Systems toxicology assessment of potential toxicity of e-vapor aerosols compared with cigarette smoke following 7-month inhalation exposures in C57BL/6 mice

Ulrike Kogel[1], Ashutosh Kumar[2], Marja Talikka[1], Yang Xiang[1], Bjoern Titz[1], Keyur Trivedi[1], Emmanuel Guedj[1], Sam Harbo[3], Kathy M Gideon[3], Nikolai Ivanov[1], K Monica Lee[2], and Julia Hoeng[1]

[1] PMI R&D, Philip Morris Products S.A., Neuchâtel, Switzerland, [2] Altria Client Services LLC, Richmond, VA, USA, [3] Battelle, West Jefferson, OH, USA

Introduction and Objectives

Smoking cessation remains the best approach to minimize the risk of smoking-related respiratory diseases, such as chronic obstructive pulmonary disease. Nonetheless, it is estimated that more than 1 billion people will continue to smoke in the foreseeable future. Nicotine-containing e-cigarettes (e-vapors) are being developed as alternatives to cigarettes to reduce tobacco-related health risk. Various e-vapor products are available; however, the long-term biological effects following exposure to e-vapors alone or after switching from cigarettes have not been studied. In this study, the aerosol from the *MarkTen*® e-vapor product (nicotine 4%) was compared with cigarette smoke (CS) from the 3R4F reference cigarette in a 7-month nose-only inhalation study in C57BL/6 mice. The impact of switching to e-vapor aerosols or cessation after the first 3 months of exposure to CS was compared with that of cessation at months 4 and 7. Here, we report on the gene expression analysis conducted on the nasal epithelium, lung, and liver to gain mechanistic understanding on any changes that are caused by exposure to e-vapor aerosol.

Experimental Design & Methods

Female C57BL/6 mice were exposed to 3R4F CS or e-vapor aerosol by nose-only inhalation for up to 4 hours/day, 5 days/week, for 7 months. Additional groups of mice were included to explore the impact of switching to e-vapor exposure or cessation (filtered air) after the first 3 months of exposure to 3R4F (Figure 1).

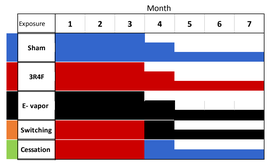

Figure 1. Schematic overview of the study design

For the 3R4F exposure the overall mean gravimetric wet total particulate matter (TPM) target concentration was 550 µg/L, and for the e-vapor exposure the overall mean gravimetric wet TPM target concentration was 1100 µg/L. With this, the nicotine nose-port concentrations of the e-vapor exposure atmosphere were about 30% lower. However, the plasma nicotine and cotinine concentrations were 2- to 3- fold higher in animals exposed to e-vapor aerosol versus those exposed to 3R4F CS[1].

The housing and animal care practices at the testing facility (Battelle, West Jefferson, OH) met the Association for Assessment and Accreditation of Laboratory Animal Care standards and the requirements stated in the Guide for the Care and Use of Laboratory Animals[2] and were approved by the Institutional Animal Care and Use Committee.
Results of a few selected endpoint are tabulated in Figure 2[1].

Exposure		Sham			3R4F smoke			E-Vapor aerosol			Switching		Cessation	
Month of exposure		3	4	7	3	4	7	3	4	7	4	7	4	7
	Body weight in g (mean ± SD) (number of animals)	22.6 ± 1.1 (12)	22.7 ± 1.1 (12)	24.9 ± 1.2 (12)	20.5 ± 1.6 (11)	21 ± 1.4 (12)	21.6 ± 1.7 (11)	22.7 ± 0.8 (12)	22.7 ± 1.5 (12)	23.4 ± 1.1 (12)	22.5 ± 1.0 (12)	23.5 ± 2.0 (12)	22.4 ± 0.8 (12)	23.4 ± 1.2 (10)
NOSE	Histo: Squamous metaplasia, respiratory epithelium (incidence/total animal number (mean severity score))	0/12	0/12	0/12	0/11	8/12 (1.6)	5/11 (1.4)	0/12	0/12	0/12	3/12 (1.0)	0/11	0/12	0/12
LUNG	Histo: Infiltrate cellular, macrophage, alveolus (incidence/total animal number (mean severity score))	0/12	0/12	0/12	11/11 (2.3)	12/12 (2.3)	11/11 (2.8)	0/12	0/12	0/12	12/12 (2.0)	11/11 (1.6)	12/12 (1.8)	12/12 (1.1)
	Organ weight relative to body weight % (mean ± SD) (number of animals)	0.97 ± 0.17 (12)	1.06 ± 0.19 (12)	0.86 ± 0.11 (12)	1.23 ± 0.13 (11)	1.15 ± 0.14 (12)	1.05 ± 0.08 (11)	0.99 ± 0.12 (12)	0.91 ± 0.20 (12)	0.83 ± 0.09 (12)	1.01 ± 0.21 (12)	0.83 ± 0.09 (11)	1.13 ± 0.23 (12)	0.84 ± 0.05 (10)
LIVER	Organ weight relative to body weight % (mean ± SD) (number of animals)	5.07 ± 0.29 (12)	5.00 ± 0.31 (12)	4.84 ± 0.52 (12)	4.77 ±0.45 (11)	4.38 ±0.15 (12)	4.07 ± 0.24 (11)	5.19 ± 0.23 (12)	5.25 ±0.36 (12)	5.17 ±0.66 (12)	5.15 ± 0.34 (12)	5.34 ±1.14 (11)	5.08 ±0.30 (12)	4.85 ± 0.18 (10)

