Long term exposure to cigarette smoke impacts genes involved in cardiac muscle structure and function in Apoe-/- mouse while exposure to a candidate modified risk tobacco product, the Tobacco Heating System 2.2 (THS 2.2) aerosol does not

Justyna Szostak[1], Stéphanie Boué[1], Marja Talikka[1], Emmanuel Guedj[1], Florian Martin[1], Blaine Phillips[2], Nikolai V. Ivanov[1], Manuel C. Peitsch[1], and Julia Hoeng[1]

[1]Philip Morris International R&D, Philip Morris Products S.A., Quai Jeanrenaud 5, 2000 Neuchâtel, Switzerland
[2]Philip Morris International Research Laboratories Pte Ltd, Science Park II, Singapore
1 and 2 Part of Philip Morris International group of companies.

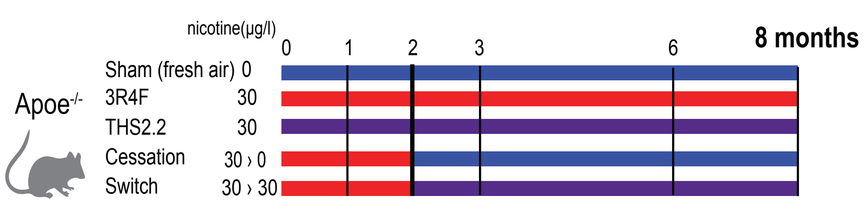
Introduction

Heart failure is reported to affect over 15 million people in Europe (1) and over 5 million people in the United States (2), and is a major cause of hospitalization and morbidity (3). Smoking is a major cause of the high incidence of cardiovascular disease (4-5) and is highly associated with endothelial dysfunction, atherosclerosis and heart failure. Although heart failure is a serious complication of atherosclerosis, other stressors such as diabetes, hypertension, and toxic compounds can impact cardiac contraction and favor the development of cardiomyopathy, eventually causing heart failure. Candidate modified risk tobacco products have been developed to reduce the harm of smoking linked to cardiovascular and other smoking-related diseases. One such product, the Tobacco Heating System 2.2 (THS2.2), which heats the tobacco instead of burning it, was developed to decrease significantly the levels of harmful and potentially harmful constituent levels, such as aldehydes and polycyclic aromatic hydrocarbons (5). To investigate the relative impact of exposure to an aerosol from THS2.2 compared with smoke from the 3R4F reference cigarette, as well as the impact of cessation or switching to THS2.2 after 2 months exposure to 3R4F smoke, we have conducted an inhalation study on Apoe-/- mice with a number of toxicological and disease-related endpoints (5).

Because there is substantial evidence that smoking is one of a risk factors of the development of cardiovascular pathologies, we also analyzed heart tissue of Apoe-/- mice from this study. A transcriptomics approach was chosen to identify at least some of the molecular mechanisms underlying the biological effects of exposure to 3R4F smoke and THS2.2 aerosol on the heart.

Study design and methods

Study design



Female Apoe-/- mice, (age 8–10 weeks) were exposed to 3R4F smoke or to THS2.2 aerosol for up to 8 months.

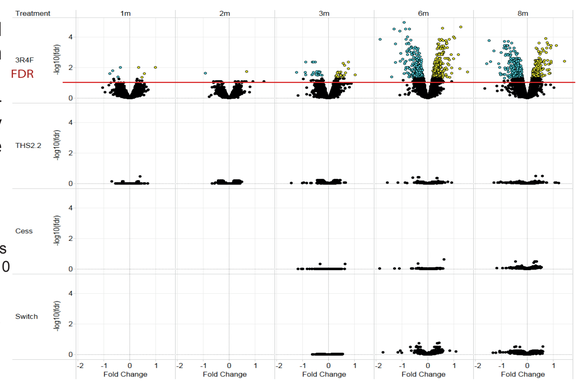
Diluted mainstream smoke extracted from 3R4F cigarettes (600 mg total particulate material/m3, equivalent to 29.9 mg nicotine/m3), THS2.2 aerosol (nicotine-matched to 3R4F, 29.9 mg/m3), or filtered air where used to expose mice (whole body exposure), during 3 hours per day, 5 days per week, for up to 8 months. To avoid a buildup of excessive carboxyhemoglobin concentrations in the 3R4F group, intermittent daily exposure to fresh filtered air for 30 min after the first hour of smoke exposure and for 60 min after the second hour of exposure was provided. Apoe-/- mice exposed to fresh air (sham) were considered as a control group. After two months of exposure to 3R4F smoke, subsets of mice were exposed to fresh air to mimic smoking cessation or were switched to THS2.2 (5).

