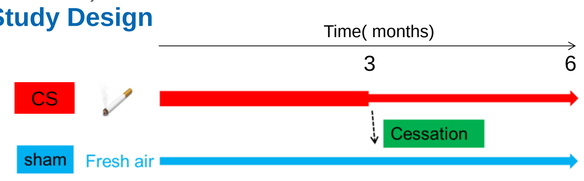
Systems toxicology in ApoE$^{-/-}$ mice demonstrates smoking cessation benefits for both cardiovascular and lung disease-related endpoints.

Stéphanie Boué, Hector De Leon, Stefan Lebrun, Walter Schlage, Gregory Vuillaume, Florian Martin, ***Julia Hoeng****, Manuel C. Peitsch.*
Philip Morris International R&D, Quai Jeanrenaud 5, CH-2000 Neuchâtel, Switzerland

Abstract

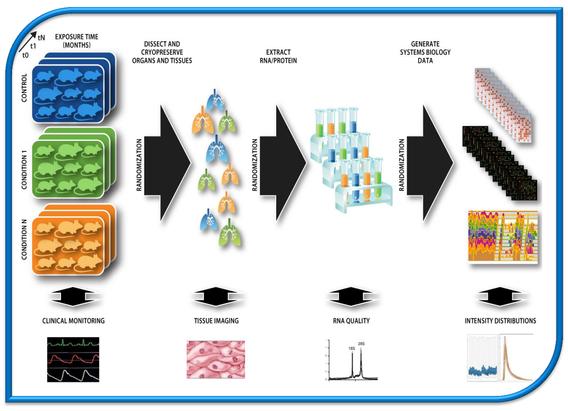
Cigarette smoking is the primary etiology of chronic obstructive pulmonary disease (COPD) and a risk factor for cardiovascular diseases. Smoking cessation results in a rapid decline of cardiovascular disease risk, but lung disease risk remains higher in former smokers compared to never smokers. Studying both pathologies in a single model is important, as they may have related causes and interactions. Therefore, we exposed ApoE$^{-/-}$ mice, which are prone to both premature atherosclerosis and emphysema, to either fresh air (sham), mainstream cigarette smoke (CS) for 6 months, or CS for 3 months followed by fresh air for 3 months (cessation group). Plasma, liver, and aorta samples were extracted for lipids and analyzed by mass spectrometry. While CS exposure increased most lipids, smoking cessation resulted in lower levels of many lipids in plasma and aortic arch. In parallel, gene expression profiles of lung parenchyma were obtained on microarrays. Findings obtained by lipidomics and transcriptomics were compared to standard toxicity assessments. For example, development of atherosclerosis in the aorta was assessed by plaque size in the aortic arch, while lung disease was evaluated by bronchoalveolar fluid (BALF) analysis and histological assessment of lung tissue. Gene set enrichment analysis of expression data from lungs of CS-exposed mice showed activation of pathways involved in cell proliferation and tissue remodeling that correlated with the general inflammation and emphysema observed in the lungs on histological evaluation. Interestingly, a progressive deactivation of these toxicity pathways was observed following CS exposure cessation. The potential of using animal models to study comorbidities associated with cigarette smoking and to develop mechanistic understanding of the impact of smoking cessation, was demonstrated. The study supports the applicability of this approach as a powerful tool to investigate disease mechanisms *in vivo* and to develop a systems biology-based risk assessment strategy for Modified Risk Tobacco Products (MRTPs) (FSPTCA 2009)

Study Design



Methods

In vivo Systems Biology Workflow



Animals and Inhalation

All animal experimental procedures were in conformity with the AALAS Policy on the Humane Care and Use of Laboratory Animals (AALAS, 1996) and were approved by the Institutional Animal Care and Use Committee (IACUC). Female ApoE$^{-/-}$ mice (ApoE/Bom, B6.129P2-Apoe^{tm1Unc}N$_{11}$) aged 8-10 weeks were obtained from Taconic (Denmark & USA). Animals were fed a normal chow diet based on soybeans with 0.003% cholesterol and 4% fat (2014 Teklad) from Harlan (Oxon, UK).

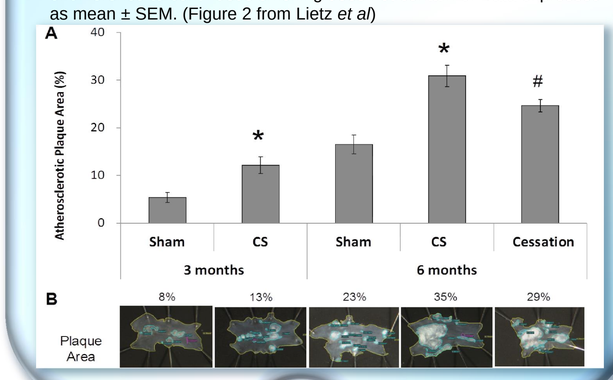
The exposure regimen consisted of 3x1-hour periods a day and 30-minute intervals with fresh filtered air, 5 days a week. Total particulate matter (TPM) levels for CS-exposed (reference cigarette 3R4F obtained from University of Kentucky, KY, USA) groups were targeted at 600 µg TPM/m^3.

