Systems Toxicology meta-analysis: Impact of a candidate Modified-Risk Tobacco Product aerosol compared with cigarette smoke on organotypic aerodigestive tract cultures

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Introduction & Overview

Systems biology combines comprehensive molecular analyses with apical endpoints 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 a Systems Toxicology meta-analysis[1] in the context of the *in vitro* assessment of a candidate modified-risk tobacco product (MRTP) using three human organotypic cultures of the aerodigestive tract (buccal[4], bronchial[3], and nasal[2] epithelia). 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"[5].

The objectives included to demonstrate (1) consistency, robustness and reproducibility of systems biology data obtained from organotypic in vitro cultures related to tobacco-smoke exposed tissues; (2) suitability of this approach to demonstrate reduced toxicological impact in the comparative risk assessment of aerosols from MRTPs compared with cigarette smoke; and (3) the benefit of including complementary data modalities such as target 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 cigarette smoke (CS) at similar nicotine concentrations. For nasal cultures, the tissue response was measured by a targeted proteomics approach.

Lower cytotoxicity was observed in all cultures following exposure to THS2.2 aerosol compared with 3R4F CS. Because of their morphological differences, a lesser exposure impact was observed in the buccal (stratified epithelium) compared with the bronchial and nasal (pseudostratified epithelium) organotypic cultures. The causal network enrichment approach supported a similar mechanistic impact of CS across the three cultures, including the impact on xenobiotic, oxidative stress, and inflammatory responses. At comparable nicotine concentrations, THS2.2 aerosol elicited reduced and more transient effects on these processes than CS. A targeted mass-spectrometry marker panel further confirmed the reduced cellular stress responses elicited by THS2.2 aerosol compared with 3R4F CS in the nasal culture.

Transcriptomics and Network-Based Analysis of the Exposure Impact

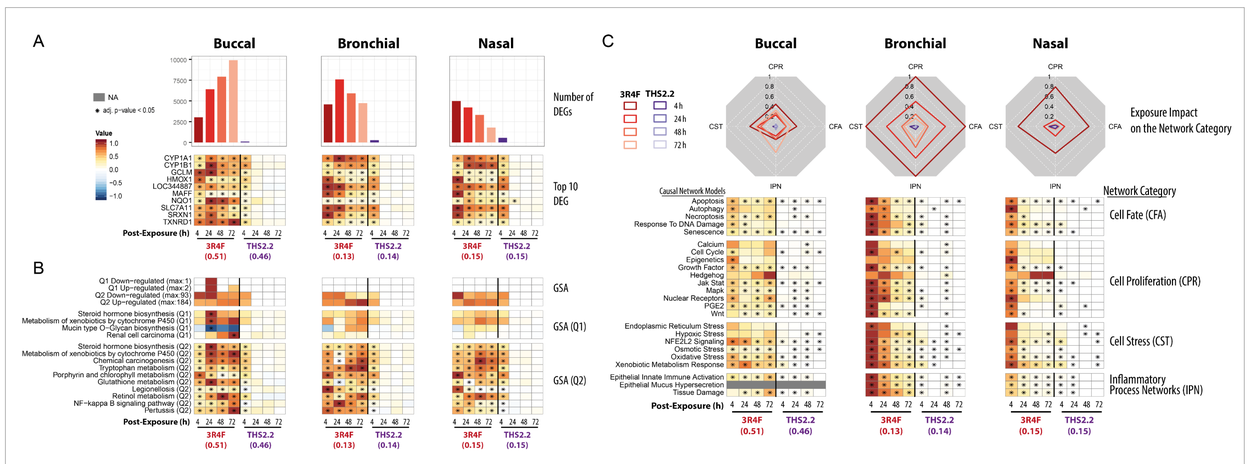

Figure 3: Mechanistic investigation of the exposure impact based on the transcriptomic data

(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[6] and have undergone a crowd-sourced review[7]. The heatmaps show the network perturbation amplitudes for each network in the collection, across all conditions. Figure from Iskandar et al. (2017)[1].

