Estimation of smoking cessation benefits on cardiovascular risk via lipidomics and transcriptomics analysis of ApoE$^{-/-}$ mice liver

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Abstract

Aim: We investigated the effect of mainstream cigarette smoke (CS) exposure and a protocol of discontinuation of smoke exposure mimicking smoking cessation on liver lipid and transcriptome profile in the ApoE$^{-/-}$ mouse, a well-established model for human atherogenesis.

Methods: Livers obtained from ApoE$^{-/-}$ mice exposed to either (i) mainstream smoke of the 3R4F reference research cigarette for six months (CS), (ii) fresh air for six months (sham), or (iii) CS for 3 months followed by fresh air for 3 months (cessation), were extracted for lipids using a robotic-assisted method and analyzed on six different mass spectrometric platforms. Targeted and non-targeted mass spectrometry methods allowed quantification of more than 200 lipid species. In addition, the liver transcriptome was profiled on Affymetrix GeneTitan:GeneChip® HT MG-430 PM plates.

Results: In liver, exposure to cigarette smoke elevated several lipid species including free and esterified cholesterol, triacylglycerols, phospholipids, sphingomyelins, and ceramides. The concentration of triacylglycerols further increased upon smoke exposure cessation, correlating with the increase of body weight, whereas the concentration of phosphatidylglycerols and sphingomyelins was reduced. Gene set enrichment analysis of the transcriptomes between the 3 conditions revealed biological functions that are regulated by CS exposure in the liver of ApoE$^{-/-}$ mice: glutathione metabolism (detoxification), oxidoreduction (energy), coenzyme biosynthesis, phosphorus metabolic process and lipid biosynthesis. The enrichment of these gene sets was clearly reduced in the cessation setup.

Conclusions: In an established mouse model of atherogenesis, the detailed investigation of lipidomic and transcriptomic profiles was coupled. After taking into account other variables such as total body weight and body weight progression in the weeks before profiling; this study appears to highlight lipid species which may be differentially regulated by cigarette smoke exposure and smoking cessation when compared to sham.

Materials and Methods

Study. Partial results previously published (grey box) [1, 2] are included here to serve as a comparison anchor. Results obtained in liver (lipidomics and transcriptomics) of an experimental group mimicking smoking cessation are presented here for the first time.

Animals. 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). Filtered tap water was supplied *ad libitum* and changed daily. Exposure room was maintained at 21.8 ± 0.5 ºC and at a relative humidity of 54.7 ± 3.5%. Mice were observed daily for mortality, morbidity, and signs of overt toxicity or injury. Body weight was measured at least once per week during the exposure period.

Smoke Generation and Animal Exposure. The exposure regimen consisted of 3 1-hour periods a day and 30-minute intervals with fresh filtered air, 5 days a week. Total particulate matter (TPM) levels for 3R4F-exposed groups were targeted at 600 µg TPM/m^3.

Atherosclerotic Plaque Measurements in the Aortic Arch. These results were published in [2] and reproduced here to highlight the effect of smoking cessation on the atherogenesis endpoint, as compared to continuous smoking.

Lipidomics Analysis. Molecular lipids from liver were extracted and quantified by Zora Biosciences (Espoo, Finland) using synthetic non-endogenous standards as described in [8]. Lipids were normalized to their respective internal standard and tissue weight. The concentrations of molecular lipids are presented nmol/mg wet tissue.

Transcriptomics analysis. RNA preparation. Total RNA was extracted from homogenized liver using TRIzol® and quantified using the Nanodrop ND-1000 (peqlab); RNA integrity was determined using the Agilent 2100 Bioanalyzer. Microarray preparation. Transcriptome analysis was done following the manufacturer's recommendations in the GeneChip® HT 3' IVT Express Kit (Santa Clara, CA) guide. Ten µg of biotinylated fragmented cRNA was hybridized to Affymetrix GeneTitan:GeneChip® HT MG-430 PM for 16 hours. HT Array Plates were scanned using the HT Scanner (Affymetrix GeneChip® HT). Scanned image files were visually inspected for artifacts before being analyzed. Gene expression data analysis. Raw RNA expression data for each data set were analyzed using the affy and limma [3, 4] packages of the Bioconductor suite of microarray analysis tools [3] available for the R statistical environment [5]. Robust Microarray Analysis (RMA) background correction and quantile normalization were used to generate microarray expression values [6]. An overall linear model was fit to the data for all sample groups, and specific contrasts of interest were evaluated to generate raw p-values for each probe set on the expression array [4]. The Benjamini-Hochberg false discovery rate (FDR) method was then used to correct for multiple testing effects. Gene Set Enrichment Analysis (GSEA) was performed using the Confero platform [7]. Functional analysis of the differentially expressed genes, including prediction of potential upstream regulators, was performed using DAVID 6.7 [8]. While significant results were obtained for the CS vs. sham comparison at 6 month with a FDR<0.01, no significant results was obtained for the cessation vs. sham comparison [1]. Therefore, we present here GSEA results with a more permissive FDR threshold (<0.1).

