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SYSTEMS TOXICOLOGY ASSESSMENT OF AEROSOL FROM A CANDIDATE MODIFIED RISK TOBACCO PRODUCT COMPARED WITH CIGARETTE SMOKE ON ORGANOTYPIC GINGIVAL EPITHELIAL CULTURES [1]

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SUMMARY

Introduction. Smoking is one of the major lifestyle-related risk factors for periodontal diseases [1]. Smoking can affect the structure of the epithelial mucosa, impair the inflammatory response, and change the redox status of the oral cavity. Tobacco harm reduction through the development of candidate Modified Risk Tobacco Products (MRTP) provides a promising opportunity for adult smokers who would otherwise continue cigarette smoking. An MRTP is defined by the U.S. Family Smoking Prevention and Tobacco Control Act as "any tobacco product that is sold or distributed for use to reduce harm or the risk of tobacco related disease associated with commercially marketed tobacco products". The Tobacco Heating System (THS) 2.2 is a candidate MRTP based on a heat-not-burn technology that uses a precisely controlled heating device into which a specially designed tobacco stick is inserted and heated to generate an aerosol [2].

Objectives. The objective of the study was to assess – using a systems toxicology approach— how aerosol from THS2.2, compared to reference (3R4F) cigarette smoke (CS), affects human gingival epithelial organotypic cultures.

EXPERIMENTAL DESIGN / METHODS

Human gingival epithelial organotypic cultures. EpiGingival™ (MatTek corp., Ashland USA) derived from a 46 year old male donor, non-smoker.

Histological analysis. Tissue sections were stained with Hematoxylin & Eosin (HE). For immunohistochemical staining, the slides were incubated with an E-cadherin antibody (Leica Biosystem PA0387, undiluted) and counterstained with hematoxylin.

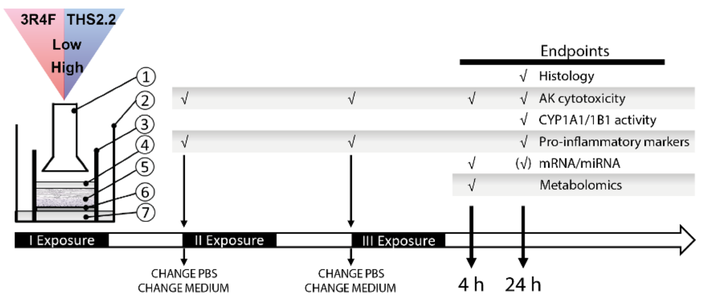
Adenylate Kinase (AK)-based cytotoxicity. The activity of AK was measured in the basolateral medium using the ToxiLight™ bioassay kit (Lonza, Rockland, MA, USA).

Pro-inflammatory mediators. Pro-inflammatory mediators were measured in the basolateral medium using a Luminex®-based technology (Luminex, Austin, TX, USA).

Microarray data processing and analysis. Transcriptomics data were analyzed in the context of hierarchically structured network models as described in [3]. The effects of exposure were quantified by scoring the impact on each subnetwork (referred to as "network perturbation amplitude", NPA) [4] .

Metabolomic analysis. Metabolites were analyzed in collaboration with Metabolon inc. (Durham, USA) [1].

A



B

Group	Name reported	Smoke/aerosol concentration (%)	Nicotine concentration measured in PBS (mg/L; average ± SEM)	Nicotine dose deposited in 100 µL PBS (µg/insert/28 min; average ± SEM)
3R4F low concentration	3R4F (Low)	25	49.4 ± 1.89	4.94 ± 0.189
3R4F high concentration	3R4F (High)	35	84.6 ± 1.43	8.46 ± 0.143
THS2.2 low concentration	THS2.2 (Low)	75	54.6 ± 2.60	5.46 ± 0.260
THS2.2 high concentration	THS2.2 (High)	100	100.4 ± 4.83	10.04 ± 0.483

