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Summary of Evidence on the Cytotoxic Effects on Bronchial Epithelial Cells of *IQOS*

Response to the article entitled

"Cytotoxic effects of heated tobacco products (HTP) on human bronchial epithelial cells"

by Leigh et al., 2018[1]

by Gizelle Baker, Patrick Picavet, Maurice Smith, Patrick Vanscheeuwijck, Emilija Veljkovic and Manuel C. Peitsch[2]

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1 EXECUTIVE SUMMARY

The Department of Department of Health Behavior, Roswell Park Comprehensive Cancer Center, Buffalo, New York, USA has recently published a Research Paper in *Tobacco Control* (Leigh, 2018) claiming that "emissions from heated tobacco products (HTP) damaged bronchial epithelial cells, and their cytotoxic effect was higher compared with e-cigarettes but lower compared with tobacco cigarettes."

Independent studies, like this one, are important to assess the potential health impacts of smoke-free products. This study presents a valuable contribution to further understand the effects of different smoke-free alternatives to cigarettes. We have reviewed and assessed those conclusions of this study, based on the methodology used and the results presented. The conclusions drawn by the authors are far reaching, and perhaps go beyond what the study results support. Methodological limitations with the conduct of the study should be considered when interpreting the results and drawing conclusions (e.g., the study did not follow the established guidelines for conducting cytotoxicity assays, or performing tests in an acute setting).

We have therefore prepared a point-by-point assessment of the conclusion drawn by the authors, and this detailed analysis can be found below.

Overall, based on an analysis of our *in vitro* studies performed according to international standards of Good Laboratory Practice (GLP), the Tobacco Heating System (THS, marketed under the brand name *IQOS*) presents less risk of harm and can reduce the risk of smoking-related diseases compared with continued smoking. This includes a significant reduction in cytotoxicity when compared to effects of cigarette smoke generated by combustion.

Although *THS* is not risk-free, switching completely to *THS* presents less risk and can reduced the risk of smoking related diseases for adult smokers than continuing to smoke cigarettes.

2 INTRODUCTION

A number of studies were conducted and published by PMI aiming to thoroughly characterize its heat-not-burn product, the Tobacco Heating System (THS, marketed under the brand name *IQOS*). The totality of scientific evidence gathered to date demonstrates that THS presents less risk of harm compared to continuing to smoke. The authors of the recently published article "*Cytotoxic effects of heated tobacco products (HTP) on human bronchial epithelial cells" Leigh et al.* (Leigh, 2018), rightly pointed out that there is a lack of independent research to evaluate the health impact of the THS and compare it to the effects of other nicotine containing products such as conventional cigarettes or e-cigarettes. More specifically, the authors stated that there is insufficient scientific evidence addressing the cytotoxic potential of THS which motivated them to conduct a study to assess the cytotoxicity of the aerosol of THS (using Amber HEETS - Philip Morris International) and compare it to the aerosol of a MarkTen e-cigarette (Altria, Philip Morris USA) as well as the mainstream smoke of Marlboro Red combusted cigarettes (Altria, Philip Morris USA). The neutral red uptake (NRU) and Trypan Blue cytotoxicity assay results on bronchial epithelial cells H292 cultivated on air-liquid interface (ALI) and acutely exposed to the three different nicotine containing products referenced above are presented in this article. Additionally, to evaluate inflammatory responses, the levels of cytokines released were determined using an ELISA assay.

The NRU assay results show a certain level of cytotoxicity caused by the THS aerosols and the authors concluded that '*emissions from THS caused damage to human bronchial epithelial cells relative to air controls. At the same time, the THS aerosol showed lower cytotoxicity compared with cigarettes but higher cytotoxicity compared with e-cigarettes*'. Cell viability was reduced to 65%, 80% and 90% for the comparator cigarette, THS and the comparator e-cigarettes exposures, respectively compared to the viability of air exposed cells (100%). The results of the Trypan Blue assay (shown in supplementary material only) showed marked reduction of cellular viability only in smoke-exposed cells, while the THS, the comparator e-cigarette and air exposed cell viability were nearly identical. Furthermore, the authors stated that the '*use of IQOS products may lead to increased risk of respiratory health impairment, and although this risk may be reduced compared with smoking tobacco cigarettes, it is likely to be higher than risk from vaping e-cigarettes'* (Leigh, 2018). The authors did not find detectable levels of 8 out of 10 analysed inflammatory cytokines regardless of the exposure. For the two inflammatory cytokines (IL-1β and IL-6) detected, the Leigh *et al*. study results did not show any differences between THS aerosol and air or the comparator e-cigarette vapor exposed cells. Smoke generated from cigarettes significantly increased cytokine levels compared with air controls, as well as with the two other products Leigh et al. investigated. The authors also reported that the data presented have shown the relative effects of acute exposure to three different tobacco products studies at PMI are performed according to international standards where validated Organization for Economic Co-operation and Development (OECD) test methods exist and in compliance with the principles of GLP, where relevant. PMI has conducted six *in vitro* studies relevant for this document, to compare the effects of THS aerosol with those of cigarette smoke. At comparable concentrations THS aerosol had no toxic effects and an overall lower biological impact, indicating that THS aerosol is markedly less toxic than 3R4F smoke. To elicit cytotoxic effects similar to cigarette exposure, THS concentrations had to be much higher (10 or more times higher, concentrations matched to nicotine) compared to reference cigarette exposure (see PMIs Modified Risk Tobacco Product [MRTP] Application for IQOS, sections 6.1.4.4.1.1 – In Vitro Models)

This report aims to clarify findings published by Leigh *et al*. and to provide additional context by summarizing the scientific data available on THS to date with regards to cytotoxicity and inflammation in epithelial cells.

3 METHODS

After the careful examination of the article by Leigh *et al*., several methodological limitations were identified:

Concentration Response

In the study by Leigh *et al*. they did not generate data for the concentration-response for the tested products, and this is fundamental and essential concept in toxicology. It correlates exposures and the spectrum of induced effects. Knowledge of the concentration-response relationship establishes causality that the tested item has induced the observed effects, establishes the lowest dose where an induced effect occurs and determines the rate at which the effects develop. The selected concentration in Leigh *et al.* study could have been excessive showing high cytotoxicity for all conditions without a possibility to discriminate between the products. For example, if the cytotoxicity of a product A is ten times lower than that of product B, but the test is performed at a single high concentration that already shows cytotoxicity for product A, then the outcome will be the same for product A and B.

Dosimetry

The authors used 'one smoking session' for the three different products tested and exposed cells only once, which can be considered as an acute exposure. There are several challenges with such an approach. First, translating one human product consumption session directly to exposure of cell cultures using exposure chambers does not represent a valid surrogate for the human exposure. This is an experimental system in which specific dosimetry parameters should be used that are common to the different products used (e.g., use of nicotine exposure level as dose parameter). Such an approach would allow the comparison of the effects of exposure to different products. In addition, it is important to provide a detailed characterization of the smoke/aerosol generated and delivered to the proximity of the cells, which was not provide by the authors (i.e., validation of the exposure system and the data on the aerosol characterization from the published study). Second, the nicotine concentration which was determined in the previous study actually is not matching between different products tested, which makes the comparison of the exposure conditions difficult. Third, the authors did not indicate the duration of the acute exposure, which is an important element for the subsequent cell viability assays they performed.

Neutral Red Uptake and Trypan Blue assay

While recognizing the NRU assay as a standard tool for the determination of the test item/compound cytotoxicity where the uptake of dye by lysosomes indicates viability of the cells, this test is typically not used as a measure of metabolic capacity of the cell, as indicated by the authors. Other *in vitro* tests more suitable for the evaluation of the metabolic status of cells such as glycolysis, oxidative phosphorylation or lipid metabolism determination are available from various distributors such as Abcam and Agilent, although not yet adapted for cell cultured in an ALI system. The statement that the NRU assay determines metabolic activity of cells is somewhat misleading and gives the impression that the two different cytotoxicity assays used in this study address different cellular functionalities, while both of them are ultimately addressing cellular viability.

The acute effects of smoke/aerosol exposure on viability of cells were measured 2.5 hours after the exposure was completed. The loading of the NR dye in the exposed cells was commenced immediately following the exposure as opposed to 24 hours after the exposure start as per generally accepted protocol of NRU performance and guidelines (INVITTOX, 1990). The observation by the authors of a significant reduction in the neutral red signal from all aerosols tested may simply reflect a short-term adaptive change of the membrane of the lysosome to such aerosols (which the authors demonstrated was not cytotoxic in Trypan Blue exclusion test). A measurement following a 24 hour recovery period would be appropriate, according to the guidelines and may show different results. Moreover, the Trypan Blue test results did not significantly differentiate cytotoxicity of exposure to THS and the comparator e-cigarette from air control exposures, while the acute exposure to the comparator cigarette smoke caused marked reduction of cellular viability. The Trypan Blue test was performed after the 2.5 hours of the incubation of cells in fresh medium, which allowed cells to recover. The authors decided not to show the results of the Trypan Blue test in the main text of the article, but in the supplementary material. More suitable comparative approach would commence the dye loading at equivalent time-points for different methods.

4 SUMMARY OF RELEVANT PMI RESULTS

PMI has conducted six *in vitro* studies relevant for this document to compare the effects of THS aerosol with those of cigarette smoke and fresh air. Results of those studies have been submitted to the FDA as a part of the MRTPA for IQOS.

Using a systems toxicology approach combining computational methods to analyze a broad array of comprehensive molecular measurements (transcriptomics, proteomics, lipidomics), in addition to the standard measurements used in toxicity studies, PMI studied the impact of direct exposure to whole 3R4F smoke and THS aerosol of human organotypic tissue cultures of oral, nasal, small airway and bronchial epithelia grown at the air-liquid interface. At comparable concentrations, THS aerosol had no toxic effects and an overall lower biological impact, indicating that THS aerosol is markedly less toxic than 3R4F reference cigarette smoke (see PMIs Modified Risk Tobacco Product [MRTP] Application for IQOS, sections 6.1.4.4.1.1 – In Vitro Models) and (Gonzalez-Suarez, 2016; Iskandar A, 2017; Iskandar, 2017a; Iskandar, 2017b; Zanetti, 2016)[3]. Relevant for the purpose of this document, those studies showed a consistent reduction in cytotoxicity, tissue injury and the response of inflammatory mediators to THS aerosol compared with cigarette smoke. To elicit cytotoxic effects similar to combusted cigarette exposure, THS concentrations had to be much higher (10 or more times higher, concentrations matched to nicotine) compared to reference cigarette exposure.

