Systems Toxicological Assessment of two Modified Risk Tobacco Products on Cardiovascular and Respiratory Effects in ApoE-/- mice - a six-month inhalation study

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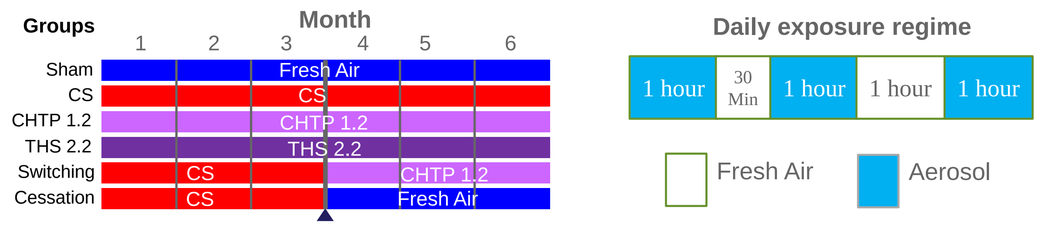
Introduction and Objectives

Cigarette smoking causes adverse health effects that may occur shortly after smoking initiation and lead to the development of cardiovascular disease (CVD), respiratory disease (e.g., chronic obstructive pulmonary disease (COPD)), and various cancers. Philip Morris International is developing Reduced Risk Products (RRP) to which adult smokers can switch and that have the potential to present less risk of harm versus continued smoking because they do not burn tobacco.

In this study, the effects of a six-month exposure to cigarette smoke (CS) or to aerosols from two RRPs, the Carbon Heated Tobacco Product (CHTP) and the Tobacco Heating System (THS), were investigated in ApoE-/- mice.

CHTP 1.2 is a potential modified risk tobacco product (pMRTP) in which the tobacco plug in a specially designed stick is heated to less than 350°C using a carbon heat source. THS 2.2, a candidate MRTP, utilizes an electronically controlled heating system to heat tobacco. The operating temperature in both systems is below the combustion temperature of tobacco, resulting in generation of aerosol with significant reduction in levels of harmful and potentially harmful constituents (HPHC) compared with CS. In a systems toxicological approach combining physiological, histological, and -omics endpoints, respiratory and cardiovascular effects of these two products were assessed and compared with CS in a six-month inhalation study. In addition, the impact of cessation or switching to CHTP aerosol exposure after three months of CS exposure was evaluated.

Study Design and Endpoints

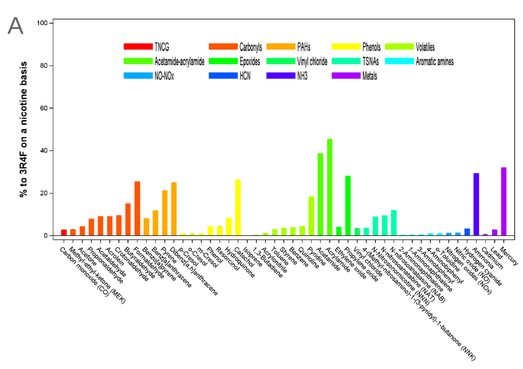


Female ApoE$^{-/-}$ mice were exposed to mainstream smoke (CS) from the 3R4F reference cigarette (600 mg/m3 total particulate matter (TPM)), aerosols from CHTP 1.2 or THS 2.2 (matched to the nicotine in 3R4F – 28 µg/l), or filtered air for three hours per day, five days per week, for up to six months. After three months of 3R4F exposure, switching and cessation groups were exposed to CHTP 1.2 aerosol or filtered air, respectively. Animals were observed on a daily basis, body weight progression was monitored twice per week, and exposure and uptake parameters (including nicotine metabolites in urine) were measured three times during the study. Dissections were performed after two, three, four, and six 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 and morphometry of lungs, lung function, plaque surface area determination, and an extensive molecular high-throughput analysis (transcriptomics, proteomics, and lipidomics).

Results – Exposure and Uptake

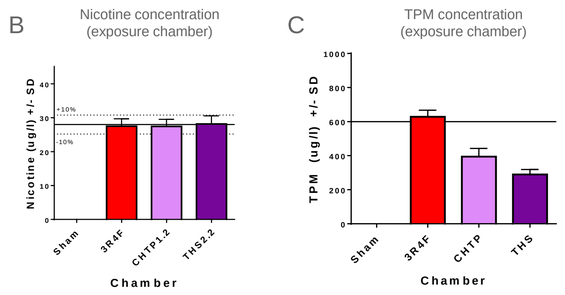
HPHC yield in CHTP 1.2 relative to 3R4F mainstream smoke

The controlled tobacco-heating approach of CHTP 1.2 reduces the delivery of HPHCs as compared with conventional burning tobacco products (**A**), as shown for 49 priority toxicants.



