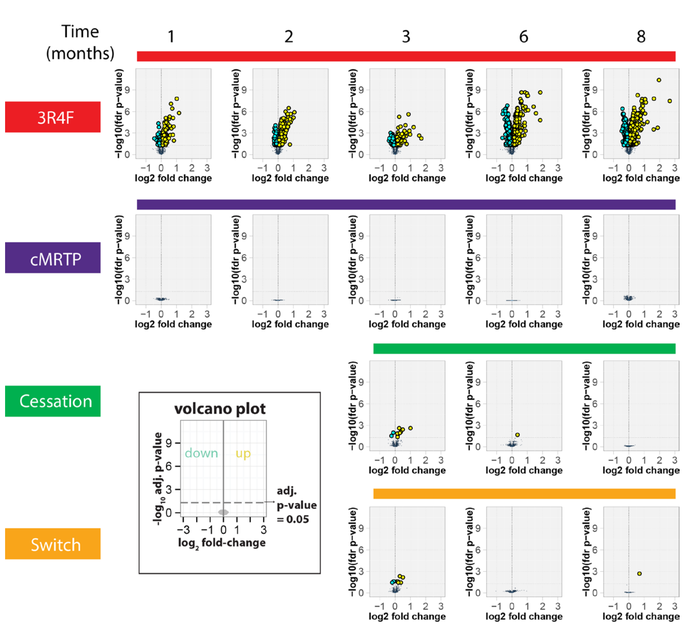

Figure 2. Volcano plots representing the proteome response profiles in lungs for the Apoe$^{-/-}$ study. For each protein, the protein expression change, calculated as the log2 fold change, is plotted on the x-axis and the statistical significance, proportional to the negative log10-adjusted *P*-value, is plotted on the y-axis. Yellow and blue dots highlight proteins that are statistically significantly up- or down-regulated, respectively, compared with the sham group at each respective time point (Benjamini-Hochberg adjusted p-value <0.05).

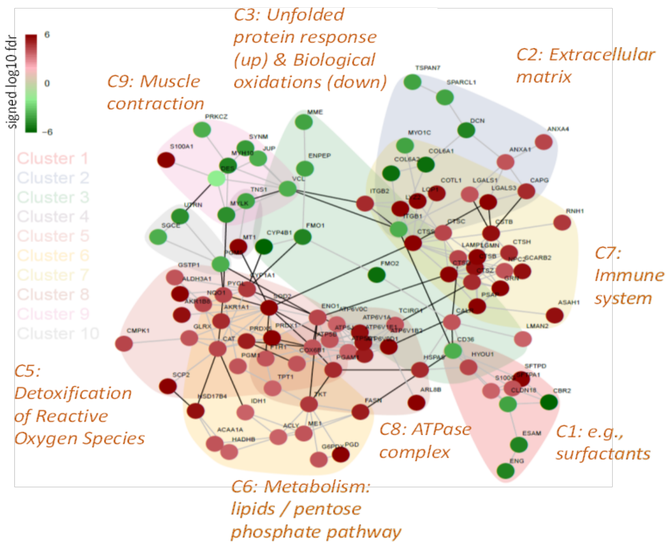

Figure 3. Functional clusters impacted by exposure to conventional cigarette smoke in the Apoe$^{-/-}$ study. Functional protein network for differentially expressed proteins upon 8 months of 3R4F exposure was identified with the dnet approach to highlight regulated biological functions (Fang et al.). Each node in the network corresponds to a regulated protein, each edge to a functional link reported in the String database (Franceschini et al., 2013). Clusters of regulated protein functions were identified and functionally annotated. Node colors show the significance of regulation upon 8 months of cigarette smoke (3R4F) exposure (maximum signed -log$_{10}$ adjusted p-value for both time points).

C57BL/6

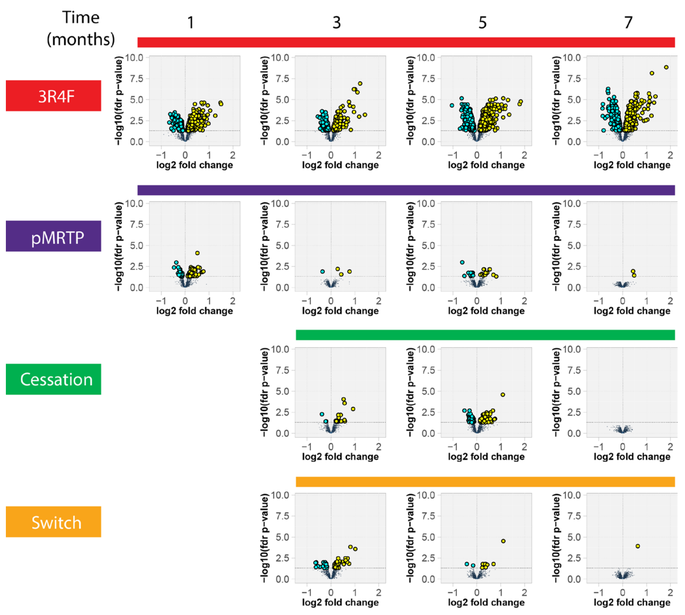

Figure 4. Volcano plots representing the proteome response profiles in lungs for the C57BL/6 study. For each protein, the protein expression change, calculated as the log2 fold change, is plotted on the x-axis and the statistical significance, proportional to the negative log10-adjusted *P*-value, is plotted on the y-axis. Yellow and blue dots highlight proteins that are statistically significantly up- or down-regulated, respectively, compared with the sham group at each respective time point (Benjamini-Hochberg adjusted p-value <0.05).