In the following section, results from studies conducted by PMI that are relevant to discuss findings from Leigh *et al.* are outlined. When possible (e.g., NRU assay) results were directly compared and discussed. However, due to differences in methodological approaches and methodological limitations of the Leigh *et al*. study outlined above, a direct comparison between PMI results from all relevant studies and Leigh *et al*. is not possible.

Neutral Red Uptake

To evaluate the cytotoxicity of THS aerosols, PMI conducted the NRU assay (see PMIs Modified Risk Tobacco Product [MRTP] Application for IQOS, sections 6.1.2.2.1). Exposure was performed by incubating BALB/c 3T3 cells for 23 hours +/- 1 hour with total particulate matter (TPM) or gas vapor phase (GVP). Three independent batches of TPM and GVP fractions from THS and reference 3R4F cigarette were tested. Three independent tests, meeting the assay acceptance criteria, were required for each item and for each fraction. At the end of the exposure, the culture medium containing the aerosol fractions was replaced by medium containing the vital dye neutral red. After a 3 hour incubation period, the neutral red dye, which was taken up only by viable cells, was determined photometrically from cell lysates. The concentrations that reduced the number of viable cells by 50% (EC50) were determined for the TPM and GVP from THS. Cytotoxicity of the THS aerosol fractions was compared with that from the reference item smoke fractions. A clear dose-dependent decrease in cell viability for both the aerosol fractions generated from THS and 3R4F smoke was observed. Furthermore, the concentrations used in the tests achieved a range spanning from the maximum to no or little cytotoxicity, allowing an accurate determination of the EC50 and consequently a direct comparison of the cytotoxicity of THS aerosol and 3R4F smoke fractions. Cytotoxicity was determined on a per-test-item basis, and using the nicotine concentration in the TPM fraction. The cytotoxicity on a per mg nicotine basis was calculated. On a per mg nicotine basis, the TPM and GVP *in vitro* cytotoxicity of

[3] Note: in all PMI published articles the term ‘THS2.2’ was used for THS

THS was reduced by approximately 90% (91.7 % for TPM and 90.2 % for GVP) compared with the 3R4F reference cigarette (Figure 1 and Table 1Error! Reference source not found.).

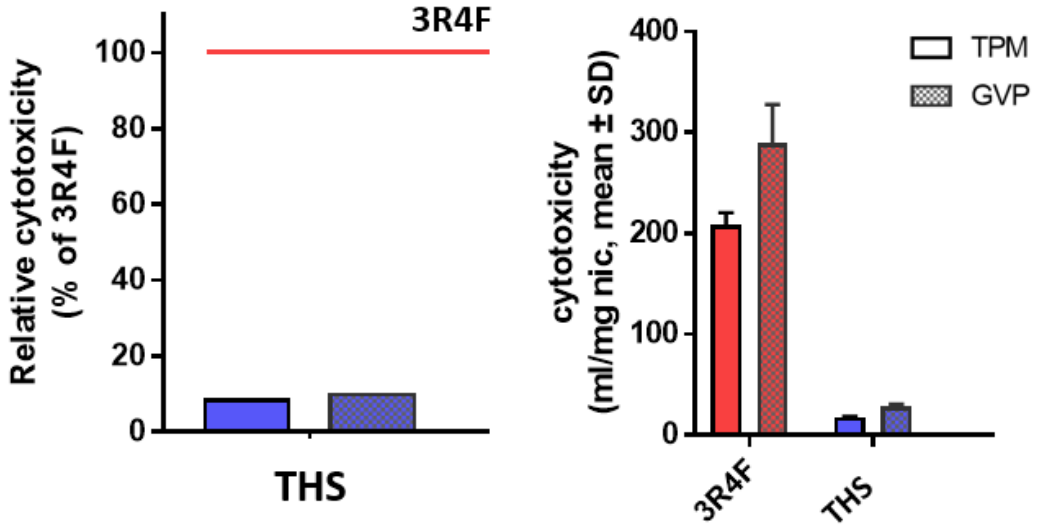

Figure 1. Relative (left) and on a per mg nicotine basis cytotoxicity (right) of TPM and GVP of THS compared to 3R4F, unpublished data

Table 1. 1/EC_{50} of 3R4F and THS on a per mg nicotine basis. M= mean; SE = standard error. From PMIs Modified Risk Tobacco Product [MRTP] Application for IQOS, sections 6.1.2.2.1

Cytotoxicity THSR		Stat. Parameter	TPM	GVP	Cytotoxicity 3R4F		Stat. Parameter	TPM	GVP
1/EC_{50} (ml/mg)		M	17.34	28.4	1/EC_{50} (ml/mg)		M	208.55	289.06
		SE	0.52	1.2			SE	6.92	22.38

Cytotoxicity of THS and the comparator e-cigarette aerosol compared to the mainstream smoke of a comparator cigarette was measured by NRU and reported by Leigh *et al*., in the Figure 1 of their article appears higher compared to PMI data. The discrepancies may be attributed to the methodological limitation of the study by Leigh *et al*., outlined above. In addition, different cell lines cultivated in a different way (ALI by Leigh *et al*. vs. submerged cell culture by PMI) and exposed to whole aerosol (Leigh *et al*.) vs. exposure to TPM and GVP (PMI) were used for those tests. Since the authors tested only one dose and the comparative parameter was not used (e.g., nicotine in the aerosol), the open question remains where in the cytotoxicity range of different products the concentrations used by Leigh *et al*. reside. It is interesting to note that the cell viability determined by the Trypan Blue assay (supplementary Figure 1, Leigh *et al*.) did not show reduced viability in cells exposed to THS or the comparator e-cigarette compared to air exposed cells. However, this finding was not commented on by the authors.

Effects of THS Aerosol on Oral Epithelial Cells

To compare the biological impact of THS aerosol with smoke from 3R4F reference cigarette on physiologically relevant systems representative of the human upper airway, human organotypic tissue cultures of the oral epithelium (EpiOral™, MatTek Corp., Ashland, MA, USA) were exposed for 28 minutes to air (control) and increasing concentrations of 3R4F smoke and THS aerosol. 3R4F smoke was applied at dilutions of 15% and 24% (v/v in air), equivalent to target nicotine concentrations of 0.32 and 0.54 mg/L respectively. THS aerosol was applied at dilutions of 25%, 34% and 69% (v/v in air), corresponding to target nicotine concentrations of 0.31, 0.46 and 1.09 mg/L respectively. To determine target concentrations of nicotine in the smoke or aerosol, various dilutions were applied to the organotypic cultures. Prior to starting the experimental repetitions, a dose range finding (DRF) experiment was performed to find the maximum tolerable concentration of 3R4F smoke. We used nicotine as the internal reference compound to compare and normalize the concentrations of THS aerosol to those of 3R4F smoke. Two 3R4F smoke and THS aerosol concentrations were selected for the comparative analysis, each pair matched for nicotine concentrations, In addition, another group to test a higher THS aerosol exposure was included. Four experimental repetitions and experiments associated with DRF phase were performed over a period of 4 months. For each repetition, 3 independent exposures (28min. each) were performed. This duration was selected according to a previous finding regarding the sensitivity of organotypic bronchial cultures following 3R4F smoke exposure: a 28 min exposure to 3R4F smoke induced the highest concentration of secreted matrix metalloproteinase (MMP)-1 in bronchial epithelial organotypic cultures (Mathis, 2013).

Cytotoxicity and gene expression (not shown) responses were assessed 4, 24, 48 and 72 hours after the exposure. Several inflammatory mediators released into the culture medium were quantified at 24, 48 and 72 hours and tissue integrity was evaluated at 48 and 72 hours after the exposure.

Exposure of EpiOral™ tissues to 3R4F smoke resulted in tissue injury, as evidenced by keratinization and desquamation, which was most prominent at the higher concentration and later time point (Figure 2, left lowest panel). In contrast, THS aerosol-exposed cultures showed a strongly attenuated response, even when nicotine concentration in aerosol was two times higher than in 3R4F smoke. Cytotoxicity following exposure to 3R4F smoke or THS aerosol was assessed by measuring the activity of adenylate kinase (AK) released from the organotypic cultures into the basolateral medium (Figure 3).

3R4F smoke exposed cultures showed more cytotoxicity than air-exposed controls (48 and 72 h post-exposure).

The THS aerosol-exposed cultures showed no cytotoxicity, as in the air-exposed controls, regardless of exposure, concentration, or time.

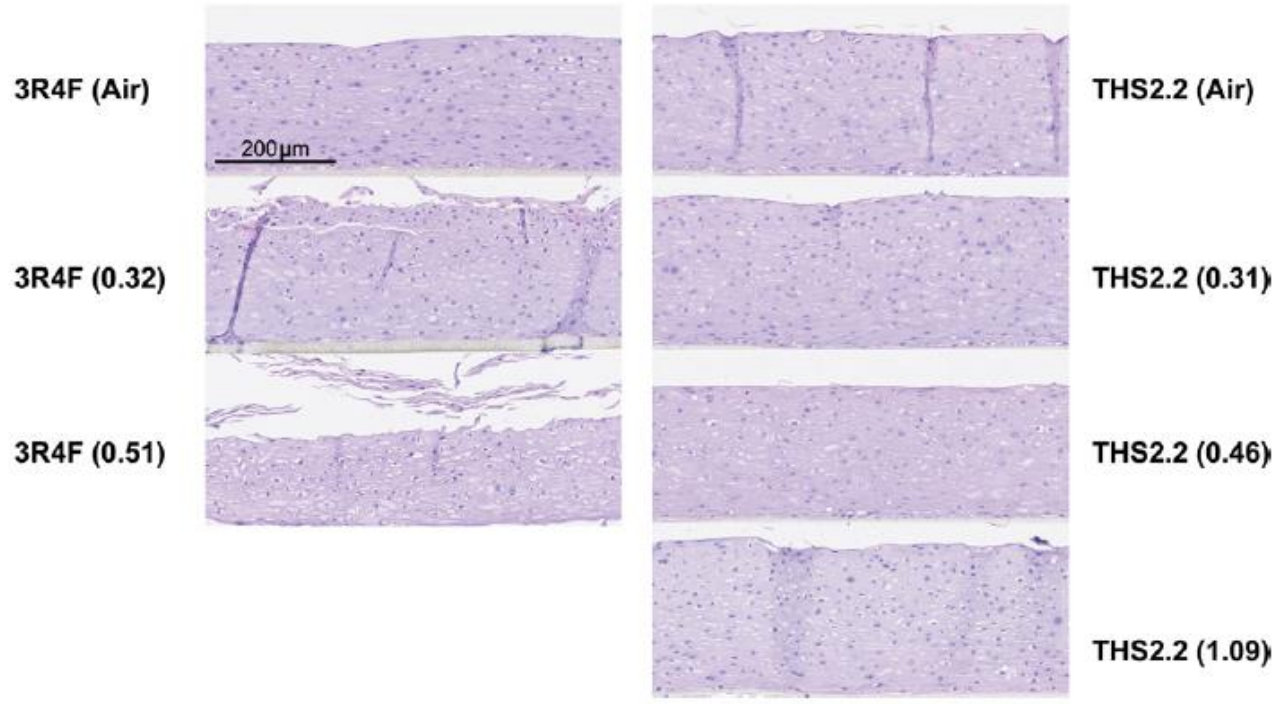

Figure 2. Tissue morphology after 72 h of exposure to 3R4F smoke or THS aerosol. The applied nicotine concentration (mg/L) for each condition is shown in parentheses; 20× magnification. N = 12. (Zanetti, 2016)