Aerosol concentrations in the exposure chambers

Aerosol concentrations in the exposure chambers (breathing zone) showed that the nicotine concentration approached the target concentration of 28 µg/l (**B**). This corresponds to approximately 600 µg/l TPM in the CS exposure group (**C**).

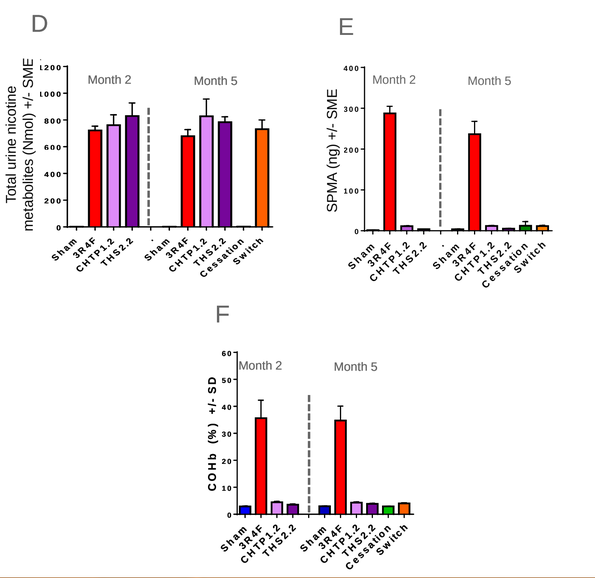


Aerosol uptake

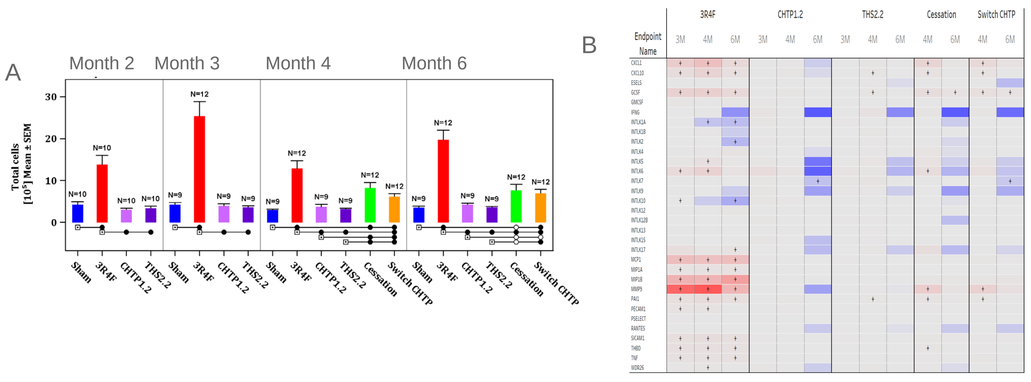
<u>Urinary nicotine metabolites (**D**)</u>: total of five main nicotine metabolites (3'-hydroxycotinine, norcotinine, cotinine, nicotine-N'-oxide, and nornicotine) was similar in mice from groups exposed to the nicotine-containing aerosols (3R4F, CHTP 1.2, or THS 2.2).

<u>Urinary S-phenylmercapturic acid (SPMA) (**E**):</u> urinary SPMA, a metabolite of benzene, was mainly detected in the 3R4F-exposed animals.

<u>Blood carboxyhemoglobin (**F**):</u> detected mainly in the CS-exposed mice, with other groups showing background levels.