C

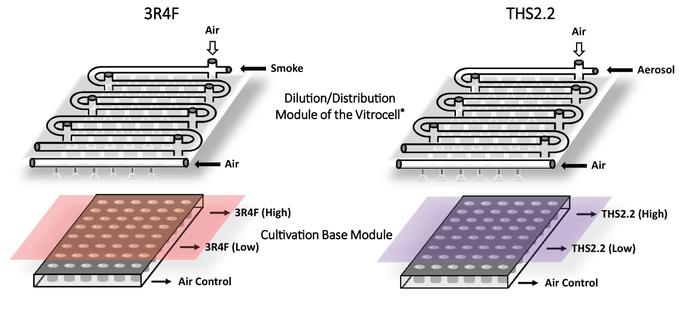


Figure 1. Study design and exposure system. (**A**) Human gingival epithelial organotypic cultures were exposed for 3 consecutive days to 28 min 3R4F CS or THS2.2 aerosol at two matching concentrations. Before each exposure, basolateral medium was collected for different assays (AK and cytokine assays) and replaced with fresh medium; apical PBS was replaced before each exposure. Different endpoints were analyzed at the indicated time points during three experimental repetitions (with three independent exposure runs each, n=9 total). (√) for 24 h mRNA/miRNA endpoint indicates that only one experimental repetition (n=3) was performed (see [1] for 24h results). 1, aerosol inlet; 2, culture well; 3, culture insert; 4, apical PBS; 5, organotypic culture; 6, membrane; 7, medium. (**B**) Selected CS/aerosol concentrations and matching delivered nicotine doses. (**C**) Vitrocell dilution and exposure system.

RESULTS

CYTOTOXICITY AND TISSUE MORPHOLOGY

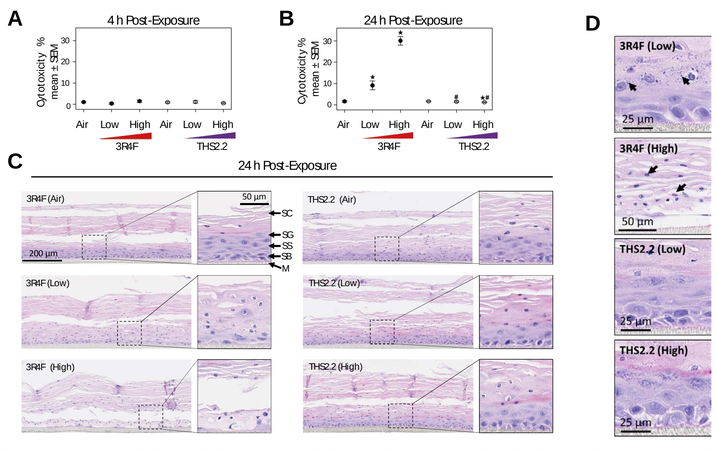


Figure 2. Cytotoxicity and tissue morphology of organotypic gingival cultures exposed to 3R4F CS and THS2.2 aerosol. Mean AK levels were assessed at 4 h (**A**) and 24 h (**B**) post exposure. AK levels were normalized to values of the positive control (Triton-X-treated cultures, considered 100% cytotoxicity). Error bars indicate SEM. * p<0.05, compared with the corresponding air control; # p<0.05, compared with matching concentrations of 3R4F CS. n=9. (**C**) Representative images of HE-stained gingival cultures. (**D**) Arrows indicate keratohyalin granules (3R4F (Low)) or parakeratosis (3R4F(High)). n=9. Abbreviations: M, membrane; SB, stratum basale, SS, stratum spinosum; SG, stratum granulosum; SC, stratum corneum.