Tissue morphology of organotypic cultures exposed to the THS aerosol showed no relevant signs of toxicity at any of the concentration tested, except for a light desquamation with exposure to the higher doses (0.46 and 1.09).

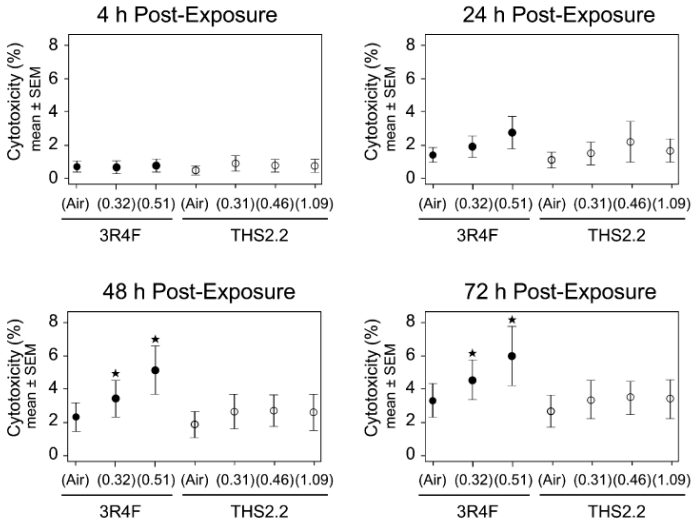

Figure 3. Cytotoxicity in organotypic cultures exposed to 3R4F smoke or THS aerosol. Mean cytotoxicity levels were determined using the AK assay at various post exposure time points. The AK levels were normalized relative to those in the positive control (Triton-X-treated cultures considered to represent 100% cytotoxicity).

Error bars indicate SEM (N = 11−13). Nicotine concentrations in 3R4F smoke or THS aerosols are indicated for each group (mg/L, x-axis). ★ p < 0.05, compared with the corresponding air control (Zanetti, 2016).

Cultures exposed to 3R4F smoke secreted higher levels of inflammatory mediators than those exposed to air. In contrast, at comparable nicotine concentrations, THS aerosol exposure resulted in significantly lower MMP-1 (-45.8%) and IL-1β (-88.8%) levels than 3R4F smoke exposure (Figure **4**). Far fewer and less pronounced changes in secretion of these mediators were observed in oral cultures exposed to THS aerosol at comparable or even higher nicotine exposure levels.

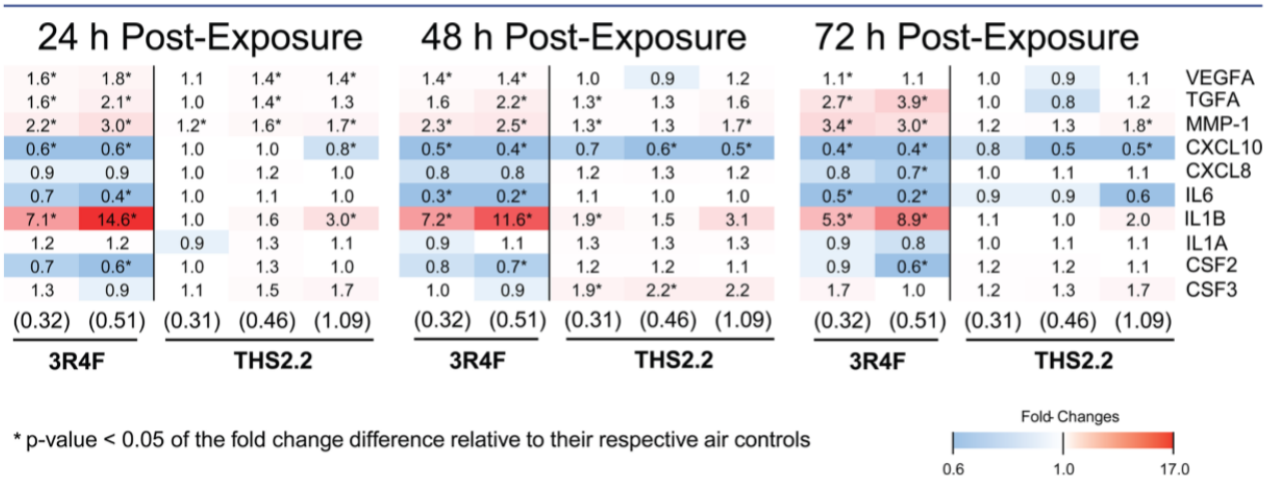

Figure 4. Profiles of secreted proinflammatory mediators following exposures. Heatmap showing the fold-changes of the mean concentrations of proinflammatory mediators in exposed cultures relative to those in their corresponding air controls at 24, 48, or 72 h post-exposure for each group. Blue and red indicate negative or positive fold-changes, respectively, in the 3R4F smoke and THS aerosol-exposed, as compared with air-exposed, samples. Nicotine concentrations in the smoke or aerosol are indicated for each group (mg/L, x-axis). N = 12. (Zanetti, 2016)

Together, these results indicated that exposure to THS aerosol caused less cytotoxicity, tissue damage and inflammation in oral organotypic models than exposure to 3R4F smoke (and PMIs Modified Risk Tobacco Product [MRTP] Application for IQOS, section 6.1.4.4.1.1.3).

Effects of THS Aerosols on Small Airway Epithelial Cells

Human organotypic cultures of small airway tissues grown at the air–liquid interface (SmallAir™ 9) was used to evaluate and compare the impact of the exposure to 3R4F smoke and THS aerosol. A set of small airway cultures was exposed to two dilutions of 3R4F smoke and to air, simultaneously, in one exposure plate. A 3R4F smoke dilution (7%) corresponding to a nicotine concentration of 0.14 mg nicotine per L, was used to expose samples termed “3R4F (0.14)”, a 3R4F smoke dilution (13%) corresponding to a nicotine concentration of 0.26 mg nicotine per L, was used to expose samples termed “3R4F (0.26)” and 0% 3R4F smoke exposure, representing the air-exposed controls for the 3R4F exposure was used to expose samples termed “3R4F (Air)”. The concentrations of 3R4F smoke were selected based on previous studies using nasal and bronchial organotypic cultures described below (Iskandar, 2017a; Iskandar, 2017b). A set of cultures was exposed to THS aerosol dilutions at nicotine concentrations matched to those of the diluted 3R4F smoke (at least for the two concentrations) and to air, simultaneously, in one exposure plate. A THS aerosol dilution (14%)

corresponding to a nicotine concentration of 0.14 mg nicotine per L was used to expose samples termed "THS2.2 (0.14)", THS aerosol dilution (24%) corresponding to a nicotine concentration of 0.30 mg nicotine per L was used to expose samples termed "THS2.2 (0.30)", THS aerosol dilution (31%) corresponding to a nicotine concentration of 0.45 mg nicotine per L was used to expose samples termed "THS2.2 (0.45)", and 0% THS aerosol exposure, representing the air exposed controls for the THS exposure was used to expose samples termed "THS2.2 (Air)".

The organotypic small airway cultures were exposed to cigarette smoke or THS aerosol for 28 min. This duration was selected according to a previous finding regarding the sensitivity of organotypic bronchial cultures following smoke exposure: a 28 min exposure to 3R4F smoke induced the highest concentration of MMP-1 in bronchial epithelial organotypic cultures (Mathis, 2013). Different test/analysis were performed at various post-exposure time points.

The relative cytotoxicity levels were mainly observed in samples exposed to 3R4F (0.26) smoke (Figure 5).

