Integration of Targeted Proteomics into Systems Toxicology Approach: Candidate Modified-Risk Tobacco Product Assessment using Nasal Cultures

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Introduction and Objectives

Systems biology combines comprehensive molecular analyses and quantitative modeling to understand the characteristics of a biological system as a whole. Leveraging a similar approach, Systems Toxicology aims to decipher complex biological responses following exposures.

This work reports parts of a Systems Toxicology analysis in the context of the *in vitro* assessment of a candidate modified-risk tobacco product (cMRTP) using a human nasal organotypic culture model[1,2]. The term "modified risk tobacco product" means any tobacco product that is sold or distributed for use to reduce harm or risk of tobacco-related diseases associated with commercially marketed tobacco products"[3].

The objectives of this study included to demonstrate (1) robustness and reproducibility of systems biology data obtained from an organotypic *in vitro* nasal culture model for cigarette smoke (CS) and aerosol exposure assessment; (2) relevancy of this approach in a comparative risk assessment of aerosols from a cMRTP compared with CS; and (3) the benefit of including complementary data modalities such as targeted proteomics in this assessment approach.

Complementing a series of functional measures, a causal network enrichment analysis of transcriptomic data was used to compare quantitatively the biological impact of aerosol from the Tobacco Heating System (THS) 2.2, a candidate MRTP, with 3R4F CS at similar nicotine concentrations. In addition, the tissue response was measured by a mass-spectrometry based targeted proteomics approach, parallel reaction monitoring (PRM), and antibody-based multi-analyte profiling (MAP).

Methods

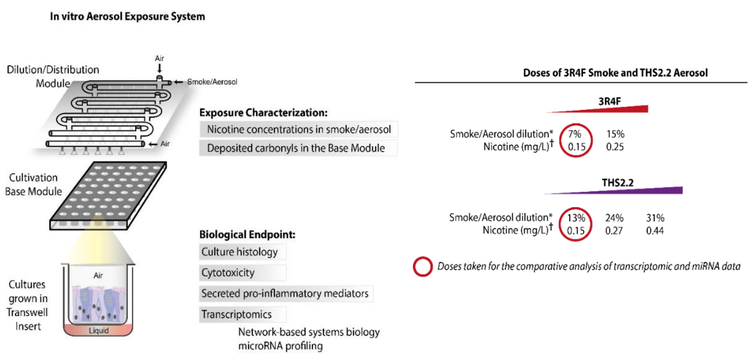

Figure 1. Study overview
*An organotypic nasal culture model was exposed (acute, for 28 min) to 3R4F CS or THS2.2 aerosol at similar nicotine concentrations in an Exposure System (Vitrocell 24/48®). * Dilution refers to the percent 3R4F smoke or THS2.2 aerosol diluted with air in the Dilution/Distribution Module of the Exposure System. † Nicotine concentration (mg/L) refers the corresponding concentration to the specific dilution of smoke/aerosol determined by trapping the diluted smoke/aerosol in the EXtrelut® 3NT column. The nasal organotypic cultures were reconstituted from the primary nasal epithelial cells of 30 year-old non-smoker male. Figure adapted from Iskandar et al. (2017)[1].*

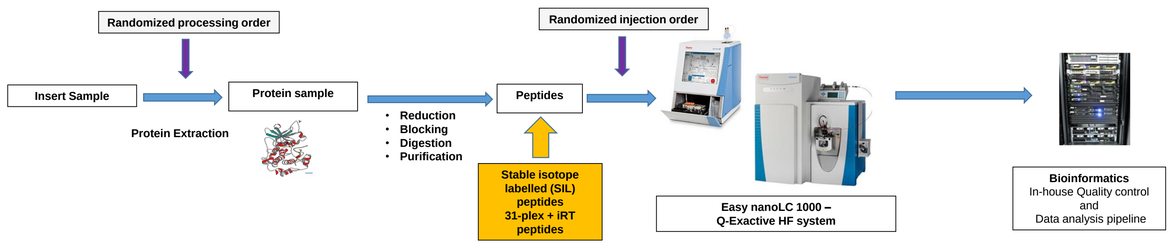

Figure 2. Schematic overview of the parallel reaction monitoring (PRM) workflow. *Absolute quantification of target proteins was achieved by spiking stable isotope labelled (SIL) peptides in known concentration as reference standard. For analysis quality-control and retention time scheduling indexed retention time (iRT)-kit was used[4].*