Main Biological Endpoints

Endpoint	Assay	Assay Description
Test atmosphere characterization		Smoke generation and test atmosphere characterization
Markers of exposure	Nicotine metabolites	Trans-3'-Hydroxycotinine (3'HOCOT), nornicotine (NNIC), norcotinine (NCOT), cotinine (COT), and nicotine-N'-oxide (NN'O) were quantified by HPLC in urine
	COHb	Carboxyhemoglobin measurement
In life observation	Body weight	Measure of body weight
CVD	Plaque size	Planimetry of aortic arch
	Lipoproteins	Lipoproteins concentrations in plasma and in the plaque measured by high-performance liquid chromatography
	Lipidomics	Mass-spectrometry based lipidomics (outsourced to Zora Biosciences, Finland)
COPD	BALF	- Cells counts in BALF (FACS analysis) - Mediator analysis in BALF outsourced to Rules Based Medicine (USA)
	Lung histology and morphometry	Histopathological and morphometrical analyses of lung tissues
	Lung function	Evaluation of respiratory mechanics
All	Transcriptomics	- mRNA levels measured using Affymetrix GeneTitan microarrays - Computational analysis
	Statistics	Sample number and Statistical Analysis

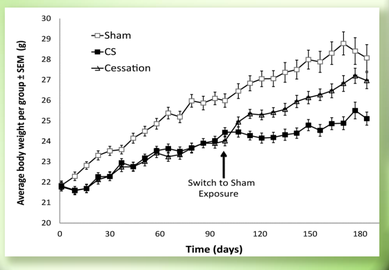
Results

Plaque Morphometric Analysis. A. CS exposure discontinuation (cessation) resulted in a significantly smaller plaque size when compared to plaque areas from CS. However, plaque area in these aortas was still higher than plaque areas from aortas of sham mice. **B. Representative images of each group at 3 and 6 months are shown.** *: 95%-CI of difference to sham does not contain 0; #: 95%-CI of difference to continuous smoking does not contain 0. Data expressed as mean ± SEM. (Figure 2 from Lietz *et al*)



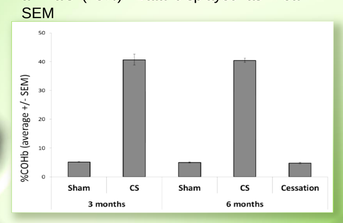
Animal body weight.

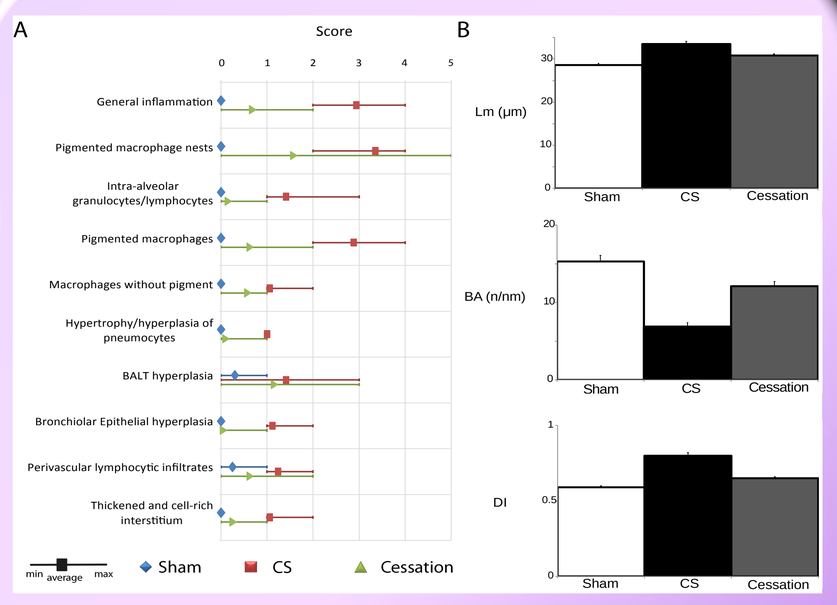
CS-exposed animals grew at a slower rate compared to sham mice. Mice exposed to CS for 3 months and then switched to regular filtered air (cessation) resumed a higher growth rate and approached the weight attained by sham animals at the end of the observation period. Data displayed as mean ± SEM. (Figure 1 from Lietz *et al*)



Carboxyhemoglobin Levels After 3 or 6 Months Exposure.