Figure 2. Selected endpoints
The intensity of the color indicates a relative value for each endpoint (0–100%) . For additional endpoints see also Kumar et al., SOT 2019, Baltimore, abstract #1935/ P321"

RNA samples of the respiratory nasal epithelium (RNE), lung and liver were analyzed on whole genome Affymetrix microarrays (GeneChip® Mouse Genome 430 2.0). Systems response profiles were measured as differential gene expression by pairwise comparisons. Using causal biological network models[3-7], differential gene expressions were transformed into differential numeric values for each node of the network. The differential node values were in turn summarized into a quantitative measure of network-level perturbation amplitude (NPA)[8].

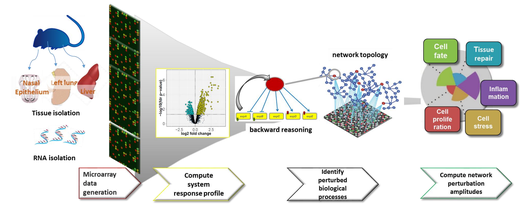

Figure 3. Systems Toxicological sample analysis strategy

Results

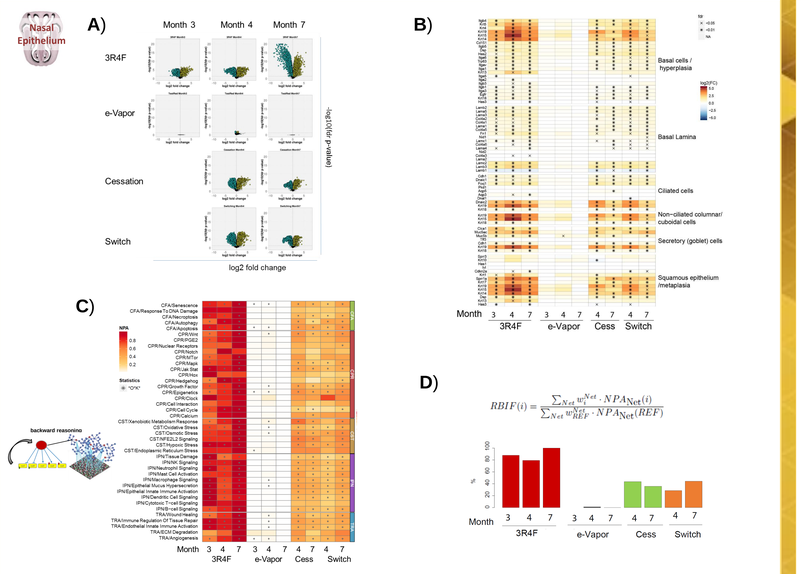

Figure 4. Gene expression analysis of respiratory nasal epithelium (RNE)
A) *Systems Response Profile of the RNE transcriptome.* Each dot represents a gene plotted as its log2(fold-change) against its significance, as indicated in the graph; increased when >0 and decreased when <0. **B)** *Expression profiles for marker panel of epithelium cell types and basal lamina components.* Gene expression fold-changes compared with the respective sham groups. x, fdr p-value < 0.05; *, fdr p-value < 0.01. **C)** *Heatmap of Network Perturbation Amplitude (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) are < 0.05 (marked with *). CFA, Cell fate; CST, Cell stress; CPR, Cell proliferation; IPN, Inflammatory processes network; TRA, Tissue repair and angiogenesis. **D)** *Biological Impact Factor Analysis.* The percentages give the relative biological impact (RBIF), 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). Only the significant networks enter the BIF calculation, no further statistics are applied.