Cytotoxicity

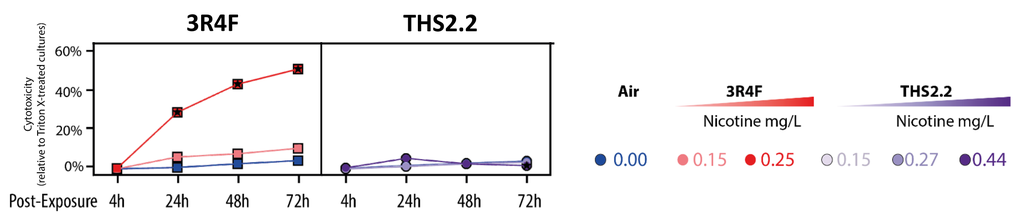

Figure 3. Assessment of cytotoxicity. *Cytotoxicity levels following exposure were measured based on the levels of adenylate kinase activity in the basolateral media (adenylate kinase release assay) at various post-exposure time points in the nasal cultures.*

Transcriptomics and Network-Based Analysis of the Exposure Impact

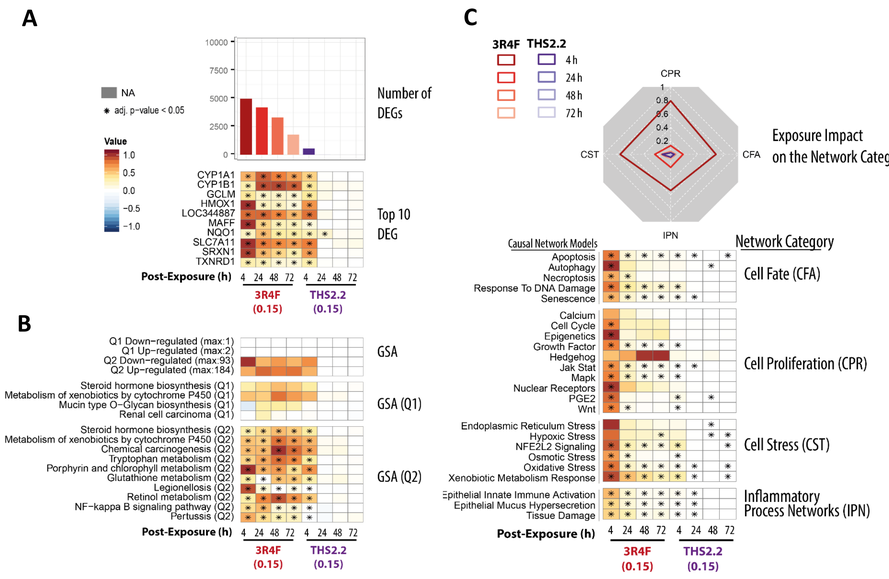

Figure 4: Mechanistic investigation of the exposure impact based on the transcriptomic data
(A) Barplots showing the number of significantly differentially expressed genes (DEGs) across the exposure conditions (FDR-adjusted p-value < 0.05). The heatmaps indicate the expression profiles of the top ten genes (sorted first by the number of significant conditions and then by the mean of the absolute fold-changes). The log2(fold-changes) compared with the respective air control groups are color-coded and the statistical significance level is indicated (FDR-adjusted p-value). (B) Gene set analysis (GSA) was performed with the KEGG gene-set collection using absolute log2(fold-changes) as the gene-level and the mean as the gene-set level statistics. Significance with respect to the treatment effect (Q2, compared with the air control) and dominant effects of individual gene sets (Q1) was assessed with Benjamini-Hochberg based FDR adjustment (FDR adj. p-value < 0.05). The numbers of significantly up- and down regulated gene sets for Q1 and Q2 are shown in the top panel, and the top gene sets, first sorted by the number of significant conditions and then by their average absolute scores, are shown in the bottom panels. (C) The causal network enrichment approach for the analysis of the transcriptomic datasets. For each network category, the relative biological impact factor is shown in radar plots (CFA, Cell Fate; CPR, Cell Proliferation; CST, Cell Stress; IPN, Inflammatory Process Networks). These causal networks describe relevant physiologic cell responses[5]and have undergone a crowd-sourced review[6]. The heatmaps show the network perturbation amplitudes for each network in the collection, across all conditions. Figure adapted from Iskandar et al. (2017)[1].