Study Design and Endpoints

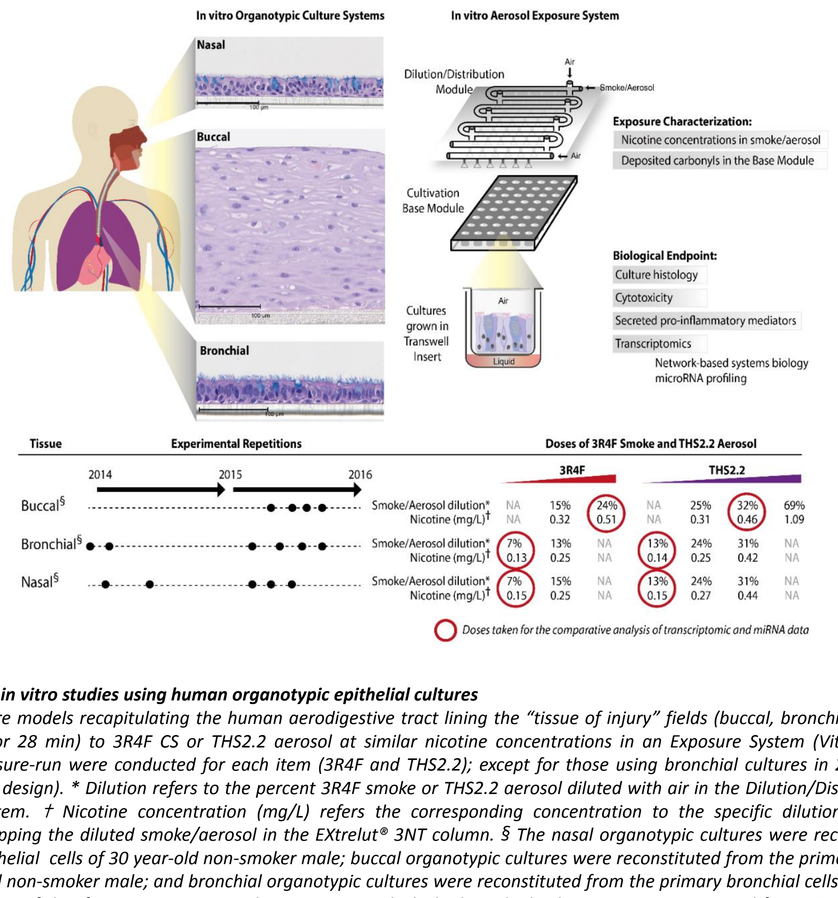

Figure 1. Series of in vitro studies using human organotypic epithelial cultures

*Organotypic culture models recapitulating the human aerodigestive tract lining the "tissue of injury" fields (buccal, bronchial, and nasal) were exposed (acute, for 28 min) to 3R4F CS or THS2.2 aerosol at similar nicotine concentrations in an Exposure System (Vitrocell 24/48®). • 3 independent exposure-run were conducted for each item (3R4F and THS2.2); except for those using bronchial cultures in 2014 (which used a different exposure design). * 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; buccal organotypic cultures were reconstituted from the primary buccal epithelial cells of 46 year-old non-smoker male; and bronchial organotypic cultures were reconstituted from the primary bronchial cells of 28 year-old non-smoker male (except of the first two experimental repetitions in which the bronchial cultures were reconstituted from 23 year-old non-smoker male). NA: not available. Figure from Iskandar et al. (2017)[1].*

