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RNA-seq-based toxicogenomics shows limited impact of e-cigarette vapor on airway cells compared with cigarette smoke when matching for nicotine delivery

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Introduction

The use of electronic cigarettes (e-cigarettes) has increased significantly in recent years and potentially offer a safer alternative to conventional tobacco products. A variety of approaches have been used for the toxicological testing of e-cigarettes including *in vitro* models. Some have reported cytotoxicity and inflammatory responses, however, a number of *in vitro* studies have been designed with little or no data on the aerosol chemistry, limited consideration for dose, or utilizing an exposure system or matrix not relevant to the inhalation context.

Objective

We compared the transcriptional response of a primary 3D airway model acutely exposed to e-cigarette aerosol and cigarette (3R4F) smoke and included in our design:

(i) products with known aerosol chemistry at the selected smoking regime
(ii) dose normalization between the tested products based on the nicotine delivered to the exposure chambers and the cell inserts

Approach and Methods

Reconstituted primary airway epithelium grown at the air liquid interface (MucilAir™) obtained from one donor (#MD058501) were exposed to air, 3R4F reference cigarette smoke, and Vype ePen e-cigarette (18 mg nicotine/ml Blended Tobacco flavour e-liquid) aerosol using a Borgwaldt RM20S smoke machine. 3R4F were smoked at the Health Canada Intense (HCI) regime and the Vype ePen were smoke at the CORESTA recommended CRM81 regime for 1 hr. Two e-cigarette aerosol dilutions (1/3 and 1/7) were tested for equivalent and higher nicotine delivery compared to 3R4F (1/30). Nicotine was quantified by mass spectrometry in the exposure chambers media and in the cell inserts. Integrity of the tissue after treatments was assessed using a series of functional respiratory epithelia and protein markers. These included (i) TEER (trans-epithelial electric resistance), (ii) FOXJ1 and MUC5AC quantitative immunohistochemistry, and (iii) LDH release (cytotoxicity). RNA was extracted at 24hrs and 48hrs post exposure for RNA-seq. The experiments were performed 3 times independently with 3 cell inserts for each condition. qRT-PCR validation was performed with the fast PCR 7500 Applied Biosystems platform using RNA from 3 MucilAir™ donors (#MD058501, #MD008301, #MD009101) and independent exposure runs. Sets of 20 genes per treatment were selected from the RNA-seq data for qPCR screening in MucilAir™. The experiments and chambers design is summarized in **Figure 1**.

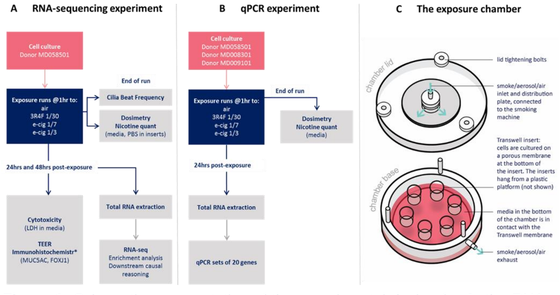

Figure 1: Schematic representation of the experimental design for **A.** the RNA-seq exposure runs, **B.** the qPCR exposure runs, and **C.** the format of an exposure chamber with cell inserts.

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Dosimetry – Nicotine delivered to the chambers' media and cell inserts during the 3R4F and e-cigarette aerosol exposure runs

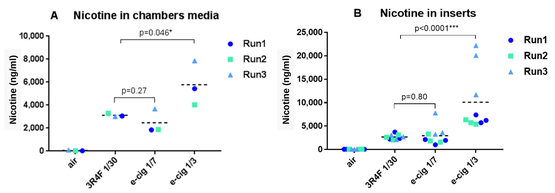

Figure 2: Scatter dot plot of nicotine delivered during the exposure runs in the basal media of the chambers (one measurement per chamber and run) (A) and in PBS filled inserts on the apical side (3 inserts per chamber and run) (B). The nicotine value points for each independent experimental run (Run1, 2, 3) have been labelled in different colors. The mean value is shown in each chart by the horizontal line. * and *** denote a t-test significance at p<0.05 and p<0.001, respectively.