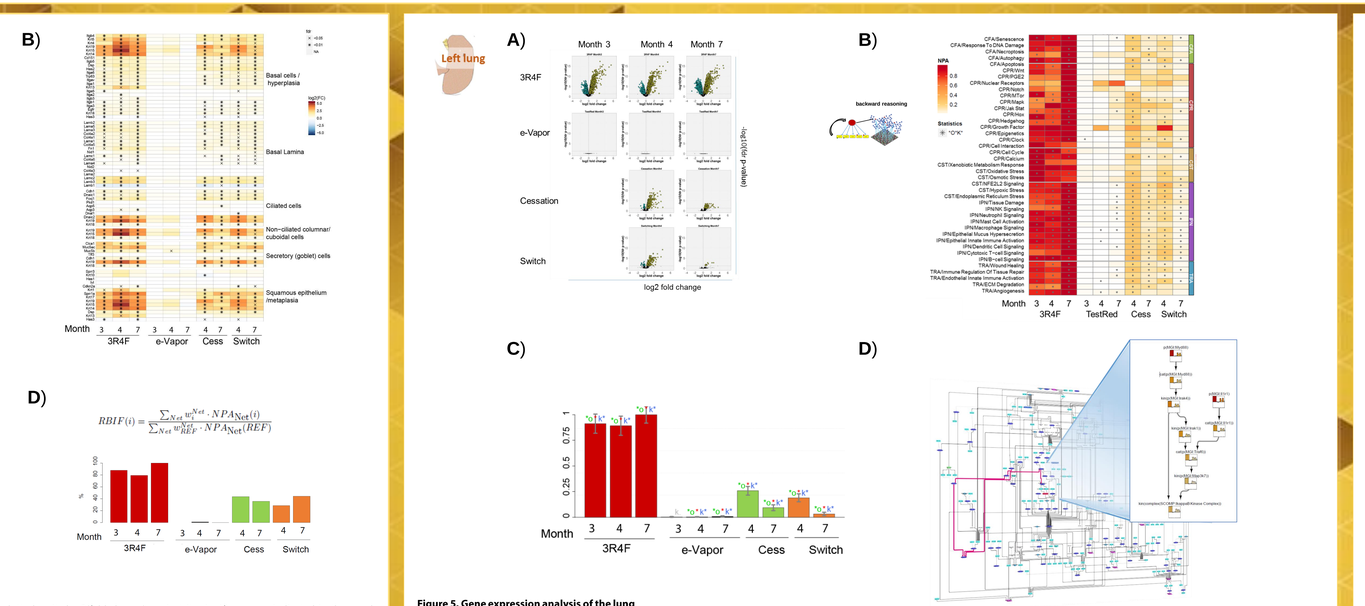

Figure 5. Gene expression analysis of the lung
A) *Systems Response Profile of the lung transcriptome.* Each dot represents a gene plotted as its log2(fold-change) against its significance, as indicated in the graph; increased when >0 and decreased when <0. **B)** *Heatmap of NPA scores.* A network is 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) are significant. *, O and K <0.05. CFA, Cell fate; CST, Cell stress; CPR, Cell proliferation; IPN, Inflammatory processes network; TRA, Tissue repair and angiogenesis. **C)** *Quantitative scores of exposure effects on the macrophage signaling.* Three statistics are shown: the red star indicates statistical significance with respect to the biological replicates, the green star (O statistic) indicates significance with respect to permutation of the downstream genes of the network nodes, and the blue star (K statistic) indicates significance with respect to permutation of the network topology (*, p < 0.05). **D)** *Macrophage signaling network backbone.* The region of the network model that most contributed to the observed impact is shown in the inset and the network perturbation amplitude of each single node are displayed in the small bar chart.