Transcriptomics analysis

The systems response profiles are illustrated as volcano plots depicting differentially expressed genes

The transcriptomes of the heart tissues from all treatment groups were compared with those from sham-exposed mice at each time point.
Exposure to 3R4F smoke resulted in a time-dependent increase in the number of differentially expressed genes in the heart compared with the sham groups.

Volcano plot representing the change in expression for each gene, calculated as the log2 fold change, is plotted on the x-axis and the statistical significance, proportional to the negative log10 raw p-value, is plotted on the y-axis. Genes that met the FDR (false discovery rate) of p <0.05 are indicated by colored dots. Yellow indicates significantly upregulated genes, and cyan indicates significantly downregulated genes.



Heatmap of the genes significantly affected by 3R4F smoke exposure at the 1–3-month time points. Genes that met the FDR (false discovery rate) of p <0.05 are indicated in color. Orange indicates significantly upregulated genes, and blue indicates significantly downregulated genes.

One month after exposure, only eight genes were significantly deregulated (FDR <0.05): Gkn3, Car14, Gm16793, and Snca were upregulated, and Phf11d, Itih5, Col6a1, and Pdzrn were downregulated. After 2 months of 3R4F smoke exposure, only two genes were significantly impacted: Gkn3 was upregulated, and Rbp7 was downregulated. After 3 months of 3R4F smoke exposure, 44 genes were significantly differentially expressed in response to 3R4F smoke exposure. Together these results demonstrated that the impact was modest until 3 months of smoke exposure. By 6 months, the number of differentially expressed genes was considerably higher, and this number remained relatively stable at 8 months of smoke exposure. In the heart tissues from mice exposed to THS2.2 aerosol or those switched to fresh air or THS2.2 after 2 months of 3R4F smoke exposure, no differentially expressed genes were detected compared with the sham groups at all time points evaluated.

Results

IPA analysis was performed to identify the main biological processes and functions impacted by the exposure (6)

Biological functions and processes related to muscle structure and function, inflammation, and cardiovascular disease were predicted to be impacted in response to 3R4F smoke exposure at 6 and 8 months.

INGENUITY PATHWAY ANALYSIS

	Biological Function	Sub Function	3R4F6m	3R4F8m
Muscle structure and function	Cell Morphology, Cellular Assembly and Organization, Cellular Function and Maintenance	formation of cellular protrusions	*	*
	Cellular Assembly and Organization, Cellular Function and Maintenance	organization of cytoskeleton	*	*
		organization of cytoplasm	*	*
		microtubule dynamics	*	*
	Embryonic Development, Organ Development, Organismal Development, Skeletal and Muscular System Development and Function, Tissue Development	formation of muscle	*	
	Cell Morphology, Cellular Assembly and Organization, Cellular Development, Cellular Function and Maintenance, Nervous System Development and Function, Tissue Development	neuritogenesis		*
		formation of plasma membrane projections		*
	Developmental Disorder, Skeletal and Muscular Disorders	congenital anomaly of musculoskeletal system		*
Inflammatory response	Inflammatory Response	inflammatory response	*	
	Cellular Movement	migration of cells	*	*
		cell movement	*	*
	Cellular Movement, Hematological System Development and Function, Immune Cell Trafficking	cell movement of mononuclear leukocytes	*	*
		cell movement of lymphocytes	*	*
		migration of mononuclear leukocytes	*	*
	Cellular Movement, Immune Cell Trafficking	leukocyte migration		*
	Hematological System Development and Function, Immune Cell Trafficking, Inflammatory Response, Tissue Development	accumulation of myeloid cells		*
Cardiovascular disease	Cardiovascular Disease	atherosclerosis		*
	Cardiovascular Disease, Hematological Disease	thrombosis	*	

Heatmap of the most significantly affected biological functions in hearts of mice exposed to 3R4F for 6 and 8 months. Differences in biological functions of the 3R4F-exposed groups compared with the corresponding sham-exposed groups that met the FDR (false discovery rate) of p <0.05 and with z scores >2 or <−2 were considered statistically significant (activated (red) or inhibited (blue)) are marked with an asterisk (*).

Z Score
-4.4 2.6
* Z Score < -2 > 2

Specifically, biological processes such as “Cell Morphology”, “Cellular Assembly and Organization”, “Embryonic Development, Organ Development”, and “Nervous System Development and Function” were predicted to be downregulated. “Inflammatory response,” “Cellular movement”, and “Hematological system development” functions were also predicted to be downregulated in response to 3R4F smoke exposure.

In contrast, “Skeletal and muscular disorder” and its associated sub-function “Congenital anomaly of musculoskeletal system” were predicted to be significantly upregulated in response to 3R4F smoke exposure. Together these data suggest that 3R4F significantly affected the expression of genes involved in heart muscle function and structure, the inflammatory response, and cardiovascular disease.