Plasma levels of COHb, an indicator of inhaled smoke, increased rapidly in mice in the CS group. Both three and six months after exposure, COHb levels in the CS group were around 40%. COHb levels in animals in the sham group remained around 5%. Mice in the cessation group exhibited levels comparable to those observed in sham animals (~5%). Data displayed as mean ± SEM



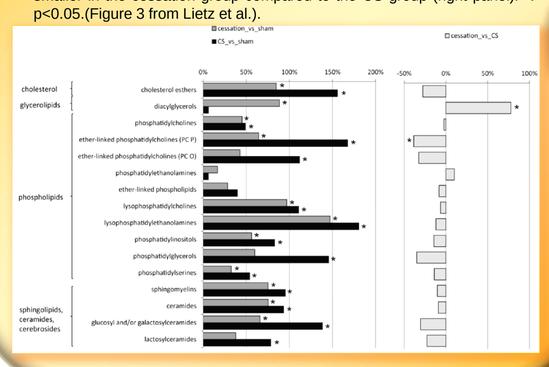


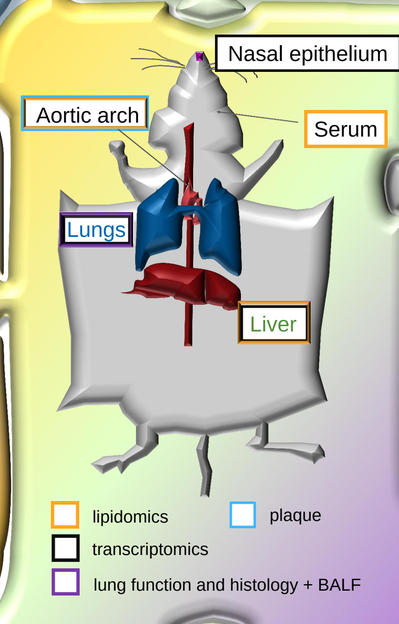
Histopathological evaluation of lung tissue after CS exposure and smoking cessation.

(A) Histopathological scoring. Ten histopathological parameters scored 0-5 to assess lung inflammation and cell hyperplasia were obtained. Lungs of CS-exposed mice (red) showed a mixed inflammatory infiltrate (general inflammation, intra-alveolar granulocytes/lymphocytes). Alveolar septa were thickened and exhibited a high cellularity. A hyperthropic/hyperplastic pattern was observed in the bronchiolar epithelium. Scores of all histopathological parameters were higher in CS group (red) compared to sham group (blue). All histopathological scores were curtailed in cessation group (green) compared to CS group (red). Data are plotted as average of score measurement per group and the error bar delineates the spread from minimum to maximum value per group.

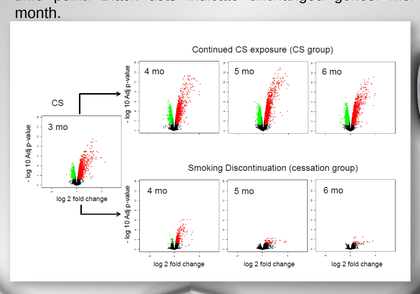
(B) Histomorphometric assessment of alveolar emphysema in ApoE$^{-/-}$ mice at six months. Mean chord length (Lm) and destructive index (DI) were larger in mice exposed to CS than in sham-exposed mice or mice from the cessation protocol, whereas less bronchiolar attachments (BA) were visible. These 3 parameters highlight lung damage in CS-exposed mice, which is much less pronounced in the cessation group, even though the levels are still significantly different from the sham groups. Lm: mean chord length; BA: bronchiolar attachments; DI: destructive index; CS: mainstream cigarette smoke. Data expressed as mean ± SD. DI is a unitless parameter.

Aorta lipidomics after 6 months of CS exposure.
Data are expressed as percent change compared to a reference group. Black bars represent percent change of lipid classes in the CS group compared to the sham group. Dark gray bars on the left panel represent percent change in the cessation group compared to the CS group. Light gray bars on the right panel represent changes in the cessation group compared to the CS group. Lipid classes within each representative group were significantly larger after CS exposure or cessation compared to sham (left panel), whereas only ether-linked phospholipid levels (PC O) were smaller in the cessation group compared to the CS group (right panel). *: p<0.05.(Figure 3 from Lietz et al.).