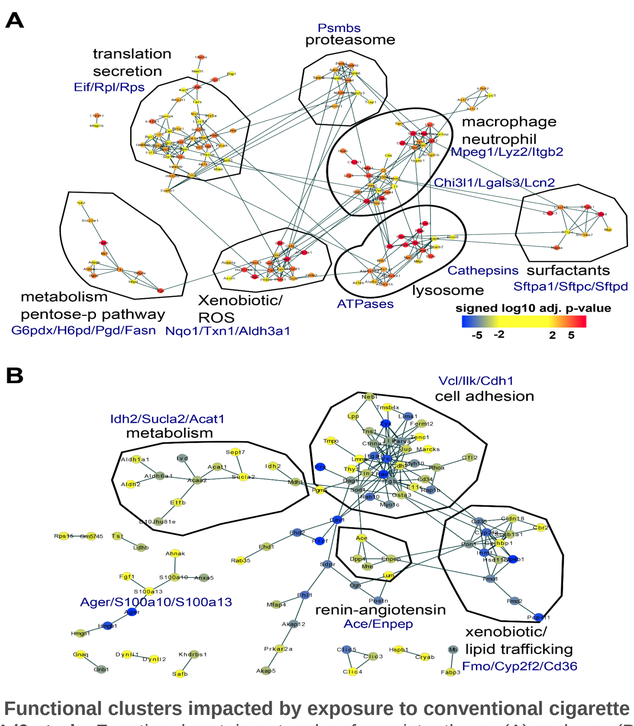

Figure 5: Functional clusters impacted by exposure to conventional cigarette smoke for the C57BL/6 study. Functional protein networks of consistently up- (A) or down- (B) regulated proteins upon 5 and 7 months of 3R4F exposure highlight regulated biological functions. Each node in the network corresponds to a regulated protein, each edge to a functional link reported in the String database (Franceschini et al., 2013). Clusters of regulated protein functions were identified and functionally annotated. Node colors show the significance of regulation upon 5/7 months of cigarette smoke (3R4F) exposure (maximum signed -log$_{10}$ adjusted p-value for both time points).

Combined Datasets

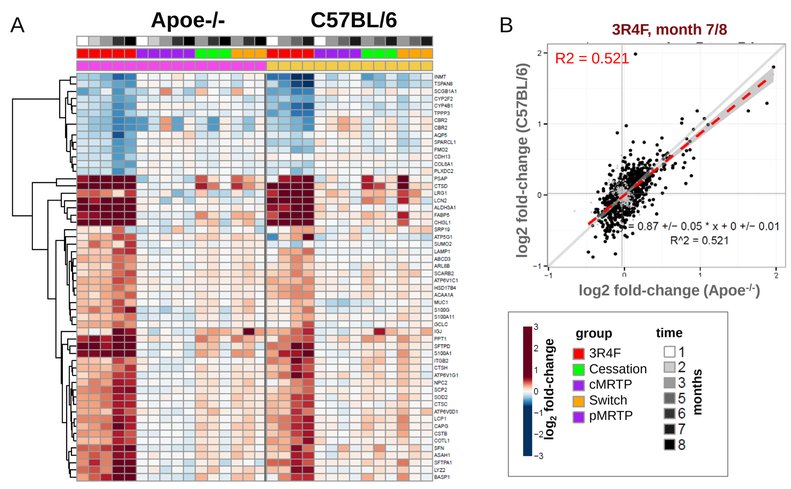

Figure 6. Comparison of exposure response in Apoe$^{-/-}$ and C57BL/6 studies. (A) Protein expression matrix for more strongly regulated proteins in Apoe$^{-/-}$ study with comparison to response in C57BL/7 study. Differentially expressed proteins (fdr-adj. p-value < 0.05) and abs(log$_2$ fold-change) > log$_2$1.3 in any contrast of Apoe$^{-/-}$ study are included. **(B)** Fold-change scatter plot comparing the response for the last 3R4F time point (vs. sham) for the Apoe-/- and C57BL/6 study.

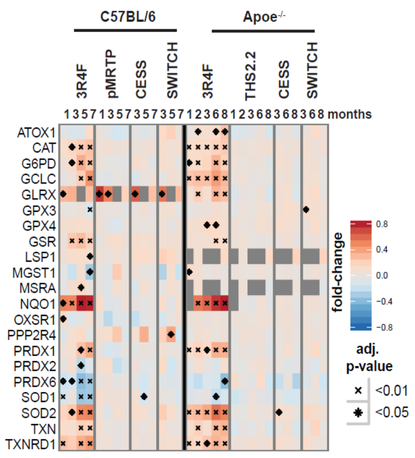

Figure 7. Response of proteins implicated in reactive oxygen species response. The human orthologs of differentially expressed proteins of the "HALLMARK REACTIVE OXI(Y)GEN SPECIES PATHWAY" are shown (www.broadinstitute.org/gsea/msigdb).