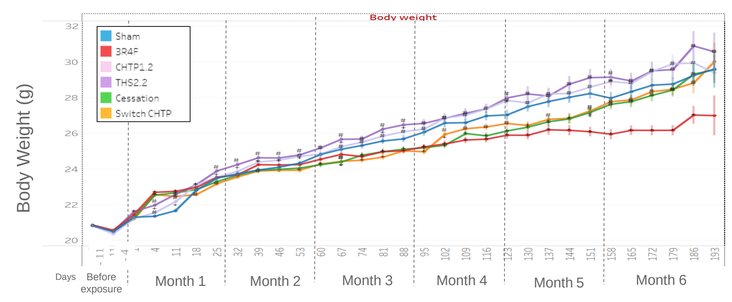


Results – Pulmonary Inflammation



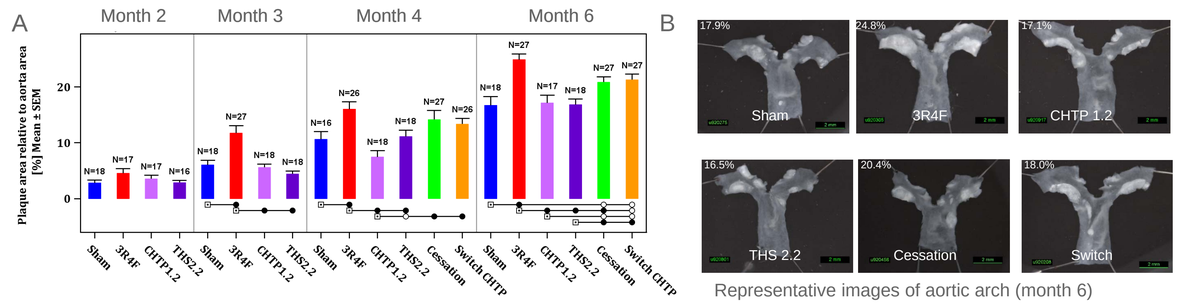
The absolute number of inflammatory cells, as determined by flow-cytometry-based analysis of free lung cells (A), and the levels of inflammation-related cytokines detected in cell-free BALF (B) were increased dramatically as early as two months following CS exposure. Both inflammatory cells and cytokines largely reverted back towards levels obtained with continuous exposure to fresh air within one month after cessation or switching. Statistics, ● p≤0.05, ○ p ≤ 0.01. Abbreviations: M, months of exposure; N, number of samples.

Results – Body Weight, Aortic Plaque Cholesterol and Development



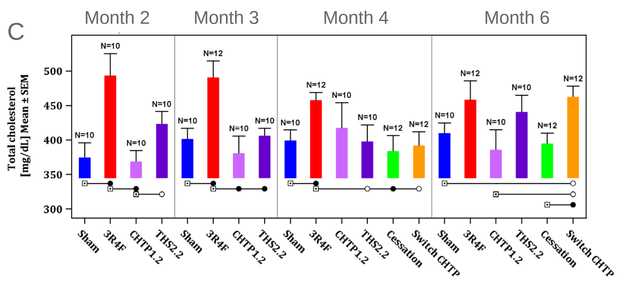
Aerosol exposure was well tolerated by the animals. Animals in all groups gained weight progressively over time.

After six months, the body weight of CS-exposed ApoE-/- mice was significantly lower compared with sham- or MRTP-exposed mice.



Representative images of aortic arch (month 6)

Exposure to CS resulted in increased plaque formation in the aortic arch of ApoE$^{-/-}$ mice compared with the sham exposure, starting from Month 3 and onwards (A, B). Cessation or switching to CHTP resulted in a slowing of the plaque formation, as the plaque area in these groups was trending lower than the continuous CS-exposed group in Month 4 and continuing to Month 6, where it was significantly lower. Even three months after CS exposure, the cessation and switch groups did not return to baseline (continuous fresh air) plaque area levels. There was no difference in plaque area in animals exposed to CHTP 1.2 or THS 2.2 for six months compared with the fresh air-treated animals. Statistics, ● p≤0.05, ○ p ≤ 0.01.



Serum samples were collected during necropsy, and total cholesterol was measured (**C**). The CS-exposed animals had higher total cholesterol compared with the other exposure groups up to Month 4. By Month 6, there was a higher variability in the total cholesterol levels in the groups, and treatment differences were less obvious.

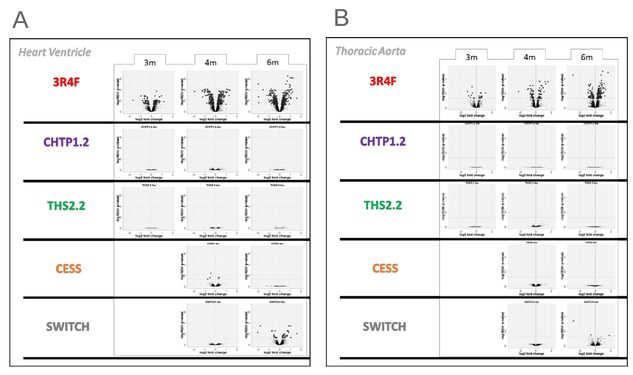
Results – Transcriptomics, Heart Ventricle, and Thoracic Aorta

Exposure to CS resulted in a time-dependent increase in the number of differentially expressed genes (compared with the sham-exposed mice) in the heart ventricle and the thoracic aorta.

The most differentially expressed gene profile was observed after six months CS exposure in both tissues (**A**-**B**).

In mice exposed to the heat-not-burn (CHTP 1.2, THS 2.2) aerosol, no differentially expressed genes were detected compared with the sham groups at all time points evaluated (FDR)<0.05.