Cellular Stress and Pro-Inflammatory Responses

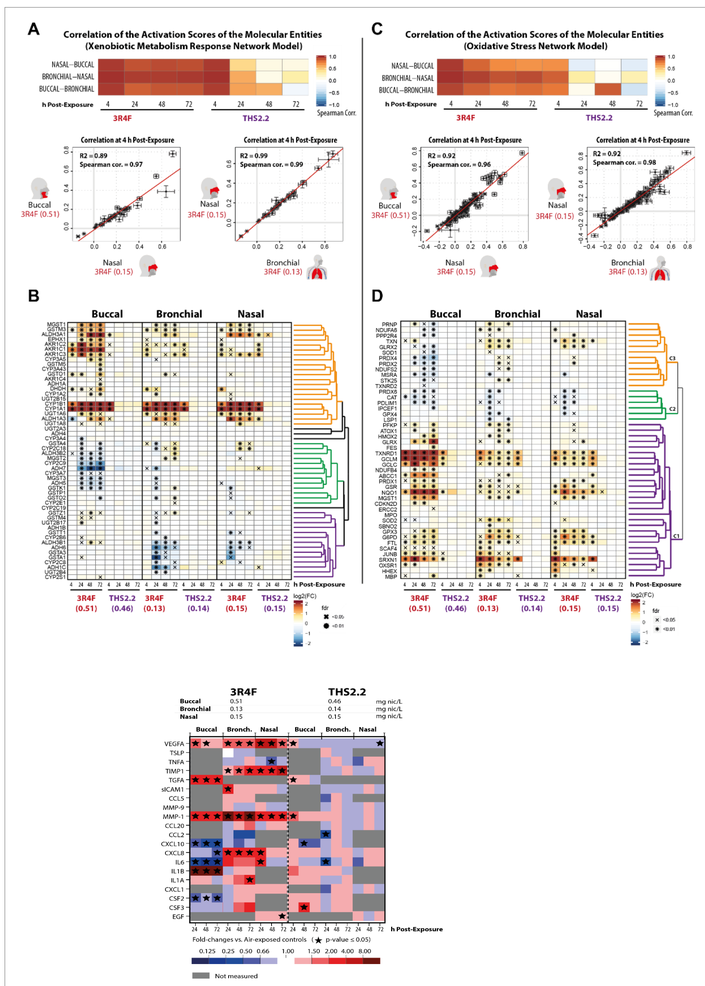

Figure 4: Induction of xenobiotic metabolism and oxidative stress responses across the three organotypic epithelium cultures.

(A) Correlation of the perturbation scores of the molecular entities in the Xenobiotic Metabolism Response network across the three organotypic cultures. The color of the heatmap indicates the Spearman correlation coefficients. Scatter plots for each culture pair show the correlation of the activation values of the molecular entities in the Xenobiotic Metabolism Response network. (B) Clustered gene expression matrix for genes of a xenobiotic metabolism gene set (KEGG collection42, 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). (C) As in A, but for the Oxidative Stress network. (D) As in B, but for the oxidative stress response gene set (Reactive Oxygen Species Pathway of the hallmark collection of mSigDB). Figure from Iskandar et al. (2017)[1].

Aerosol Characterization, Cytotoxicity, and Cilia Beating

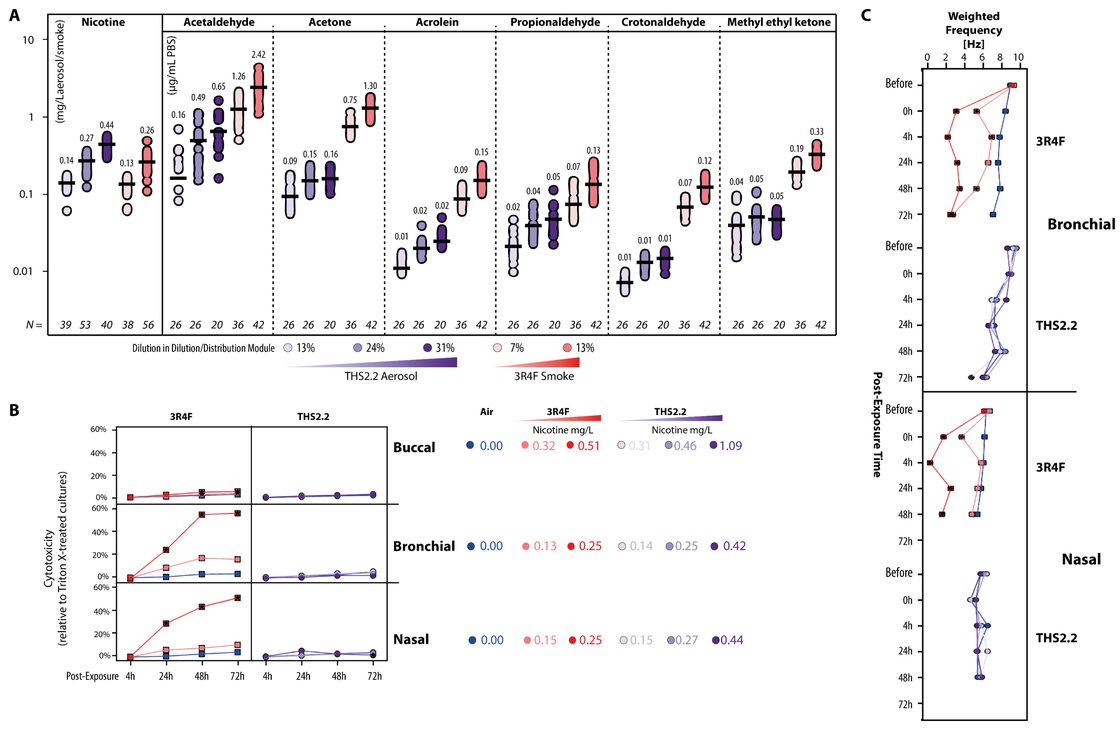

Figure 2. Characterization of the CS/THS2.2 aerosol in the exposure system and assessment of cytotoxicity and ciliary beating

