P3-217 Systems toxicology assessment of the effect of repeated cigarette smoke exposure on human respiratory tract tissue cultures

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INTRODUCTION

Cigarette smoke (CS) is a complex mixture of chemicals eliciting oxidative stress and inflammation, which are linked to the adverse effects of smoking in respiratory tissues. These effects were investigated using two human organotypic bronchial and nasal tissue models grown at the air/liquid interface (Epithelix MucilAir™-HF). These two *in vitro* systems mimic human nasal and bronchial tissue:

- with the morphological characteristics of a pseudo-stratified epithelium composed of basal cells, mucus-releasing cells and ciliated cells
- with the advantage of a co-culture with fibroblasts.
- with active ion transport
- with the capacity to secrete inflammatory markers upon challenge.

Human organotypic bronchial tissue cultures mimic the clinical situation more closely than primary monolayer cultures, thus providing more meaningful risk assessment tools [1].

The aim of our study was to investigate the impact of repeated whole CS exposure (Fig. 1) on human bronchial and nasal tissue cultures and to determine the effect in both tissue types which are in primary contact with CS upon inhalation *in vivo*.

The air/liquid interface of each culture allows a direct exposure to whole smoke using the Vitrocell® system connected to a smoking robot (Fig. 2). Both tissues have been exposed in parallel to various CS concentrations ranging from 8% to 35% (diluted with humidified air (vol/vol) or to only humidified air (control) in order to establish the highest dose of CS which does not induce more than 20% cell death in both nasal and bronchial tissue cultures. Consequently we selected two doses of CS (low dose: 10% and high dose: 16%) and exposed both tissue cultures in parallel as shown in Fig. 1. Various biological endpoints (Table 1) were captured at different post-exposure time points in triplicates. Using different approaches, we compared the CS response at the gene expression level for: (i) *in vivo*[3] versus *in vitro* systems (Fig. 7), and (ii) CS-exposed nasal and bronchial organotypic tissue cultures (Fig. 8).

MATERIALS & METHODS

Figure 1: The smoking behavior of a light smoker during one day was mimicked by exposing repeatedly the tissues to a total of 4 cigarettes (mean of Total Particles Matter deposition measured after each cigarette = 2.8 microgram/cm2 +/- SEM = 0.5 with n=24 cigarettes) with one hour interval between each cigarette.

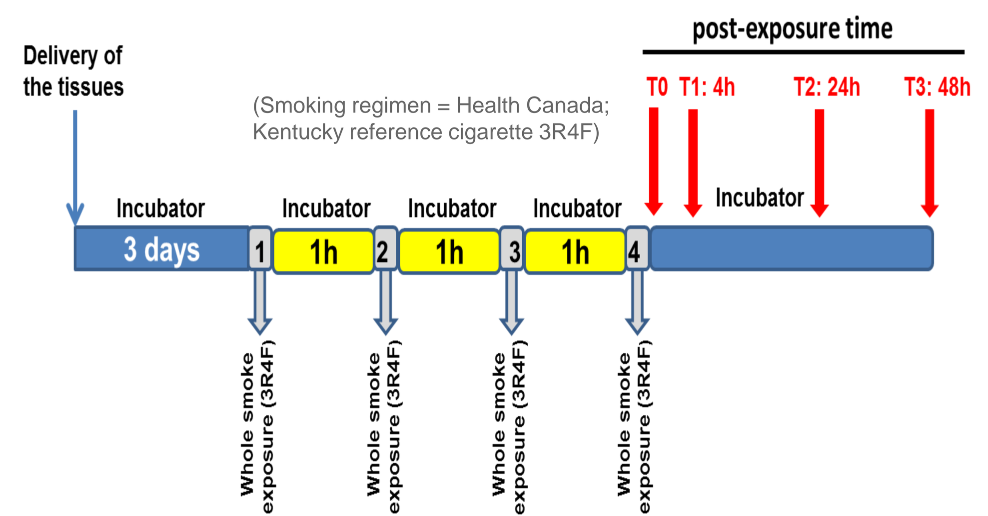


Figure 2: Schematic view of the whole CS exposure system and the exposure chamber (Vitrocell®).