Correlation between lipidomics and transcriptomics results.
Since samples for transcriptomics and lipidomics were obtained from the same animals, profiles were correlated at the animal level. Genes of interest were studied using NextBio [9].

Discussion & Outlook

In ApoE$^{-/-}$ mouse liver, approximately 500 molecular lipid species from 20 different lipid classes were quantified.
Although the plaque size in aorta suggested a decreased lipid accumulation in the smoking cessation group as compared to the continuous smoking group, results in liver showed different patterns, with an overall increased concentration of lipids measured in the cessation group. This increase was mostly due to TAGs, whereas the concentrations of other species, such as free cholesterol and sphingomyelin, were decreased.
Gene expression profiling in the liver indicate a much lower number of differentially regulated genes in the cessation group compared with the continuous smoking group. It is interesting to note that with little exception, the gene sets predicted to increase in the cessation group (compared to sham) are linked to lipid metabolism and oxidative phosphorylation, suggesting that lipids may be transported to the liver for further processing.
The expression profiles of a few genes correlated well with the levels of lipids measured in the same organs, making them interesting candidates for further mechanistic investigations. For example, the mouse gene 1810053B23Rik has a yet unknown function, but its expression patterns as shown in NextBio [9] seem relevant to the digestive system physiopathology.
The results of this study suggest that the approach may be a powerful tool for the investigation of disease mechanisms in vivo. This could have applications in the development of a systems biology-based assessment for comparison of the biological impact of Modified Risk Tobacco Products (As defined by the US FDA) with conventional cigarettes.

References

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Results

Body Weight Progression of Mice Is Slower in Group Exposed to 3R4F Reference Cigarette Compared to Sham

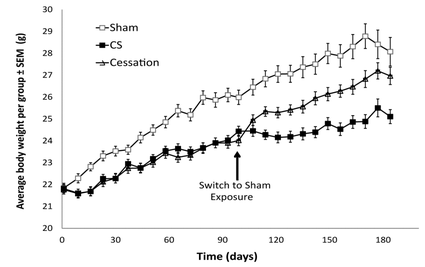


Figure 1 from Lietz *et al* [2]

3R4F Reference Cigarette Exposure Increases Atherogenesis (Aorta)

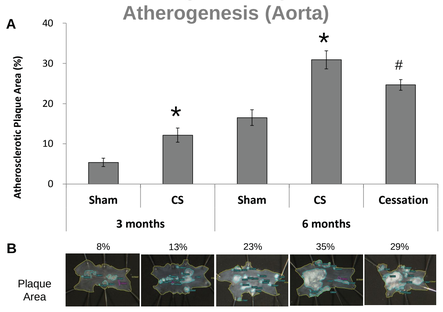


Figure 2 from Lietz *et al* [2]