Mapping of the differentially expressed genes onto KEGG “Focal adhesion” pathways (7)

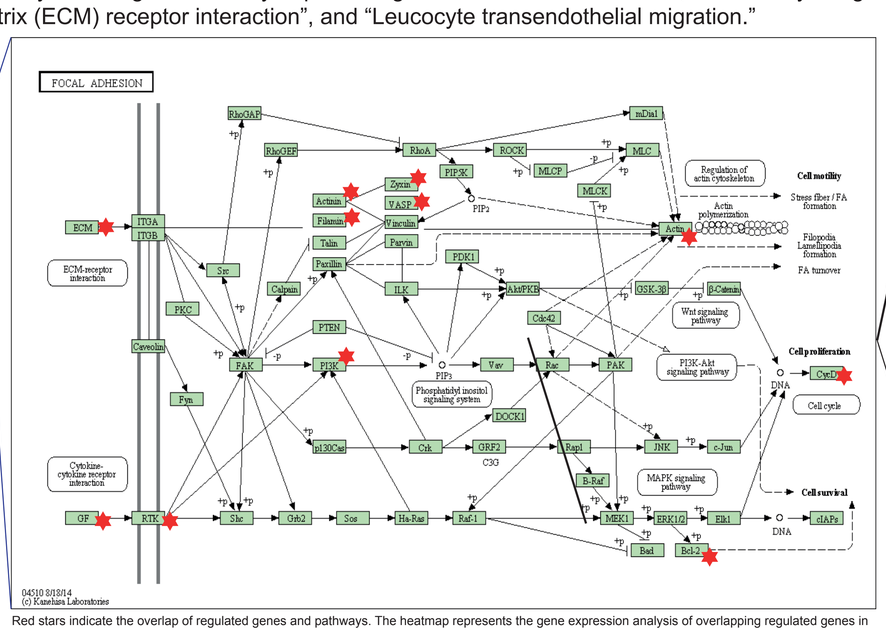
The top regulated KEGG pathway involving differentially expressed genes was “Focal adhesion”, followed by “Regulation of actin cytoskeleton,” “Extracellular matrix (ECM) receptor interaction”, and “Leucocyte transendothelial migration.”

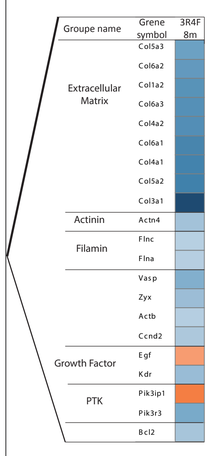
“Focal adhesion”

“Regulation of actin cytoskeleton”

“Extracellular matrix (ECM) receptor interaction”

“Leucocyte transendothelial migration.”





Red stars indicate the overlap of regulated genes and pathways. The heatmap represents the gene expression analysis of overlapping regulated genes in the focal adhesion pathway. Blue and orange indicate downregulated and upregulated genes, respectively.

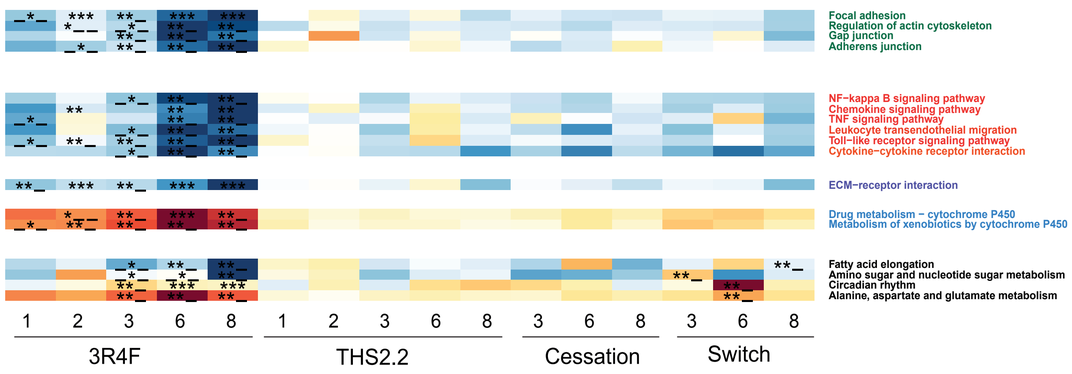
The mapping of genes that were regulated in response to 3R4F after 8 months of exposure to the “Focal adhesion” pathway suggested that the regulation of the actin cytoskeleton was disrupted in CS-exposed mouse heart. Genes involved in the regulation of cytoskeleton and signal transduction, such as filamin (Flnc), zyxin (Zyx), vasodilator-stimulated phosphoprotein (Vasp), and beta actin (Actb), were downregulated in response to 3R4F exposure. Additionally, genes that encode proteins involved in the regulation of cytokine-cytokine receptor interaction and ECM-receptor interaction, such as collagen (Col3a1, Col5a2), epidermal growth factor, FMS-like tyrosine kinase 1, and p21 protein (Cdc42/Rac-activated kinase 1), were also differentially regulated in response to 3R4F smoke exposure.