GENE EXPRESSION IMPACT OVERVIEW

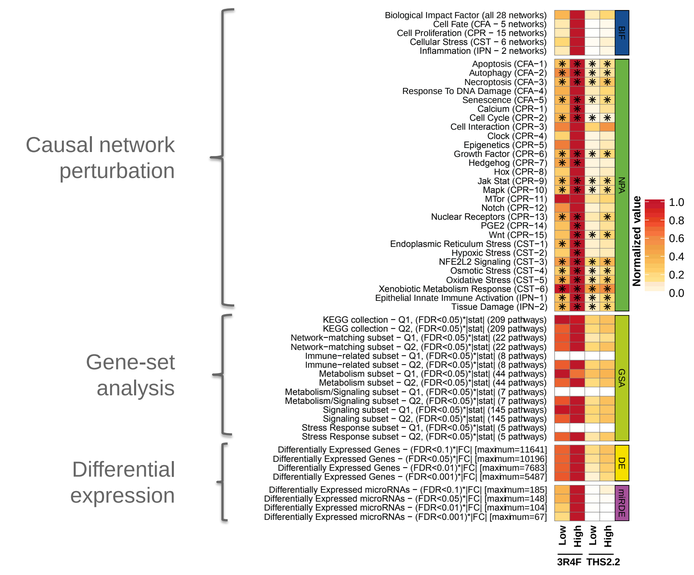


Figure 3. Overview of the impact of 3R4F CS or THS2.2 aerosol exposures on differential expression of genes. Values are normalized to the interval [0, 1] in a row-wise manner. The uppermost panel displays the overall biological impact factor (**BIF**) and the relative BIFs for four network families (cell fate and angiogenesis (CFA), cell proliferation (CPR), cellular stress (CST), and pulmonary inflammation (IPN)) assessed by a causal network approach [4,5]. The network perturbation amplitudes (**NPA**) for the individual networks are shown in the next panel. The "*" indicates statistically significant network perturbations. Overall results of gene set analyses (**GSA**) are displayed in the next panel for the KEGG collection and the two standard statistical tests (Q1 and Q2). Also shown are specific subsets of the KEGG collection: first, the 22 pathways matching the mechanistic networks, and second, the five broad categories of the 228 pathways contained in the KEGG collection. Displayed values were defined as sums of absolute values of gene set-level statistics (i.e. fold-change mean) for the statistically significant gene sets in each category. The two lower panels show the number of differentially expressed genes (DE) and miRNAs (miRDE) for four distinct statistical significance thresholds, to identify possible threshold effects. Again, sums of absolute values of fold-changes of statistically significant genes or miRNAs are displayed. n=6-9.

OXIDATIVE STRESS

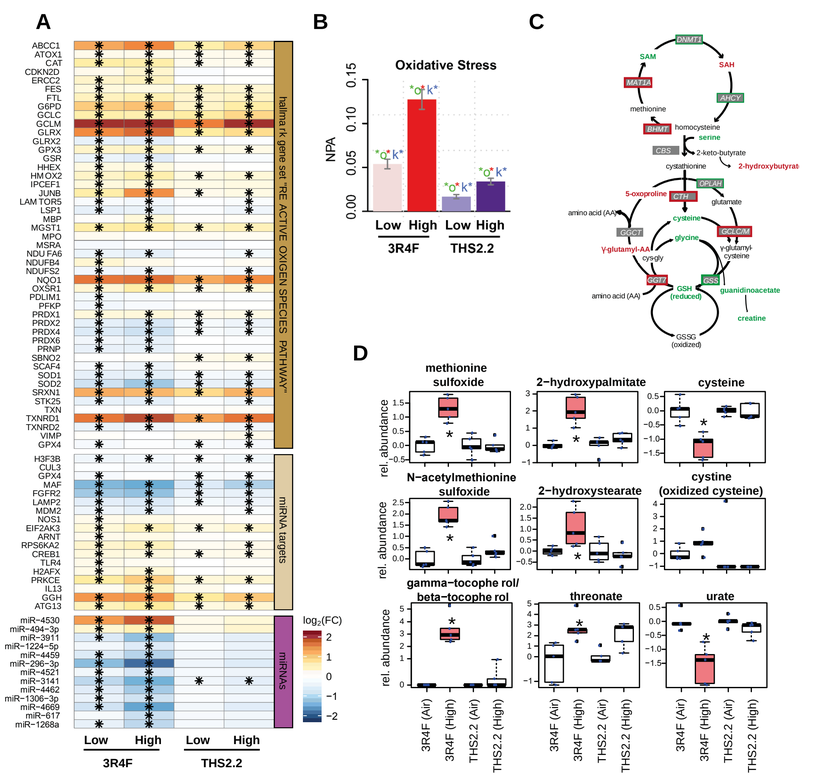


Figure 4. Differential induction of oxidative stress by 3R4F CS and THS2.2 aerosol (**A**) Induction of oxidative stress response program: differential expression heatmap for genes of the reactive oxygen species pathway (HALLMARK_REACTIVE_OXIGEN_SPECIES_PATHWAY; M5938), as well as for genes and miRNAs belonging to the "Oxidative Stress" candidate miRNA-mRNA network (see [1] for details). The "*" indicates statistically significant differential expression (FDR <0.05). n=6-9. (**B**) Assessment of exposure effects on the "Oxidative Stress" network. Bars show overall network perturbation amplitudes (NPA scores) based on transcriptomics data. Error bars delimit their 95% confidence intervals. Three statistics are shown: the red star indicates statistical significance with respect to the biological replication (i.e. 95% confidence intervals do not contain the 0 value), while the green and red stars indicate significant specificity statistics with respect to the network structure ("O" and "K" statistics). n=6-9. (**C**) Summary of exposure effects on glutathione and related metabolic reactions (see [1] for details). Significantly up- or down-regulated metabolites and genes are marked with red and green, respectively. (**D**) Metabolomics profiling was conducted 4 h after exposure of the tissue to high 3R4F CS and THS2.2 aerosol concentrations. Boxplots summarize the response of metabolites sensitive to oxidative stress (blue dots indicate individual samples, n=5). Significant differences between exposed groups and their respective sham groups are indicated by filled colored boxes and a star ("*" means FDR <0.05).