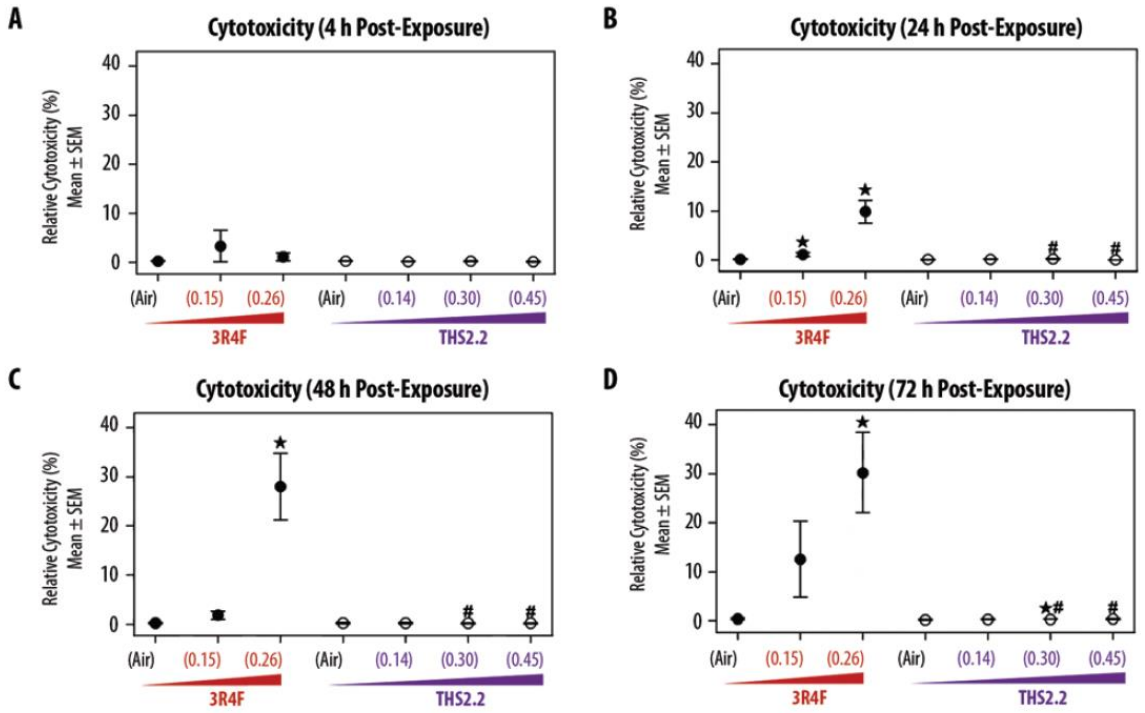

Figure 5. Cytotoxicity following exposure. Mean cytotoxicity levels evaluated by an adenylate kinase (AK) release assay at various time points post-exposure. AK levels were normalized relative to the positive and negative controls (see Materials and methods section). Nicotine concentrations in 3R4F smoke or THS aerosol are indicated for each group (mg L−1, x-axis). ★ indicates $p \leq 0.05$ compared with the corresponding air controls. # indicates a $p \leq 0.05$ difference from 3R4F smoke exposure at a similar nicotine concentration. In the case of THS (0.45), 3R4F (0.26) was used as the comparison group (Iskandar A, 2017).

THS aerosol exposure at any concentration was not associated with alterations in relative cytotoxicity at all post-exposure time points tested. One statistically significant difference was observed between the samples exposed to THS (0.30) aerosol and air (at 72 h post-exposure); however, this change was considered not biologically relevant because the relative toxicity level was only 0.34%.

Compared with the samples exposed to air, the samples exposed to 3R4F (0.15) smoke had exhibited reduced cilia numbers and increased frequency of empty spaces between cells (arrowheads in Figure 6), which could be attributed to a lower cell–cell adherence, and induced detachment at the suprabasal layer.

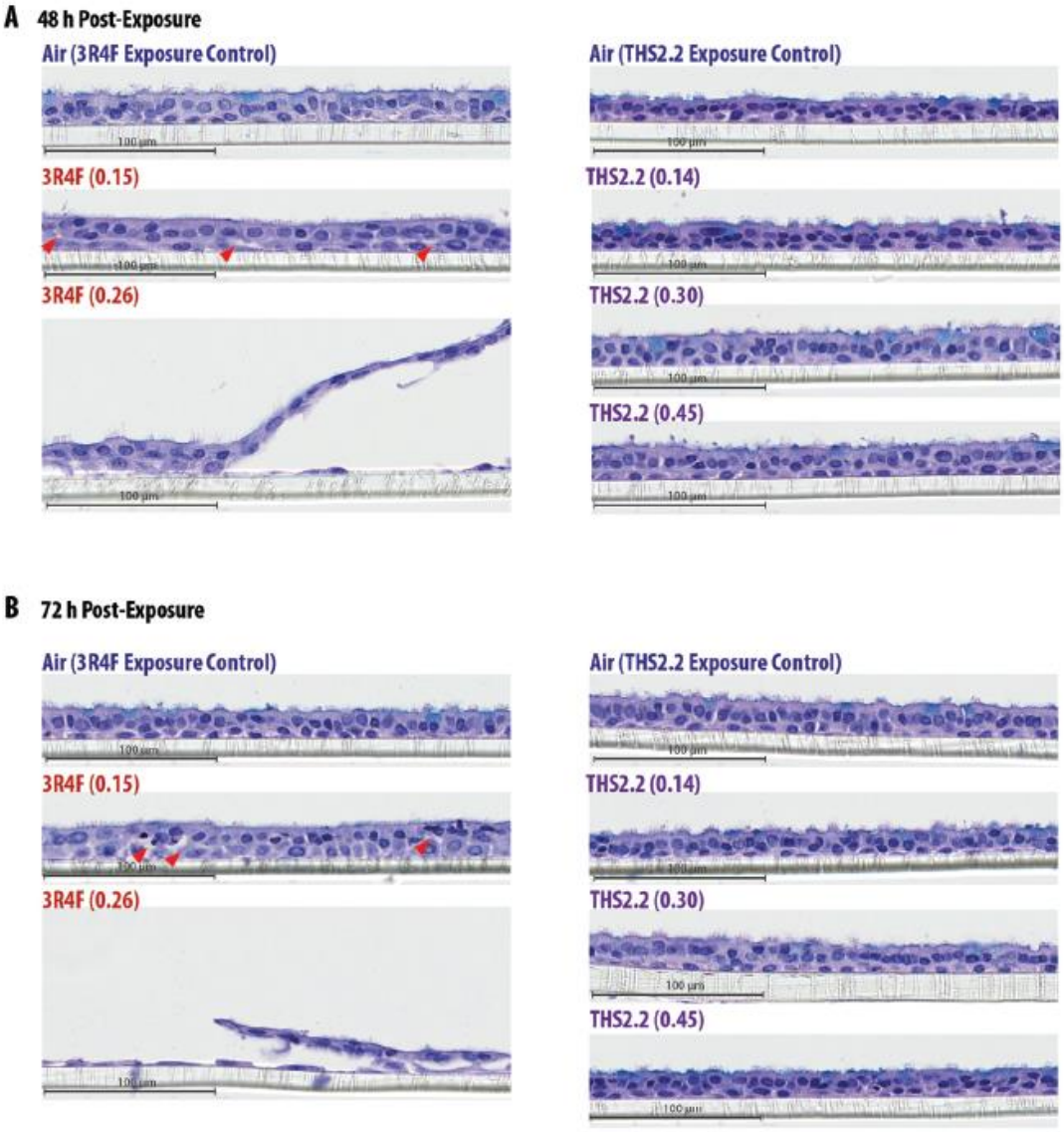

Figure 6. Culture morphology 48 h and 72 h post-exposure. Representative images of hematoxylin and eosin (H&E) and alcian blue (AB)-stained small airway culture sections observed 48 h (A) and 72 h (B) after exposure. Arrowheads indicate empty spaces between cells (Iskandar A, 2017).

Squamous cells and apoptotic cells were also detected in the 3R4F (0.15) smoke-exposed samples. When the small airway tissue cultures were exposed to the highest concentration of 3R4F smoke (0.26 mg nicotine per L), more pronounced damage was observed 48 h (Figure 6A) and 72 h (Figure 6B) after exposure. In contrast, THS aerosol-exposed samples (at all concentrations tested) did not

exhibit any apparent morphological alterations compared with the air-exposed controls at either post-exposure time point.

The concentrations of secreted pro-inflammatory mediators from the basolateral media of the cultures collected at various post-exposure time points were measured (Figure 7). Generally, an increased mediator levels following 3R4F smoke exposure, and smaller changes following THS aerosol exposure, relative to the levels seen in the air-exposed samples were observed.

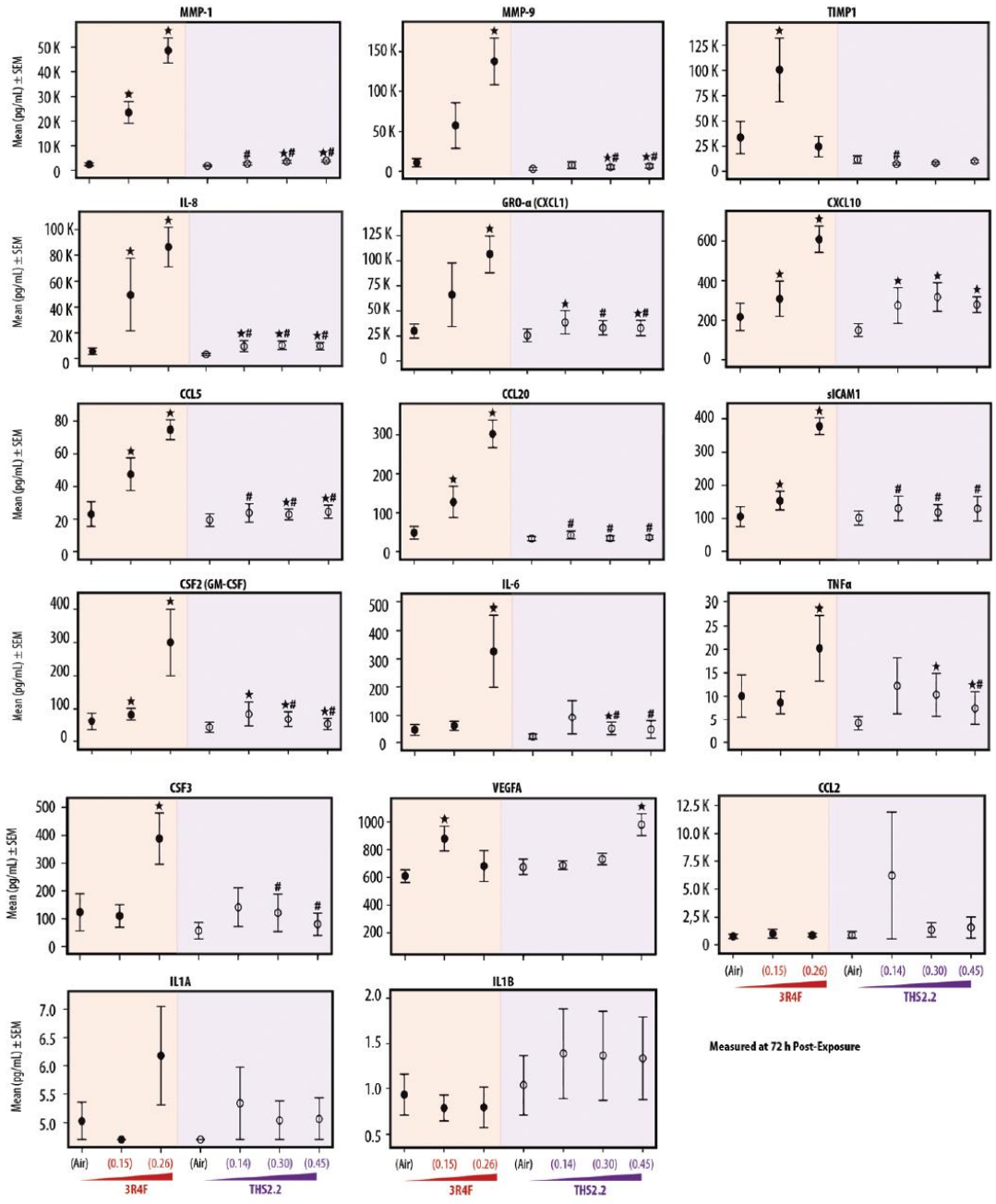

Figure 7. Profiles of secreted pro-inflammatory mediators following exposure. Mean concentrations of pro-inflammatory mediators measured in the basolateral media of the cultures 72 h after exposure. ★ indicates p ≤

0.05 compared with the corresponding air controls. # indicates a p ≤ 0.05 difference from 3R4F smoke exposure at a similar nicotine concentration. In the case of THS (0.45), 3R4F (0.26) was used as the comparison group (Iskandar A, 2017).

