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Cigarette smoking (CS) is the main risk factor for the development and progression of a series of diseases, including cardiovascular disease (CVD) and chronic obstructive pulmonary disease (COPD). Suitable animal models play an important role in understanding of smoke-induced pathogenesis. This study examined the development of hallmarks of both COPD and CVD in ApoE<sup>-/-</sup> mice exposed to either CS or to an aerosol from a candidate modified risk tobacco product, the tobacco heating system (THS2.2) over an 8-month period. In addition to chronic exposure regimes, a comparison of exposure cessation or switching to THS2.2 after 2 months of exposure to CS was performed using a battery of assays (physiological, morphological and molecular). Biological interpretation of various endpoints enables to understand the biological effects of cessation/switching as compared to continuous smoking.

The diagram illustrates the experimental timeline for five groups: Sham (fresh air), 3R4F, candidate MRTP, Cessation, and Switch. The x-axis represents time in months (0, 1, 2, 3, 6, 8 months) and nicotine concentration in µg/l (0, 1, 2, 3, 6, 8 months). A vertical arrow at month 2 indicates the 'Switch/cessation' point. The groups are color-coded: red for 3R4F/THS2.2 and blue for Fresh air. The Cessation group transitions from 30 µg/l to 0 µg/l at month 2. The Switch group transitions from 30 µg/l to 30 µg/l at month 2. The legend indicates that red segments represent 1 hour of 3R4F/THS2.2 exposure, blue segments represent 1 hour of Fresh air exposure, and a 30 min segment represents 30 minutes of exposure.

Female ApoE<sup>-/-</sup> mice were exposed to 3R4F (600 mg/m<sup>3</sup> TPM), THS2.2 (matched to the nicotine in 3R4F – 30 µg/l) or filtered air for 3 hours per day, 5 days per week, for up to 8 months. After 2 months of exposure to 3R4F, switching and cessation groups were exposed to aerosol from THS2.2 or filtered air, respectively. Animals were observed on a daily basis, body weight progression was monitored twice per week, exposure parameters (carboxyhemoglobin (COHb) in blood and nicotine metabolites in urine) were measured 3 times during the study. Dissections were performed after 1, 2, 3, 6, and 8 months of exposure. At each time point animals were allocated for the following end points: 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 determination and an extensive molecular high throughput analysis (transcriptomics, proteomics and lipidomics).

**A**

CO<sub>2</sub>H (%)

Month 2      Month 4      Month 7

Sham THF 2.5 THF 5.0 Switch Sham THF 2.5 THF 5.0 Switch Sham THF 2.5 THF 5.0 Switch

**B**

Total Metabolites (exc. nicotine) nmol/g (s.d.)

Month 2      Month 3      Month 6      Month 8

Sham THF 2.5 THF 5.0 Switch Sham THF 2.5 THF 5.0 Switch Sham THF 2.5 THF 5.0 Switch Sham THF 2.5 THF 5.0 Switch

After switching to the THS2.2 aerosol or filtered air, levels of COHb in blood (A) of exposed animals were substantially lower compared to those found in samples of mice continuously exposed to 3R4F. Measured concentration of nicotine metabolites in urine (B) (3'-hydroxycotinine, norcotinine, cotinine, nicotine-N'-oxide and nornicotine) confirmed that mice received equivalent nicotine concentrations in the different groups exposed to 3R4F or THS2.2.

**A**

Line graph showing Total cytokine ( $\times 10^3 \pm$  SEM) over 8 months. The Sham group (blue diamonds) remains relatively stable around 0.5. The TMS2.2 group (purple triangles) shows a slight decrease from 1.0 to 0.5. The 3R4F group (red squares) shows a significant increase, peaking at month 6 (approx. 2.2) before decreasing. The Cessation group (green inverted triangles) shows a decrease from 1.0 to 0.5. Statistical significance is indicated by asterisks: \*\*\* p < 0.001.