Transcriptomics - Cellular Stress Response

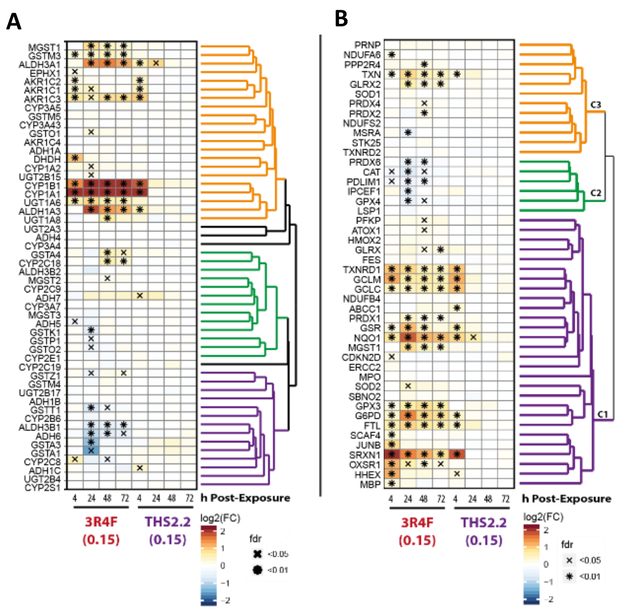

Figure 5: Induction of xenobiotic metabolism and oxidative stress responses in the nasal organotypic epithelium cultures.
(A) Clustered gene expression matrix for genes of a xenobiotic metabolism gene set (KEGG collection, Metabolism of Xenobiotics by Cytochrome P450). The log2 fold-changes compared with the air control are color-coded and FDR-adjusted significance is indicated for the FDR-adjusted p-value < 0.05 (x) and < 0.01 (*) levels. Gene clustering based on the pair-wise correlation between the fold-changes and the clustering results are shown as a dendrogram (clusters are marked in different colors). (B) As in B, but for the oxidative stress response gene set (Reactive Oxygen Species Pathway of the hallmark collection of mSigDB). Figure adapted from Iskandar et al. (2017)[1].

Targeted Proteomics – Cellular Stress and Pro-Inflammatory Responses

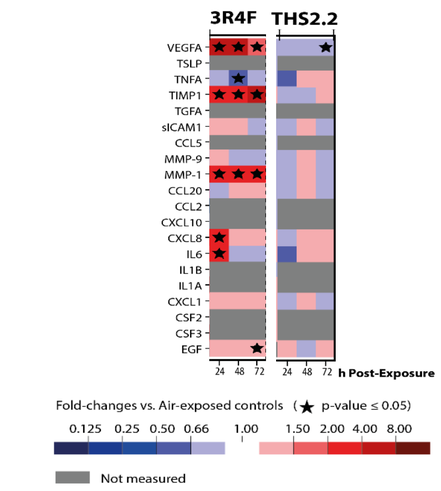

Figure 6. Exposure-induced pro-inflammatory responses.
Multianalyte profiling (MAP) data for secreted pro-inflammatory mediators measured at various post-exposure time points. Figure adapted from Iskandar et al. (2017)[1].

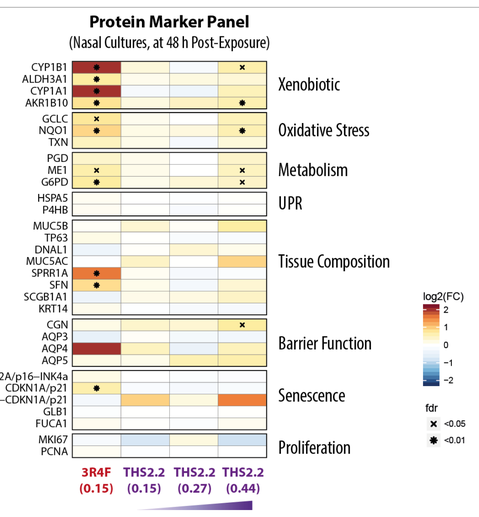

Figure 7. Alterations of proteins in the nasal organotypic cultures following exposure measured by parallel-reaction monitoring (PRM).
The log2(fold-changes) compared with the air control are color-coded and the FDR-adjusted p-values are indicated (UPR, unfolded protein response). Figure from Iskandar et al. (2017)[1].