Immunohistochemistry markers and TEER measured at 24hrs and 48hrs post-exposure to 3R4F and e-cigarette aerosols

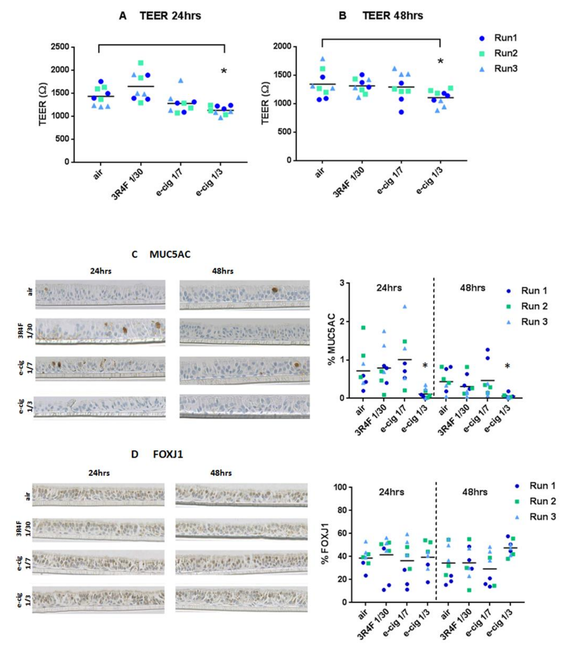

Figure 3: MucilAir™ functional markers after exposure to air, 3R4F smoke 1/30 and e-cigarette aerosol 1/7, 1/3. TEER at 24hrs (A) and 48hrs (B) post treatment. MUC5AC (C) and FOXJ1 (D) quantitative immunohistochemistry at 24hrs and 48hrs post treatment with the micrographies on the left and values plots on the right with the mean indicated by the horizontal line. * denotes a significant difference versus air control at p<0.05.

RNA-seq differential expression of MucilAir™ cells exposed to 3R4F smoke and e-cigarette aerosol

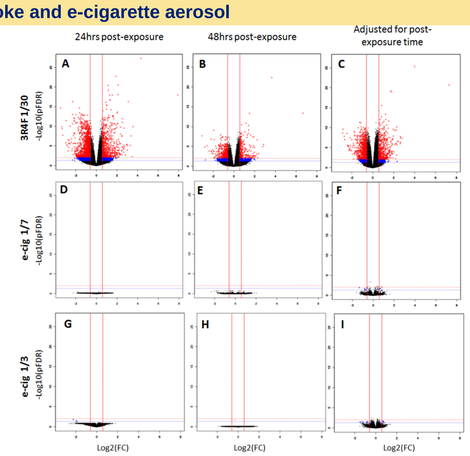

Figure 4: Volcano plots for 9 RNA-seq contrasts. On top, air vs 3R4F (1/30) at 24hrs (A), 48hrs (B) post exposure, and adjusted for post exposure time (C). In the middle, air vs e-cigarette (1/7) at 24hrs (D), 48hrs (E) post exposure, and adjusted for post exposure time (F). At the bottom, air vs e-cigarette (1/3) at 24hrs (G), 48hrs (H) post exposure, and adjusted for post exposure time (I). The horizontal red line shows the 0.01 pFDR threshold. The vertical red lines show the +1.5 and -1.5 fold change thresholds.

Hierarchical clustering and GOlden eye plots for the top enriched genesets and selected GO terms for MucilAir™ exposed to 3R4F smoke and e-cigarette aerosol

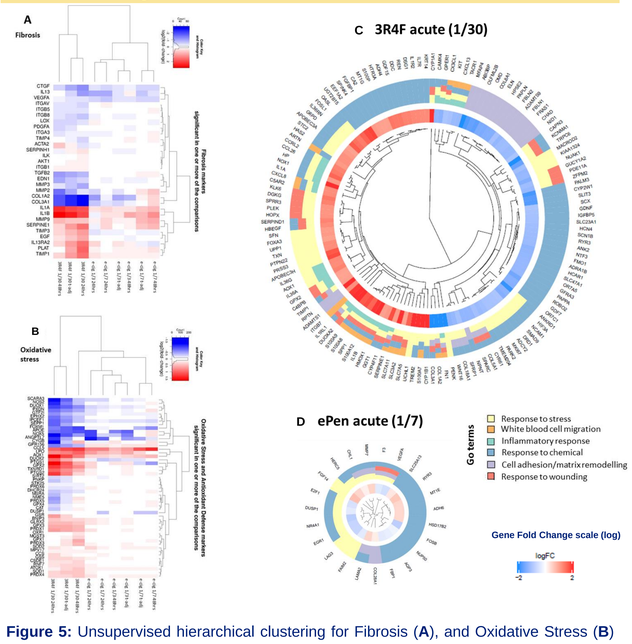

Figure 5: Unsupervised hierarchical clustering for Fibrosis (**A**), and Oxidative Stress (**B**) genesets. Gene markers significant at pFDR<0.05 in one or more of the treatment contrasts are shown. GOlden eye plots for six GO categories for each treatment adjusted for time. 3R4F 1/30 smoke dilution (pFDR<0.01, FC>2, adjusted for time) (C), e-cigarette aerosol exposure at 1/7 dilution (pFDR<0.05, adjusted for time) (D) are presented.