**B**

Line graph showing MMP activity (mU/ml)  $\pm$  SEM over 8 months. The Sham group (blue diamonds) is stable around 1.0. The TMS2.2 group (purple triangles) is stable around 1.0. The 3R4F group (red squares) shows a significant increase, peaking at month 2 (approx. 2.2) and month 8 (approx. 2.4). The Cessation group (green inverted triangles) shows a decrease from 1.0 to 0.5. Statistical significance is indicated by asterisks: \*\*\* p < 0.001.

**C**

Heatmap showing the ratio of treated mice over median of sham mice at same time point (truncated scale) for 45 cytokines and chemokines across 16 treatment groups. The color scale ranges from 0.5 (light pink) to 2.0 (dark red). The 3R4F groups (3R4F-1m to 3R4F-6m) show high ratios (dark red) for many cytokines, while the TMS2.2 and Cessation groups show lower ratios (light pink).

The absolute number of inflammatory cells in lung as determined by flow cytometry based analysis of free cells in BALF (A), the activity of matrix metalloproteinase (B) and the levels of inflammation-related chemokines detected in bronchoalveolar lavage fluid (C) were dramatically increased even after 1 month of exposure to 3R4F. Most of the inflammatory parameters measured reverted to the levels obtained with continuous exposure to filtered air or THS2.2-exposed animals after the cessation or the switch.

**A**

sham 3R4F THS2.2 cess switch

**B**

Alveolar emphysema

1m 2m 3m 6m 8m

sham 3R4F THS2.2 Cessation Switch

**C**

Lung volume, relative (g)/BW (g)

Sham 3R4F THS2.2 Cessation Switch

Month

Panel A – representative haematoxylin and eosin stained lung sections; panel B – scoring of alveolar emphysema; panel C – lung volume. The level of emphysema was evaluated by the scoring of severity and by morphometrical measurements performed in a blinded manner by an external pathologist. After the switch or cessation, the evaluation revealed a certain level of reduction of severity compared to the emphysema seen in lungs from continuously exposed animals. However, even 6 months after the switch some tissue degradation was still evident (A and B). Those changes are well reflected in the liquid displacement based lung volume measurement (C).

Figure 1 consists of five line graphs showing VpI (mV) +/- SEM versus Ppl (cmH2O) for five groups: Sham (blue diamonds), THS2.2 (purple squares), 3R4F (red circles), Switch (orange triangles), and Cessation (green inverted triangles). The graphs are for Month 1, Month 2, Month 3, Month 6, and Month 8. In all months, VpI increases with Ppl. The 3R4F group consistently shows the highest VpI values, while the Sham group shows the lowest. The Switch and Cessation groups show intermediate values, with the Cessation group generally having higher VpI than the Switch group in later months. Error bars represent SEM.

Month	Ppl (cmH2O)	Sham	THS2.2	3R4F	Switch	Cessation
Month 1	0	0.00	0.00	0.00	0.00	0.00
	5	0.10	0.10	0.15	0.10	0.10
	10	0.25	0.25	0.40	0.30	0.30
	20	0.40	0.40	0.55	0.45	0.45
	30	0.55	0.55	0.65	0.55	0.55
Month 2	0	0.00	0.00	0.00	0.00	0.00
	5	0.05	0.05	0.10	0.05	0.05
	10	0.15	0.15	0.30	0.20	0.20
	20	0.30	0.30	0.50	0.40	0.40
	30	0.45	0.45	0.65	0.50	0.50
Month 3	0	0.00	0.00	0.00	0.00	0.00
	5	0.05	0.05	0.10	0.05	0.05
	10	0.15	0.15	0.30	0.20	0.20
	20	0.30	0.30	0.50	0.40	0.40
	30	0.45	0.45	0.65	0.50	0.50
Month 6	0	0.00	0.00	0.00	0.00	0.00
	5	0.05	0.05	0.10	0.05	0.05
	10	0.15	0.15	0.30	0.20	0.20
	20	0.30	0.30	0.50	0.40	0.40
	30	0.45	0.45	0.65	0.50	0.50
Month 8	0	0.00	0.00	0.00	0.00	0.00
	5	0.05	0.05	0.10	0.05	0.05
	10	0.15	0.15	0.30	0.20	0.20
	20	0.30	0.30	0.50	0.40	0.40
	30	0.45	0.45	0.65	0.50	0.50