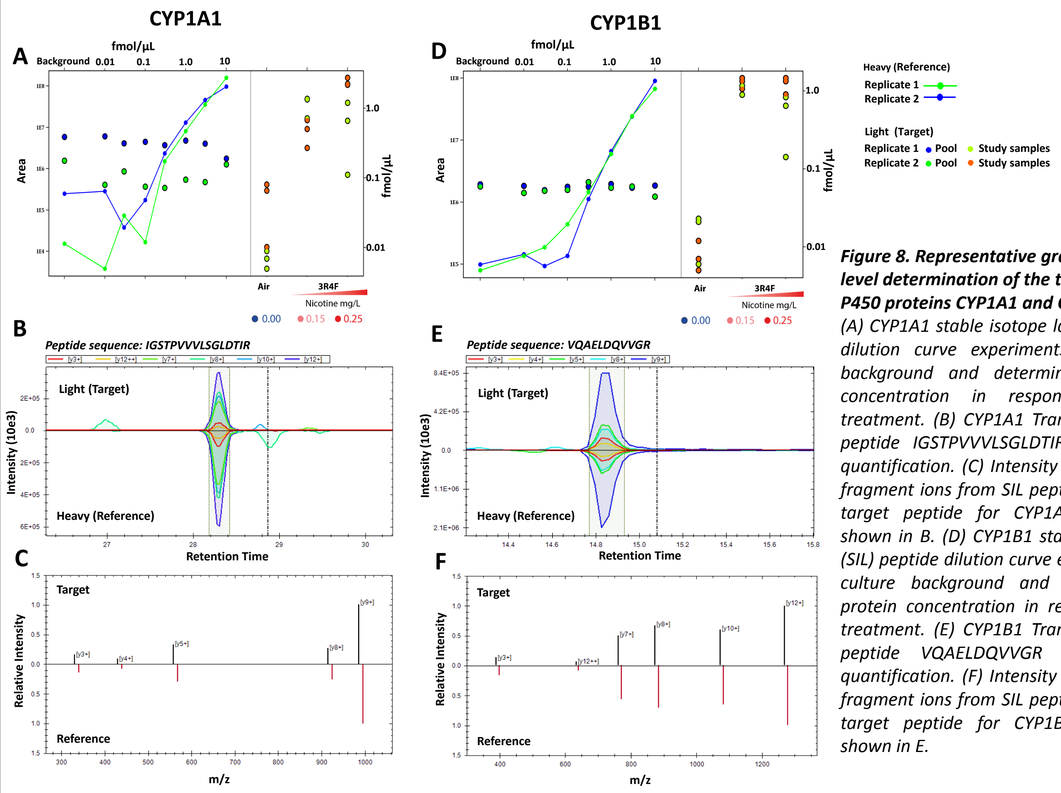

Figure 8. Representative graphs for expression level determination of the two cytochrome P450 proteins CYP1A1 and CYP1B1.
(A) CYP1A1 stable isotope labelled (SIL) peptide dilution curve experiments in nasal culture background and determined target protein concentration in response to 3R4F CS treatment. (B) CYP1A1 Transition ion-traces of peptide IGSTPVVVLSGLDTIR used for protein quantification. (C) Intensity correlation chart of fragment ions from SIL peptide (Reference) and target peptide for CYP1A1 specific peptide shown in B. (D) CYP1B1 stable isotope labelled (SIL) peptide dilution curve experiments in nasal culture background and determined target protein concentration in response to 3R4F CS treatment. (E) CYP1B1 Transition ion-traces of peptide VQAELDQVVGR used for protein quantification. (F) Intensity correlation chart of fragment ions from SIL peptide (Reference) and target peptide for CYP1B1 specific peptide shown in E.

Conclusions

- Lower cytotoxicity was observed in the nasal organotypic cultures following exposure to THS2.2 aerosol compared with 3R4F CS.
- The causal network enrichment approach demonstrated a broad mechanistic impact of 3R4F CS in the nasal organotypic culture model including xenobiotic, oxidative stress, and inflammatory responses. At comparable nicotine concentrations, THS2.2 aerosol elicited reduced and more transient effects on these processes than CS.
- Demonstrated applicability of the Systems Toxicology approach to quantify and compare the effects of 3R4F CS and THS 2.2 aerosol exposure on the level of pertinent biological mechanisms, including cellular stress and pro-inflammatory responses.
- Developed a 31-plex protein targeted panel for PRM relevant to biological processes perturbed by 3R4F CS exposure such as Xenobiotic and oxidative stress response, metabolism, unfolded protein response, barrier function, senescence and cell proliferation.
- Exemplified how targeted proteomics using PRM and antibody-based multi-analyte-profiling can strengthen the conclusions from other endpoints: The targeted marker panels further confirmed the reduced cellular stress responses elicited by THS2.2 aerosol compared with 3R4F CS in the nasal organotypic culture model.

References
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September 17-20, 2017

Competing Financial Interest

All authors are employees of Philip Morris International (PMI) or worked for PMI under contractual agreements. PMI is the sole source of funding and sponsor of this project.