Gene set enrichment analysis (GSEA) (8)

The GSEA showed significant downregulation of genes sets involved in muscle structure and function, affecting the actin cytoskeleton, focal adhesion sites, gap junctions, and adherent junctions in the hearts of 3R4F smoke exposed mice. No such downregulation was detected for the same gene sets in the THS2.2, cessation, and switching groups.

The GSEA analysis predicted a significant downregulation of inflammatory pathways associated with “NF-Kappa B signaling”, “Chemokine signaling”, “TNF signaling”, “Leucocyte transendothelial migration”, “Toll-like receptors”, and “Cytokine-cytokine receptor interactions”. 3R4F smoke exposure additionally upregulated genes involved in “Drug metabolism-cytochrome P450” and “Metabolism of xenobiotics by cytochrome p450” and downregulated genes involved in ECM-receptor interaction.

Differential gene regulation in the hearts of mice in the switching groups was observed. At the 8-month time point, the gene set related to fatty acid elongation was downregulated in the hearts of mice in the switching group; however, the regulation was at a much lower level than in mice continuously exposed to 3R4F smoke. The regulation of gene sets related to amino sugar and nucleotide sugar metabolism, and alanine, aspartate, and glutamate metabolism, as well as circadian rhythm, seemed to be more up-regulated in the switching group at the 3-and 6-month time points compared with the 3R4F group at the same time points.



Significance is indicated by an asterisk (*). In each rectangle, the first character symbol is for the overall adjusted Fisher p-value, the second symbol indicates significance for GSEA Q2, and the third symbol indicates significance for the over-representation analysis.

Conclusion

Exposure to 3R4F smoke in Apoe-/- mice, resulted in a time-dependent increase in the number of differentially expressed genes in the heart compared with the sham groups.
In the heart tissues from mice exposed to THS2.2 aerosol or those switched to fresh air or THS2.2 after 2 months of 3R4F smoke exposure, no differentially expressed genes were detected compared with the sham groups at all-time points evaluated in accordance to the FDR<0.05.

Analysis of differentially expressed genes in the heart tissue indicated that 3R4F smoke exposure induced the downregulation of genes involved in cytoskeleton organization and the contractile function of the heart, notably genes that encode beta actin (Actb), actinin alpha 4 (Actn4), and filamin C (Flnc). This was accompanied by the downregulation of genes related to the inflammatory response suggestive of an immunomodulatory response in the heart.

References

1. Dickstein, K., et al., ESC Guidelines for the diagnosis and treatment of acute and chronic heart failure 2008: the Task Force for the Diagnosis and Treatment of Acute and Chronic Heart Failure 2008 of the European Society of Cardiology. Developed in collaboration with the Heart Failure Association of the ESC (HFA) and endorsed by the European Society of Intensive Care Medicine (ESICM). Eur Heart J, 2008. 29(19): p. 2388-442.
2. Mozaffarian, D., et al., Heart disease and stroke statistics--2015 update: a report from the American Heart Association. Circulation, 2015. 131(4): p. e29-322.
3. Sequeira, V., et al., The physiological role of cardiac cytoskeleton and its alterations in heart failure. Biochimica et Biophysica Acta (BBA)-Biomembranes, 2014. 1838(2): p. 700-722.
4. Gairola, C.G., et al., Sidestream cigarette smoke accelerates atherogenesis in apolipoprotein E-/- mice. Atherosclerosis, 2001. 156(1): p. 49-55.
5. Smith, M.R., et al., Evaluation of the Tobacco Heating System 2.2. Part 1: Description of the system and the scientific assessment program. Regul Toxicol Pharmacol, 2016.
6. Kramer, A., et al., Causal analysis approaches in Ingenuity Pathway Analysis. Bioinformatics, 2014. 30(4): p. 523-30.
7. Huang, D.W., et al., The DAVID Gene Functional Classification Tool: a novel biological module-centric algorithm to functionally analyze large gene lists. Genome Biol, 2007. 8(9): p. R183.
8. Varemo, L., J. Nielsen, and I. Nookaew, Enriching the gene set analysis of genome-wide data by incorporating directionality of gene expression and combining statistical hypotheses and methods. Nucleic Acids Res, 2013. 41(8): p. 4378-91.

PMI RESEARCH & DEVELOPMENT

This work was funded by Philip Morris International

52nd Congress of the European Societies of Toxicology,
4th to the 7th September, 2016, Seville