XENOBIOTIC METABOLISM

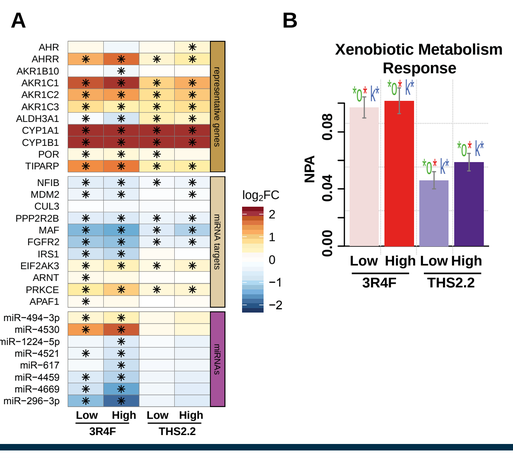
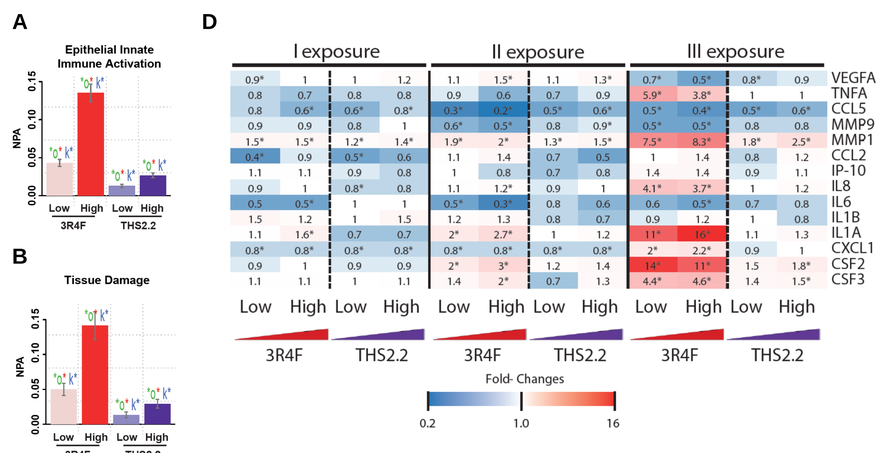


Figure 5. Xenobiotic metabolism in 3R4F CS- and THS2.2-exposed gingival cultures. (**A**) Heatmap shows differential expression of genes representative of xenobiotic metabolism as well as of genes and miRNAs belonging to the "Xenobiotic Metabolism" candidate miRNA-mRNA network. The "*" indicates statistically significant differential expression (FDR <0.05). n=6-9. (**B**) Assessment of exposure effects on the "Xenobiotic Metabolism Response" network (for details on the statistics see Figure 4B legend). n=6-9.

INFLAMMATION



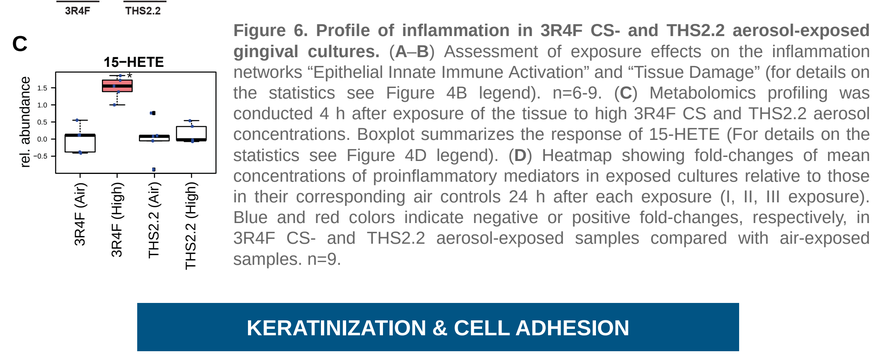


Figure 6. Profile of inflammation in 3R4F CS- and THS2.2 aerosol-exposed gingival cultures. (**A**–**B**) Assessment of exposure effects on the inflammation networks "Epithelial Innate Immune Activation" and "Tissue Damage" (for details on the statistics see Figure 4B legend). n=6-9. (**C**) Metabolomics profiling was conducted 4 h after exposure of the tissue to high 3R4F CS and THS2.2 aerosol concentrations. Boxplot summarizes the response of 15-HETE (For details on the statistics see Figure 4D legend). (**D**) Heatmap showing fold-changes of mean concentrations of proinflammatory mediators in exposed cultures relative to those in their corresponding air controls 24 h after each exposure (I, II, III exposure). Blue and red colors indicate negative or positive fold-changes, respectively, in 3R4F CS- and THS2.2 aerosol-exposed samples compared with air-exposed samples. n=9.

