Molecular changes in mice following 3 weeks of inhalation exposure to e-vapor aerosols in comparison with cigarette smoke

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Objectives

The objective of this systems toxicology study was to compare the acute molecular effects in the lung following 3-week exposure to aerosols generated from electronic vapor (e-vapor) products in comparison with cigarette smoke (CS) from the reference cigarette 3R4F [1].

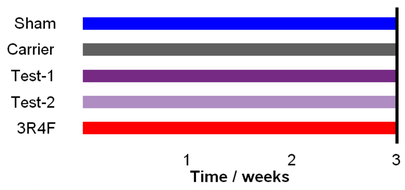

Figure 1: Exposure groups

The e-vapor formulations contained aerosol formers (propylene glycol, glycerol), water and 4% nicotine (Carrier fluid) and two different flavor mixtures (Test-1, Test-2) (**Figure 1**). As control, mice were exposed to filtered air (Sham). For comparison, mice were exposed to the Carrier aerosols and to 3R4F CS, matched to the test product aerosols by nicotine concentration at the nose ports (~41 µg/L).

Here, we report on the molecular effects of these exposures on the lung, as measured by transcriptomic and proteomic analyses. In addition, release of inflammatory mediators into the bronchoalveolar lavage fluid (BALF) was assessed by multi-analyte profiling (MAP) using Luminex® technology.

Methods

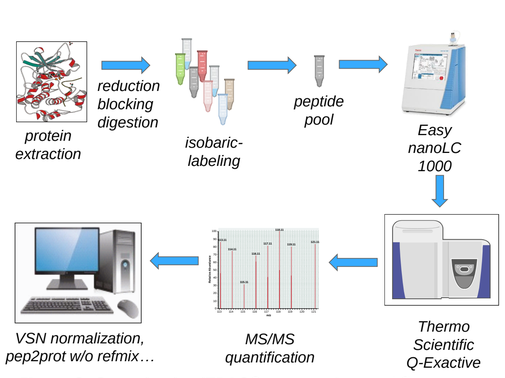

Figure 2: Quantitative iTRAQ® proteomics workflow.

C57BL/6 mice were exposed via nose-only inhalation for up to 4 hours/day, 5 days/week for 3 weeks. Aerosols were generated from e-vapor products using CORESTA reference method number 81 puffing regimen (3-sec puffing; 55 mL/puff, 2 puffs/min) and from 3R4F using modified Canadian intense puffing regimen (2-sec puffing; 55 mL/puff, 2 puffs/min). Aerosol concentrations for 3R4F and e-vapor exposures were set to match the nicotine concentration at the nose ports (~41 µg/L) [1].

Perfused left lungs were collected and cryosliced for the transcriptomic (Affymetrix microarrays) and proteomic (iTRAQ®-based quantification [2], **Figure 2**) analyses (N=8). Bronchoalveolar lavage fluid (BALF) was analyzed for inflammatory mediators by multi-analyte profiling (MAP) using Luminex® technology (N=15).

Results – Transcriptome

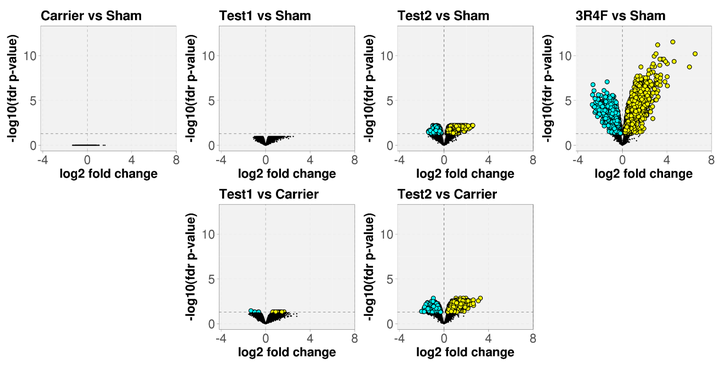

Figure 3: Volcano plots of the mRNA response profiles in lung tissue.
For each gene, the gene expression change, calculated as the log2 fold change, is plotted on the x-axis and the statistical significance (fdr<0.05), proportional to the negative log10-adjusted p-value, is plotted on the y-axis. Yellow and cyan dots highlight genes that are statistically significantly up- and down-regulated, respectively.

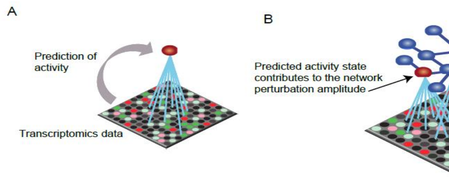

Figure 4: Network perturbation amplitude analysis (NPA).
(A) Analogous to gene set enrichment, genes are grouped under their common regulators. When the levels of the corresponding mRNAs are changed in the sample, the activity of the common regulator can be inferred [3,4]. (B) When modeled under a given biological process with causal connections, these inferred molecular activities determine whether the biological network is perturbed as a whole. The network models are available for view in http://www.causalbionet.com/ [5]. The figure is from Talikka et al. (submitted to Clinical Medicine Insights Circulatory Respiratory and Pulmonary Medicine).