In cessation and switching groups, the number of dysregulated genes was decreased strongly in comparison with those observed following CS exposure.



Volcano plots representing the systems response profiles in heart ventricle and thoracic aorta. For each gene, the gene expression change, calculated as the $\log_2$ 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. Upper right panel highlights genes that are statistically significantly up-regulated, and upper left panel highlights genes that are down-regulated.

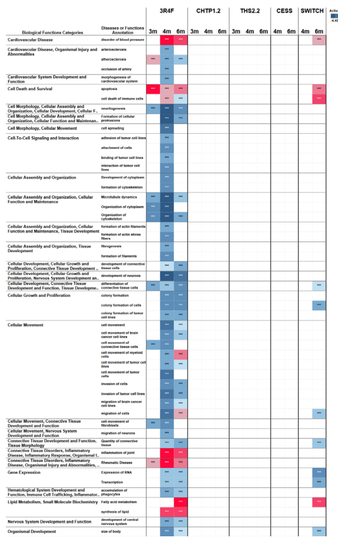
Number of differentially expressed genes (FDR<0.05) per contrasts in Heart Ventricle.

Group of Mice		Sham			3R4F			CHTP1.2			THS2.2			CESS		SWITCH	
Heart Ventricle	TimePoint	REF	REF	REF	3	4	6	3	4	6	3	4	6	4	6	4	6
	UP	REF	REF	REF	73	208	359	0	0	0	0	0	0	2	0	0	31
	DOWN	REF	REF	REF	-42	-235	-219	0	0	0	0	0	0	-3	0	0	-14

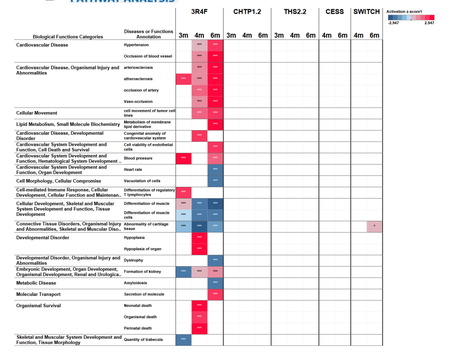
Number of differentially expressed genes (FDR<0.05) per contrasts in Thoracic Aorta.

Group of Mice		Sham			3R4F			CHTP1.2			THS2.2			CESS		SWITCH	
Thoracic Aorta	TimePoint	REF	REF	REF	3	4	6	3	4	6	3	4	6	4	6	4	6
	UP	REF	REF	REF	13	48	151	0	0	0	0	0	0	0	0	0	1
	DOWN	REF	REF	REF	-9	-39	-66	0	0	0	0	0	0	0	0	0	-2

C INGENUITY PATHWAY ANALYSIS — Heart ventricle- Biological functions and processes



D INGENUITY PATHWAY ANALYSIS — Thoracic aorta- Biological functions and processes



Ingenuity® Pathway Analysis (IPA) of the transcriptomics results from the heart (**C**) and the thoracic aorta (**D**) confirmed that mainly, a CS exposure-mediated effect was present in both tissues. Based on IPA of gene expression data, biological processes related to "Cardiovascular system development and function," "occlusion of artery," "atherosclerosis," "Connective Tissue Development and Function, Tissue Morphology," and "Cellular Assembly and Organization" were affected significantly by CS exposure in the thoracic aorta and heart ventricle but not by the exposure to aerosol from CHTP 1.2. Cessation or switching decreased atherosclerotic plaques and restored transcriptomic profiles to profiles similar to those observed in air-exposed animals. Statistics: *** p<0.01.

Discussion and Conclusion

- Exposure to CS resulted in significant levels of pulmonary inflammation and decline in pulmonary function and caused adverse effects on aortic plaque formation and a dysregulation of the heart and lung transcriptome.
- Continuous exposure to two heat-not-burn tobacco products (CHTP 1.2 and THS 2.2) resulted in a very small difference in all measured parameters related to COPD and CVD when compared with fresh air-exposed animals.
- The biological responses to switching to CHTP 1.2 (after three months of CS exposure) were similar to those observed in the cessation group across the spectrum of endpoints assessed and showed a generally positive effect with respect to continuous smoke exposure.
- Differential "omics" profiles associated with CS exposure returned to sham air levels following switching to CHTP 1.2 or fresh air (cessation).
- These data collectively indicated a halting or regression of cardiovascular or emphysematous disease parameters following switching from CS exposure to CHTP 1.2 aerosol in ApoE$^{-/-}$ mice.

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