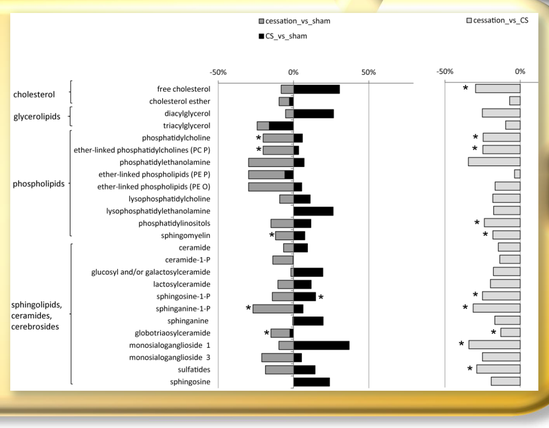


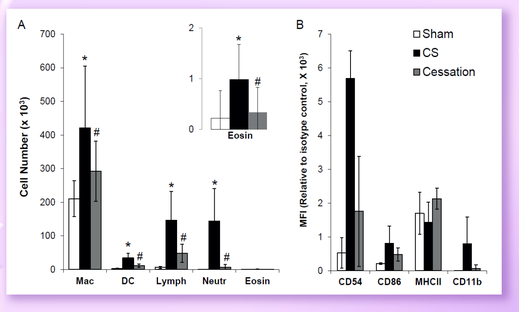


Significance vs. fold-change volcano plots showing time course gene expression data in lung parenchyma.
CS-exposed ApoE$^{-/-}$ mice (CS) showed a time-dependent up- (red dots) and down-regulation (green dots) of the expression levels of multiple genes. Discontinuing CS exposure after 3 months (cessation) resulted in a dramatic reduction of most up- and down-regulated genes. ApoE$^{-/-}$ mice from the sham group were used as reference at each time point. Black dots indicate unchanged genes. mo: month.



Plasma lipidomics at 6 months of CS exposure.
Data are expressed as percent change compared to a reference group; three comparisons are shown. Black bars represent percent change of lipid classes in the CS group compared to the sham group. Dark gray bars on the left panel represent percent change in the cessation group compared to sham. Light gray bars on the right panel compared cessation vs. CS groups. Lipid classes within each representative group were significantly smaller after smoking cessation in comparison to the CS group, whereas only sphingosine-1-P levels were significantly higher in the CS group compared to the sham group. Multiple molecular species were lower in the cessation group when compared to the CS group.
(*: p<0.05).(Figure 3 from Lietz et al.)





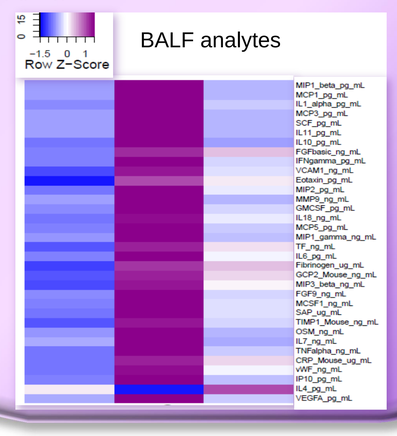
Differences between groups were estimated by their 95% CI. *: compared to sham, #: compared to CS. MHCII a protein expressed at higher levels in dendritic cells showed slightly higher levels in cessation group compared to CS group. Mac: alveolar macrophages, DC: dendritic cells, Lymph: lymphocytes, Neutr: neutrophils, Eosin: eosinophils, MFI: mean fluorescence intensity shift relative to isotype control. Data expressed as mean ± SD.

BALF cells

(A) Inflammatory cell subpopulations in BALF. All cell types including, macrophages, dendritic cells, lymphocytes, neutrophils and eosinophils were found in larger numbers in CS-exposed mice compared to sham. A cessation protocol (cessation) resulted in smaller number of cells compared to the smoke-exposed group.

(B) Markers of macrophage activation (CD54, CD86 and CD11b) revealed larger numbers of alveolar macrophages expressing activation markers in the CS-exposed group compared to sham. Cessation animals exhibited lower numbers of activated cells compared to CS-exposed mice.

Multianalyte profiling (MAP) of BALF
The protein abundance in BALF of most analytes examined in BALF were increased after CS exposure. Smoking cessation resulted in significantly lower levels of 68% of the analytes examined in BALF.



Discussion & Outlook

- CS (3R4F) exposure increases atherogenesis in (ApoE)mice, as seen on plaque size and lipid levels (especially in aorta)
- CS increases lung inflammation and induces histopathological changes in lung.
- Cessation generally induces a lowering of atherogenic lipid molecules and results in reduced plaque size compared to continuous smoking.
- Lung inflammation is markedly reduced after smoke exposure is discontinued based on histological and molecular findings.

The results obtained from a comprehensive list of endpoints in this study suggest that the systems toxicology approach is powerful for the simultaneous investigation of lung and cardiovascular disease mechanisms *in vivo*. This could add mechanistic insight in support of an MRTP Application (FDA MRTPA 2012) using a systems biology-based risk assessment benchmarked against smoking cessation.

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Philip Morris International Research & Development, Quai Jeanrenaud 5, 2000 Neuchâtel, Switzerland
T: +41 58 242 21 11, **F**: +41 58 242 28 11, **W**: www.pmi.com