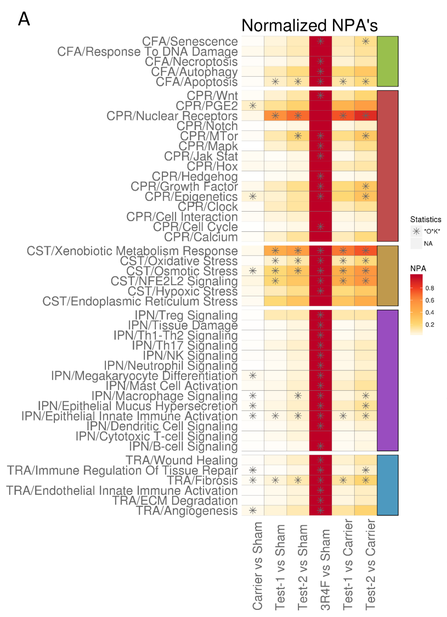

Figure 5: Network perturbation amplitudes and biological impact factor for lung tissue.
(A) Heatmap of NPA scores. A network is considered as perturbed if, in addition to the significance of the NPA score with respect to the experimental variation, the two companion statistics (O and K) derived to inform on the specificity of the NPA score with respect to the biology described in the network, are significant ($p < 0.05$). (B) Relative biological impact factor. The percentages give the relative biological impact which is derived from the cumulated network perturbations caused by the treatment relative to the reference (defined as the treatment comparison showing the highest perturbation). For each treatment comparison, the value (-1 to 1) indicates how similar the underlying network perturbations are with respect to the reference (i.e. REF). A value of 1 indicates that all the networks are perturbed by the same mechanisms. Pie charts indicate contributions of different network families: CST, Cell Stress; IPN, Inflammatory Process network; CPR, Cell Proliferation; TRA, Tissue Repair and Angiogenesis; CFA, Cell Fate.

Results – Proteome

Figure 8: Volcano plots of the protein response profiles in lung tissue.
For each protein, the protein expression change, calculated as the log2 fold change, is plotted on the x-axis and the statistical significance (fdr<0.05), proportional to the negative log10-adjusted p-value, is plotted on the y-axis. Yellow and cyan dots highlight proteins that are statistically significantly up- and down-regulated, respectively.