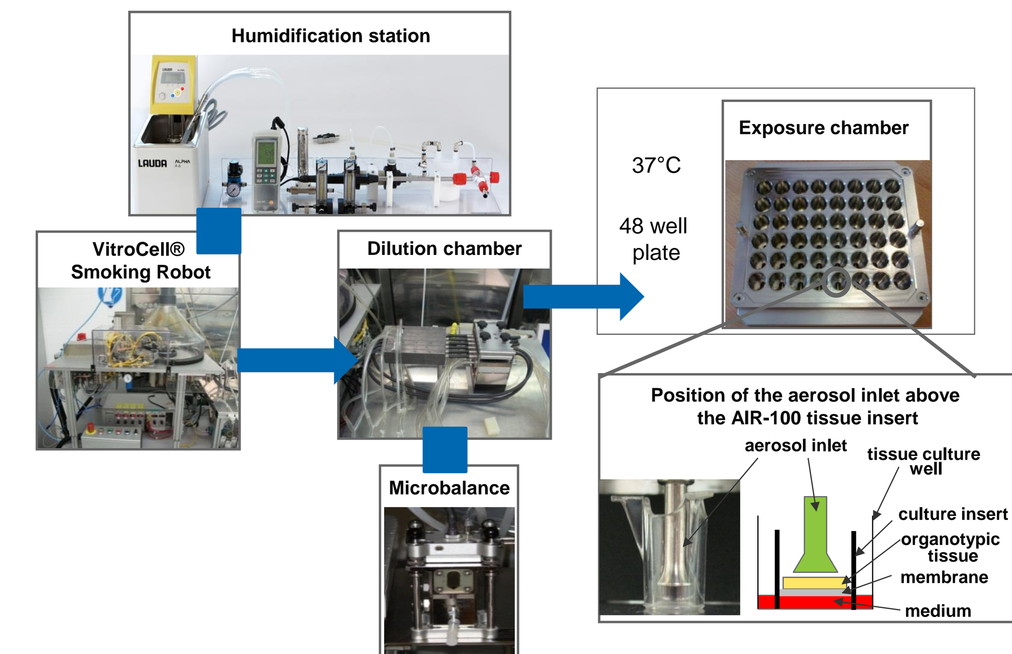


Table 1: List of the biological endpoints measured at different post-exposure time points.

Biological endpoints	Post-exposure time points			
	0	4h	24h	48h
Cytotoxicity (TEER, LDH assay, Resazurin)			X	X
Gene expression profiles (Affymetrix human genome U133 plus 2 array)	X	X	X	X
MicroRNA expression profiles (GeneChip miRNA 3.0 array)	X	X	X	X
Histology (Hematoxylin/Eosin & Alcian Blue)			X	
Immunohistology (P63, Ki67, beta-Tubulin, Muc5AC)			X	
Release of pro-inflammatory markers (MAP)			X	X

RESULTS

Figure 3: (A) Cytotoxicity (LDH assay) and (B) tissue integrity (TEER measurement) of nasal and bronchial organotypic tissue cultures were measured 24 h after exposure to humidified air or to 10% or 16% CS. For the LDH assay, a positive (2h treatment with 10% Triton X-100) and a negative (2h treatment with PBS) control were included.