THS aerosol exposure (at all concentrations tested) was linked to fewer changes in mediator levels (relative to the air exposure) for the majority of mediators, compared with the changes associated with 3R4F smoke exposure. In general, we did not observe a concentration dependent alteration in mediator concentrations following THS aerosol exposure except for vascular endothelial growth factor A (VEGFA), for which a significant difference between THS (0.45) aerosol exposure and the air-exposure was observed.

The results showed that THS aerosol exposure at the tested doses elicited markedly lower cytotoxicity levels, caused no morphological tissue changes and much lower levels of secreted pro-inflammatory mediators than 3R4F smoke in small airway cells cultivated in organotypic culture. A description of an extensive systems biology analysis is beyond scope of this document and can be found in the published article by Iskandar *et al*. (Iskandar A, 2017).

Effects of THS Aerosol on Nasal Epithelial Cells

The impact of exposure to 3R4F smoke and THS aerosol were also studied in human organotypic tissue cultures of the nasal epithelium (Nasal MucilAir™, Epithelix Sarl, Geneva, Switzerland). In this study, the cultures were exposed for 28 minutes to air (control); 8% or 15% (v/v in air) 3R4F smoke, equivalent to target nicotine concentrations of 0.15 and 0.25 mg/L respectively; or to 15%, 24% and 31% (v/v in air) aerosol from THS, corresponding to target nicotine concentrations of 0.15, 0.27 and 0.44 mg/L respectively. Cytotoxicity and tissue integrity (Figure 8), as well as gene expression responses (not shown), were assessed 4, 24, 48 and 72 hours following exposure. Inflammatory mediators released into the culture supernatants were measured at 24, 48 and 72 hours post-exposure. While exposure to 8% 3R4F smoke induced little to no cytotoxicity at any time point, exposure to 15% 3R4F smoke elicited substantial cytotoxicity at 24, 48 and 72 hours post-exposure. However, exposure to various concentrations of THS aerosol did not induce any cytotoxicity. Histological examination confirmed that exposure to 3R4F smoke resulted in more damage than did exposure to THS aerosol, with tissues exhibiting less damage at 48 than at 72 hours post-exposure. THS aerosol exposure with a nicotine concentration at least four times higher than that of 3R4F smoke was needed to elicit similar morphological changes and cytotoxicity in the nasal cultures (Figure 9). Multi-analyte profiling revealed that cultures exposed to THS aerosol secreted lower levels of various inflammatory mediators, including CXCL-8 (-52.9%) and soluble intercellular adhesion molecule 1 (sICAM-1) (-80.6%), at 24 hours after exposure than those exposed to 3R4F smoke (Figure 10). In contrast, nasal tissue cultures exposed to THS aerosol at nicotine levels comparable to those in the smoke exposures, showed no significant increase in secretion of inflammatory mediators (Iskandar, 2017a).

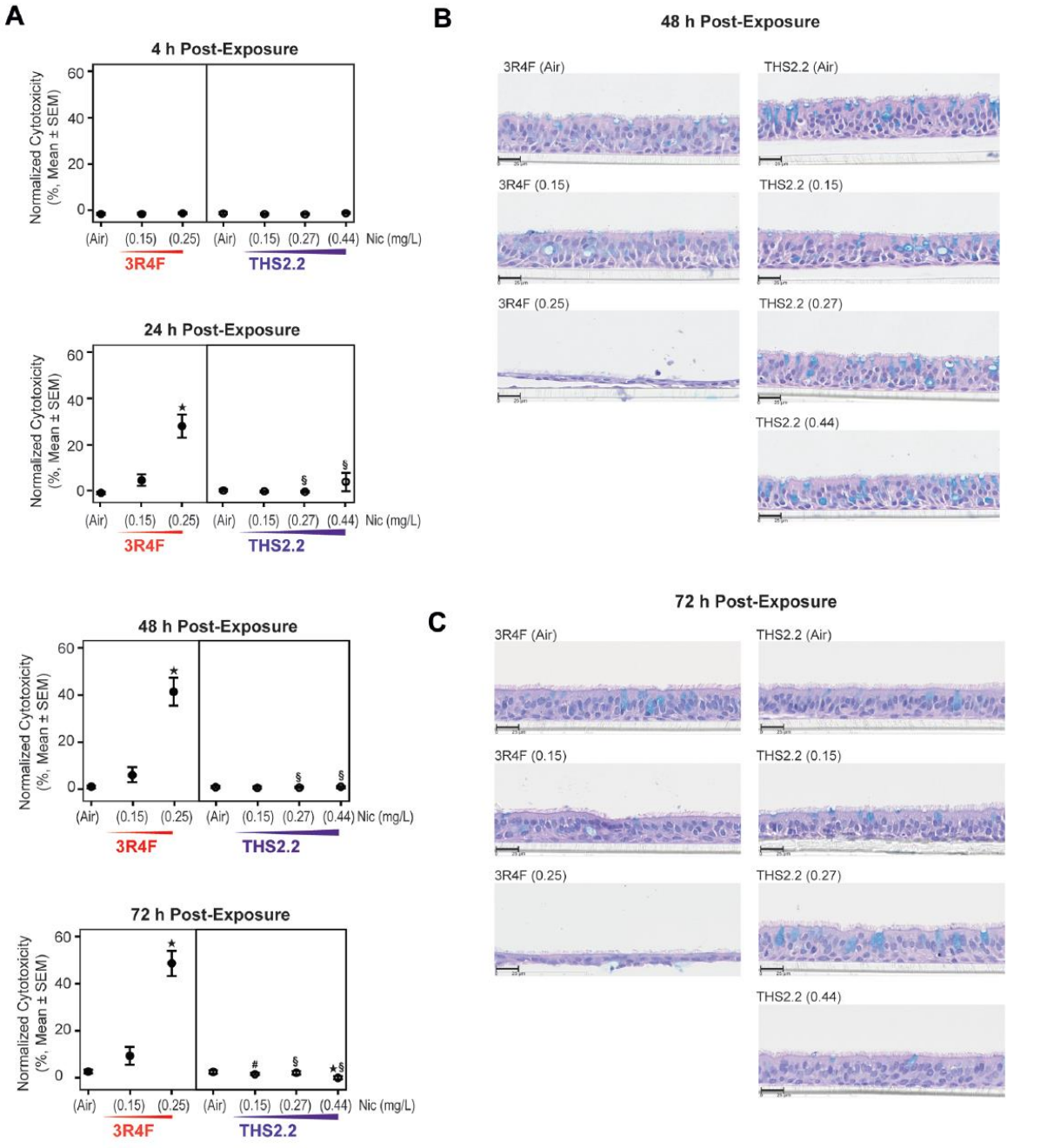

Figure 8. Cytotoxicity and tissue morphology after exposure (A) Mean cytotoxicity levels evaluated by AK assay at various time points post-exposure. The AK levels were normalized relative to the positive and negative controls. Nicotine concentrations in 3R4F smoke or THS aerosols are indicated for each group (mg/l, x-axis). ★ indicates $p < 0.05$ compared with their corresponding air controls. # indicates $p < 0.05$ differences with 3R4F (0.15) and § indicates the difference with 3R4F (0.25). (B) and (C) Representative images of H&E/Alcian Blue-stained nasal culture sections at 48 and 72 h post-exposure, respectively. Abbreviations: AK, adenylate kinase; H&E, hematoxylin and eosin; Nic, nicotine; SEM, standard error of the mean (Iskandar, 2017a).