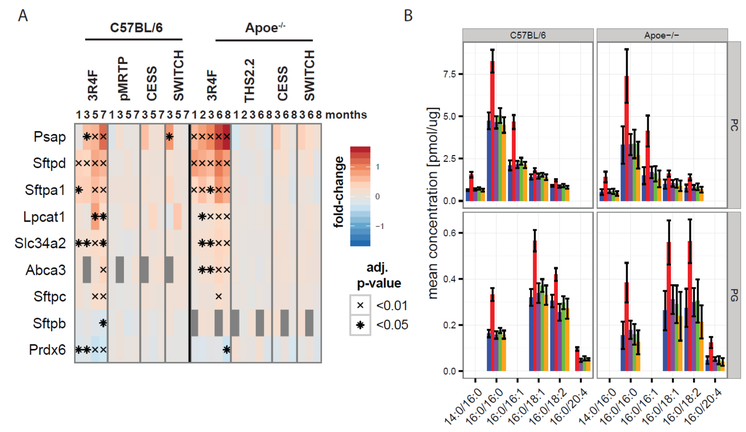

Figure 8. Surfactant protein and surfactant lipid responses in C57BL/6 and *Apoe*$^{-/-}$ mice. CS exposure strongly affected both protein (A) and lipid (B) components of surfactant, while pMRTP and cMRTP exposure did not induce such changes and the cessation and switching groups rapidly returned to sham levels of these proteins and lipids.

CONCLUSIONS

- Quantitative proteomics of lung tissue captured the important biological process that are associated with smoking-induced diseases such as xenobiotic response, oxidative stress, immune-response and metabolic alterations.
- Generated proteomics datasests showed reproducibility and robustness between the 2 mice strains in the 2 different studies.
- Exposure to 3R4F smoke induced a strong effect in a time-dependent manner with significant changes in differential protein expressions in both the ApoE^{-}/- and C57BL/6 mice, where switching to and continuous exposure to both cMRTP and pMRTP resulted in significant decrease or no change in the differential protein expressions of , reaching similar levels of cessation in both the ApoE^{-}/- and C57BL/6 mice.
- Integration of proteomics with other data modalities such as lipidomics showed its success in verifying the surfactant-related protein and lipid response (**Figure 8)**.
- The integration of proteomics datasets with transcriptomics, lipidomics and histopathological datasets proved to be valuable in the systems toxicology approach to perform product assessment of MRTPs and reduced risk products (RRPs).

REFERENCES

- Phillips, B. et al. **An 8-Month Systems Toxicology Inhalation/Cessation Study in Apoe-/- Mice to Investigate Cardiovascular and Respiratory Exposure Effects of a Candidate Modified Risk Tobacco Product, THS 2.2, Compared with Conventional Cigarettes**. Toxicological sciences : an official journal of the Society of Toxicology, doi:10.1093/toxsci/kfv243 (2015).
- Titz, B. et al. **Effects of cigarette smoke, cessation and switching to two heat-not-burn tobacco products on lung lipid metabolism in C57BL/6 and Apoe-/- mice - an integrative systems toxicology analysis.** Toxicological sciences : an official journal of the Society of Toxicology, doi:10.1093/toxsci/kfv244 (2015).
- Phillips, B. et al. **A 7-month cigarette smoke inhalation study in C57BL/6 mice demonstrates reduced lung inflammation and emphysema following smoking cessation or aerosol exposure from a prototypic modified risk tobacco product.** Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association 80, 328-345, doi:10.1016/j.fct.2015.03.009 (2015).
- Titz, B. et al. **Proteomics for systems toxicology.** Computational and structural biotechnology journal 11, 73-90, doi:10.1016/j.csbj.2014.08.004 (2014).
- Fang, H. et al. **The 'dnet' approach promotes emerging research on cancer patient survival.** Genome medicine 6, 64, doi:10.1186/s13073-014-0064-8 (2014).
- Hoeng, J. et al. **A network-based approach to quantifying the impact of biologically active substances.** Drug discovery today 17, 413-418, doi:10.1016/j.drudis.2011.11.008 (2012).
- Hoeng, J. et al. **Case study: the role of mechanistic network models in systems toxicology.** Drug discovery today 19, 183-192, doi:10.1016/j.drudis.2013.07.023 (2013).

PMI RESEARCH & DEVELOPMENT

Systems Toxicology 2016- Real World Applications and Opportunities.
Las Diablerets, Switzerland
January 27-29, 2016.

Philip Morris International Research & Development, Quai Jeanrenaud 5, 2000 Neuchâtel, Switzerland
T: +41 58 242 21 13, **F**: +41 58 242 28 11, **W**: www.pmi.com