Exposure of mice to 3R4F cigarette smoke resulted in a leftward shift of the P-V loops as compared to the results obtained with filtered air exposed animals. Measurements from THS2.2 and filtered air exposed animals revealed very similar values during the course of the study. After the switch to the aerosol from THS2.2 or cessation, P-V loops shifted right, but did not reach values obtained from animals continuously exposed to filtered air or THS2.2

**A**

Sham 3R4F THS 2.2

25.0 % 38.5 % 27.5 %

Cessation Switch

28.1 % 29.9 %

Plaque area (mm<sup>2</sup>) × 10<sup>3</sup> ± SEM

Month	Sham	3R4F	THS 2.2	Cessation	Switch
Month 1	~1.5	~1.5	~1.5	~1.5	~1.5
Month 2	~1.5	~1.5	~1.5	~1.5	~1.5
Month 3	~1.5	~1.5	~1.5	~1.5	~1.5
Month 6	~1.5	~1.5	~1.5	~1.5	~1.5
Month 8	~1.5	~1.5	~1.5	~1.5	~1.5

**B**

Plaque Volume

Plaque Volume (mm<sup>3</sup>)

Aortic Arch Occlusion

Mean Occlusion (%)

Plaque Surface

Plaque Surface (mm<sup>2</sup>)

Plaque Volume (mm<sup>3</sup>)

Group	Plaque Volume (mm <sup>3</sup> )
Sham	~1.0
3R4F	~1.5
THS 2.2	~1.2
Cessation	~1.1
Switch	~1.1

Mean Occlusion (%)

Group	Mean Occlusion (%)
Sham	~1.0
3R4F	~1.5
THS 2.2	~1.2
Cessation	~1.1
Switch	~1.1

Plaque Surface (mm<sup>2</sup>)

Group	Plaque Surface (mm <sup>2</sup> )
Sham	~1.0
3R4F	~1.5
THS 2.2	~1.2
Cessation	~1.1
Switch	~1.1

Exposure to 3R4F resulted in increased plaque formation in aortic arch of ApoE<sup>-/-</sup> mice compared with sham exposure. These changes were alleviated by smoking cessation or switching to THS2.2 within one month, and nearly reached control levels at the 8-month time point. Panel A – oil red O stained plaque, two-dimensional quantification, percentages indicate the surface of aorta under plaque; panel B –  $\mu$ CT scanned aorta after the staining, 3D reconstruction showing position and the thickness of the plaque (collaboration with an external partner)