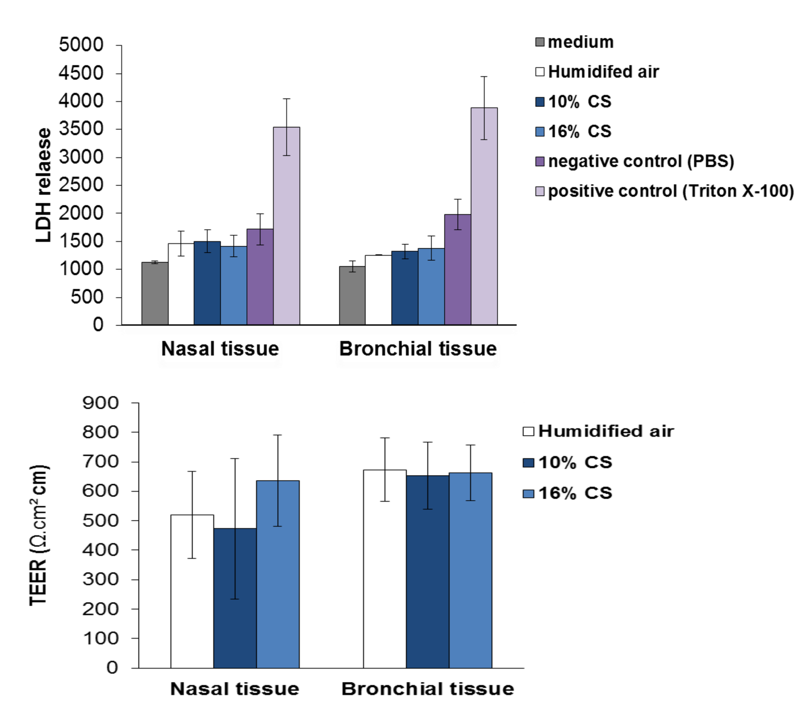


Figure 4: Comparison of the release of IL-8, VEGF and MMP-1 protein in the culture medium of air- and CS-exposed nasal (NE) and bronchial (BR) tissue cultures after 24h and 48h post-exposure. Measurements were performed on three biological replicates for each condition using a multiplex kit (Millipore). Nasal and bronchial tissue cultures exposed for 24h with 10ng/ml TNF-α and 10ng/ml IL-1β were used as positive controls (data not shown). P value: *<0.05; **<0.01; ***<0.001; ***<0.0001.

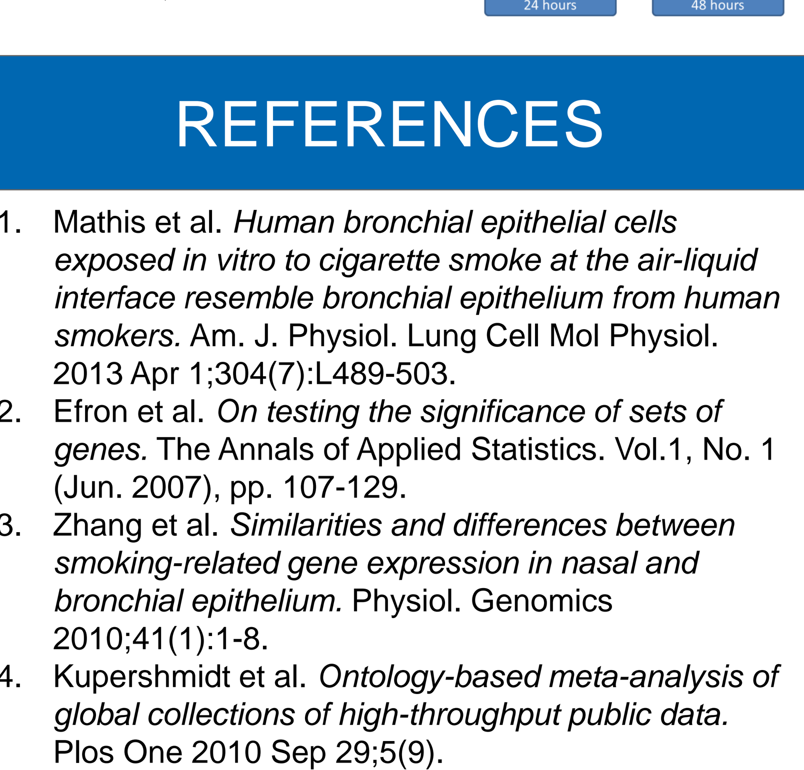


Figure 5: Volcano plot performed from the whole gene expression array demonstrating the global gene expression changes related to 16% CS which occurred during different post-exposure times (0h, 4h, 24h, 48h) in nasal and bronchial tissue cultures. Coefficient presents log2 based fold change.