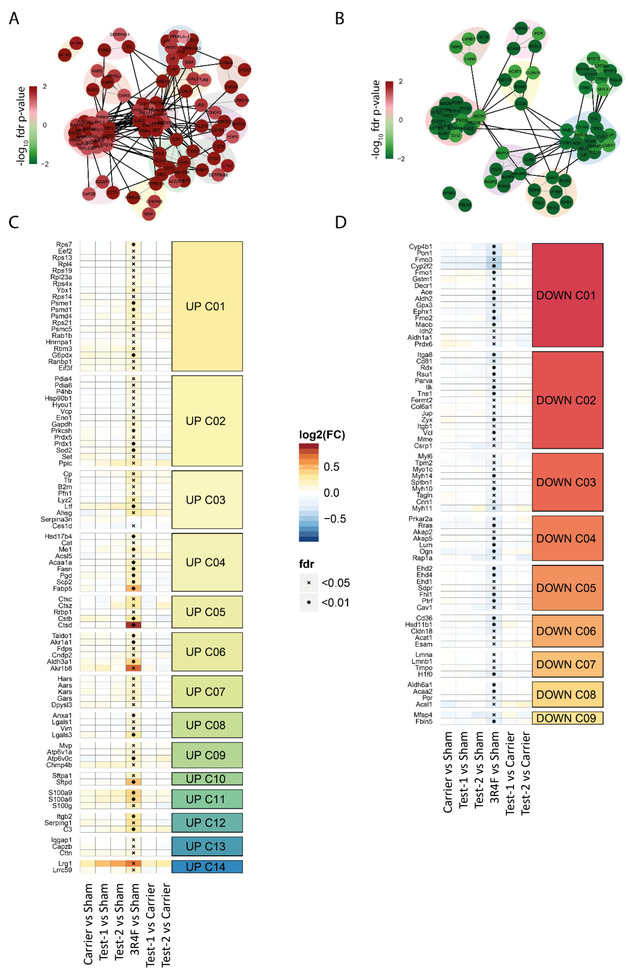

Figure 9: Protein expression response clusters in lung tissue.
Functional association network analysis was conducted and association clusters for the significantly up- (A) or down- (B) regulated proteins were identified (functional associations obtained from the STRING database (Szklarczyk et al., 2015)). Nodes in the networks represent proteins (red, upregulated; green, downregulated), edges in the network represent functional associations, and identified clusters are marked. Heatmaps show the expression response profiles for the proteins in the upregulated (C) and downregulated (D) clusters. The log2 fold-changes are color-coded (see key) and statistically significant differential expression is marked (fdr adjusted p-value < 0.05 ("x") or < 0.01 ("")). The panels on the right side demark the clusters. The identified clusters can be broadly assigned to the following biological functions: UP C01, protein translation; UP C02, oxidative stress and unfolded-protein response; UP C04, metabolic adaptations; UP C05, cathepsins; UP C06, xenobiotic metabolism; UP C07, tRNA biosynthesis; UP C10, lung surfactant; UP C11, immune-related; DOWN C01, xenobiotic metabolism; DOWN C02, extracellular matrix interactions.*

Results – BALF

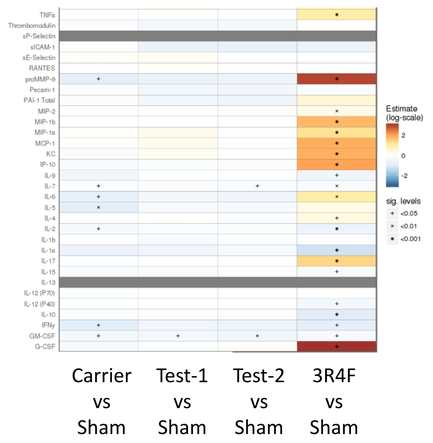

Figure 6: Multi-analyte profiling in bronchoalveolar lavage fluid
The color-scale reflects the magnitude of the estimated differences between groups, while the text symbols (star, cross, bullet) correspond to p-values resulting from testing for significant differences between the groups at the 5%, 1% and less than the 1% significance levels. Colors ranging from beige to red depict increase in endpoints in the treatment groups compared with Sham, while colors ranging from skyblue to deep blue depict decreasing values for the targeted endpoints in the treatment groups compared with Sham. Grey lines correspond to endpoints on which statistical testing could not be executed due to the majority of data points being below detection limits.

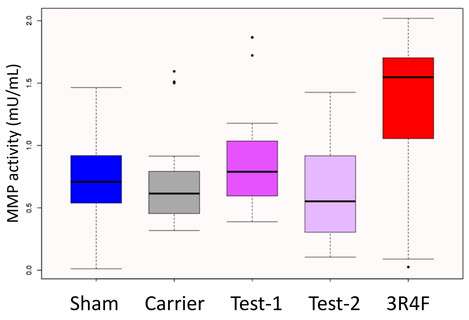

Figure 7: Matrix-Metalloproteinase (MMP) activity boxplots by treatment group.
BALF (first lavage cycle) was analyzed for MMP activity by a gelatinolytic activity assays using fluorescence-labeled gelatin (EnzChek® Gelatinase/Collagenase Assay Kit; Invitrogen, Karlsruhe, Germany). Horizontal lines inside the boxplots indicate medians. Boxes include 50% of the observed data.

Summary & Conclusions

- In the lung tissue of the 3R4F group, more than 8,000 genes were significantly up- or down-regulated compared with Sham (fdr<0.05). Carrier and Test-1 exposures did not result in significant changes in gene expression but Test-2 exposure resulted in more than 2,000 differentially expressed genes compared with Sham exposure (fdr<0.05). The lung proteome showed changes after 3R4F exposure (about 200 proteins were up- or down-regulated) but no significant changes were detected after exposure to Carrier or aerosol from any of the two e-vapor test products (Test-1 or Test-2).
- The magnitude of gene expression-based network perturbations in the e-vapor test groups were >94% less than the 3R4F group. Molecular effects after 3R4F CS exposure included perturbation of cellular stress, inflammation, and tissue repair networks and protein clusters.
- In BALF a number of mediators were significantly upregulated in the 3R4F-exposed group, for example ProMMP9 and G-CSF were highly upregulated compared with Sham ($p<0.05$), while no mediator was upregulated after Test-1 or Test-2 exposure. Protease (MMP) activity of gelatinases and collagenases was slightly higher in 3R4F exposed ($p<0.1$) but showed no difference in e-vapor groups compared with Sham.
- In summary, the molecular impact on the lung tissues after 3 weeks of e-vapor exposures was substantially lower compared with the impact found after 3R4F exposure. Test-2 showed a relatively higher impact on differential gene expression than Test-1, however, higher impact was not confirmed in proteomics or BALF analysis.

References:
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Competing Financial Interest

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