(A) Concentrations of nicotine in the diluted 3R4F CS and THS2.2 aerosol (mg nicotine/L) were measured by trapping the diluted smoke/aerosol in EXtrelut® columns and detection by gas chromatography-flame ionization (samplings were done throughout the study period, in N replicates). In addition, concentrations of deposited carbonyls in the PBS-filled Cultivation Base Module of the Exposure System were determined. (B) Cytotoxicity levels following exposure were measured based on the levels of adenylate kinase activity in the basolateral media (adenylate kinase released assay) at various post-exposure time points in the buccal, bronchial, and nasal cultures. (C) Ciliary beating functionality of the ciliated pseudostratified epithelium (bronchial and nasal) cultures (the buccal epithelium is non-ciliated) was assessed longitudinally before, immediately after (0 h) and 4 h, 24 h, and 48 h after exposure (as well as 72 h for the bronchial culture only). Weighted frequency (Hz) is the mean frequency over the pixel, weighted by the FFT power at the pixel dominant frequency. Figure adapted from Iskandar et al. (2017)[1].

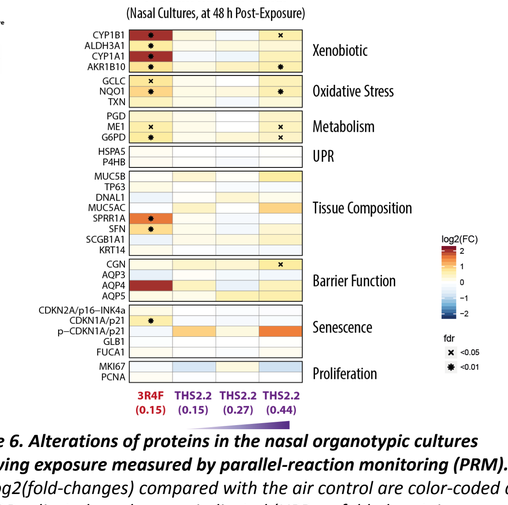

Figure 6. Alterations of proteins in the nasal organotypic cultures following exposure measured by parallel-reaction monitoring (PRM).

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 5. Exposure-induced pro-inflammatory responses across the buccal, bronchial, and nasal cultures.

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

Conclusions

- Meta-analysis included functional measurements (cytotoxicity, ciliary beating functionality, and secreted pro-inflammatory mediator profiles) and advanced computational approaches leveraging gene set analyses and causal network enrichment to comprehensively assess the biological impact of 3R4F CS and THS2.2 aerosol exposures on *in vitro* human organotypic buccal, bronchial, and nasal cultures.
- Demonstrated applicability of the Systems Toxicology approach to quantify and compare the effects of CS and THS2.2 aerosol exposure at the level of pertinent biological mechanisms, including cellular stress and pro-inflammatory responses, across three organotypic culture models.
- Demonstrated that the 21st century toxicology approach may further corroborate the robustness and reliability of organotypic in vitro models with respect to the "3Rs": to reduce, refine, and/or replace animal testing.
- Exemplified how targeted proteomics can strengthen the conclusions from other endpoints, including transcriptomics.
- Showed consistently across all three *in vitro* models—buccal, bronchial, and nasal—that THS2.2 aerosol exposure had a considerably reduced and more transient biological impact on these *in vitro* models compared with equivalent nicotine concentration exposures to 3R4F CS.

References

[1] Iskandar et al. (2017), Systems Toxicology Meta-Analysis of In Vitro Assessment Studies: Biological Impact of a Candidate Modified-Risk Tobacco Product Aerosol Compared with Cigarette Smoke on Human Organotypic Cultures of the Aerodigestive Tract, *Toxicol. Res.*, Accepted, doi: 10.1039/C7TX00047B

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Competing Financial Interest

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