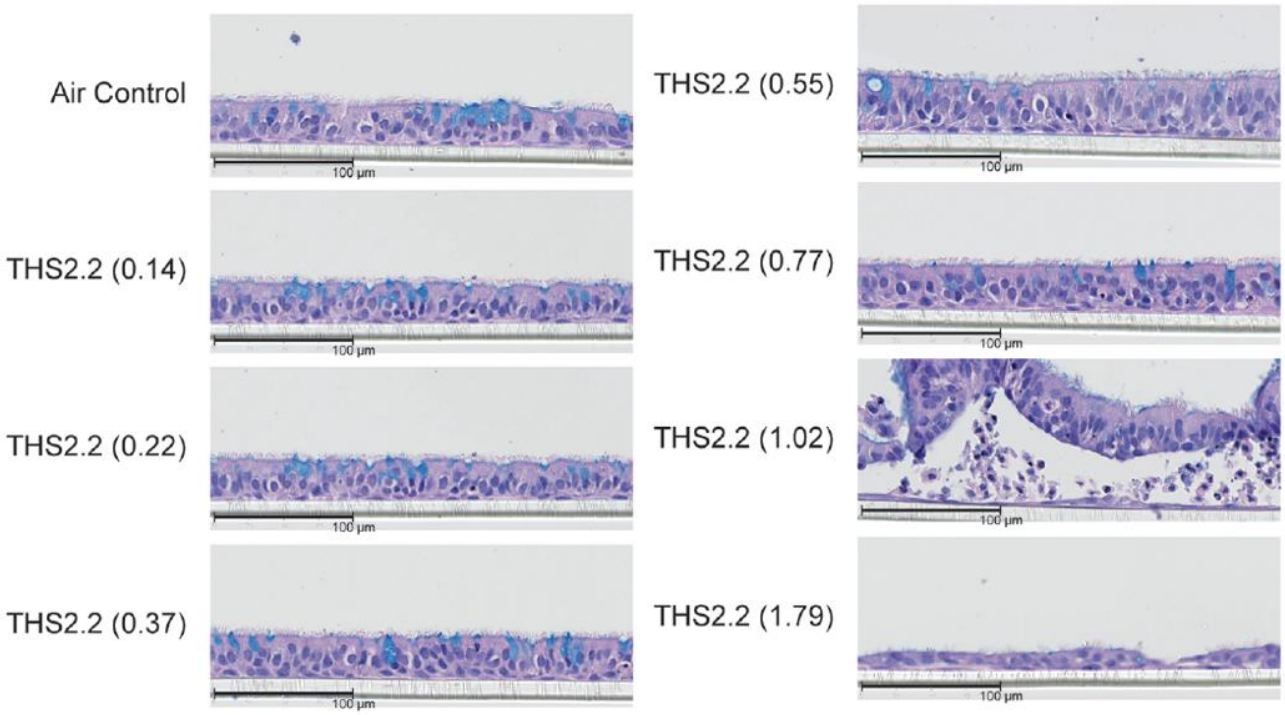

Figure 9. Dose range assessment of THS aerosol. Haematoxylin and eosin stained tissue sections at 72 h post-exposure. Numbers in parentheses indicate the concentration of nicotine in the THS aerosol (mg/L) (Iskandar, 2017a).

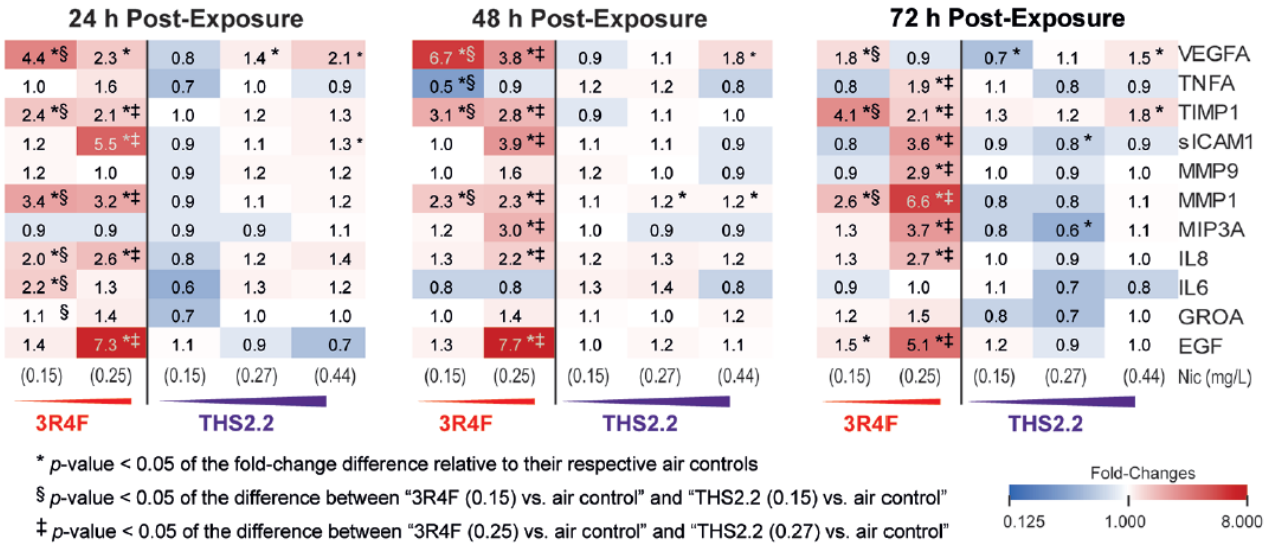

Figure 10. Heatmaps showing the fold-changes of the mean concentrations of pro-inflammatory mediators measured at basolateral media of the cultures at 24, 48 and 72 h post-exposure relative to their corresponding air control. Red and blue represent the degree of increased and decreased fold-changes, respectively. Nicotine concentrations in the smoke or aerosol are indicated for each group (mg/L) (Iskandar, 2017a).

In conclusion, these data demonstrated that exposure of human organotypic nasal epithelial tissues to whole smoke generated from 3R4F reference cigarettes resulted in time- and dose-dependent cytotoxicity, tissue damage and increased secretion of inflammatory mediators.

In contrast, exposure of the cultures to the corresponding concentrations of THS aerosol (matched by nicotine levels) had effects more comparable to those of fresh air exposures, with negligible cytotoxicity or tissue damage. A description of an extensive systems biology analysis can be found in the published article by Iskandar *et al*. (Iskandar, 2017a).

Effects of THS Aerosol on Bronchial Epithelial Cells

Similar studies were conducted in human organotypic tissue cultures of the bronchial epithelium (bronchial MucilAir™, Epithelix Sarl, Geneva, Switzerland or bronchial EpiAirway™, MatTek Corporation, Ashland, MA, USA). In these studies, the cultures were exposed under the same conditions as the human nasal epithelium cultures described above. These cultures were then examined for exposure-related effects at 4, 24, 48 and 72 hours post-exposure. At the earliest time point, no cytotoxicity was noted, independent of the type of exposure. However, cultures exposed to 3R4F smoke at 0.25 mg/L nicotine showed approximately 40% cytotoxicity at 48 and 72 hours post-exposure, while THS aerosol exposure did not cause cytotoxicity in bronchial tissues, independent of dose or post-exposure time point (Figure 11). These results were confirmed by histological evaluation (Figure 12), highlighting that the morphology of THS aerosol-exposed cultures resembled that of those exposed to air. 3R4F smoke exposure induced a strong inflammatory response in bronchial organotypic cultures, as indicated by significantly higher levels of soluble mediators released into the cell culture medium, in particular at the higher smoke dose. With exposure to THS aerosol, however, this response was much lower (Figure 13). For example, secretion of CXCL-8 and sICAM-1 was significantly reduced (-56.5% and -91.2%, respectively) in the basolateral medium of the THS aerosol-exposed bronchial tissue cultures than in those of the 3R4F smoke exposed cultures at 24 hours after the exposure (Iskandar, 2017b).

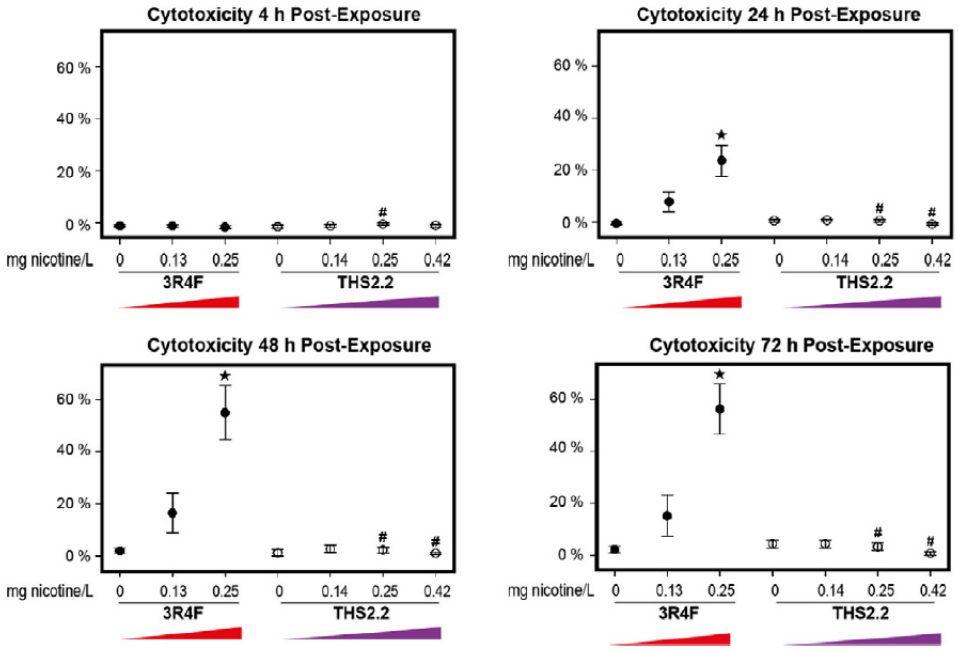

Figure 11. Cytotoxicity levels after exposure. Mean cytotoxicity levels were assessed using an adenylate kinase (AK) assay at various time points of post-exposure following 3R4F smoke and THS aerosol exposures applied at (at least) two comparable nicotine concentrations. AK levels were normalized relative to the positive and negative control (taken as 100% and 0% cytotoxicity, respectively, as described in Materials and Methods). ★p-value b 0.05 vs. its corresponding air control. # p-value b 0.05 vs. 3R4F (0.25) (Iskandar, 2017b).

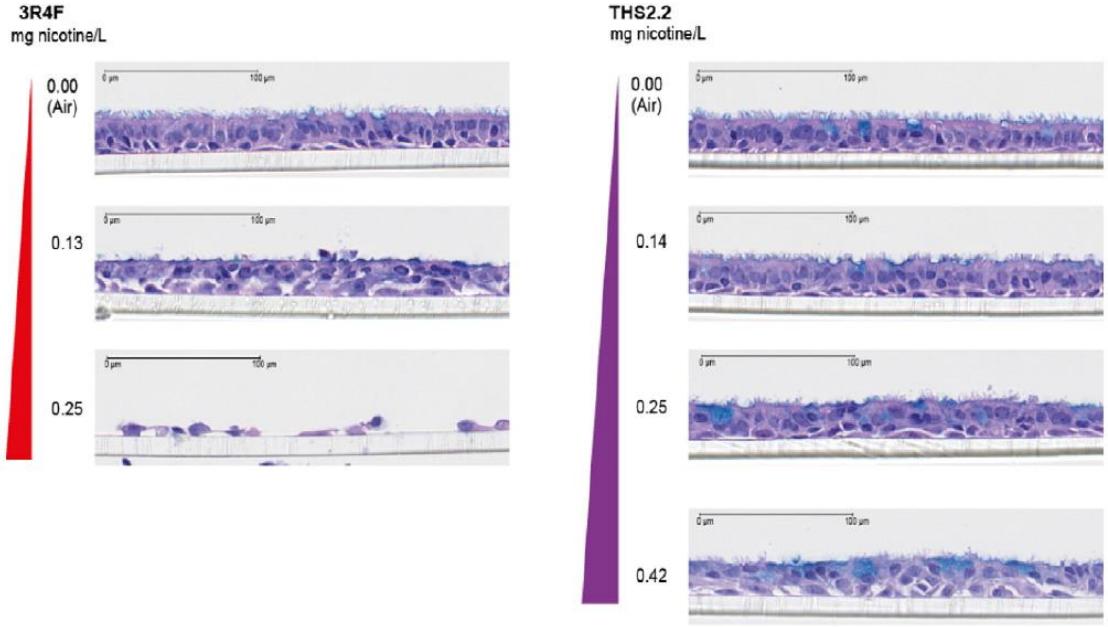

Figure 12. Histology 72 h post-exposure. Representative pictures (taken from experimental repetition 3) of the haematoxylin and eosin (H&E) and Alcian blue-stained culture sections at 72 h post-exposure to 3R4F smoke (left) and THS aerosol (right) applied at (at least) two comparable nicotine concentrations. Representative pictures for the assessment at 48 h post-exposure are given in Supplementary Fig. 2. (Iskandar, 2017b).