KERATINIZATION & CELL ADHESION

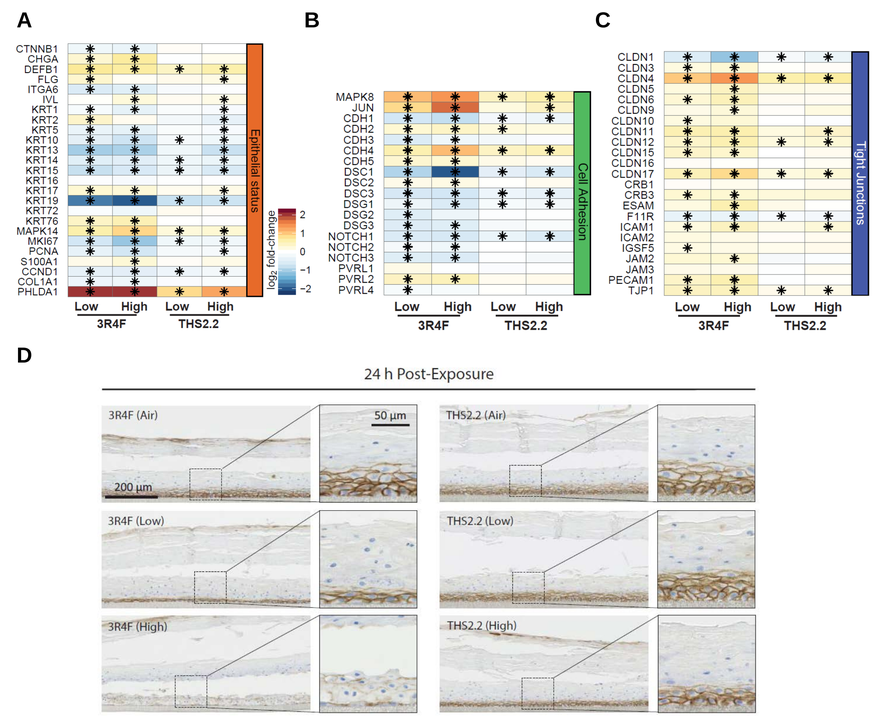


Figure 7. Keratinization and cell–cell adhesion in 3R4F CS- and THS2.2 aerosol-exposed samples. (**A**, **B**, **C**) Heatmaps showing differential expression of genes related to epithelial status/cell type, cell adhesion, or tight junctions. (for details on the statistics see Figure 4B legend). N=6-9. (**D**) Representative images of E-cadherin-stained gingival culture sections observed 24 h after the last exposure. Magnification is set at 20× and 63× for the insets. n=9.

CONCLUSIONS

- Systems toxicology approach was applied for the assessment of THS2.2 aerosol compared to CS on an organotypic gingival epithelium model. Multiple endpoints (e.g., cytotoxicity, transcriptomics, and metabolomics) were combined toward a comprehensive assessment of the exposure effects.
- Major morphological alterations (loss of cell adhesion, keratinization, Figure 7) and cytotoxicity (max. ~30%, Figure 2) were observed after CS exposure but were limited, if none, upon exposure to THS2.2 aerosol.
- Transcriptomic and metabolomic analysis indicated a general reduction of the impact in THS2.2 aerosol-exposed samples with respect to CS (~79% lower biological impact for the high THS2.2 aerosol concentration compared to CS, and 13 metabolites significantly perturbed upon THS2.2 aerosol exposure vs. 181 for 3R4F CS).
- Proinflammatory mediator analysis showed a higher impact in CS-exposed cultures compared to THS2.2 aerosol, with 11 analytes significantly altered by CS *vs.* 5 (common to CS-exposure condition) by THS2.2 aerosol, showing a reduced fold-change with respect to CS (Figure 6).
- Biological effects induced by CS, such as oxidative stress, xenobiotic metabolism, and inflammation-related processes, are relevant to the pathophysiology of periodontal diseases.
- Overall, THS2.2 aerosol had a statistically significantly lower impact on molecular processes associated with the pathophysiology of human gingival organotypic cultures compared to CS.

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