GSEA: CS vs. sham - Liver - 6 months – FDR<0.01

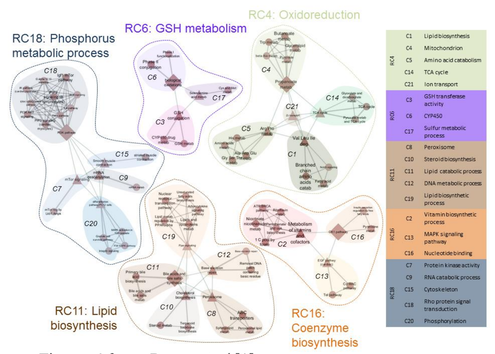
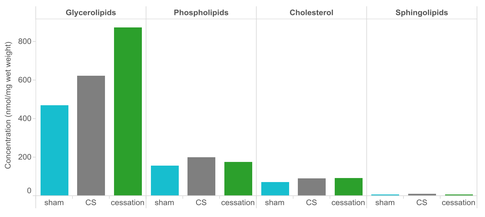
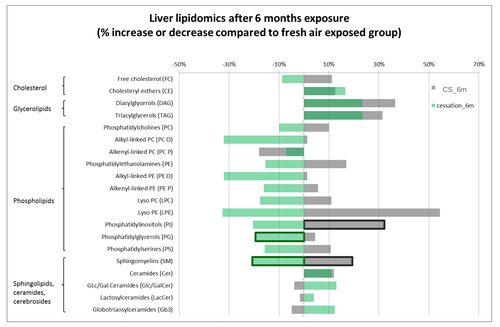


Figure 4 from Boue *et al* [1]

Total Lipid Concentration in Liver is Increased by CS and even more in Cessation Group Compared to Sham Group.

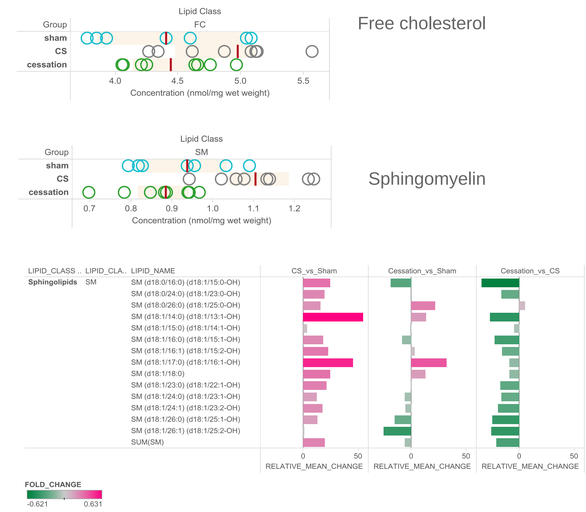


Increase in Lipid Concentration in Cessation Group is Mainly Driven by Triacylglycerols (TAGs)

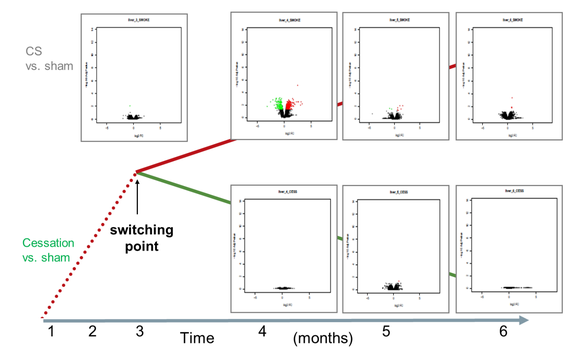


Lipidomics – Changes per lipid class between 3R4F reference cigarette or cessation-mimicking exposure protocol and sham exposure in liver of ApoE$^{-/-}$ mice. Changes reaching statistical significance of p<0.05 are marked with thick borders.

Quantification of Individual Lipid Species in Liver Shows Clearer Discrimination Between the Groups

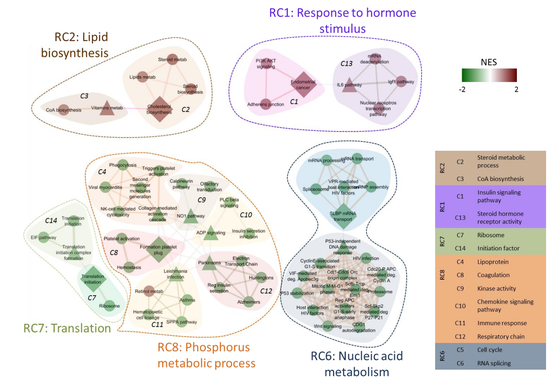


Gene Expression Profiles of Liver Samples in Cessation Group Return to Sham Levels



GSEA Reveals no Significant GeneSet in Cessation vs. Sham at 6 months (FDR<0.01)

GSEA: Cessation vs. Sham (6 months) - Liver – FDR<0.1



Lipid profiles can be correlated with transcriptomics profiles. For example, 1810053B23Rik expression levels increase with increased SM levels.
The exact function of this gene product is unknown and warrants further investigation.