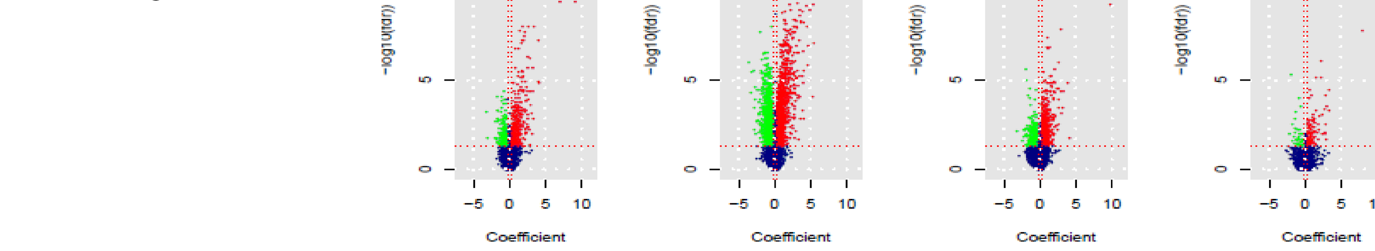


Figure 7: Using gene set analysis (GSA[2]) approach, a comparison between *in vivo* (GSE16008, Zhang et al.[3]) and *in vitro* response to CS exposure was performed in both nasal epithelial tissue (A) and in bronchial epithelial tissue (B). GS.UP and GS.DN denotes sets of significantly up- and downregulated genes in *in vivo* nasal or bronchial epithelium tissue when comparing smokers vs. non-smokers. NES = Normalized enrichment score. ***: FDR<0.001; **: FDR<0.01; *: FDR<0.05. The GSA approach was used to assess the enrichment of GS.UP and GS.DN in the transcriptomic profiles of *in vitro* tissue after different post-exposure times.

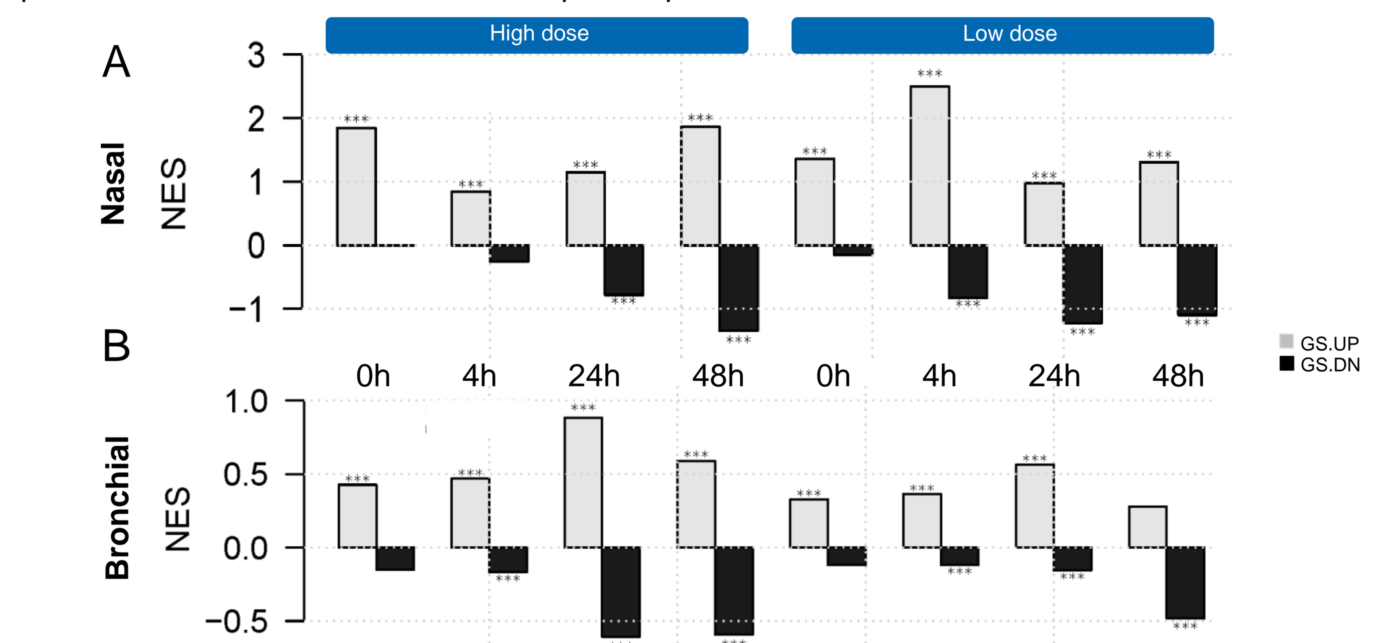


Figure 6: Untreated bronchial and nasal organotypic cultures were immunostained with A) anti-p63 antibody to detect basal cells; B) with anti-MUC5AC antibody to label mucus secreting cells and C) after Hematoxylin & Eosin staining, fibroblasts layer (arrows) and ciliated cells (stars) were visible.