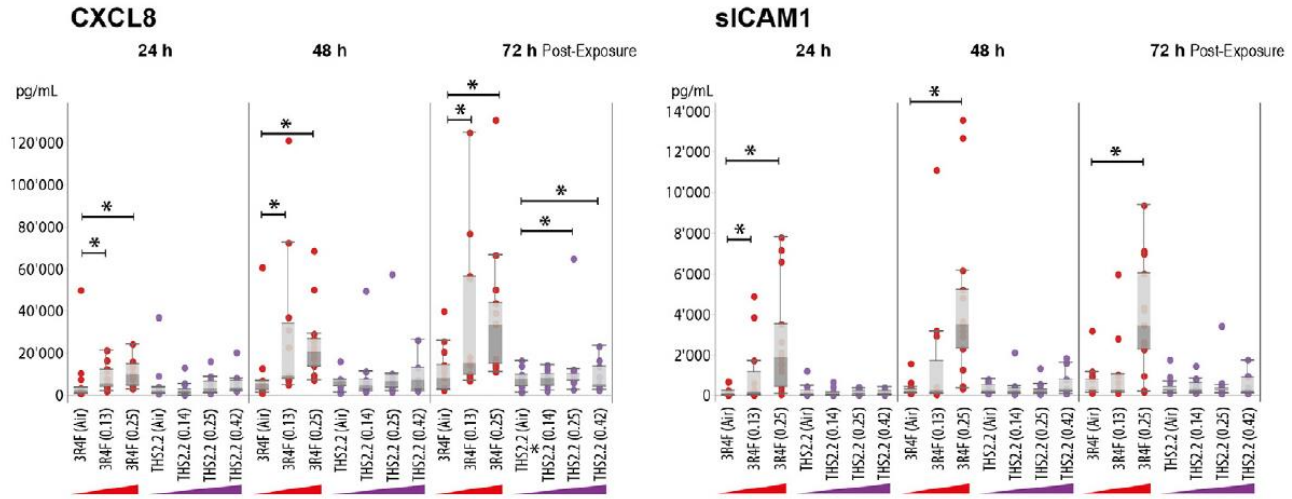

Figure 13. Concentration of pro-inflammatory mediators released in the basolateral media after exposure. Each dot indicates the concentrations detected in each sample measured in a total of six experimental repetitions. The box plots shows the upper whisker (1.5 times the interquartile range), upper quartile, median, lower quartile, and lower whisker (1.5 times the interquartile range). *indicates p-value b 0.05 vs. its corresponding air control. For all mediators measured following exposure, their fold-changes (exposed vs. their respective air controls) are reported in Supplementary Fig. 3. (Iskandar, 2017b).

Together, these results demonstrate that exposure to THS aerosol, in contrast to 3R4F smoke, caused little to no cytotoxicity, tissue damage or inflammation in organotypic bronchial epithelium tissue cultures.

A description of an extensive systems biology analysis and other results of this study can be found in the published article by Iskandar *et al*. (Iskandar, 2017b).

Effects of THS Aerosol on Normal Primary Human Bronchial Epithelial Cells

Primary normal human bronchial epithelia cells (NHBE) cells, cultivated in 2 dimensional culture, were exposed, in a time- and dose dependent manner, to three different smoke/aerosol fractions generated from 3R4F and THS, aqueous extract (AE), TPM and GVP. Multiparametric indicators of cellular toxicity were measured via high content screening analysis. The study was complemented with a microarray-based transcriptomics analysis followed by a quantitative systems biology-based approach leveraging mechanistic network models.

Exposure of cells to 3R4F smoke resulted in a dose-dependent response in most toxicity end points. Moreover, a significant level of perturbation in multiple biological pathways, particularly in those related to cellular stress were detected.

By contrast, exposure to THS resulted in an overall lower biological impact including cellular viability (Figure 14). A toxic response was observed for THS in some functional end points, but the responses occurred at doses between 3 and 15 times higher than those of 3R4F. **(**Gonzalez-Suarez, 2016**)**.

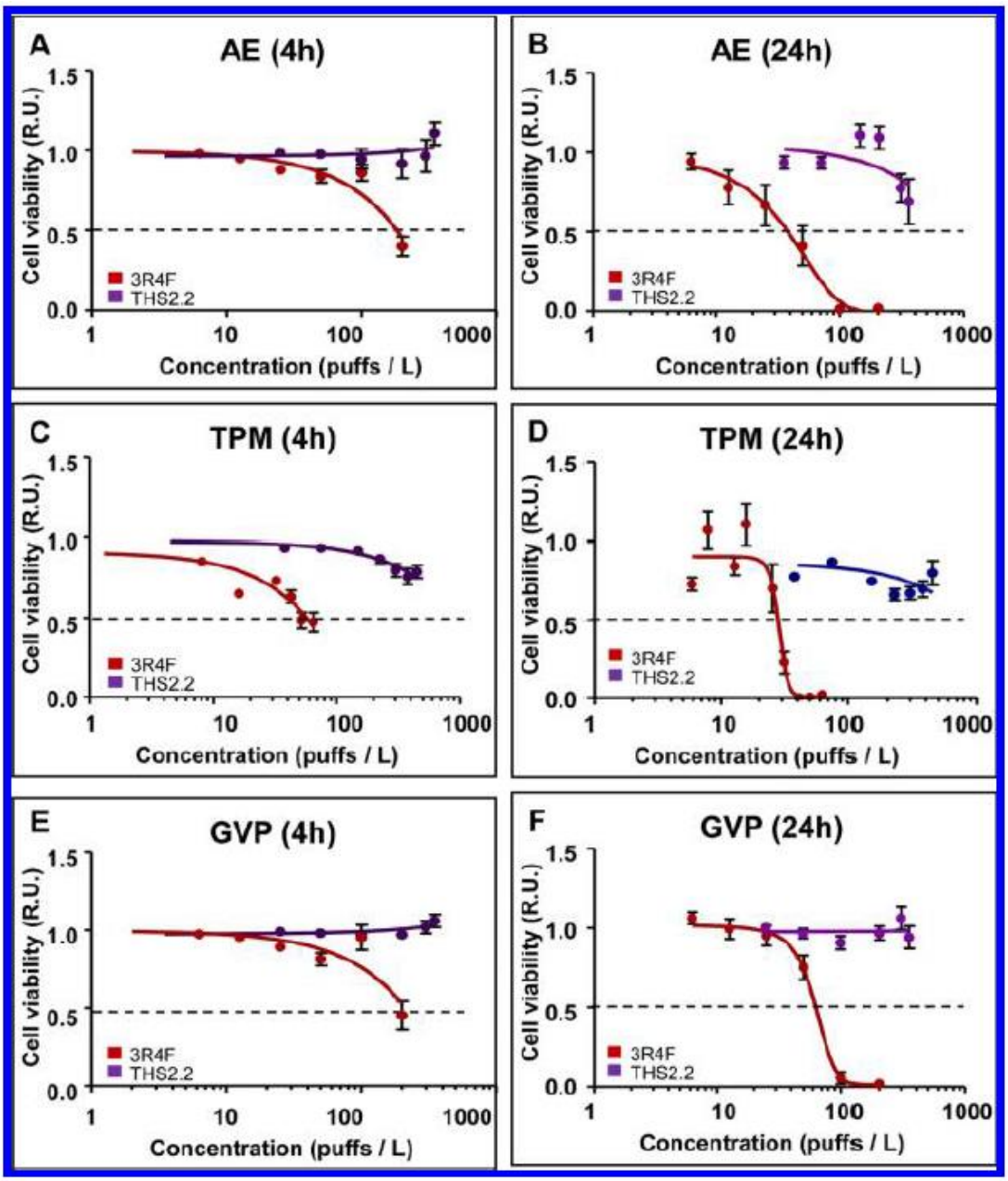

Figure 14. Cell viability in NHBE cells exposed for 4 h or 24 h to AE (A and B), TPM (C and D), or GVP (E and F) fractions from 3R4F smoke or THS aerosol. Values are normalized to the vehicle control and represent the average ± SEM of at least three independent experiments. The dotted line indicates 50% cell viability. R.U. relative units (Gonzalez-Suarez, 2016).

5 DISCUSSION

Leigh *et al*. acutely exposed the lung cancer cell line H292 to smoke/aerosol of three nicotine-containing products:

1. smoke from the cigarette Marlboro Red (the comparator cigarette)
2. aerosol from THS and
3. aerosol (vapor) from an e-cigarette MarkTen (the comparator e-cigarette).

Based on the results of the NRU assay the authors concluded that "*emissions from heated tobacco products (HTP) damaged bronchial epithelial cells, and their cytotoxic effect was higher compared with e-cigarettes but lower compared with tobacco cigarettes*."

However a number of methodological limitations that should be considered when interpreting the results and drawing conclusions.