**Lung tissue response to smoke exposure**

Responses to diverse cell stresses  
xNPA scores

Cell fate: live or die  
xNPA scores

Cell Proliferation  
xNPA scores

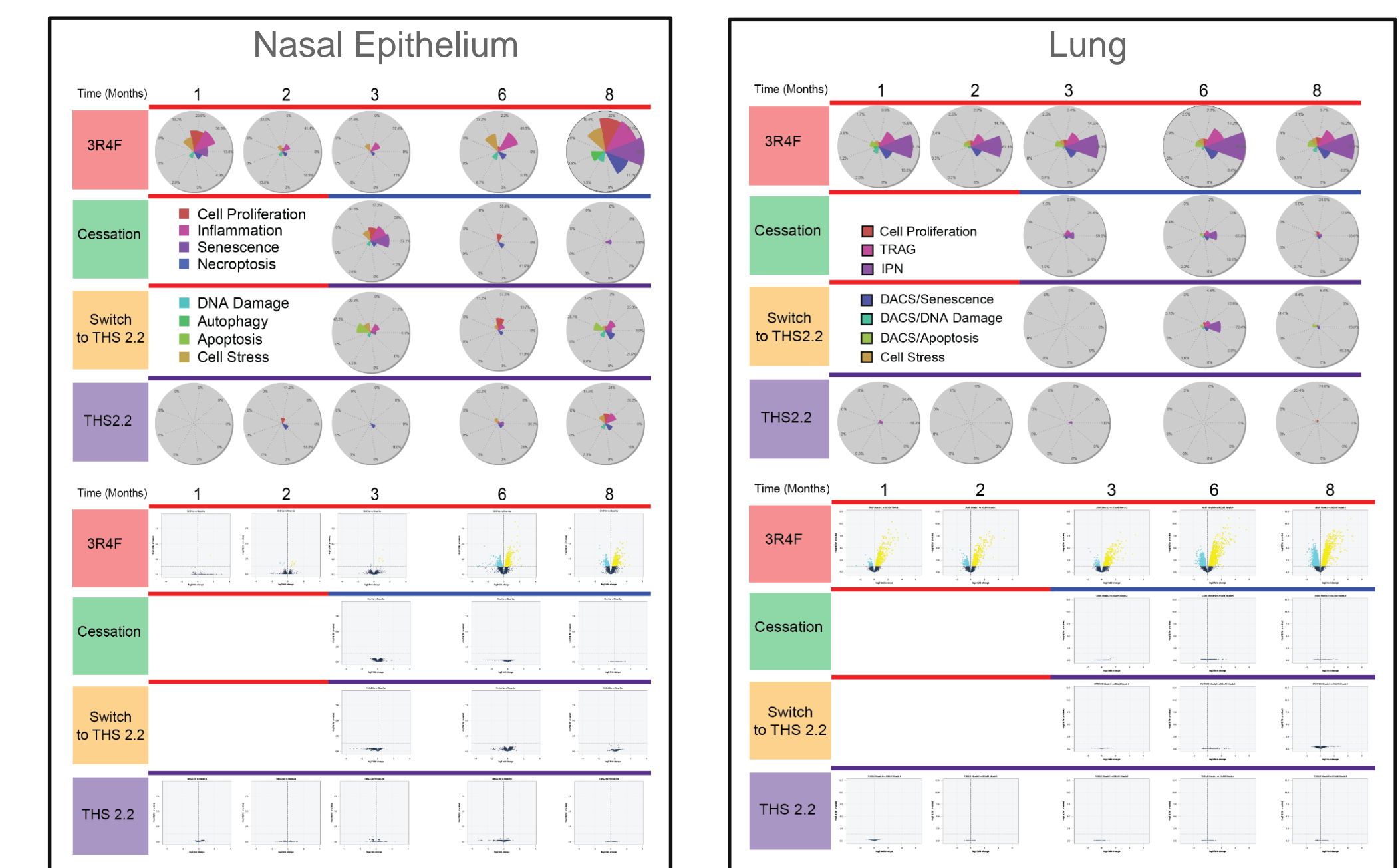
Tissue repair and angiogenesis  
xNPA scores

Inflammatory response  
xBiological network scores

Biological Network Model

Aggregation:  
Per system response profile:  
1 Biological Impact Factor (BIF)  
taking into account all 5

system response profile



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  $\log_{10}$ -adjusted *P*-value, is plotted on the y-axis. Yellow and blue dots highlight genes that are statistically significantly up- or down-regulated, respectively, compared with the sham group at each respective time point. Star plots illustrate the decomposition of the overall transcriptional changes into its eight mechanistic components (from cell proliferation to inflammation, indicated by colours) for each treatment group.

- The exposure to 3R4F cigarette smoke resulted in significant levels of pulmonary inflammation, decline in pulmonary function, and histopathological changes. These phenotypic changes were coherent with the molecular data.
- Chronic exposure to an aerosol from the THS2.2 resulted in very little difference in all measured parameters related to COPD and CVD when compared to the filtered air-exposed animals.
- The biological response to switching to a THS2.2 aerosol or filtered air following 2 months of 3R4F cigarette smoke exposure were very similar between the two conditions across the spectrum of endpoints assessed, and showed a generally positive effect.

- Differential 'omics' profiles associated with 3R4F exposure returned to nearly filtered air-like level following either switching to a THS2.2 aerosol or filtered air.
- Histopathological assessment also showed a marked effect of switching, in which a partial or complete (depending on the inflammatory cell type) reversal of pulmonary inflammation was observed.
- These data collectively indicate a halting or regression of the disease genesis following switching from conventional cigarette to THS2.2 aerosol in Apoe<sup>-/-</sup>.