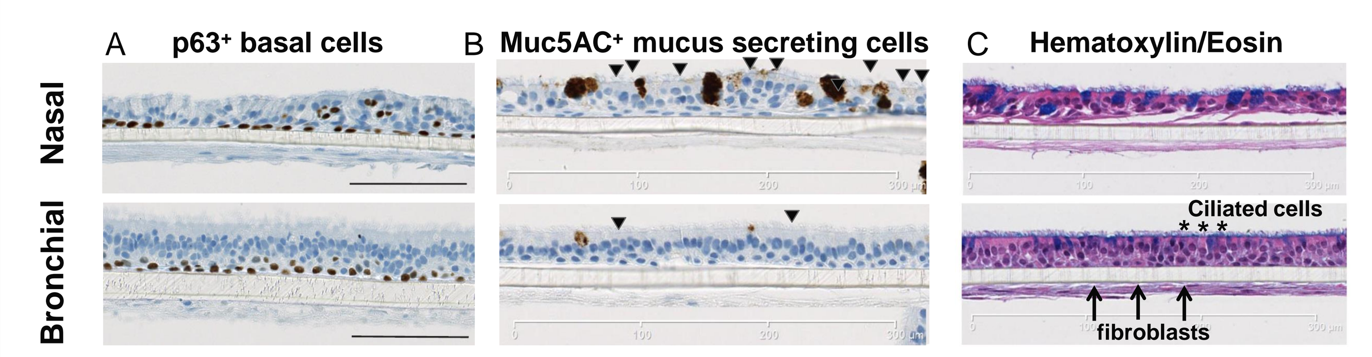
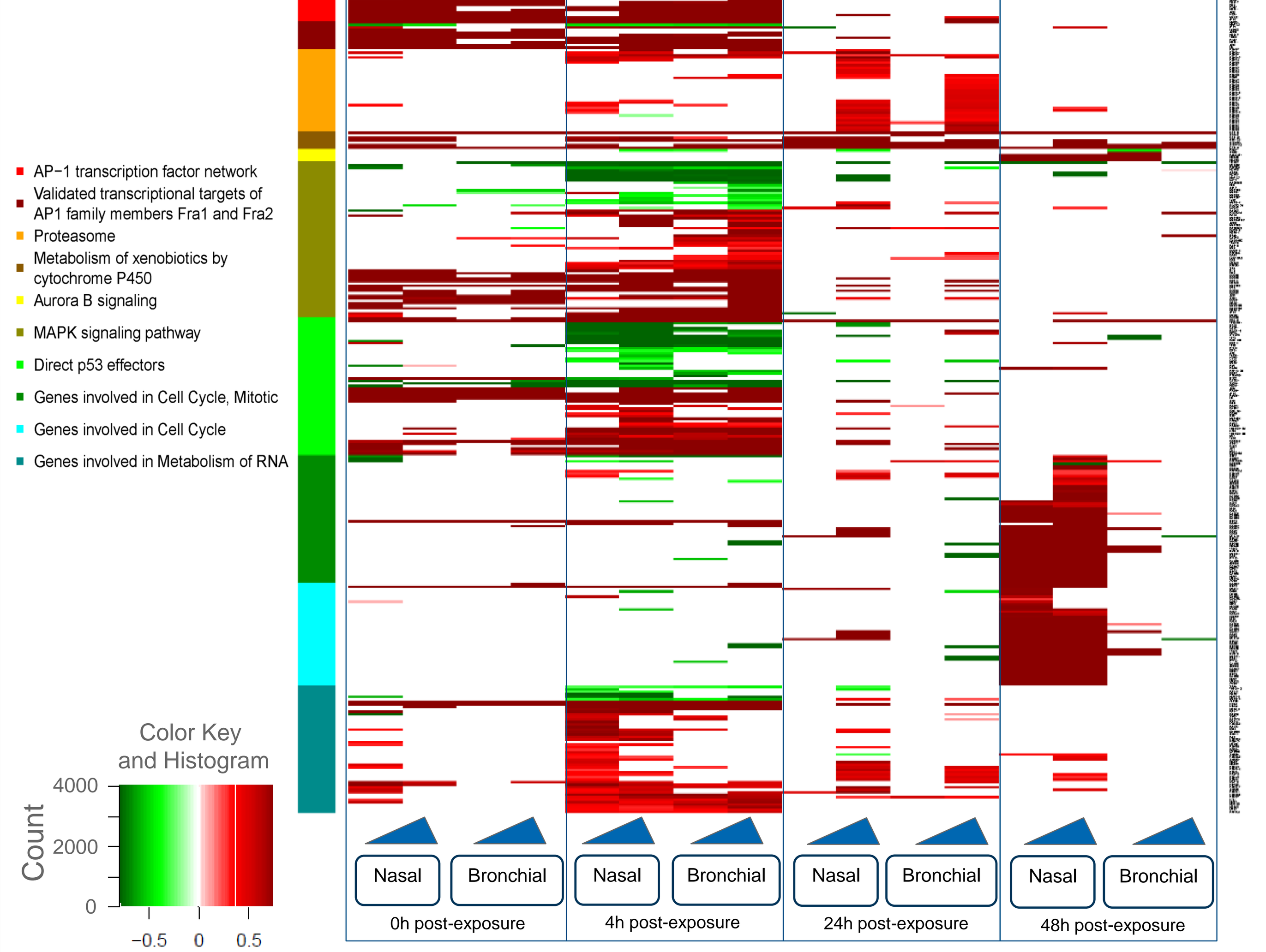