For example, dose range finding has not been performed, which is a basic principles of toxicological evaluation. Building a concentration-response assessment allows understanding the dynamic range of toxicity of the test item which is not possible with this study as the authors only performed a single dose treatment. Translating one human product consumption session ('one smoking session') directly to exposure of cell cultures using exposure chambers does not represent a valid surrogate for the human exposure. Furthermore, the exposure atmosphere has not been characterized, especially determination of levels of nicotine in the exposure atmosphere indicated in the Material and Methods section were not generated. The nicotine concentration is also not matching between different products tested, which makes the comparison of the exposure conditions difficult.

Recognized, standard protocols (INVITTOX, 1990) were deviated from such as the Neutral Red incubation that was performed immediately after the exposure, without allowing cells to recover from the smoke/aerosol treatment. The Trypan Blue assay was performed 2.5 h after the exposure was completed and cells recovered in fresh cell medium. These deviations might explain the discrepancies between the results of those two assays.

For a complete interpretation of the results outcomes that support the significant reduction in toxicity of the THS such as the outcome of the Trypan Blue assay are not discussed in the main paper and shown in the supplementary data only. Although in this case the methodological approach should also be questioned for the reasons mentioned above, the study showed that a significant reduction in cellular viability was observed only in smoke-exposed cells, while THS and the comparator e-cigarette aerosol did not cause additional reduction of cellular viability compared with the air exposed cells.

The authors also suggested that the rapid death of cells exposed to tobacco smoke may have resulted in low levels of cytokines measured in their study. However, according to the NRU assay, ~70 % of cells are still viable, which makes this explanation rather unlikely.

Finally, the comparison between THS and e-cigarettes is based on one e-cigarette only – MarkTen. Therefore, the authors should link their conclusion to the tested product only, not to e-cigarettes in general.

Since all our studies relevant for the work published by Leigh *et al*. are published in peer reviewed scientific journals and the protocols, data and results of other relevant studies are available as part of PMIs MRTPA for IQOS a full review and discussion should have been considered by the authors. Furthermore, the literature available on the *in vitro* exposure study design, conduct, protocols,

standards, data generation and interpretation of results should have been considered for the design and interpretation of obtained results.

PMI *in vitro* studies relevant for this document were conducted according to International Standards, GLP, good toxicological practices and fulfilling statistical requirements to achieve robustness and relevance of the results.

Summarizing all PMI's *in vitro* data on epithelial cells, it can be stated that for THS aerosol to exhibit cytotoxic, tissue damaging effects and responses of inflammatory cytokines, the doses applied exceed those of cigarette smoke multiple times and are far from representing a real life THS consumption scenario. THS is not the only smoke-free product we offer. We are developing and offering a range of products designed to meet different needs of consumers. So far we did not perform a thorough assessment of the potential health impact of other potentially less harmful products such as e-cigarettes and compare them with heat-not-burn products and combusted cigarettes and the authors rightly pointed out that such research (also independent) is missing and is urgently needed. However, to allow direct comparison of scientific results the use of recognized, standard protocols and matching exposure concentrations is mandatory.

6 CONCLUSION

There is growing scientific consensus expressed by leading health organizations that the main cause of the harm from smoking results from the exposure to the harmful chemicals found in smoke, most of which are produced from burning the tobacco (Mallock, 2018; McNeill A, 2018). Non-combustible smoke-free products, such as heated tobacco and e-vapor products, have been shown to reduce the levels of harmful chemicals formed in their aerosols by more than 90 % on average compared to the levels found in cigarette smoke (Jaccard, 2017; Schaller, 2016). Switching completely to such smoke-free products has the potential to reduce the risk of harm for smokers who would otherwise continue to smoke. Comparing different types of smoke-free products with each other in terms of their risk reduction potential is complex as the risk of harm associated with the use of a specific product largely depends on the quality of the product, how the product is used and if the product provides the level of satisfaction to the user enabling them to completely switch from cigarette smoking.

The motivation of Leigh *et al*. to conduct a study to compare THS to e-cigarettes and cigarettes is well intended and appreciated as more independent verification on the harm reduction potential of different smoke-free products is needed, especially engaging cutting edge approaches such as systems toxicology.

The study contributes to further understand the effects of different products alternative to combusted cigarettes. Although the authors selected a standard method (NRU) to determine levels of cytotoxicity, the conclusion that are drawn from this study are far reaching, and may go beyond what the study results support, due to methodological limitations with the conduct of the study. Furthermore some important data was unfortunately only presented in the supplementary material. The methodological limitations as well as the supplementary data however should be taken into consideration when interpreting the results and drawing conclusions for this study.

Based on an analysis of our *in vitro* studies performed according to international standards of GLP, the THS presents less risk of harm and can reduce the risk of smoking-related diseases compared with continued smoking. This includes a significant reduction in cytotoxicity when compared to effects of cigarette smoke generated by combustion.

We welcome independent research, an open dialogue about the results and the knowledge exchange in a form of collaborative work resulting in scientifically substantiated findings. And when results differs from those from those in our studies, we conduct a thorough review of the methodology used and results generated, and compare where possible with PMI data and other relevant studies available in public domain and share our findings.

7 ABBREVIATIONS

AB	Alcian Blue
AE	Aqueous Extract
AK	Adenylate Kinase
ALI	Air-Liquid Interface
CXCL-8	Chemokine (C-X-C motif) ligand 8
DRF	Dose Range Finding
EC	Effective concentration
ELISA	Enzyme-linked immunosorbent assay
FDA	U.S. Food and Drug Administration
GVP	Gas Vapor Phase
GLP	Good Laboratory Practice
H&E	Hematoxylin and Eosin
HTP	Heated Tobacco Product
IL	interleukin
MMP	secreted Matrix Metalloproteinase
MRTP	Modified Risk Tobacco Product
MRTPA	Modified Risk Tobacco Product Application
NHBE	Normal Human Bronchial Epithelia Cells
NRU	Neutral Red Uptake
OECD	Organisation for Economic Co-operation and Development
PMI	Philip Morris International
SEM	Standard Error of the Mean
sICAM-1	Soluble Intercellular Adhesion Molecule 1

THS	Tobacco Heating System (commercialized as *IQOS*)
TPM	Total Particulate Matter
VEGFA	Vascular Endothelial Growth Factor A
3R4F	Reference cigarette

8 REFERENCES AND RELATED DOCUMENTS

Gonzalez-Suarez, I., Martin, F., Marescotti, D., Guedj, E., Acali, S., Johne, S., Dulize, R., Baumer, K., Peric, D., Goedertier, D., *et al.* (2016). In Vitro Systems Toxicology Assessment of a Candidate Modified Risk Tobacco Product Shows Reduced Toxicity Compared to That of a Conventional Cigarette. Chem Res Toxicol *29*, 3-18.

INVITTOX (1990). Protocol No. 3a: the FRAME modified neutral red uptake cytotoxicity test.

Iskandar A, A.R., Martinez, Y., Martin, F., Schlage, W.K., Leroy, P., Sewer, A., Torres, L.O., Majeed, S., Merg, C., Trivedi, K., *et al.* (2017). Comparative effects of a candidate modified-risk tobacco product Aerosol and cigarette smoke on human organotypic small airway cultures: a systems toxicology approach. Toxicol Res (Camb) *6*, 930-946.

Iskandar, A.R., Mathis, C., Martin, F., Leroy, P., Sewer, A., Majeed, S., Kuehn, D., Trivedi, K., Grandolfo, D., Cabanski, M., *et al.* (2017a). 3-D nasal cultures: Systems toxicological assessment of a candidate modified-risk tobacco product. ALTEX *34*, 23-48.

Iskandar, A.R., Mathis, C., Schlage, W.K., Frentzel, S., Leroy, P., Xiang, Y., Sewer, A., Majeed, S., Ortega-Torres, L., Johne, S., *et al.* (2017b). A systems toxicology approach for comparative assessment: Biological impact of an aerosol from a candidate modified-risk tobacco product and cigarette smoke on human organotypic bronchial epithelial cultures. Toxicol In Vitro *39*, 29-51.

Jaccard, G., Tafin Djoko, D., Moennikes, O., Jeannet, C., Kondylis, A., and Belushkin, M. (2017). Comparative assessment of HPHC yields in the Tobacco Heating System THS2.2 and commercial cigarettes. Regul Toxicol Pharmacol *90*, 1-8.

Leigh, N.J., Tran, P.L., O'Connor, R.J., and Goniewicz, M.L. (2018). Cytotoxic effects of heated tobacco products (HTP) on human bronchial epithelial cells. Tob Control *27*, s26-s29.

Mallock, N., Böss, L., Burk, R., Danziger, M., Welsch, T., Hahn, H., Trieu, H.-L., Hahn, J., Pieper, E., Henkler-Stephani, F., *et al.* (2018). Levels of selected analytes in the emissions of "heat not burn" tobacco products that are relevant to assess human health risks. Archives of Toxicology *92*, 2145-2149.

Mathis, C., Poussin, C., Weisensee, D., Gebel, S., Hengstermann, A., Sewer, A., Belcastro, V., Xiang, Y., Ansari, S., Wagner, S., *et al.* (2013). Human bronchial epithelial cells exposed in vitro to cigarette smoke at the air-liquid interface resemble bronchial epithelium from human smokers. Am J Physiol Lung Cell Mol Physiol *304*, L489-503.

McNeill A, B.L., Calder R, Bauld L and Robson D (2018). Evidence review of e-cigarettes and heated tobacco products 2018 (London: Public health England).

Schaller, J.P., Keller, D., Poget, L., Pratte, P., Kaelin, E., McHugh, D., Cudazzo, G., Smart, D., Tricker, A.R., Gautier, L., *et al.* (2016). Evaluation of the Tobacco Heating System 2.2. Part 2: Chemical composition, genotoxicity, cytotoxicity, and physical properties of the aerosol. Regul Toxicol Pharmacol *81 Suppl 2*, S27-S47.

Zanetti, F., Sewer, A., Mathis, C., Iskandar, A.R., Kostadinova, R., Schlage, W.K., Leroy, P., Majeed, S., Guedj, E., Trivedi, K., *et al.* (2016). Systems Toxicology Assessment of the Biological Impact of a Candidate Modified Risk Tobacco Product on Human Organotypic Oral Epithelial Cultures. Chem Res Toxicol *29*, 1252-1269.

https://www.agilent.com/en/products/cell-analysis/seahorse-analyzers
https://www.abcam.com/kits/measure-cell-metabolism-on-your-plate-reader