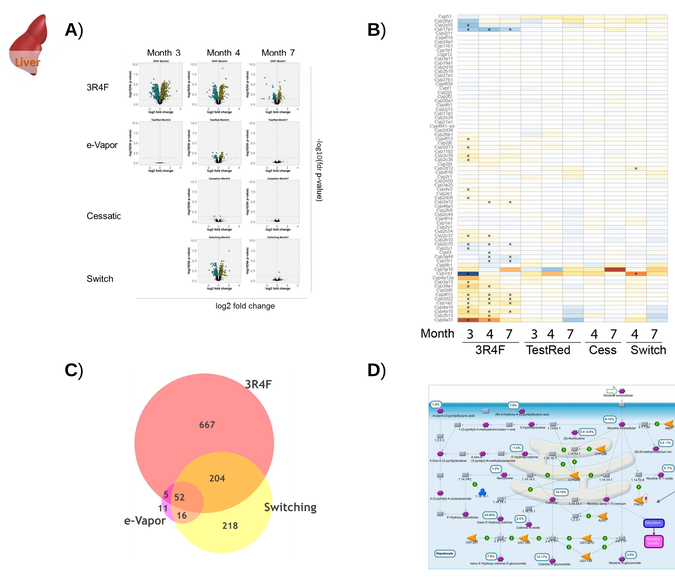

Figure 6. Gene expression analysis of the liver
A) *Systems Response Profile of the liver transcriptome.* Each dot represents a gene plotted as its log2(fold-change) against its significance, as indicated in the graph; increased when >0 and decreased when <0. **B)** Cytochrom P450 (*CYP*) *differential gene expression in liver.* Exposure groups versus sham visualized as a heatmap, x indicate significance for fdr p-value. **C)** *Venn Diagram of differential expressed genes derived from liver samples after 4 month of exposure*. Note the cessation group is not included in the graph. **D)** *Metacore pathway maps analysis.* 4 month data set analysis showed nicotine metabolism in liver as one of the best hits.

Conclusions

While there were many more differential expressed genes in the RNE than in lung and liver of 3R4F CS-exposed mice, very few were detected in the RNE exposed to e-vapor only at 4 months. Exposure to e-vapor triggered also differential gene expression only after 4 months of exposure in the liver. The RNEs from both the cessation and switching groups showed nearly as many differential expressed genes as the RNEs from mice exposed continuously to 3R4F CS at 4 and 7 months, indicating slower recovery from exposure than in the lung. The number of differential expressed genes in the liver of the mice in the switching group was approximately half of that in the group continuously exposed to 3R4F for four months.

While 3R4F CS exposure impacted the biology in the majority of the network models in the RNE and lung, the effect was negligible in response to e-vapor compared with the sham animals at the corresponding time point. The impact in the cessation and switching groups at 4 and 7 month time points was significant but much lower than in mice continuously exposed to 3R4F CS. Aligned with analysis of the differentially expressed genes, the difference in NPA between 3R4F and cessation/switching groups was less in RNE than in the lung.

In line with the histopathological observation, gene expression analysis of the RNE suggests an increase in basal cell and squamous cell markers, strongest in the 3R4F exposure groups, and lung gene expression analysis could likewise confirm involvement of macrophage signalling. In contrast, gene expression analysis of the liver could not capture xenobiotics/nicotine metabolism very well in all exposure groups. Likely, those processes are transient on the gene expression level and cannot be measured when tissue is dissected 24 hours after the last exposure.
In conclusion, the gene expression analysis showed a minimal impact of e-vapor aerosol on RNE, lung, and liver compared with 3R4F CS exposure.

References

1 Lee K.M., et al, (2019) 7-month nose-only inhalation switching study of an e-vapor product and 3R4F reference cigarette aerosol in female C57BL/6 mice; in preparation.
2 [NRC] National Research Council. 2011. Guide for the care and use of laboratory animals. 8th Edition. [accessed 2018 July 4]. https://grants.nih.gov/grants/olaw/guide-for-the-care-and-use-of-laboratory-animals.pdf.
3 Westra, J.W. et al. (2011) Construction of a computable cell proliferation network focused on non-diseased lung cells, BMC Syst Biol., 5: 105.
4 Schlage, W.K. et al. (2011) A computable cellular stress network model for non-diseased pulmonary and cardiovascular tissue, BMC Syst Biol., 5: 168.
5 Westra, J.W. et al. (2013) A modular cell-type focused inflammatory process network model for non-diseased pulmonary tissue, Bioinformatics and biology insights 7:1-26.
6 Gebel, S. et al. (2013) Construction of a computable network model for DNA damage, autophagy, cell death, and senescence, Bioinformatics and Biology Insights 7, 97-117.
7 Park, J.S. et al. (2013) Construction of a computable network model of tissue repair and angiogenesis in the lung. J. Clin. Toxicol., S12, 002.
8 Martin, F. et al. (2014) Quantification of biological network perturbations for mechanistic insight and diagnostics using two-layer causal models, BMC Bioinformatics 15, 238.

Society of Toxicology, Annual Meeting March 10-14, 2019, Baltimore

Competing Financial Interest – The research described in this poster was jointly sponsored by Philip Morris International and Altria Client Services LLC.