qPCR validation of genes candidates identified in the RNA-seq approach and applied to MucilAir™ from 3 donors

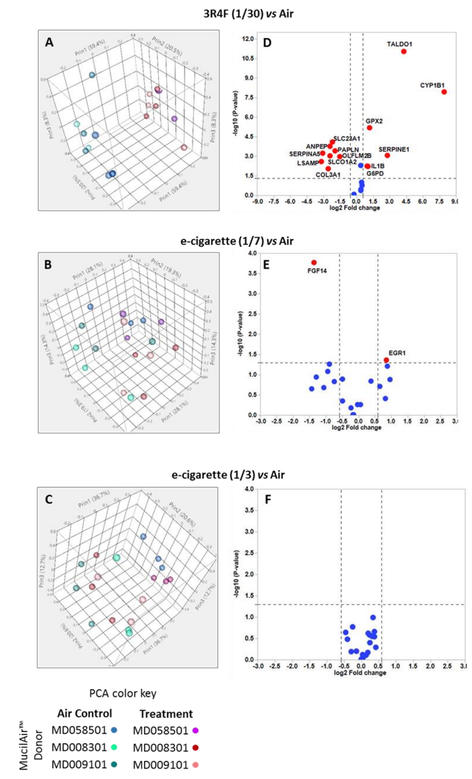

Figure 6: qPCR validation of RNA-seq data in MucilAir™ from 3 different subjects at 24hrs post-exposure. 20 genes for each treatment were selected from the RNA-seq data. PCA plots of qPCR data comparing treatments (air vs 3R4F 1/30 (A), e-cigarette 1/7 (B) and e-cigarette 1/3 (C)) and subjects (#MD058501, #MD008301, #MD009101). Corresponding volcano plots showing the response for each individual gene using the data from the 3 donors (3R4F 1/30 (D), e-cigarette 1/7 (E) and 1/3 (F)). Dots colored in red indicate differentially expressed genes significant at p<0.05 and with a FC>[1.5]. The red dots are labelled with the gene name.

Conclusions

- Based on aerosol dilutions delivering equivalent or higher nicotine, Vype ePen e-cigarette had limited or no impact on transcriptional regulation compared to 3R4F at the tested smoking regime
- 3R4F exposure enrichment analysis identified perturbations for oxidative stress response, inflammation and tissue remodelling response pathways
- A qPCR validation of representative genes selected from the RNA-seq data using cells from three different subjects confirmed the RNA-seq results
- In perspective, repeated exposures to the different aerosols could give us further insights into the differential response triggered by 3R4F and e-cigarettes

Conflict of interest statement: All the authors were employed by British American Tobacco (Investments) Ltd at the time this study was conducted, and the research was funded by British American Tobacco (Investments) Ltd. Elements of this work were conducted Fios Genomics Ltd. as part of a commercial contract.

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Introduction: There is increasing evidence from *in vitro* testing that electronic cigarettes (e-cigarettes) cause minimal damage to cell systems, however some studies reported cytotoxicity and inflammatory responses. These discrepancies arise from the use of different cell models, products, exposure matrices, times, and doses. Ideally, a comprehensive *in vitro* assessment of e-cigarettes should use a relevant cell model and a well-considered exposure strategy.

Aim: In this study we compared the transcriptional response of a primary 3D airway model (MucilAir™) exposed for one hour to e-cigarette (Vype ePen) vapor and smoke from a reference combustible cigarette (3R4F). The originality of this work is a careful consideration for the dose where instead of matching the exposure between different products based on puff number, we used nicotine as a surrogate for dose matching.

Results: The average nicotine delivered to the cells from 3R4F smoke at a 1:30 dilution was matched at a dilution of 1:7 for the e-cigarette. One additional e-cigarette dilution of 1:3 was also tested for higher nicotine delivery. RNA was extracted for RNA-seq from the tissues at 24hrs and 48hrs post exposure. 873 and 205 RNA features were differentially expressed for 3R4F smoke at 24hrs and 48hrs post exposure using a pFDR<0.01 and a fold change>2 threshold, respectively. Differentially expressed RNA from e-cigarettes (49 RNA at 1:7 dilution and 113 RNA at 1:3 dilution) could only be identified using a looser threshold of pFDR<0.05, no fold change filter, and by pooling the two time points to increase statistical power. Gene set enrichment analysis revealed a clear response from lung cancer and fibrosis associated genes after 3R4F smoke exposure. Using the less robust thresholds, glucagon metabolism pathway and processes relating to the extracellular matrix were identified for e-cigarette exposures, albeit with a low degree of confidence.

Conclusion: Based on equivalent or higher nicotine delivery, an acute exposure to Vype ePen vapor has very limited impact on gene expression compared to 3R4F smoke exposure.

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