Figure 8: Heatmap of fold change for significantly regulated genes that overlap with known canonical pathways in NextBio® platform[4]. The gene list were identified in the highest ranked pathways for each contrast. All contrasts are scored against the selected genes. Red and green colors denote significant up and down regulation (FDR ≤ 0.05) when compared to sham condition.



CONCLUSION

- We took advantage of the availability of well differentiated human bronchial and nasal organotypic cultures (Fig. 6), that mimic *in vivo* tissues, to assess and compare across both tissue models the impact of repeated direct exposure of whole CS (Fig. 1) using the Vitrocell® system (Fig. 2). CS doses were chosen to cause less than 20% cytotoxicity (Fig. 3) and various biological endpoints (Table 1) were captured over different post-exposure time points (0h, 4h, 24h and 48h).
- At the highest CS dose (16%), the global gene expression changes represented by the volcano plots in Fig. 5 were more intense 4h after exposure in both tissue cultures and decreased at later post-exposure time points (24h and 48h). A stronger response of the nasal organotypic tissue was noticeable 48h after exposure compared to the exposed bronchial epithelial culture.
- We observed a similar dose- and time-dependent regulation of the release of pro-inflammatory markers (IL-8, VEGF, MMP-1) in both CS-exposed nasal and bronchial tissue cultures (Fig. 4).
- This result could reflect the effect of CS exposure on the fibroblasts present on the basal side of the culture or indicate a similar response of the bronchial and the nasal epithelium to CS.
- The GSA[2] allowed us to compare published[3] *in vivo* datasets from nasal and bronchial epithelial cells obtained from the same donors, smoker and non-smoker, with our *in vitro* data (Fig. 7). A significant enrichment of the set of genes differentially upregulated in smokers bronchial epithelial cells was noticed when compared to low and high dose of CS-exposed *in vitro* counterparts at all post-exposure time points tested (Fig. 7A). For *in vivo* down-regulated gene sets, the enrichment within the *in vitro* dataset was only significant at the latest post-exposure time points (24h and 48h) for low and high doses and 4h after exposure for the low dose. Thus, at the gene expression level, nasal epithelial culture exposed repeatedly to CS can mimic molecular changes occuring *in vivo* in smokers nasal epithelium. A similar observation was found when comparing epithelial cells obtained by bronchial brushings of the right main stem bronchus in smokers to CS-exposed bronchial organotypic cultures. This result is in agreement with our previous report [1] demonstrating the relevance of human bronchial epithelial tissue culture for investigating CS effect s well as future modified risks tobacco products.
- Finally, using the NextBio® platform[4], we further investigated the dose- and time-dependent molecular changes which are similar or different between both exposed-tissue cultures (Fig. 8). Similar responses to CS were observed in both types of tissue cultures, such as the activation of AP-1 pathway or of the xenobiotic metabolism at early post-exposure time points (0h and 4h). The most obvious difference observed between the two tissue response was found 48h after exposure and was related to the up-regulation of genes involved in cell cycle in nasal tissue culture, probably indicating a recovery process specific to the nasal epithelium.

REFERENCES

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